Imperial College London

Department of Surgery and Cancer, Faculty of Medicine

Genomics and Metabonomics in Severe Alcoholic Hepatitis

PhD Thesis

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2014 – 2018

Statement of Originality

I, Stephen R Atkinson, confirm that the work presented in this thesis is my own. Where others have contributed or information, data or ideas are derived from other sources I confirm that this has been indicated.

Stephen R Atkinson

Copyright Declaration

Table of contents

Acknowledgements

Completion of the work towards this doctoral thesis has allowed me to develop an enormous debt of gratitude to a large number of people for their help, support and guidance. Foremost among them I must thank my primary supervisor, Prof Mark Thursz. Not only for providing me with the opportunity to undertake this work and trusting me with its completion but also the encouragement, mentorship, collaborations and robust academic discussion required to yield what I hope will be judged a high-quality academic treatise. Second, Prof Elaine Holmes, has provided me with opportunity and access to cutting-edge scientific techniques.

This work would simply not have been possible without an incredibly valuable and rewarding collaboration with researchers at University College London. Prof Marsha Morgan and Dr Andrew McQuillin were kind enough to permit access to vital control samples for genetic studies but their contributions to the work run far beyond this. Prof Morgan has provided invaluable mentorship and guidance throughout my thesis and beyond in addition to a thorough schooling in scientific writing and English grammar. Likewise, Dr McQuillin has freely given time, of which he has scarcely little, advice, encouragement and direction which have been vital to completing that the genetic studies described. I must also thank Dr Michael Way for his friendship and assistance with many aspects of the genetic work described here – from the basics of genotyping through to collaboratively writing scripts to perform bioinformatics analyses.

I am grateful for a budding collaboration with Prof Ramon Bataller and Dr Josepmaria Argemi at the University of Pittsburgh who provided the data for the whole liver RNAseq analysis reported in this thesis. I must also acknowledge the kind provision of control samples used in metabonomics analyses by Prof Simon Taylor-Robinson, Dr Vish Patel and the Imperial College Gastroenterology and Hepatology biobank.

The post-doctoral researchers who have taken the time to teach me various laboratory techniques are legion: Dr Suzanne Knapp for assistance with DNA extraction from the STOPAH samples; Drs Alex Pechliavanis, Jia Li and Maria Romero-Gomez for their help, guidance, support and patience with metabonomics data acquisition and analysis. Special mention is necessary for Dr Fouzia Sadiq who's skill, knowledge and apparently limitless patience was critical to the cell culture work described herein and to Moe Kimura for assistance in performing these experiments.

The research office and, more importantly the individuals within it, acted as a unique environment for the generation, incubation and execution of various research ideas, some of which were successful. I must thank Nikhil for his friendship, assistance and our frequent, enjoyable and occasionally heated debates on the subject of severe alcoholic hepatitis, in particular bacterial DNA. I am grateful to James, James, Ben, Julie and the other occupants of the research office for their tolerance of these discussions in addition to their friendship and academic input. I must also thank Imperial College staff including Larry Koomson, Claire Parsonage, Amanda Ledlie, Suze Farrell and Dawn Campbell for the huge and varied assistance they have provided in order to help get things done!

I am enormously indebted to my wife, Karen, for her love, help and support throughout this period, particularly in the latter stages of writing-up. Our daughter, India, has been an inspiration and delight without which this thesis may have been completed in half the time! I would never have reached this point without the support, encouragement and sacrifice of my parents, Rita and Alan. Together with my sister, Sophie, they have been an inspiration, not least of all not to be the only member of the family without a PhD.

None of this work would have been possible without the generous financial support of the Medical Research Council and, perhaps most importantly, the willing participation of many thousands of patients.

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Table of tables

Table of figures

Abbreviations

ABV	Alcohol by volume
ABIC	Age, bilirubin, INR and creatinine score
ADH	Alcohol dehydrogenase
AHHS	Alcoholic hepatitis histological score
ALD	Alcohol-related liver disease
ALDH	Acetaldehyde dehydrogenase
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AMPK	5'-adenosine monophosphate-activated protein kinase
ApoE	Apolipoprotein A
ASH	Alcoholic steatohepatitis
AST	Aspartate transaminase
ATF4	Activating transcription factor 4
ATG	Autophagy-related protein
ATP	Adenosine triphosphate
AUDIT	Alcohol use disorders identification test
B	Beta
BCAA	Branched chain amino acid
BMI	Body mass index
BP	Base position
CADD	Combined annotation dependent depletion score
CD14	Cluster of differentiation 14
C/EBPB	CCAAT/enhancer-binding protein beta

CI	Confidence interval
CHOP	CCAAT/enhancer-binding homologous protein
ChREBP	Carbohydrate-responsive element-binding protein
CK	Cytokeratin
CPA	Collagen proportionate area
CRF	Case report form
CTLA4	Cytotoxic T lymphocyte associated protein 4
CXCL1	CXC motif ligand 1
CYP2E1	Cytochrome P450 family 2 member E1
DALY	Disability adjusted life year
DDIT3	DNA damage-inducible transcript 3
DF	Maddrey's discriminant function
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
DSM	Diagnostics and Statistics Manual
ECBL	Early change in bilirubin level
EDTA	Ethylenediaminetetraacetic acid
EIF2α	Eukaryotic initiation factor 2α
eIF4E-BP1	Eukaryotic initiation factor 4E binding protein 1
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
eQTL	Expression quantitative trait locus
FasL	Fas-ligand
FBS	Fetal bovine serum
FDR	False discovery rate

FPA	Fat proportionate area
FUMA GWAS	Functional Mapping and Annotation of GWAS
GADD153	Growth arrest and DNA damage-inducible protein 153
GAHS	Glasgow alcoholic hepatitis score
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GCN	General control non-derepressible
G-CSF	Granulocyte colony stimulating factor
GISAH	Global information system on alcohol and health
GSTM1	Glutathione S-transferase 1
GSTP1	Glutathione S-transferase pi 1
GSTT1	Glutathione *S*-transferase theta 1
GTEx	Genotype-tissue expression project
GWAS	Genome-wide association study
HCC	Hepatocellular carcinoma
HPLC	High-performance liquid chromatography
HR	Hazard ratio
HSC	Hepatic stellate cell
IBD	Identity-by-descent
ICD	International classification of disease
IL	Interleukin
INR	International normalised ratio
IQR	Interquartile range
JPEG	Joint Photographic Experts Group
KASPar	K-Biosciences Competitive Allele Specific PCR

Kb	Kilobase
LD	Linkage disequlibrium
LLOQ	Lower limit of quantification
LPS	Lipopolysaccharide
MAF	Minor allele frequency
MAT	Methionine adenosyltransferase
Mb	Megabase
MBOAT7	Membrane-bound O-acyltransferase domain containing 7
MDS	Multi-dimensional scaling
MELD	Model for end-stage liver disease
MMP3	Matrix metalloproteinase 3
MRIS	Medical research information service
MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
MTP	Microsomal triglyceride transfer protein
MyD88	Myeloid Differentiation primary response 88
NAD	Nicotinamide adenine dinucleotide
NAFLD	Non-alcoholic fatty liver disease
NAT	N-acetyltransferase
NDPI	Nanozoomer Digital Pathology Image
NFKB1	Nuclear factor kappa B subunit 1
NHS	National health service
NIAAA	National Institute on Alcohol Abuse and Alcoholism
NMR	Nuclear magnetic resonance

NO	Nitric oxide
NOS	Nitric oxide synthetase
OPLS(-DA)	Orthogonal projection to latent structures (discriminant analysis)
OR	Odds ratio
PBS	Phosphate buffered saline
PC	Principal component
PCA	Principal components analysis
PCR	Polymerase chain reaction
PNPLA3	Patatin-like domain-containing lipase 3
PolyPhen	Polymorphism phenotyping
PPAR	Peroxisome proliferator activated receptor
PS	Penicillin-streptomycin
PT	Prothrombin time
QQ	Quantile-quantile
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SAH	*S*-adenosylhomocysteine
SAM	*S*-adenosylmethionine
SAMM50	Sorting and assembly machinery component 50
sFas	Soluble Fas
shRNA	Short hairpin RNA
SIFT	Sorting intolerant from tolerant mutations
SLC38A4	Solute-carrier family 38 member 4
SNP	Single nucleotide polymorphism
SOD2	Superoxide dismutase

SREBP-1c	Sterol regulatory element binding protein 1c
STAR	Spliced Transcripts Alignment to a Reference alignment algorithm
STAT3	Signal transducer and activator of transcription 3
STOPAH	Steroids or Pentoxifylline for Severe Alcoholic Hepatitis
TGFβ	Transforming growth factor β
TLR	Toll-like receptor
TM6SF2	Transmembrane 6 superfamily 2
TNFα	Tumour necrosis factor α
TPM	Transcripts per million
tRNA	Transfer RNA
TSH	Thyroid stimulating hormone
UCSC	University of California Santa Cruz
UHPLC	Ultra-high-performance liquid chromatography
ULOQ	Upper limit of quantification
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoproteins
WHO	World health organization
WT	Wild type

Glossary of terms

Discriminant function (DF) a commonly used scoring system in severe alcoholic hepatitis, calculated as 4.6 × (patient prothrombin time [s] − control prothrombin time [s]) + (serum bilirubin [µmol/l]/17.1); scores > 32 indicate severe disease[1]

Dysbiosis a term used to describe an imbalance or maladaptation of commensal microbial communities either inside (e.g. enteric) or on (e.g. dermal) the body; typically when compared to that seen in healthy individuals without overt signs of disease. Dysbiosis may occur in response to multiple external stimuli and is postulated to contribute to the pathogenesis of many diseases[2]

Glasgow alcoholic hepatitis score (GAHS) a commonly used scoring system in severe alcoholic hepatitis derived by assignment of scores based upon age, blood urea nitrogen, international normalised ratio and bilirubin; ranges from 5 to 12, higher scores indicate worse prognosis[3]

Heritability for complex inherited traits, such as alcohol-related liver disease, the heritability refers to the degree of variation in the phenotype which can be attributed to inherited genetic factors

Heterozygosity refers to the presence of different alleles at a genetic locus. In the context of genome-wide association study quality control procedures it refers to the proportion of non-missing genotypes which are heterozygous, particularly high or low levels may indicate sample contamination or poor-quality genotyping

Lille score Composite scoring system incorporating age, serum albumin and bilirubin levels at baseline and 7 days after the start of treatment. A score of > 0.45, 7 days after initiation of treatment predicts an adverse outcome[4]

Model for end-stage liver disease (MELD) a commonly used scoring system in end-stage liver disease, derived from international normalised ratio, serum bilirubin and creatinine using the formula

3.78×ln[serum bilirubin (mg/dL)] + 11.2×ln[INR] + 9.57×ln[serum creatinine (mg/dL)] + 6.43; scores range from 6 to 40, higher scores indicate worse prognosis[5]

Pi-Hat ($\hat{\pi}$) calculated as the fraction of alleles in a linkage-disequilibrium pruned dataset which are the same in a pair of samples thereby giving an estimate of relatedness. Duplicate samples or twins would be expected to have a $\hat{\pi}$=1, siblings $\hat{\pi}$=0.5 and so forth

Population stratification describes systematic differences in allele frequency as a function ethnicity, this may be hidden at a phenotypic level and confound genetic association studies if it also segregates with the trait of interest.

Abstract

Severe alcoholic hepatitis is a florid presentation of alcohol-related liver disease and is associated with very high short-term mortality, in excess of 20% within 28 days. Severe alcoholic hepatitis occurs in a minority of patients who develop alcohol-related liver disease. A combination of genetic and environmental factors is likely to predispose to severe alcoholic hepatitis. To date the clinical phenotype has not been extensively examined in candidate gene studies and has been the subject of a single, small genome-wide association study. A genome-wide association study of severe alcoholic hepatitis identified two loci potentially associated with the risk of developing severe alcoholic hepatitis: i) A strong association with *PNPLA3*, a well-recognised risk locus for alcohol-related liver disease, and ii) a novel but weaker association with *SLC38A4*, an amino acid transporter. The primary genetic variant at each locus was evaluated to determine whether there was an influence on disease phenotype or outcome. The primary variant in *PNPLA3*, rs738409, is a missense variant. Analyses indicated a deleterious effect of homozygosity on medium-term survival in addition to more severe disease on baseline histology and a slower recovery in liver function over the short-term period; consistent with established literature in alcohol-related cirrhosis. In contrast the primary variant in *SLC38A4*, rs11183620, is intronic with no clear evidence for an effect on gene expression or function. Analyses did not indicate an influence on histology, clinical phenotypes or outcomes. In light of the locus' novelty further work was undertaken to determine any potential contribution to disease pathogenesis. *SLC38A4* was down-regulated in whole liver tissue in severe alcoholic hepatitis. Experiments with cell lines in culture suggested the pro-inflammatory cytokine IL-1β as a potential driver. *SLC38A4* knockdown resulted in upregulation of some cellular responses associated with nutrient deprivation. There was no influence of the variant on serum amino acid profiles. The functional significance of *SLC38A4* down-regulation remains the subject of ongoing work.

Contributions

General

I was predominantly responsible for the collation and curation of the data and samples collected as part of the STOPAH trial after its use in the primary trial analysis. Consequently, I was responsible for assimilating the data and compiling an extensive phenotypic database for use in subsequent analyses.

Chapter 2 – A two-stage genome-wide association study of severe alcoholic hepatitis

The conceptualization and design of the study was undertaken by myself in conjunction with Prof Mark Thursz, Prof Marsha Y Morgan and Dr Andrew McQuillin. I was involved in extraction and preparation of DNA samples from patients with severe alcoholic hepatitis with the assistance of Dr Susanne Knapp. Genotyping was performed at the Wellcome Trust Sanger Institute, Cambridge. Control samples were performed quality control of the genotyping data, all analyses and imputation of variants. I was also responsible for conducting the replication genotyping of selected variants. Variants in *PNPLA3*, *TM6SF2* and *MBOAT7* were genotyped by Dr Michael J Way.

Chapter 3 – The influence of genetic variation on presentation with severe alcoholic hepatitis

The design and conceptualization of these studies was performed by Prof Marsha Y Morgan and I. Histological scoring was performed by Prof Rob Goldin and Dr Alberto Quaglia in conjunction with Drs Gemma Petts and Kirsty Lloyd. Histological quantitation of fibrosis and steatosis was performed by Drs Pinelopi Manousou and Roberta Forlano. Serum quantitation of CK18 fragments and sFas was performed by Prof Guru Aithal and Dr Jane Groves. Genotyping of rs738409 in *PNPLA3* was performed by Dr Michael J Way. I performed genotyping of rs11183620 in *SLC38A4*. I conducted all data processing and statistical analyses.

Chapter 4 – The influence of genetic variation on outcomes from severe alcoholic hepatitis

The design and conceptualization of these studies was performed by Prof Marsha Y Morgan and I. Genotyping of rs738409 in *PNPLA3* was performed by Dr Michael J Way. I performed genotyping of rs11183620 in *SLC38A4*. I conducted all data processing and statistical analyses.

Chapter 5 – The potential role of *SLC38A4* in severe alcoholic hepatitis

Prof Mark Thursz, Dr Fouzia Sadiq and I conceptualised and designed the series of analyses and experiments described in this chapter. Whole liver RNAseq data were obtained and processed by Dr Josepmaria Argemi and Prof Ramon Bataller. Extracted data were analysed by myself. Primary human hepatocytes were kindly donated by Tomasz Kostrzewski (CNBio, Thame, UK). Cell lines were donated by Dr Marcus Dorner (Imperial College London, London, UK). Cell culture experiments, including cytokine and serum stimulation experiments and knockdown cell line construction were conducted by Dr Fouzia Sadia, Ms Moe Kimura and I. All data were analysed by myself.

Chapter 6 – Serum amino acids in severe alcoholic hepatitis

The conceptualization and design of the study was undertaken by myself and Dr Alex Pechliavanis. Preparation and mass spectroscopic analysis of samples was conducted by the Clinical Phenome Centre, Imperial College London. I was responsible for the analysis of all data.

CHAPTER 1

INTRODUCTION

1 Introduction

1.1 Overview

The consumption of alcohol is prevalent across the globe and often has significant cultural and social meaning. It is, however, associated with adverse health outcomes. The development of liver disease is one of the most well recognised complications of alcohol misuse.

Alcohol-related liver injury comprises a spectrum of disease ranging from comparatively benign fatty infiltration through to cirrhosis, or end-stage liver disease. However, its development is not invariable and the severity and progression of disease with ongoing alcohol consumption show significant heterogeneity. Alcoholic hepatitis is perhaps the most florid presentation of alcohol-related liver disease and severe disease is associated with significant short-term mortality.

This section provides an overview of i) alcohol consumption and its relation to health; ii) the global burden, spectrum and pathogenesis of alcohol-related liver disease including the factors believed to affect disease progression; and, iii) the condition of alcoholic hepatitis and the clinical dilemmas which it gives rise to.

1.2 Alcohol

In chemistry alcohols are a family of organic compounds defined by the presence of a hydroxyl group (-OH) bound to one or more saturated carbon atoms. In the vernacular, and throughout this thesis, alcohol is synonymous with ethanol (C_2H_5OH) the predominant species of alcohol found in alcoholic beverages.

Alcohol within beverages is generated by fermentation, the biological process through which sugars are anaerobically metabolised to yield carbon dioxide and alcohols. Although this process may occur in mammalian, bacterial and fungal cells, in the context of alcoholic drinks it refers to the conversion

of sugars, such as glucose, to carbon dioxide and ethanol by yeast (Figure 1.1). The final concentration of alcohol produced by fermentation in this manner is limited by the inherent toxicity of alcohol to the yeast which produce it. However additional processes such as distillation are used to yield final products with significantly greater alcohol concentrations.

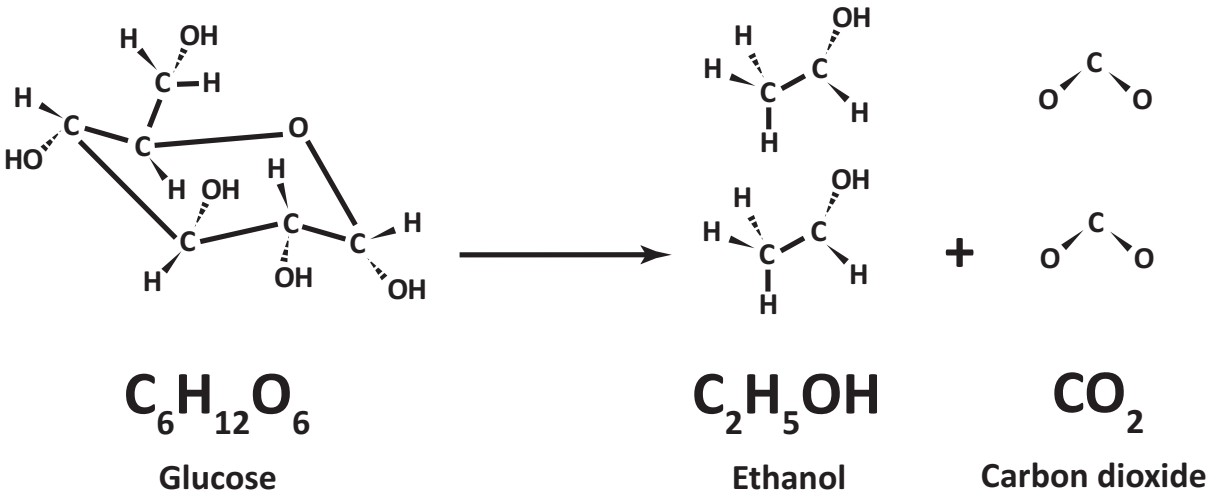


Figure 1.1 The chemical reaction of fermentation

Fermentation is the process by which sugars, such as glucose, are anaerobically converted to alcohols, in this case ethanol, and carbon dioxide. Typically, fermentation only occurs in anaerobic environments, however at high sugar concentrations certain yeast, e.g. *Saccharomyces* spp. preferentially metabolise sugars by fermentation.

The alcohol content of drinks is quantified by the percentage alcohol by volume (ABV). There is wide variation in the ABV between types of alcoholic beverage – commercially available drinks may vary in strength between 3 and 60% ABV. Even within a "class" of beverage the strength may vary widely, as an example a typical lager may contain around 4% ABV but "super-strength" variants may exceed 8% ABV (Figure 1.2).

This variation in alcohol content, in combination with variation in standard container size of different beverages, engenders a requirement for standardised methodologies for quantifying and comparing alcohol intake between individuals and populations[6, 7]. The most commonly used and useful methodology is the calculation and reporting of alcohol consumption as the amount of pure ethanol consumed per unit time (typically grams/day or litres/year). The amount of ethanol may be converted

to “units” which, in the United Kingdom, equates to 10 millilitres (or 8 grams) of pure ethanol; this is the definition of a unit used in this thesis.

Type of alcohol	Typical alcohol content (%, ABV)	Standard container size	Alcohol contained in a ‘drink’
Cider			
	*Standard strength* **4.5%**	*Bar or pub* **568mls**	**2.5 - 26 units**
	Super strength **8.5%**	*Shop bought* **up to 3L**	**20 - 200 grams**
Lager and bitter			
	*Standard strength* **4%**	*Bar or pub* **568mls**	**2 - 5 units**
	Super strength **9%**	*Shop bought* **500mls**	**2.5 - 6.4 grams**
Wine and fortified wine			
	*Standard strength* **14%**	*Bar or pub* **250mls**	**3.5 - 15 units**
	Fortified **20%**	*Shop bought* **750mls**	**4.4 - 19 grams**
Spirits			
	*Standard strength* **40%**	*Bar or pub* **25mls**	**1 - 45 units**
	Overproof **60%**	*Shop bought* **750mls**	**1.25 - 56 grams**

Figure 1.2 The alcohol content of common beverages

Alcohol content differs markedly between and within classes of drinks as do “standard serving measures”. These differences mean it is essential to carefully characterise an individual’s alcohol consumption and express it in a standardised way, such as units or grams of alcohol consumed, in order to derive meaningful estimates of alcohol consumption which are comparable between individuals and populations.

Abbreviations: ABV: Alcohol by volume

1.3 Patterns and levels of alcohol consumption

The associations between alcohol consumption and physical, social and societal wellbeing have led to attempts to categorise drinking behaviour based upon the likelihood of related harms. Categorisation may be based upon either the absolute amount of alcohol consumed or using questionnaires designed to identify problem drinking. The Alcohol Use Disorders Identification Test (AUDIT) incorporates several questions regarding alcohol drinking behaviours and the occurrence of withdrawal symptoms on cessation of drinking and has been extensively validated[8, 9]. Using these tools drinking behaviour may be broadly categorised as:

A. 'Low risk' – unlikely to lead to adverse health outcomes. Defined either as drinking in line with guidance issued by public health bodies or by an AUDIT score <7[10]. In the United Kingdom, current guidance for low risk levels of alcohol consumption is <16 g/day of ethanol on no more than 3 days of the week, equivalent to 14 units/week,[11];
B. 'Hazardous' – levels of alcohol consumption which pose an increased risk of adverse physical or mental health outcomes. This equates to alcohol consumption or an AUDIT score above 'low risk' but below 'harmful' thresholds[10, 12];
C. 'Harmful' drinking constitutes the consumption of alcohol at levels clearly associated with adverse health outcomes and is defined in both the Diagnostics and Statistics Manual, 4th edition (DSM-IV), and the International Classification of Disease 10 (ICD-10). It typically equates to alcohol consumption >40 g/day (~35 units/week) in women and >60 g/day (~50 units/week) in men or an AUDIT score >15[12];

Individuals may also demonstrate dependency on alcohol, manifest as clinical features of anxiety, agitation, autonomic activation, hallucinations and even seizures upon its withdrawal. Dependent drinkers typically have an AUDIT score ≥20 and disclose the highest levels of alcohol consumption[13].

The Global Information System on Alcohol and Health (GISAH) was set up and is administered by the World Health Organization (WHO) to collect data on alcohol consumption related harm and policy

from 194 nations. The most recent data indicate that worldwide around 40% of the population regularly consume alcohol. Total annual per capita alcohol consumption, by those at least 15 years of age, was estimated at 6.13 and 6.2 litres of pure alcohol in 2005 and 2010, respectively[14, 15]. These figures belie significant variations in consumption between regions as a function of cultural, economic and health policy influences[15] (Figure 1.3).

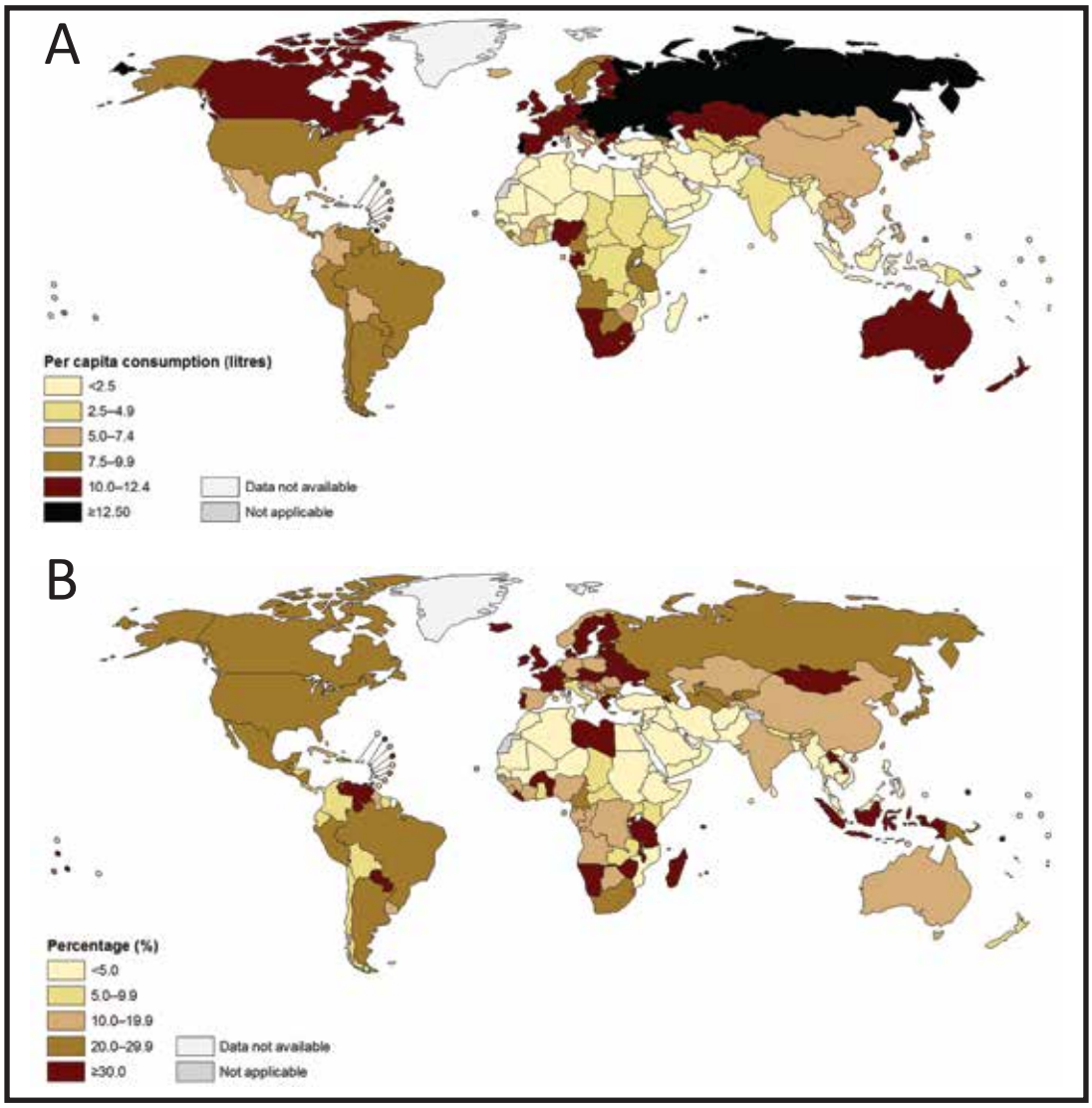


Figure 1.3 Global representations of (A) total per capita alcohol consumption and (B) proportion of individuals reporting heavy episodic drinking

Data are for individuals over the age of 15. Heavy episodic drinking is defined as >60 g pure ethanol on >1 occasion per month. Figures are derived based upon data reported in 2010 *via* the Global Information System on Alcohol and Health. Areas of high per capita alcohol consumption and high prevalence of heavy episodic drinking are typically co-located in countries with higher levels of national wealth and relative affordability of

alcohol. Adapted from images produced by Health Statistics and Information Systems (HIS) for the WHO, obtained from **http://www.who.int/gho/alcohol/consumption_levels/adult_recorded_percapita/en/**

Within the United Kingdom only a minority of adults, around 18%, abstain from the consumption of alcohol though, in line with global data, abstinence is more common in women than men (21% *vs*. 15% respectively)[12, 14, 15]. English data indicate that a significant minority of individuals, some 22% and 16% of male and female drinkers, respectively, consume alcohol in excess of levels considered to be "safe" (168 grams per week for men, 98 grams per week for women, based upon government guidelines at the time of publication). Whilst 5% of male and female drinkers consume alcohol at harmful levels[12]. White ethnicity, regional location (South West and East of England) and both increasing age (up to around 65 years old) and income were associated with higher prevalence of harmful alcohol consumption[12].

Recent survey data for England collected using the AUDIT tool indicate that 16.6% of adults drink at hazardous levels whilst around 3% had scores indicating possible or probable dependence[10]. At a population level this equates to 9 million adults drinking at levels with the potential to damage their health and 1.6 million displaying a degree of dependence[16]. The economic costs of alcohol misuse are estimated at a staggering £21 billion per annum whilst the annual cost to the National Health Service has been calculated at £3.5 billion[16].

1.4 Alcohol and health

Alcohol consumption has myriad, and almost exclusively deleterious, effects on health with well-recognised effects on the neurological, cardiac, hepatological, immunological and pancreatic organ systems. Alcohol is a recognised risk factor for not only aerodigestive cancers but also malignancies in other organs as well, such as breast cancer.

In addition to categorising alcohol-related health effects based upon their positive or negative impact on health, they may also be considered in terms of their temporal relationship to alcohol consumption

(i.e. acute or chronic). The potential major adverse health consequences of alcohol on health are summarised in Table 1.1. In addition, the consumption of alcohol may cause individuals to come to harm as a function of impaired ability or judgement leading to accidents, trauma or violence, not infrequently as either as the victims or commissioners of criminal acts. The risk of adverse alcohol-related health outcomes may be potentiated by co-morbid behaviour such as smoking. Whilst a potential beneficial impact of moderate alcohol consumption on cardiovascular health[17, 18] and even progression of non-alcoholic fatty liver disease[19] has been reported, the magnitude, and even existence, of these effects remains debated and cannot constitute reasons to encourage patients to increase their alcohol consumption[20-22].

Table 1.1 A summary of major adverse health outcomes associated with alcohol misuse

Organ System	Acute	Chronic
Cardiovascular	Arrhythmias	Arteriovascular disease Cardiomyopathy Hypertension
Gastrointestinal	Gastritis and oesophagitis Pancreatitis	Cirrhosis Malnutrition Oral, laryngeal and oesophageal cancers Pancreatic insufficiency
Metabolic	Hypoglycaemia	Osteoporosis
Neuromuscular	Accidental injury, including head trauma and intracranial bleeding Acute alcohol poisoning Neuropraxia Rhabdomyolysis Seizures	Alcohol-related dementia Cerebellar degeneration Korsakoff's psychosis Peripheral neuropathy Wernicke's encephalopathy
Reproductive	Sexual dysfunction	Breast cancer Infertility Foetal alcohol syndrome Sexual dysfunction
Respiratory	Aspiration pneumonia	

1.5 Alcohol-related liver disease

The development of liver disease is probably the most recognised long-term health complication of alcohol misuse. Alcohol-related liver disease comprises a spectrum of lesions differing in clinical presentation, severity and significance. End-stage liver disease, or cirrhosis, is the culmination of progressive liver injury caused by chronic alcohol misuse and represents an irreversible disease state.

1.5.1 Global burden of alcohol-related liver disease

Cirrhosis is a major contributor to global mortality and morbidity. The 2010 Global Burden of Disease study estimated over 1 million deaths per annum and 31 million disability-adjusted life years (DALYs) were attributable to cirrhosis[23, 24]. Almost half of both deaths and DALYs, 47.9% and 46.9% respectively, are attributable to alcohol[25]. Whilst many areas of Western Europe have successfully reduced age standardised cirrhosis mortality rates, the United Kingdom has seen a dramatic 31.2% increase in attributable deaths over the period 1980 – 2010[26].

1.5.2 The spectrum of alcohol-related liver disease

Alcohol-related liver disease encompasses a spectrum of histopathological lesions comprising comparatively benign simple steatosis, active inflammation and cirrhosis. These findings exist on a dynamic continuum with the possibility that all may occur contemporaneously or over a period of time in a given individual. Alcohol-related liver disease begins with steatosis with super-imposed inflammation leading to fibrosis and eventually cirrhosis. Abstinence from alcohol of sufficient duration may lead to resolution of inflammation and steatosis; fibrosis is typically less dynamic.

1.5.2.1 Steatosis

Fatty liver is an early and virtually inevitable development in patients who misuse alcohol. Steatosis was observed in 90% of liver biopsies from a series of patients admitted to secondary care with a recent history of heavy alcohol use; a small number had evidence of periportal fibrosis but none had

developed histological or clinical evidence of cirrhosis[27]. Hepatic steatosis whilst reversible[28] is, however, a precursor to more severe liver injury. Studies using serial liver biopsies report the development of cirrhosis in as many as 10-15% of patients with alcohol-related steatosis at presentation[29-31]. A Danish population-based study indicated that 7% of patients with a diagnosis of pure steatosis progressed to cirrhosis over 5 years[32].

1.5.2.2 Steatohepatitis and fibrosis

In a subset of individuals accumulation of fat within the hepatic parenchyma is associated with inflammation. The histological appearances on liver biopsy in this cohort are referred to as alcoholic steatohepatitis (ASH; Table 1.2). ASH is a histopathological diagnosis and whilst no universal criteria exist to define it, steatosis, hepatocyte ballooning and an inflammatory infiltrate are considered pre-requisites[33]. Persistent inflammation and cycles of tissue repair and regeneration lead to collagen deposition and the accumulation of fibrosis. The pattern of fibrosis associated with alcohol-related liver disease is initially perivenular, progressing to pericellular ("chicken-wire") fibrosis[33]. The prevalence of alcoholic steatohepatitis amongst individuals who misuse alcohol is not well defined. The wide estimate of 10-35% quoted in international guidelines reflects this[34]. In a large series of 1,407 patients admitted for treatment of alcohol-related disorders, biopsies from 12% of patients without cirrhosis demonstrated alcoholic steatohepatitis rising to 44% of those with cirrhosis[35]. A more recent, but somewhat smaller, study reported the presence of steatohepatitis in a fifth of patients with a history of chronic alcohol misuse undergoing liver biopsy[36]. In both studies only a minority of patients had simple steatosis, the majority, around 70%, had more advanced histopathological lesions.

1.5.2.3 Cirrhosis

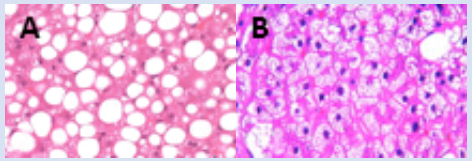
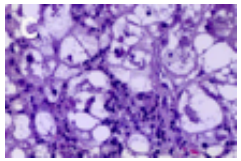
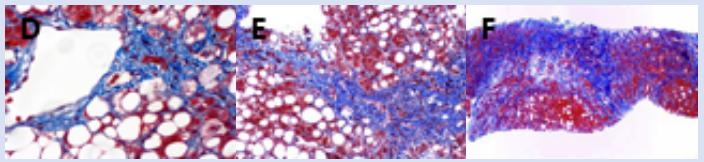
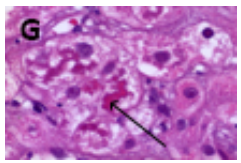
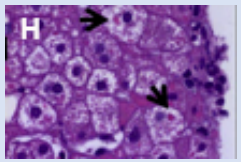
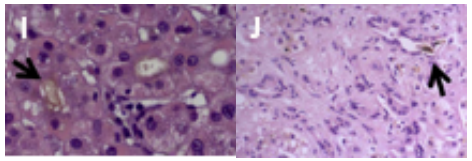
Eventually persistent collagen deposition leads to the formation of regenerative nodules bounded by thick fibrous septa – the histological appearance of cirrhosis. The overall proportion of individuals who

chronically misuse alcohol and proceed to develop cirrhosis varies between studies but is likely in the region of 15-20%[37, 38].

1.5.2.4 Hepatocellular carcinoma

Alcohol-related cirrhosis has been associated with a two-fold increase in the risk of developing any cancer compared to the general population. Whilst some of this increase may be attributed to the carcinogenic effects of alcohol and co-morbid tobacco use the dramatic 40-70-fold increase in the incidence of hepatocellular carcinoma (HCC) is indicative of the nature of cirrhosis as a pre-malignant state[39, 40]. In the context of alcohol-related liver disease, HCC occurs almost exclusively in patients who have developed cirrhosis with very low incidence in patients with alcohol misuse but without cirrhosis[40]. A recent United Kingdom population-based study estimated a 1.2% 10-year cumulative incidence rate for HCC in patients with alcohol-related cirrhosis[41]. Other estimates of cumulative incidence in alcohol-related cirrhosis range from 2.6% over 1 year to 1% over 15 years[40, 42]. Differences in the estimates of cumulative incidence may be attributable, in part, to different selection criteria for the populations studied and stringency for making diagnoses of cirrhosis and cancer. It is generally accepted that 3-10% of patients with alcohol-related cirrhosis will develop HCC during follow-up[34].

Table 1.2 Histopathological features of alcoholic steatohepatitis, and their appearance

Feature	Description	Histological appearance
Steatosis	Typically macrovesicular (A), progressing from small to large-droplet. Uncommon microvesicular form affecting virtually all hepatocytes – "alcoholic foamy degeneration" (B), similar appearances to other liver diseases characterised by mitochondrial dysfunction.	
Inflammatory infiltrate	Associated with alcoholic steatohepatitis, typically neutrophil rich (C) though may be mixed or predominated by mononuclear cells; distribution is typically lobular.	
Fibrosis	Initially perivenular extending in a pericellular fashion to result in "chicken-wire" fibrosis pattern (D). Extension and progression over time leads to formation of fibrous septa (E) and cirrhosis (F).	
Hepatocyte ballooning	Swollen hepatocytes with rarefied cytoplasm and often degenerate nuclei (G). Indicative of cellular damage. Necrosis and apoptosis are also prominent features.	
Mallory-Denk bodies	Aggregations of misfolded keratin filaments which coalesce in the cytoplasm to form inclusion bodies (G, arrow).	
Megamitochondria	May be seen in conjunction with steatosis alone in the absence of other features described above. Swelling or fusion of mitochondria as a function of changes in their membrane leads to their formation as a potential cell survival mechanism in the face of oxidative stress (H, arrows).	
Bilirubinostasis	The retention of bile within liver tissue which may occur in one of two patterns. Hepatocellular (I), where bile is seen within hepatocytes, or canalicular, where plugs of bile may be seen in the canaliculi or even bile ducts (I and J, arrows).	

Images adapted from Alitmirano et al., 2014[43], Bataller et al., 2011[44], Kleiner et al., 2015[45]

1.5.3 Pathogenesis of alcohol-related liver disease

The development of alcohol-related liver disease, particularly that which is progressive in nature, is the result of a complex interplay between host and environmental factors driven by continued alcohol consumption[34, 46]. A number of pathogenetic mechanisms, largely derived from cellular and animal models, are believed to drive liver disease:

i) Alcohol-mediated disruption of hepatic lipid metabolism;

ii) Toxic effects of intermediates and by-products of hepatic metabolism of alcohol;

iii) Alcohol-mediated effects on gut barrier function;

iv) Inflammation associated liver injury;

Each of these is discussed, in turn, below.

1.5.3.1 Alcohol-mediated disruption of lipid metabolism

Alcohol works *via* a number of mechanisms to interfere with hepatic lipid metabolism. Evidence from rat models indicates an increase in dietary lipid absorption with acute alcohol administration. However, this effect appears to diminish and even reverse with chronic administration of ethanol raising questions regarding its contribution to chronic liver injury[47]. Although the severity of hepatic steatosis induced by alcohol increases with greater proportions of dietary fats, extreme restriction is insufficient to prevent its development[48]. These latter findings have been replicated in man[49]. Thus, although dietary fats, and their absorption, appear contributory to the development of alcohol-related steatosis, additional factors must play a role.

Alcohol increases hepatic lipid availability by promoting absorption from the gut, mobilisation from adipose tissue and hepatic uptake of lipid species[50]. Reductions in the availability of nicotinamide adenine dinucleotide (NAD), up-regulation of sterol regulatory element binding protein 1c (SREBP-1c) and impaired peroxisome proliferator activated receptor α (PPAR-α) activity lead to increased synthesis and impaired oxidation of fatty acids[51-53]. An alcohol-mediated reduction in 5'-adenosine

monophosphate-activated protein kinase (AMPK) results in alterations in activity of enzymes involved in fatty acid metabolism[54]. The net result of this plethora of effects is increased synthesis and decreased β-oxidation of fatty acids. Under normal circumstances an increase in hepatocellular free fatty acids would be offset by increased fatty acid oxidation. Failure of this homeostatic mechanism means that excess free fatty acids are available as substrates for triacylglycerol synthesis[55]. Excess hepatocellular triacylglycerides would normally be exported within very low-density lipoproteins (VLDL) for storage in adipocytes. Ethanol inhibits hepatocellular secretion of VLDL[56-58]. The net effect of these processes is hepatocellular accumulation of triglycerides, cholesterol and phospholipids.

1.5.3.2 Hepatotoxic effects of alcohol metabolism

Ethanol is metabolised in hepatocytes to acetaldehyde by the enzymes alcohol dehydrogenase (ADH) and Cytochrome P450 2E1 (CYP2E1). Acetalydehyde in turn is metabolised to acetate by acetaldehyde dehydrogenase (ALDH). This metabolic process results in the generation of reactive oxygen species (ROS) with resultant lipid peroxidation and depletion of antioxidants (e.g. glutathione, *S*-adenosylmethionine) and impairment of mitochondrial function[59-63]. Acetaldehyde has direct toxic effects at cellular level through the formation of DNA and protein adducts which serve to exacerbate antioxidant depletion[64]. Inhibition of its production through inhibition of CYP2E1 can ameliorate certain aspects of experimental liver injury[65, 66]. The net effect is the generation of significant oxidative stress which in turn results in cellular dysfunction and potentially death, by necrosis or apoptosis[67]. Hepatocyte injury and death leads to the release of several damage associated molecular proteins which promotes an inflammatory response. Free-radical generation in immune cell may also lead to their activation[60].

1.5.3.3 The effect of alcohol on gut barrier function

The gut microbiome is diverse and complex. Alterations in its composition in terms of both diversity and the presence, or absence of specific organisms, have been implicated in the pathogenesis of many

gastrointestinal and hepatological diseases[2]. These alterations in the gut microbiota associated with pathological stimuli or the development of disease, when compared to healthy states, are broadly termed dysbiosis. Data from murine models indicate that consumption of alcohol causes gut microbial dysbiosis, disrupts gut barrier function and encourages the translocation of bacterial products, particularly lipopolysaccharide (LPS), into the portal circulation[68]. LPS stimulates resident tissue macrophages in the liver, Kupffer cells, *via* signalling through toll-like receptor 4 (TLR4). TLR4 signalling through the Myeloid Differentiation primary response (MyD88)-independent pathway leads to the induction of expression of pro-inflammatory cytokines such as tumour necrosis factor α (TNFα) and interleukin-6 (IL-6)[69-71]. The degree of endotoxaemia correlates with the severity of the observed liver injury[72, 73]. Alcohol also plays a role in the activation of complement[74, 75]. The combination of these effects produces a pro-inflammatory environment which directly damages hepatocytes and exacerbates oxidative stress.

Elevated plasma levels of endotoxin are noted in patients who consume alcohol to excess including those with alcohol-related liver disease[76-78]. In the setting of severe alcoholic hepatitis, they have been noted to predict the development of organ failure and death[79]. The relevance of these data to the human condition is further supported by evidence of microbial dysbiosis in individuals who consume excess alcohol with additional alterations in the microbiome seen in the subset of patients with evidence of significant liver disease[80-82]. However, the cross-sectional cohort nature of these studies makes it challenging to discern whether such changes are a cause or effect of the development of cirrhosis[83]. However, a recent study indicating that faecal microbial transfer from humans to germ-free mice can transmit both susceptibility and resistance to alcohol-mediated liver injury indicates a causative link[84].

1.5.3.4 Inflammation associated liver injury

The hepatic environment in patients with alcoholic steatohepatitis is characterised by a rich, pro-inflammatory milieu generated not only by activation of innate and adaptive immune cells but

parenchymal cells as well[85-87]. A number of chemokines, such as interleukin 8 (IL-8) and CXC motif ligand 1 (CXCL1), are potent chemoattractants for neutrophils and lead to formation of an acute inflammatory infiltrate[86-88]. The activated infiltrate causes direct cellular damage and exacerbates oxidative stress[89]. The inflammatory response is also important however in the induction of wound-healing responses designed to promote hepatic repair and regeneration. Anti-inflammatory cytokines such interleukins 10 (IL-10) and 22 (IL-22) have hepatoprotective effects[90-92]. Reactive oxygen species, inflammatory cytokines and growth factors (transforming growth factor β, TGFβ; vascular endothelial growth factor; VEGF) released by inflammatory, parenchymal and activated endothelial cells cause hepatic stellate cell (HSC) activation to myofibroblasts and inhibit anti-fibrotic mechanisms[90, 93-96]. The net result is collagen deposition and accumulation leading to the development of liver fibrosis.

1.5.4 Factors influencing the progression of alcohol-related liver disease

1.5.4.1 Overview

The development of advanced liver injury in patients who misuse alcohol, even those who consume the greatest quantities, is not guaranteed (Figure 1.4). Indeed, only a comparative minority of heavy drinkers progress to cirrhosis[37, 38]. Furthermore, the rate of progression shows significant heterogeneity[31, 97].

Progressive liver injury is typically contingent upon continued ethanol consumption but is highly variable. Significant effort has been invested in trying to determine the environmental and endogenous factors which influence the development of advanced alcohol-related liver disease.

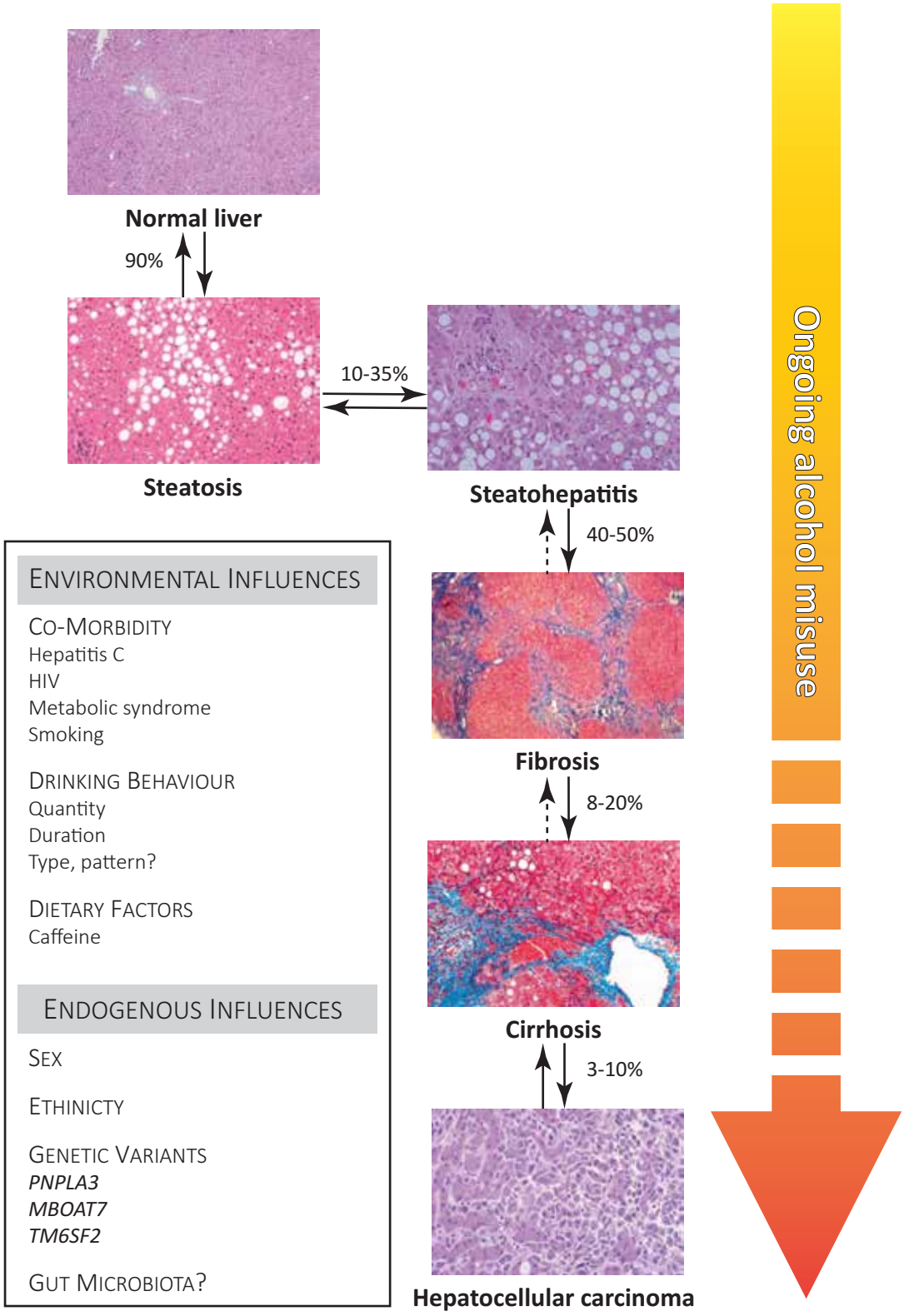


Figure 1.4 Progression of alcohol-related liver disease

The progression of alcohol-related liver disease from uncomplicated steatosis to hepatocellular carcinoma is primarily dependent, at least in the early stages, on continued misuse of alcohol. In spite of this progression of disease is highly variable and influenced by a range exogenous (environmental) and endogenous (host) factors. Image adapted from[98].

1.5.4.2 Alcohol consumption

Steatosis is reversible with the cessation of alcohol consumption. A study examining serial biopsies from patients with fatty liver secondary to alcohol reported resolution and normal liver histology on cessation of alcohol intake[28]. The accumulation and disappearance of hepatic fat is comparatively rapid; developing within as few as 8 days of starting drinking and resolving within a month of abstinence[99]. Unsurprisingly continued alcohol consumption critically modulates the risk of progression. In the subgroup of patients with steatosis who continue to misuse alcohol the rate of development of significant fibrosis or cirrhosis rises to 37-64% with comparatively negligible rates in their counterparts who are either abstinent or consuming alcohol at "safe" levels[28, 31]. Even fibrosis, which is classically regarded as irreversible, has been reported to regress with abstinence from alcohol[97]. Abstinence, albeit self-reported, does not however guarantee that disease will not progress with one study reporting progression to cirrhosis in 18% of abstinent patients and persistent steatohepatitis in 55%[100].

As suggested by studies based upon biopsies[28, 31] long-standing evidence indicates a dose-dependent relationship between amount of alcohol consumption and the risk of liver disease development and progression[101, 102]. At a population level this is evidenced by a strong link between per capita levels of alcohol consumption and mortality from cirrhosis of the liver[103]. Cohort studies demonstrate that at an individual level the risk of cirrhosis increases dramatically with escalating levels of daily alcohol consumption. A significant increase in risk is evident above 30 grams of ethanol per day[104, 105]. The precise nature of the dose-response relationship is debated. Individual studies and a meta-analysis support a linear relationship across the spectrum of alcohol consumption[105, 106]. Conversely others suggest a "step-change" in risk with consumption of more than 30-60 grams of ethanol per day[107-109]. A recent meta-analysis provides additional support for the existence of a threshold effect[110] (Figure 1.5).

There appears to be some influence of other parameters of alcohol consumption, over and above the absolute amount, on the risk of developing liver disease[111]. Studies have variously indicated that only consuming alcohol with food[104], drinking intermittently rather than daily[112, 113] and wine in preference to beer or spirits[113] may all reduce the risk of alcohol-related complications. A comparatively recent study suggests that binge-drinking is associated with a significantly increased risk of liver disease[114].

Illicitly produced and distributed ("bootlegged") alcohol or surrogate alcohol (products containing alcohol not intended for human consumption) may contain significant levels of hepatotoxic contaminants[115-117]. However, the available evidence indicates that their consumption does not influence the risk of developing cirrhosis independently of the quantity of alcohol consumed, except in certain specific instances[118, 119].

1.5.4.3 Sex

Several studies examining the relationship between alcohol consumption and the risk of liver disease have sought to additionally determine whether these risks differ in men and women. An increased risk of liver disease in women for a given level of alcohol consumption has been broadly consistently reported in individual studies[104, 107, 108, 120, 121](Figure 1.5). A meta-analysis of 17 studies incorporating data from 1,477,887 individuals confirms an increased risk of both liver disease and liver-related mortality in female drinkers for any given level of alcohol consumption[110]. The thresholds above which alcohol consumption should be considered harmful are, accordingly, widely considered to be lower in women than men and generally accepted to be >60 and >80 grams of ethanol per day respectively. Sex-related differences in susceptibility to the hepatotoxic nature of excess alcohol consumption are likely to relate, predominantly, to systematic differences in male and female body mass and composition. The overall effect is a lower volume of distribution in the female population leading to greater blood alcohol concentrations for the same absolute amount of alcohol consumed.

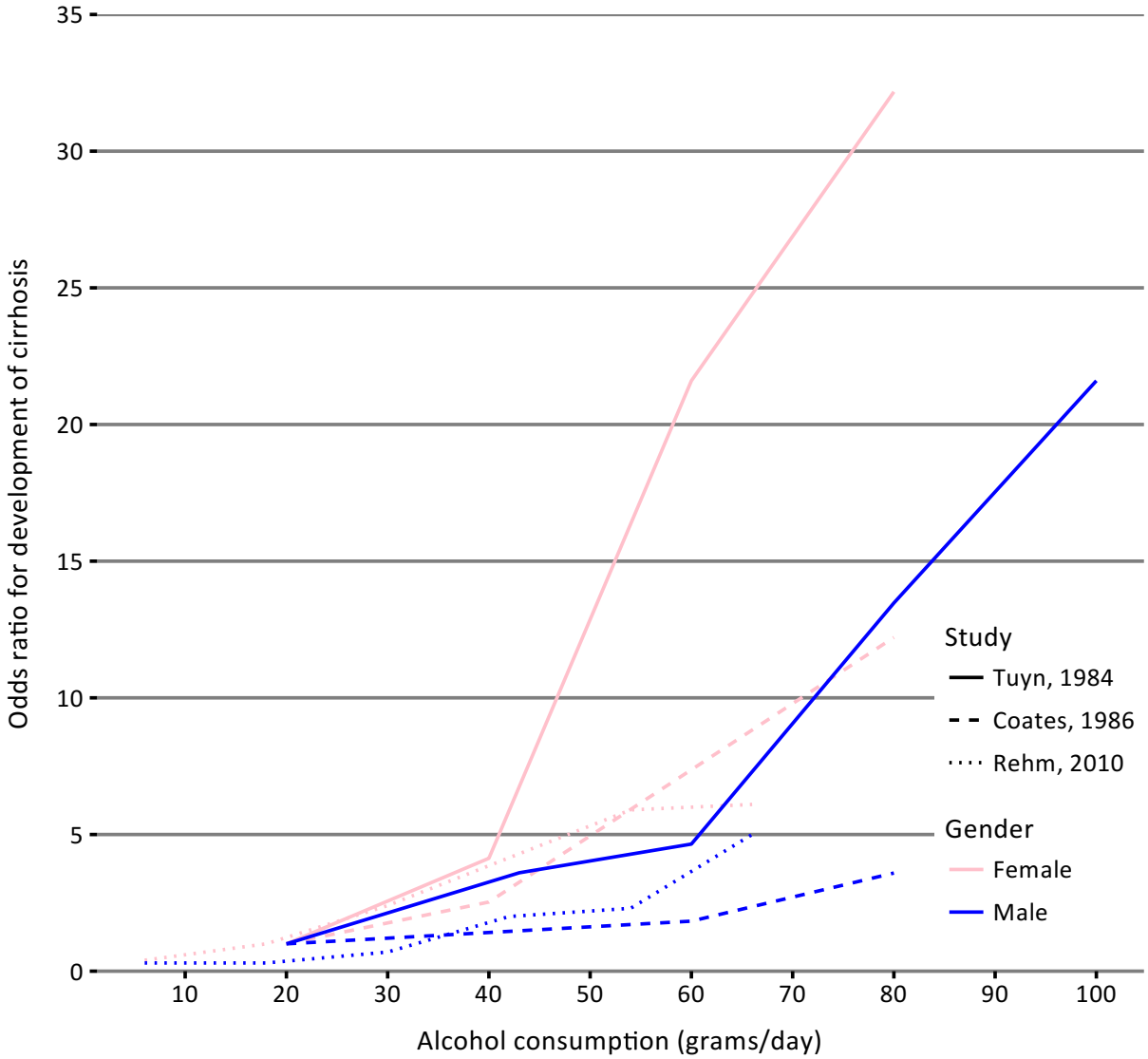


Figure 1.5 Odds ratios for the development of cirrhosis for differing levels of alcohol consumption, by gender
Data are drawn from two case-control studies[107, 108] and a meta-analysis[110]. Although odds ratios vary between studies, potentially as a function of estimation of alcohol consumption and case acquisition, within studies the risk of cirrhosis for a given level of alcohol consumption is consistently higher in females compared to males.

1.5.4.4 Ethnicity

Epidemiological studies suggest that there may be ethnic differences in susceptibility to the development of alcohol-related cirrhosis. In the United Kingdom, a study comparing the ethnic make-up of patients with alcohol-related cirrhosis in secondary care and the local population from which they were drawn described an over-representation of male patients of South Asian descent and an under-representation of male patients of Afro-Caribbean descent[122]. A retrospective analysis of

mortality data in the United States reported substantially higher cirrhosis mortality in individuals of 'Hispanic' origin[123]. In particular, it was noted that, based upon the most recent data from 1997, Hispanic males disclosed a mortality rate from cirrhosis with a mention of alcohol 114% greater than the overall male rate. The equivalent figure for Hispanic females was 16%. In both studies a significant issue arises due to an inability to incorporate and adjust for the potential confounding arising from differences in attitudes to alcohol and its consumption in different ethnic groups. They do however provide an indicator that ethnicity may modulate the risk of developing alcohol-related cirrhosis.

1.5.4.5 Pre-existing liver injury

In the context of alcohol-related liver disease, the occurrence of liver injury predicts the development of more advanced disease. The presence of significant liver injury, *viz.* steatohepatitis and fibrosis, on index biopsy is associated with an increased risk of progressive disease[97]. This is supported by more recent population-based data indicating that the presence of alcoholic steatohepatitis on liver biopsy was associated with a 16% risk of developing cirrhosis within 5 years compared to 7% in those with pure steatosis and 0.3% in matched population controls[32]. Steatohepatitis was also associated with a significant increase in liver-related mortality[32]. This may reflect a combination of self-perpetuation of and/or inherent predisposition to develop liver injury.

1.5.4.6 Co-morbid liver disease

Alcohol acts synergistically with other causes of liver injury to increase the risk of significant liver disease and accelerate the rate of progression. Patients who consume alcohol to excess in the context of also having features of the metabolic syndrome[35, 114, 124], chronic viral hepatitis B and C[125-128] or HIV infection[129] disclose a significantly increased risk of progression of liver fibrosis or excess liver-related death. The combination of chronic viral hepatitis C and alcohol misuse is particularly potent in causing significant liver disease. Heterozygosity for the *Z* allele of α1-antitrypsin is a predisposing factor for the development of cirrhosis in heavy drinkers[130, 131]. Patients with hereditary

haemochromotosis who drink harmful levels of alcohol have greater evidence of iron overload and higher serum transaminases[132] and, consequently, a greater risk of cirrhosis[133, 134]. However, it is not clear that carriage of variants associated with hereditary hemochromatosis confers additional risk of progression of alcohol-related liver disease[135-137].

1.5.4.7 Additional lifestyle factors

Effects of other lifestyle factors on the progression of alcohol-related liver disease have been reported. There is generally a high prevalence of smoking among patients who misuse alcohol; co-morbid smoking has been associated with an increased risk of liver disease[138-140]. Conversely caffeine consumption has been shown to protect against liver fibrosis[128, 138, 141].

1.5.4.8 Genetic factors

Observation of the epidemiological patterns of alcohol-related liver disease including familial, sex and ethnicity biases in susceptibility to disease, combined with significant variation in disease prevalence and progression between individuals, provide the foundation for the hypothesis that genetic factors significantly influence disease. Genetic studies in alcohol-related liver disease are considered below.

1.5.5 Genetic studies in alcohol-related liver disease

1.5.5.1 Twin and family studies

Alcohol-related liver disease is modelled as a complex inherited trait resulting from an interaction between environment and several individual genetic factors. Heritability is a statistical concept which aims to describe the proportion of variation in a phenotype which can be attributed to inherited genetic variation between individuals. Twin studies provide a means of trying to discern the relative contributions of genetics and environment in complex inherited diseases and thus provide estimates of heritability.

A study in 15,924 male twin pairs registered in the National Academy of Sciences-National Research Council Twin Registry in the United States reported a three-fold higher concordance of alcohol-induced cirrhosis in monozygotic as opposed to dizygotic twins (14.6% *vs*. 5.4%), indicating a genetic contribution[142]. A follow-up study of the same cohort reported that although the number of diagnoses of alcohol-related cirrhosis had increased within the cohort, the ratio between concordance rates remained stable[143]. Estimates of the heritability of alcohol-related liver disease in these two studies were between 21 and 67%. However, the second study questioned whether the genetic liability for end-organ damage was independent of the heritability of alcohol dependence.

Family studies seek to discern the heritability of a disease and genetic risk loci by examining patterns of familial aggregation and transmission of disease from parents to offspring. No formal family studies have been conducted in alcohol-related liver disease. A single, prospective study has examined the prevalence of alcohol-related liver disease in family histories reported by individuals who misuse alcohol with and without cirrhosis. It reported that alcohol misusers with liver cirrhosis were more likely to report a father who had died from liver disease than those without liver disease[144]. Whilst such studies are subject to potential bias due to diagnosis of a new case stimulating the flow of information within a family[145] these findings are potentially supportive of a genetic contribution to the development of alcohol-related liver disease.

1.5.5.2 Candidate gene studies in alcohol-related liver disease

Candidate gene studies are hypothesis driven and examine associations between genetic variants within pre-specified genes of interest and disease traits. Loci, and the variants within them, are chosen based upon biological plausibility and thus typically focus on a small number of functional variants in one or two genes implicated in the pathogenesis of disease.

Drawing upon knowledge related to its pathogenesis, candidate gene studies in the field of alcohol-related liver disease have predominantly focussed upon variants in loci related to alcohol and lipid

metabolism, oxidative stress, fibrogenesis, immune responses and the generation of inflammation (Table 1.3). Such studies have, with a small number of exceptions, failed to yield robust associations. There are several reasons for this including, but not limited to: i) limited statistical power due to small populations; ii) differing definitions used to define cases and controls; and, iii) study population admixture and stratification. Studies have generally focussed upon cases with alcohol-related cirrhosis and control populations drawn from healthy or alcohol-dependent individuals without evidence of liver disease. A handful of studies have included cohorts of patients with alcoholic hepatitis but, where specified, such groups are usually small in number and potentially defined according to the presence of alcoholic steatohepatitis on liver biopsy rather than the clinical syndrome of severe alcoholic hepatitis[146]. Data from candidate gene studies including significant populations of patients with severe alcoholic hepatitis do not indicate a role for polymorphisms in *TNFA* or *Interleukin-10* (*IL10*)[147] but do suggest an association between rs738409 in *Patatin-like phospholipase domain-containing 3* (*PNPLA3*) and disease development[148].

Overall, data from candidate gene studies indicate fairly conclusively that rs738409 in *PNPLA3* is associated with an increased risk of developing alcohol-related liver disease. Data also suggest that polymorphisms in the genes *TNFA* and *Glutathione S-transferase 1* (*GSTM1*) may contribute to an increased risk of developing alcohol-related liver disease[149, 150]. However, these polymorphisms have not been highlighted in a subsequent genome-wide association study (GWAS)[151].

Table 1.3 A summary of candidate gene studies and meta-analyses in alcohol-related liver disease

Process	Gene	Findings	Summary
Alcohol metabolism	*ADH1B*	R48H (rs1229984) strongly associated with risk of alcohol dependence on meta-analysis, particularly in Asian populations[152]; indirectly protects against alcohol-related liver disease[152-154]	Variants in *ADH1B*, *ADH1C* and *ALDH2* associated with alcohol misuse phenotypes in GWAS studies. A meta-analysis of alcohol-related liver disease phenotypes indicated variants in genes encoding enzymes involved in alcohol metabolism appear to associate with the risk of alcohol dependence and thus offer indirect rather direct protection against the development of alcohol-related liver disease *per se*.
	ADH1C	Protective effect of non-synonymous variants in *ADH1C* for alcohol dependence but not cirrhosis *per se*[154, 155]	
	CYP2E1	Conflicting results from several studies[156-159]. Meta-analyses have failed to demonstrate associations between the *CYP2E1* variants (Rsa-I, Pst-I) and risk of liver disease[152, 160]	
	ALDH2	E504K (rs671) significantly associated with a reduction in alcohol consumption due to a marked reduction in the capacity to metabolize acetaldehyde[157, 161], on meta-analysis it is strongly associated with the risk of alcohol misuse but not liver disease however its rarity outside Asian populations precluded analysis in other ethnic populations[152]	

Abbreviations: ADH1B: Alcohol dehydrogenase 1B; ADH1C: Alcohol dehydrogenase 1C, ALDH2: Aldehyde dehydrogenase 2; CYP2E1: Cytochrome P450 family 2 subfamily E member 1; GWAS: Genome-wide association study

Process	Gene	Findings	Summary
Oxidative stress	*GSTM1*	An association has been described between the *GSTM1* "null" alleles and liver disease risk[162, 163]. Despite some negative studies[156, 157] a meta-analysis incorporating data from 8 studies suggests an association between the null allele and an increased risk of alcohol-related liver disease[150]	Individual studies examining the role of genetic polymorphisms in genes related to oxidative stress generally demonstrate conflicting results. There is some evidence for a role of polymorphisms in *GSTP1* and an increased risk of developing alcohol-related cirrhosis but for other genes the data remains conflicting and the overall impact unclear.
	GSTP1	A meta-analysis incorporating data from 6 studies suggests an association between homozygosity for I105V with an increased risk of alcohol-related liver disease[150]	
	GSTT1	Individual studies have failed to demonstrate any significant association[157, 163, 164] and none was revealed on meta-analysis[150]	
	SOD2	An initial study indicated an association between a missense variant and increased risk of alcohol-related cirrhosis[165] though this has not been consistently replicated in subsequent studies[166, 167]	
	NAT	A variant in the *N-acetyltransferase* gene conferring slower acetylation activity has been reported as significantly less frequent in patients who misuse alcohol but do not develop liver disease compared to those who develop cirrhosis[156], though this finding has not been consistently replicated[168, 169]	

Abbreviations: GSTM1: Glutathione *S*-transferase mu 1; GSTP1: Glutathione *S*-transferase pi 1; GSTT1: Glutathione *S*-transferase theta 1; NAT: N-acetyltransferase; SOD2: Superoxide dismutase

Process	Gene	Findings	Summary
Immune response and inflammation	*TNFA*	The polymorphism G238A in *TNFA* has been associated with ALD in a number of studies one of which included a small number of patients with alcoholic steatohepatitis[146, 170]. Other studies have been negative[166, 171, 172]. One study failed to find an association with alcoholic hepatitis[147]. A meta-analysis examining the potential role of several variants demonstrated a potential role for the G238A polymorphisms[149]	Results of studies examining associations between polymorphisms in genes associated with inflammatory responses have failed to produce consistent associations. Meta-analysis indicates a potential role for G238A in *TNFA*.
	IL10	A variant in the *IL10* promoter was initially associated with an increased risk of developing liver disease[173] but was not subsequently replicated[166, 171], other studies have suggested an association between other variants and disease risk[172]. A study incorporating novel data into a meta-analysis failed to demonstrate a robust association between the -592C>A promoter polymorphism and liver disease[174]. One study failed to find an association with in alcoholic hepatitis[147].	
	IL1RA	A polymorphism in the *IL1RA* gene has been associated with hepatic fibrosis in two small studies in Japanese and Chinese patients with alcohol-related liver disease, the Japanese study included a small number of patients with alcoholic hepatitis[175, 176]. Studies in Caucasian populations have been negative but also conducted in small numbers of patients[172, 177].	
	IL1B	An initial study in a Japanese population suggested a positive association between a promoter polymorphism and the risk of alcohol-related cirrhosis[178] though this was not replicated in a Caucasian population[172].	

Process	Gene	Findings	Summary
	IL6	Whilst one study suggested an association of disease with an *IL6* polymorphism which alters the transcription of the gene[172] this was not replicated in a subsequent Spanish study[179]	
	CD14	Variation in the *CD14* promoter has been associated with an increased risk of developing alcohol-related liver disease in some studies[180, 181] but not others[166]	
	CTLA4	Homozygosity for the A49G polymorphism was associated with an increased risk of alcohol-related cirrhosis in a single study[182]	
	NFKB1	A single study has demonstrated an association between a functional variant in *NFkB1* (-94 ins/del), and the risk of developing alcohol-related liver disease[183]	

Abbreviations: ALD: Alcohol-related liver disease; CD14: Cluster of differentiation 14; CTLA4: Cytotoxic T lymphocyte associated protein 4; IL10: Interleukin 10; IL1B: Interleukin 1 beta; IL1RA: Interleukin 1 receptor antagonist; IL6: Interleukin 6; NFKB1: Nuclear factor kappa B subunit 1; TNFA: Tumour necrosis factor alpha

Process	Gene	Findings	Summary
Fibrosis and steatosis	*TGFB1*	A study testing for associations between three variants likely to affect *TGFB1* expression and alcohol-related liver disease failed to demonstrate any significant associations[184]	Generally, these genes have only been evaluated in small populations and in single or a small number of studies. These studies have failed to demonstrate positive associations and where evaluated in more than one study, data is conflicting. The variant rs738409 in *PNPLA3* is the one exception, having been examined in multiple studies with consistent, strong associations with an increased risk of developing alcohol-related liver disease
	MMP3	A single study using two independent cohorts failed to demonstrate any association between a functional promoter variant and the risk of developing alcohol-related cirrhosis[185]	
	PPARG	A single study evaluating a missense variant in (34C>G) failed to demonstrate an association with alcohol-related cirrhosis[183]	
	MTP	A single small study suggested an association between a missense variant and the risk of developing alcohol-related liver disease[186]	
	ApoE	Conflicting data exists from two very small studies[157, 187]	
	PNPLA3	I148M (rs738409) consistently associated with an increased risk of developing alcohol-related cirrhosis in individual studies[188-190]. The association has been confirmed on meta-analysis[191] and subsequently a genome-wide study[151]. A small study, reported in abstract form only, indicates an association in patients with severe alcoholic hepatitis[148]	

Abbreviations: ApoE: Apolipoprotein A; MMP3: Matrix metalloproteinase 3; MTP: Microsomal triglyceride transfer protein; PNPLA3: Patatin-like phospholipase domain containing 3; PPARG: Perioxisome proliferator-activated receptor gamma; TGFB1: Transforming growth factor beta 1

1.5.5.3 Genome-wide association studies

Genome-wide association studies (GWAS) are agnostic in design and conducted in the absence of any *a priori* hypothesis other than that a trait has a genetic component. Several hundred thousand genetic variants, typically single nucleotide polymorphisms (SNPs), are genotyped in parallel. Allelic and genotypic associations with either qualitative or quantitative phenotypic data are then tested.

Several liver disease phenotypes have been investigated in genome-wide association studies including autoimmune liver disease, drug-induced liver injury and haemochromatosis. Conduct of genome-wide association studies in non-alcoholic fatty liver disease (NAFLD) yielded the locus *Patatin-like Phospholipase domain-containing protein 3* (*PNPLA3*)[192]. Evaluation of the causative variant, rs738409, in candidate gene studies in alcohol-related liver disease has confirmed this as a major risk locus in alcohol-related liver disease, both in individual studies and on meta-analysis[189-191]. Only comparatively recently has alcohol-related liver disease been the subject of genome-wide investigation[151]. This study confirmed *PNPLA3* (I148M, rs738409) and identified *Transmembrane 6 Superfamily 2* (*TM6SF2*, rs58542926) and *Membrane Bound O-Acyltransferase Domain Containing 7* (*MBOAT7*, rs641738) as risk loci for the development of alcohol-related cirrhosis. *MBOAT7* is implicated in arachidonic acid metabolism and inflammatory responses in neutrophils[193]. However, rather than being implicated in inflammatory responses *PNPLA3* and *TM6SF2* have been linked to roles in lipid metabolism[194, 195]. This, combined with identification of these loci as risk factors for non-alcoholic fatty liver disease[192, 196, 197], suggests that genetic variation predisposing to alcohol-related liver disease may be found in genes associated metabolic functions.

A single GWAS study has examined the phenotype of severe alcoholic hepatitis[198]. This study was reported as exploratory due to the very small number of subjects included, around 180 in total split between cases and controls, leading to significant limitations in statistical power. No genome-wide significant associations were reported, though a modest signal was noted between rs738409 in *PNPLA3* and an increased risk of developing severe alcoholic hepatitis[198].

1.5.6 Clinical presentations of alcohol-related liver disease

Clinical presentations associated with alcohol-related liver disease generally mirror the histopathological lesions which may been on liver biopsy (Figure 1.6). The early stages of alcohol-related liver disease, steatosis and steatohepatitis, are typically asymptomatic. Indeed, even patients with cirrhosis but without significant impairment of liver function may not exhibit outward evidence of disease. Thus, the presence of early liver disease is frequently detected incidentally as abnormalities on imaging or blood tests in individuals undergoing investigation or monitoring of other health issues. Symptomatic presentation of alcohol-related liver disease is typically with decompensation of established cirrhosis – i.e. the development of ascites, encephalopathy, jaundice or variceal haemorrhage; often precipitated by infection.

The vast majority of patients who develop alcohol-related cirrhosis progress along a clinical path from steatosis through steatohepatitis and culminating in cirrhosis with an eventual decompensation. The clinical syndrome of alcoholic hepatitis sits outside this usual progression of disease. A Danish population-based study estimated the annual incidence of alcoholic hepatitis in 2008 at 46 and 34 per 1,000,000 in men and women, respectively[199]. In contrast, data for alcohol-related liver disease in general, covering a similar period, indicated an annual incidence of 311-343 per 1,000,000 and overall prevalence of 0.2% of the adult population[200]. Whilst these estimates are likely to be affected by misdiagnosis and incomplete case acquisition it is clear that, particularly in comparison to the overall burden of alcohol-related liver disease, severe alcoholic hepatitis only occurs in a minority of patients. Histological data indicate that cirrhosis is highly prevalent, in the region of 60-80%, but not invariant in patients presenting with severe alcoholic hepatitis[43, 201, 202]. Patients who recover from an episode of severe alcoholic hepatitis may, on resumption of drinking, re-present with the same clinical syndrome which may be more severe and associated with poorer outcomes[203].

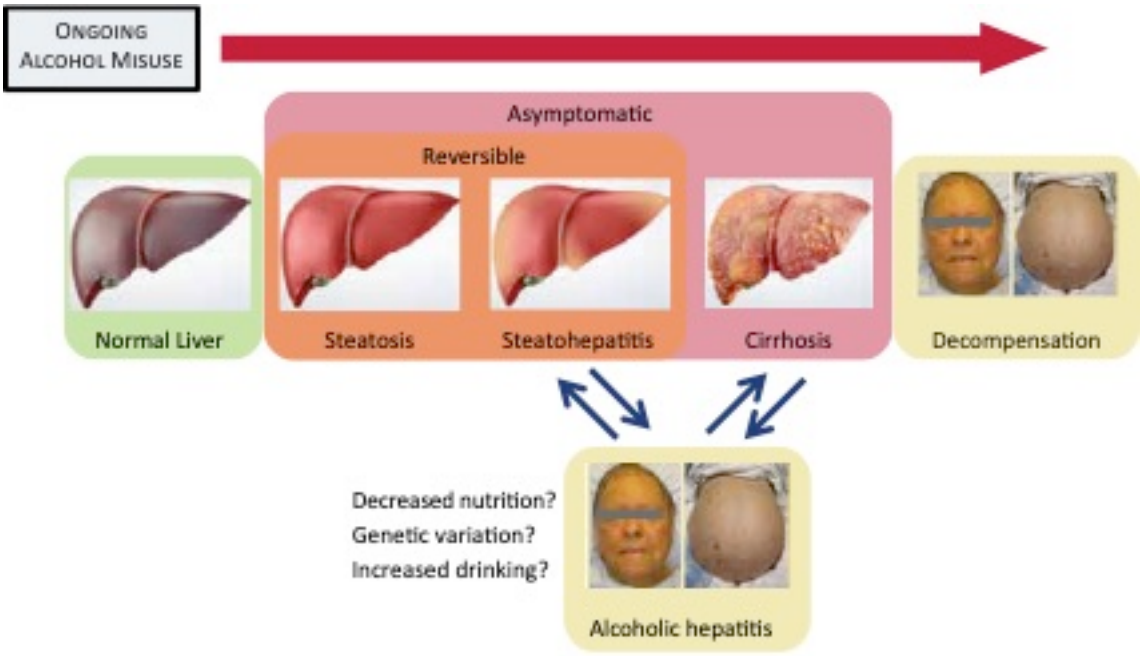


Figure 1.6 Disease progression and clinical presentations associated with alcohol-related liver disease

Severe alcoholic hepatitis sits outside the usual pathway of disease progression and may develop in patients who have not already developed cirrhosis. The precise factors which drive patients to develop severe alcoholic hepatitis remain unclear.

1.6 Severe alcoholic hepatitis

1.6.1 Presentation

Alcoholic hepatitis is a florid presentation of alcohol-related liver disease, manifesting as the recent onset of, often profound, jaundice in patients with a history of chronic, heavy and ongoing alcohol misuse[34, 204, 205]. It is usually accompanied by other features of liver failure including hepatic encephalopathy, coagulopathy and ascites. Affected individuals frequently present with concomitant gastrointestinal haemorrhage or infection[206-209]. Severity is determined based upon the Maddrey's discriminant function (DF), a score calculated from the prothrombin time and serum bilirubin[210]. A value greater than or equal to 32 portends a poor prognosis and defines the group of patients with severe disease[210].

1.6.2 Diagnosis

Whilst alcoholic hepatitis is defined as a clinical syndrome the presence of alcoholic steatohepatitis (ASH) on liver biopsy is the histological hallmark of the condition. However, ASH may be seen in patients with no outward features of liver disease and thus is insufficient in its own right to make the diagnosis[35, 211]. Indeed, significant debate centres upon the requirement to obtain liver histology.

Due to coagulopathy, thrombocytopaenia and/or ascites performing a liver biopsy in patients with severe alcoholic hepatitis necessitates use of the transjugular route. Time, expense, availability and potential complications limit its widespread availability and use. Proponents argue that obtaining histology is necessary to both confirm the diagnosis and exclude mimics. Detractors argue that the high prevalence of alcoholic steatohepatitis in patients meeting strict clinical criteria renders biopsy unnecessary.

A retrospective case series incorporating patients with a full spectrum of alcohol-related liver injury reported a high concordance between the clinical classification and histological diagnosis, including in patients with alcoholic hepatitis[212]. A wider review of diagnoses in patients enrolled into clinical trials, but only published in abstract form, indicated that when a serum bilirubin level >80 µmol/L was required as a criterion for entry, 96% of individuals had steatohepatitis confirmed on liver biopsy[201]. A recent retrospective cohort study indicated that only 13% of patients with a clinical suspicion of severe alcoholic hepatitis had no evidence of steatohepatitis on liver biopsy[202]. In contrast, a study evaluating histology in heavy drinkers with evidence of liver disease reported the histological diagnosis may not be alcohol-related in as many as 20% of cases[213]. A study examining liver biopsies in patients presenting with a deterioration of alcohol-related cirrhosis suggested that only around half of patients had steatohepatitis on biopsy, even when restricted to those displaying features of the systemic inflammatory response syndrome[214]. This tallies with recent data indicating that only 62% of patients with clinically suspected alcoholic hepatitis had steatohepatitis on biopsy[215]. However, both studies included patients in whom a clinical diagnosis of severe alcoholic hepatitis would be questioned. The

former defined a deterioration of cirrhosis as progression of pedal oedema and ascites as well as worsening jaundice[214], whilst the latter included patients with comparatively modest levels of bilirubinaemia[215]. Indeed, in the latter study the presence of steatohepatitis on biopsy was associated with significantly higher serum bilirubin levels[215]. It is also important to consider the possibility of sampling error and consequent misdiagnosis as a limitation of liver biopsy, particularly as steatohepatitis is likely to be diffuse but not homogenous though there is no data to support or refute this.

A recent consensus guideline has proposed a three-group categorisation of patients presenting with a potential diagnosis of alcoholic hepatitis[204]. A definite diagnosis of alcoholic hepatitis requires histology. In the absence of histology, application of strict clinical criteria permits a diagnosis of probable alcoholic hepatitis. Remaining cases pose diagnostic dilemmas and require histology in order to confirm, or refute, possible alcoholic hepatitis. This proposal has been broadly adopted into the most recent guidance issued by the European Association for Study of the Liver and American College of Gastroenterology such that both bodies recommend reserving the use of liver biopsy for cases of diagnostic uncertainty[34, 205].

The benefits of obtaining liver tissue are not restricted to diagnosis. Studies indicate that histological features may have prognostic significance and may even predict response to treatment[43, 216-218]. However, it is not clear that, on its own, this additional information is sufficient to justify routine recommendation of liver biopsy[43].

1.6.3 Pathogenesis of severe alcoholic hepatitis

The pathogenesis of the clinical syndrome of alcoholic hepatitis is not well understood. Much is extrapolated from data from animal models of alcohol-related liver injury which has been described above. However, whilst these models do produce hepatic inflammation they do not recapitulate the key features of severe alcoholic hepatitis, in particular the profound jaundice. Clinicians often relate

anecdotally that patients presenting with severe alcoholic hepatitis have recently increased their alcohol intake or started additional binge drinking. This is supported by data indicating that the addition of acute binge ethanol feeds to chronic ethanol- or high-fat diet fed mice exacerbates neutrophilic inflammation[219-221]. Recent efforts to recapitulate the syndrome of severe alcoholic hepatitis in a rodent model have evaluated a combination of a high fat diet, chronic alcohol feeding and additional binge alcohol feeds[222]. Animals develop hepatic inflammation and fibrosis alongside a transaminitis and a degree of hyperbilirubinaemia with features of portal hypertension. Interestingly without the addition of the binge feeds the more severe aspects of the phenotype do not develop[222]. However, a single case-control study comparing patients with alcoholic hepatitis to heavy-drinking matched controls suggested that patients with alcoholic hepatitis disclosed lower levels of binge drinking and that age, sex and body mass index did not increase the risk of developing the condition[223]. Ultimately the host and environmental factors which predispose this sub-group of patients to develop the clinical syndrome of severe alcoholic hepatitis are unknown.

1.6.4 Prognosis

Those with non-severe disease have a comparatively good short-term prognosis with spontaneous 28-day survival of at least 90% with supportive care alone[224, 225]. In contrast those with severe disease disclose a much greater short-term mortality risk – typically 20-30% at 28 day and as high as 50% at a year[224, 226-229]. A recent analysis encompassing a significant number of studies suggests that actually there has been no significant reduction in mortality from severe alcoholic hepatitis over time[228]. Given the significantly elevated mortality risk in those with severe disease, interventional trials have predominantly focussed on this group.

Significant effort has been devoted to developing scoring systems which permit a clearer division of patients into good and poor prognostic groups, as close to the initiation of therapy as possible. Whilst the DF is sensitive for mortality it lacks specificity[230]. Attempts to improve prognostication have involved re-purposing of existing models such as the Model for End-stage Liver Disease (MELD)[5] and

new disease-specific models, such as the Glasgow Alcoholic Hepatitis score (GAHS)[3] and Age, Bilirubin, INR and Creatinine (ABIC)[231], have been proposed. The Lille score[4] is a further disease-specific prognostic model which represents a development of the Early Change in Bilirubin Level (ECBL)[232]. These differ from the other models as they include a dynamic component. Although the respective weightings may differ, each score generally incorporates terms for age, liver dysfunction and renal dysfunction underlying the importance of these parameters in determining outcome. For each of the scores, thresholds have been proposed which define sub-populations with substantially different mortality risks as a means of guiding clinical management[3] [205, 231].

Irrespective of these thresholds and predicted outcomes it is clear that all scores are associated with and predictive of short-term outcome[233-235]. Recent analysis indicates that the severity of liver dysfunction at baseline, and its improvement over the initial treatment period, are key determinants of short-term outcome[236]. In contrast longer term outcomes appear predominantly related to the risk of alcohol relapse which increases mortality risk in a dose-dependent fashion[236, 237].

1.6.5 General management principles

Patients presenting with severe alcoholic hepatitis typically disclose multiple clinical complications of both their liver disease and the underlying alcohol misuse disorder. The treatment and management of the condition consequently requires a multifaceted approach. The main principles are explored below.

1.6.5.1 Management of fluid status

Patients presenting with severe alcoholic hepatitis frequently have issues related to their fluids status – intravascular depletion, peripheral oedema and ascites may be present individually or in combination. Development of acute kidney injury, typically secondary to acute tubular necrosis or type 1 hepatorenal syndrome, confers an increased risk of mortality[238]. Early and appropriate fluid resuscitation and management is therefore important in management of these patients.

1.6.5.2 Nutritional status

Deficiencies of both macro- and micronutrients are common in this cohort of patients[239, 240]. Protein-energy malnutrition has been described as almost universal[241] and its presence is associated with poorer outcomes[242]. Individual studies of nutritional supplementation have failed to consistently demonstrate a mortality benefit to intensive nutrition[227, 243, 244] including when administered as more specific nutritional supplementation, such as amino acids[245-247]. These are supported by results of a meta-analysis[248]. However, a post-hoc analysis of the most recent trial indicated that, irrespective of treatment arm, those patients who achieved a nutritional intake in excess of 21.5 kcal/kg per day had significantly greater survival rates and a lower incidence of complications[227]. Thus, it may be that adherence to and complications related to its implementation may limit the benefit associated with intensive nutritional support. Consequently, whilst intensive nutritional support is not routinely recommended for this group of patients, individual assessment for and correction of nutritional deficiencies is required[34, 205].

Vitamin deficiencies are also common particularly vitamins B and K. Deficiencies of the former may lead to Wernicke's encephalopathy whilst deficiency of the latter may prolong the prothrombin time meaning it does not solely reflect hepatic synthetic dysfunction. Parenteral replacement, as recommended in national guidelines, should be instituted[249].

1.6.5.3 Monitoring for and treatment of alcohol withdrawal

Patients require close-monitoring for symptoms related to alcohol withdrawal and prompt and appropriate treatment, typically with short-acting benzodiazepines, if these occur[249]. Hepatic impairment alters drug metabolism and increases the risks of toxicity – injudicious administration may lead to obtundation either as a result of drug toxicity or *via* precipitation of hepatic encephalopathy.

A return to drinking is a key determinant of medium- and long-term survival in patients who survive the acute admission[250-253]. Appropriate assessment and treatment of underlying alcohol misuse

disorders is therefore a key principle of ongoing management, however recidivism rates remain stubbornly high[252, 253].

1.6.5.4 Screening for and treatment of infection

Infection is a common complication of severe alcoholic hepatitis. Data indicate that up to a quarter of patients have an infection at presentation[206, 254, 255]. Severe alcoholic hepatitis may give rise to features of the systemic inflammatory response in the absence of infection; conversely patients with infection may not mount a strong clinical response. Thus, identifying infection requires a high index of suspicion and a systematic and thorough approach to its diagnosis. Institution of antibiotic therapy and control of sepsis is essential prior to consideration of treatment, particularly with corticosteroids or other immunosuppressive agents. Spontaneous bacterial peritonitis, bacteraemias and urinary tract infections are particularly common at presentation[206, 255]. In clinical trials around a further quarter of patients will develop an infection in the 28-day period following the start of treatment[255, 256]. The development of infection, particularly in the first 7 days of steroid treatment, is associated with the development of additional organ failure and adverse outcomes, independently of the degree of liver failure[209, 254, 255]. Chest infections are common in those on treatment[206, 254, 255].

The relationship between prednisolone and infection in severe alcoholic hepatitis is complex. Infection at presentation does not appear to be a contraindication to treatment with prednisolone[206, 255]. However, decisions regarding the timing of commencing steroids and discontinuing antibiotics may have a significant impact on outcomes[255]. In the STOPAH trial prednisolone was associated with an increased risk of developing an infection reported as severe (defined as grade 3 or higher according to the Common Terminology Criteria for Adverse Events v4)[208]. An increase in invasive fungal disease has also been reported in steroid-treated patients[208, 257]. Pre-treatment circulating levels of bacterial products such as lipopolysaccharide or bacterial DNA may predict the development of infection in patients subsequently prescribed steroids and provide a potential means to identify patients requiring alternative management strategies[79, 255].

1.6.6 Specific therapies

1.6.6.1 Prednisolone

The use of steroids to treat alcoholic hepatitis was first trialled in the 1970s[258, 259]. Since this time many trials have sought to evaluate its efficacy in this setting. Mixed results from early trials were potentially attributable to heterogenous patient populations, particularly in terms of disease severity, and small sample sizes[1, 210, 224, 260-262]. An initial meta-analysis indicated that although there was no benefit to prednisolone therapy across all patients with alcoholic hepatitis a reduction in 28-day mortality was seen in those with a DF ≥32[263]. These findings were supported by a further meta-analysis using individual patient data[264].

It was within this context that the Steroids or Pentoxifylline for severe Alcoholic Hepatitis (STOPAH) trial was performed[208]. It sought to definitively answer whether steroids offered therapeutic benefit in patients with severe alcoholic hepatitis, defined by a DF ≥32[265]. However, the numerical reduction in mortality at 28 days did not reach statistical significance[208]. Subsequent meta-analyses of both trial and individual patient level data have confirmed a beneficial effect of prednisolone on 28-day mortality; though it is clear that any benefit does not extend beyond this time[229, 266].

A key concept in the use of prednisolone in severe alcoholic hepatitis is that of 'response'. A response to steroids is defined based upon the Lille index; a score derived from the change in bilirubin over 7 days and additional prognostic factors such as age, renal function, serum albumin and the prothrombin time. In patients with severe alcoholic hepatitis the score is strongly associated with survival. In its original description a cut-off of 0.45 defined two populations with markedly different survival at 6 months (<0.45: 85% *vs.* ≥0.45: 25%)[4]. Subsequent analysis suggested the therapeutic benefit of prednisolone may differ in sub-groups defined by Lille scores[264]. While this finding has not been replicated and a 'response' may be seen in patients treated with placebo it is generally accepted

that further steroid therapy should be stopped in Lille 'non-responders'[34, 205]. Alternative therapies are, however, limited.

1.6.6.2 Pentoxifylline

Pentoxifylline is a weak tumour necrosis factor alpha (TNFα) antagonist. An initial placebo-controlled trial indicated an improvement in mortality in patients with severe alcoholic hepatitis, potentially related to a reduction in the incidence of hepatorenal syndrome[267]. Subsequent studies comparing pentoxifylline to prednisolone or placebo reported conflicting results[268-270]. Investigators have been unable to demonstrate a benefit combination of pentoxifylline with steroid therapy or a change to pentoxifylline where steroids were deemed to have failed[271-273]. Pentoxifylline was included, alone and in combination with steroids, in the STOPAH trial[265]. The primary analysis did not demonstrate any benefit of pentoxifylline alone or in combination with prednisolone, nor was there an interaction between prednisolone and pentoxifylline in relation to 28-day mortality[208]. These findings are supported by subsequent meta-analyses indicating that pentoxifylline is not an effective treatment for severe alcoholic hepatitis[229, 266].

1.6.6.3 N-acetylcysteine

N-acetylcysteine has not demonstrated efficacy in the treatment of severe alcoholic hepatitis on its own or when combined with a cocktail of antioxidants[244, 274]. Although one of these trials did include patients treated with steroids[274] only a single trial has compared prednisolone with or without five days of N-acetylcysteine[275]. The results indicated a significant reduction in mortality at 28 days and a trend to a reduction in mortality at 3 and 6 months. Infections were also less frequent in those treated with N-acetylcysteine in combination with steroids[275]. Data indicating that incubation with N-acetylcysteine reverses monocyte dysfunction seen in patients with severe alcoholic hepatitis support this as a potential mechanism of action[276].

1.6.6.4 Granulocyte colony stimulating factor (G-CSF)

Data from animal models indicate that G-CSF may promote hepatic regeneration potentially through the mobilisation and integration of haemopoietic stem cells into the liver[277-279]. An initial study in patients with alcoholic hepatitis indicated that administration of G-CSF was safe and appeared to induce hepatic regenerative responses[280]. Two recent, but comparatively small trials, have demonstrated a survival advantage from the addition of G-CSF to standard of care[281, 282]. The latter trial also included an arm which received N-acetylcysteine. Thus, initial results of trials examining the role of G-CSF in the treatment of severe alcoholic hepatitis are encouraging but require confirmation in larger trials.

1.6.6.5 Anti-TNF therapy

In light of the role of TNFα as a mediator of liver damage in severe alcoholic hepatitis and the potential associated with pentoxifylline therapy trials of anti-TNFα monoclonal antibodies were conducted in severe alcoholic hepatitis. Initial studies evaluating safety and using a surrogate endpoint of improvement in DF were promising[283-286]. However larger studies with mortality-based endpoints were curtailed due to an excess of infections and deaths in the treatment arms[287, 288]. Thus, anti-TNFα monoclonal antibodies should not be used in the treatment of severe alcoholic hepatitis.

1.6.6.6 Antioxidants and other agents

In view of the role of oxidative stress in the initiation and perpetuation of liver injury associated with alcohol consumption a number of compounds with antioxidant activity have been trialled, alone and in combination. These include vitamin E[289], silymarin (milk thistle)[290] and “cocktails” of anti-oxidants[274, 291]. Investigators have also evaluated the role of anabolic steroids[224, 292, 293] and the antithyroid drug propylthiouracil[294]. None of these agents has shown consistent benefit nor is their use supported by the results of meta-analyses[295-297]. Other agents which have been trialled and found

ineffective include ursodeoxycholic acid[298], calcium channel blockers[299], colchicine[300] and insulin-dextrose infusions[301].

1.6.6.7 Liver transplantation

In the absence of effective pharmacological treatments for a significant proportion of patients, liver transplantation has been trialled as a treatment for patients with severe alcoholic hepatitis. A European pilot study indicated that, in highly selected patients who were non-responders to steroid therapy, transplantation was able to dramatically improve survival compared to matched historical controls[302]. Centres in the United States have also reported favourable outcomes in patients undergoing liver transplantation for severe alcoholic hepatitis[303-305]. However, a pilot programme of early transplantation for severe alcoholic hepatitis in the United Kingdom was terminated early due to a lack of uptake. This experience highlights the cultural and ethical challenges that surround the use of liver transplantation in patients who have been actively drinking up until presentation and underlines the importance of careful patient selection[306]. This, combined with the limited availability of donor organs, underlines that whilst likely to be effective, liver transplantation will never be a treatment option for the vast majority of patients with severe alcoholic hepatitis.

1.7 The steroids or pentoxifylline for severe alcoholic hepatitis (STOPAH) trial

1.7.1 Overview

The STeroids Or Pentoxifylline for severe Alcoholic Hepatitis (STOPAH) trial was a double-blind, randomised placebo-controlled trial conducted across 65 centres throughout the United Kingdom[208]. The study was designed with the specific aim of providing a definitive answer to the question of whether prednisolone, pentoxifylline or a combination of the two agents was effective in reducing twenty-eight day mortality from severe alcoholic hepatitis[265].

1.7.2 Trial design

Trial inclusion was based upon a positive history of recent and long-standing alcohol misuse; compatible clinical, laboratory and/or liver biopsy features of alcoholic hepatitis; no other identified causes for of liver disease; and a Maddrey's discriminant function (DF) ≥32. Patients presenting with infection, gastrointestinal haemorrhage or renal failure were eligible for enrolment at the point that this had resolved or was clinically controlled; full inclusion and exclusion criteria are displayed in Table 1.4[208, 265].

Table 1.4 Inclusion and exclusion criteria for the Steroids of Pentoxifylline for Severe Alcoholic Hepatitis (STOPAH) trial[208, 265]

Inclusion	Exclusion
Age > 18 years	Abstinence >2 months
Clinical diagnosis of alcoholic hepatitis	Duration of clinical jaundice >3 months
Serum bilirubin >80 µmol/L	AST >500 IU/L or ALT >300 IU/L
Discriminant function (DF)[1] * ≥32	Creatinine >500 µmol/L or renal replacement therapy
Excess alcohol consumption (>80 g/day male, >60 g/day female) to within 2 months of randomisation	Other causes of liver disease (viral hepatidides, biliary obstruction, hepatocellular carcinoma)
Hospital admission <4 weeks	Active gastrointestinal haemorrhage or uncontrolled sepsis†
Informed consent	Active use of vasopressors
	Previous entry into the study, or use of either prednisolone or pentoxifylline within the prior 6 weeks
	Current malignancy (except non-melanotic skin cancer)
	Prior adverse reaction to prednisolone, pentoxifylline or other methyl xanthine
	Previous history of cerebral or retinal haemorrhage, recent acute myocardial infarction (within 6 weeks) or severe cardiac arrhythmia (not atrial fibrillation)
	Pregnancy

* Discriminant function (DF) calculated as 4.6 × (patient prothrombin time [s] − control prothrombin time [s]) + (serum bilirubin [µmol/l]/17.1); scores > 32 indicate severe disease[1]

†Patients presenting with either gastrointestinal haemorrhage or sepsis were eligible for inclusion once these were considered controlled by the treating physician

Patients were randomized to treatment with prednisolone or pentoxifylline for 28 days using a double-blind, double-dummy design, leading to the creation of four treatment groups:

1. Placebo-placebo;
2. Prednisolone-placebo;
3. Pentoxifylline-placebo;
4. Prednisolone-pentoxifylline;

Randomization was block designed and stratified by geographical region and dichotomous risk status. The presence of sepsis, gastrointestinal bleeding or renal failure prior to randomization was used to define high risk. A pre-trial power calculation estimated that recruitment of 1,026 patients would provide 90% power to detect a reduction in 28-day mortality from 30% to 21% (30% relative risk reduction)[265].

1.7.3 Trial data and sample collection

Extensive data collection was built into the trial visit structure and design. Information relating to demography, prior medical history, current clinical status, haematological and biochemical data and alcohol usage were collected at pre-defined time points during the trial. The occurrence and cause of mortality and adverse events were reported during the trial in accordance with Good Clinical Practice and using the Common Terminology Criteria for Adverse Events v4 standard. Patients were consented for follow-up *via* the NHS Information Centre Data Linkage service using the Medical Research Information Service (MRIS) ensuring ongoing follow-up and reliable capture of mortality data both within and beyond the period over which the trial was conducted. The visits undertaken and data collected at each are summarised below in Table 1.5; study case report forms (CRFs) are included in Supplementary Methods. Due to funding limitations, the trial was terminated at the point in time when the last patient enrolled in the study had completed 28 days of follow-up.

The STOPAH trial was primarily a clinical study and not designed, *a priori*, for the evaluation genetic factors predisposing to the development of severe alcoholic hepatitis. However, it was envisaged that it could provide the opportunity to collect samples which could be included in either genome-wide or candidate gene association studies at a subsequent point in time. Consequently the trial protocol also incorporated the collection of biological samples: i) whole blood anticoagulated with ethylenediaminetetraacetic acid (EDTA) and stored without further sample preparation, and, ii) serum was isolated from whole blood by centrifugation at 1200*g* for 10 minutes. All samples were collected at baseline, prior to the institution of treatment. Once processed they were stored at -80°C pending use in subsequent analyses. Whole blood and serum samples were collected from 898 and 872 patients, respectively.

Histological demonstration of alcoholic steatohepatitis (ASH) to confirm the clinical diagnosis of alcoholic hepatitis was not mandated by the trial protocol as a pre-requisite for enrolment. Where participating centres did perform liver biopsy, either as part of routine clinical care or in the context of diagnostic uncertainty, samples were obtained for central, independent histological review and analysis.

Table 1.5 STOPAH study trial visit and data collection structure

Visit	Timing	Samples[$]	Demography	Medical history	Clinical exam	Laboratory data	Alcohol usage
Screening	At admission or referral to study team		X	X	X	X	X
Baseline	Start of treatment	X			X	X	
Treatment[*]	Weekly during hospital admission				X	X	
Discharge	At or immediately prior to discharge				X	X	
90-day[†]	90 days after the start of treatment				X	X	X
1 year[†]	1 year after the start of treatment			X	X	X	X

*On treatment visits were conducted weekly whilst patients were admitted to hospital. If the patient was discharged prior to the completion of the study medication a telephone follow-up visit was conducted at 28 days.

†The trial was terminated at the point in time at which the final patient enrolled in the study had completed 28 days of follow-up.

[$]Whole blood (anti-coagulated with ethylenediaminetetraacetic acid, EDTA) and serum samples were collected from patients at baseline

1.7.4 Trial results

In total 1,103 patients were enrolled in the trial and underwent randomisation. Eleven (1%) patients withdrew from the study and refused permission for use of any of their data, leaving 1,092 patients for inclusion in subsequent analyses. Due to early trial termination 39 and 222 patients did not reach their 90-day and 1-year follow-up visits respectively.

The trial failed to show any statistically significant effect of either pentoxifylline (odds ratio [OR] 1.07, 95% confidence interval [CI] 0.77 – 1.49, p=0.69) or prednisolone (OR 0.72, 95% CI 0.52 – 1.01, p=0.06) on mortality at 28 days[(208)]. No interaction was detected between the trial medications and consequently no benefit, or harm, from a combination of the two agents. However, multivariable analysis adjusting for baseline factors associated with short-term outcome revealed an independent,

statistically significantly reduction in 28-day mortality risk associated with prednisolone (OR 0.61, 95% CI 0.41 – 0.91, p=0.02) but not pentoxifylline. Analysis of the 90- and 365-day mortality secondary endpoints did not demonstrate any evidence of a benefit from prednisolone at either of these time points.

Secondary analyses confirmed previous reports that infection at baseline, if controlled, was not associated with an adverse outcome with prednisolone treatment[206]. In contrast, the trial did report prednisolone significantly increased the risk of developing an infection reported as a serious adverse event (13% *vs*. 7%, p=0.002)[208].

1.8 Aims of thesis

The predominant aim of this thesis was to perform a hypothesis-generating genome-wide association study in order to identify genetic loci associated with an increased risk of developing severe alcoholic hepatitis. Beyond this, work sought to evaluate how these genetic loci might contribute to the development and outcomes from severe alcoholic hepatitis.

The specific aims of this thesis were thus:

1. Perform a genome-wide association study of severe alcoholic hepatitis;
2. Examine how identified genetic loci may contribute to the clinical phenotype and outcomes from severe alcoholic hepatitis;
3. Evaluate how a novel locus *Solute carrier family 38 member 4* (*SLC38A4*), identified through the genome-wide association study, is affected in severe alcoholic hepatitis and could thus potentially contribute to its pathogenesis;

CHAPTER 2

GENOME-WIDE ASSOCIATION STUDY OF SEVERE ALCOHOLIC HEPATITIS

2 A two-stage genome-wide association study of severe alcoholic hepatitis

2.1 Overview

This chapter describes the conduct of a genome-wide association study of severe alcoholic hepatitis. The study was conceived and conducted as a two-stage process comprising exploratory and validation cohorts and identifies *Patatin-like phospholipase domain-containing 3* (*PNPLA3*) and *Solute carrier family 38 member 4* (*SLC38A4*) as risk loci for the development of severe alcoholic hepatitis.

2.2 Aim

To conduct a genome-wide association study of severe alcoholic hepatitis.

2.3 Background

2.3.1 Conduct and primary analysis of genome-wide association studies

In genome-wide association studies (GWAS) several hundreds of thousands of genetic variants are simultaneously assayed and their allelic and genotypic frequencies are tested against quantitative or qualitative phenotypes; they are undertaken in the absence of *a priori* hypotheses. In order to avoid reporting spurious associations, studies require careful design and conduct.

Genome-wide genotyping is typically performed using high density beadchips which permit simultaneous determination of genotypes for up to several million variants or single nucleotide polymorphisms (SNPs). Sample DNA undergoes whole genome amplification followed by enzymatic fragmentation. The resultant DNA is applied to the beadchips which are made up of hundreds of thousands of silica beads uniformly arranged on a silicon wafer (Figure 2.1). Each bead is related to a different variant and is coated with copies of a 50-base long oligonucleotide designed to capture a specific DNA sequence (Figure 2.1). Each probe selectively hybridises the locus of interest stopping one base short of the variant in question. Enzymatic single base extension leads to incorporation of a

fluorescently labelled base with subsequent colour detection permitting determination of genotype (Figure 2.1).

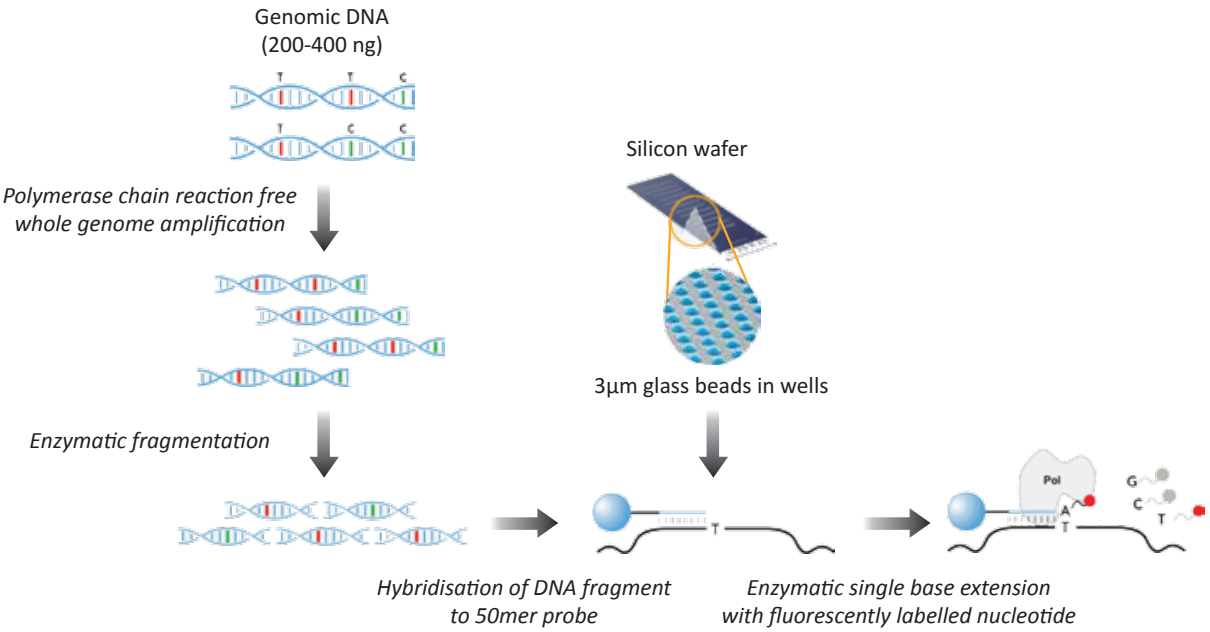


Figure 2.1 Genome-wide genotyping using BeadChips

Sample DNA undergoes amplification and fragmentation. Genome-wide genotyping beadchips consist of silica beads each coated with a different oligonucleotide which captures a specific fragment of DNA. Sample DNA fragments hybridize to the bead-bound oligonucleotides before undergoing single base extension with fluorescent nucleotides. Subsequent detection of fluorescence permits determination of genotype which may be "called" using specifically designed algorithms. Adapted from http://www.illumina.com/.

The different nucleotides used in the final single base extension step are labelled with different fluorophores. The vast majority of markers assayed are biallelic with the two possible alleles arbitrarily referred to as allele A and allele B. Differential hybridisation of bases, based upon the genotype of the DNA being assayed, leads to differences in the fluorescent intensity which may be plotted graphically. Simultaneously plotting the different fluorescent intensities of multiple samples on the same axes leads to clustering of points based upon the genotype – AA, AB and BB. Manual determination of genotypes for all the markers on a genotyping chip would be prohibitively time-consuming. Consequently, statistical models have been used as the basis for algorithms designed to automate the process of "genotype calling".

Illumina's proprietary software (Beadstudio) incorporates their proprietary calling algorithm, GenCall. This provides multi-sample, single SNP calling and delivers robust genotype calling for common variants[307]. Rare variants on a genotyping chip present challenges to calling algorithms due to the small size or even absence of clusters corresponding to individuals heterozygous or homozygous for the less frequent (minor) allele. The zCall algorithm has been developed in order to overcome this and is implemented as a post-processing step after initial genotype calling using GenCall. It provides improved detection of rare alleles[308].

Data are analysed by fitting statistical models for each variant in turn to determine whether allelic frequencies or genotypes associate with the phenotype of interest. Associations between genetic variants and disease may also be examined under different models of inheritance. The multiplicity of statistical tests performed in the association testing of genome-wide genotypic data requires stringent α thresholds in order to avoid reporting large numbers of false positive associations. The appropriate threshold to use has been a subject of debate; several have been proposed. A threshold of $p<5x10^{-8}$ was originally proposed in 1996[309] and supported by subsequent permutation modelling[310]. It has become standard in the field although thresholds used to define 'suggestive' associations are more variable and arbitrary[311, 312].

In light of the requirement to control for multiple testing, it is important to ensure that genome-wide studies are designed with adequate statistical power to detect associations. Several factors need to be taken into account, including: the frequency of the risk allele, the effect size on disease risk, the model of inheritance applied, the genotyping platform used and the accepted error rate[313, 314]. Smaller sample sizes are required to detect strong effect sizes exerted by more common risk variants[314].

The clarity of phenotyping is also an important consideration when undertaking genome-wide association studies. Misclassification of controls as cases, and vice versa, will lead to a reduction in power. Conversely clarity of phenotyping and selection of those most likely to have a high genetic

loading provides a means to increase power for a given sample size. This has been proposed as a means of detecting rare variants with large effect sizes in smaller populations[315, 316] but may also increase statistical power for more common variants[317]. Consequently, a comparison of cases with early-onset or severe disease with controls displaying 'super normal' or disease-resistant phenotypes would generally have greater statistical power than one of the same size using populations with less extreme phenotypes[312].

In addition to study design the quality control and analysis of data are important in ensuring that spurious associations are not reported. In particular, poor quality genotyping, of variants or samples, unexpected sample relatedness and population stratification, must be sought and excluded[318].

The genetic variation included on any given genotyping chip represents only a small fraction of the variation present across the entire human genome. It is possible to statistically infer, i.e. impute, unmeasured genotypes with confidence[319]. This process leverages pre-existing data on known linkage disequilibrium patterns within the human genome and large, high-quality, densely genotyped or sequenced reference datasets[320].

The results from genetic studies of the same phenotype may be considered separately as replications of results in independent populations or combined using the statistical process of meta-analysis in order to yield greater statistical power.

Table 2.1 Potential issues influencing the successful conduct of genome-wide association studies

Issue	Solutions
Statistical power	Adequate sample size for given risk variant effect size and minor allele frequency under specified model of inheritance. Sufficient density of genotyping with adequate data to permit imputation.
Population stratification	Ethnically homogenous populations; principal components analysis and specification as covariates if associated with phenotype. Exclusion of related individuals.
Phenotypic overlap	Narrowly defined phenotypes matched for confounding features, e.g. alcohol misuse, with a predicted component of genetic risk.
Genotyping quality	Extraction of high-quality DNA samples. Application of stringent quality control procedures, both per sample and per variant, to genotyping data prior to analysis.
False positive association reporting	Application of stringent significance thresholds; replication of findings in independent validation cohorts

2.3.2 Determining the biological significance of significantly associated variants

Variants significantly associated with disease risk should be evaluated for their biological significance. Tools such as the Sorting Intolerant From Tolerant (SIFT)[321] and Polymorphism Phenotyping (PolyPhen)[322] algorithms can be used to indicate whether variants are likely to have an adverse effect on protein structure and thus function. However, they can only be used to assess the effect of variants in protein-coding regions.

Genetic variation may influence the development of disease not just through effects on protein structure and function but also due to alterations in expression. Such considerations are particularly pertinent when considering the mechanisms by which intronic variants may result in disease. The term expression quantitative trait locus (eQTL) is used when a genetic variant influences the expression of a gene[323]. eQTLs that map to the approximate location of their gene-of-origin are referred to as local eQTLs whereas those that map far from the location of their gene of origin, often on different chromosomes, are referred to as distant eQTLs. These two types of eQTLs are referred to as *cis*- and *trans*-, respectively, but these terms are best reserved for instances when the regulatory mechanism of the underlying sequence has been established.[324]. Large-scale, tissue-specific genome-wide mapping of eQTLs has been made possible by high throughput technologies[325]. In addition publicly-

accessible data repositories have made it possible for individual investigators to test for specific eQTLs of interest[326].

A number of scores have been developed in order to predict the likely effects, particularly in terms of pathogenicity, of single nucleotide variants. The Combined Annotation Dependent Depletion (CADD) score aims to summarise data from a range of prediction tools in order to derive a single score reflective of the likely pathogenicity of a given SNP[327]. The higher the CADD score the greater the likelihood of pathogenicity. RegulomeDB combines annotations from multiple sources, e.g. analyses of epigenomes and predicted transcription factor binding sites, and assigns a categorical score reflective of the likelihood that a variant will alter gene expression[328]. The lower the RegulomeDB score the more likely it is that the variant will have an effect on gene expression. Unlike tools such as SIFT[321] and PolyPhen[322], CADD and RegulomeDB scores can be assigned to intronic and non-coding variants.

2.3.3 Additional analyses of GWAS data

Epistasis was first coined as a term in 1909 by Bateson and describes an interaction between genes such that the effect of a variant at one genetic locus in the causation of disease may be altered, either by way of suppression or enhancement, by another[329, 330]. Due to the number of variants assayed in genome-wide association studies, systematic, pairwise testing of all epistatic interactions leads to issues with computational resource and lack of statistical power. Rationalisation of tests to pairs of variants with marginal genome-wide significance or on a hypothesis-driven basis obviates these issues[331]. Pairwise epistatic interactions may be specified and tested for using logistic regression models implemented in open source programmes such as PLINK[332, 333].

It has been proposed that methodologies which test for associations using all SNPs within a gene or pre-defined biological pathway may provide a means of increasing statistical power and gaining insight into the genetic architecture of a disease trait[334]. Such techniques may be employed using either

direct genotyping data or the summary statistics of a primary GWAS analysis[335-338]. Such methods are not without their limitations. These include accurate mapping of SNPs to genes, annotation of gene function, the requirement to adjust for differing gene and pathway size and computational demand[334].

2.4 Study design

The study was conceived and performed in two distinct stages. An exploratory phase in which a minority of carefully phenotyped samples underwent genome-wide genotyping, followed by a replication stage where candidate loci were examined in a second, larger, cohort. Loci were chosen for replication based upon a combination of statistical significance of association (p-value), proximity to a coding region of the genome and biological plausibility (Figure 2.2).

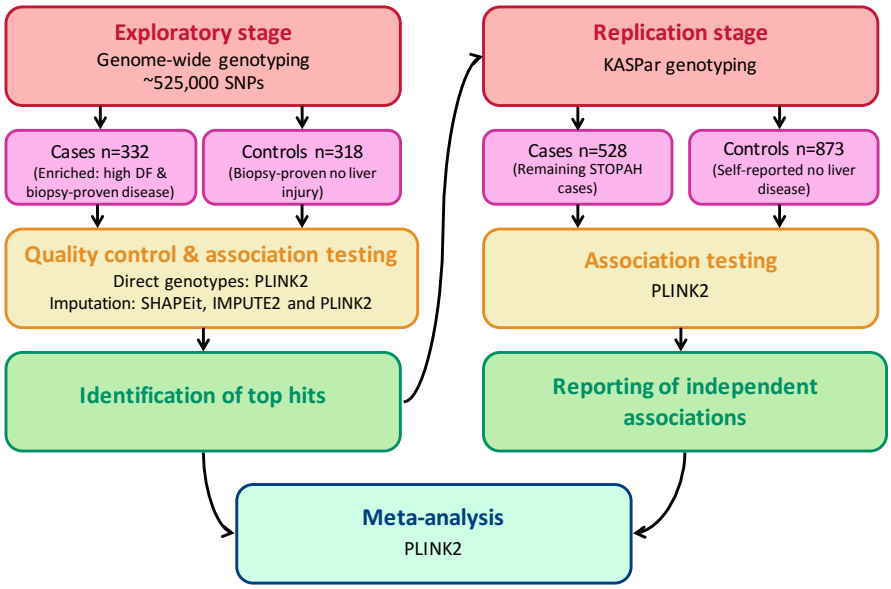


Figure 2.2 Design and conduct of the two-stage genome wide association study of severe alcoholic hepatitis
Putative causative genetic variants were identified in a primary population who underwent genome-wide genotyping. Top hits were defined based upon statistical strength of association ($p<5.10^{-5}$), proximity to a coding region and, related to this, biological plausibility. Replication was attempted in a larger secondary population. Abbreviations: DF: Discriminant function, a measure of disease severity in severe alcoholic hepatitis[210]; IMPUTE2: program for inference of genotypes from phased haplotypes using a reference population[319]; KASPar: K-Biosciences Competitive Allele Specific PCR, a platform for genotyping single nucleotide polymorphisms; PLINK2: whole genome association analysis toolset[333]; SHAPEit: Haplotype phasing program[339]; SNP: Single nucleotide polymorphism

2.5 Statistical power

The variant rs738409 in *PNPLA3* which is a risk factor for the development of alcohol-related cirrhosis was used as an exemplar to inform the design of the initial phase of the study. The study was primarily designed to detect common variants (minor allele frequency, MAF, ≥10%) conferring at least a moderately increased risk of disease development (odds ratio, OR, ≥2). Power calculations were performed using the CaTS power calculator for genetic studies[340]. Statistical power was determined based upon a case:control ratio of 1, disease prevalence of 20% and a significance level of $p<5x10^{-8}$ using an additive model of inheritance as the base case. In light of the likelihood that only a small number of markers could be genotyped in the second stage, power was calculated using a one-stage design. Using these parameters a sample size of 650 would have approximately 80% power to detect a risk variant with a minor allele frequency of 15% conferring a genotype relative risk of 2. The variation in statistical power with changes in the disease model and sample size are illustrated in Figure 2.3. In this scenario, statistical power is sensitive to changes in the genotype relative risk and minor allele frequency, with substantial increases in power with a rise in the disease allele frequency towards 20% and genotype relative risk above 2. The effect of the underlying inheritance model on statistical power is illustrated in Figure 2.4. Reasonable statistical power is seen for additive, dominant and multiplicative inheritance models. However, there is no appreciable statistical power to detect variants exerting an effect under a recessive inheritance model.

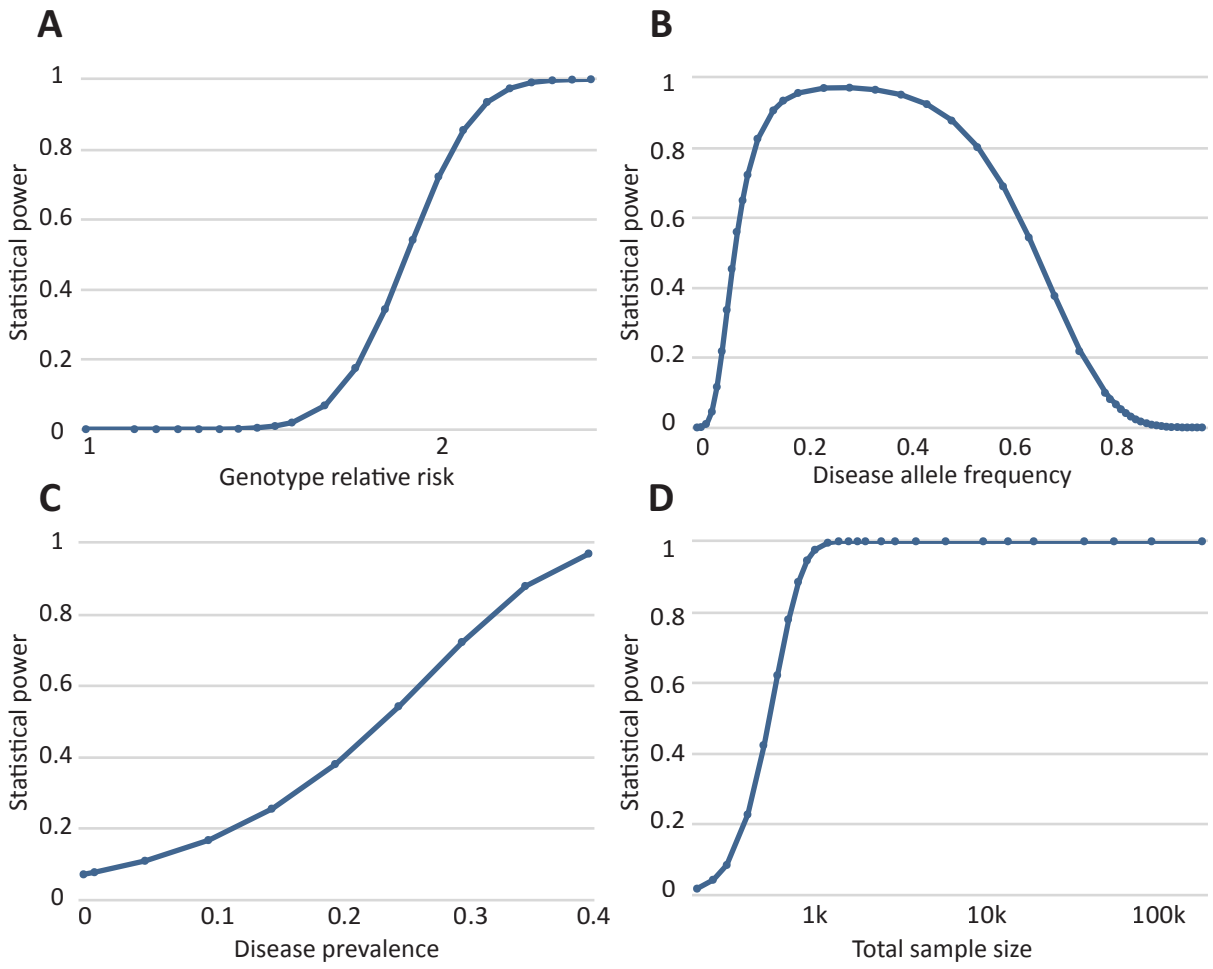


Figure 2.3 Power curves demonstrating the effect of (A) genotype relative risk, (B) disease allele frequency, (C) disease prevalence and (D) samples size on statistical power

In the base case the genotype relative risk was 2, disease prevalence 20%, minor allele frequency 10% and sample size 650. Each curve illustrates statistical power as a function of alteration of one of these parameters. The case:control ratio was fixed at 1:1 and the threshold for significance was held at p< 5x10-8. Curves were generated using the Genetic Association Study power calculator available at http://csg.sph.umich.edu/abecasis/gas_power_calculator/index.html which is based upon the CaTS algorithm[340].

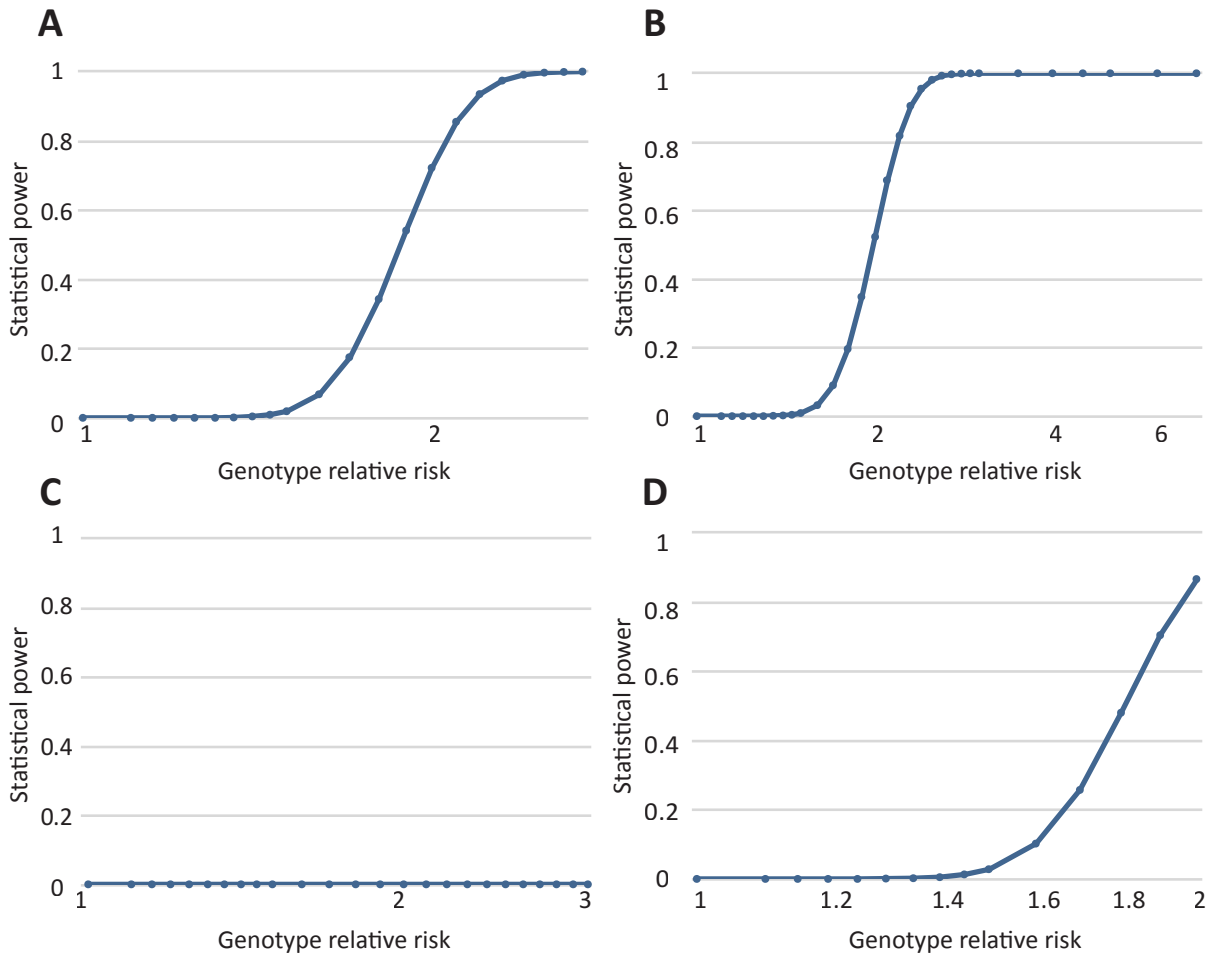


Figure 2.4 Power curves demonstrating the effect of (A) additive, (B) dominant, (C) recessive and (D) multiplicative inheritance models on statistical power for different levels of genotype relative risk

Disease prevalence, minor allele frequency and sample size were set at 20%, 10% and 650 respectively. The case:control ratio was fixed at 1:1 and the threshold for significance was held at $p< 5x10-8$. Reasonable statistical power is demonstrated for (A) additive, (B) dominant and (D) multiplicative inheritance models where the genotype relative risk is ≥2. The study design had no power to detect variants associated with disease risk under a (C) recessive model. Curves were generated using the Genetic Association Study power calculator available at http://csg.sph.umich.edu/abecasis/gas_power_calculator/index.html which is based upon the CaTS algorithm[340].

2.6 Patients, materials and methods

2.6.1 Severe alcoholic hepatitis patient cohort

Patients with alcoholic hepatitis were recruited through the steroids or pentoxifylline for alcoholic hepatitis (STOPAH) trial[208]. Inclusion was based upon a clinical diagnosis of alcoholic hepatitis, Maddrey's discriminant function (DF) ≥32, current excess alcohol consumption, recent onset of jaundice and exclusion of other causes of decompensated liver disease[265]. Although histological confirmation of the diagnosis was not required for enrolment a subset of 188 patients (17%) underwent transjugular liver biopsy. The sample was split into exploratory (n=332) and replication (n=521) cohorts. In order to maximise phenotypic differences in the exploratory stage, in accordance with the study design, patients with biopsy-proven disease and the most severe liver injury, as indicated by the DF, were preferentially selected for inclusion in the exploratory cohort. In order to reduce population admixture only patients with self-reported 'white' ethnicity were included in the initial genetic studies (n=853).

2.6.2 Alcohol dependence without significant liver injury cohort

Controls with a background of alcohol dependence but with no evidence of liver injury (n=1,090) were recruited via the University College London Consortium. The majority had been drinking hazardously for over 15 years and were actively drinking at the time of enrolment. In approximately one-third the absence of significant alcohol-related liver injury was confirmed on liver biopsy, these individuals were preferentially included in the exploratory stage of the study. The remainder had no historical, clinical or radiological features suggestive of significant liver injury either at presentation or during prolonged follow-up. All were of English, Scottish, Welsh or Irish descent with a maximum of one grandparent of white European Caucasian origin. None of the individuals was related. The sample was split into exploratory (n=318) and replication cohorts (n=772) cohorts; those without liver injury on biopsy were preferentially included in the exploratory cohort.

2.6.3 Alcohol-related cirrhosis patient cohort

A further control group of patients with alcohol-related cirrhosis but without a current or historical presentation consistent with severe alcoholic hepatitis (n=327) were also recruited *via* the Centre for Hepatology at the Royal Free Hospital, London. The diagnosis of cirrhosis was made either based on histological features on liver biopsy (n=224, 69%) or the presence of historical, clinical, biochemical, endoscopic and/or radiological features indicative of cirrhosis.

2.6.4 DNA extraction

Genomic DNA from the patients with severe alcoholic hepatitis was extracted from 400ul of EDTA anticoagulated whole blood using Qiamp DNA Blood mini kits (Qiagen, Hilden, Germany), using an in-house optimisation of the manufacturer's protocols (Supplementary Methods). The resulting DNA was quantified fluorometrically using the Qubit platform (Invitrogen, Carlsbad, USA) and spectrophotometrically using the NanoDrop platform (Thermo Scientific, Waltham, USA) to ensure sufficient quantity and quality (A260:A280 ≥1.8). Genomic DNA from individuals with alcohol dependence and no liver injury included in the exploratory and replication cohorts and the separate cohort of patients with alcohol-related cirrhosis was extracted from EDTA anticoagulated whole blood samples at University College London. The protocol used was developed from a commercial Gentra Puregene kit and adopted as a local laboratory standard operating procedure (Supplementary Methods). The resulting DNA was quantified fluorometrically using the Qubit platform (Invitrogen, Carlsbad, USA).

2.6.5 Genome-wide genotyping

The Illumina HumanCoreExome Beadchip v12-1 (Illumina, San Diego, USA) was used in the exploratory phase of the study. The chip is designed to include variants that: i) are predominantly located within coding regions of the genome; ii) have previously been associated with disease phenotypes in other genome-wide association studies; and, iii) are likely to have a functional implication (i.e. missense variants). Though, as a result, the chip has a comparatively greater proportion of uncommon and rare

variants it retains a "backbone" of common variants widely and evenly spread across the genome. This facilitates imputation of common variants throughout the genome. Genome-wide genotyping was performed at the Wellcome Trust Sanger Institute (Cambridge, United Kingdom).

2.6.6 Genotype calling

Genotypes were initially called using the GenCall algorithm[307] as implemented in Illumina's proprietary software (Beadstudio). The zCall algorithm was implemented as a post-processing step after initial genotype calling using GenCall[308]. Genome-wide data were called using GenCall and post-processed using zCall by the Wellcome Trust Sanger Institute and supplied as two separate datasets for analysis. In accordance with the original intended usage of the zCall algorithm, zCall derived genotypes were used to update missing GenCall genotypes for rare variants and were integrated during quality control procedures[308].

2.6.7 Genotyping quality control procedures

Stringent quality control procedures were applied to genotyping. Quality control criteria and thresholds were based upon the established literature in the field[318, 341]. Variant data was controlled for call rate, Hardy-Weinberg equilibrium and conflicting genotypes for the same sample for markers assayed in duplicate. Sample data was controlled for call rate, heterozygosity and concordant genotypic and phenotypic sex, the latter potentially revealing sample handling errors. Criteria were iteratively applied with increasingly stringent thresholds – the parameters applied and final thresholds used are detailed in Table 2.2. Quality control procedures were implemented in PLINK v1.9 (open source)[333].

Table 2.2 Overview of quality control criteria and associated thresholds applied to genotyping data

Marker quality control	
Call rate	≥98%
Hardy-Weinberg equilibrium	$p<1x10^{-6}$
Conflicting genotypes in duplicate markers	Set to missing
Case-control differential missingness	$p<1x10^{-4}$
Sample quality control	
Call rate	≥98%
Heterozygosity	<3 standard deviations from the mean
Sex check	Genotypic and phenotypic sex concordant

2.6.8 Population quality control procedures

Additional quality control procedures were applied in order to obviate issues of population admixture. A dataset for population quality control procedures was generated in PLINK v1.9 by removing extended regions of high linkage disequilibrium (e.g. HLA loci) and retaining only common variants (MAF >5%) not in linkage disequilibrium (r^2<0.2).

Identity-by-descent (IBD) analysis was performed in PLINK v1.9. Related samples equivalent to second cousins, corresponding to $\hat{\pi}$≥0.185, were removed. The HapMap3 dataset contains genome-wide genotyping data from 1,184 reference individuals from 11 well-defined global populations[342]. Genetic homogeneity of the study population was assessed by merging it with the HapMap3 dataset and conducting multi-dimensional scaling (MDS) analysis. Briefly, HapMap3 genotypes were downloaded (ftp://ftp.ncbi.nlm.nih.gov/hapmap/) and genomic coordinates were updated from hg18/build36 to hg19/build37. Genotype harmonizer[343] was used to merge this and the study dataset. Multidimensional scaling was performed in PLINK v1.9 using the --cluster option. The resulting plots were inspected and outliers, with respect to the Western European (CEU) and study cohort clusters, identified and removed.

Principal components analysis was performed in PLINK v1.9 using the --pca option and a linkage disequilibrium pruned dataset of common variation. The top 10 principal components were examined

in subsequent analyses. Linear regression modelling was used to test for independent associations between population principal components and case-control status using linear regression modelling in R[344]. Outliers, defined as those lying greater than 3.5 standard deviations from the mean of a principal component, were removed and the principal components recalculated. Outlier detection was repeatedly performed in an iterative manner along the top three principal components.

2.6.9 Primary association analyses

Primary association analyses were tested using logistic regression under an additive model of inheritance and were performed in PLINK v1.9[333]; models were specified with those principal components associated with case-control status as a covariate. In light of the very limited power to detect associations for rare variants those with a minor allele frequency <1% were excluded. Gender was not specified as a covariate in the initial discovery analysis due to a potential reduction in statistical power[345].

2.6.10 Gene-based association tests

Gene-based association tests were performed on GWAS summary data using the MAGMA algorithm[338] implemented in the Functional mapping and annotation of associations (FUMA) package, a web-based platform created to facilitate understanding of the biological significance of genetic associations by performing additional analyses and drawing bioinformatic data from multiple sources[346]. The algorithm was run using a list of lead SNPs identified using the suggestive significance threshold of $p<5x10^{-5}$ in the primary analysis. The 1000 genomes phase 3 European population was specified as the reference panel. All other settings were left as default including exclusion of SNPs with a minor allele frequency <1% or association P-value >0.05[346].

2.6.11 Imputation of genotypes

Imputation was performed against the 1000 genomes project reference panel of haplotypes. Study haplotypes were pre-phased using ShapeIt v2.r790 prior to imputation using IMPUTE v2.3.2[319, 339].

Genotypes were imputed from haplotypes in 5 megabase chunks using a 250 kilobase flanking region. Imputed genotypes were controlled for quality of imputation (info score ≥0.8), missingness (<5%), minor allele count (≥1) and Hardy-Weinberg equilibrium using QCTOOL v1.3 (http://www.well.ox.ac.uk/~gav/qctool). Genotype probabilities >0.9 were hard-called and tested using the same models specified for directly genotyped data in PLINK v1.9[333]. Analyses were conducted in an embarrassingly parallel manner on the Imperial College high performance computing cluster, cx1.

2.6.12 Triage of variants for replication genotyping

Single nucleotide polymorphisms were selected for genotyping in the replication population based upon a combination of the statistical probability of association and additional bioinformatic data derived from a number of sources. Thresholds defining suggestive significance were chosen to balance the number of variants for follow-up against the risk of signal loss. For directly genotyped data a P-value threshold of $5x10^{-5}$ was used; in the imputed dataset, a marginally more stringent threshold of $1x10^{-5}$ was applied due to the substantially greater number of SNPs and the uncertainty associated with genotype probabilities. Additionally, the following were considered in replication selection and prioritisation:

1. Variants in or near to (<5kb) genes;
2. Variants near genes with a known or predicted effect potentially related to the generation of alcohol-related liver disease;
3. Variants with a predicted deleterious effect on gene function (e.g. missense variants) or expression;

The data used to inform these considerations were drawn from several sources:

1. The University of California Santa Cruz (UCSC) human genome browser[347] was used to inspect genomic loci and determine the closest recognised gene in the RefSeq database[348]. Variants within or close to coding regions of the genome were prioritised;
2. The allele frequency was drawn from the HapMap Western European (CEU) population (http://www.sanger.ac.uk/resources/downloads/human/hapmap3.html);
3. Entries were sought in the GWAS Catalog[349] and the Genetic Association Studies of Complex Diseases and Disorders database[350];
4. Coding variants were also assessed in SIFT[321, 351] and Polyphen-2[322] to determine their predicted effect on protein structure and function;
5. An expression quantitative trait locus (eQTL) association described in the genotype-tissue expression project database (GTEx)[352];

2.6.13 Replication genotyping

Custom primers were designed for each selected SNP to be interrogated using PrimerPicker Lite v0.27 (KBiosciences, Hoddeson, UK). Replication genotyping was performed using the K-Biosciences Competitive Allele Specific PCR (KASPar) platform (LGC Genomics, Hoddesdon, UK) with amplification and detection undertaken using a LightCycler® 480 real-time PCR system (Roche Molecular Diagnostics, Burgess Hill, UK). Genotype calling was performed automatically using proprietary software with minor manual editing of genotype calls. All assays were optimised with positive genotype controls. Samples genotyped on the Illumina beadchip platform were genotyped again using the KASPar assay to ensure concordance between the two techniques.

Replication genotypes were compiled into a PLINK binary fileset and analysed using the same statistical methodology as the primary dataset.

2.6.14 Candidate marker genotyping

In a recent study the variants rs58542926 in *TM6SF2* and rs626283 in *MBOAT7* were reported to be associated with the risk of developing alcohol-related cirrhosis at genome-wide significance level[151]. Neither of these variants was included on the Illumina HumanCoreExome beadchip. Therefore, they were prospectively evaluated in both the exploratory and replication cohorts on a hypothesis-driven basis. Genotyping was performed using the KASPar platform (LGC Genomics, Hoddesdon, UK) using the same methodology as the single marker replication experiments but with variant-specific primer sets.

2.6.15 Meta-analysis

Variants genotyped in both the exploratory and replication cohorts were meta-analysed using both a fixed and random effects model in PLINK 1.9[333]. Meta-analysis of GWAS studies has typically been performed using fixed effects models[353]. Where heterogeneity exists (I^2>25%) random effects models may be more appropriate, though potentially overly conservative[354]. For each variant both models are presented, the random effects model is preferred where I^2 exceeds 25%.

2.6.16 Post-hoc adjusted analyses

For variants independently identified as significantly associated with disease in the exploratory and replication cohorts a post-hoc analysis was conducted adjusted for age, gender, body mass index and type 2 diabetes due to prior identification in the literature as potential co-factors for the development of alcohol-related liver disease. Additional post-hoc analyses were performed in PLINK1.9[333] conditioning on lead SNPs at loci demonstrating replicated associations and testing for epistatic interactions between replicated SNPs.

2.6.17 Alcohol-related cirrhosis population comparison

Variants selected for replication genotyping were further examined in a population of patients with alcohol-related cirrhosis but no historical episodes of severe alcoholic hepatitis. This population was

compared with the exploratory severe alcoholic hepatitis population. Associations with severe alcoholic hepatitis were performed in PLINK 1.9 using logistic regression[333].

2.6.18 Expression quantitative trait locus (eQTL) testing

The Genotype-Tissue Expression (GTEx) project is an open-source resource comprising tissue-specific matched RNA expression and genotyping data from many individuals[326]. Fifty-three tissue types are represented including healthy liver samples (n=153). A 'Test Your Own' function, accessible *via* the project website (https://www.gtexportal.org/home/testyourown), allows researchers to test for eQTLs between specific SNPs and genes within a single, specified tissue. This function was used to test for eQTLs for SNPs which were associated with an increased risk of severe alcoholic hepatitis in both the exploratory and replication cohorts. eQTLs were tested for each SNP for its nearest gene and other genes containing replicated SNPs. eQTLs were only tested for liver tissue.

2.7 Results

2.7.1 Population characteristics

The population studied was predominantly male, all cohorts had a mean age in the 5th decade of life (Table 2.3). These features are consistent with the epidemiology of both alcohol dependence and alcohol associated liver disease. The selection criteria applied to split the alcoholic hepatitis patients into exploratory and replication cohorts led to significant differences in disease severity (mean DF 67 *vs.* 57, p<0.0001) and the proportion of patients with biopsy-proven disease (31% *vs.* 14%, p<0.0001) between the two groups. A non-statistically significant greater proportion of patients with diabetes was seen in the alcoholic hepatitis exploratory cohort compared to the no liver injury exploratory cohort (8.1% *vs.* 4.3%, p=0.08).

Table 2.3 Population characteristics of genome-wide association study populations

Variable	Exploratory		Replication	
	Cases (n=332)	Controls (n=318)	Cases (n=528)	Controls (n=772)
Gender (male, n [%])	223 (67%)	241 (76%)	321 (61%)	504 (65%)
Age (years)	47.6 ± 9.3	48.4 ± 10.5	49.7 ± 10.7	45.7 ± 10.5
Albumin (g/l)	25 ± 6.3	N/A	25 ± 5.8	N/A
Bilirubin (µmol/l)	324 ± 161§	N/A	270 ± 148§	N/A
INR	1.9 ± 0.5	N/A	1.8 ± 0.4	N/A
DF	67 ± 28*	N/A	57 ± 25*	N/A
MELD	22 ± 6.4	N/A	20 ± 5.9	N/A
Biopsy-proven (n, %)	103 (31%) *	100%	73 (14%) *	N/A
Diabetes	27/313 (8.6%) ¶	9/210 (4.3%) ¶	35/493 (7.1%)	N/A

¶ p>0.05, § p<0.001, * p<0.0001

Abbreviations: DF: discriminant function; INR: international normalised ratio; MELD: model for end-stage liver disease.

2.7.2 Quality control of genome-wide dataset

The raw dataset comprised of 542,585 variants and 650 individuals (332 cases and 318 controls). The quality of genotyping was good with an overall genotyping rate of 99.6%. In total 11,976 variants and five samples had a genotyping rate <98% whilst five samples demonstrated excess heterozygosity (Figure 2.5). All were excluded from downstream analyses in accordance with quality control criteria. There were no discordances between phenotypically reported and genetically inferred sex. Identity-by-descent analysis detected a potential duplicate or twin pair of samples. Inspection of recorded phenotypic data indicated this was a single participant recruited twice to the study – the sample with the lower base call rate was excluded. A further more distantly related sample pair was identified of which one sample was excluded at random (Figure 2.6).

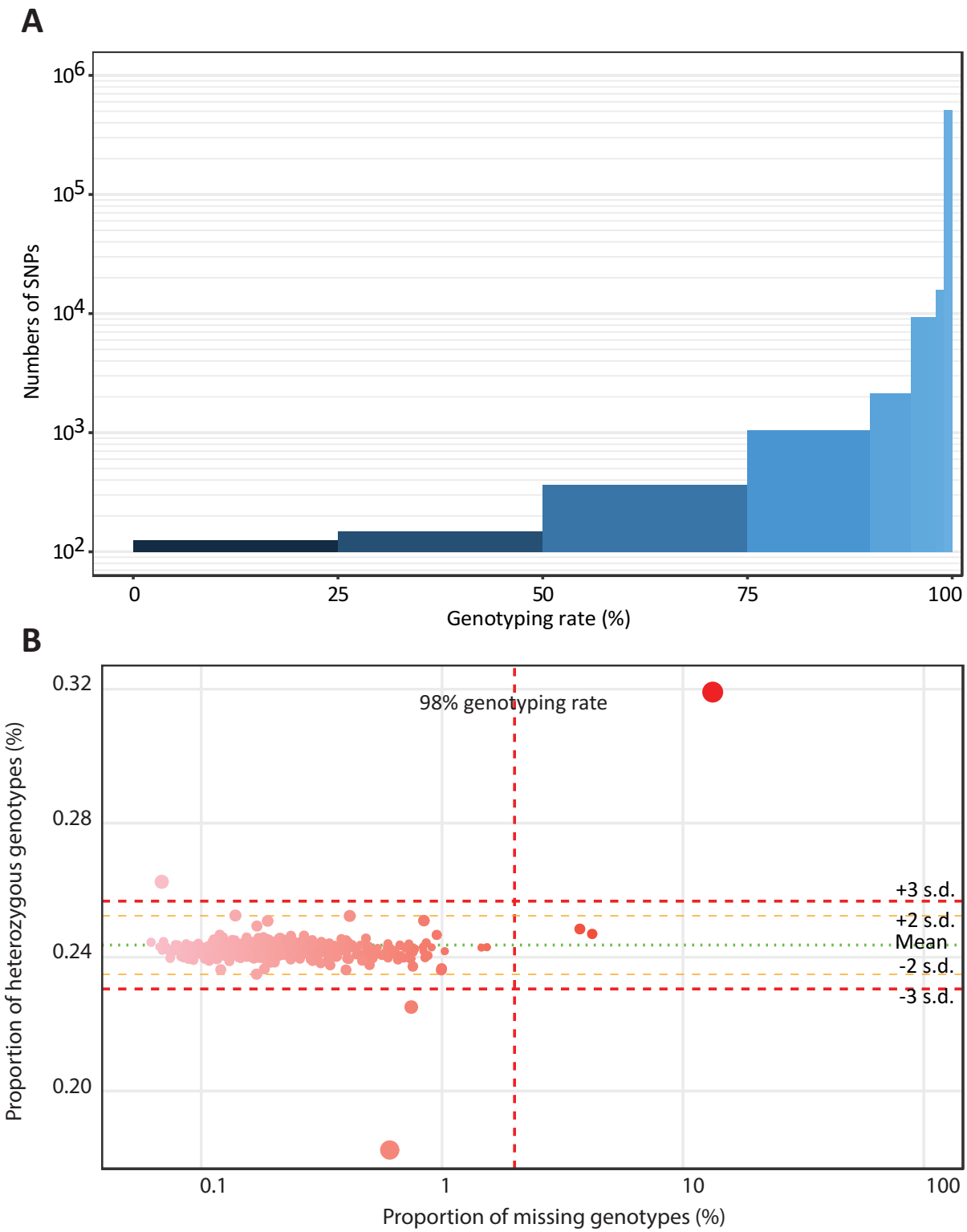


Figure 2.5 Graphical representation of (A) Per marker and (B) per sample genotyping quality

The vast majority of markers had a genotype call rate in excess of 98%, while the genotyping rates for individual samples was also generally in excess of 98% (vertical dotted line)[318, 341]. A small number of samples demonstrated extreme heterozygosity and were excluded (more than three standard deviations from the mean, horizontal red dotted lines). Two samples had profoundly low genotype call rates <85% and extreme heterozygosity, likely to secondary to genotyping failure.

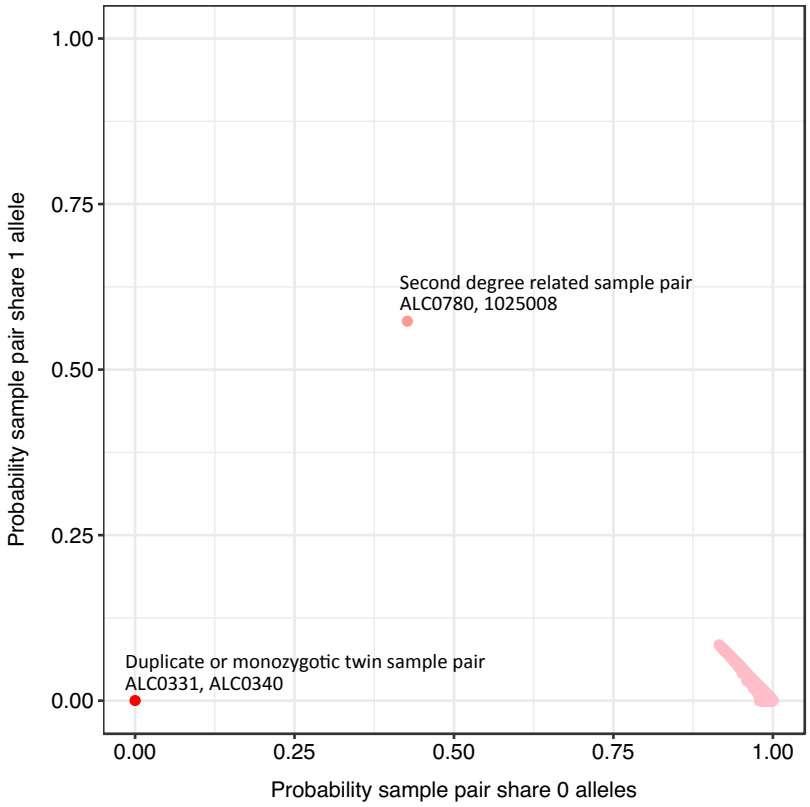


Figure 2.6 Identity-by-descent analysis of quality-controlled dataset

The majority of samples demonstrate very little relatedness and cluster in the bottom right corner of the plot. An identical sample pair is shown (bottom left) while a pair of samples with relatedness equivalent to second degree relatives lies in the centre of the plot.

The HapMap3 multidimensional scaling (MDS) plot (Figure 2.7) demonstrated both study populations clustered together with Utah residents with European ancestry and a population of individuals of Italian descent. A small number (n=4) lay outside the main clusters of the study population and, despite this being their closest cluster, were excluded.

The first population principal component was significantly associated with case-control status (Table 2.4, Figure 2.8). Two outliers were identified and removed from the dataset. After outlier exclusion and recalculation of principal components the first principal component remained associated with case-control status (Table 2.4, Figure 2.9); no further outliers were identified.

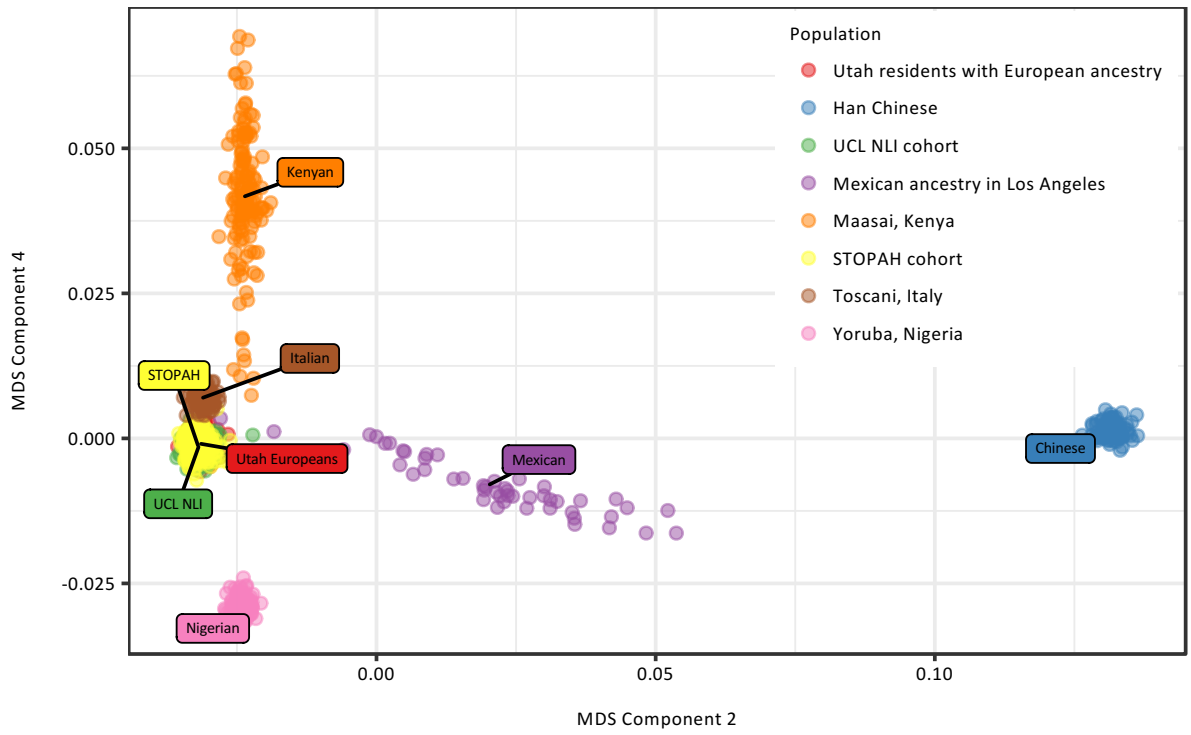


Figure 2.7 A multidimensional scaling plot of the merged study and HapMap datasets

The study population clusters tightly in a single area of the plot indicating similar and homogenous genetic background; the closest HapMap population is Utah residents with European ancestry[342].

Table 2.4 Associations between population genetic principal components and case-control status

Component	Before outlier removal		After outlier removal	
	P	R^2	P	R^2
1	**1.39e-11**	**0.069**	**3.95e-11**	**0.0666**
2	0.305	0.0617	0.479	0.0578
3	0.191	-0.927	0.97	-0.933
4	0.827	-0.927	0.742	-0.933
5	0.275	-0.925	0.781	-0.932
6	0.668	-0.925	0.0811	-0.928
7	0.223	-0.923	0.847	-0.928
8	0.629	-0.922	0.701	-0.928
9	0.608	-0.922	0.88	-0.928
10	0.205	-0.92	0.51	-0.927

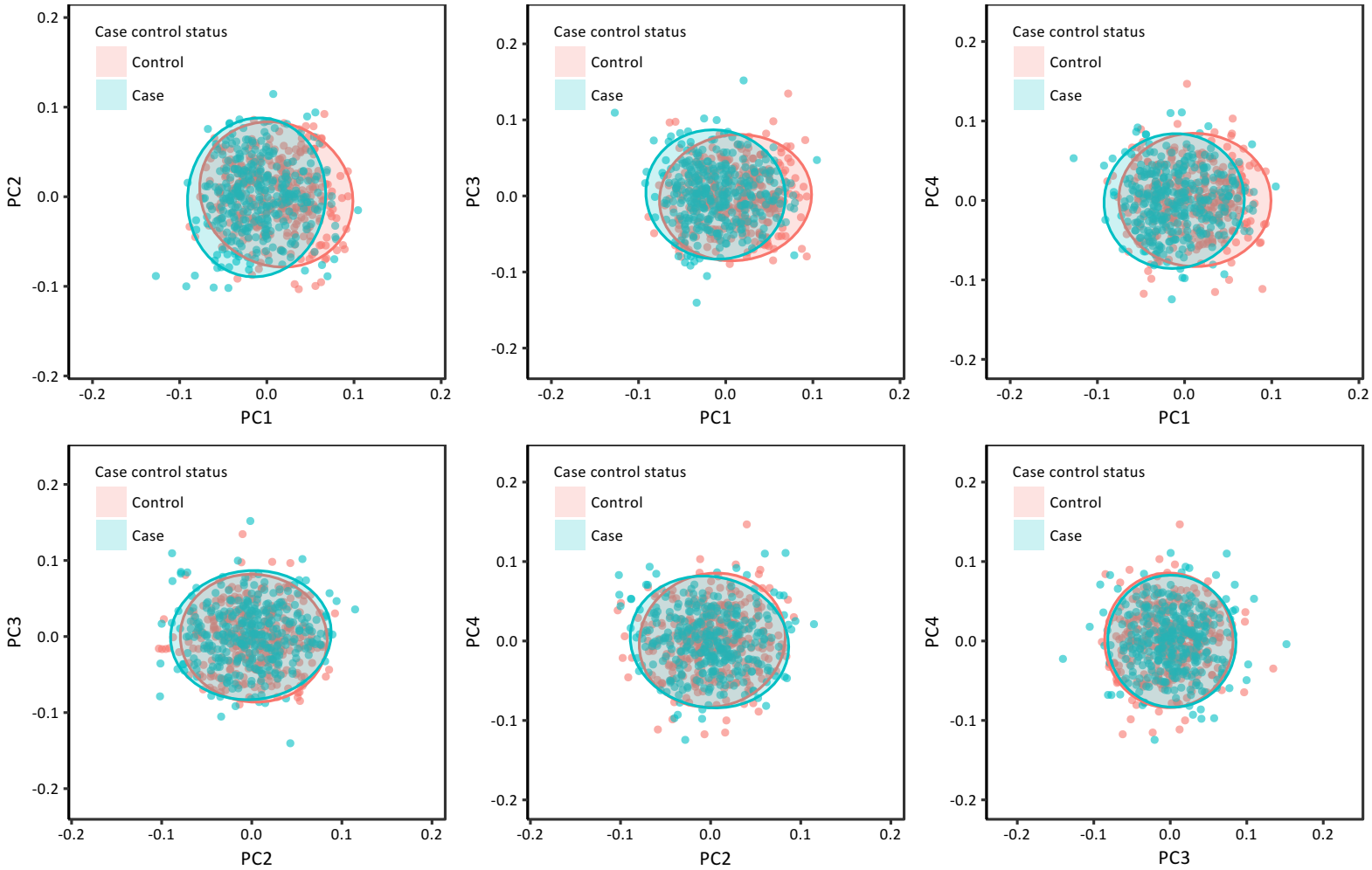


Figure 2.8 Scatterplots of the top four principal components, prior to outlier exclusion

Principal components are plotted against each other in turn. Point colour denotes case-control status, ellipses represent population-specific 95% confidence intervals. The ellipses separate along the first principal component, corresponding to its association with disease status. No significant shifts were observed along any of the other axes.

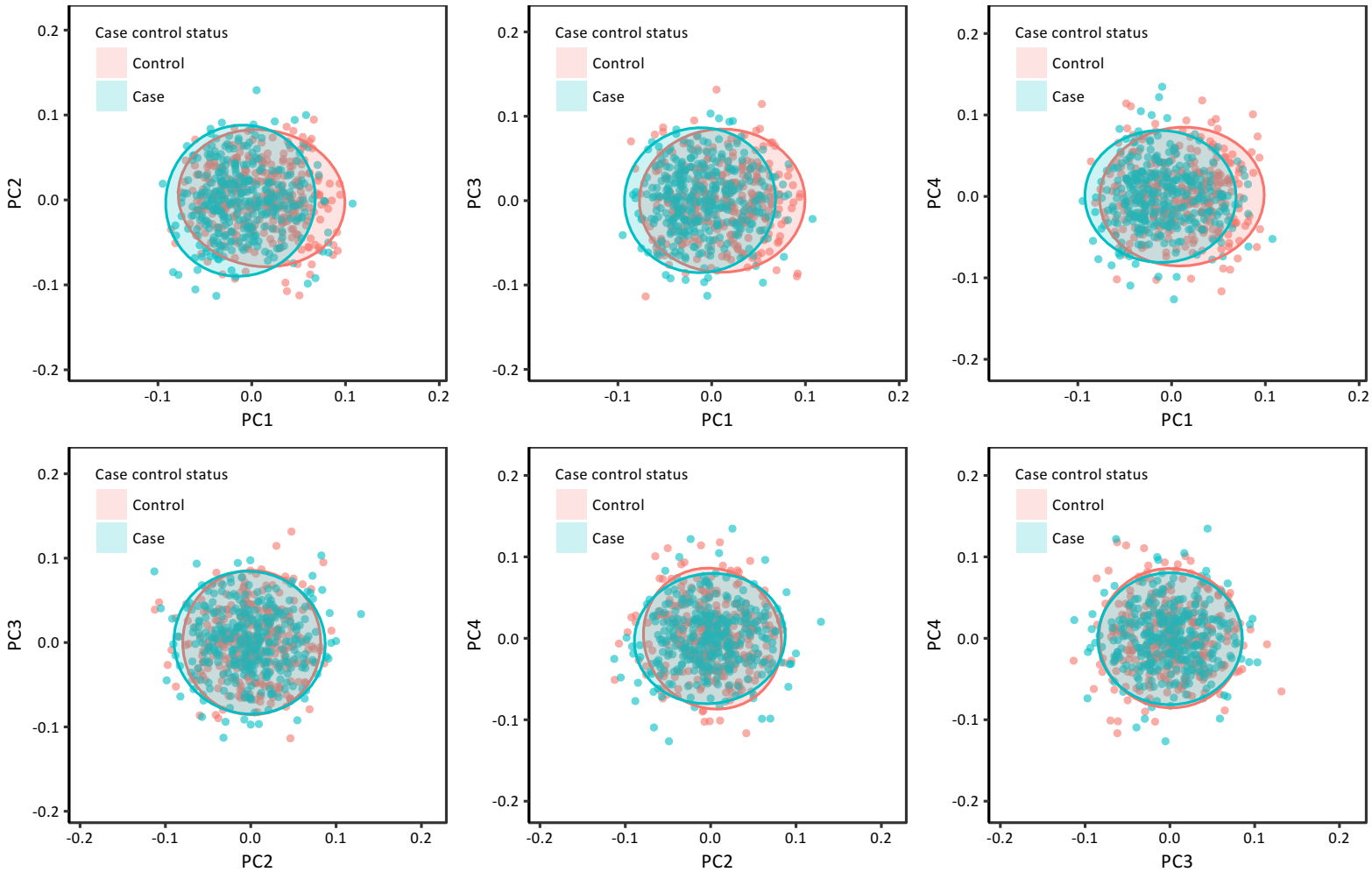


Figure 2.9 Scatterplots of the top four principal components, after outlier exclusion

Ellipses represent population-specific 95% confidence intervals. Despite outlier removal and a tighter clustering of samples, a shift remains in the locations of the ellipses along the first principal component, corresponding to its persistent association with disease status.

2.7.3 Primary association analysis

The final quality-controlled dataset comprised 524,215 variants and 636 individuals (323 cases and 313 controls). The number of variants with a minor allele frequency ≥1% was 268,209. Application of a strict Bonferroni correction using α=0.05 and n=268,209 gave a study-specific threshold of significance p=1.9x10^{-7}.

Primary association analysis revealed the variant most significantly associated with disease was rs738409 in *PNPLA3* (odds ratio [OR] 2.09, 95% confidence interval [95% CI] 1.58 – 2.77, p=2.7x10^{-7}). This, however, did not reach either the genome-wide or study significance threshold. A further 10 variants demonstrated an association with disease below the suggestive threshold of significance (p<5x10^{-5}) (Table 2.5, Figure 2.10). The quantile-quantile (QQ) plot of log-transformed P-values did not show systematic deviation from the identify line and the genomic inflation factor (λ) was 1.02 counting against population stratification (Figure 2.12).

Table 2.5 Directly genotyped variants meeting suggestive significance threshold for association

SNP name	Chromosome	BP	Risk allele	MAF Cases	MAF Controls	Odds ratio	95% CI	P
rs738409	22	44324727	G	31%	18%	2.09	1.58 – 2.77	2.7x10^{-7}
rs6028984	20	38883710	T	25%	36%	0.57	0.44 – 0.73	1.3x10^{-5}
rs11573	19	51359497	C	37%	48%	0.60	0.48 – 0.76	2.4x10^{-5}
rs6444127	3	186143744	C	61%	51%	1.67	1.32 – 2.13	2.6x10^{-5}
rs2246129	13	44826634	T	46%	57%	0.60	0.48 – 0.76	2.7x10^{-5}
rs9472138	6	43811762	T	35%	25%	1.72	1.34 – 2.22	2.8x10^{-5}
rs3959632	22	36668884	C	25%	16%	1.89	1.40 – 2.54	3.0x10^{-5}
rs985975	16	60812501	C	9%	16%	0.46	0.32 – 0.66	3.1x10^{-5}
rs4241122	2	113678856	G	25%	36%	0.59	0.46 – 0.76	4.1x10^{-5}
rs11183620	12	47212370	G	45%	57%	0.60	0.47 – 0.77	4.2x10^{-5}
rs505347	6	103600715	G	52%	63%	0.60	0.47 – 0.77	4.6x10^{-5}

Study-specific suggestive threshold of significance was p<5x10^{-5}

Abbreviations: BP: Base position; CI: confidence interval; MAF: Minor allele frequency; SNP: Single nucleotide polymorphism

2.7.4 Gene-based association analyses

Input SNPs were mapped to 17,969 protein-coding genes. Application of a strict Bonferroni correction yielded a genome-wide significance threshold of $p<2.83 \times 10^{-5}$. Gene-based association tests revealed two genes associated with an increased risk of developing severe alcoholic hepatitis – *PNPLA3* and *Kallikrein-3* (*KLK3*) (Table 2.6, Figure 2.11). The QQ plot did not demonstrate systematic deviation from the identify line and the genomic inflation factor (λ) was 0.81 (Figure 2.12).

Table 2.6 Top ten genes most significantly associated with the risk of developing severe alcoholic hepatitis

Gene	Chromosome	Start	Number of SNPs	P
PNPLA3	**22**	**44309619**	**14**	**2.83×10^{-7}**
KLK3	**19**	**51348171**	**10**	**2.47×10^{-6}**
KLK2	19	51354824	14	1.48×10^{-5}
SAMM50	22	44341301	12	2.88×10^{-5}
OR56A1	11	6037901	3	8.73×10^{-5}
SLC38A4	12	47148546	11	0.00016
TMPRSS2	21	42826478	23	0.00020
PARVB	22	44385091	32	0.00033
PLSCR4	3	145900126	10	0.00036

Gene set analyses were conducted using MAGMA[338]; genes highlighted in bold met the threshold for genome-wide significance ($p<2.83 \times 10^{-5}$)

Abbreviations: SNP: Single nucleotide polymorphism

2.7.5 Analysis of imputed variants

After quality control procedures and removal of polymorphisms invariant within the dataset a total of 8,753,529 variants were successfully imputed and underwent association testing. No loci were associated with disease state at genome-wide significance ($p<3 \times 10^{-8}$). A threshold for suggestive significance of $p<5 \times 10^{-5}$ was applied to the imputed dataset. The only locus containing variants meeting this threshold was *PNPLA3*. No additional loci were revealed. The variant most significantly associated with the risk of developing severe alcoholic hepatitis remained rs738409 in *PNPLA3*.

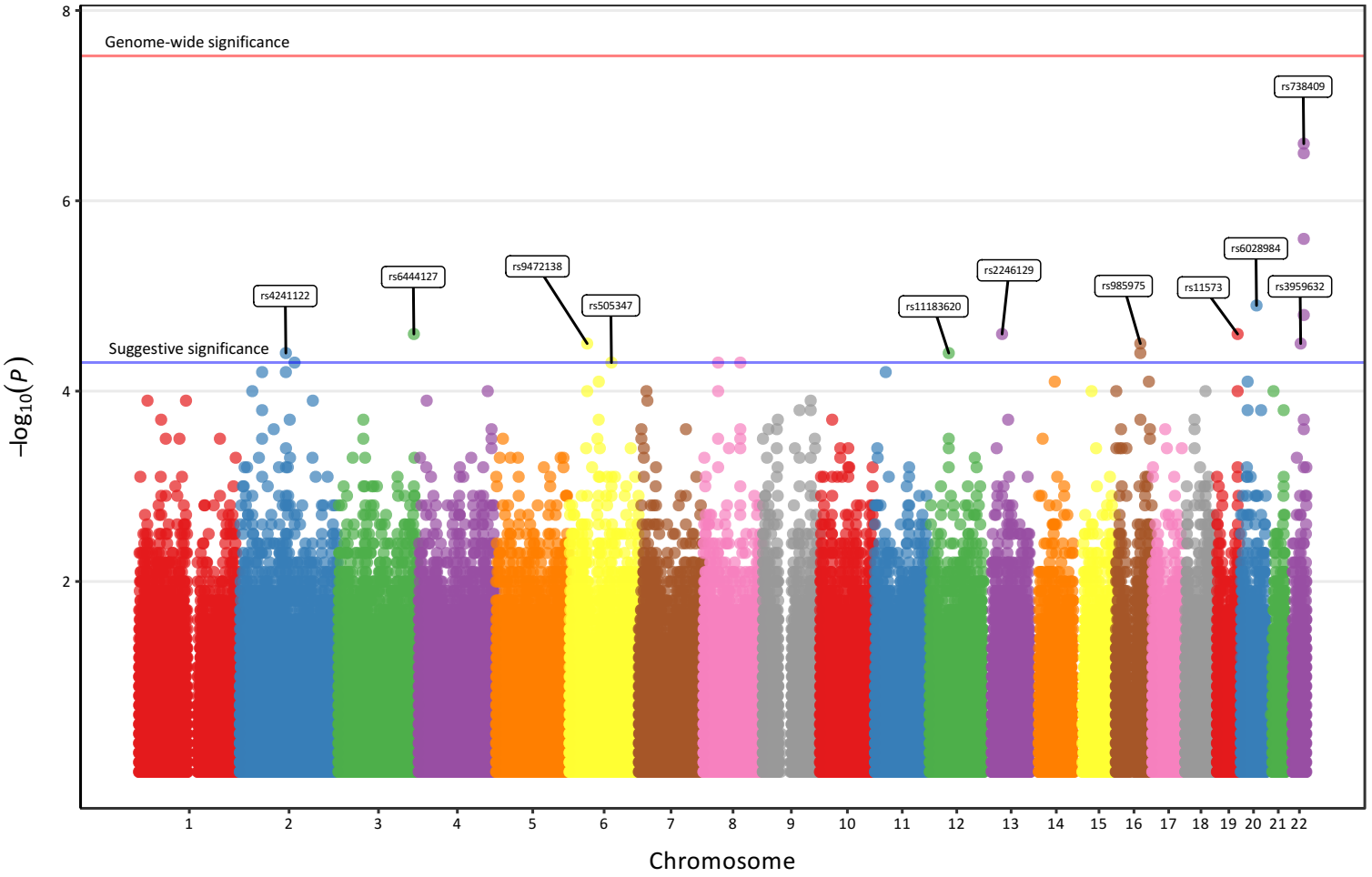


Figure 2.10 Manhattan plot depicting the results of the primary association analysis

Genetic variants are plotted based on their genetic position and statistical significance of association (p-value). Horizontal lines indicate the specified thresholds for genome-wide (red) and suggestive (blue) significance. The variant rs738409 in *PNPLA3* (labelled) was most significantly associated with the risk of disease. A further ten variants (labelled) were associated above the suggestive significance threshold and considered as potential candidates for replication genotyping.

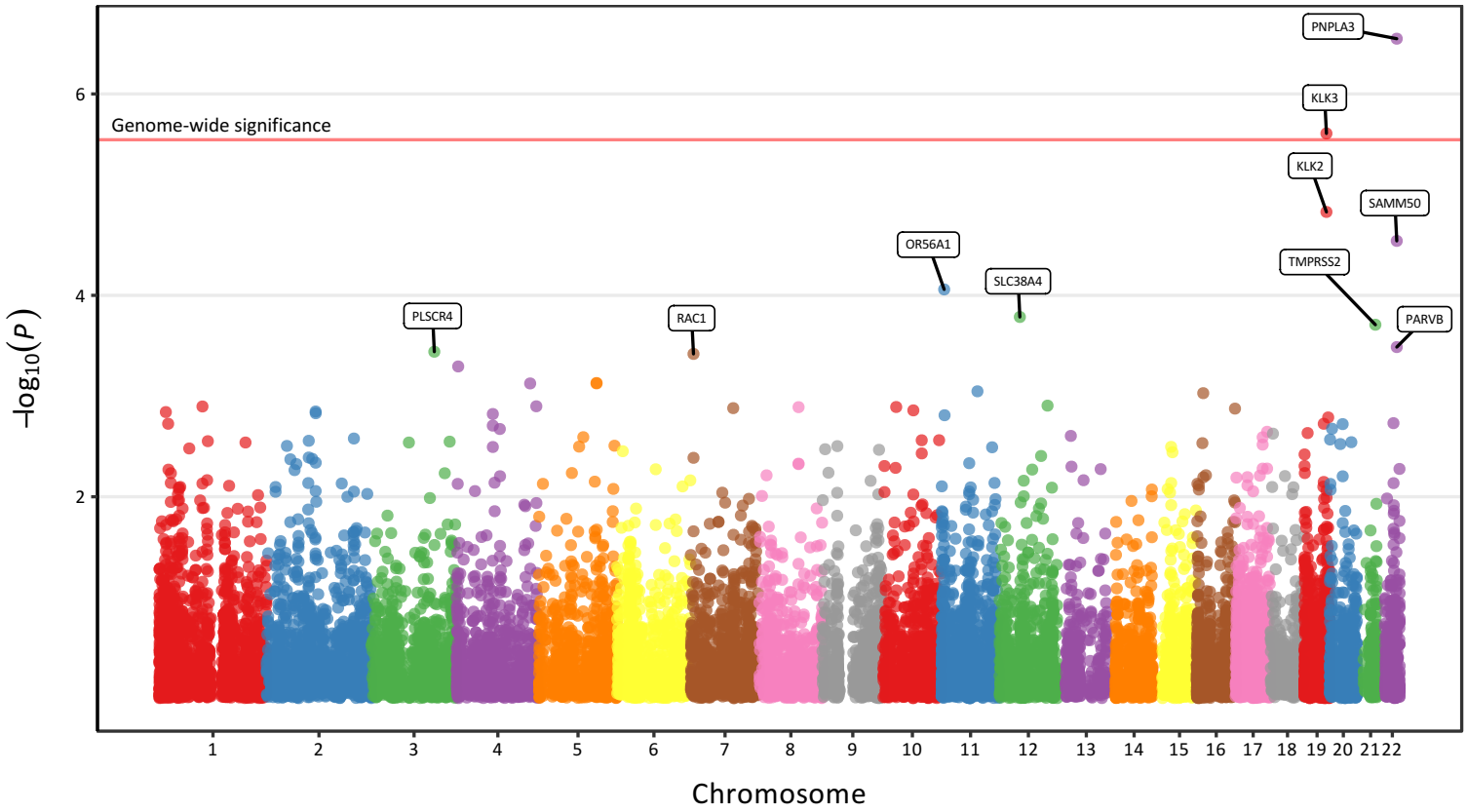


Figure 2.11 Manhattan plot depicting the results of the gene-based association analysis in severe alcoholic hepatitis

Gene-based association analysis was performed using MAGMA[338]. Gene loci are plotted based upon their genetic position and statistical significance of association (P-value). The threshold for genome-wide significant (horizontal red line) is set using α=0.05, corrected for 17,558 tests using the Bonferroni method. The top ten most significantly association gene loci are labelled. The gene *PNPLA3* (labelled) reached the threshold for genome-wide significance. Gene-based association tests were conducted to explore the potential underlying genetic architecture of the condition. Loci were chosen for replication based upon a single lead variant rather than the combined effect of several variants within a gene.

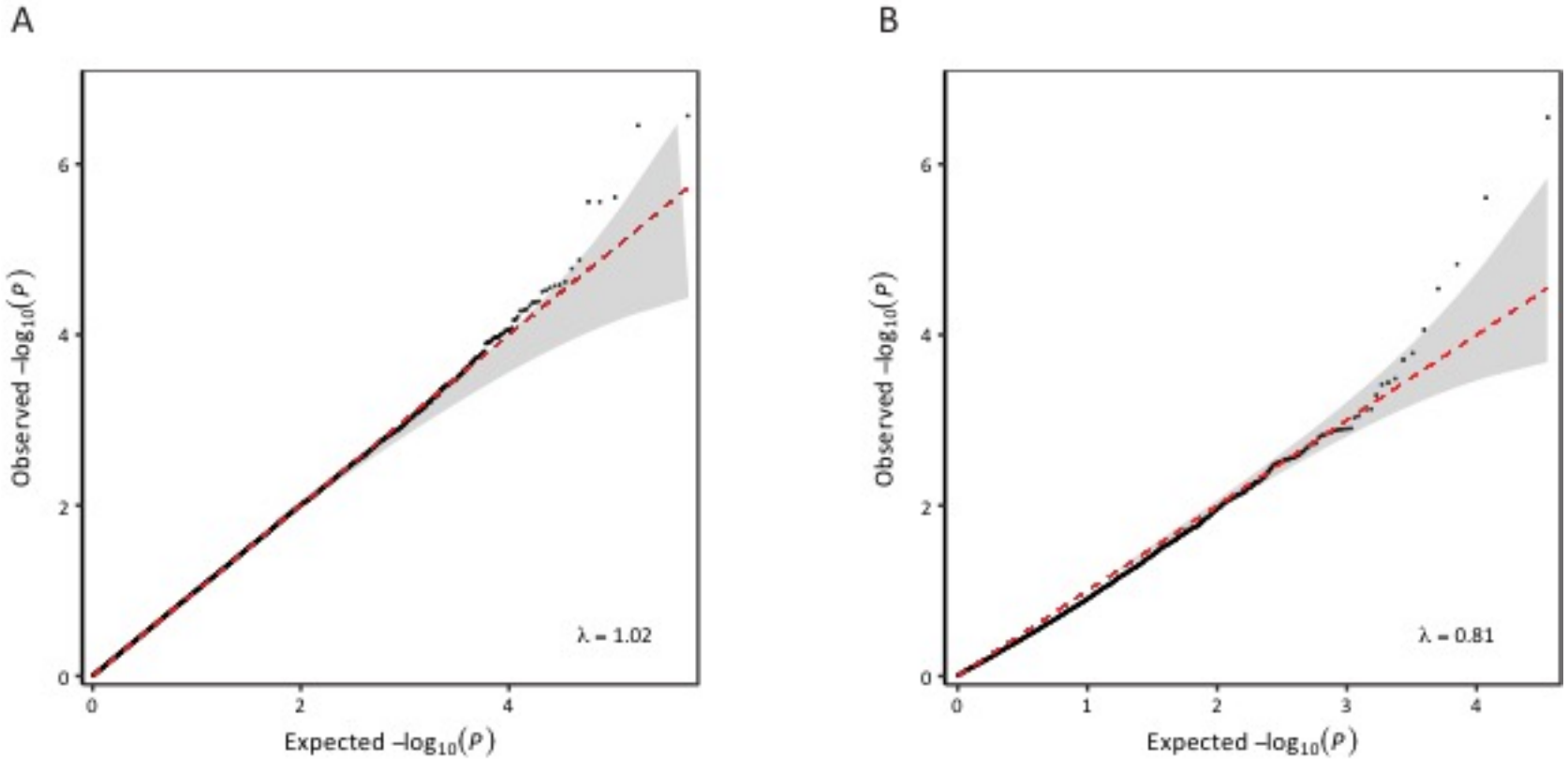


Figure 2.12 Quantile-quantile plots of log transformed P-values for a normal distribution against those observed in (A) SNP- and (B) gene-based association analyses of data in severe alcoholic hepatitis

The grey shaded areas indicate a 95% confidence interval around the identity line (red dashed). Neither plot shows significant systematic deviation from the identity line, particularly at larger P-values, and in both instances the genomic inflation factor (λ) is < 1.05 counting against significant population stratification.

2.7.6 *Triage of variants for replication genotyping*

Five variants associated with suggestive significance were chosen for replication based upon pre-specified criteria (Table 2.7).

Table 2.7 Bioinformatic data relating to variants associated at or beyond suggestive significance threshold in the GWAS of severe alcoholic hepatitis

SNP	Chromosome	Base position	Nearest gene (distance)	Alternate allele and frequency*	Comments
rs4241122¶	2	113678856	*IL37* (2.4kb)	G – 28%	Highly induced by LPS stimulation; Suppresses innate inflammation; Variant associated with lower expression levels in skin
rs6444127	3	186143744	*LINC02052* (30kb)	C – 58%	
rs9472138	6	43811762	*VEGFA* (58kb)	T – 25%	Genome-wide significant association with serum TSH levels
rs505347	6	103600715	*GRIK2* (1.1Mb)	G – 59%	
rs11183620¶	12	47212370	*SLC38A4* (intronic)	G – 49%	Hepatically expressed solute carrier (amino acids)
rs2246129	13	44826634	*SMIM2* (91kb)	T – 48%	
rs985975	16	60812501	*CDH8* (0.9Mb)	C – 9%	
rs11573¶	19	51359497	*KLK3* (coding)	C – 39%	Synonymous, splice region variant; encodes a serine protease; aka Prostate specific antigen; Associated with risk of developing prostate cancer
rs6028984	20	38883710	*LINC01370* (0.25Mb)	T – 33%	
rs3959632¶	22	36668884	*APOL1* (5kb)	C – 20%	Downstream gene variant; positive eQTL in liver (p=0.01)
rs738409¶	22	44324727	*PNPLA3* (coding)	G – 22%	Missense variant; significantly associated with alcohol-related cirrhosis; SIFT prediction – damaging; PolyPhen prediction – probably damaging

*The reference allele is defined by the positive strand allele in the human genome build hg19. Alternate allele frequency is taken from the Western European (CEU) population of the HapMap study

¶Denotes variants selected for replication genotyping

Abbreviations: Kb: kilobase; LPS: Lipopolysaccharide; Mb: Megabase; SNP: Single nucleotide polymorphism; TSH: Thyroid stimulating hormone

2.7.7 Replication genotyping

Twenty-three samples (five cases, 18 controls) were excluded because genotyping failed in more than two variants. The genotyping rate in the remainder was 98% or greater for all variants, except rs4241122 where the genotyping rate was 97.9%. In total 603 individuals were genotyped using both the genome-wide BeadChip and KASPar genotyping platforms. The overall concordance between the platforms for non-missing genotypes across all variants was 99.6% with a maximum of four non-missing conflicting genotypes for rs11573 and rs3959632.

The variant rs738409 in *PNPLA3* was associated at genome-wide significance in the replication cohort (OR 1.75, 95% CI 1.44 – 2.13, $p=1.78x10^{-8}$, Table 2.8)). In addition, one other locus, *SLC38A4*, contained a variant which was significantly associated with disease using a significance threshold of $p<0.05$ (OR 0.83, 95% CI 0.71 – 0.98, p=0.02, Table 2.8).

Table 2.8 Results of replication genotyping

SNP name	Chromosome	BP	Risk allele	MAF Cases	MAF Controls	Odds ratio	95% CI	P
rs738409	22	44324727	G	29%	19%	1.75	1.44 – 2.13	$1.8x10^{-8}$
rs11183620	12	47212370	G	49%	54%	0.83	0.71 – 0.98	0.02
rs11573	19	51359497	C	43%	41%	1.06	0.91 – 1.25	0.45
rs4241122	2	113678856	G	29%	30%	0.95	0.79 – 1.13	0.54
rs3959632	22	36668884	C	21%	21%	1.01	0.83 – 1.22	0.95

Abbreviations: BP: Base position; CI: confidence interval; SNP: Single nucleotide polymorphism

2.7.8 Meta-analysis of replicated variants

The G allele of rs738409 in *PNPLA3* was strongly associated with an increased risk of developing severe alcoholic hepatitis, exceeding genome-wide significance ($p=4.1x10^{-14}$, Table 2.9). The variant rs11183620 in *SLC38A4* remained associated with the risk of developing severe alcoholic hepatitis on fixed effects meta-analysis but did not reach the genome-wide significance threshold ($p=3.7x10^{-5}$, Table 2.9).

Table 2.9 Results of meta-analysis of variants genotyped in the replication and exploratory cohorts

Chr	BP	SNP	A1	A2	OR(F)	P(F)	OR(R)	P(R)	I^2
22	44324727	rs738409	G	C	1.85	$4.1x10^{-14}$	1.86	$2.3x10^{-13}$	5.0
12	47212370	rs11183620	G	A	0.76	$3.7x10^{-5}$	0.72	0.04	79
2	113678856	rs4241122	G	A	0.81	0.004	0.75	0.23	89
22	36668884	rs3959632	C	G	1.21	0.02	1.36	0.32	92
19	51359497	rs11573	C	T	0.89	0.08	0.81	0.45	93

Abbreviations: A1: Risk allele; A2: Reference allele; BP: Base position; Chr: Chromosome; F: Fixed effects; OR: Odds ratio; R: Random effects

2.7.9 Post-hoc adjusted analyses

For the variants which were associated with disease in both the exploratory and replication cohorts post-hoc adjusted analyses were conducted in the exploratory cohort to ensure that associations were robust to adjustment for age, gender, body mass index and diabetes, in addition to the population principal components associated with case-control status. The variants at both loci remained significantly associated with disease ($p<0.05$). Despite some shift in the statistical certainty of association the estimated size of effect (odds ratio) was broadly unchanged in the unadjusted and adjusted analyses (Table 2.10).

Table 2.10 Post-hoc adjusted associations of *PNPLA3* and *SLC38A4* in the exploratory cohort

Locus	SNP	$P_{EXPLORATRY}$	$OR_{EXPLORATORY}$	$P_{ADJUSTED}$	$OR_{ADJUSTED}$
PNPLA3	rs738409	$2.7x10^{-7}$	2.09	$6.2x10^{-6}$	2.45
SLC38A4	rs11183620	$4.2x10^{-5}$	0.60	0.004	0.63

Abbreviations: MAF: minor allele frequency; OR: Odds ratio; SNP: Single nucleotide polymorphism

Data adjusted for age, gender, body mass index and diabetes

Additional analyses were performed for each locus conditioned on the respective lead SNPs. No independent associations were identified at either *PNPLA3* or *SLC38A4* ($p<0.05$) within a window +/- 50kb from the lead marker.

Epistasis testing did identify a significant interaction between rs738409 in *PNPLA3* and rs11183620 in *SLC38A4* in the exploratory cohort ($OR_{INTERACTION}$ 0.65, 95% CI 0.44 – 0.97, p=0.03). Statistical

significance was borderline when the analysis was also adjusted for the first principle component. This finding was not seen in the replication cohort (Table 2.11).

Table 2.11 Epistasis testing between rs738409 in *PNPLA3* and rs11183620 in *SLC38A4*

Term	Test	OR	95% CI	P
Exploratory cohort				
rs11183620	Additive	0.47	0.34 – 0.66	$1.3x10^{-5}$
rs738409	Additive	3.23	1.94 – 5.38	$6.9x10^{-6}$
rs11183620xrs738409	Interaction	0.66	0.44 – 1.00	0.05
PC1	Covariate	$8x10^{-7}$	$4.4x10^{-10}$ – $1.4x10^{-3}$	$2.3x10^{-4}$
Replication cohort				
rs11183620	Additive	0.85	0.69 – 1.05	0.14
rs738409	Additive	1.61	1.17 – 2.22	$3.7x10^{-3}$
rs11183620xrs738409	Interaction	1.09	0.82 – 1.43	0.56

Abbreviations: CI: confidence interval; OR: odds ratio; PC: Principle component

2.7.10 Comparison with alcohol-related cirrhosis without severe alcoholic hepatitis

The five variants genotyped in the replication population were also examined in the cohort of patients with alcohol-related cirrhosis but without a history of severe alcoholic hepatitis. The allelic frequencies were compared with the exploratory cohort of patients with severe alcoholic hepatitis (Table 2.12). The variant rs11183620 in *SLC38A4* was associated with the risk of developing severe alcoholic hepatitis when compared between the groups (OR 0.78, 95% CI 0.62 – 0.97, p=0.03). Importantly the minor allele frequency of the alternative allele (G) was 52% in patients with alcohol-related cirrhosis but no history of severe alcoholic hepatitis, this lay between those with no liver injury and severe alcoholic hepatitis (57% and 45% in the exploratory cohort, respectively). In contrast, the risk allele of rs738409 in *PNPLA3* was not further enriched in the cohort of patients with severe alcoholic hepatitis (OR 0.98, 95% CI 0.78 – 2.34, p=0.87).

Table 2.12 Replication variants in cases with severe alcoholic hepatitis compared to controls with alcohol-related cirrhosis

SNP name	Chromosome	BP	Risk allele	MAF AH	MAF Cirrhosis	Odds ratio	95% CI	P
rs11573	19	51359497	C	37%	45%	0.72	0.57 – 0.90	0.004
rs11183620	12	47212370	G	45%	52%	0.78	0.62 – 0.97	0.03
rs3959632	22	36668884	C	25%	20%	1.31	0.99 – 1.72	0.05
rs4241122	2	113678856	G	25%	29%	0.84	0.66 – 1.06	0.14
rs738409	22	44324727	G	31%	31%	0.98	0.78 – 1.24	0.87

Abbreviations: AH: Alcoholic hepatitis; BP: Base position; CI: confidence interval; MAF: Minor allele frequency; SNP: Single nucleotide polymorphism

2.7.11 Genotyping of candidate variants

Neither rs58542926 in *TM6SF2* nor rs626283 in *MBOAT7* was significantly associated with disease state in either the exploratory or replication cohorts (Table 2.13).

Table 2.13 Analyses for variants associated with alcohol-related cirrhosis in *TM6SF2* and *MBOAT7*

		EXPLORATORY			REPLICATION		
Locus	Chromosome	MAF Cases	MAF Controls	P	MAF Cases	MAF Controls	P
TM6SF2	19	7.4%	6.2%	0.36	9.2%	7.6%	0.15
MBOAT7	8	40%	47%	**0.01**	45%	44%	0.49

Abbreviations: MAF: minor allele frequency

2.8 eQTL analyses

There was no significant change in expression of *PNPLA3* with genotype of rs738409 (p=0.49, Figure 2.12) nor of *SLC38A4* with rs11183620 genotype (p=0.47, Figure 2.13). However, a reduction in *SLC38A4* expression was seen with heterozygosity and homozygosity for the risk (G) allele of rs738409 suggesting a possible distant eQTL (p=0.04, Figure 2.13).

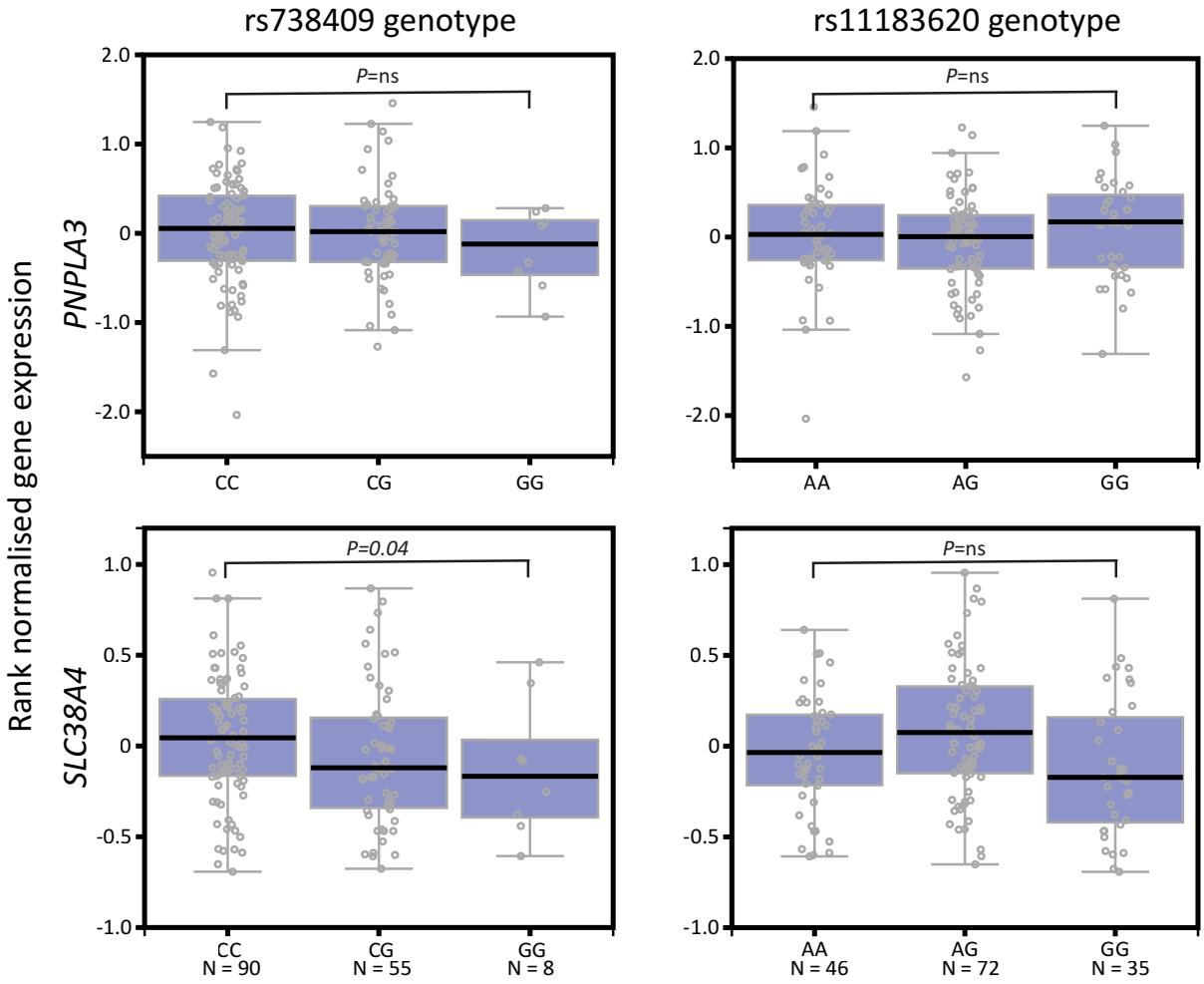


Figure 2.13 Rank normalised *PNPLA3* and *SLC38A4* gene expression in healthy liver tissue (n=153), by rs738409 or rs11183620 genotype (eQTL analyses)

No statistically significant association was noted between rs11183620 genotype and either *SLC38A4* or *PNPLA3* expression. However, whilst there was no statistically significant association between rs738409 genotype and *PNPLA3* expression, a significant reduction in *SLC38A4* expression was seen with heterozygosity and homozygosity for the G allele of rs738409. However, the numbers of individual included within these analyses are comparatively small and limits statistical power. This is especially true of those homozygous for the G allele of rs738409 (n=8). eQTL analyses were performed using data from the Genotype-Tissue expression (GTEx) project using their online platform at https://www.gtexportal.org/home/testyourown[326].

2.9 Discussion

This two-stage genome-wide association study has identified two loci, *PNPLA3* and *SLC38A4*, associated with the risk of developing severe alcoholic hepatitis.

The variant most strongly associated with disease at the *PNPLA3* locus, and in fact at genome-wide significance, is rs738409. It lies within the coding region of the *PNPLA3* gene in an area of linkage disequilibrium that spans both this gene and another, *Sorting and assembly machinery component 50* (*SAMM50*). In this study any association signal was bound by the recombination spikes upstream of *PNPLA3* and downstream of *SAMM50* (Figure 2.14). The rs738409 polymorphism is a missense variant which results in the substitution of an isoleucine for a methionine residue at position 148 of the protein (PNPLA3 I148M). Combined with the lack of an independent association with disease state for any variant lying within a 100kb window centred on rs738409 this provides strong evidence that this is the only variant driving the association at this locus.

This finding is perhaps unsurprising in the context of the pre-existing literature. The variant rs738409 was originally described as associated with hepatic fat content and inflammation in a genome-wide association study of non-alcoholic fatty liver disease[192]. Since its discovery in this context individual candidate gene studies and meta-analyses have implicated it as having a role in increasing the risk of developing alcohol-related cirrhosis[188-191, 355]. Most recently a genome-wide association study of alcohol-related cirrhosis provided further confirmation of this association[151]. Whilst severe alcoholic hepatitis is very much a distinct presentation of alcohol-related liver disease the vast majority of patients also have cirrhosis at presentation[43, 201, 202]. The association between rs738409 in *PNPLA3* and the development of severe alcoholic hepatitis is perhaps to be expected and attributable, in part, to the phenotypic overlap that exists between the two conditions. The comparison between patients with severe alcoholic hepatitis and alcohol-related cirrhosis did not demonstrate any additional enrichment of the variant in patients presenting with severe alcoholic hepatitis suggesting that its role

may lie in the predisposition to alcohol-related liver disease and potentially the development of fibrosis rather than further influencing phenotype by increasing the risk of developing severe alcoholic hepatitis.

Whilst an association between rs738409 in *PNPLA3* and the risk of developing both alcohol and non-alcohol-related fatty liver disease is well described its precise functional implications and mechanistic contribution to the development of liver disease require further elucidation. The protein belongs to a larger family of lipid hydrolases with significant homology to the protein patatin, found abundantly in the potato tuber[356]. Mammalian patatins appear to show specificities for a diverse range of molecules including triacylglycerols, phospholipids and retinol esters[356]. *In vitro* and *in vivo* the effect of the I148M variant, encoded by rs738409, is to promote hepatic steatosis mediated by an accumulation of triglycerides within hepatocytes. The missense variant leads, *in vitro*, to a reduction in hydrolytic activity[357, 358] potentially as a function of decreased substrate accessibility to the active site[359]. A reduction in triacylglycerol hydrolysis by mutant PNPLA3, exacerbated by increased synthesis[55] and impaired export as VLDL[56-58] would explain, at least in part, the association between the variant and development of hepatocellular steatosis.

However, the link between this and liver disease does not appear to be as straightforward as a simple loss of function. In mice, knockout of the *PNPLA3* gene has no impact on lipid homeostasis or development of hepatic steatosis and injury[360, 361], rather it appears that over-expression or knock-in of the mutant protein is required to develop fatty liver disease[194, 362]. However, *PNPLA3* is also strongly expressed by hepatic stellate cells where its retinol palmitase activity, reduced in the variant protein, may be important in controlling cell activation and differentiation[358]. Ultimately the mechanism by which the variant rs738409 in PNPLA3 predisposes to alcohol-related liver disease remains incompletely elucidated.

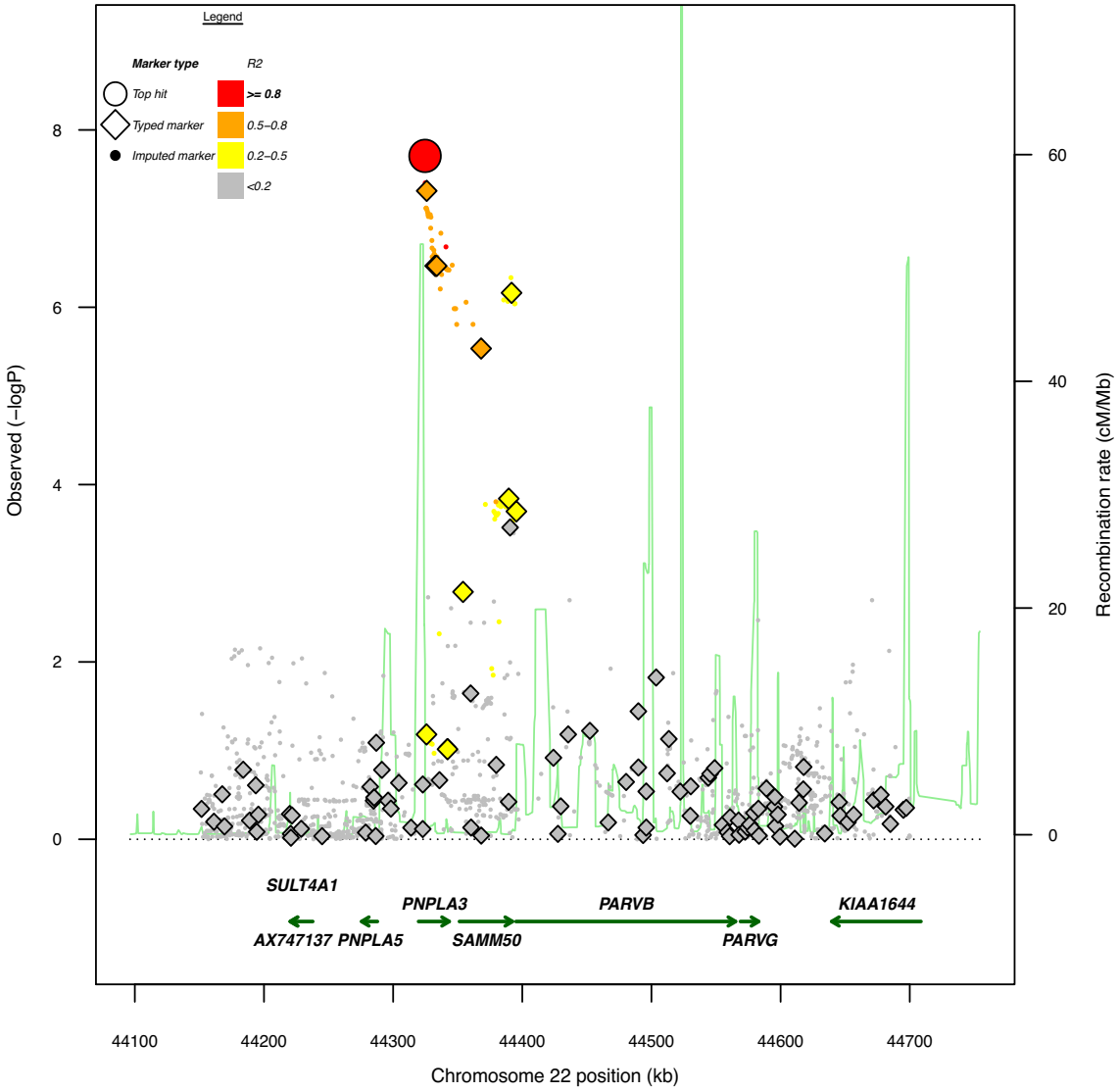


Figure 2.14 Locus plot of typed and imputed markers at the *PNPLA3* locus in the exploratory cohort

Markers are plotted based upon chromosomal position and the statistical significance of association with disease state. Marker colour denotes the strength of linkage disequilibrium with the most significantly associated marker (red circle, rs738409), the frequency of recombination events is represented by the green line and gene reading frames are plotted in the table beneath the graph. Associations between variants and disease at this locus are driven by linkage disequilibrium with the top associated variant, rs738409.

The genome-wide association study of alcohol-related cirrhosis identified associations between variants at two further loci, *TM6SF2* and *MBOAT7*, and the risk of developing alcohol-related cirrhosis both replicated and at genome-wide significance[151]. The former had already been associated with the risk of developing non-alcoholic fatty liver disease[196] whilst the latter was subsequently linked to

the condition[197]. These findings support an argument that both alcoholic and non-alcoholic steatohepatitis share a common genetic background. Although specifically examined in this study neither of the previously described lead variants at each locus demonstrated a robust association with an increased risk of developing severe alcoholic hepatitis. This may perhaps indicate that these variants are more important in driving steatosis and/or fibrosis rather than the inflammation and hepatocellular dysfunction which appear to characterise severe alcoholic hepatitis.

TM6SF2 has been implicated as having an important role in hepatic lipid handling with carriage of the risk variant associated with a reduction in levels of TM6SF2 protein[196], potentially mediated by an alteration in the rate of protein turnover[363]. The result appears to be a reduction in triglyceride secretion from hepatocytes potentially as a function of impaired lipidation and/or secretion of very low-density lipoproteins[195, 363, 364]. The net effect is a reduction in plasma lipid levels but a substantial increase in hepatic steatosis[363]. Given the apparent similarities between the biological effects of the variants associated with alcohol-related cirrhosis in *PNPLA3* and *TM6SF2* it is perhaps odd that only one is associated with the risk of developing severe alcoholic hepatitis. An explanation for this may lie, in part, in statistical underpowering of this study to detect associations for less common variants. The minor allele frequency for rs58542926 in *TM6SF2* is approximately 7% in Western European populations, this contrasts strongly with rs738409 in *PNPLA3* (22%) and demands a dramatically increased sample size in order to achieve adequate statistical power. *MBOAT7* encodes a membrane-bound protein which has been implicated in lysophospholipid remodelling and arachidonic acid metabolism, including in inflammatory cells[193, 365]. The variant rs626283 lies in an area which has known expression quantitative trait loci (eQTL) associations with *MBOAT7*[366]. It is perhaps more surprising then, given the intense inflammatory changes that are seen histologically in severe alcoholic hepatitis, that evaluation of this variant in this study failed to demonstrate a reproducible association. The risk allele for this variant is common in Western European populations, approximately 37%, making statistical power in this study >90% and indicating that, in this population rs626283 in *MBOAT7* is not associated with an increased risk of developing severe alcoholic hepatitis.

In contrast, this study reports an association between severe alcoholic hepatitis and a novel locus, *SLC38A4*. This gene encodes a sodium-coupled solute carrier protein[367]. Gene expression studies indicate that, in man, the liver is the dominant site of expression[368] (Figure 2.15). The gene lies on chromosome 12 and spans a 46.5kb region from 47.17Mb to 47.22Mb, it contains 16 exons the first of which is untranslated. The final 547 amino acid protein contains 10 transmembrane domains with a molecular mass of 60.8 kDa.

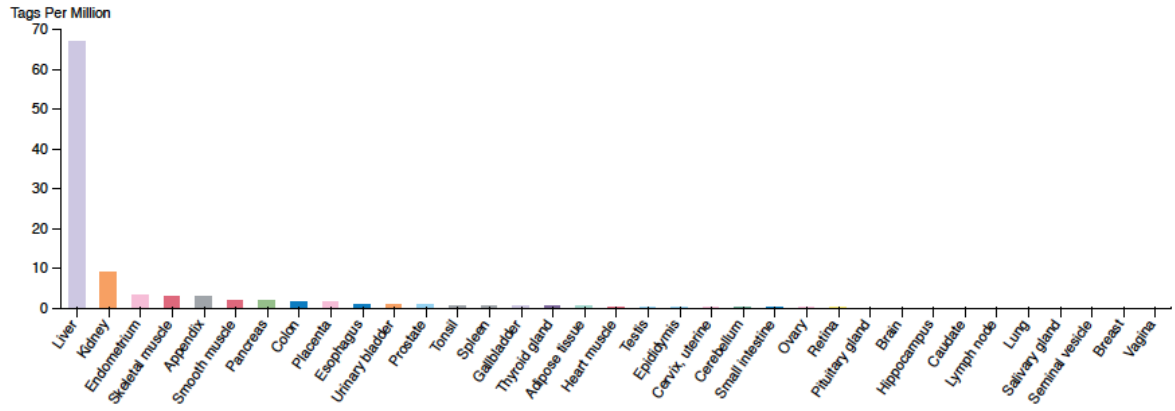


Figure 2.15 Tissue-specific expression of *SLC38A4*

Data are drawn from the FANTOM5 dataset and analysed and graphed using the human proteome atlas online tool (http://www.proteinatlas.org/ENSG00000139209-SLC38A4/tissue)[368]. Expression of *SLC38A4* is significantly enriched in liver tissue with comparatively little expression at other sites.

The lead variant at this locus is rs11183620 which lies in in the first intron and the alternate allele (G) potentially confers a degree of protection against the risk of developing severe alcoholic hepatitis. Whilst the effect of variants in protein-coding regions may be comparatively easy to discern (e.g. missense or nonsense variants), intronic variants such as rs11183620 in *SLC384A* may exert their effects *via* a number of mechanisms. These include alterations in gene expression due to splice site variation or changes in regulatory elements which may alter transcription factor binding or post-transcriptional modifications. Such modifications may alter both mRNA and protein targeting and stability[369-371].

However, intronic SNPs may not change the level of gene expression, but alternatively result in the formation of a novel protein isoform. This can happen when the SNP affects binding of spliceosomal factors to a nearby splice site, resulting in use of alternative sites elsewhere. This eventually results in inclusion of additional sequences in the mRNA, or exclusion of some of the original sequences, most often through skipping of an entire exon. If the change in sequence length is divisible by 3, the mRNA will still be in-frame, and may code a protein with an altered (possibly negative dominant) function. In some cases a (truncated) protein may also be produced even if the reading frame is changed, giving rise to a premature stop codon, although usually such mRNAs are cleared by nonsense-mediated decay.

The variant itself does not appear to confer any impact on protein function, structure or expression – as indicated by a low Combined Annotation Dependent Depletion (CADD) score and RegulomeDB score of 7 (Figure 2.16). Mapping of the locus using the Functional Mapping and Annotation of GWAS (FUMA GWAS) web server application[372] indicates that, in Western European populations, it is in strong linkage disequilibrium (r^2>0.7) with two variants of potential interest – rs4491335 and rs7953215 (Figure 2.16).

Rs4491335 (r^2=0.98) has a CADD score suggestive of potential pathogenicity (10.89) and rs7953215 (r^2=0.69) has a RegulomeDB score of 2b indicating it is likely to affect transcription factor binding (Figure 2.16). Though both were successfully imputed, neither demonstrated a greater significance of association with the risk of disease than the lead SNP (rs4491335: OR 1.58, 95% CI 1.25 – 2.01, $p=1.8x10^{-4}$; rs7953215: OR 1.54, 95% CI 1.21 – 1.96, $p=5.4x10^{-4}$).

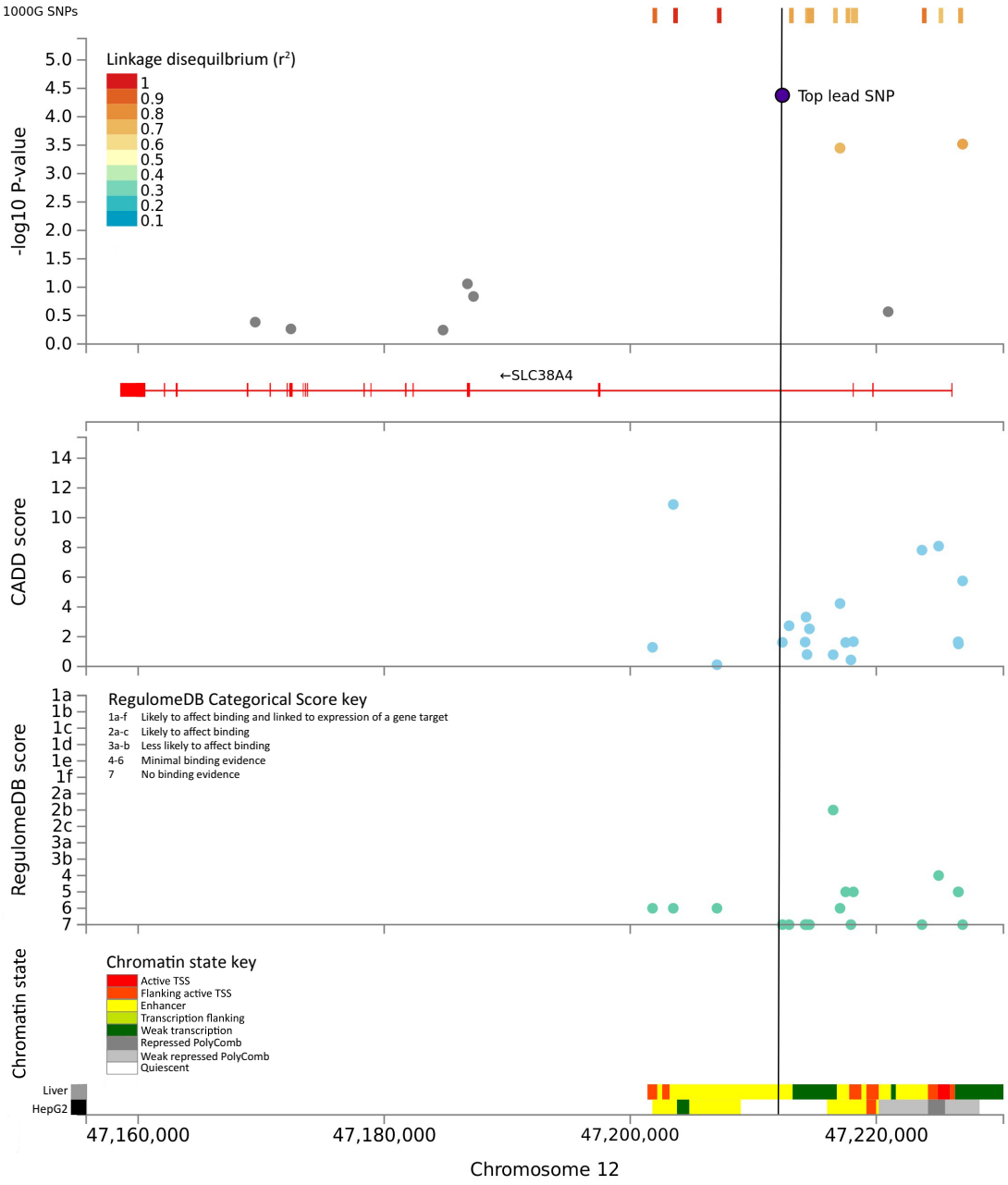


Figure 2.16 A locus plot for the lead SNP, rs11183620, in *SLC38A4*

Genome-wide association results are graphed in the top panel based upon genomic position and p-value for association. Known SNPs from the 1000 genomes project in moderate to strong LD (r^2>0.5) with rs11183620 are displayed in the top row. The *SLC38A4* reading frame is illustrated beneath – vertical bars represent exons and splice sites. The Combined Annotation Dependent Depletion (CADD)[327] and RegulomeDB[328] scores for variants in moderate to strong LD are displayed below whilst. the final panel illustrates the chromatin state at each genomic location, drawn from the NIH Roadmap Epigenomics Consortium reference epigenomes[373] for healthy liver and the hepatocellular carcinoma derived cell line, HepG2. This plot was generated using the Functional Mapping and Annotation of GWAS (FUMA GWAS) web server application[372].

The lead variant identified in this study lies within 80bp of predicted binding sites for the transcription factors CCAAT/enhancer-binding protein beta (C/EBPB) and Signal transducer and activator of transcription 3 (STAT3; Figure 2.17). These transcription factors are induced by inflammatory responses and have been identified as important for liver regeneration in animal models[374-376]. A study examining the genetic regulatory architecture in CD4+ T lymphocytes in patients with rheumatoid arthritis identified a significant *trans*-eQTL for rs11183620 and *STAT3* expression (beta=-0.18, $p=9.5x10^{-6}$), though this was not robust to correction for multiple testing[377]. These findings raise the possibility that rs1183620, or variants with which it is in linkage disequilibrium, are associated with eQTLs which are only seen in the context of active inflammation due to alterations in transcription factor binding or expression. Analyses of epistasis suggest a potential interaction between rs738409 and rs1183620 with a potential *trans*-eQTL revealed on examining hepatic expression data. These results however demonstrate only borderline statistical significance and, especially in the case of the latter, include limited numbers of patients, particularly those homozygous for the G allele of rs738409. Consequently the results must be regarded with caution.

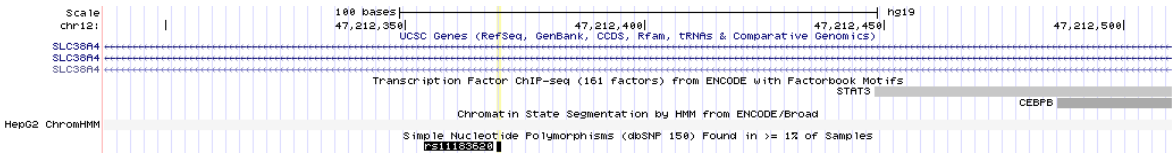


Figure 2.17 Transcription factor binding sites in *SLC38A4* near rs11183620

The variant rs11183620 lies close to predicted binding sites for the transcription factors STAT3 and C/EBPB, both of which are implicated in inflammatory responses. It is possible that any eQTL of rs11183620, or variants with which it is in strong LD, may be only be seen in the context of active inflammation. Plot generated using the University of California Santa Cruz Human Genome Browser (https://genome-euro.ucsc.edu/index.html).

SLC38A4 has a reasonably broad specificity for amino acid transport but with a higher affinity for cationic (arginine, lysine, histidine and glutamate) over neutral (alanine, glycine, serine) amino acids[367]. Liver expression of cationic amino acid transporters is comparatively low suggesting that SLC38A4 may be the predominant transporter responsible for arginine uptake into hepatocytes[367]. Nitric oxide synthases (NOS) use L-arginine as a precursor to synthesis nitric oxide (NO). NO is a small

pleiotropic signalling molecule with a variety of biological functions including regulation of vascular tone, inhibition of platelet aggregation bacterial killing by phagocytes and as a neurotransmitter. In the liver NO can be produced by both resident tissue macrophages (Kupffer cells) and hepatocytes and may influence both blood flow through effects on endothelial cells as well as hepatocyte metabolic function[378, 379]. Experiments in animal models indicate that NO production, particularly by hepatocytes, protects against ischaemia-reperfusion[380, 381] and LPS-mediated liver injury[382, 383]. In the context of alcohol-related liver disease NO may represent a double-edged sword depending on the mechanism of production, with apparently beneficial effects of constitutive NOS derived NO and harmful effects of inducible NOS derived NO[384-386]. This apparent dichotomy in action may be a function of the microenvironment within which the NO is generated, however a beneficial effect of arginine supplementation on the severity of alcohol-related liver injury in animal models is seen[384, 385]. Reduced uptake of arginine by cells in alcohol-related liver disease, potentially mediated by a reduction in *SLC38A4* expression, may contribute to disease progression.

An analysis of genome-wide expression data from colonic biopsies in patients with Crohn's disease described significant down-regulation of *SLC38A4* expression associated with active inflammation[387, 388]. If this phenomenon was also seen in liver as a function of the inflammation associated with severe alcoholic hepatitis it could be envisaged that this would reduce intracellular availability of several amino acids, including arginine, cysteine, methionine and glutamine impairing both NO production, protein synthesis and cellular capacity to manage oxidative stress.

This study represents the first GWAS of the phenotype of severe alcoholic hepatitis with sufficient statistical power to detect moderate effects of common variants. The populations used in the exploratory GWAS were carefully phenotyped with a significant proportion having supportive histological data. Evaluation of genetic variants in a larger, independent cohort permitted replication of findings. In total, the inclusion of 1,950 individuals makes this the single largest study of the genetics of severe alcoholic hepatitis.

This study is not without its limitations. In comparison to many other GWAS the exploratory cohort used for the initial genome-wide association analysis is small meaning there is only sufficient statistical power to detect common variants associated with at least a moderate increased risk of developing disease. Consequently, it is not possible to draw any conclusions about the existence or potential role of uncommon or rare variants with a lower minor allele frequency or even more common variants exerting a small effect. Discovery of such variants, and demonstration of association at genome-wide significance would require a dramatic expansion in the size of the discovery cohort. The sample size required to demonstrate genome-wide significance with the approximate effect size of rs11183620 (OR=1.5) and minor allele frequency of rs738409 (MAF=30%) would comprise 1,000 cases and the same number of controls. Conducting such a study by performing genome-wide genotyping in the replication cohort used here is the subject of ongoing work. However, the comparative rarity of severe alcoholic hepatitis and practical difficulties that exist in recruiting these patients to studies[389] is highly likely to preclude recruitment of cohorts of sufficient size to adequately power studies of this kind seeking to discover rarer variants with more modest effects and prohibit attempts to replicate them. Furthermore it may be questioned what the biological and clinical relevance of such variants might be. Consequently it seems unlikely that the expenditure to time and resources on even larger GWAS studies of severe alcoholic hepatitis will yield worthwhile results. Criticism may also be levelled at the choice of control group. A comparison between patients with alcohol-related cirrhosis and no history of severe alcoholic hepatitis and those who do develop severe alcoholic hepatitis may be argued to be more appropriate. The challenge with this comparison is the comparative instability of the alcohol-related cirrhosis phenotype. Even in those with a long history of cirrhosis a small but significant fraction will subsequently develop severe alcoholic hepatitis. Conversely the challenges that exist in making a reliable diagnosis of severe alcoholic hepatitis, compounded by issues relating to obtaining a reliable clinical history from this cohort of patients, mean that it is difficult to definitively exclude a prior episode of severe alcoholic hepatitis in a proportion of patients. Given the overlap in clinical phenotype between alcohol-related cirrhosis and severe alcoholic hepatitis one would expect that this

would be mirrored in a less pronounced difference in the burden of genetic risk variants and thus necessitate larger sample sizes in order to achieve genome-wide significance in GWAS studies. Thus, given the comparatively small size of the exploratory study here, whilst individuals with a history of alcohol misuse may not be the ideal control population they do facilitate comparison of phenotypic extremes whilst controlling for the major confounding factor of genetic variation associated with alcohol misuse and dependence. Evaluation of candidate variants in a population of patients with alcohol-related cirrhosis but no history of severe alcoholic hepatitis was performed on a hypothesis-driven basis in order to mitigate against this criticism. A combination of the datasets from this study and the published GWAS of alcohol-related cirrhosis would permit a case-case comparison and is the subject of ongoing work.

The rates of biopsy-proven liver disease, or the absence thereof, are comparatively low amongst the cases in the exploratory cohort and both cases and controls in the replication population. Although careful clinical criteria were applied in the selection of both cohorts a small, but potentially not insignificant, rate of misdiagnosis cannot be excluded. The selection of the exploratory cohort was non-random and sought to preferentially include those with biopsy-proven and the most severe disease at presentation. Whilst this is a potential methodology to increase power in GWAS experiments by selecting those individuals with the greatest genetic burden[315, 316] it does risk introducing a degree of bias[390]. Such bias is recognised as a particular problem in the evaluation of quantitative traits[390]. Whilst it may be less of an issue in studies with a case-control design, it may explain some of the less significant associations seen in the replication group and the apparent heterogeneity seen on meta-analysis of the exploratory and replication results. Although the association of *SLC38A4* with disease was confirmed by independent replication in a second cohort of cases and controls the degree of statistical certainty in this group, and on meta-analysis, fell short of genome-wide significance. Results of genome-wide association studies may be confounded by differences in population genetic sub-structure co-segregating with the phenotype of interest, termed population stratification. In the exploratory cohort significant steps were taken to minimise the risks

of this confounding results including calculation of genetic principle components and inclusion of those associated with case-control status as a covariate in analysis, examination of the study population in relation to the HapMap populations using multi-dimensional scaling and ensuring that the genomic inflation factor was <1.05. A fully adjusted analysis was also performed in the exploratory cohort to control for additional potential confounders such as age, gender, body mass index and a diagnosis of diabetes. However, whilst the same inclusion and exclusion criteria in relation to ethnicity were applied to the replication cohort, an absence of phenotypic and genome-wide genotypic data means that the presence of population stratification, or other sources of confounding, cannot be guaranteed. The ethnicity selection criteria also mean that the findings of this study cannot be immediately generalised beyond a Western European population.

In summary, the results of this two-stage genome-wide association study in severe alcoholic hepatitis identify *PNPLA3* rs738409 as a risk factor for the development of disease. A further locus, *SLC38A4* (rs11183620), is further implicated in disease. The biological function and predominant site of tissue expression of this protein lend support to the implication that it may be involved in disease pathogenesis and warrants further investigation.

CHAPTER 3

GENETIC VARIANTS AND PRESENTATION WITH SEVERE ALCOHOLIC HEPATITIS

3 The influence of genetic variation on presentation with severe alcoholic hepatitis

3.1 Overview

The G allele of rs738409 in *PNPLA3* is associated with the risk of developing severe alcoholic hepatitis (Chapter 2). It has also, previously, been associated with more severe histological lesions in individuals with alcohol-related liver disease. In this chapter the impact of rs738409 genotype on the presentation of severe alcoholic hepatitis, including disease severity and histological appearances, is examined. In light of the association between rs11183620 in *SLC38A4* genotype and the risk of developing severe alcoholic hepatitis the impact of this variant on the same aspects of severe alcoholic hepatitis is also examined.

3.2 Introduction

The missense variant, rs738409, in the gene *Patatin-like phospholipase domain containing protein 3* (*PNPLA3*) results in substitution of an isoleucine residue for methionine at position 148 of the protein (Ile148Met; I148M). The role of rs738409 genotype in liver disease was first described in the field of non-alcoholic fatty liver disease as an association with hepatic triglyceride levels, measured by ^{1}H-MRS[192]. Additionally, *PNPLA3* rs738409 genotype has been shown to influence histology in non-alcoholic fatty liver disease with carriers of the minor allele developing greater steatosis and fibrosis and an increased prevalence of steatohepatitis[391-394]. Rs738409:G has also been associated with the development of increased steatosis and inflammation in people with chronic hepatitis B and C infection[395-397]. An influence of the variant on fibrosis stage and the presence of cirrhosis has also been described in patients with hereditary haemochromatosis and a normal body mass index[398].

Extensive evidence from individual studies[188-190, 355], a meta-analysis[191] and, most recently, a genome-wide association study[151] links carriage of the risk allele, rs738409:G, to increased risk for the development of alcohol-related cirrhosis. In the setting of alcohol-related liver disease an

association between carriage of rs738409:G and more advanced fibrosis and extensive steatosis, including in cirrhotic patients has been described[190].

Histologically alcoholic hepatitis is associated with the presence of alcoholic steatohepatitis (ASH). This is defined by the presence of hepatocellular steatosis, ballooning degeneration of hepatocytes and the presence of an acute inflammatory infiltrate. It is only comparatively recently that a histological scoring system for severe alcoholic hepatitis has been described and validated[43]. The Alcoholic Hepatitis Histological Score (AHHS) incorporates a number of histological features which are semi-quantitatively assessed *viz.* fibrosis, bilirubinostasis, neutrophilic infiltration and megamitochondria, which have been independently associated with short-term outcome[43]. A number of other systems exist for semi-quantitatively grading the degree of fibrosis in a liver biopsy specimen[399]. These include the METAVIR and Ishak scores in viral hepatitis[400, 401]; Brunt score in non-alcoholic fatty liver disease[402] and the more generally applicable Laennec fibrosis score[403, 404]. The latter has the advantage of having sub-classifications for cirrhosis which correlate with the clinical disease stage and degree of portal hypertension[405] and has been applied to alcohol-related cirrhosis[406]. Fibrosis may be quantified by digital image analysis of a liver section stained using Sirius Red in order to produce the amount of collagen-straining as a fraction of total liver tissue, termed the Collagen Proportionate Area (CPA). There are generally strong but imperfect correlations between manual staging of fibrosis using semi-quantitative grading systems and digital image analysis of the CPA[407, 408]. Inter-observer variability in fibrosis staging and quantification[409-411] means that techniques employing digital image analysis are preferable for accurate assessment of the degree of fibrosis and may reveal otherwise hidden associations[408]. It should however be recognised that many of the semi-quantitative scores consider not only the quantity but also the morphology of fibrosis. This may partially explain why correlations between the two are imperfect and also means that relying solely on quantitation may miss pathologically relevant information.

Hepatocellular injury and death are cardinal histopathological features of alcoholic steatohepatitis and is seen on liver biopsy in patients with severe alcoholic hepatitis. Hepatocyte death in alcohol-related liver disease may occur *via* a multiplicity of mechanisms. Necrosis and apoptosis are considered the major mechanisms[67, 412, 413], however, more recent data suggests necroptosis[414, 415] and pyroptosis[416] are also likely to play a role. Cytokeratin 18 (CK18-M65) is a constituent protein of epithelial cells, including hepatocytes, and is released upon epithelial cell death[417, 418]. When the mode of cell death is apoptosis, a caspase-cleaved fragment of the CK18 molecule (CK18-M30) is released and can be specifically detected. Elevated circulating CK18 levels have been associated with the presence of inflammation and fibrosis in patients with non-alcoholic fatty liver disease[419, 420], viral hepatitis[421, 422] and cholestatic liver injury[423]. In the setting of alcohol-related liver disease elevated CK18 levels have been described in patients with alcoholic steatohepatitis on biopsy[424]. Serum levels of CK18 seem to be correlated with the presence of Mallory-Denk bodies and hepatocyte necrosis[424] and apoptosis[425]. Increased levels of CK18 in patients who misuse alcohol compared to healthy controls raise questions as to whether excess alcohol consumption alone can cause sufficient cell death to cause elevated CK18 levels however this study failed to exclude the presence of alcoholic steatohepatitis on biopsy in patients with alcohol misuse[426]. Recent work suggests that circulating CK18 may serve as a diagnostic marker of the presence of alcoholic steatohepatitis on biopsy in patients with a clinical presentation consistent with severe alcoholic hepatitis[215] and may also be predictive of outcome[427]. One mechanism for the initiation of apoptosis is stimulation of the 'death receptor' Fas to its ligand (Fas-ligand, FasL)[428]. Fas is expressed by hepatocytes in healthy livers and patients with alcohol-related cirrhosis, but is dramatically upregulated in the livers of patients with cirrhosis secondary to viral hepatitis[429]. FasL is present on the surface of natural killer cells and cytotoxic T lymphocytes. The Fas receptor may also be present in serum as soluble Fas (sFas) and serves to antagonise the interaction of cell-surface Fas with FasL, thus inhibiting apoptosis mediated by this mechanism[430]. Elevated levels of sFas have been described in patients with non-alcoholic steatohepatitis as opposed to those with simple steatosis[431]; and in viral hepatitis where it has been

related to the stage of fibrosis and development of hepatocellular carcinoma[432, 433]. Raised serum sFas levels have also been described in alcoholic cirrhosis with an apparent further elevation in patients with alcoholic hepatitis[434].

Thus, while carriage of rs738409:G has been associated with more severe histological lesions in patients with alcohol-related liver disease, it remains unknown whether this effect is also seen in individuals with very severe liver injury, such as severe alcoholic hepatitis. If so, an influence may also be seen upon clinical features at presentation and serum markers of epithelial cell death.

3.3 Aim

The aim of the analyses conducted and described in this chapter was to determine, in the context of severe alcoholic hepatitis, the influence of rs738409 genotype upon:

1. Clinical features at presentation;
2. Histological features on biopsy;
3. Serum markers of epithelial cell death;

In light of the association between the genotype of rs11183620 in *SLC38A4* and the risk of developing severe alcoholic hepatitis, the same analyses were also conducted for this variant on an exploratory basis in the sub-group of patients with available rs11183620 genotypes.

3.4 Patients, materials and methods

3.4.1 Severe alcoholic hepatitis patient cohort

The cohort comprised of the 898 patients with severe alcoholic hepatitis recruited *via* the STOPAH trial for whom clinical data and genomic DNA were available. All had a history of long-standing alcohol misuse; compatible clinical, laboratory and/or liver biopsy features of alcoholic hepatitis; no other identified causes for their liver disease; and a DF ≥32. All were British but additional data were collected on self-reported ethnicity. Eight-hundred and sixty patients (95.8%) identified themselves as White, three (0.3%) as Black or Black British, 23 (2.6%) as Asian or Asian British, five (0.6%) as mixed origin and seven (0.8%) as 'other' or not stated. Cases were included irrespective of self-reported ethnicity.

3.4.2 Clinical and laboratory data

Clinical data including demographics were collected routinely as part of the STOPAH trial protocol (Chapter 1, Supplementary Methods). Basic demography was recorded at screening and baseline, pre-treatment, haematological and biochemical data were used in these analyses. If patients remained admitted to hospital, data were additionally collected at day 7 in accordance with the study protocol and incorporated.

3.4.3 Drinking data

Data regarding self-reported pre-admission drinking behaviour were collected at screening as the maximum weekly consumption of alcohol in the preceding two months expressed in units.

3.4.4 Histological data

Liver biopsy specimens collected as part of the STOPAH trial were obtained *via* the transjugular route. Liver cores were fixed in formaldehyde and embedded in paraffin wax, in accordance with standard diagnostic laboratory practice. Two sections were provided by the treating centres to St. Mary's

hospital, London; one was stained with haematoxylin and eosin, the other Sirius red. In total sections from 208 patients were sent by the treating centre for central pathological review. Stained sections were reviewed independently by two consultant histopathologists, blinded to the clinical data. Biopsy specimens were deemed adequate for histological evaluation if they displayed a minimum of five portal tracts. The Laennec system was used to grade the degree of fibrosis as it provides better definition in those who have already developed cirrhosis (Table 3.1)[403-405]. Features of alcoholic steatohepatitis were noted and scored in line with the Alcoholic Hepatitis Histological Score (AHHS, Table 3.2[43]).

Additionally, digital image analysis of the stained sections was performed in order to quantify both steatosis and fibrosis. Images were captured using a 20x objective lens on a digital whole slide scanner (Hammatsu Photonics, Shizuoka, Japan) in Nanozoomer Digital Pathology Image (NDPI) format. Images were exported at 2x magnification to Joint Photographic Experts Group (JPEG) format using NDP.view Nanozoomer Viewer Software (Hammatsu Photonics, Shizuoka, Japan). These JPEG images were then processed by automated image analysis, using a proprietary machine-learning algorithm[435], to quantify the degree of fibrosis (Sirrus red) and steatosis (H&E) and derive the collagen and fat proportionate areas (CPA and FPA, respectively) (see Supplementary Methods).

Categorisation according to the criteria defined in the derivation of the AHHS was used for hepatocyte ballooning, neutrophil infiltration and bilirubinostasis.

Histological specimens were evaluated independently by two senior histopathologists. Where there were discrepancies between the assigned scores the relevant sections and parameters were reviewed jointly and a consensus decision reached.

Table 3.1 Laennec fibrosis score, adapted from[404]

Grade	Category	Description
0	No definite fibrosis	No fibrosis
1	Minimal fibrosis	No septa or rare thin septa; may have portal expansion or mild sinusoidal fibrosis
2	Mild fibrosis	Occasional thin septa
3	Moderate fibrosis	Moderate thin septa; up to incomplete cirrhosis
4A	Mild cirrhosis	Definite or probable
4B	Moderate cirrhosis	At least 2 broad septa
4C	Severe cirrhosis	At least one very broad septum or many minute nodules

Table 3.2 Categorisations and criteria evaluated for inclusion in the Alcoholic Hepatitis Histological Scoring system (AHHS), adapted from[43]

Feature	Categorisation
Fibrosis	None or portal fibrosis
	Expansive fibrosis
	Bridging fibrosis or cirrhosis
Bilirubinostatsis	None
	Hepatocellular
	Canalicular or ductular
	Canalicular or ductular plus hepatocellular
Neutrophil infiltration	Minimal
	Mild – few neutrophils around a small cluster of hepatocytes
	Severe – neutrophils readily seen at low magnification
Megamitochondria	Absent
	Present
Hepatocyte ballooning	Occasional – focal and dispersed ballooned hepatocytes
	Marked – ballooned hepatocytes easily seen at low magnification

Due to the potential influence of both time and treatment on histological appearances, liver biopsies were included in or excluded from analyses based on time thresholds determined by their likely influence on the relevant histological feature. Neutrophil infiltration, steatosis, hepatocyte ballooning and the presence of Mallory-Denk bodies or megamitochondria were all likely to change over a short time frame whereas fibrosis was felt to be comparatively immutable. Additional, more liberal,

parameters were set for sensitivity analyses in order to assess the robustness of the findings. The following thresholds were used:

1. Fibrosis – 28 days before or after the start of treatment, widened to 90 days pre- or post-treatment for the sensitivity analyses;
2. Steatosis, inflammation, ballooning and the presence of Mallory-Denk bodies and megamitochondria – 14 days before or after the start of treatment, widened to 28 days pre- or post-treatment for the sensitivity analyses;

Comparatively little paired biopsy data exists upon which to firmly based these thresholds. Fibrosis whilst potentially dynamic evolves over many years [32], consequently a conservative timeframe of 28 days, equivalent to the treatment period for severe alcoholic hepatitis and widened to a maximum of three months seemed reasonable on the basis that significant changes in its severity within such a period were highly unlikely. The degree of if fibrosis is unlikely to alter as a function of the presence or absence of steatohepatitis. In contrast steatosis and inflammation are more dynamic and an effect of prednisolone on the presence of inflammation would be expected. Limited data indicate that steatosis may resolve within a month of alcohol cessation[99]. This was used as a proxy for other features of steatohepatitis and thus a conservative threshold of 14 days either side of the start of treatment was set with widening to 28 days in sensitivity analyses. In any biopsy taken before or after this window it was considered that the clinical relevance and accuracy of the diagnosis would need to be questioned.

3.4.5 Genotyping

Genotyping for rs738409 in *PNPLA3* and rs11183620 in *SLC38A4* was performed using the K-Biosciences Competitive Allele Specific Polymerase Chain Reaction (PCR) (LGC Genomics, Hoddesdon, UK) platform with amplification and detection undertaken using a LightCycler® 480 real-time PCR system (Roche Molecular Diagnostics, Burgess Hill, UK). Genotype calling was performed

automatically using proprietary software with minor manual editing of genotype calls. Approximately 28% of all the samples, randomly selected *a priori*, were genotyped in duplicate to ensure consistent genotype calling. The primer sequences used in KASPar genotyping are given in Table 3.3.

Table 3.3 Polymerase chain reaction primers used for genotyping on the KASPar platform

Primer	Sequence
rs738409 in *PNPLA3*	
Allele specific (C)	GAAGGTGAACCAAGTTCATGCTCCTTGGTATGTTCCTGCTTCATC
Allele specific (G)	GAAGGTCGGAGTCAACGGATTCCTTGGTATGTTCCTGCTTCATG
Reverse	CGCCTCTGAAGGAAGGAGGGAT
rs11183620 in *SLC38A4*	
Allele specific (A)	GAAGGTGACCAAGTTCATGCTGCCAGCTGAGGGTTCCTTATATTA
Allele specific (G)	GAAGGTCGGAGTCAACGGATTCCAGCTGAGGGTTCCTTATATTAC
Reverse	CTTAGTTTTGGTAATATAGTACAACTCATT

3.4.6 Serum markers of epithelial cell death

Cytokeratin-18 M30 (Apoptosense, cat 10011, Bioaxxess Peviva, Tewkesbury, UK) and M65 (EpiDeath, cat 10040, Bioaxxess Peviva, Tewkesbury, UK) subtypes were used as markers of epithelial cell death and measured in baseline, pre-treatment serum samples by enzyme-linked immunosorbent assay (ELISA). Serum sFas was used as a marker of cell death by apoptosis and was also measured by ELISA (Thermo Fisher, Massachusetts, USA).

3.4.7 Data processing

3.4.7.1 Haematological and biochemical data

Prognostic scores were calculated at baseline (DF[1], Model for End-stage Liver Disease [MELD][5] and Glasgow Alcoholic Hepatitis Score [GAHS][3]) and after 7 days of treatment (Lille score[4]) were derived.

3.4.7.2 Histological data

Whilst manual evaluation of histopathological features was achieved in all specimens, a technical failure rate was seen in the automated image analysis of fibrosis and steatosis for some samples. In order to address the problem of missing data, imputation was performed. A decision was made, *a prioi*, to employ methods of imputation utilising measure of central tendency on the basis of simplicity. For the CPA, missing data were imputed using the median CPA value for the histopathologist-assigned Laennec grade for the sample in question. Missing FPA data were imputed using mean value imputation. Analyses were conducted in both only measured and also imputed data to ensure findings were consistent and not soley a function of the imputation methodology.

In light of high CPA values the FPA was adjusted for the CPA in order to derive a value reflective of the proportion of hepatocellular tissue which was steatotic rather than the entire section. Adjustment was performed using the formula:

$$FPA_{adjusted} = \frac{FPA_{raw}}{100 - CPA}$$

3.4.8 Statistical analyses

Statistical analyses were performed using SPSS version 22 (IBM, Armonk, USA), intraclass correlation coefficients were calculated in R[(344)] using the psych package. Survival curves and plots were generated in R[(344)] using the packages ggplot2, survival, gridExtra, reshape and plyr.

The influence of genotype on clinical and histological features of severe alcoholic hepatitis at presentation, as well as markers of epithelial cell death, was tested by comparison of features across all three genotypic groups, using linear regression (continuous data), Jonckheere-Terpstra test (ordinal and continuous data) or Chi-square or Fisher's exact test (categorical data), as appropriate. In linear regression analyses residual distribution and normal probability plots were inspected to ensure model validity, where these were violated or a significant regression model could not be found the

Spearman's rank test was used to test for correlations between continuous variable, the Jonckheere-Terpstra test was used to compare across genotypic groups and the Mann-Whitney U or Kruskal-Wallis tests to compare two or more groups, respectively. Agreement between Laennec grade and CPA was tested using the Kendall tau rank test and generation of an intraclass correlation coefficient[436].

Additional analyses were conducted to detect any potential confounding influence of body mass index (BMI) and diabetes on the histological appearances in light of their known associations with disease progression in alcohol-related and non-alcohol-related fatty liver disease. A fully adjusted analysis was conducted using linear regression analysis, residual distribution and normal probability plots were inspected to ensure validity.

3.5 Results

3.5.1 Genotyping

PNPLA3 rs738409 genotypes were successfully called in 867 (97%) of 898 cases. Two samples (<0.05% of total) demonstrated conflicting genotypes for either marker and were excluded. *SLC38A4* rs11183620 were additionally available in 860 (99.2%) of the 867 cases with *PNPLA3* rs738409 genotypes. The markers followed Hardy-Weinberg equilibrium in both case and control populations (p>0.05). For *PNPLA3* rs738409 the distribution of genotypes was – CC 425/867 (49%), CG 372/867 (43%) and GG 70/867 (8%) with a minor allele frequency of 30%. For *SLC38A4* rs11183620 the distribution of genotypes was: AA 236/860 (27.4%), AG 424/860 (49.3%) and GG 200/860 (23.3%), with a minor allele frequency of 48%.

3.5.2 Association with baseline demography and assessment variables

There were no clinically significant differences in age, gender distribution, alcohol consumption, or the majority of the clinical or laboratory variables at baseline in relation to rs738409 genotype (Table 3.4). A comparatively lower prevalence of overt hepatic encephalopathy was noted in the heterozygote group (CC: 30.0%, CG: 19.4%, GG:24.3%, p<0.005). A statistically, but not clinically, significant difference

in the distribution of INRs between genotypic groups was noted. There were no differences in the distributions of the prognostic scores calculated at baseline in relation to rs738409 genotype (Table 3.4).

Similarly, there were no clinically significant differences in age, gender distribution, alcohol consumption, any of the clinical or laboratory variables or the prognostic scores calculated at baseline in relation to rs11183620 genotype (Supplementary Results, Table 10.1).

Table 3.4: Baseline characteristics of cases with severe alcoholic hepatitis, by rs738409 genotype

Characteristics	*PNPLA3* rs738409 Genotype			Significance (p)
	CC (n = 425)	CG (n = 372)	GG (n = 70)	
Baseline demographics				
Age (years)	48 (41 – 56)	49 (42 – 56)	51 (42 – 58)	0.36
Male gender	262 (61.4%)	240 (64.5%)	52 (74.3%)	0.11
Alcohol consumption (units/week)	140 (84 – 210)	126 (84 – 210)	126 (84 – 200)	0.89
Overt hepatic encephalopathy	128 (30.0%)	72 (19.4%)	17 (24.3%)	**0.003**
Baseline laboratory variables				
White cell count (x10^6/mm^3)	8.8 (6.1 – 12.1)	8.7 (6.2 – 12.8)	9.3 (5.7 – 11.9)	0.89
Bilirubin (µmol/l)	282 (177 – 416)	271 (165 – 419)	205 (153 – 373)	0.09
Albumin (g/l)	25 (21 – 29)	25 (21 – 29)	25 (22 – 29)	0.93
Aspartate transaminase (IU/l)	120 (85 – 158)	124 (90 – 177)	128 (83 – 158)	0.43
Alkaline phosphatase (IU/l)	171 (131 – 231)	168 (130 – 231)	153 (114 – 214)	0.09
International normalised ratio	1.7 (1.5 – 2.0)	1.7 (1.5 – 2.0)	1.8 (1.6 – 2.1)	**0.03**
Urea (mmol/l)	3.4 (2.1 – 5.5)	3.1 (2.1 – 4.8)	3.8 (2.5 – 4.8)	0.26
Creatinine (µmol/l)	63 (52 – 83)	66 (54 – 84)	64 (54 – 90)	0.71
Prognostic scores				
DF	54 (43 – 70)	56 (44 – 74)	58 (44 – 77)	0.52
MELD	20 (17 – 24)	21 (17 – 24)	19 (17 – 25)	0.59
GAHS	8 (7 – 9)	8 (7 – 9)	8 (7 – 9)	0.46

Data expressed as median (IQR) or as number (%)

Abbreviations: DF: Discriminant function; MELD: Model for End-Stage Liver Disease; GAHS: The Glasgow Alcoholic Hepatitis Score

3.5.3 Suitability of samples for histological analysis

In total 208 biopsies were returned for central review of histology. Twenty-five (12.0%) were excluded as they failed to meet the minimum quality required for analysis. In those which could be assessed 7

samples has been obtained more than 90 days prior to the start of treatment and a further sample more than 90 days after the start of treatment. In a further three instances the timing of the biopsy in relation to the start of treatment was unclear and so these were also excluded. Nineteen (11.0%) of the 172 biopsies potentially eligible for inclusion on the basis of timing were considered non-diagnostic for alcoholic steatohepatitis and were excluded. *PNPLA3* rs738409 and *SLC38A4* rs11183620 genotypes were available in 135/153 cases (88.2%). In both cases the distribution of genotypes and the minor allele frequency in the biopsy-proven cohort were similar to that seen in the overall alcoholic hepatitis cohort (rs738409: CC: 63/135 (47%); CG: 58/135 (43%); GG 14/135 (10%), MAF 32%; and, rs11183620: AA 35/135 (26%); AG 69/135 (52%); GG 30/135 (22%), MAF 48%). The number of biopsies and time to biopsy for the different analysis populations are show in Table 3.5.

Table 3.5 Numbers of cases and time to biopsy in populations for histological analyses

Feature	Analysis	Cases (n)	Time to biopsy (days)
Fibrosis	Primary*	129	4 (0.5 – 9)
	Sensitivity¶	135	5 (1 – 10)
All other features	Primary§	115	3 (0 – 7)
	Sensitivity*	129	4 (0.5 – 9)

Data expressed as median (interquartile range)

*Time window with respect to treatment start ±28 days

¶ Time window with respect to treatment start ±90 days

§ Time window with respect to treatment start ±14 days

The baseline characteristics of the biopsy cohort were similar to the overall STOPAH population (Table 3.6).

Table 3.6 Baseline characteristics of the biopsy cohort in comparison to the overall STOPAH population

Characteristic	Biopsy population (n=135)	Overall population (n=1092)
Age (years)	48 (41 – 55)	48 (41 – 56)
Sex (male)	91 (67%)	685 (63%)
Prednisolone	71 (53%)	547 (50%)
Discriminant function	54 (41 – 75)	55 (43 – 74)
MELD	23 (21 – 26)	23 (21 – 26)
White blood cell count (x10^9/l)	9.2 (6.2 – 12.2)	9.0 (6.3 – 12.6)
Bilirubin (µnol/l)	340 (205 – 461)	274 (172 – 418)
Albumin (g/l)	25 (21 – 30)	25 (21 – 29)
Aspartate transaminase (IU/l)	131 (91 – 159)	124 (87 – 169)
Urea (mmol/l)	3.4 (2.0 – 5.9)	3.3 (2.2 – 5.2)
Creatinine (µnol/l)	66 (57 – 94)	64 (53 – 85)
INR	1.69 (1.50 – 2.00)	1.73 (1.54 – 2.00)
PNPLA3* rs738409 genotypes	CC 63 (47%) CG 58 (43%) GG 14 (10%)	CC 425 (49%) CG 372 (43%) GG 70 (8%) N/A 225

Data are shown as median (IQR) or n (%).

**PNPLA3* rs738409 genotypes reported as percentage of valid observations

Abbreviations: N/A: not available; INR: International normalised ratio; MELD: Model for end-stage liver disease

3.5.4 Relationship between Laennec grade and collagen proportionate area

The Laennec fibrosis grade was successfully determined in all 135 cases included in the sensitivity analysis of fibrosis. The majority of cases (120/135, 89%) had at least definite or probable cirrhosis (Laennec grade 4A and above) with severe cirrhosis the single largest category (52/135, 39%). The collagen proportionate area was successfully determined in 105/135 (78%) cases. There was a strong correlation between the Laennec grade and CPA ($\tau = 0.50$, $p<0.00001$). Significant overlap was seen in CPA ranges for the different Laennec grades (Figure 3.1, Table 3.7) and thus the intraclass correlation coefficient (ICC) was accordingly low (ICC = 0.101).

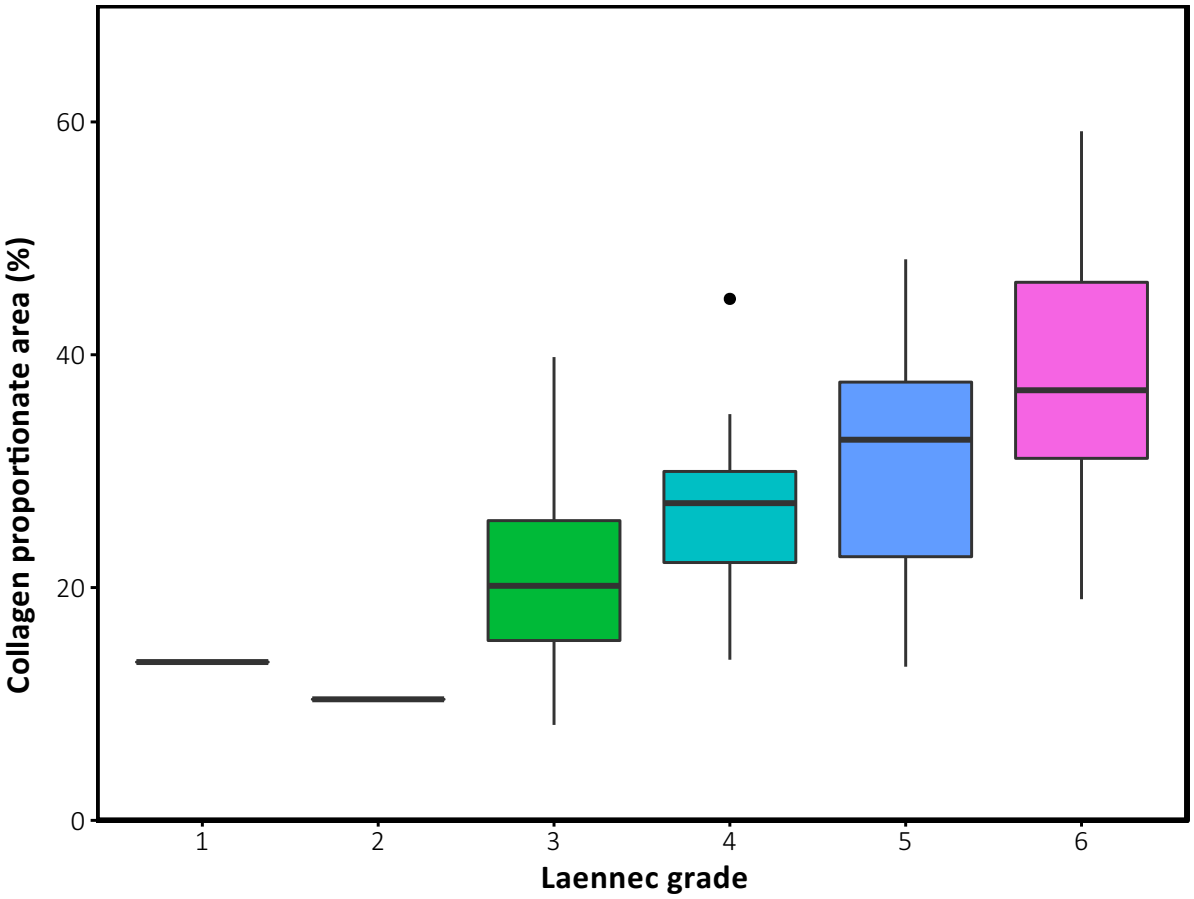


Figure 3.1. Measured collagen proportionate area (CPA) measurements, by Laennec fibrosis grade

Data are displayed as the median (solid bar), interquartile range (box) and 95% confidence interval (whiskers); dots indicate outliers.

Table 3.7 Collagen proportionate area, by Laennec fibrosis grade

Laennec grade	Cases (n)	CPA (IQR,%)
1	1	13.6 (-)
2	1	10.4 (-)
3	8	20.2 (15.0 – 32.3)
4A	16	27.3 (21.5 – 30.5)
4B	36	32.8 (22.5 – 38.9)
4C	43	37.4 (31.0 – 46.3)

Abbreviations: CPA: Collagen proportionate area; IQR: Interquartile range

3.5.5 Genetic variation and collagen proportionate area

Although the proportion of patients with moderate or severe cirrhosis (Laennec grades 4B and 4C) was greater in those homozygous for rs738409:G (CC: 70%, CG: 68%, GG: 85%; Table 3.8), there was no significant relationship between Laennec fibrosis grade and *PNPLA3* rs738409 genotype (p=0.36). This finding was consistent on sensitivity analysis (p=0.26). A significant regression model was found (F (1,127) = 8.67, p<0.01, R^2=0.06) with a positive relationship between *PNPLA3* rs738409:G and the collagen proportionate area (Beta=3.86, 95% CI 1.27 – 6.45, Table 3.8), this was robust on sensitivity analysis (p=0.001) and evaluation of only unimputed data (p=0.01).

There was no correlation between the Laennec fibrosis grade and number of risk alleles (A) for rs1183620 (rho = -0.06, p=0.53), nor could a significant regression model be fitted relating rs11183620 genotype and the CPA (F (1,127) = -1.58, p=0.21, R^2=0.01).

3.5.6 Genetic variation and fat proportionate area

The fat proportionate area was successfully determined in 119/129 (92%) of cases included in the sensitivity analysis of steatosis. The median fat proportionate area was 8.1% (IQR 4.8 – 13.0%). Following adjustment for the collagen proportionate area this was 11.9% (IQR 7.8 – 19.0%). A significant regression model was found (F (1,113) = 5.65, p=0.02, R^2=0.05) with a negative relationship between *PNPLA3* rs738409:G and the adjusted fat proportionate area (Beta= -3.03, 95% CI -5.56 – -0.50, Table 3.8), this was robust on sensitivity analysis (p=0.03) and evaluation of only unimputed data (p=0.02). However, there was no relationship between rs11183620 genotype and the FPA (F (1,113) = 2.02, p=0.16, R^2=0.02). These findings were also confirmed on sensitivity analyses.

Table 3.8 Distribution of Laennec fibrosis grades, CPA and adjusted FPA, by rs738409 genotype

	rs738409 genotype		
	CC (n=60)	CG (n=56)	GG (n=13)
Laennec grade			
0	0	0	0
1	1 (2%)	0 (0%)	0 (0%)
2	0 (0%)	1 (2%)	0 (0%)
3	5 (8%)	7 (13%)	1 (8%)
4A	12 (20%)	10 (18%)	1 (8%)
4B	21 (35%)	17 (30%)	3 (23%)
4C	21 (35%)	21 (38%)	8 (62%)
Collagen proportionate area (%)			
	29.7 (22.4 – 36.0)	32.9 (24.4 – 37.4)	42.2 (28.6 – 48.3)
Adjusted fat proportionate area (%)*			
	13.2 (10.0 – 21.5)	10.9 (7.9 – 17.5)	8.5 (6.3 – 12.4)

Data are shown as n (%) except CPA and FPA which are median (IQR)

*For the adjusted fat proportionate area, numbers per genotypic group were: CC: 53; CG: 50; GG: 12

3.5.7 Influence of diabetes and body mass index on CPA and FPA

Valid regression models could not be fitted for any of the variables considered in relation to the CPA or adjusted FPA. No difference was seen in the CPA in groups defined by the presence or absence of diabetes (median: 32.0% (IQR: 23.5 – 37.4%) *vs*. 30.4% (21.0 – 36.2%), p=0.78). Similarly, there was no correlation between the CPA and BMI (rho = 0.06, p=0.57). These findings were all robust to sensitivity analysis.

There was no difference in the adjusted FPA in groups defined by the presence or absence of diabetes (median: 11.7% (IQR: 8.2 – 19.8%) *vs*. 12.3% (8.3 – 19.6%), p=0.99). Similarly, there was no correlation between adjusted FPA and BMI (rho = 0.002, p=0.99). These findings were all robust to sensitivity analysis.

3.5.8 Post-hoc adjusted analyses of CPA and FPA

Incorporation of body mass index and diabetes status with *PNPLA3* rs738409 genotype into multiple linear regression models produced non-significant regression models for both CPA (F (4, 96) = 1.49, p=0.21, R^2=0.06) and the adjusted FPA (F (4, 83) = 1.32, p=0.27, R^2=0.06). Thus although the relationships between rs738409:G and both CPA and adjusted FPA were apparently independent of body mass index and diabetes these results must be interpreted with caution (Tables 3.9 and 3.10). These findings were consistent on sensitivity analysis. In the absence of a significant association on initial analysis, adjusted analyses were not performed for rs11183620.

Table 3.9 Multiple linear regression analysis of *PNPLA3* rs738409 genotype and CPA

Variable	B	95% CI	P
rs738409:G	3.68	0.62 – 6.70	0.02
Diabetes	-0.56	-7.85 – 6.74	0.88
Body mass index (kg/m^2)	0.01	-0.35 – 0.37	0.94

Abbreviations: B: Beta; CI: Confidence interval

Table 3.10 Multiple linear regression analysis of *PNPLA3* rs738409 genotype and adjusted FPA

Variable	B	95% CI	P
rs738409:G	-3.08	-6.21 – 0.06	0.05
Diabetes	0.40	-6.68 – 7.48	0.91
Body mass index (kg/m^2)	0.05	-0.32 – 0.42	0.80

Abbreviations: B: Beta; CI: Confidence interval

3.5.9 Genetic variation and other features of steatohepatitis

There was a significant positive association between *PNPLA3* rs738409:G and the presence of severe inflammation (p=0.003, Table 3.11). There was no association of genotype and the presence of severe hepatocyte ballooning (p=0.43), megamitochondria (p=0.30) or Mallory–Denk bodies (p=0.06); this was confirmed in the sensitivity analyses except for the Mallory bodies which showed a positive association with genotype within the extended time frame (Table 3.11). No differences were observed in the presence of severe inflammation, severe ballooning, megamitochondria or Mallory-Denk bodies based upon *SLC38A4* rs11183620 genotype (Supplementary Results, Table 10.2).

Table 3.11 Histological features of alcoholic steatohepatitis, by rs738409 genotype

PNPLA3 rs738409 genotype	CC	CG	GG	Significance (p)
Primary analysis	**n=53**	**n=50**	**n=12**	
Severe inflammation	4 (8%)	16 (32%)	4 (33%)	0.003
Severe hepatocyte ballooning	29 (55%)	31 (62%)	5 (42%)	0.43
Megamitochondria	24 (45%)	21 (42%)	8 (67%)	0.30
Mallory-Denk bodies	26 (49%)	36 (72%)	7 (58%)	0.06
Sensitivity analysis	**n=60**	**n=56**	**n=13**	
Severe inflammation	7 (12%)	18 (32%)	4 (31%)	0.02
Severe hepatocyte ballooning	34 (57%)	35 (63%)	5 (39%)	0.29
Megamitochondria	25 (42%)	22 (39%)	8 (62%)	0.38
Mallory-Denk bodies	30 (50%)	41 (72%)	7 (54%)	0.03

Data are shown as n (%)

3.5.10 Serum markers of epithelial cell death and histological features

Sufficient serum was available to estimate serum markers of epithelial cell death in a subset of 97 patients from the fibrosis primary analysis population, of these 86 were in the primary analysis population of other features of alcoholic steatohepatitis. Serum sFas, CK18-M65 and CK-M30 were successfully determined in 98%, 100% and 100% of samples assayed, respectively. Unsurprisingly CK18-M30 and CK18-M65 were strongly correlated ($r=0.86$, $p<0.001$). CK18-M65 but not CK18-M30 was weakly correlated with sFas ($r=0.25$, $p=0.01$ and $r=0.11$, $p=0.28$, respectively).

Serum CK18-M30 and M65 were strongly associated with the severity of inflammation and hepatocyte ballooning, as well as the presence of Mallory-Denk bodies and megamitochondria (Table 3.12). A weak negative correlation was noted between both CK18-M30 and M65 and the adjusted fat proportionate area but not the collagen proportionate area. Serum sFas was not associated with the severity or presence of any of inflammation, hepatocyte ballooning, Mallory-Denk bodies or

megamitochondria. It was, however, weakly positively correlated with the adjusted fat proportionate area and negatively correlated with collagen proportionate area (Table 3.12).

3.5.11 Genetic variation and serum markers of epithelial cell death

Carriage and homozygosity for rs738409:G was associated with increased serum levels of CK18-M30 (CC: median 771 U/L (IQR 435-1111); CG: 1000 (612 – 2886), GG: 1349 (761 – 5776), p=0.01). A similar trend was seen for CK18-M65 with the inverse the case for sFas however neither of these reached statistical significance (p=0.06 and p=0.08, Table 3.12).

In contrast carriage or homozygosity for rs11183620:G was not associated with any significant difference in serum levels of CK18-M30 (AA: 873 U/L (557 – 2413); AG: 941 (434 – 3121); GG: 863 (570 – 3636), p=0.60), CK18-M65 (AA: 2335 U/L (1377 – 3880); AG: 3102 (1296 – 4887); GG: 2026 (1308 – 16244), p=0.72) or sFas (AA: 13364 U/L (10566 – 17728); AG: 11934 (9973 – 15915); GG: 12546 (9460 – 15466), p=0.25).

Table 3.12 Associations between serum markers of epithelial cell death, histological features of alcoholic steatohepatitis and *PNPLA3* rs738409 genotype

Feature (n)	CK18-M30 (U/L)	CK18-M65 (U/L)	sFas (pg/ml)
Inflammation			
Mild (n=17)	771 (495 – 2129)	2031 (1275 – 4587)	12027 (9845 – 16181)
Severe (n=69)	1726 (1000 – 6702)	5000 (3428 – 23952)	12873 (11835 – 17162)
Significance (p)	<0.001	<0.001	0.23
Ballooning			
Occasional (n=40)	675 (495 – 1743)	1954 (1051 – 3957)	12121 (9593 – 14871)
Marked (n=46)	1000 (828 – 3598)	34667 (1699 – 15532)	12611 (10122 – 17754)
Significance (p)	0.005	0.01	0.21
Mallory-Denk bodies			
Absent (n=36)	650 (437 – 945)	1706 (909 – 2704)	12215 (9971 – 14751)
Present(n=50)	1000 (878 – 3328)	3970 (2021 – 17263)	12671 (10026 – 18206)
Significance (p)	<0.001	<0.001	0.37
Megamitochondria			
Absent (n=46)	1000 (482 – 2037)	2951 (1298 – 4179)	12712 (10120 – 16728)
Present (n=40)	1000 (622 – 2736)	2361 (1462 – 14704)	11895 (9972 – 16035)
Significance (p)	<0.001	<0.0001	0.37
Adjusted fat proportionate area (n=86)			
Correlation (r)	-0.25	-0.26	0.29
Significance (p)	0.02	0.02	<0.01
Collagen proportionate area (n=97)			
Correlation (r)	0.05	0.02	-0.28
Significance (p)	0.64	0.89	<0.01
***PNPLA3* rs738409 genotype (n=97)**			
CC (n=43)	771 (435 – 1111)	2091 (1452 – 3749)	12709 (10566 – 17761)
CG (n=44)	1000 (612 – 2886)	3608 (1116 – 15291)	12225 (9971 – 15898)
GG (n=10)	1349 (761 – 5776)	3769 (1301 – 23855)	9845 (7649 – 13963)
Significance (p)	0.01	0.06	0.08

Data shown as median (interquartile range)

3.6 Discussion

The variant rs738409:G in *PNPLA3* has been consistently associated with the risk of developing alcohol-related cirrhosis[151, 190, 191, 437]. Additionally carriage of rs738409:G has been associated with more advanced fibrosis, extensive steatosis and florid steatohepatitis in patients with a history of alcohol misuse, including in those with cirrhosis, suggesting that it may further influence the severity of any histological lesion within a cohort of patients[190, 437, 438]. An association between rs738409:G in *PNPLA3* and the risk of developing severe alcoholic hepatitis has been described in one small series, published in abstract form[148] and confirmed through the work described in this thesis. In addition, this work describes an association between a variant (rs11183620) at a novel locus (*SLC38A4*) and the risk of developing severe alcoholic hepatitis. To date, the potential impact of genetic polymorphisms on disease presentation and histology in severe alcoholic hepatitis has not been evaluated. The results presented in this chapter help clarify some of these associations.

First: there is no evidence that rs738409 genotype plays a role in determining the onset, mode of presentation or severity of alcoholic hepatitis. Thus, the age, gender distribution, quantity of alcohol consumed and disease severity, assessed using the available scoring systems, were similar in all subgroups defined, by genotype.

Second: analysis of histological data from patients presenting with severe alcoholic hepatitis indicates that carriage and homozygosity for rs738409:G are associated with increased fibrosis, hepatocyte ballooning and inflammation. This corroborates previous reports[190, 437{Rausch, 2016 #294)] and indicates that even in those patients with the most advanced and severe forms of alcohol-related liver disease the variant continues to exert some influence on histological appearances. The association with fibrosis and steatosis appears to persist even after adjustment for known confounders. The association between rs738409:G and increasing levels of CK18-M30 provides a form of objective corroboration of the association with more severe steatohepatitis unaffected by issues such as timing with respect to

treatment, sampling error and the subjective grading of histological features. The finding that rs738409:G is apparently associated with less marked steatosis than the wild type allele is surprising as previous reports have associated the variant with increased steatosis[192, 394, 439]. A potential explanation is that the severity of fibrosis in this cohort of patients means that hepatic steatosis is reduced simply as a function of decreased parenchymal tissue. However, the relationship persists even after adjustment of the FPA for the degree of fibrosis. A link has previously been drawn between higher *PNPLA3* mRNA and more extensive steatosis[397]. Consequently, epigenetic modifications associated with *PNPLA3* rs738409 genotype and severe fibrosis[440] may lead to a downregulation of *PNPLA3* expression in advanced liver disease and alter the association between genotype and hepatic steatosis seen in milder disease. Studies in the field of non-alcoholic fatty liver disease indicate that the development of more severe fibrosis is associated with changes in the pattern and quantity of steatosis[441, 442]. Thus, the reduction in steatosis seen with carriage of rs738409:G here may be driven by its association with more advanced fibrosis rather than any direct causal link.

Finally: the genetic variant rs11183620 in *SLC38A4* does not appear to influence either the clinical features at presentation or the histopathological appearances of ASH on biopsy in patients with severe alcoholic hepatitis.

This study has a number of limitations *viz.*: (i) although the overall cohort size was large, the number of patients undergoing liver biopsy was small; as a consequence the reduction in the numbers of patients heterozygous or homozygous for rs738409:G does lead to limitations of statistical power; (ii) the requirement to perform transjugular liver biopsy results in small, often fragmented samples which may be more difficult or inadequate to interpret, either manually or using image analysis, and increase the risks of sampling error; (iii) all biopsies were not taken contemporaneously with the start of treatment, thus an effect of treatment or time on histological appearances cannot be definitively excluded. Clear, pre-defined time windows for inclusion and the performance of sensitivity analyses

have been used to mitigate against this, whilst baseline pre-treatment serum markers provide a degree of corroboration.

In conclusion: Although rs738409:G in *PNPLA3* does not appear to influence the onset, mode of presentation or severity of disease in severe alcoholic hepatitis, there is an apparent association with the severity of steatohepatitis and fibrosis evidence on liver biopsy. This is reflected in higher serum levels of biomarkers of hepatocellular injury. These differences could translate into an effect on clinical outcomes including treatment response and mortality.

CHAPTER 4

GENETIC VARIANTS AND OUTCOMES FROM SEVERE ALCOHOLIC HEPATITIS

4 The influence of genetic variation on outcomes from severe alcoholic hepatitis

4.1 Overview

The G allele of rs738409 in *PNPLA3* is associated with the risk of developing severe alcoholic hepatitis (Chapter 2). Furthermore, it also appears to be associated with more severe histological lesions at presentation (Chapter 3). Carriage of the variant has also been associated with a number of negative outcomes in people with chronic liver disease of varying aetiologies including time to decompensation of liver disease and overall survival. In this chapter the impact of rs738409 genotype on the outcomes from severe alcoholic hepatitis, including short- and medium-term survival and treatment interactions, is examined. In light of the association between rs11183620 in *SLC38A4* genotype and the risk of developing severe alcoholic hepatitis the impact of this variant on the same aspects of severe alcoholic hepatitis is also examined.

4.2 Introduction

The role of genetic polymorphisms, especially rs738409 in *PNPLA3*, in determining outcomes in liver disease has received considerable attention in recent years. Growing evidence indicates that rs738409:G influences not only the development but also the clinical trajectory of alcohol-related liver disease. Thus, carriage of the G allele is associated with earlier development of cirrhosis, independently of the age of onset of at-risk alcohol consumption[443]; more rapid progression towards decompensated disease[444]; and a reduction in transplantation-free survival[444]. A recent study has reported that homozygosity for rs738409:G is associated with an increased risk of decompensation events and mortality in patients with chronic liver disease and portal hypertension due to fatty liver disease[445]. An association between rs738409:G and the development of hepatocellular carcinoma (HCC) in patients with non-alcoholic fatty liver disease has been described[446]. This tallies with several individual studies[447, 448, 449 , 450], and a meta-analysis[451] which indicate that, in patients with alcohol-

related cirrhosis, carriage of the risk allele, rs738409:G, is significantly associated with the development of HCC and with poorer outcomes following its development[452].

In a large Caucasian population of heavy drinkers carriage of rs738409:G was associated with higher levels of liver stiffness at presentation. Following the withdrawal of alcohol heterozygotes (CG), but not those homozygous for the minor allele (GG), displayed a reduction in liver stiffness to levels comparable to those homozygous for the major allele (CC) during the follow-up period[438]. These data suggest that *PNPLA3* rs738409 genotype may also influence the recovery of alcohol-related liver injury.

An association between *PNPLA3* rs738409 and the development of severe alcoholic hepatitis has been described in a small population of patients with severe alcoholic hepatitis but published in abstract form only[148]. Through the work described in this thesis this association has been replicated with a very high degree of confidence. Poor short-term prognosis in severe alcoholic hepatitis is known to be associated with high serum bilirubin and creatinine, significant prolongation of the prothrombin time, hepatic encephalopathy, hypoalbuminaemia and ascites[1, 208, 453]. Over the longer term, additional factors including gender, the presence of or evolution to cirrhosis and relapse in alcohol consumption come in to play[224, 236, 237, 250, 251, 454, 455]. It remains unknown whether the rs738409 genotype has influence over disease course and outcome in the setting of severe alcoholic hepatitis.

4.3 Aim

The aim of the analyses conducted and described in this chapter was to determine, in the context of severe alcoholic hepatitis, the influence of rs738409 genotype upon:

1. Short-term survival and response to treatment with prednisolone (defined by the Lille model);
2. Short-term changes in liver function – defined as 90 days from the start of treatment;
3. Medium-term survival;

In light of the association between the genotype of rs11183620 in *SLC38A4* and the risk of developing severe alcoholic hepatitis, the same analyses were also conducted for this variant on an exploratory basis in the sub-group of patients with available rs11183620 genotypes.

4.4 Patients, materials and methods

4.4.1 Severe alcoholic hepatitis patient cohort

The cohort of patients with severe alcoholic hepatitis used in these analyses are the same as those described in Chapter 3.

4.4.2 PNPLA3 rs738409 genotyping

Genotyping for rs738409 in *PNPLA3* and rs11183620 in *SLC38A4* was performed using the K-Biosciences Competitive Allele Specific Polymerase Chain Reaction (PCR) (LGC Genomics, Hoddesdon, UK) platform as described in Chapter 3.

4.4.3 Return to drinking data

Data regarding current drinking behaviour were collected at the day 90 and 1-year follow-up visits. Patients self-categorised their current drinking behaviour at day 90 as (i) abstinent; (ii) drinking at low levels: men ≤24g/day; women: ≤16 g/day; (iii) drinking at moderate levels: men >24 but ≤60g/day; women >16 but ≤40 g/day; (iv) drinking at high levels: men >60g/day; women >40g/day.

4.4.4 Data processing

4.4.4.1 Haematological and biochemical data

Prognostic scores were calculated at baseline (DF[1], Model for End-stage Liver Disease [MELD][5] and Glasgow Alcoholic Hepatitis Score [GAHS][3]) and after 7 days of treatment (Lille score[4]). Changes in bilirubin, albumin and the international normalised ratio (INR) between baseline and the day 90 visit were calculated and corrected for the number of days which had elapsed between the two measurements to give a rate of change.

4.4.4.2 Return to drinking data

Information on drinking behaviour post hospital discharge was available in 397 (46%) of the 867 cases who were successfully genotyped at day 90 and in 174 (20.1%) at one year. For purposes of genetic statistical analysis patients were classified as either abstinent (i) or drinking (ii-iv) due to comparatively small numbers of individuals returning to drinking in the various categories. In view of the relatively high incidence of missing data on drinking behaviour at the day 90 and 1-year time points additional sensitivity analyses were undertaken based upon the following principles:

1. Reclassification of drinking behaviour at day 90 in light of additional information at 1 year, where available;

2. The assumption that individuals in whom information on drinking behaviour was not available at day 90, for any reason, had returned to drinking;

4.4.4.3 Survival data

Survival times, and mortality endpoints, were calculated as the time elapsed between the treatment start date or, if not recorded, the date of randomization and the date on which the NHS Information Centre Data Linkage service database was queried. A data cut-off of 450 days, corresponding to 1 year after the day 90 visit, was applied because the large variation in follow-up times engendered a risk of informative censorship, disproportionate censorship between genotypic groups and the likely impact of additional factors such as delayed return to drinking and development of co-morbid disease on longer-term survival, about which little or no information was available. Thus, cases were censored at the time of liver transplantation, the limit of follow-up or day 450, whichever occurred first.

4.4.5 Statistical analyses

Statistical analyses were performed using SPSS version 22 (IBM, Armonk, USA). Survival curves and plots were generated in R[344] using the packages ggplot2, survival, gridExtra, reshape and plyr.

Cox proportional hazards models were used to test associations between explanatory variables and survival. Interactions between explanatory variables in relation to survival were tested by introduction of multiplicative interaction terms into the Cox proportional hazards model. Interactions were specifically sought between genotypes and:

1. Treatment with prednisolone in relation to 28-day survival;
2. Haematological and biochemical parameters and drinking behaviour at day 90 in relation to 450-day survival;

Where significant interactions were found univariate and multivariate analyses were undertaken in relevant population sub-groups to better understand the main effects of the covariates on outcome.

Tests of genotypic association were performed using three models of inheritance: additive (P_{ADD}), recessive (P_{REC}) and dominant (P_{DOM}). The model showing the greatest statistical significance was used in subsequent multivariable analysis. Separate models were fitted for clinically relevant features and biochemical parameters. Variables demonstrating marginal statistical significance ($p<0.1$) in univariate analyses were included in multivariable analyses in order to refine the number of explanatory variables included in the model whilst avoiding reporting spurious associations unique to this dataset. These models were fitted by backward elimination with a cut-off ($p=0.05$). Where a composite variable and its constituents were both associated with outcome only the most significantly associated was incorporated into the multivariable analysis in order to reduce co-linearity.

For recovery of liver function analyses the Mann-Whitney U test was used to test for differences between groups dichotomised by prednisolone treatment status. Correlations between the level of alcohol relapse and recovery in liver function were tested using the Spearman's rank test. The influence of rs738409 genotype on rates of recovery in liver function was tested by comparison of features across all three genotypic groups, using linear regression. Residual distribution and normal probability plots were inspected to ensure model validity, where these were violated or a significant

regression model could not be found the Jonckheere-Terpstra test was used to compare across genotypic groups. In light of the known impact of drinking on recovery in liver function, genotypic groups were also compared separately in groups dichotomised by a self-reported return to drinking at day 90.

4.5 Results

4.5.1 Genotyping

The population and genotyping data used in these analyses were the same as in Chapter 3. Thus, the distribution of genotypes for rs738409 was: CC 425/867 (49%), CG 372/867 (43%) and GG 70/867 (8%) with a minor allele frequency of 30%. For *SLC38A4* rs11183620 the distribution of genotypes was: AA 236/860 (27%), AG 424/860 (49%) and GG 200/860 (23%), with a minor allele frequency of 48%.

4.5.2 Impact of genetic variation on short-term survival and treatment response

One-hundred and thirty-one (15.0%) of the 867 cases with severe alcoholic hepatitis had died by day 28 while a further three were lost to follow-up. There was no significant relationship between 28–day mortality and rs738409 genotype (P_{ADD} = 0.95, P_{DOM} = 0.88, P_{REC} = 0.64; Figure 4.1). Treatment with prednisolone was associated with a decreased risk of mortality compared with placebo (Hazard Ratio [HR]=0.67; 95% CI 0.48–0.95, p=0.03). No interaction was detected between rs738409 genotype and any of the scoring systems in relation to 28-day mortality.

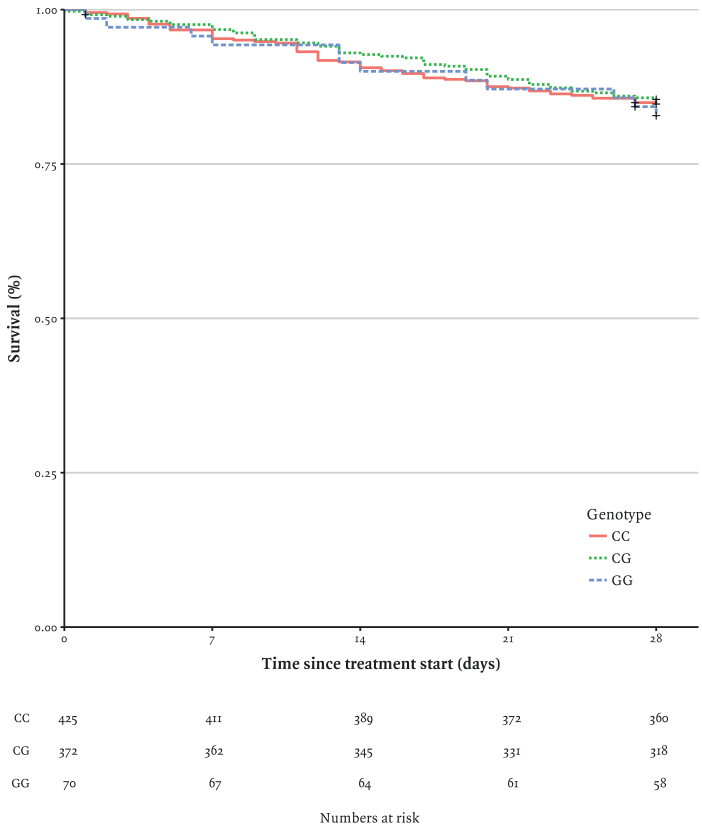


Figure 4.1 Kaplan-Meier survival functions illustrating 28-day survival for patients with severe alcoholic hepatitis, by rs738409 genotype

There was also no association between 90-day mortality and rs738409 genotype (P_{ADD} = 0.79, P_{DOM} = 0.76, P_{REC} = 0.85; Table 4.1).

Similarly, there was no association between rs11183620 genotype and 28-day (Hazard ratio (HR) 0.94, 95% Confidence Interval (CI) 0.74 – 1.20), P_{ADD} = 0.64; HR 1.19, 95% CI 0.80 – 1.77, P_{DOM} = 0.39 and HR 0.68, 95% CI 0.43 – 1.07, P_{REC} = 0.09) or 90-day mortality (P_{ADD} = 0.62, P_{DOM} = 0.99, P_{REC} = 0.39; Supplementary Results, Table 10.3).

Table 4.1 Ninety-day mortality in cases with severe alcoholic hepatitis, by treatment allocation and rs738409 genotype

Treatment allocation	Cases (n)	Overall deaths (n: %)	Deaths, by rs734809 genotype (n: %)		
			CC (n=425)	CG (n=372)	GG (n=70)
Prednisolone	429	105 (24.5%)	47 (22.3%)	46 (25.6%)	12 (30.8%)
No prednisolone	438	111 (25.3%)	57 (26.6%)	48 (24.9%)	6 (19.4%)
Total	**867**	**216 (24.9%)**	**104 (24.4%)**	**54 (25.2%)**	**18 (25.7%)**

Data presented as number (%)

No significant interaction was detected between either rs738409 or rs11183620 genotype and prednisolone treatment in relation to 28-day or 90-day mortality. Lille scores were calculable in 575 patients. There was no significant difference in the distribution of Lille scores or proportion of responders (Lille score <0.45), by rs738409 genotypic group (Table 4.2). There was no difference in the distribution of Lille scores (AA: median 0.44, IQR [0.20 – 0.78]; AG: 0.37 [0.12 – 0.79); GG: 0.37 [0.14 – 0.74], p=0.19) or the proportion of Lille responders (AA: 76/157, 48%; AG: 157/294, 53%; GG: 77/139, 55%, p=0.22), by rs11183620 genotype. No interaction was observed between rs11183620 genotype and prednisolone in relation to Lille response.

Table 4.2 Distribution of Lille scores and proportion of Lille responders, by rs738409 genotype

	CC (n = 292)	CG (n = 246)	GG (n = 37)	Significance (p)
Lille score	0.37 (0.12 – 0.79)	0.45 (0.14 – 0.79)	0.33 (0.11 – 0.74)	**0.55**
Lille response (<0.45)	158 (54.1%)	119 (48.3%)	23 (62.2%)	**0.19**

Data presented as median (interquartile rang) or number (%)

Randomisation risk, treatment with prednisolone, age, the presence of overt hepatic encephalopathy, total white blood cell and neutrophil counts, blood urea, INR, serum bilirubin and creatinine were significantly associated with 28-day mortality (Table 4.3). Multivariable analysis, incorporating the variables associated on univariate analysis (p<0.1), together with a term for homozygosity for rs738409:G, confirmed significant, independent associations with 28-day survival for many of the variables identified in univariate analyses, including prednisolone treatment; homozygosity for rs738409:G was not independently associated (Table 4.3). When adjusted for the same factors no independent association between homozygosity for rs11183620:G and 28-day survival was observed (adjusted HR 0.64, 95% CI 0.39 – 1.05, p=0.078).

Table 4.3 Univariate and multivariable Cox proportional hazards regression analyses for factors associated with 28-day survival in cases with severe alcoholic hepatitis

Variable	Univariate			Multivariable		
	HR	95% CI	Significance (p)	HR	95% CI	Significance (p)
Age	1.05	1.04 – 1.07	<0.001	1.04	1.02 – 1.07	<0.001
Gender	0.88	0.74 – 1.06	0.19			
Alcohol consumption§	1.00	0.99 – 1.00	0.10			
Overt hepatic encephalopathy	2.85	2.02 – 4.02	<0.001	2.46	1.55 – 3.90	<0.001
White cell count* ($x10^6/mm^3$)	1.08	1.06 – 1.11	<0.001			
Neutrophils ($x10^6/mm^3$)	1.09	1.06 – 1.12	<0.001	1.06	1.02 – 1.09	0.001
Bilirubin (µmol/l)	1.003	1.002 – 1.005	<0.001	1.001	1.000 – 1.003	0.09
AST (IU/l)§	1.002	1.000 – 1.005	0.09			
ALP (IU/l)	0.999	0.997 – 1.001	0.45			
Albumin (g/l)	0.99	0.97 – 1.02	0.67			
Urea (mmol/l)	1.09	1.07 – 1.12	<0.001	1.11	1.07 – 1.15	<0.001
Creatinine (µmol/l)§	1.01	1.008 – 1.013	<0.001			
International normalised ratio	1.21	1.06 – 1.38	0.004	1.27	1.06 – 1.51	0.009
Randomisation risk§	1.51	1.26 – 1.81	<0.001			
rs738409:G homozygosity§	1.15	0.64 – 2.09	0.64			
Prednisolone	0.67	0.48 – 0.95	0.03	0.59	0.37 – 0.93	0.02

Abbreviations**:** ALP: Alkaline phosphatase; AST: Aspartate transaminase; HR: Hazard Ratio; CI: Confidence Intervals

* Variable not entered into the multivariable analysis due to co-linearity

§ Variable excluded by backward elimination due to lack of significant independent association

4.5.3 *Prednisolone and recovery of liver function*

Paired information on rs738409 genotype and laboratory variables at baseline and the day 90 visit was available in 377 cases. The median time between treatment start and completion of the day 90 visit was 92 days (IQR 89 – 103). There was no impact of prednisolone on the rate of recovery of any of bilirubin (non-prednisolone: -1.89 µmol/l/day [IQR -1.02 – -3.23] *vs.* prednisolone: -1.96 µmol/l/day [IQR -0.80 – -3.35], p=0.79), albumin (-0.063 g/l/day [-0.12 – -0.02] *vs.* -0.067 [-0.13 – -0.02], p=0.97) or the international normalised ratio (0.003 U/day [0.001 – 0.006] *vs*. 0.003 [0.001 – 0.006], p=0.50).

4.5.4 *Drinking and recovery of liver function*

Information on drinking behaviour was available in 365 (97%) of the 377 patients for whom there was paired biochemical data. Moderate correlations were noted between the level of alcohol relapse and rate of change in serum bilirubin (rho = -0.22, p<0.0001), serum albumin (rho = 0.19, p<0.0001) and international normalised ratio (rho = -0.19, p=0.0001, Figure 4.2, Table 4.4).

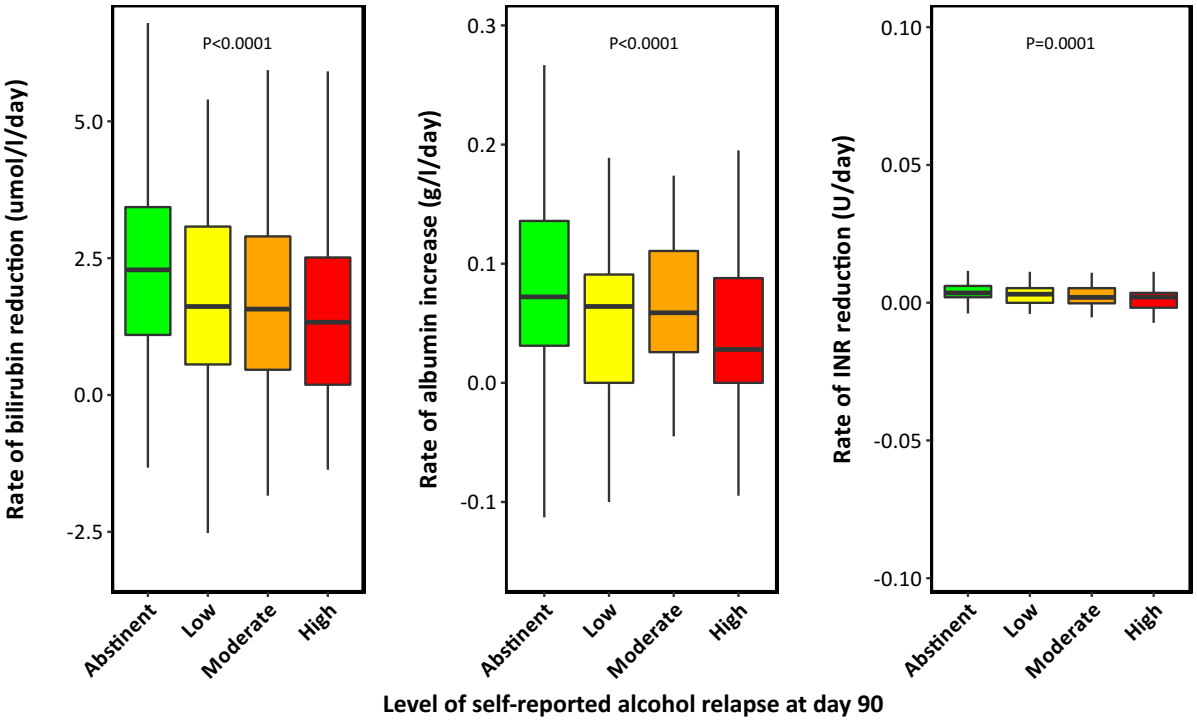


Figure 4.2 The rate of change in markers of liver function between the start of treatment and the day 90 visit, by the level of self-reported alcohol relapse

Data are displayed as the median (solid bar), interquartile range (box) and 95% confidence interval (whiskers).

Table 4.4 Rates of recovery of serum bilirubin and albumin and the international normalised ratio, by level of self-reported alcohol relapse at day 90

Drinking category	Alcohol consumption (g/day)	Fall in serum bilirubin (µnol/l/day)	Increase in serum albumin (g/l/day)	Reduction in INR (U/day)
Abstinent	Men: 0 Women: 0	2.29 (1.09 – 3.44)	0.07 (0.03 – 0.14)	0.004 (0.002 – 0.006)
Low	Men: ≤24 Women: ≤16	1.62 (0.55 – 3.19)	0.06 (0.00 – 0.09)	0.003 (0.000 – 0.005)
Moderate	Men: 24 to ≤60 Women: 16 to ≤40	1.57 (0.43 – 3.05)	0.06 (0.03 – 0.11)	0.002 (0.000 – 0.006)
High	Men: >60 Women: >40	1.33 (0.16 – 2.55)	0.03 (-0.01 – 0.09)	0.002 (-0.002 – 0.004)

Data shown as median (interquartile range)

4.5.5 Impact of rs738409 genotype on recovery of liver function

Carriage of rs738409:G was associated with a slower decline in median serum bilirubin concentration (CC: 2.47 µmol/l/day [IQR 1.31 – 3.44]; CG: 1.65 [0.75 – 3.32]; GG 1.16 [0.63 – 1.68], p<0.001) and a slower recovery in serum albumin concentration (CC: 0.073 g/l/day [0.025 – 0.139]; CG: 0.064 [0.011 – 0.117]; GG 0.040 [0.089 – 0.010], p=0.04), but did not appear to influence recovery of the INR (CC: 0.0033 U/day [IQR 0.0011 – 0.0054]; CG: 0.0031 [0.0012 – 0.0062]; GG 0.0022 [-0.0004 – 0.0057], p=0.90) (Figure 4.3). These findings were robust to normalisation of the change in the biochemical parameter to the baseline value in order to give an effective percentage change. There was no association between rs11183620 genotype and the recovery in any of the INR, serum bilirubin or albumin (p=0.94, p=0.60 and p=0.45, respectively, Supplementary Results, Figure 10.1).

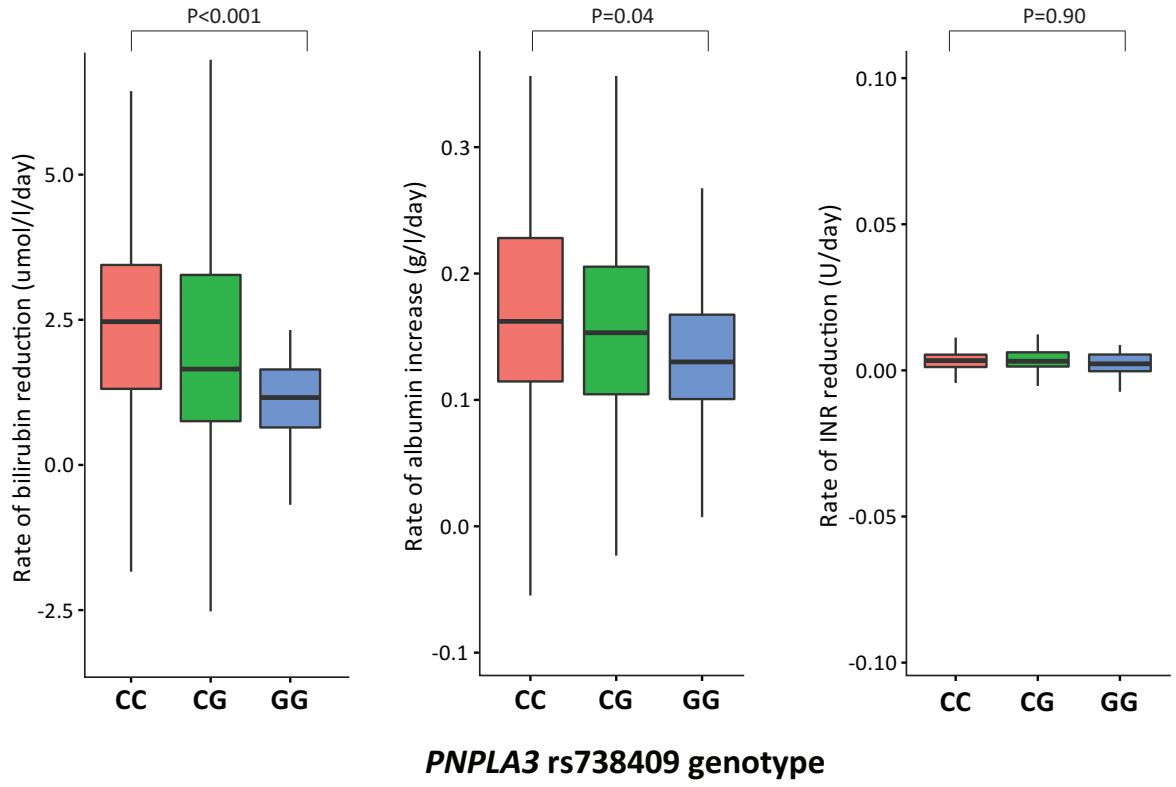


Figure 4.3 Rate of recovery in biomarkers of liver function over the 90 days since the start of treatment in patients with severe alcoholic hepatitis, by *PNPLA3* rs738409 genotype

Data are displayed as the median (solid bar), interquartile range (box) and 95% confidence interval (whiskers).

Significant linear regression models were successfully fitted for both the rate of recovery in serum bilirubin ((F (2, 362) = 16.1, p<0.001, R^2=0.08) and albumin (F (2, 356) = 7.14, p=0.001, R^2=0.04). In both cases there was an independent effect of the level of alcohol relapse and rs738409:G (Table 4.5). In the absence of an unadjusted association, adjusted analyses were not conducted for rs11183620 genotype and the rates of recovery of bilirubin, albumin or INR.

Table 4.5 Multiple linear regression analysis of recovery in serum bilirubin and albumin

Variable	B	95% CI	P
Recovery in serum bilirubin			
rs738409:G	-0.56	-0.85 - -0.28	<0.001
Level of alcohol relapse*	-0.38	-0.55 - -0.21	<0.001
Recovery in serum albumin			
rs738409:G	-0.02	-0.031 – 0.004	0.01
Level of alcohol relapse*	-0.01	-0.020 - -0.004	<0.01

*Level of alcohol relapse coded 0-3 corresponding to abstinence, low, moderate and high levels of alcohol relapse

4.5.6 *Impact of drinking behaviour on medium-term survival*

Reported abstinence rates at day 90 and 1 year were 65% and 57% respectively. In the 138 patients who had returned to drinking at day 90 the distribution between the three drinking categories was broadly equal (Table 4.6). Significant differences in survival to day 450 were observed in relation to drinking behaviour recorded at day 90 with a dose-dependent increase in the HR for mortality (Figure 4.4, Table 4.6).

Table 4.6 Cox regression analysis of level of drinking reported at day 90 and survival to day 450 after presentation with severe alcoholic hepatitis

Drinking category	Alcohol consumption (g/day)	Cases (n)	HR	95% CI	Significance (p)
Abstinent	Men: 0 Women: 0	259	Reference	-	-
Low	Men: ≤24 Women: ≤16	51	2.09	1.13 – 3.88	0.02
Moderate	Men: 24 to ≤60 Women: 16 to ≤40	44	3.00	1.69 – 5.35	<0.001
High	Men: >60 Women: >40	43	3.31	1.86 – 3.90	<0.001

Abbreviations: CI: Confidence interval; HR: Hazard ratio

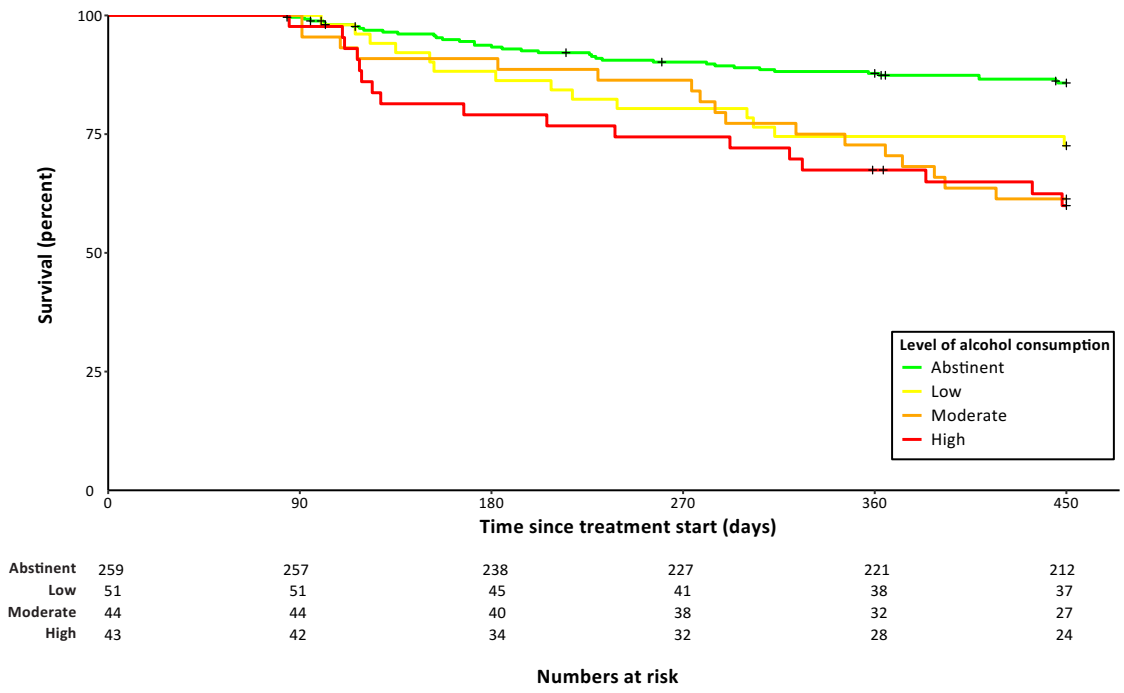


Figure 4.4 Survival in patients with severe alcoholic hepatitis alive at 90 days, by reported drinking behaviour
A clear dose-dependent increase in the risk of mortality at day 450 is seen with low, moderate and high-level alcohol relapse when compared with abstinence.

When reduced to a binary classification, mortality at day 450 in those who were drinking was 35.3% (47/133) *vs.* 14.3% (35/244) in those reporting abstinence (HR 2.77, 95% CI 1.79–4.29; p<0.00001, Figure 4.5B). This association was robust to the incorporation of the additional data on drinking behaviour collected at 1 year and remained when all cases with missing data at day 90 were assumed to have resumed drinking (Table 4.7).

Table 4.7 Cox regression analysis, including sensitivity analyses, of the association between drinking status at day 90 and survival to day 450 after presentation with severe alcoholic hepatitis

Term	HR	95% CI	Significance (p)
Observed cases only			
Return to drinking	2.77	1.79 – 4.29	<0.0001
Reclassification using 1 year data			
Return to drinking	2.34	1.52 – 3.61	0.0001
Reclassification of unknown status as drinking			
Return to drinking	2.21	1.51 – 3.24	<0.0001

Abbreviations: CI: Confidence interval; HR: Hazard ratio

4.5.7 *Impact of rs738409 genotype on medium-term survival*

In the cohort of patients surviving beyond day 90, homozygosity for rs738409:G was associated with a significant decrease in survival at day 450 (GG: 34.7% (17/49); CG: 21.8% (53/243); CC: 25.1% (74/295); P_{REC} = 0.04; [HR_{REC} 1.69, 95% CI 1.02 – 2.81]; P_{ADD} = 0.62; P_{DOM} = 0.67) (Figure 4.5A).

The association between rs738409 homozygosity and 450-day survival was independent of a return to drinking (Table 4.8).

Table 4.8 Multivariable Cox regression analysis of the association between drinking status at day 90, homozygosity for rs738409:G and survival to day 450 after presentation with severe alcoholic hepatitis

Term	HR	95% CI	Significance (p)
Homozygosity for rs738409:G	2.15	1.11 – 4.17	0.02
Return to drinking	2.87	1.85 – 4.45	<0.0001

Abbreviations: CI: Confidence interval; HR: Hazard ratio

Statistically significant interactions were identified between drinking behaviour and both serum bilirubin (p=0.004) and neutrophil count (p=0.002) at day 90 in relation to medium-term survival. Interactions between drinking behaviour and homozygosity for rs738409:G (p=0.11) and the INR at day 90 (p=0.09) did not reach statistical significance (Table 4.9). In view of these interactions, factors influencing medium-term survival were examined separately in groups defined by drinking status.

Table 4.9 Associations of interactions between day 90 variables and survival to day 450

Interaction term	HR	95% CI	Significance (p)
Age*Drinking	0.99	0.95 – 1.03	0.62
Gender*Drinking	1.66	0.73 – 3.75	0.23
Overt encephalopathy*Drinking	1.54	0.49 – 4.82	0.46
White cell count*Drinking	0.86	0.78 – 0.96	0.005
Neutrophils*Drinking	0.84	0.76 – 0.93	0.001
Bilirubin *Drinking	0.99	0.98 – 0.99	<0.001
Aspartate transaminase*Drinking	0.99	0.98 – 1.01	0.42
Alkaline phosphatase*Drinking	0.99	0.99 – 1.00	0.23
Albumin *Drinking	1.05	0.99 – 1.11	0.14
Urea*Drinking	1.01	0.99 – 1.22	0.09
Creatinine*Drinking	1.01	0.99 – 1.02	0.5
INR*Drinking	0.82	0.66 – 1.02	0.08
rs738409:GG*Drinking	0.26	0.05 – 1.32	0.11

Abbreviations: CI: Confidence interval; HR: Hazard ratio; INR: International normalised ratio

Tests for interaction were performed using Cox proportional hazards regression analysis; models incorporated the lower order effects but these are not shown within the table for ease of display. Each line represents the interaction term from a different Cox regression model.

In cases reporting drinking at day 90, homozygosity for rs738409:G had no statistically significant effect on survival; mortality rates were around 30% in all three genotypic groups over the 90 to 450 day period (Figure 4.5C). This lack of effect was confirmed on multivariable regression (Table 4.10). However, in cases reporting abstinence at day 90 homozygosity for rs738409:G was associated with a significantly higher mortality during the follow-up period (GG: 36.4% (8/22); CG 12.1% (13/107); CC 12.2% (14/115); HR 3.40, 95% CI 1.54–7.49, p=0.002) (Figure 4.5D). Cox multivariable regression analysis confirmed that homozygosity for rs738409:G was significantly and independently associated with reduced survival in this group (HR 2.56, 95% CI 1.03–6.34, p=0.04) (Table 4.11). These differences were maintained when drinking behavior was further refined based on the data collected at 1 year. Analyses undertaken assuming that patients in whom data on drinking behaviour at day 90 were not available had resumed drinking confirmed significant independent associations with 450-day survival

for both homozygosity for rs738409:G and drinking behaviour, as well as revealing a statistically significant interaction between the two (Table 4.12).

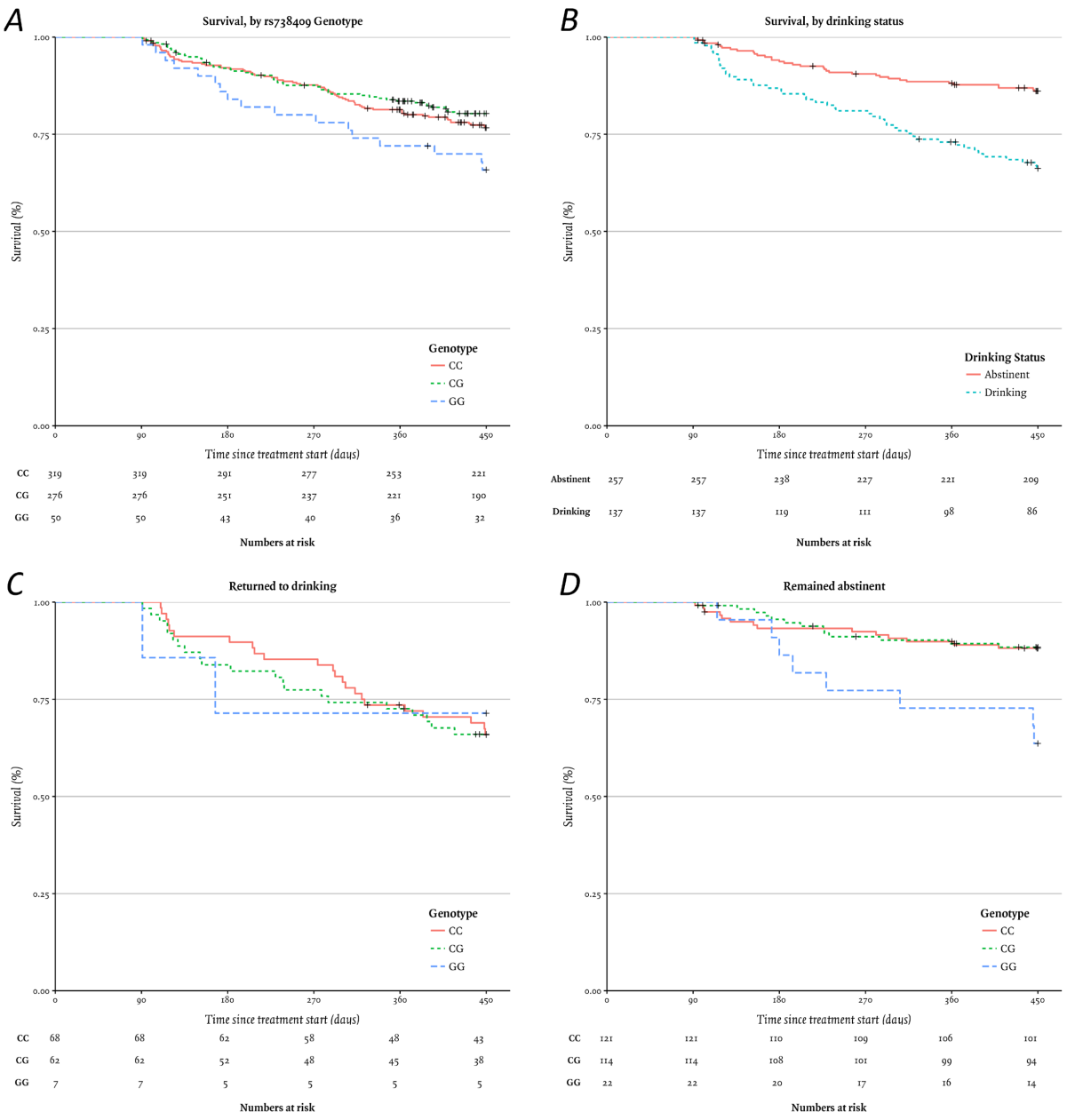


Figure 4.5. Medium-term survival in cases with severe alcoholic hepatitis surviving at least 90 days.

(A) Mortality was increased in cases homozygous for rs738409:G (GG: 34.7%; CG: 21.8%; CC: 25.1%; HR 1.69, 95% CI 1.02 – 2.81; P_{REC}=0.04). (B) Patients reporting alcohol consumption at day 90 have increased mortality at day 450 compared to those reporting abstinence (35.3% *vs.* 14.3% (HR 2.77, 95% CI 1.79–4.29; p<0.00001). (C) In cases who resumed drinking genotype did not affect outcome; (D) In cases who attained abstinence, survival was reduced in rs738409:G homozygotes (GG: 36.4%; CG 12.1%; CC 12.2%; HR 3.40, 95% CI 1.54–7.49, P_{REC}=0.002).

Table 4.10 Univariate and multivariable Cox proportional hazards regression analyses for factors associated with 450-day survival in cases with severe alcoholic hepatitis who resumed alcohol consumption

Variable	Univariate			Multivariable		
	HR	95% CI	Significance (p)	HR	95% CI	Significance (p)
Age	1.04	1.00 – 1.07	0.04			
Gender	1.68	0.95 – 2.98	0.08	2.02	1.05 – 3.90	0.04
Overt hepatic encephalopathy	2.34	1.12 – 4.90	0.02			
White cell count* ($x10^6/mm^3$)	1.07	1.00 – 1.13	0.04			
Neutrophils§ ($x10^6/mm^3$)	1.09	1.02 – 1.17	0.01			
Bilirubin (µmol/l)	1.004	1.002 – 1.006	<0.001	1.005	1.002 – 1.007	<0.001
AST (IU/l)†	1.01	1.001 – 1.011	0.01			
ALP (IU/l)	1.002	1.000 – 1.004	0.03	1.002	1.000 – 1.005	0.03
Albumin (g/l)	0.94	0.90 – 0.99	0.01			
Urea (mmol/l)	1.22	1.10 – 1.35	<0.001	1.23	1.10 – 1.38	<0.001
Creatinine§ (µmol/l)	1.02	1.01 – 1.03	0.005			
International normalised ratio	1.00	0.81 – 1.24	0.98			
Randomisation risk	0.71	0.42 – 1.18	0.19			
rs738409 homozygosity§	0.88	0.21 – 3.63	0.86			
Prednisolone	0.75	0.42 – 1.33	0.32			

Abbreviations: HR: Hazard Ratio; CI: Confidence Intervals; AST: Aspartate transaminase; ALP: Alkaline phosphatase

* Variable not entered into the multivariable analysis due to co-linearity

§ Variable excluded by backward elimination due to lack of significant independent association

†Variable not entered into multivariable analysis due to significant missing information (>10%)

Table 4.11 Univariate and multivariable Cox proportional hazards regression analyses for factors associated with 450-day survival in cases with severe alcoholic hepatitis who remained abstinent

Variable	Univariate			Multivariable		
	HR	95% CI	Significance (p)	HR	95% CI	Significance (p)
Age§	1.06	1.03 – 1.10	0.001			
Gender	0.91	0.45 – 1.83	0.79			
Overt hepatic encephalopathy	2.11	0.81 – 5.46	0.13			
White cell count* ($x10^6/mm^3$)	1.25	1.13 – 1.38	<0.001			
Neutrophils ($x10^6/mm^3$)	1.33	1.19 – 1.49	<0.001	1.22	1.06 – 1.41	0.005
Bilirubin (µmol/l)	1.01	1.01 – 1.02	<0.001	1.007	1.002 – 1.012	0.006
AST (IU/l)	1.01	0.99 – 1.03	0.17			
ALP§ (IU/l)	1.006	1.001 – 1.010	0.02			
Albumin (g/l)	0.90	0.86 – 0.94	<0.001	0.92	0.88 – 0.97	0.002
Urea§ (mmol/l)	1.25	1.14 – 1.37	<0.001	1.15	1.03 – 1.29	0.02
Creatinine§ (µmol/l)	1.01	1.005 – 1.023	0.003			
International normalised ratio	1.23	1.10 – 1.39	0.001	1.24	1.08 – 1.42	0.003
Randomisation risk§	1.36	0.94 – 1.96	0.1			
rs738409 homozygosity	3.40	1.54 – 7.49	0.002	2.56	1.03 – 6.34	0.04
Prednisolone	1.29	0.66 – 2.52	0.46			

Abbreviations**:** HR: Hazard Ratio; CI: Confidence Intervals; AST: Aspartate transaminase; ALP: Alkaline phosphatase

* Variable not entered into the multivariable analysis due to co-linearity

§ Variable excluded by backward elimination due to lack of significant independent association

Table 4.12 Sensitivity analysis for association between homozygosity for rs738409:G, drinking behaviour and medium-term survival, using assumption of drinking where unknown

Term	HR	95% CI	Significance (p)
Homozygosity for rs738409:G	3.47	1.58 – 7.64	0.002
Return to drinking	2.61	1.70 – 3.99	<0.0001
rs738409:G homozygosity-by-return to drinking	0.35	0.12 – 0.99	0.05

Abbreviations: HR: Hazard Ratio; CI: Confidence Intervals

4.6 Impact of rs11183620 on medium-term survival

In the cohort of patients surviving beyond 90 days there was an apparent increase in mortality associated with carriage of the alternate (G) allele (Figure 4.6). However, this did not reach statistical significance under an additive (HR 1.16, 95% CI 0.92 – 1.45, p=0.21), dominant (HR 1.39, 95% CI 0.94 – 2.07, p=0.098) or recessive (HR 1.08, 95% CI 0.74 – 1.57, p=0.70) model of inheritance.

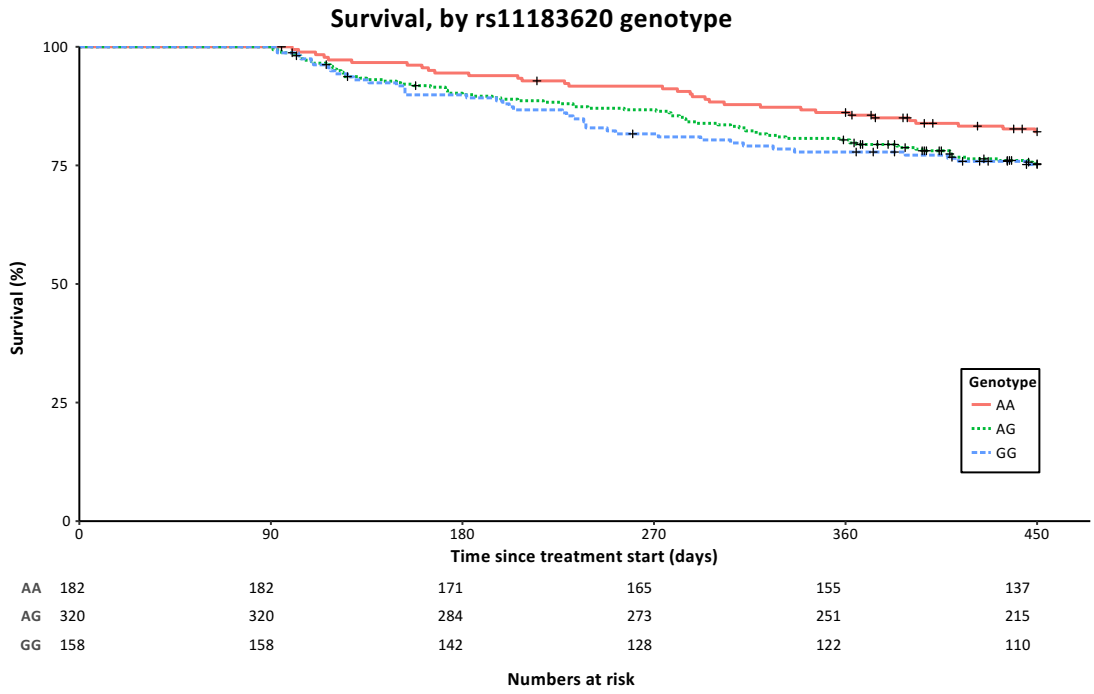


Figure 4.6 Medium-term survival in cases with severe alcoholic hepatitis surviving at least 90 days

Although there was an apparent reduction in survival in patients homozygous for rs11183620:A – AA: 17.6% (32/150), GA: 24.1% (77/320), GG: 24.7% (39/158), this did not reach statistical significance.

In light of the known influence of drinking on medium-term survival the association between rs11183620:G and medium-term survival was tested under a dominant model adjusted for a return to drinking, including under an assumption of a return to drinking where actual drinking behaviour was unknown.

Carriage of rs11183620:G was not independently associated with medium-term survival in the population of patients with known drinking behaviour (Table 4.13). However, a statistically significant independent association was revealed in the sensitivity analysis assuming a return to drinking (Table 4.13). This was not, however, robust to adjustment for homozygosity for rs738409:G.

Table 4.13 Cox regression analysis of the association between rs11183620 and drinking status at day 90 and survival to day 450 after presentation with severe alcoholic hepatitis

Term	HR	95% CI	Significance (p)
Observed cases only			
Homozygosity for rs11183620:A	1.30	0.79 – 2.16	0.30
Return to drinking	2.77	1.79 – 4.29	<0.0001
Reclassification of unknown status as drinking			
Homozygosity for rs11183620:A	1.49	1.01 – 2.21	0.045
Return to drinking	2.28	1.56 – 3.32	<0.001

Abbreviations: CI: Confidence interval; HR: Hazard ratio

4.7 Discussion

The variant rs738409:G in *PNPLA3* has been implicated in influencing the clinical trajectory of alcohol-related liver disease, including more rapid progression and an increased risk of hepatocellular carcinoma[437, 443, 447, 449-451]. In an unselected population of patients attending a secondary care centre, *PNPLA3* rs738409:G was associated with greater liver stiffness values at presentation and a significantly lower degree of normalisation over the follow-up period[438]. These data indicate that rs738409:G may influence the recovery and thus ultimately the outcome from an alcohol-related liver injury. To date no studies have examined whether rs738409:G influences the response to treatment or clinical outcomes in patients with severe alcoholic hepatitis.

The work presented in this thesis indicates that rs738409:G influences the histological severity of steatohepatitis in patients presenting with severe alcoholic hepatitis. The analyses presented in this chapter have helped to determine whether this translates into an impact upon treatment response and disease outcomes.

First: there is no evidence that the rs738409 genotype is associated with short-term mortality in patients with severe alcoholic hepatitis, nor does it interact with the severity of liver disease, prednisolone treatment or early improvement in liver function, as measured by the Lille score, in relation to short-term mortality.

Second: carriage of rs738409:G appears to be associated with a slower rate of normalisation of parameters indicative of hepatic excretory (bilirubin) and synthetic (albumin) function, in a dose-dependent fashion. This may relate to slower resolution of the underlying steatohepatitis as postulated in other work looking at normalisation of liver stiffness after cessation of drinking[438] and supported, to a degree, by histological analyses presented in Chapter 3.

Third: these analyses provide clear evidence supporting the primacy of drinking behaviour as a determinant of medium-term outcome in patients with severe alcoholic hepatitis who survive the

initial illness[236, 250, 251]. Individuals who maintain abstinence have significantly lower mortality than individuals who resume drinking, at any level. There is a clear dose-dependent relationship between the level of alcohol relapse and medium-term recovery in liver function and mortality which confirms similar findings reported by others[236]. However, the finding that there is an increased risk of mortality even at levels of alcohol consumption which might normally be considered non-hazardous indicates that complete abstinence from alcohol must be advised in patients who survive an episode of severe alcoholic hepatitis. Unsurprisingly, resumption of alcohol consumption also influences the rate of recovery of markers of liver function over the medium-term, this may provide an explanation as to why it appears to influence the relative associations of several variables with survival, particularly the serum bilirubin.

Fourth: rs738409 genotype influences medium-term survival. Thus, in the entire population surviving beyond day 90 taken as a whole, mortality was significantly higher in individuals homozygous for the G allele. Given the dominant impact of alcohol consumption on many aspects of liver disease it is important to note that the observed effects of rs738409:G on both recovery of liver function and medium-term survival are independent of the return to drinking. That being said the relationship between rs738409:G and the clinical trajectory of patients with severe alcoholic hepatitis does not appear straight forward. There is evidence that the genotypic effect on both recovery of liver function and medium-term survival is modulated by drinking behaviour. Thus, while there was no difference in the rate of recovery of liver function or mortality, by genotype, in individuals who continued to drink, abstinence from alcohol was associated with improved survival and improvement in liver function in heterozygote carriers of rs738409:G or non-carriers but not in patients homozygous for rs738409:G. This suggests the effect of rs738409 genotype is subservient to drinking behaviour in those who continue to drink.

The analyses presented here do not support a role for rs11183620 genotype in determining the response to treatment and either short- or medium-term outcomes in patients with severe alcoholic hepatitis.

This study has a number of limitations *viz.*: (i) The information on drinking behaviour was based on self-reported estimates of alcohol intake collected on day 90 and these data were only available for 46% of the cases; data were only available in 21% of survivors at one year. Sensitivity analyses were conducted to evaluate the potential effect on findings based upon adjustment of drinking status based upon 1-year data and assumption of resumed drinking in those of unknown status. The results of the subsequent analyses show clear differentiation in the direction expected supporting this stance and indicate the findings are robust. However this cannot completely obviate the fact that a substantial amount of this data is missing and the assumption used to perform the sensitivity analysis is very coarse. (ii) A small proportion of cases were of non-British ancestry (n=38, 4.2%). There are ethnic differences in the frequency of rs738409:G but its association with an increased risk of developing alcohol-related liver disease is consistent across ethnic groups. Thus, inclusion of these individuals in the analyses is unlikely to have confounded the results to any appreciable degree; (iii) survival data were captured using the NHS database of registered deaths but registration is often delayed, and deaths occurring outside the UK are not registered; thus the number of deaths may have been underestimated; (iv) data on the number of cases undergoing liver transplantation were only captured for the duration of the STOPAH trial, although it is likely that numbers transplanted beyond this immediate time-point would have been small; (v) although the number of cases was large the number of individuals homozygous for rs738409:G was relatively small and this may have limited the power.

In conclusion: individuals with severe alcoholic hepatitis who survive the acute event and carry rs738409:G in *PNPLA3* demonstrate a slower rate of recovery of liver function up to 90 days. This may be explained, in part, by apparent differences in the severity of steatohepatitis and fibrosis evident on liver biopsy. Beyond the 90 day threshold those individuals homozygous for rs738409:G would appear

to be at increased risk of mortality in the subsequent year, even if they attain and maintain abstinence from alcohol. Genotyping rs738409 in *PNPLA3* will identify these individuals and could play a role in clinical decision-making, potentially facilitating stratification of individuals for liver consideration of transplantation or novel therapies. The need to employ measures to assist patients with severe alcoholic hepatitis to attain and maintain abstinence is highlighted as of critical importance. In the absence of any association between rs11183620 genotype and clinical features or outcomes in severe alcoholic hepatitis further investigation is warranted to determine how it might potentially contribute to the development of disease.

CHAPTER 5

THE POTENTIAL ROLE OF *SLC38A4* SEVERE ALCOHOLIC HEPATITIS

5 The potential role of *SLC38A4* in severe alcoholic hepatitis

5.1 Overview

Two SNPs were found to be associated with the risk for developing severe alcoholic hepatitis in the previously described genome-wide association study (Chapter 2). One of these, rs738409 in *PNPLA3,* was also adversely associated with the clinical course of the disease and its outcome; these additional associations have provided insight into how this variant might be linked to disease pathogenesis. The other SNP, rs11183620 in *SLC384A* shows no associations with disease course and outcome, making its link to disease pathogenesis less obvious (Chapters 3 and 4).

Whilst the effect of variants in protein-coding regions may be comparatively easy to discern (e.g. missense or nonsense variants), intronic variants such as rs11183620 in *SLC384A* may exert their effects *via* a number of mechanisms. These include alterations in gene expression due to splice site variation or changes in regulatory elements which may alter transcription factor binding or post-transcriptional modifications. Initial bioinformatic analyses do not indicate that rs11183620 has an effect on protein structure, function or expression. It does, however, lie in strong linkage disequilibrium with another intronic variant (rs7953215) which is predicted likely to affect transcription factor binding based upon its RegulomeDB score. Whilst such a variant might alter *SLC38A4* expression there is no data to indicate whether this would, in fact, have any biological significance. Thus, this chapter describes work done to ascertain whether *SLC38A4* expression is altered in severe alcoholic hepatitis and, if so, what the consequences of an alteration in expression might be.

5.2 Introduction

The gene *SLC38A4* encodes a sodium-coupled amino acid transporter with almost exclusive hepatic expression. The transporter is reported to preferentially transport alanine but also transports several

other amino acids including valine, histidine, methionine and threonine[456]. It belongs to the SLC38 family of amino acid transporters

SLC38A4 is down-regulated in the colonic epithelium of patients with active inflammation due to Crohn's disease[387, 388] but is unchanged when the condition is quiescent[388]. Active Crohn's disease is characterised by increased serum and intestinal epithelial levels of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β) and interleukin 8 (IL-8) which have been implicated in disease pathogenesis[457-461]. Severe alcoholic hepatitis is also characterised by increased serum concentrations of the same pro-inflammatory cytokines[70, 462]. Higher serum concentrations have been associated with more severe disease and poorer outcomes[78, 463-467].

In animal models of alcohol-related liver injury, translocation of bacterial products, particularly lipopolysaccharide (LPS), into the portal circulation[68] stimulates resident tissue macrophages to produce pro-inflammatory cytokines such as TNFα and interleukin-6 (IL-6)[69-71]. The degree of endotoxaemia correlates with the severity of the observed liver injury in rodents[72, 73] and clinical outcomes in humans with severe alcoholic hepatitis[79]. In a rodent model of renal injury, murine podocytes increased *SLC38A3* expression in response to LPS. No effect was observed on *SLC38A4* expression though this transporter is not normally expressed in the kidney; hepatic changes were not evaluated[468]. Treatment of rat vascular smooth muscle cells with LPS leads to upregulation of pathways relating to amino acid transport of which SLC38A4 is a member[469]. Consequently, changes in *SLC38A4* expression in patients with severe alcoholic hepatitis could potentially be mediated by a direct effect of LPS on hepatocytes as well as *via* the inflammatory response it induces.

A reduction in *SLC38A4* expression and protein levels could, theoretically, impair amino acid uptake by cells leading to intracellular nutrient deprivation and consequent metabolic stress. Amino acid deprivation results in activation of a number of compensatory adaptive changes in protein synthesis and metabolism. Restriction of amino acids leads to an increase in the abundance of uncharged transfer RNAs (tRNAs) which mediate the phosphorylation of the kinase general control non-

derepressible 2 (GCN2)[470]. GCN2, in turn, phosphorylates eukaryotic initiation factor 2α (eIF2α)[471], a protein which forms part of a complex that mediates ribosomal selection of the messenger RNA start site during translation[472]. Phosphorylation reduces the activity of eIF2α resulting in a general suppression of protein synthesis[472]. In contrast, the translation of certain mRNAs containing a particular upstream open reading frame is promoted. The mRNAs of the transcription factors general control non-derepressible 4 (GCN4), in yeast, and activating transcription factor 4 (ATF4), in mammals, contain such open reading frames[473, 474]. These transcription factors upregulate the expression of genes involved in processes such as amino acid transport and biosynthesis, and also cellular responses to stress[475, 476]. One such gene is the transcription factor *DNA damage-inducible transcript 3 (DDIT3*), which has two aliases – *CCAAT/enhancer-binding homologous protein* (*CHOP*) and *growth arrest and DNA damage-inducible protein 153* (*GADD153*)[477]. *DDIT3* activation leads to cell death[478, 479] possibly by promoting protein synthesis and exacerbating oxidative stress in the endoplasmic reticulum of cells already under metabolic duress[480, 481]. Oxidative stress and hepatocyte apoptosis are strongly implicated in the pathogenesis of alcoholic steatohepatitis.

ATF4 also interacts with the mammalian target of rapamycin (mTOR) pathway, another system by which cells seek to respond to changes in amino acid availability. mTOR acts as a central mechanism by which cellular metabolism, growth and survival are co-ordinated. Under normal circumstances the mTOR complex 1 (mTORC1) positively regulates protein synthesis *via* phosphorylation of p70-S6 kinase and eukaryotic initiation factor 4E binding protein 1 (eIF4E-BP1)[482]. The net effect is a global promotion of mRNA transcription and translation. mTORC1 also negatively regulates autophagy, the process by which cells catabolise organelles in order to support anabolic processes during periods of nutrient deprivation[483]. mTORC1 activation is maintained by influx of amino acids into cells, in particular the exchange of L-glutamine for essential amino acids such as L-leucine[484].

Amino acids promote interaction of mTORC1with the Rag family of GTPases with and its relocalisation to an intracellular compartment where it interacts with its activator Rheb[485]. Deprivation of amino

acids leads to a reduction in this interaction and consequently mTORC1 activity with a consequent rapid dephosphorylation of both p70-S6 kinase and eIF4E-BP1[482]; this, in turn, results in a reduction in protein synthesis and an increase in autophagy. Overexpression of *ATF4* results in a reduction of mTORC1 activity mediated *via* increased expression of *Redd1*, a small protein which acts as a negative regulator of mTORC1[486, 487]. Thus, an increase in *ATF4* expression would be expected to lead to an increase in autophagy.

Autophagy is mediated by a family of autophagy-related proteins (ATG). Within this family ATG4 is the only protease whilst ATG8 is a ubiquitin-like protein which plays a pivotal role in the formation of autophagosomes and trafficking of organelles and proteins into them for degradation[488, 489]. Cleavage of ATG8 by ATG4 leads to its activation in formation of phagolysosomes[490]. Inhibition of ATG4 effectively disrupts autophagy at the primary stage of autophagosome formation[491]. Hence, ATG4 expression and activity are important measures of the activation of autophagy.

5.3 Aim

The aims of the analyses conducted and described in this chapter were to:

1. Determine whether *SLC38A4* expression is altered in severe alcoholic hepatitis;
2. Elucidate potential drivers of any alteration in *SLC38A4* expression;
3. Determine whether alterations in *SLC38A4* expression lead to alterations in expression of genes associated with intracellular nutrient sensing and cellular stress;

5.4 Patients, material and methods

5.4.1 Whole liver RNAseq analysis

Liver tissues were obtained from the National Institute on Alcohol Abuse and Alcoholism (NIAAA) - funded InTeam consortium biorepository core. Human liver tissue was obtained from patients across a spectrum of alcohol-related liver disease *viz.* alcoholic steatohepatitis without established cirrhosis (n=12) and severe alcoholic hepatitis (n=27). Ten of the samples from patients with severe alcoholic hepatitis were from explants of patients who had undergone transplantation. The remaining samples from patients with severe alcoholic hepatitis were obtained by transjugular liver biopsy at presentation, prior to treatment. Nine (53%) of these 17 were Lille non-responders. All patients had i) a history of recent alcohol misuse ii) histological evidence of alcoholic steatohepatitis, iii) negative serological markers for viral hepatitis and autoimmune liver disease, iv) body mass index (BMI) <30 and v) liver function tests compatible with alcohol-related liver injury. None of the patients with early alcoholic steatohepatitis had clinical evidence of decompensation. All patients with severe alcoholic hepatitis had a DF ≥32 and were treated with prednisolone. Samples of 'normal' liver tissue (n=10) were obtained from patients undergoing hepatic resection of liver metastases from areas which were macroscopically unaffected. DNA was not available from any of the patients for genotyping.

For RNA sequencing, total RNA was extracted from liver tissues using Trizol21 and was analysed using the Agilent 2100 Bioanalyzer system (Agilent Biotechnologies, Palo Alto, CA). High quality RNA was used for library construction using the Illumina TruSeq Stranded Total RNA Ribo-Zero Gold kit (Illumina, San Diego, California). Multiplexed samples were sequenced using the Illumina HiSeq2000 platform (Illumina) using a read length of 2 x 100 bases. Short read alignment was performed using the Spliced Transcripts Alignment to a Reference (STAR) alignment algorithm with default parameters[492]. Normalization of gene expression level across samples was computed as transcripts per million mapped reads (TPM)[493]. Normalised gene expression data for the two genes of interest, *PNPLA3* and *SLC38A4*, were extracted from the whole dataset for use in analyses.

The Kruskal-Wallis test was used to compared expression across normal liver, early alcoholic steatohepatitis and severe alcoholic hepatitis patients. Early alcoholic steatohepatitis was compared, in turn, to normal liver and severe alcoholic hepatitis using Dunn's multiple comparisons test. Expression levels in patients with severe alcoholic hepatitis were subsequently compared in groups defined by Lille response and, in turn, to expression in explanted livers. Normally distributed data were compared using a two-tailed t-test; non-normally distributed data were compared using the Kuskal-Wallis test with post-hoc analyses performed using Dunn's multiple comparisons test.

5.4.2 Cell culture techniques

Cell lines were cultured in Dulbecco's modified Eagle medium (DMEM, cat 41966052, Life Technologies, Paisley, UK) supplemented with penicillin-streptomycin (PS, cat 15070063, Life Technologies, Paisley, UK) at a final concentration of 50 U/ml and 10% fetal bovine serum (FBS, cat 10500-064, Life Technologies, Paisley, UK) – referred to as D10-P/S. For experiments examining the effects of human serum on cell culture, DMEM was supplemented with PS (50 U/ml) but not FBS (D-P/S). Cells were incubated in a humidified environment at 37˚C with 5% CO_2, the media was exchanged every 48-72 hours. Prior to use in experiments cells were plated at pre-specified densities and incubated overnight in fresh media (D10-P/S).

At specified time points during experimental protocols samples were harvested to obtain samples for RNA and/or protein extraction. Samples for RNA were collected by removing residual growth media, washing the cells with ice-cold 1X phosphate buffered saline (PBS, cat 10010056, Life Technologies, Paisley, UK) and lysing with buffer "RLT", with added ß-mercaptoethanol, from the RNeasy kit (cat 74104, Qiagen, Crawley, UK). Lysates were placed in RNase-free microcentrifuge tubes; if RNA extraction was not performed immediately, lysates were stored at -80˚C for subsequent processing. Cell pellets for protein extraction were obtained by removing residual growth media and trypsanising the adherent cells (cat 25200072, Life Technologies, Paisley, UK). Trypsin activity was quenched by the addition of D10-P/S. The cell suspension was then transferred to a 15ml falcon tube, centrifuged at

500*g* for 5 minutes before removal of the supernatant and re-suspension in 1X PBS. This cell suspension underwent further centrifugation at 500*g* for 5 minutes; subsequently the supernatant was aspirated and the washed cell pellet stored at -80˚C for subsequent use.

5.4.3 Primary human hepatocyte and cell line expression of SLC38A4

Expression of *SLC38A4* was determined by reverse transcription quantitative polymerase chain reaction (RT-qPCR) in primary hepatocytes of human origin and the two hepatocyte-derived cell lines – HepG2 and Huh-7. Total RNA was isolated directly from cell pellets of primary human hepatocytes containing $1x10^6$ cells from four different donors. HepG2 and Huh-7 cells were plated in 24-well plates at a density of 150,000 cells in 500ul of D10-P/S. Samples for RNA were collected after overnight incubation and then again at further 6 and 24 hour time points.

RNA was isolated from cell pellets or lysates using the Qiagen RNeasy Mini Kit in accordance with the manufacturer's guidelines. Lysates were homogenised using QIAshredder columns (cat 79656, Qiagen, Crawley, UK) and an optional on-column DNAse digestion (cat 79254, Qiagen, Crawley, UK) was employed. The concentration and quality of RNA was analysed by spectrophotometry on a Multiskan™ GO microplate reader (Thermo Scientific, Waltham, USA). cDNA was synthesised using the RETROscript Reverse Transcription kit (cat AM1710, Life Technologies, Paisley, UK).

Quantitative PCR was performed on an Applied Biosystems StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, California, USA) using TaqMan™ chemistry (Taqman™ Gene Expression Master mix, cat 4369016, Applied Biosystems, Foster City, California, USA). An off-the-shelf Taqman™ gene expression probe was used to detect *SLC38A4* expression (probe Hs00394339_m1, Applied Biosystems, Foster City, California, USA). Target gene expression was normalised to that of the house-keeping gene *GAPDH* (ΔC_T) determined simultaneously (probe Hs02786624_g1, Applied Biosystems, Foster City, California, USA). A minimum of three biological replicates and two technical replicates were performed for each condition and assay, respectively. The level of expression of

SLC38A4 in hepatocyte-derived cell lines was determined relative to the expression in the primary human hepatocytes. Statistical comparisons between groups were made using a two-tailed t-test for normally distributed data and a Mann-Whitney U test for non-normally distributed data. The $2^{-\Delta\Delta C_T}$ method was used to determine relative expression[494].

5.4.4 Cell line serum stimulation experiments

Huh-7 cells were seeded in a 48-well plate at a density of 100,000 cells/well and incubated overnight in 200ul of FBS-supplemented media (D10-P/S). The media was removed and the cells were washed with 1X PBS and then incubated for 4 hours in 200ul of serum-free media (D-P/S). Human serum, from healthy donors (n=12) or patients with severe alcoholic hepatitis (n=12), was diluted 1:3 with D-P/S to a final concentration of 25% human serum. Serum-free media was aspirated from the wells. The cells were then rinsed with 1X PBS and finally incubated in 200ul of human-serum supplemented media for 24 hours. At the end of the incubation samples were harvested for RNA extraction with subsequent determination of *SLC38A4* expression as previously described. Experiments were performed with four biological replicates and two technical replicates. Statistical comparisons between groups were made using a two-tailed t-test for normally distributed data and a Mann-Whitney U test for non-normally distributed data.

Serum pro-inflammatory cytokines, TNF-α, IL-1β, IL-6 and IL-8, were measured using a custom multiplex ELISA run in accordance with the manufacturer's instructions (cat K151A0H-1, MSD, Maryland, USA) in eight of the patients with severe alcoholic hepatitis used in the experiments above and four of the healthy controls. Sera from the healthy controls were run without dilution whilst sera from patients with severe alcoholic hepatitis were run at 2- and 8-fold dilutions in light of local experience and previously published data[467]. All samples, including standards, were run in duplicate. Standard curves were constructed and unknown values interpolated in Prism v7.0 (GraphPad Software, California, USA). The differences in serum cytokine concentrations in groups defined by disease state were tested using the Mann-Whitney U test. Serum cytokine concentrations were

correlated with expression of *SLC38A4,* normalised to *GAPDH* (dCT), in Huh-7 cells cultured for 24 hours in media supplemented with the respective serum sample. Correlations were performed using the Spearman's rank test.

5.4.5 Single cytokine stimulation cell culture experiments

Huh-7 cells were seeded in a 24-well plate at a density of 150,000 cells/well and incubated overnight in D10-P/S. The media was then replaced with 500ul of D10-P/S supplemented with one of the pro-inflammatory cytokines, prednisolone or LPS to a pre-determined final concentration (Table 5.1). The cytokine concentrations used were determined based upon both values reported in the literature[495-499] and the concentrations measured by ELISA in the serum samples from patients with severe alcoholic hepatitis (see above). Cells were stimulated for 24 hours prior to harvesting of samples for RNA extraction and subsequent analyses of *SLC38A4* expression. Cells cultured in D10-P/S media only were used as a control; each condition was run with its own control. Experiments with IL-1β, IL-6, IL-10, TNFa and prednisolone were performed twice, while the IL-8 and LPS experiments were performed only once. In each experiment for every condition a minimum of two biological and two technical replicates were performed. Normalised expression of *SLC38A4* was compared between control and every test condition using the Student's t-test. Relative expression of *SLC38A4* was determined by raising 2 to the power $-\Delta\Delta C_T$ with confidence intervals derived from the statistical tests applied.

Table 5.1 Cell culture conditions for single agent cell culture stimulation experiments in Huh-7 cells

Agent	Supplier	Preparation	Final concentration in culture
TNFa	Cat PHC3015, Life Technologies, Carlsbad, USA	Dissolved in deionised water	15 ng/ml
IL-6	Cat PHC0064, Life Technologies, Carlsbad, USA	Dissolved in 100mM acetic acid	25 ng/ml
IL-8	Cat PHC0084, Life Technologies, Carlsbad, USA	Dissolved in deionised water	20 ng/ml
IL-10	Cat PHC0104, Life Technologies, Carlsbad, USA	Dissolved in deionised water	50 ng/ml
IL-1β	Cat PHC0814, Life Technologies, Carlsbad, USA	Dissolved in deionised water	20 ng/ml
Prednisolone	Actavis Generics, New Jersey, USA	Tablet (5mg) crushed and dissolved in DMSO	0.5 ug/ml
LPS	Cat 00-4976-03, eBioscience, San Diego, USA	Diluted in media	100 ng/ml

All cytokines used were recombinant proteins generated from *Escherichia coli* and intended for use in cell culture. The LPS used was derived from *Escherichia coli* 026:B6.

Abbreviations: LPS: Lipopolysaccharide

5.4.6 IL-1β blocking experiments

An antibody against IL-1β (Anti-hIL-1β-IgG, cat mabg-hil1b-3, Invivogen, San Diego, California) was used to neutralise IL-1β in either serum from patients with severe alcoholic hepatitis or recombinant protein added to cell culture media. Cells grown in media alone, media supplemented with antibody and IL-1β (20ng/ml) were used as controls.

Huh-7 cells were seeded in a 48-well plate at a density of 100,000 cells/well and incubated overnight in 200ul of FBS-supplemented media (D10-P/S). The media was then removed and replaced with 500ul D10-P/S alone or 500ul D10-P/S supplemented with i) anti-IL-1β antibody (10 ug/mL), ii) IL-1β (20 ng/mL) or iii) anti-IL-1β antibody (10 ug/mL) and IL-1β (20 ng/mL). Cells were incubated under these conditions for 24 hours. Experiments were performed with four biological replicates and two technical replicates. In additional serum experiments, Huh-7 cells were seeded in a 48-well plate at a density of 100,000 cells/well and incubated overnight in 200ul of FBS-supplemented media (D10-P/S). FBS-

supplemented media was removed, cells were washed with 1X PBS and then incubated for 4 hours in 200ul of serum-free media (D-P/S). IL-1β blocking antibody was added to half the human serum available from patients with severe alcoholic hepatitis (n=4) at a concentration of 10ug/mL and incubated for 2 hours at 37°C with gentle agitation every 30 minutes. Human serum was diluted 1:3 with D-P/S to give a final concentration of 25% human serum. Serum-free media was aspirated from the cells which were then washed in PBS and finally incubated in 200ul of human-serum supplemented media for 24 hours. Experiments were performed with three biological replicates and two technical replicates. At the end of the incubation samples were harvested for RNA extraction with subsequent determination of *SLC38A4* expression as previously described. Statistical comparisons between groups were made using a paired Student's t test.

5.4.7 SLC38A4 knockdown cell line construction and characterisation

Stable *SLC38A4* knockdown cell lines were constructed by transfecting short hairpin RNA (shRNA) encoding genes into wild type Huh-7 cells using a lentivirus plasmids. A set of six pre-designed shRNA constructs encoding shRNAs targeting the *SLC38A4* gene were obtained as *Escherichia coli* glycerol stocks (cat RHS4531-EG55089, Dharmacon, GE Healthcare, Chalfont, UK). A plasmid containing an shRNA with a scrambled target sequence, and thus not targeted to affect expression of any gene, was used as a control (cat RHS4349, Dharmacon, GE Healthcare, Chalfont, UK). To prepare plasmid DNA 10ul of each glycerol stock was inoculated into 5ml of Luria-Bertani media and cultured overnight at 37°C. The culture was centrifuged to obtain a bacterial pellet and plasmid DNA was isolated using a Qiagen Plasmid DNA mini kit in accordance with the manufacturer's guidelines (cat 12123, Qiagen, Crawley, UK).

5.4.7.1 Lentivirus particle production

Lentivirus particles were generated in T293 cells. Cells were seeded at a density of $2.2x10^6$ cells in $10cm^2$ plates and incubated overnight in DMEM media with 10% FBS. A plasmid DNA mix consisting of 6ug of *SLC38A4* shRNA plasmid DNA, 1.2ug of pCMV-VsVg and 4.8ug of pHIV-Gag/Pol, made up to a total volume of 1.5mls with Opti-MEM reduced serum media (cat 11058021, Thermo Scientific, Waltham, USA), was incubated at room temperature for 5 minutes. Lipofectamine 2000 (cat 11668-019, Invitrogen, Carlsbad, USA) was diluted 1:25 in Opti-MEM and incubated at room temperature for 5 minutes. Each of the plasmid DNA mixes was combined with 1.5mls of diluted Lipofectamine 2000 to a final volume of 3mls and incubated at room temperature for 20 minutes to produce transfection complexes. The transfection complexes were applied to separate plates of T293 cells and incubated for 48 hours in a humidified environment at 37˚C with 5% CO_2. Efficiency of transfection was confirmed, but not quantified, by visual determination of the expression of green fluorescent protein in the T293 cells. Media and transfection complexes were removed from the cells and replaced with 7.5mls pre-warmed DMEM supplemented with 1.5% FBS. After 12 hours this media, containing lentiviral particles, was harvested into a 15ml tube and stored at 4˚C until required. Cells were incubated overnight in a further 7.5mls of pre-warmed DMEM supplemented with 1.5% FBS. The media was harvested again and added to that already collected. Harvested media was passed through a 0.2um syringe filter. Hepes and Polybrene were added to the filtered lentiviral particles to give final concentrations of 20mM and 4ug/ml, respectively. Lentivirus particles were stored at -80˚C until required.

5.4.7.2 Transduction

Huh-7 and HepG2 wild type cells were seeded in a six-well plates at a density of 450,000 cells/well in DMEM with 10% FBS and incubated overnight. Media was replaced with 1ml of DMEM with 10% FBS, supplemented with Hepes and Polybrene at final concentration of 20mM and 4µg/ml, respectively. One millilitre of lentivirus particles for each short hairpin RNA was pipetted into a designated well and

plates were incubated for 24-48h at 37°C with 5% CO_2. Puromycin (Life Technologies, Carlsbad, USA) was added to the growth media at a final concentration of 1 ug/ml to select stably transduced cells. Transduction was validated by observation of green fluorescent protein expression. Cells were cultured under puromycin selection pressure for 14 days in order to generate stable cell lines; the incubation media was exchanged every 48 to 72 hours and cells were maintained at <90% confluence.

5.4.7.3 Cell line characterisation

Two stably transfected shRNA cell lines, together with wild type and non-targeting shRNA controls were plated in a 24-well plate at a density of 150,000 cells/well and cultured in D10-P/S for 24 hours prior to harvesting of samples for RNA extraction. RT-qPCR was used to quantify expression of the genes *SLC38A4*, *ATF4*, *ATG4A* and *DDIT3*, normalised to *GAPDH*. Experiments were performed with four biological replicates and two technical replicates. Normalised expression levels were compared between wild type cells and those transfected with a non-targeting shRNA and between cells transfected with the two *SLC38A4*-specific shRNAs and those transfected with a non-targeting shRNA. Statistical comparisons between groups were made using a two-tailed t-test for normally distributed data and a Mann-Whitney U test for non-normally distributed data.

5.5 Results

5.5.1 Whole liver RNAseq analysis

Transcripts of both *SLC38A4* and *PNPLA3* were identified in liver tissue from all patient groups. The distribution of normalised gene expression levels was non-normal for several of the comparator groups.

Expression of *SLC38A4* differed significantly between normal liver, early alcoholic steatohepatitis and severe alcoholic hepatitis ($p<0.0001$). No difference was apparent between normal liver tissue compared to patients with early alcoholic steatohepatitis (median 10.2 [IQR 10.02 – 10.23] *vs*. 10.25 [9.52 – 10.58] TPM, $p=0.99$, Figure 5.1A). However, *SLC38A4* expression was significantly lower in the entire cohort of patients with severe alcoholic hepatitis (8.38 [IQR 7.84 – 8.78] TPM) compared to early alcoholic steatohepatitis ($p<0.0001$). There was no significant difference in the expression of *SLC38A4* in the groups defined by Lille response (responders: 8.58 [7.98 – 8.78] TPM *vs*. non-responders: 8.18 [7.48 – 8.73], $p=0.99$. *SLC38A4* expression in tissue from patients with severe alcoholic hepatitis undergoing transplantation (7.42 [6.93 – 8.25] TPM) was not significantly to that seen in either Lille responders ($p=0.079$) or non-responders ($p=0.63$, Figure 5.1A).

PNPLA3 expression differed significantly between normal liver samples and patients with early alcoholic steatohepatitis and severe alcoholic hepatitis ($p=0.003$). Whilst expression levels in normal liver did not differ from patients with early alcoholic steatohepatitis (5.96 [5.81 – 6.12] *vs*. 5.80 [5.24 – 6.69] TPM, $p=0.99$, Figure 5.1B). *PNPLA3* expression was lower in patients with severe alcoholic hepatitis (4.74 [4.43 – 5.11] TPM) compared to early alcoholic steatohepatitis ($p=0.0014$). There was no difference in *PNPLA3* expression between Lille responders and non-responders (4.82 [4.47 – 5.22] *vs*. 4.68 [4.38 – 4.94] TPM, $p=0.99$, Figure 5.1B). Nor was there any additional change in expression between liver tissue from explants (5.00 [4.27 – 5.24]) and either Lille responders ($p=0.99$) or non-responders ($p=0.99$).

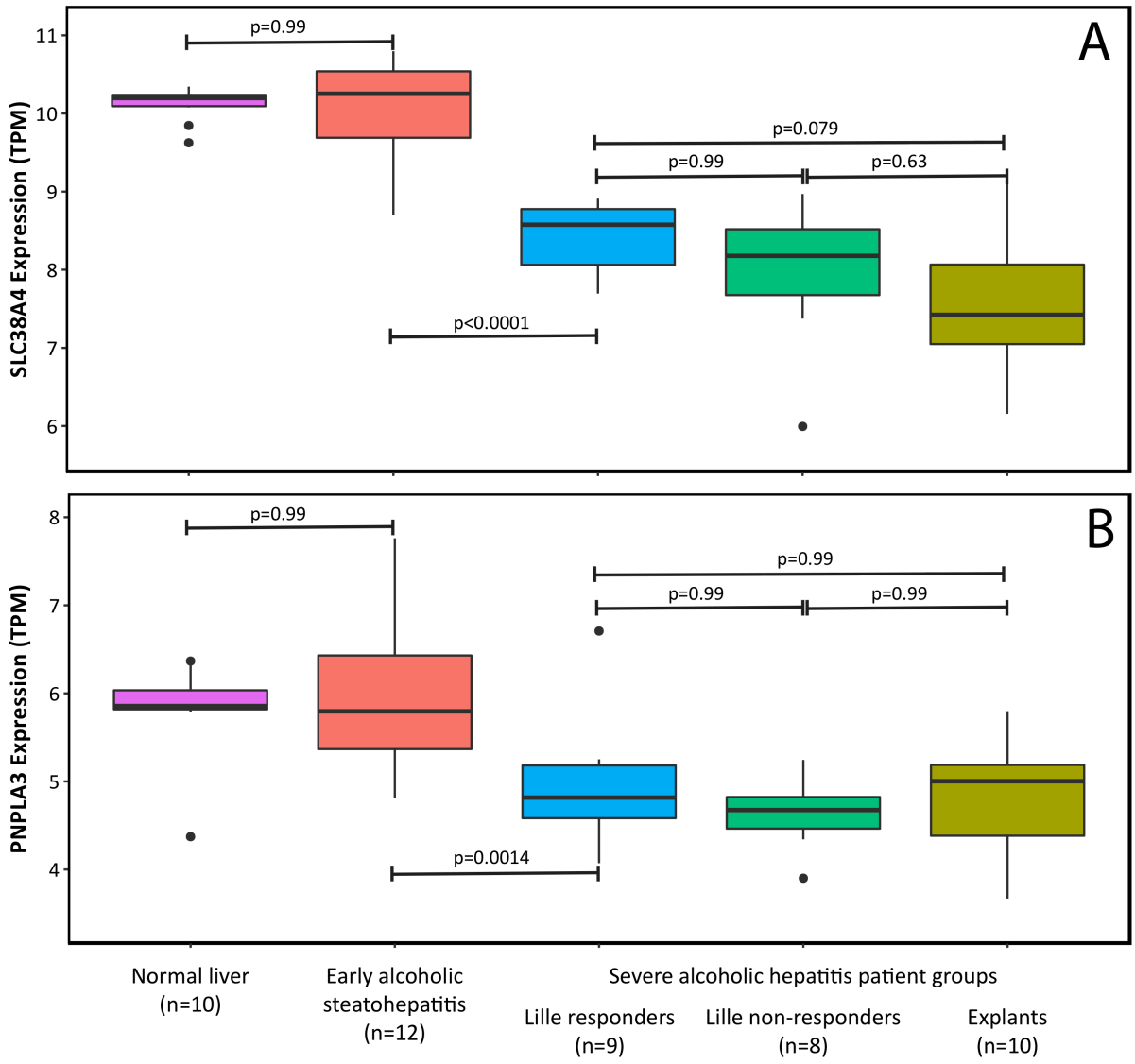


Figure 5.1 Hepatic expression of (A) *SLC38A4* and (B) *PNPLA3* in patients with alcohol-related liver disease

Expression levels were normalised and are displayed as tags per million (TPM).

Data are displayed as the median (solid bar), interquartile range (box) and 95% confidence interval (whiskers).

5.5.2 Primary human hepatocyte and cell line expression of SLC38A4

SLC38A4 was expressed in primary human hepatocytes and both the hepatocyte-derived cell lines. Relative expression was lower in both cell lines than primary hepatocytes but was comparatively greater in Huh-7 compared to HepG2 cells (Table 5.2).

Table 5.2 Expression of *SLC38A4* in primary hepatocytes and cell lines, normalised to *GAPDH*

Cell line	N	Mean ΔCT (95% CI)	Relative expression (95% CI, $2^{-\Delta\Delta CT}$)
Primary human hepatocytes	4	6.3 (4.7 – 7.8)	100
Huh-7	8	10.0 (9.5 – 10.5)*	7.7 (3.8 – 14.4)
HepG2	9	15.6 (15.3 – 15.8)*†	0.16 (0.1 – 0.26)

Difference in normalised expression of *SLC384A* between primary human hepatocytes and cell lines *p<0.0001

Difference in normalised expression of *SLC384A* between Huh-7 and HepG2 cell lines †p<0.0001

There was no statistically significant change in expression of *SLC38A4* after 6 or 24 hours in culture compared to baseline for Huh-7 cells. In HepG2 cells there was an apparent decrease in *SLC38A4* expression at 6 hours equivalent to a relative expression of 73% (Figure 5.2). RNA was isolated directly from cell pellets of primary human hepatocytes; they were not incubated.

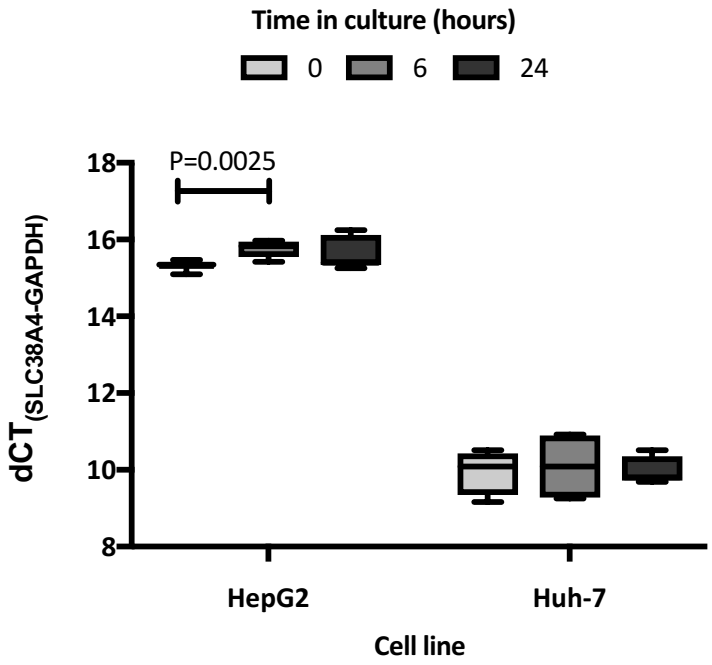


Figure 5.2 HepG2 and Huh-7 expression of *SLC38A4* normalised to *GAPDH* as a function of time in culture

Data are displayed as the median (solid bar), interquartile range (box) and 95% confidence interval (whiskers).

In view of the significantly higher level of expression of *SLC38A4* in Huh-7 cells further experiments were undertaken in this cell line.

5.5.3 Cell line serum stimulation experiments

SLC38A4 expression was significantly reduced in Huh-7 cells following culture in medium containing serum from patients with severe alcoholic hepatitis (dCT healthy: median 7.39 [IQR 7.01 – 7.64] *vs*. dCT alcoholic hepatitis: 8.23 [7.78 – 8.52]), ddCT 0.80, 95% CI 0.28 – 1.33, p=0.0029, Figure 5.3). This equated to a relative expression of 0.57 (95% CI 0.40 – 0.82).

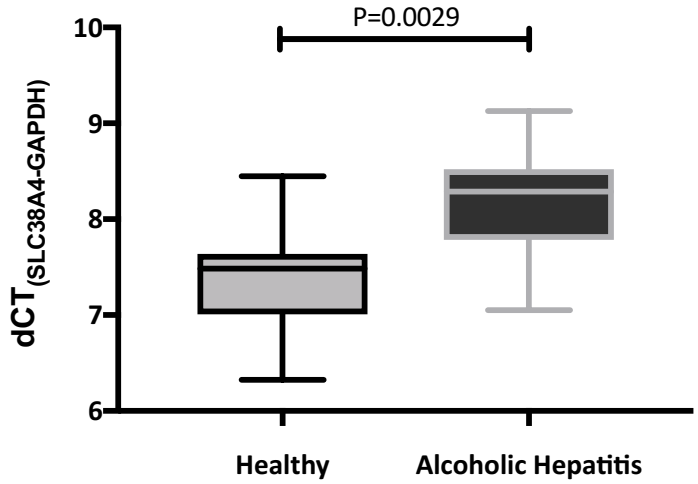


Figure 5.3 *SLC384A* expression in Huh-7 cells after 24-hour culture with serum from either healthy individuals or patients with severe alcoholic hepatitis

Data are displayed as the median (solid bar), interquartile range (box) and 95% confidence interval (whiskers).

Pro-inflammatory cytokines were detected in significantly higher concentrations in the serum of patients with severe alcoholic hepatitis than healthy controls (Figure 5.4A-D). Serum pro-inflammatory cytokine concentrations correlated significantly with the normalised expression of *SLC38A4* seen in Huh-7 cells cultured for 24 hours in media supplemented with the respective serum (Figure 5.4E-H). The correlation was strongest for IL-1β (rho = 0.73, 95% CI 0.25 – 0.92, p=0.0096).

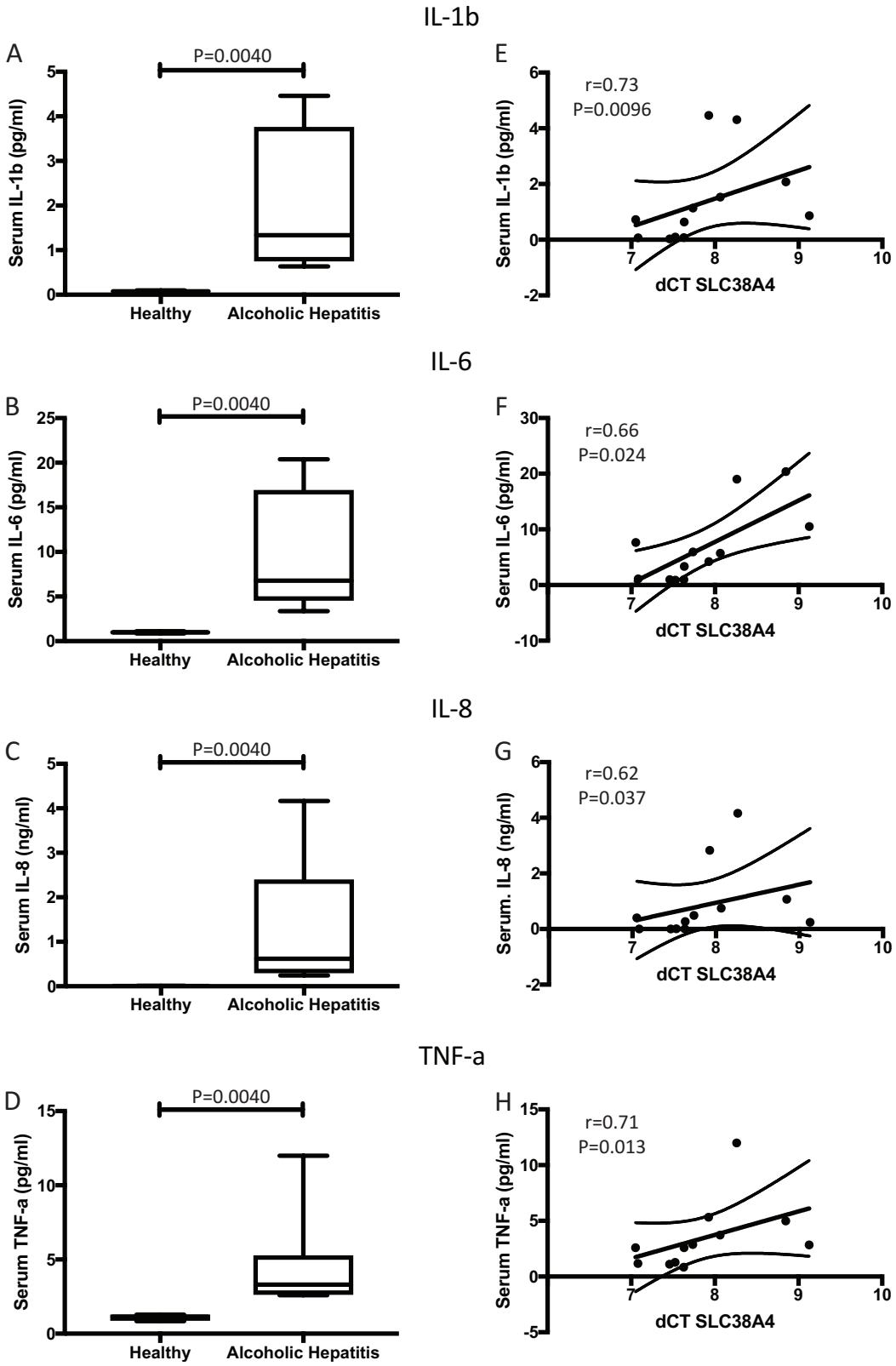


Figure 5.4 Serum pro-inflammatory cytokines and correlation with expression of *SLC38A4* in cell culture

Left hand panel A-D Serum levels of pro-inflammatory cytokines in patients with severe alcoholic hepatitis compared to healthy controls. Data are displayed as the median (solid bar), interquartile range (box) and 95% confidence interval (whiskers). Right hand panel E-H Correlations between serum cytokine concentrations and the normalised expression of *SLC38A4* in Huh-7 cell cultures

5.5.4 Single cytokine stimulation and blocking cell culture experiments

Huh-7 cells cultured in the presence of IL-1β showed a significant reduction in expression of *SLC38A4* expression compared to cells cultured under control conditions (ddCT mean -1.70, 95% CI -0.84 – -2.55, p=0.0003; Figure 5.5). Culture of cells in the presence of prednisolone was associated with a relative increase in *SLC38A4* expression (ddCT 1.76, 95% CI 0.61 – 2.91, p=0.0040; Figure 5.5). These findings were both replicated in the second set of experiments (Figure 5.5, Table 5.3). None of the other experimental conditions was associated with a significant alteration in the relative expression of *SLC38A4* (Figure 3.5, Table 3.3).

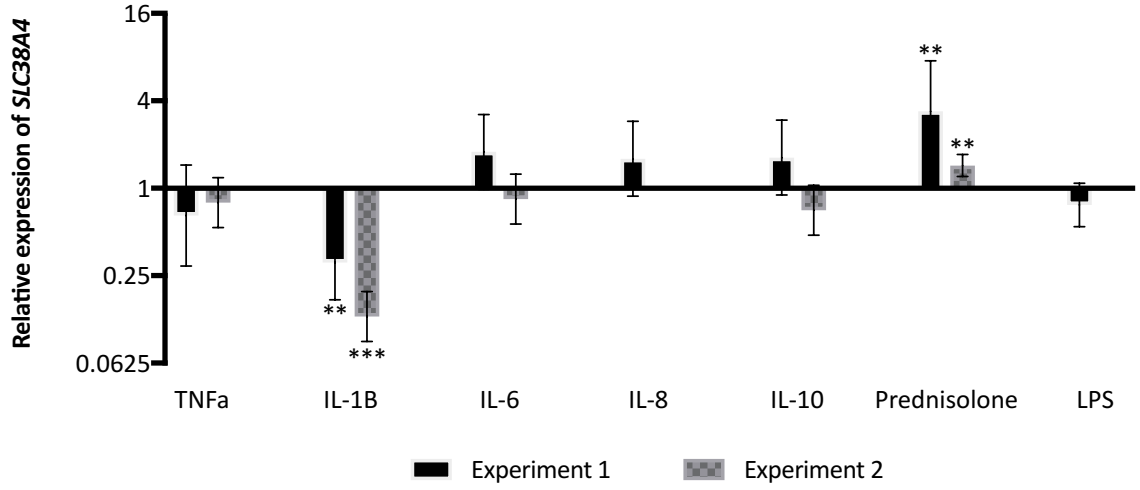


Figure 5.5 Changes in Huh-7 expression of *SLC38A4* induced culture with pro- and anti-inflammatory stimuli
Expression is calculated relative to that in Huh-7 cells cultured under identical conditions but without the addition of pro- or anti-inflammatory stimulus to media. Changes induced by IL-8 and LPS were only evaluated on a single occasion, all other experiments were conducted in duplicate.
Data displayed as mean (bar) and 95% confidence interval (whiskers).
** p<0.001, *** p<0.0001

The expression of *SLC38A4* in Huh-7 cells cultured in the presence of IL-1β was only 15-30% of the level seen Huh-7 cells cultured under control conditions (Table 5.3). The increase in *SLC38A4* expression induced by prednisolone varied substantially between the two experiments – 3.39 (1.12 – 10.2) and 1.43 (1.21 – 1.71).

Table 5.3 Changes in *SLC38A4* expression in cultured Huh-7 cells following cytokine stimulation

Cytokine	Experiment 1			Experiment 2		
	N	Relative expression	p	N	Relative expression	p
IL-1β	4	0.31 (0.17 – 0.56)	0.0003	3	0.13 (0.09 – 0.19)	<0.0001
IL-6	4	1.78 (0.98 – 3.22)	0.057	3	0.84 (0.57 – 1.25)	0.37
IL-10	4	1.62 (0.90 – 2.94)	0.11	3	0.71 (0.48 – 1.05)	0.081
TNFa	2	0.65 (0.29 – 1.44)	0.28	3	0.80 (0.54 – 1.18)	0.24
Prednisolone	2	3.39 (1.52 – 7.54)	0.0040	7	1.43 (1.21 – 1.71)	0.0011
IL-8	4	1.60 (0.90 – 2.94)	0.12			
LPS	6	0.77 (0.54 – 1.08)	0.12			

Data are displayed as mean (95% confidence interval)

Abbreviations: CI: Confidence interval; LPS: Lipopolysaccharide

5.5.5 IL-1β blocking experiments

Incubation of Huh-7 cells with IL-1β significantly reduced *SLC38A4* expression compared to control conditions (Control: dCT 8.02, 95% CI 7.75 – 8.29 *vs*. IL-1β: dCT 11.26, 95% CI 9.66 – 12.85, p=0.0007, Figure 5.6). Incubation of Huh-7 cells with anti-IL-1β antibody did not alter the expression of *SLC38A4* (anti-IL-1β: dCT 7.77, 95% CI 7.35 – 8.19, p=0.16, Figure 5.6). Simultaneous incubation with IL-1β and anti-IL-1β antibody (dCT 9.28, 95% 8.96 – 9.60) attenuated the reduction in *SLC38A4* see with IL-1β alone though complete blocking was not achieved (Figure 5.6).

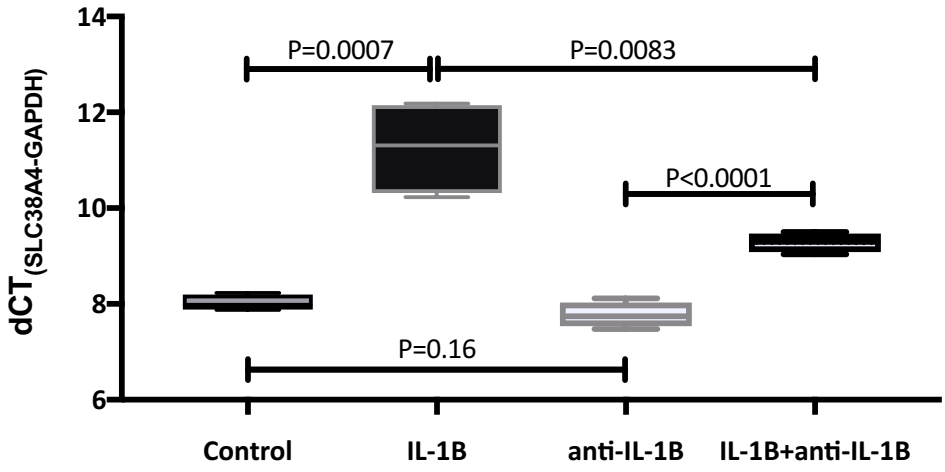


Figure 5.6 Changes in Huh-7 expression of SLC38A4 with IL-1β cytokine and anti-IL-1β antibody exposure

Data are displayed as the median (solid bar), interquartile range (box) and 95% confidence interval (whiskers).

SLC38A4 expression was significantly reduced in Huh-7 cells following addition of serum from patients with severe alcoholic hepatitis to the culture medium. Addition of anti-IL-1β antibody resulted in a significant increase in the normalised expression of *SLC38A4* (Figure 5.7). This change equated to a mean relative increase of 42% (95% CI 27 – 59%) in *SLC38A4* expression.

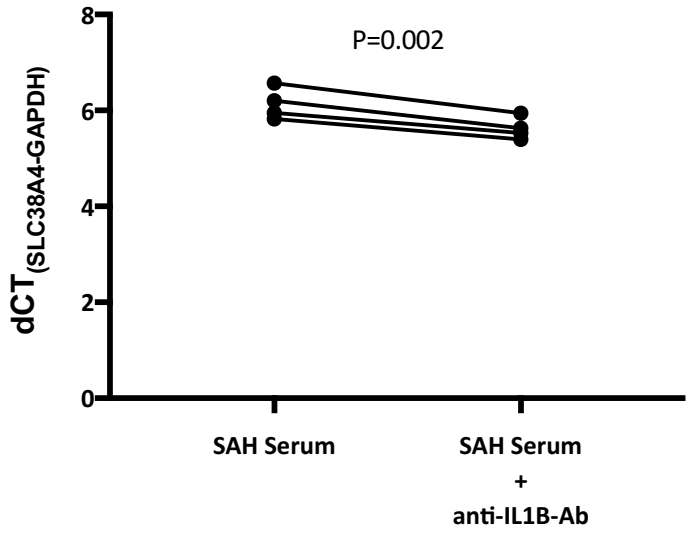


Figure 5.7 Change in *SLC38A4* expression with pre-treatment of serum with anti- IL-1β

The reduction in *SLC38A4* expression seen with the addition of serum from patients with severe alcoholic hepatitis to culture media was attenuated by pre-incubation with anti- IL-1β antibody (ddCT -0.51, 95% CI -0.35 – -0.67). Data are displayed for pairs of experiments using sera from different patients with severe alcoholic hepatitis (n=4).

Abbreviations: SAH: Severe alcoholic hepatitis

5.5.6 SLC38A4 knockdown cell line construction and characterisation

Transfection of T293 cells with SLC38A4 shRNA constructs 4 and 6 was unsuccessful as these were universally lethal to the cell line. Transfection was, however, successful using constructs 1, 2, 3 and 5; this was confirmed visually by green fluorescent protein (GFP) expression and a reduction in the relative expression of *SLC38A4* on RT-qPCR. Transfection of all four shRNAs reduced normalised expression of *SLC38A4* (Figure 5.8).

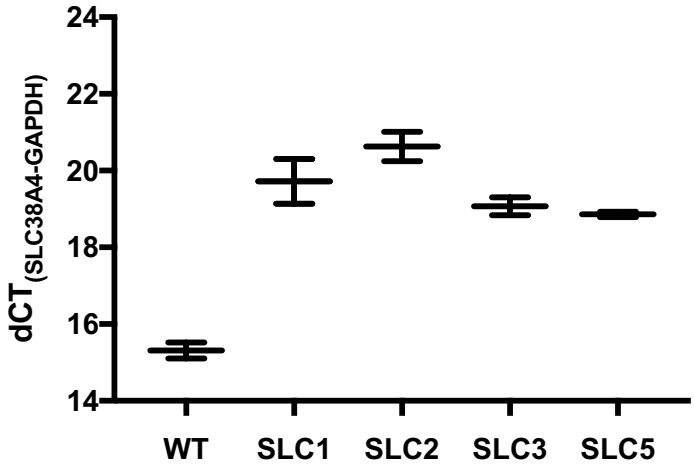


Figure 5.8 The effect of *SLC38A4* targeting shRNAs on *SLC38A4* expression, normalised to *GAPDH* in T293 cells
Data are displayed as the median (solid bar), interquartile range (box) and 95% confidence interval (whiskers). Legend: WT: Wild type (i.e. non-transfected cells); SLC: Cells transfected with *SLC38A4* targeting shRNA

Lentiviral particles were successfully produced for the non-targeting control shRNA and *SLC38A4* targeting shRNAs 1, 2, 3 and 5. As shRNAs 1 and 2 produced the greatest reduction *SLC38A4* expression, lentiviral particles for these two shRNAs, as well as the non-targeting shRNA, were transduced into Huh-7 and HepG2 cells. Successful transduction and selection of stably transduced cells with puromycin was confirmed by expression of green fluorescent protein (Figure 5.9).

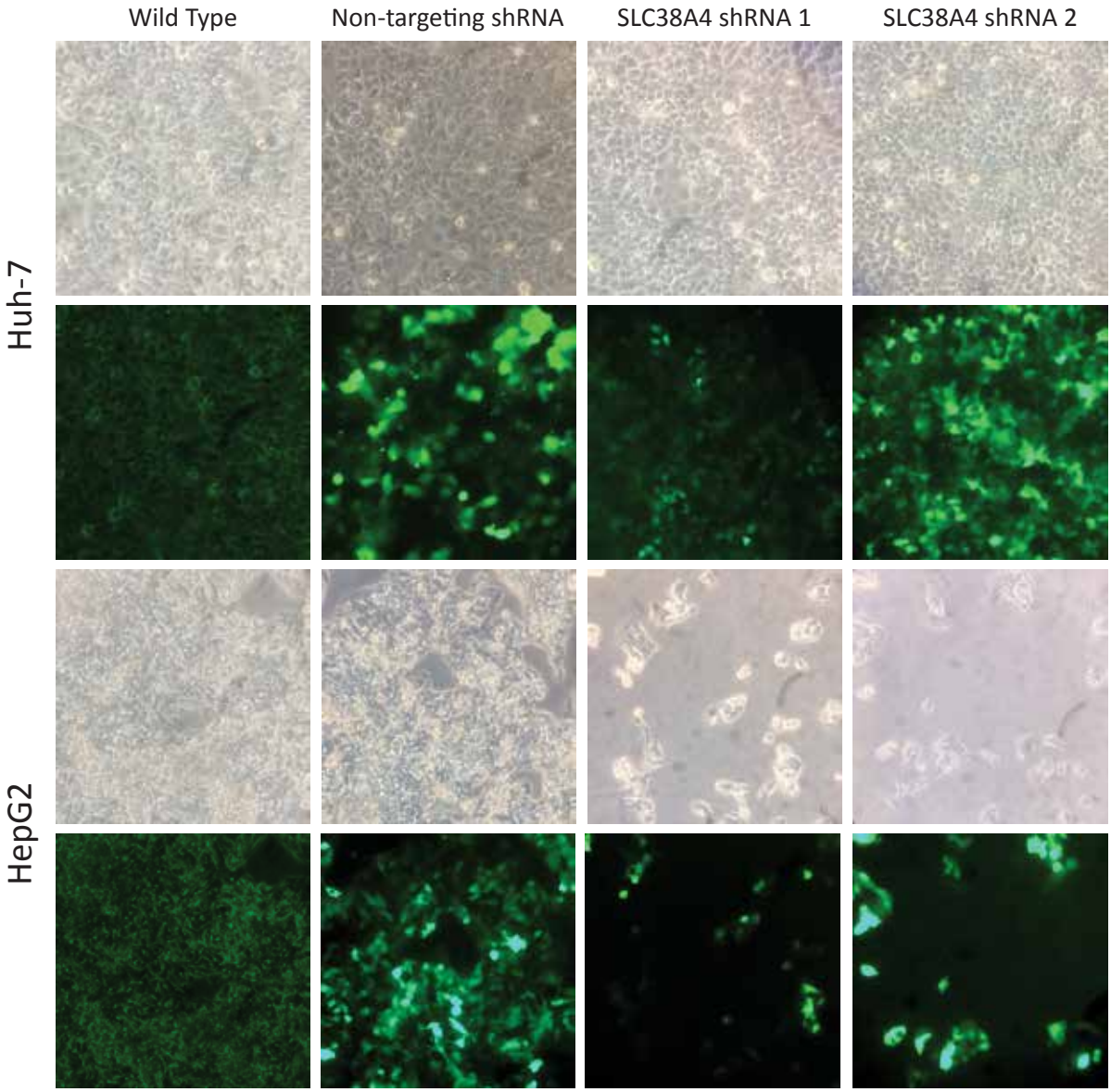


Figure 5.9 Green fluorescent protein expression by Huh-7 and HepG2 cells transduced with shRNA constructs
A degree of auto-fluorescence was seen in wild type cells (untransduced cells). Cellular expression of green fluorescent protein was seen with successful transduction with the non-targeting shRNA and both shRNAs targeting *SLC38A4*. Green fluorescent protein expression was noted to be stronger and more universal in cells transfected with *SLC38A4* shRNA 2 compared to shRNA 1.

Transfection of cells with a non-targeting shRNA was not associated with a statistically significant alteration of expression of any of the genes of interest examined in either cell line (Figure 5.10). In HepG2 cells significant reductions in expression of *SLC38A4* were seen with transfection of both shRNA SLC1 (ddCT median 0.58, 95% CI 0.23 – 2.53, p=0.007) and shRNA SLC2 (ddCT 2.1, 95% CI 1.51 – 3.28, p=0.0002). This was equated to a reduction in *SLC38A4* expression of 33% (95% CI 15 – 83%) and 77%

(95% CI 65 – 90%) for shRNAs SLC1 and SLC2 respectively compared with cells transduced with the non-targeting shRNA. No reduction in expression of *SLC38A4* was observed in Huh-7 cells transduced with shRNA SLC1 (ddCT 0.98, 95% CI-0.45 – 2.56, p=0.65). *SLC38A4* expression was significantly reduced in Huh-7 cells transduced with shRNA SLC2 (ddCT 1.6, 95% CI 0.35 – 3.30, p=0.01), equivalent to a 67% reduction (95% CI 22 – 90%)(Figure 5.10).

Normalised expression of *ATF4* was increased in HepG2 cells transfected with both shRNA SLC1 (ddCT -1.50, 95% CI -0.04 – -2.63, p=0.02) and SLC2 (ddCT -1.59, 95% CI -0.15 - -2.67, p=0.02). This equates to increases in expression of 182% and 201%, respectively. In contrast, a statistically significant increase in *ATF4* expression was not seen in Huh-7 cells transfected with either shRNA (SLC1: ddCT -0.07, 95% CI -0.35 – 0.33, p=0.81 and SLC2: ddCT -0.10, 95% CI -0.48 – 0.29, p=0.59). However, a significant increase in *DDIT3* expression was seen in both cell lines transfected with either shRNA (Figure 5.10). The normalised expression of *ATG4A* was unaltered by transfection of any of the shRNA constructs (Figure 5.10).

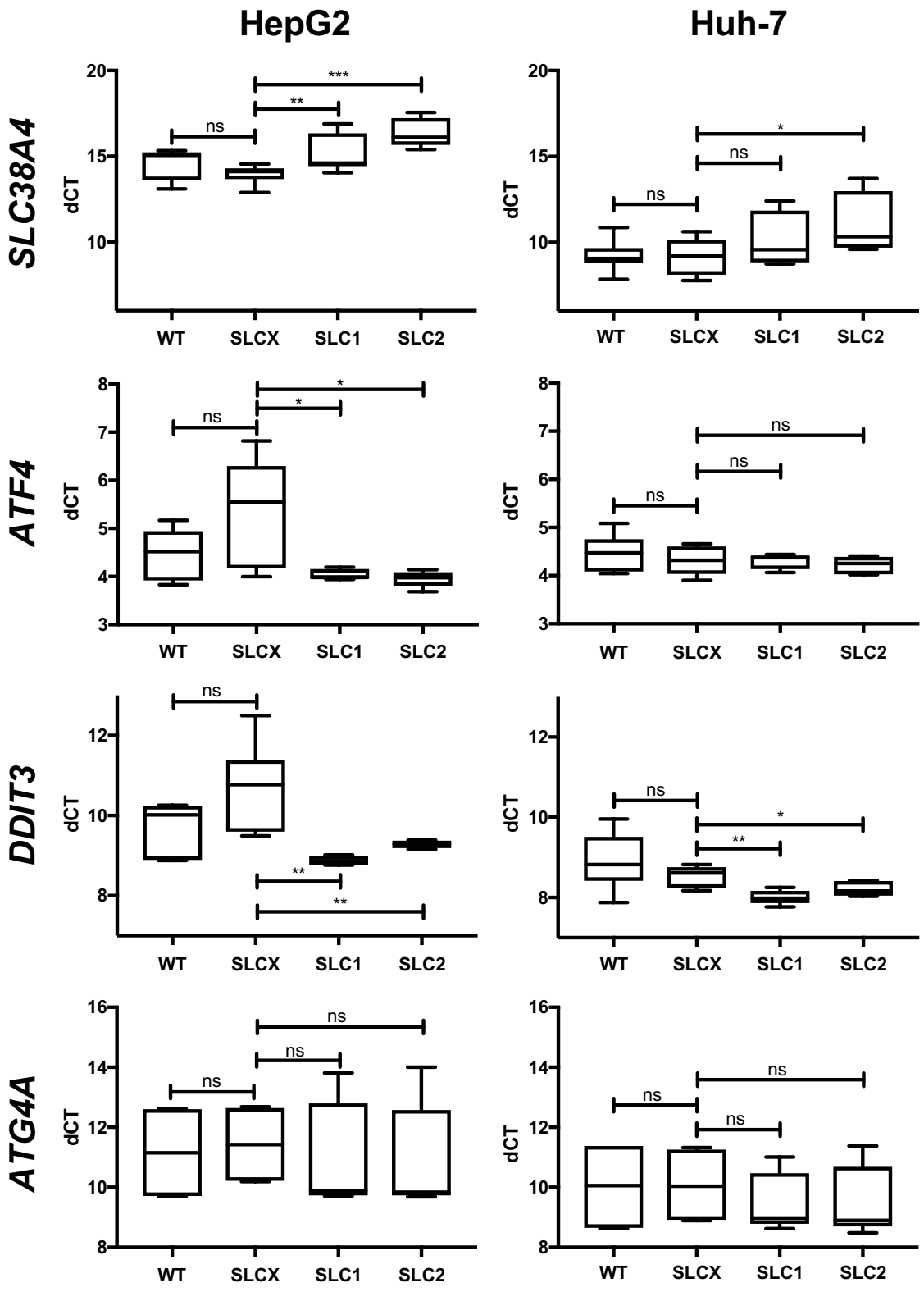


Figure 5.10 Changes in the expression of genes associated with cellular stress with knockdown of *SLC38A4*

Expression was normalised to *GAPDH* (dCT). A greater dCT indicates lower normalised expression levels. Legend: WT: untransduced cells; SLCX: Non-targeting shRNA; SLC1: SLC38A4 targeting shRNA 1; SLC2: SLC38A4 targeting shRNA 2. Data are displayed as the median (solid bar), interquartile range (box) and 95% confidence interval (whiskers). Annotation: ns not significant, * p<0.05, ** p<0.01, *** p<0.001.

5.6 Discussion

Analysis of the genome-wide association study data presented in this thesis identified an association between a commonly occurring intronic SNP, rs11183620 in *SLC38A4*, and the risk for developing severe alcoholic hepatitis. Intronic variants predisposing to disease may exert their effects *via* a number of mechanisms. These include alterations in gene expression due to splice site variation or changes in regulatory elements which may alter transcription factor binding or post-transcriptional modifications. Bioinformatic analyses do not indicate that rs11183620 has an effect on protein structure, function or expression though it does lie in strong linkage disequilibrium with another intronic variant (rs7953215) which may. Whilst such a variant might alter *SLC38A4* expression there is no data to indicate whether this would, in fact, have any biological significance. The purpose of the experiments presented in this chapter was to determine whether alterations in *SLC38A4* expression are seen in severe alcoholic hepatitis and, if so, what the potential impact of such an alteration in expression might be.

Analysis of whole liver transcriptomic data indicates that *SLC38A4* expression is not down-regulated in the livers of patients with the histological lesion of alcoholic steatohepatitis, in the absence of hepatic decompensation, compared to healthy controls. However, a profound reduction in expression was seen in those with the clinical syndrome of severe alcoholic hepatitis. Expression of *SLC38A4* did not appear to be further reduced in the explants from patients requiring transplantation for severe alcoholic hepatitis compared to those who responded to prednisolone treatment, as defined by the Lille response. Thus, alterations in expression of *SLC38A4* are seen with the development of severe alcoholic hepatitis though these data are insufficient to determine whether this is cause or effect.

SLC38A4 is expressed almost exclusively in the liver[456] and was readily detectable, in this study, using RT-qPCR in primary human hepatocytes and the hepatocyte-derived cell lines Huh-7 and HepG2. Cell line expression was noted to low compared to primary cells, particularly in HepG2 cells.

Down-regulation of *SLC38A4* expression has been reported in the colonic epithelium in the presence of active inflammation secondary to Crohn's disease; though not when the disease is quiescent[387, 388]. Pro-inflammatory mediators such as TNF-α and IL-1β, are known to be involved in the pathogenesis of Crohn's disease[458, 460, 461] and may play a role in medicating the changes in gene expression in the presence of active inflammation. The same pro-inflammatory mediators are strongly implicated in the pathogenesis of severe alcoholic hepatitis, readily detected in the serum of patients with the condition and significantly elevated compared to control populations[464, 465, 467]. Thus, the possibility that the down regulation of *SLC384A* expression observed in patients with severe alcoholic hepatitis might be a consequence of the increased proinflammatory activity was explored in the series of experiments reported here.

Culture of Huh-7 cells with serum from patients with severe alcoholic hepatitis resulted in an approximately 40% reduction in expression in *SLC38A4*. High levels of IL-1β, IL-6, IL-8 and TNF-α were confirmed in the sera from patients with severe alcoholic hepatitis compared to that from healthy controls used in these experiments. Strong correlations were noted between the levels of pro-inflammatory cytokines in the sera and the expression of *SLC38A4*. When evaluated individually, only culture with IL-1β consistently produced a reduction in *SLC38A4* expression in Huh-7 cells. The effect of exogenous IL-1β on *SLC38A4* expression was partially reversed by culture in the presence of a blocking antibody, though this was only performed at a single concentration. Neutralisation of IL-1β in serum from patients with severe alcoholic hepatitis using the same antibody resulted in greater *SLC38A4* expression in Huh-7 cells compared to the same, untreated, serum.

Culture of Huh-7 cells in the presence of prednisolone resulted in an increase in *SLC38A4* expression. Whilst the spread of results in the initial experiment was wide the finding was confirmed on replication. Prednisolone is the only agent which to have demonstrated a positive impact on survival in severe alcoholic hepatitis at 28 days[229, 263, 266]. Prednisolone signals *via* the glucocorticoid steroid receptor which dimerises and interacts with p21, the glucocorticoid response element and NF-κB.

Whilst the activated glucorticoid receptor inhibits NF-κB mediated gene transcription, IL-1β signalling mediates its effects by increasing NF-κB mediated transcription. These opposing effects on NF-κB activity provide a potential basis for the opposing effects of the two compounds on *SLC38A4* expression in Huh-7 cells. The rationale for using prednisolone in severe alcoholic hepatitis in mainly predicated on its anti-inflammatory effects on immune cells with comparatively little consideration given to its potential influence on the function of epithelial cells such as hepatocytes. Transcriptomic data from healthy mice treated with prednisolone show that, in addition to the anticipated downregulation of pro-inflammatory genes, there is an upregulation of genes promoting gluconeogenesis and amino acid transport, including a member of the SLC38 family for transporters, SLC38A2[(500)]. These changes were not seen in mice homozygous for a mutation which abrogates glucocorticoid receptor dimerization suggesting that these effects are dependent upon DNA-binding by the activated receptor[(500)]. These findings raise the possibility that the therapeutic benefit of prednisolone in patients with severe alcoholic hepatitis is mediated through both anti-inflammatory effects on immune cells and anabolic effect on hepatocytes.

Transduction of short hairpin RNAs (shRNA) designed to interfere with SLC38A4 expression into two hepatocyte-derived cell lines was successfully achieved using lentiviral vectors. Universal strong expression of the vector was more difficult to achieve in Huh-7 cells compared to HepG2 cells and this was reflected in the degree of knockdown achieved. One shRNA, SLC2, successfully reduced *SLC38A4* expression by approximately 70% in both cell lines. This was associated with a significant increase in expression of the transcription factor *DDIT3* in both cell lines and *ATF4* in HepG2 cells. These transcription factors are both induced by amino acid deprivation and endoplasmic reticulum (ER) stress. Thus, reduction in expression of *SLC38A4* in hepatocyte-derived cell lines may be sufficient to trigger these responses. Over-expression of *ATF4* in HepG2 cells has been associated with an increase in intracellular triglyceride content through stimulation of hepatic lipogenesis mediated *via* the transcription factors sterol regulatory element-binding protein 1 (SREBP-1c) and carbohydrate-

responsive element-binding protein (ChREBP)[501]. Such alterations are reminiscent of the changes in cellular metabolism seen in response to ethanol ingestion. Furthermore, *ATF4* and *DDIT3* are upregulated in HepG2 cells in response to stimuli which stimulate endoplasmic reticulum stress[502]. *SLC38A4* knockdown results in an increase in expression of both transcription factors indicating that it may induce cellular responses similar to the ER stress response. A note of caution is, however, required as a significant increase in *DDIT3* expression was seen in Huh-7 cells in the absence of a significant decrease in *SLC38A4* expression; though this effect was not seen in non-targeting shRNA control suggesting it was not simply a function of introduction of the lentiviral vector.

SLC38A4 knockdown was not associated with an increase in expression of *ATG4A* suggesting that it did not trigger autophagy. One explanation is that a multiplicity of amino acid transporters with consequent redundancy allows physiological responses to compensate for a reduction in expression of a single transporter. However, in HepG2 cells *DDIT3* activation has been noted to modify the impact of *ATF4* overexpression, switching cellular fate from autophagy to apoptosis[502]. This is corroborated by findings that *ATF4* overexpression makes cells more susceptible to apoptosis triggered by stimuli such as radiation[503]. Consequently, an alternative hypothesis is that the simultaneous activation of *ATF4* and *DDIT3* after *SLC38A4* knockdown, as seen in HepG2 cells, leads to cell death by apoptosis rather than triggering autophagy. Apoptosis is a well-recognised phenomenon in alcoholic hepatitis.

This work is not, however, without its limitations. Whilst demonstration of *SLC38A4* downregulation was demonstrated in human samples DNA was unavailable to perform genotyping for either rs738409 or rs11183620. Consequently, an effect of genotype on expression could not be evaluated. However, the comparatively small number of samples available would likely have precluded an adequately powered analysis. Subsequent work was performed in cell lines. HepG2 and Huh-7 cells are both immortalised cell lines originally derived from hepatocellular carcinomas. Tumour cells undergo a number of adaptations in order to survive and proliferate in physiological environments characterised by significant metabolic stress, including hypoxia and nutrient deprivation. Increased *ATF4* expression

has been described in tumours, especially in necrotic areas distant from tumour vasculature and consistent with hypoxic and nutrient-deprivation mechanisms of activation[504, 505]. The significance of *ATF4* upregulation in tumour cells is underlined by its implication in chemotherapeutic resistance, potentially partly mediated by upregulation of genes involved in glutathione synthesis[506]. Thus, it is likely that in both HepG2 and Huh-7 cells there is already a degree of dysregulation of the GCN2-eIF2α-ATF4 pathway. HepG2 and Huh-7 cells are known to be homozygous for the rs738409:G allele which, in turn, confers alterations in lipid metabolism[395, 507]. Genotyping for rs11183620 was not performed. Consequently, there are difficulties extrapolating the findings demonstrated here to the responses that might be seen in primary hepatocytes, either *in vitro* or *in vivo* or where the rs738409 variant is not present. Experiments using cytokines in cell culture were conducted at a single concentration. Although this concentration was chosen based upon a review of published experiments and the pathophysiological levels seen in conditions such as severe alcoholic hepatitis, a series of dose-finding experiments would help inform the range of concentrations over which any effect is seen.

Whilst a statistically significant knockdown in *SLC38A4* expression was achieved in both cell lines with at least one shRNA construct, the degree was comparatively limited and variability was noted. It is likely that this is at least partly attributable to failure to select a pure population of stably transduced cells. Whilst significant changes in gene expression were seen in this experimental model, use of a knockout model, such as might be achieved using CRISPR/Cas9 technology, would likely provide cleaner results.

Alterations in cellular responses were assessed, in these studies, using alterations in the expression of genes at the level of mRNA. Though the genes chosen for evaluation were selected in part based on the assumption that alterations in mRNA levels were their primary regulator, this should be further developed by assessment of protein levels. In addition, the phosphorylation status of other players within the relevant pathways should also be assessed. Assessment of a greater number of targets within pathways and evaluation of enzymatic activity may be particularly important in assessing any

activation of autophagy. ATG4A represents one of a family of four ATG4 enzymes. Indeed, ATG4B exhibits several-fold greater activity against ATG8-like substrates compared to ATG4A[508]; activity may be further modified by post-translational modifications dependent upon redox status[509]. Consequently an assay able to evaluate whole ATG4 activity *in vitro* may permit more accurate assessment of the extent of autophagy activation[510-512]. In addition the employment of techniques such as the Seahorse flux analyser would permit functional readouts related to oxidative stress and mitochondrial function and whether these are comprised in *SLC38A4* knockdown or knockout cells{Luz, 2015 #901}.

In summary: *SLC38A4* expression is significantly reduced in the livers of patients with severe alcoholic hepatitis. Cell culture experiments indicate a role for IL-1β in suppressing *SLC38A4* expression whilst prednisolone may increase it. Knockdown of *SLC38A4* may induce cellular stress responses leading to upregulation of *ATF4* and *DDIT3*. Whilst these are compensatory mechanisms aimed at restoring homeostasis they may simultaneously sensitise cells to stimuli which may trigger cell death *via* apoptosis. In severe alcoholic hepatitis down regulation of *SLC38A4* induced by IL-1β may contribute to disease pathogenesis by increasing hepatocellular dysfunction and death.

CHAPTER 6

SERUM AMINO ACID PROFILES IN SEVERE ALCOHOLIC HEPATITIS

6 Serum amino acid profiles in severe alcoholic hepatitis

6.1 Overview

The variant rs11183620 in *SLC38A4*, an hepatically expressed amino acid transporter of both cationic and neutral amino acids, is associated with the risk of developing severe alcoholic hepatitis (Chapter 2). Analysis of whole liver RNAseq data has shown that *SLC38A4* is downregulated in severe alcoholic hepatitis; data from cell culture experiments have shown that this downregulation may result in activation of intracellular mechanisms designed to overcome nutrient deprivation (Chapter 5). It has been shown previously that circulating amino acids patterns are disturbed in patients with alcohol-related liver disease. This chapter describes work undertaken to explore amino acid patterns in patients with severe alcoholic hepatitis and their pathological correlates.

6.2 Introduction

Amino acids are the building blocks of all proteins found in the human body. They comprise a group of organic acids characterised by an amine (-NH2) and a carboxyl group (-COOH) adjoined to a central carbon molecule with a side chain which is unique to each amino acid. In total, twenty different amino acids are encoded by the human genome[513]. Amino acids may be sub-classified based upon criteria including the structural and biochemical properties of their side chains. Two important sub-groups are the aromatic and branched chain amino acids (BCAAs). The aromatic amino acids comprise phenylalanine, tryptophan, tyrosine and histidine and are defined by the presence of a carbon ring in their side chain. The branched chain amino acids comprise leucine, isoleucine and valine and are characterised by a non-linear, hydrophobic side chain[514]. A sub-group of amino acids is additionally designated as 'essential' as they cannot be endogenously synthesised by the human body. The amino acids threonine, valine, methionine, leucine, isoleucine, phenylalanine, lysine and tryptophan are 'essential' in adults while arginine and histidine are additionally 'essential' during childhood[515].

The liver plays a central role in protein and amino-acid metabolism[513]. It processes dietary amino acids and reprocesses amino acids released from catabolism of other tissues. The liver has a significant demand for amino acids as the major site for synthesis of virtually all plasma proteins. Amino acids excess to the protein synthesis requirements of the liver and other tissues are converted into energy substrates by gluconeogenesis. This process requires hepatic deamination of amino acids and produces ammonia as a by-product; this is detoxified by the urea cycle enzymes[514]. Hepatic metabolism of hormones, including those which regulate extra-hepatic amino-acid metabolism provides a mechanism *via* which the liver influences overall body protein metabolism.

Hepatocyte uptake of amino acids is governed by transport mechanisms which are potentially stereospecific, saturable and may exhibit cross inhibition[516]. Uptake of alanine, the liver's major substrate for gluconeogenesis, exceeds that of other amino acids. Branched chain amino acids are comparatively poorly extracted by the liver and predominantly metabolised in skeletal muscle[517-519]. Work conducted in animal models of liver insufficiency, with or without encephalopathy, has described disturbances in several amino acids[520-522]. In patients with fulminant hepatic failure, plasma concentrations of all amino acids are high except those of the branched chain amino acids, which are normal or low[523]. Significant changes also occur in plasma amino-acid concentrations in patients with chronic liver disease, including alcohol-related cirrhosis, which appear to relate to the severity of liver disease, its activity, and its aetiology[524, 525]. Typically the aromatic amino acids phenylalanine and tyrosine are increased together with methionine whilst branched chain amino acid concentrations are broadly unchanged[525-527]. The former are attributable to impaired hepatic function, hyperinsulinaemia and hyperglucagonaemia whilst the latter reflects the lack of hepatic metabolism of branched chain amino acids[525]. Decreases in glycine, alanine and phenylalanine and increases in glutamic acid and proline in patients with alcohol-related cirrhosis with or without superimposed alcoholic hepatitis have also been described[528, 529].

Daily protein requirements are increased in patients with cirrhosis and estimated at 0.75–1.2 g/kg[530-532]. This compares to a recommended daily intake of approximately 0.8 g/kg in healthy individuals. It has been

suggested that a decreased ability to store glycogen leads to a compensatory increase in gluconeogenesis due to a reduction in glycogenolysis[533]. This use of amino acids as a major energy source results in depletion of tissue protein stores with a consequent increase in dietary protein requirements in order to maintain balance. This hypothesis is supported by evidence that whole body protein synthesis and breakdown rates are increased in cirrhotic patients[534]. Despite this dietary protein intakes in patients with cirrhosis, and in particular with severe alcoholic hepatitis, are often insufficient, protein energy malnutrition is common and high protein intake is required to maintain nitrogen balance[241, 293, 535, 536].

It has become increasingly clear over time that the effects of branched chain amino acids are complex and several other potentially beneficial actions have been identified particularly in relation to hepatic regeneration and malnutrition[537]. The beneficial effect of branched chain amino acid administration on liver regeneration has been demonstrated in experimental studies[538, 539] and likely pertains to their stimulation of i) protein synthesis[540, 541]; ii) secretion of hepatocyte growth factor[542-544]; and, iii) glutamine production[545] which appears pro-regenerative[546-548]. They also have an inhibitory effect on proteolysis[540]. These observations and descriptions of disturbances in circulating amino acid profiles in patients with alcohol-related liver disease led to the evaluation of amino acid supplementation as a therapy for severe alcoholic hepatitis. Initial studies indicated that addition of amino acid supplementation to a high calorie, high protein diet positively impacted short-term survival and improvement in serum bilirubin and albumin levels[245]. In a small study with a heterogenous patient population additional supplementation of BCAAs in patients receiving enteral support did not confer a survival advantage[246]. A subsequent study in patients with very severe disease, DF >85, failed to demonstrate any impact of amino acid therapy on survival but did indicate a positive impact on improvement of markers of liver dysfunction similar to a prior study in patients with mild to moderate severity disease[549, 550]. These trials indicate that amino acid supplementation improves recovery in measures of liver function in patients with alcoholic hepatitis of varying severity. However, a consistent benefit in terms of mortality is not seen, this may be, in part, a function heterogeneity in the patient population and the comparatively small number of patients evaluated.

Metabonomics is the quantitative measurement of the multiparametric metabolic changes in a complex organism in a disease state. Typically, nuclear magnetic resonance (NMR) or mass spectrometry (MS) are used as they have the capacity to measure the concentrations of many molecules simultaneously[551]. Analyses may be performed as part of untargeted 'profiling', aiming to give semi-quantitative information for a broad range of compounds. Alternatively, a 'targeted' approach may be adopted in order to derive quantitative data relating to multiple compounds within a particular class. Mass spectrometry using derivatisation, coupled reverse-phase high performance liquid chromatography, provides a suitable means for detecting amino acids in complex biological fluids. Chemical standards may be used to either to generate standard curves or, if ^{13}C-labelled, for inline absolute quantification.

The datasets generated using metabolomics techniques are often contain many variables which are closely correlated. Analysis of such datasets requires use of statistical techniques which are able to account for both the noise and correlation structures of such datasets. Such multivariate modelling techniques include unsupervised analyses such principal component analysis (PCA) and supervised techniques such as orthogonal projection to latent structures discriminant analysis (OPLS-DA).

Principal components analysis seeks to desscribe variation in a dataset by summarising information information spread across several, potentially correlated variables into unrelated latent variables termed principal components[552]. Principal components are generated such that the first component describes the greatest variance in the dataset whilst subsequent components summarise successively less variation[552]. The PCA algorithm is not given prior information regarding the classes to which observations belong and thus is termed 'unsupervised' and thus permits exploration of patterns in datasets in a similar manner to clustering[553].

Orthogonal projection to latent structures discriminant analysis is, in contrast, a supervised multivariate analytical technique[554]. The model is given prior information regarding which class each observation belongs to and then seeks to summarise the variation in the dataset into that which

predicts class (the predictive component) and that which is unrelated to class (orthogonal variation)[554]. Having devised a valid model it is then necessary to determine which variables contribute to the predictive component. A plethora of techniques exist in order to attempt to select those variables which are most important in a predictive model. These include 'hard thresholding' based upon cut-offs for either the loadings along or correlation coefficient with the predictive component and use of the variable importance in projection methodology[555] [556].

6.3 Aim

The aims of the analyses described in this chapter were to conduct an exploratory analysis in order to:

1. Examine serum amino acid profiles in patients with severe alcoholic hepatitis and to compare these profiles with those of alcohol-related liver disease and healthy controls;
2. Determine the relationships between serum amino acid profiles and demographic, clinical and histological parameters and outcome data in patients presenting with severe alcoholic hepatitis;
3. Examine the influence of *SLC38A4* rs11183620 genotype on serum amino acid profiles in patients with severe alcoholic hepatitis;

6.4 Patients, materials and methods

6.4.1 Healthy control samples

Healthy controls (n=20) were recruited *via* the Imperial College biomarkers study. None of the participants had a history of liver disease; consumed alcohol in excess of recommended limits at the time of recruitment (21 units/week for men and 14 units/week for women); had a diagnosis of diabetes or a BMI >30 kg/m^2.

6.4.2 Patients with alcohol-related cirrhosis

A disease control population of patients with decompensated alcohol-related cirrhosis (n=18) were recruited *via* the Liver Units at St Mary's and Kings College Hospitals. All patients had a history of excess alcohol consumption, though not all were actively drinking at the time of recruitment; other causes of liver disease were systematically excluded. The diagnosis of cirrhosis was made based upon a combination of clinical examination, biochemical results and clinical imaging. The severity of the liver injury was assessed using the model for end-stage liver disease s (MELD) score. None had a clinical diagnosis of alcoholic hepatitis; histological samples were not routinely obtained.

6.4.3 Patients with severe alcoholic hepatitis

Patients with severe alcoholic hepatitis were recruited through the steroids or pentoxifylline for alcoholic hepatitis (STOPAH) trial[208]. Inclusion was based upon a clinical diagnosis of alcoholic hepatitis, Maddrey's discriminant function (DF) ≥32, current excess alcohol consumption, recent onset of jaundice and exclusion of other causes of decompensated liver disease[265]. A subset of patients (n=85) with available serum were selected for inclusion in the present study. Those with histological confirmation of alcoholic steatohepatitis in biopsies obtained within 7 days of the start of treatment (68/85, 80%) were preferentially selected for inclusion.

6.4.4 Histological evaluation in patients with severe alcoholic hepatitis

Histological variables, including: neutrophilic inflammation and the presence or absence of severe ballooning degeneration were scored using the criteria defined in the Alcoholic Hepatitis Histological Scoring system (AHHS)[43]. The collagen proportionate area (CPA) was quantified across the entire biopsy specimen using a machine-learning based image analysis program. The fat proportionate area (FPA) was similarly derived but was adjusted for the CPA. Full details of the methods used, including the imputation of missing data are provided in Chapter 3 section 3.4.4 and Supplementary Methods.

6.4.5 Serum amino acid estimation

Blood was collected from participants in serum separator tubes. Once drawn the sample was allowed to settle and clot at room temperature prior to centrifugation at 1200*g* for 10 minutes. The serum was divided into 0.5-1ml aliquots and stored at -80°C for further use. Samples were processed within 2 hours of being taken.

Serum amino acid concentrations were determined by mass spectrometry at the Clinical Phenome Centre, Imperial College, in accordance with previously described methodology, summarised below[557]. Serum aliquots were thawed at 4°C; 10µl of serum was transferred to an Eppendorf tube and diluted 1:1 with Optima grade water (Fisher Scientific, Leicester, United Kingdom). Solutions of stable-isotope-labelled amino acids were combined in Optima grade water to generate an internal standard mixture with each labelled amino acid at a concentration of 10µg/ml. The diluted serum samples were spiked with 5µl of the internal standard mixture. Samples were deproteinised by: addition of 40µl of cold isopropanol (containing 1% formic acid (v/v), (Sigma-Aldrich, Gillingham, United Kingdom), vortex mixing and incubation at -20°C for 20 minutes followed by centrifugation at 13,000*g* and 4°C for 10 minutes. Ten µl of supernatant was then transferred from the Eppendorf tube to a glass high-performance liquid chromatography (HPLC) vial for derivatisation. This was achieved by adding 70µL of borate buffer (pH 8.6) to the samples, vortex mixing and then adding 20µL of AccQTag Ultra derivatizing reagent solution (Waters Corporation, Milford, USA), with further vortex

mixing, and heating at 55°C (10 min). Samples were then diluted 1:100 with Optima grade water for analysis.

Ultra-high-performance liquid chromatography (UHPLC)-MS/MS analysis was performed using an Acquity UPLC binary solvent manager, sampler manager, and column manager (Waters, Milford, USA) interfaced with a Xevo TQ-S tandem quadrupole mass spectrometer (Waters, Wilmslow, United Kingdom). MS/MS detection was performed *via* electrospray ionization (ESI) in positive ion mode using multiple reaction monitoring for the quantification of each compound. Nitrogen was used as the desolvation gas, and argon was used as the collision gas. The chromatographic separation used reversed-phase gradient chromatography on an HSS T3 2.1 × 150 mm, 1.8 µm column (Waters). The mobile phase was composed of 0.1% formic acid in water (v/v) (A) and 0.1% formic acid in acetonitrile (v/v) (B). The column temperature was maintained at 45 °C and linear gradient elution was performed at 0.6 mL/min starting at 4% B, held for 0.5 min before increasing to 10% over 2 min, then to 28% over 2.5 min, and finally increasing to 95% for 1 min, before returning to 4% B (1.3 min) for re-equilibration. The weak and the strong washes were 95:5 water/acetonitrile (v/v) and 100% isopropanol, respectively.

Study samples were randomised using disease status as a stratification factor in order to avoid the introduction of bias due to run-order effects. Samples were run in a single run. Quality control samples were interspersed at regular intervals throughout the run, in order to ensure stability of the system. The amino acids assayed, together with the dynamic ranges of the assay, are summarised in Table 6.1.

Table 6.1 Amino acids quantified by mass spectrometry with dynamic ranges of the assay

Amino acid	LLOQ (µM)	ULOQ (µM)	Amino acid	LLOQ (µM)	ULOQ (µM)
4-Hydroxyproline	1	400	**Serine**	2	400
Alanine	1	400	**Threonine**	1	400
Arginine	10	400	**Tryptophan**	1	400
Aspartic acid	1	400	**Tyrosine**	1	400
Asparagine	1	400	**Valine**	1	400
Carnosine	10	400	**β-Amino-iso-Butyric acid**	2	400
Cystine	10	400	**Citrulline**	2	400
Ethanolamine	1	400	**Cystathionine**	1	400
Glutamic acid	1	400	**3-Methylhistidine**	10	400
Glutamine	1	400	**1-Methylhistidine**	10	400
Glycine	10	400	**Hydroxylysine**	10	400
Histidine	2	400	**Ornithine**	10	400
Isoleucine	1	400	**Aminoadipic acid**	1	400
Leucine	1	400	**α-Amino-n-Butyric acid**	2	400
Lysine	1	400	**Sarcosine**	1	400
Methionine	1	400	**β-Alanine**	1	400
Phenylalanine	1	400	**γ-Amino-n-Butyric acid**	2	400
Proline	2	400			

Abbreviations: LLOQ: Lower limit of quantification; ULOQ: Upper limit of quantification

6.4.6 Data processing and statistical analyses

6.4.6.1 Amino acid data

An amino acid was considered unquantified within the study population and excluded from subsequent analysis if it was undetectable in >10% of samples. Where missing data fell below this threshold absent values were imputed as the lower limit of quantification. A non-normal distribution of data was assumed.

6.4.6.2 Healthy control and liver disease group comparisons

Serum amino acid concentrations were compared between groups using either a Mann-Whitney U or Kruskal-Wallis test, depending upon the number of groups included. Differences in serum amino acids

were tested in healthy control and liver disease cohorts between groups defined by sex and drinking status. Spearman's rank correlation test was used to assess for correlations between serum amino acids and both age and body mass index (BMI). In the cohort of patients with severe alcoholic hepatitis serum amino acid concentrations were also correlated with reported alcohol consumption.

Multivariate analysis was used to further explore between-group differences in the levels of serum amino acids. Prior to modelling amino acid data were log-transformed and mean centre scaled. Principal components analysis (PCA) was used to visualise the data and assess the ability of multivariate analyses to separate relevant clinical groups. Orthogonal projection to latent structures discriminant analysis (OPLS-DA) was performed to assess the ability of serum amino acids to distinguish between clinical cohorts. Groups were compared in a pairwise fashion. Model performance was assessed based upon i) a positive R^2 indicating explanation of a significant proportion of variation in the data; and, ii) a positive Q^2, derived using seven-fold leave one out cross-validation, indicating the ability of the model to correctly predict new data. Models were considered valid if the CV-ANOVA returned $p<0.05$ and the reported R^2 and Q^2 exceeded values determined by permutation testing (n=200) with random label-swapping. Furthermore in discriminant analysis (DA) models the misclassification table, generated in SIMCA, was used to generate a sensitivity and specificity for the model as a further marker of validity. The S-plot was used to determine which amino acids contributed significantly to valid models. Important amino acids were identified based upon a significant conitrbution the predictive component of the model (covariance, p1) and a strong correlation with phenotype (p(corr)). Given the preliminary nature of the work strict cut-offs to define metabolites of interest were not applied and were considered model by model though p(corr)>0.5 or <-0.5 and p1 >0.2 or <-0.2 were used as guides.

6.4.6.3 Severe alcoholic hepatitis groups comparisons

In the cohort of patients with severe alcoholic hepatitis the Spearman's rank test was used to correlate serum amino acid concentrations with clinical parameters, a Benjamini-Hochberg correction was

applied for a false discovery rate (FDR) of 0.05 due to the large number of comparisons made. Multivariate analyses were performed to examine the associations between serum amino acid profiles and clinical and histological parameters as well as the Lille response, 28-day and 90-day mortality. In light of the known effect of prednisolone on both Lille score and 28-day mortality a term for prednisolone treatment was included in the X matrix for these analyses. Orthogonal projection to latent structures regression analysis (OPLS) was used to assess the association between serum amino acids and continuous variables including disease severity scores and the collagen or fat proportionate areas on liver biopsy. OPLS-Discriminant Analysis (OPLS-DA) was used to determine whether serum amino acid levels could distinguish between groups defined by mortality or the presence of severe inflammation or ballooning on liver biopsy. The criteria used to assess model validity and performance are as described above. The association between *SLC38A4* rs11183620 genotype and serum amino acid concentrations was tested using the Jonckheere-Terpstra test, these analyses were restricted to amino acids known to be transported by *SLC38A4*[456] and which demonstrated significant dysregulation in patients with severe alcoholic hepatitis.

6.4.6.4 Statistical software

Statistical analyses were conducted in SPSS v24 (IBM, Armonk, USA) and R (R Foundation, Vienna, Austria). Multivariate analyses were conducted using SIMCA P+ v14 (Sartorius Stedim Biotech, Gottingen, Germany).

6.5 Results

6.5.1 Population characteristics

The cohorts of patients with alcohol-related cirrhosis and severe alcoholic hepatitis were of similar age and male predominance; healthy controls however were generally younger with a significant female preponderance (Table 6.2). Whilst all the patients with severe alcoholic hepatitis were, by definition, misusing alcohol at the time of recruitment, this was only the case for 7 (39%) of those with alcohol-related cirrhosis. Just over half of healthy controls were consuming alcohol to some degree. The distribution of MELD scores was similar between the patients with alcohol-related cirrhosis and severe alcoholic hepatitis indicating both groups had significantly decompensated liver disease. However, the relative contribution of the different parameters of liver dysfunction to these scores differed between the groups; those with severe alcoholic hepatitis tended to have higher levels of serum bilirubin but less coagulopathy and renal dysfunction (Table 6.2).

Table 6.2 Baseline characteristics of the study populations used in amino acid analyses

Characteristic	Healthy (n=20)	Alcohol-related cirrhosis (n=18)	Severe alcoholic hepatitis (n=85)
Age (years)	42 (36 – 57)	55 (49 – 61)	50 (43 – 56)
Gender (male)	4 (20%)	12 (67%)	57 (67%)
Alcohol misuse	NA	7 (39%)	85 (100%)
MELD	NA	25.8 (20.0 – 31.2)	23.5 (21.0 – 26.6)
DF	NA	NA	54.9 (40.3 – 75.4)
Haemoglobin (g/L)	NA*	94 (83 – 104)	108 (96 – 121)
White cell count ($x10^6/mm^3$)	NA*	7.5 (5.1 – 12.9)	9.0 (6.0 – 11.8)
Neutrophils ($x10^6/mm^3$)	NA*	5.9 (3.5 – 11.0)	6.0 (3.8 – 8.6)
Bilirubin (µmol/l)	NA*	156 (110 – 287)	309 (227 – 474)
Albumin (g/l)	NA*	26 (24 – 33)	24 (21 – 29)
Aspartate transaminase (IU/l)	NA*	67 (49 – 143)	131 (90 – 155)
Alanine transaminase (IU/l)	NA*	27 (16 – 47)	47 (31 – 70)
International normalised ratio	NA*	1.89 (1.49 – 2.33)	1.68 (1.50 – 2.04)
Urea (mmol/l)	NA*	8.1 (3.7 – 16.9)	3.4 (2.2 – 5.3)
Creatinine (µmol/l)	NA*	100 (62 – 150)	69 (58 – 98)
***SLC38A4* genotype**	NA*	NA	AA 19 (22.4%) AG 43 (50.8%) GG 23 (27.1%)

*Haematological and biochemical data were not available for healthy controls nor was sufficient biological material to retrospectively determine them

Data presented as median (IQR) or n (%)

Abbreviations: DF: Discriminant function; MELD: Model for end-stage liver disease; NA: Not available

6.5.2 *Serum amino acid data*

Eleven amino acids were excluded from the analysis due to missing data because data were missing in most if not all of the 123 samples (Table 6.3).

Table 6.3 Amino acids excluded from analysis due to missing data

Amino acid	Missing data (n, (%))
Carnosine	123 (100%)
Cysteine	53 (43%)
β--Amino-iso-Butyric acid	107 (87%)
Cystathionine	64 (52%)
3-Methylhistidine	122 (99%)
1-Methylhistidine	123 (100%)
Hydroxylysine	123 (100%)
Aminoadipic acid	121 (98%)
Sarcosine	93 (76%)
β--Alanine	18 (15%)
γ-Amino-n-Butyric acid	123 (100%)

Data presented as n (%)

6.5.3 *Serum amino acid concentrations*

6.5.3.1 Associations with demographic features

Significant differences were observed In healthy controls in the median (IQR) concentrations of 4-hydroxyproline (female: 6.1 (5.2– 6.7) µM *vs*. male: 9.5 (8.3– 12.1) µM, p=0.003) and glycine (female: 207 (188– 297) µM *vs*. male: 141 (126– 176) µM, p=0.003) in groups defined by sex. Serum glutamic acid concentrations were positively correlated with age (rho=0.54, p=0.02) and BMI (rho=0.54, p=0.01) whilst age was also positively correlated with serum citrulline concentrations (rho=0.61, p<0.01). However, none of these associations was seen in the group of patients with liver disease (data not shown). There were no differences in serum amino acid concentrations in healthy controls or patients with alcohol-related cirrhosis in groups defined by drinking or abstinence. In patients with

severe alcoholic hepatitis there were no significant correlations between serum amino acids and reported alcohol consumption (data not shown).

6.5.3.2 Differences between healthy controls and liver disease groups

Marked differences were observed in serum concentrations of several amino acids across the three different study groups (Table 6.4). Elevated serum 4-hydroxyproline, proline, ethanolamine, glycine, methionine and tyrosine appeared to be features related to the presence of advanced liver disease with no further elevation seen in the cohort of patients with severe alcoholic hepatitis. In contrast elevation of serum aspartic acid, glutamic acid and serine appeared to be features of severe alcoholic hepatitis. Serum concentrations of the branched chain amino acids isoleucine, leucine and valine as well as the essential amino acid tryptophan demonstrated a stepwise decrease across the three groups.

Table 6.4 Serum amino acids in healthy controls, patients with alcohol-related cirrhosis and severe alcoholic hepatitis

Amino acid	Healthy (n=20)	Alcohol-related cirrhosis (n=18)	Severe alcoholic hepatitis (n=85)	P
4-Hydroxyproline	6.4 (5.5 – 7.8)	29.8 (18.7 – 58.6)	18.2 (14.1 – 27.9)	<0.001
Alanine	314 (240 – 362)	300 (204 – 443)	303 (245 – 348)	0.98
Arginine	103 (84 – 110)	102 (72 – 117)	93 (74 – 112)	0.57
Aspartic acid	20.4 (17.0 – 24.3)	14.9 (10.6 – 22.6)	31.9 (24.5 – 41.9)	<0.001
Asparagine	33.3 (29.3 – 40.9)	47.3 (35.3 – 67.3)	54.0 (44.1 – 67.9)	<0.001
Ethanolamine	6.16 (5.26 – 6.86)	27.3 (18.8 – 44.9)	31.5 (21.1 – 47.5)	<0.001
Glutamic acid	49.9 (42.0 – 61.1)	54.6 (35.0 – 81.0)	120 (85.9 – 228)	<0.001
Glutamine	467 (430 – 569)	570 (397 – 699)	433 (346 – 553)	0.04
Glycine	194 (181 – 271)	270 (217 – 336)	298 (259 – 348)	<0.001
Histidine	71 (59 – 75)	76 (59 – 102)	75 (63 – 89)	0.16
Isoleucine	57 (42 – 65)	43 (21 – 58)	34 (26 – 47)	<0.001
Leucine	115 (85 – 123)	81 (57 – 103)	63 (53 – 88)	<0.001
Lysine	146 (122 – 173)	163 (123 – 278)	182 (144 – 225)	0.01
Methionine	18 (15 – 22)	35 (29 – 86)	41 (30 – 60)	<0.001
Phenylalanine	60 (54 – 68)	101 (81 – 169)	83 (68 – 108)	<0.001
Proline	142 (114 – 162)	233 (171 – 341)	201 (150 – 250)	<0.001
Serine	114 (102 – 130)	89 (65 – 135)	149 (118 – 177)	<0.001
Threonine	99 (75 – 114)	123 (108 – 181)	169 (136 – 217)	<0.001
Tryptophan	60 (50 – 68)	44 (28 – 57)	30 (21 – 56)	<0.001
Tyrosine	58 (47 – 77)	159 (104 – 192)	104 (68 – 146)	<0.001
Valine	182 (144 – 206)	121 (92 – 180)	100 (83 – 133)	<0.001
Citrulline	22 (19 – 28)	30 (24 – 42)	24 (18 – 31)	0.02
Ornithine	53 (39 – 57)	69 (47 – 98)	57 (43 – 77)	0.09
α-Amino-n-Butyric acid	12 (7.4 – 13)	13 (6.8 – 25)	14 (9.2 – 19)	0.23

Data presented as median (IQR)

A multivariate PCA model encompassing all three groups was successfully fitted (R^2= 0.81, Q^2 = 0.55). This demonstrated separation of the three groups, in particular along principal components (PC) 2 and 3 (Figure 6.1A), though overlap, particularly between the liver disease groups was noted. OPLS-DA models were successfully fitted for discrimination between healthy controls and both patients with alcohol-related cirrhosis (R^2X = 0.73, R^2Y = 0.88, Q^2 = 0.85, CV-ANOVA p<0.00001, sensitivity 100%, specificity 100%) and those with severe alcoholic hepatitis (R^2X = 0.75, R^2Y = 0.89, Q^2 = 0.85, CV-ANOVA

p<0.00001, sensitivity 100%, specificity 100%). OPLS-DA was also able to discriminate between patients with alcohol-related cirrhosis and severe alcoholic hepatitis ($R^2X = 0.74$, $R^2Y = 0.67$, $Q^2 = 0.48$, CV-ANOVA p<0.00001, sensitivity 98%, specificity 89%). Examination of the loadings plots (Figure 4.1B-D) confirmed the findings of the univariate analyses – changes in leucine, isoleucine, valine and tryptophan appeared to be associated with the development of liver disease. In contrast, aspartic acid and glutamic acid appeared to contribute most to discrimination between alcohol-related cirrhosis and severe alcoholic hepatitis.

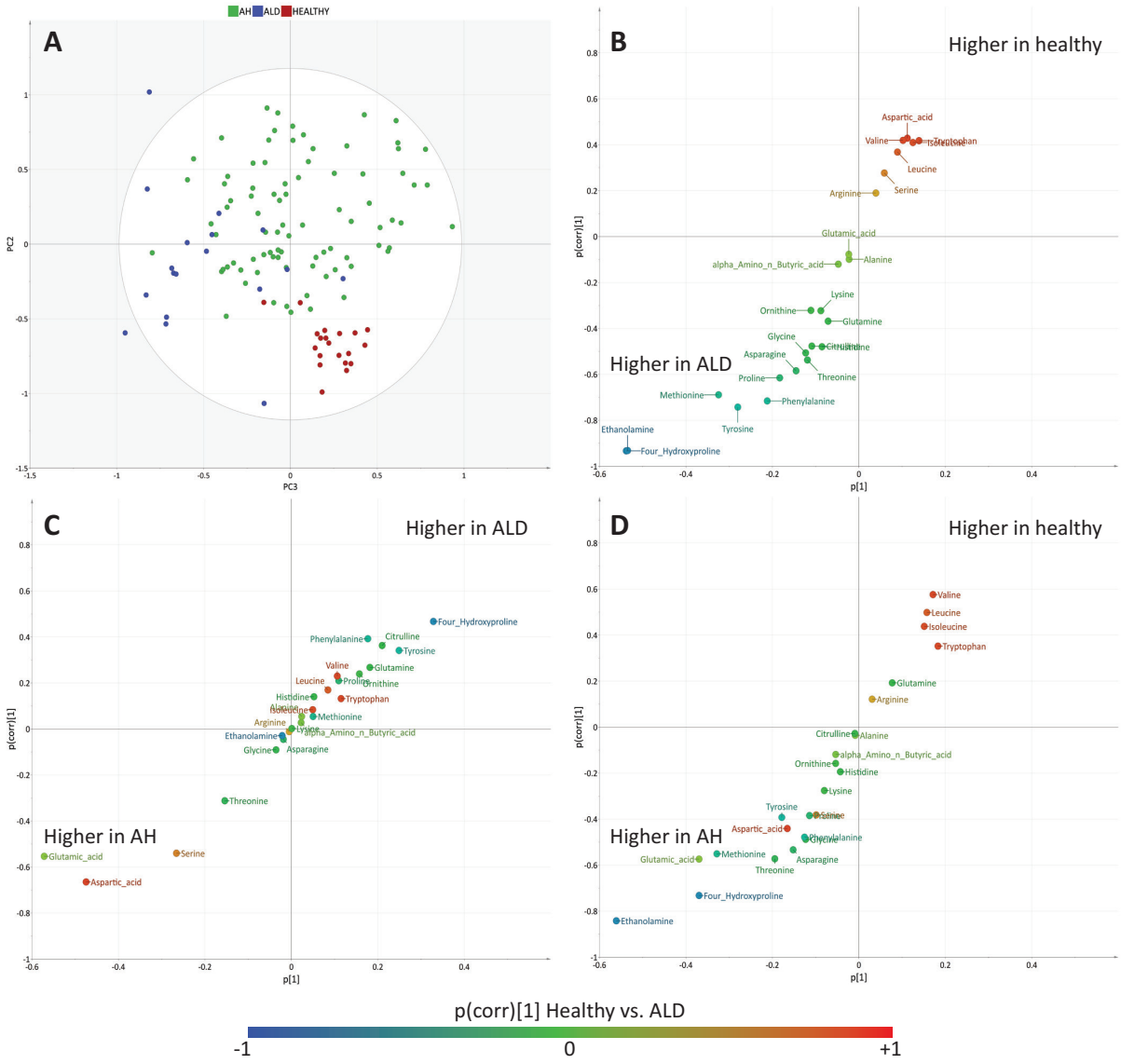


Figure 6.1 Multivariate analyses of serum amino acid profiles in healthy controls, patients with alcohol-related cirrhosis and severe alcoholic hepatitis

A. Scores plot of principal components analysis (PCA) of all three groups demonstrating clustering of healthy controls distinct from liver disease groups. Incomplete separation of liver disease groups is seen. S-plots of OPLS-DA models comparing: B. Healthy controls and alcohol-related cirrhosis; C. Alcohol-related cirrhosis and severe alcoholic hepatitis and D. Healthy controls and severe alcoholic hepatitis. Points are coloured based upon the correlation coefficient (p(corr)[1]) derived from the OPLS-DA model comparing healthy controls and alcohol-related cirrhosis. The BCAAs and tryptophan were consistently higher in healthy controls compared to liver disease groups whilst ethanolamine and 4-hydroxyproline were consistently lower (B and D). In contrast higher concentrations of serine, glutamic and aspartic acids and lower concentrations of 4-hydorxyproline appeared to differentiate severe alcoholic hepatitis from alcohol-related cirrhosis (C).

6.5.4 *Correlations with clinical parameters in severe alcoholic hepatitis*

In patients with severe alcoholic hepatitis correlations were noted between several amino acids and relevant clinical variables (Figure 6.2). Whilst serum glutamic acid (rho=0.33, q=0.04) and methionine (rho=0.35, q=0.02) were weakly correlated with the discriminant function, individual serum amino acid levels were not, in general, correlated with disease severity as measured by the DF or MELD scores. The Lille score appeared to demonstrate positive correlations with a number of amino acids and peripheral white cell and platelets counts the converse (Figure 6.2). Several amino acids were positively correlated with the serum urea whilst serum AST and ALP activities positively correlated with serum aspartic and glutamic acids.

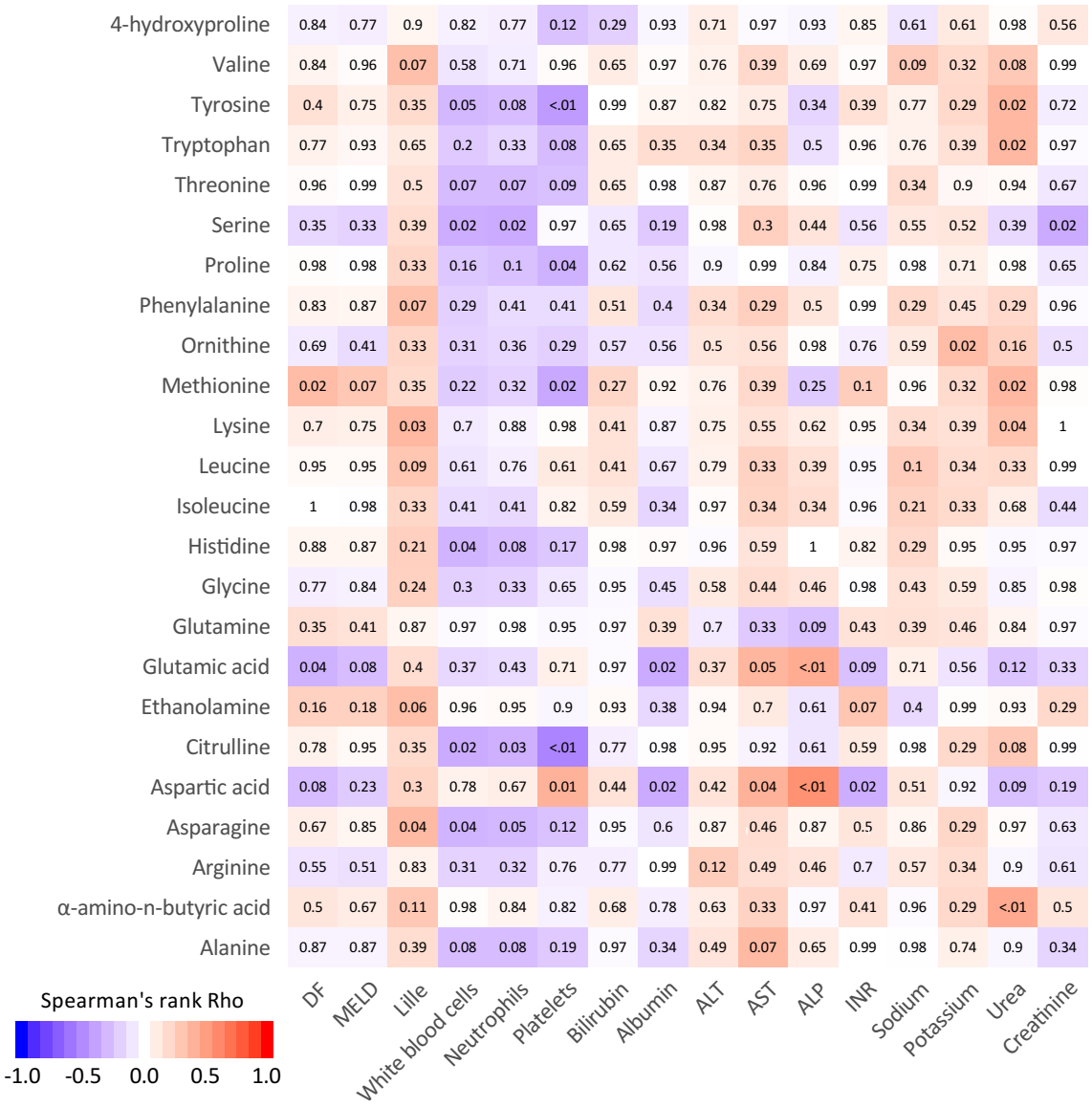


Figure 6.2 Correlation matrix for serum amino acids and clinical variables in patients with severe alcoholic hepatitis

Cells are shaded based upon the Spearman's rank correlation coefficient and annotated with the Benjamini-Hochberg adjusted p-value (q) corrected for a false discovery rate of 0.05. Strong positive correlations are noted between the serum urea and several amino acids as well as between serum ALP and AST activities and serum aspartic and glutamic acid concentrations. Peripheral neutrophil and platelet counts were negatively correlated with a number of amino acids.

Valid OPLS-DA models could not be fitted for the outcomes of Lille response ($R^2X = 0.26$, $R^2Y = 0.15$, $Q^2 = 0.06$, CV-ANOVA p=0.16, sensitivity 63%, specificity 62%), 28-day mortality ($R^2X = 0.51$, $R^2Y = 0.08$, $Q^2 = 0.04$, CV-ANOVA p=0.17, sensitivity 83%, specificity 0%) or 90-day mortality ($R^2X = 0.49$, $R^2Y = 0.23$, $Q^2 = 0.01$, CV-ANOVA p=0.93, sensitivity 81%, specificity 63%). A multivariate OPLS regression

model was successfully fitted for the DF (R^2X = 0.28, R^2Y = 0.16, Q^2 = 0.08, CV-ANOVA p=0.03) and confirmed univariate observations of positive correlations with methionine and tyrosine and negative correlations with glutamic and aspartic acid (Figure 6.3A). Although the model for MELD did not meet the threshold for significance (R^2X = 0.63, R^2Y = 0.32, Q^2 = 0.14, CV-ANOVA p=0.08), the amino acids demonstrating strong positive and negative loadings in the model were similar to those seen for DF (Figure 6.3B).

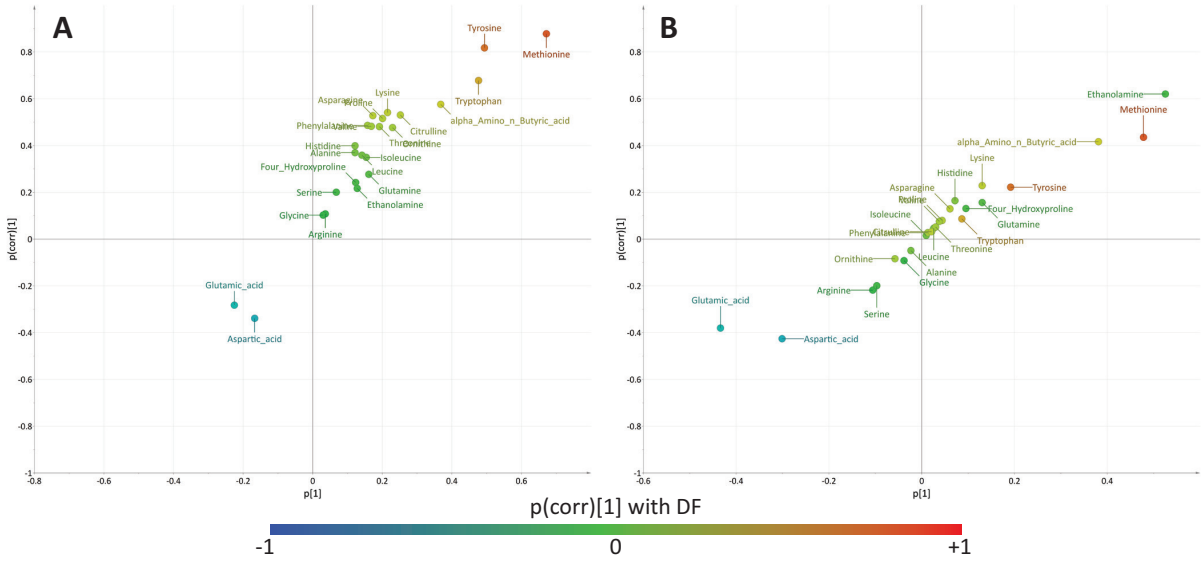


Figure 6.3 S-plots derived from OPLS regression models of serum amino acid profiles against (A) DF and (B) MELD in patients with severe alcoholic hepatitis

Points are coloured based upon the p(corr)[1] derived from the multivariable linear regression analysis against DF. A. Serum levels of most amino acids appear to be positively associated with increasing disease severity. The majority of associations were weak; those of methionine and the aromatic amino acids were stronger. In contrast only aspartic and glutamic acids were negatively correlated with disease severity, as measured by the DF. B. The OPLS regression model for MELD did not reach significance however the pattern of amino acids showing positive and negative correlations with MELD were broadly similar to that seen with the DF.

Valid OPLS-DA models could not be fitted for the presence or absence of severe inflammation or hepatocyte ballooning on liver biopsy. However, a valid OPLS regression model was fitted for both the collagen proportionate area (R^2X = 0.21, R^2Y = 0.23, Q^2 = 0.14, CV-ANOVA p<0.001, Figure 6.4A) and the Laennec fibrosis grade (R^2X = 0.27, R^2Y = 0.30, Q^2 = 0.21, CV-ANOVA p<0.001, Figure 6.4B). An OPLS

regression model for the fat proportionate area demonstrated similar performance ($R^2X = 0.49$, $R^2Y = 0.43$, $Q^2 = 0.25$, CV-ANOVA $p<0.001$) with inverse correlations (Figure 6.4C).

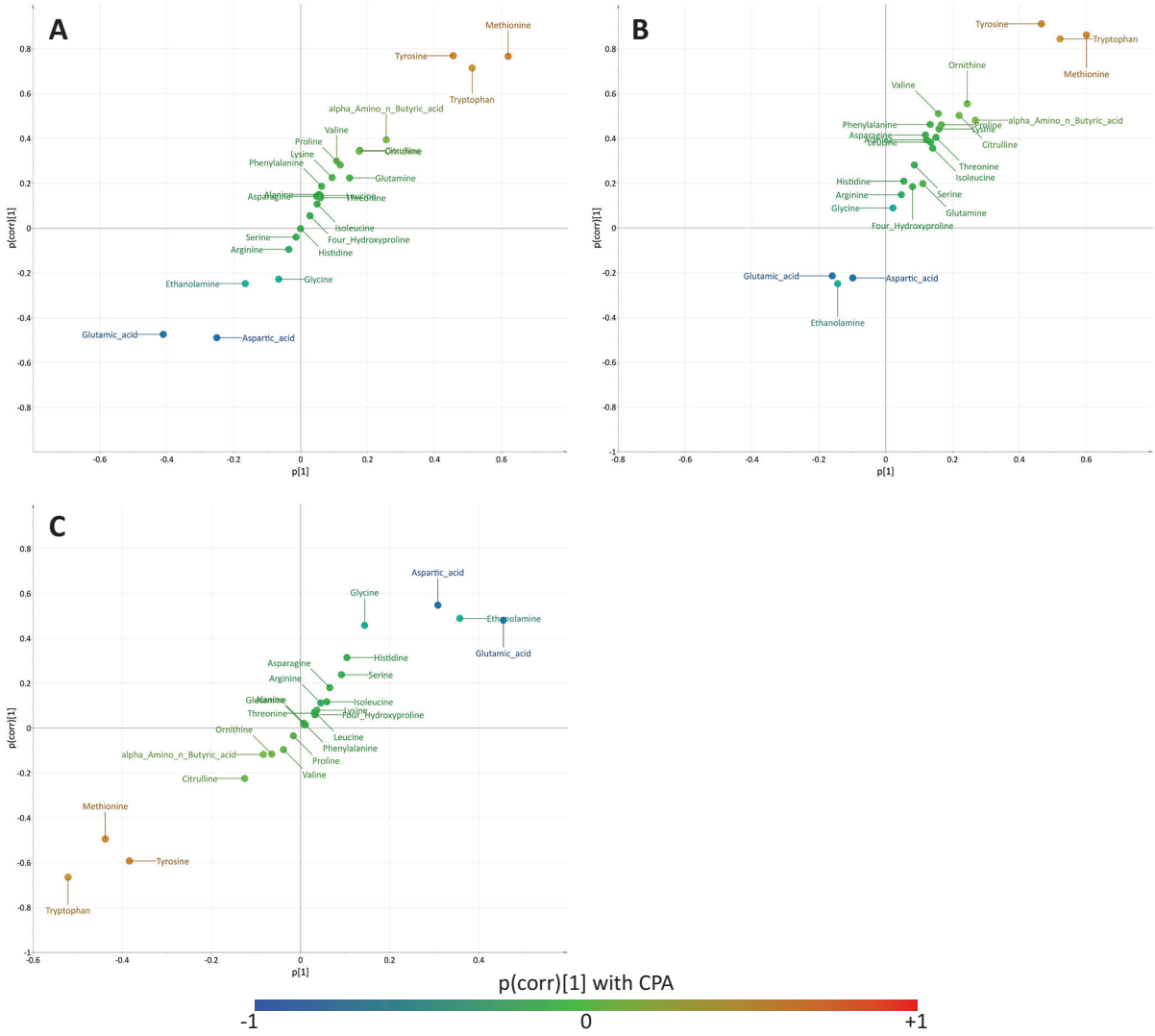


Figure 6.4 S-plots of serum amino acids derived from OPLS regression models of serum amino acid profiles against the A. Collagen proportionate area (CPA), B. Laennec grade and C. Fat proportionate area (FPA) on liver biopsy

(A) Serum levels of methionine, tryptophan and tyrosine were positively correlated with the CPA whilst glutamic and aspartic acids were negatively correlated. (B) These findings were replicated in analyses evaluating associations with the Laennec grade whilst the inverse was seen with the FPA (C).

6.5.5 Serum amino acids, by SLC38A4 rs11183620 genotype

Eight amino acids were identified as significant substrates for *SLC38A4* (alanine, serine, glycine, asparagine, threonine, proline, methionine and glutamine). All but alanine were significantly

dysregulated in severe alcoholic hepatitis compared to control groups. Serum glutamine levels demonstrated a stepwise increase with carriage and homozygosity for rs11183620:G (AA median 422 (IQR 248– 511) µM; AG: 409 (339– 550) µM; GG: 484 (393– 605) µM, p=0.04). No association was seen between any of the other amino acids and *SLC38A4* rs11183620 genotype. A valid OPLS regression model incorporating these amino acids against *SLC38A4* rs11183620 genotype could not be fitted (R^2X = 0.68, R^2Y = 0.14, Q^2 = 0.06, CV-ANOVA p=0.33).

6.6 Discussion

Derangement of circulating amino acid concentrations has been described in patients with alcohol-related liver disease and many of these previous findings are replicated here. The groups of patients with alcohol-related liver disease included in the present study both showed significant reductions in serum levels of branched chain amino acids and elevations of the aromatic amino acids phenylalanine and tyrosine. This is in line with published literature[524].

Serum 4-hydoxyproline concentrations were significantly higher in patients with alcohol-related liver disease compared to controls. 4-Hydoxyproline concentrations were also higher in patients with alcohol-related cirrhosis compared to patients with severe alcoholic hepatitis, running counter to prior reports[529]. Hydroxyproline is found almost exclusively in collagen and estimation of tissue content is widely used as a measure of the extent of fibrosis in animal models[558, 559]. Thus, as expected, liver tissue levels are increased in patients with cirrhosis[560]. However, serum hydroxyproline concentrations did not correlate with either the CPA or Laennec grade in patients with biopsy-proven severe alcoholic hepatitis, nor were they significantly correlated with either clinical variables or outcomes. These findings are consistent with prior reports that serum proline concentration is not a reliable marker of the histological status of alcohol-related liver disease[561, 562]. The explanation for this may lie in the effect of alcohol on non-hepatic tissues rich in hydroxyproline, in particular bone[563].

Serum ethanolamine concentrations were also significantly higher in patients with alcohol-related liver disease; though they did not differentiate between disease phenotypes. Ethanolamine is the second most common amine head group on cell membrane phospholipids, after choline. Culture of cells in conditions of elevated ethanolamine concentrations inhibits choline uptake and subsequent formation of phosphatidylcholine, though the converse is not observed[564-566]. In cell culture, alcohol inhibits incorporation of both ethanolamine and choline into phospholipids and alters ethanolamine metabolism through inhibition of its conversion into phosphorylethanolamine which occurs prior to

its incorporation into phospholipids[567]. Alterations in the phosphatidylcholine to phosphatidylethanolamine ratio in hepatocyte cell membranes have been described in murine models of steatohepatitis. Relative increases in the phosphatidylethanolamines are associated with a reduction in membrane integrity, increases in the serum ALT concentration and development of steatohepatitis[568]. This could relate to an inhibitory effect of both ethanolamine and phosphatidylethanolamine on mitochondrial function[569]. In animals models of liver regenerations, both administration of exogenous polyunsaturated phosphatidylcholine and raised serum levels of ethanolamine have been associated with enhanced responses[570, 571]. Consequently whilst deficiency of the former may impair regeneration, the latter are not necessarily hepatotoxic. Thus, whilst increased ethanolamine levels are clearly a feature of advanced and decompensated alcohol-related liver disease, their pathophysiological significance is unclear.

Serum methionine levels were also elevated in patients with alcohol-related liver disease, irrespective of phenotype; concentrations also correlated with scores of disease severity and the extent of fibrosis on liver biopsy. In the liver, methionine is converted to *S*-adenosylmethionine (SAM) by methionine adenosyltransferase. SAM functions as a primary methyl donor; donation of its methyl group results in its conversion to *S*-adenosylhomocysteine (SAH); a potent inhibitor of the enzyme methionine adenosyltransferase (MAT)[572, 573]. A reduction in MAT expression is associated with reduced hepatic SAM and glutathione concentrations and the spontaneous development of steatohepatitis[574]. Reduced expression of MAT isoform 1A, has been described in patients with cirrhosis, most likely driven by epigenetic factors, has been described[575]. The B vitamins, particularly thiamine, pyridoxine, folate and cobalamin, are required for normal methionine metabolism. Their deficiency in patients with alcohol-related liver disease leads to further inhibition of methionine metabolism and SAM production[576]. SAM is a precursor to glutathione synthesis and has been implicated in modulating cell proliferation, death and inflammatory responses. Consequently, deficiency may lead to or exacerbate liver injury and supplementation has been proposed as therapy for several liver diseases including alcohol-related cirrhosis[577]. However, despite promising pre-clinical data from animal models

supplementation with SAM in patients with various forms of alcohol-related liver disease has not shown clinical benefit[578]. Thus, the data presented here are consistent with the literature and that methionine metabolism is increasingly impaired as liver functional capacity decreases. There was no preferential elevation in patients with severe alcoholic hepatitis suggesting that therapies such as SAM supplementation are unlikely to demonstrate differing efficacy in this group of patients.

Three amino acids: serine and glutamic and aspartic acids, were significantly higher in patients with severe alcoholic hepatitis compared to those with alcohol-related cirrhosis. Serine is a neutral amino acid that is also involved in methyl group transfer. This occurs primarily in two different reactions i) donation of a methyl group to tetrahydrofolate; and, ii) combination with homocysteine to yield cystathionine, ultimately facilitating the regeneration of methionine. Indeed, serine may be the primary source of methyl groups for the remethylation of homocysteine[579]. In rats dietary protein restriction leads to an increase in serine synthesis and hence circulating serum concentrations. Alongside this an increase in glycine was also noted both in serum and intrahepatically[580]. Interestingly serine biosynthesis has been increasingly recognised as an important factor in the proliferation of tumours[581-583]. It is possible that in the presence of increased cellular proliferation serine metabolism may act as an alternative mechanism for generating adenosine triphosphate (ATP)[584]. Severe alcoholic hepatitis is associated with active inflammation and oxidative stress and consequent high cell turnover and regeneration. In this context alternative means of ATP generation such as serine biosynthesis might be called into play in order to meet the increased metabolic demands.

Aminotransferases catalyse the interconversion of an amino acid and alpha-ketoglutarate to a ketoacid and glutamate, this is the first step in amino acid deamination and the generation of urea in order to eliminate ammonium. AST, for example, catalyses the interconversion of oxaloacetate and glutamic acid to aspartic acid and alpha-ketoglutarate. Interestingly both glutamic and aspartic acid levels were correlated with serum AST and alkaline phosphatase (ALP) but not alanine transaminase

(ALT) activities. An elevated serum AST:ALT ratio is widely considered a hallmark of alcohol-related liver injury and a measure of hepatocellular death. Consequently, the elevation of serum glutamic and aspartic acids in patients with severe alcoholic hepatitis may be indicative of a failure of deamination due to alcohol-induced hepatocellular dysfunction and death.

Finally, no strong relationship was found between *SLC38A4* rs11183620 genotype and the serum concentrations of any of the amino acids which were both altered in severe alcoholic hepatitis patients and it is reported to transport. Given the profound nature of the hepatocellular failure seen in patients with severe alcoholic hepatitis and the likely existence of compensatory mechanisms this is, perhaps, unsurprising.

This work is not without significant limitations. A key limitation pertains to statistical power. There was significant mismatching in terms of sample size between the clinical groups and, due to the exploratory nature of the experiment, an *a priori* power calculation was not performed. Sample sizes were predominantly determined by available biological material and resources available to conduct the analyses. Furthermore, amino acid estimation was performed on serum and not tissue samples. Several other tissues, in particular skeletal muscle and the kidney have important roles in amino acid handling and, thus, serum concentrations of amino acids are not solely determined by hepatic function. Additionally, the majority of healthy controls were female whilst disease groups were predominantly male and samples were not taken consistently in relation to either the time of day or last meal. In normal healthy individuals plasma amino acid concentrations vary with age and sex[585, 586]. The amount of exercise taken is also a potential factor[587]. Whilst amino acid levels demonstrate reasonably little diurnal variation, levels may rise post-prandially, mainly in individuals consuming comparatively high protein diets (>1.5 g/kg) or change in a more complex way following periods of starvation[586, 588-590]. Linked to this, neither dietary information nor an assessment of nutritional status was recorded for any of the patients included in this study. However, many of the changes described here have also been reported in several prior studies providing a degree of validation of the data.

A number of amino acids could not be adequately quantified in a significant number of samples. In particular cysteine and cystathionine levels were unmeasurable in around half of the samples. Related to this a small number of amino acids were recorded at concentrations which fell outside the validated dynamic range of the assay used. This may pertain, in part, to the fact that within this methodology a number of amino acids have their concentrations 'monitored' rather than fully quantified. This is due to the fact that their concentrations are not sufficiently reliably measured. Achieving this standard would require significant method development which is outside the scope of this thesis. Additionally, whilst the targeted nature of the assay employed here permits quantification of several specific targets, information is lacking in relation to some potentially important intermediates. Quantification of intermediates in the one-carbon cycle as well as important co-factors such as B vitamins may have helped to unpick more precisely the location of key metabolic bottlenecks.

The failure of plasma amino acid profiles to predict either early changes in liver function, as measured by the Lille response, or mortality endpoints suggests that they are not necessarily drivers of outcome. Recent data indicate that the degree of impairment of urea synthesis is associated with both disease severity and outcome and its recovery in enhanced by prednisolone[591]. These changes likely reflect reduction in protein synthesis in the failing liver which is partially stimulated by steroids. This, in conjunction with the observation that the serum concentrations of many amino acids are actually raised rather than depressed in severe alcoholic hepatitis, indicates that the primary issue is not a lack of amino acids but, more likely a failure of their uptake and subsequent metabolism. This may explain the failure of supplementation studies and suggests that therapies targeted to the correction of metabolic failure may have greater success.

In summary, serum amino acid concentrations are grossly deranged in patients with alcohol-related liver disease; some additional changes appear specific to severe alcoholic hepatitis. There was no evidence of an influence of *SLC38A4* rs11183620 genotype on plasma amino acid profiles. However, given the generally small sample sizes, lack of standardisation of sampling, mismatching of patients

with liver disease and controls in relation to group size, demographics and absence of data relating to dietary intake, this should be considered a pilot study and firm conclusions cannot be drawn from these data.

CHAPTER 7

REVIEW OF FINDINGS AND FUTURE DIRECTIONS

7 Review of findings and future directions

7.1 Overview

Severe alcoholic hepatitis is a florid presentation of alcohol-related liver disease which occurs in a minority of individuals with alcohol-related liver disease[199]. The factors which predispose individuals to develop severe alcoholic hepatitis remain unclear[223]. The disease is associated with very high short-term mortality, in excess of 20% within 28 days[224, 226-229]. Despite modernisation of medical care this appears to have changed little over time[228]. This relates, in part, to a failure to develop new, effective drug therapies. Corticosteroids remain the mainstay of treatment, and only effective drug therapy, despite being originally proposed for use in the condition around 50 years ago[229, 258, 259].

A genetic contribution to the risk of developing alcohol-related cirrhosis is recognised[142-144]. A recent genome-wide association study successfully identified three risk loci – *PNPLA3*, *TM6SF2* and *MBOAT7*[151]. The first of these had already been widely reported as a risk factor for alcohol-related liver disease and a potential modulator of its clinical course[443-445, 451]. To date there have only been a small number of very limited genetic studies of severe alcoholic hepatitis[148, 198].

7.2 Review of findings

7.2.1 Genome-wide association study

The work presented here describes the conduct of the first genome-wide association study of severe alcoholic hepatitis with sufficient power to detect common variants conferring a moderate risk of disease. The variant rs738409 in *PNPLA3* was identified as a risk factor for the development of severe alcoholic hepatitis, consistent with a previous report and the phenotypic overlap with alcohol-related cirrhosis. However, the variants in *TM6SF2* and *MBOAT7*, for which associations with alcohol-related cirrhosis have been described, were not associated with an increased risk of severe alcoholic hepatitis in this population; indicating the overlap in genetic risk factors is incomplete. The study identified a

novel SNP, rs11183620 in *SLC38A4*, with a replicated association with the risk of developing severe alcoholic hepatitis. While this association did not reach genome-wide significance, the predominant hepatic expression and relevant biological function of the protein merited further evaluation.

7.2.2 The influence of genetic variation on histology, clinical variables and outcomes

Genetic variants associated with the risk of developing severe alcoholic hepatitis in the genome-wide association study were further evaluated to determine whether there was any additional influence on disease phenotype, clinical and histological, or outcome. The variant rs738409 in *PNPLA3* was associated with more severe histological lesions, slower short-term recovery in liver function and decreased medium-term survival[252]. In contrast no significant associations were found between the associated variant in *SLC38A4* and any of these aspects of severe alcoholic hepatitis. These analyses also highlighted the primacy of drinking behaviour in determining medium- and, by extension, longer-term outcomes in severe alcoholic hepatitis[237].

7.2.3 The potential role of SLC38A4 in severe alcoholic hepatitis

Analysis of whole liver RNAseq data indicated a reduction in *SLC38A4* expression with the development of severe alcoholic hepatitis compared to 'normal' liver and patients with alcoholic steatohepatitis but no impairment of liver function. Work in cell lines indicate that this may be driven, in part, by soluble mediators including IL-1β. Knockdown experiments conducted in cell lines suggest that a reduction in *SLC38A4* expression may be associated with an increase in expression of genes associated with cellular stress responses. However, analysis of serum amino acid profiles failed to demonstrated a link with *SLC38A4* rs11183620 genotype.

7.3 Future directions

The work described in this thesis alongside additional experiments performed during my doctoral studies have given rise to additional lines of enquiry to pursue.

7.3.1 Genetic studies

The exploratory stage of the genome-wide association study described above included only approximately one third of the total number of patients recruited to STOPAH with available DNA. Work in underway to obtain genome-wide genotyping data for the remaining patients, along with the controls. This will permit performance of a GWAS of severe alcoholic hepatitis with much greater power to detect more modest genetic effects of single variants, genes and pathways. In addition, it will facilitate performance of case-only analyses to test for a genetic influence on the clinical course of the disease and treatment effect. Further work will also seek to combine the STOPAH GWAS dataset with that of the study of alcohol-related cirrhosis[151] to facilitate a comparison of these two phenotypes.

Even with the additional statistical power afforded by inclusion of the cases and controls used in the replication phase of the GWAS reported here it is unlikely that additional variants explaining a significant proportion of the heritability of severe alcoholic hepatitis, or alcohol-related liver disease, will be discovered. It is possible that differences in epigenetic factors induced by alcohol consumption may explain differences in the susceptibility to alcohol-related liver disease and this is a field which I intend to explore.

7.3.2 The influence of rs738409 in PNPLA3

Work is underway to determine whether in addition to its influence on histology and clinical aspects of severe alcoholic hepatitis, the variant rs738409 in *PNPLA3* also has an effect on the serum lipid profile in patients with severe alcoholic hepatitis. Untargeted serum lipid profiles have been obtained using mass spectrometry, as have targeted assays of serum oxylipins. These data are currently being analysed.

7.3.3 The role of SLC38A4

The role of *SLC38A4* in the pathogenesis of severe alcoholic hepatitis remains unresolved. The data presented here are suggestive but not conclusive. Further work is underway to construct a CRISPR/Cas9 knockout cell line in order to explore the effect of down-regulation in a cleaner system. In addition to examining gene expression this work will also look at changes in protein levels, phosphorylation of eIF2α and alterations in the cellular transport of tritiated amino acids. Ultimately there is an aim to attempt to undertake some of this work in primary human hepatocytes. The available whole tissue RNA expression data will also be further interrogated to determine whether the alteration in expression of *SLC38A4* seen in severe alcoholic hepatitis is, in fact, part of a broader alteration in the expression of hepatocyte transporters and, if so, which pathways mediate this.

7.3.4 Prognostic modelling

During the course of my doctoral studies I have also evaluated the prognostic utility of a number of biomarkers including the neutrophil-to-lymphocyte ratio[592], procalcitonin[593], circulating bacterial DNA[255] and serum markers of iron metabolism[594]. Work is ongoing to derive novel prognostic models in severe alcoholic hepatitis with greater clinical utility which will potentially utilise these prognostic markers.

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SUPPLEMENTARY METHODS

9 Supplementary methods

9.1 Protocol for DNA extraction using QIAGEN QIAamp blood mini kit (Cat: 51304/51306)

1) Equilibrate samples to room temperature
2) Add 40µl of proteinase K to a microcentrifuge tube
3) Add 400µl of sample to the microcentrifuge tube, if available sample <400µl make up to a total volume of 400µl with phosphate buffered saline
4) Add 400µl of lysis buffer (AL) and mix by vortexing for ≥15s
5) Incubate at 56°C for 1 hour
6) Add 400µl of 100% ethanol and mix thoroughly by vortexing
7) Apply half of the mixture from previous step to a QIAamp Mini spin column. Centrifuge at 6000*g* for 1 minute. Discard the filtrate, load the column with the remaining mixture and centrifuge again at 6000*g* for 1 minute. Discard filtrate and place the mini spin column into a clean collection tube.
8) Apply 500µl of wash buffer AW1 to mini spin column, centrifuge at 6000*g* for 1 minute. Discard filtrate and place the mini spin column into a clean collection tube.
9) Apply 500µl of wash buffer AW2 to mini spin column, centrifuge at 20000*g* for 3 minutes. Discard filtrate and place the mini spin column into a clean collection tube.
10) Centrifuge at 20000*g* for 1 minute.
11) Place the mini spin column in a clean microcentrifuge tube. Add 200µl of elution buffer (AE) pre-warmed to 40°C and incubate at room temperature for 5 minutes then centrifuge at 6000*g* for 1 minute. Re-load the eluent onto the column, incubate for 5 minutes then centrifuge at 6000*g* for 1 minute.

9.2 UCL “Puregene” DNA extraction method

1) Thaw blood in a 37°C water bath for 30 min
2) Pour defrosted blood and 30ml 1X RBC lysis solution into a labelled 50ml Falcon tube
3) Incubate samples for 5 minutes at room temperature, invert several times during incubation
4) Centrifuge at 3000rpm and 4°C
5) Pour off the supernatant leaving the residual cell pellet
6) Repeat steps 2 to 4 until the cell pellet is clear of haemoglobin
7) Add 60 µL of Proteinase K with the white blood cell pellet and any remaining RBC lysis solution. Vortex until homogeneous
8) Add 10 mls of Cell lysis solution to each sample. Vortex for 10 secs
9) Leave in water bath at 55°C for a minimum of 2 hours. The sample should turn a straw yellow colour.
10) Place sample in ice for 5 mins. When cool add 3.33 mL of Protein Precipitation solution. Vortex for 10 secs. Leave in ice for 10 – 15 mins vortexing intermittently. (The mixture should go cloudy).
11) Spin solution @ 3,000 rpm and 4°C for 10 min
12) Whilst steps 10 and 11 are happening, relabel new falcon tubes and add 1 µL of Glycogen (Glycogen OPTIONAL – helps with DNA precipitation) and 10 mL Isopropanol
13) Carefully transfer supernatant, avoiding the precipitated protein pellet into the corresponding falcon tube containing the isopropanol
14) Invert tube several times until DNA strands precipitate
15) Spin solution @ 3,000 rpm and 4°C for 5 min to pellet DNA.
16) Carefully pour off supernatant (watch pellet).
17) Add 10 mL 70% Ethanol, vortex and leave on shaker for 10 mins.
18) Spin solution @ 3,000 rpm for 5 min to pellet DNA.
19) Carefully pour off supernatant (watch pellet). Dry tubes upside down (5 mins max)

20) Resuspend DNA pellet in 500 µL of TE buffer (depending on pellet size).

21) Incubate samples in water bath at 55 oC for 1 hour.

22) Leave in shaker at 37oC overnight.

Solutions used in extraction:

10X RBC Lysis Buffer	100mM NaCl, 10mM EDTA, 1.5M NH_4Cl.
Cell Lysis Solution	10 mM Tris-HCl pH 8.0, 25 mM EDTA, 0.5% SDS
Protein Precipitation solution	5M Ammonium Acetate

9.3 Digital image analysis of Haematoxylin and Eosin and Sirius Red stained liver sections for Steatosis and Fibrosis

9.3.1 Image analysis for steatosis

Stage 1: H&E biopsy images have a high intensity background, whilst liver tissue has a deep red color. Images were coloured in RGB colour space, so that three channels (Red, Green and Blue) could be used for visualization. To identify tissue regions in the image, the method separates the pixels of tissue from background pixels using clustering techniques. In this way, all the pixels of the image are grouped into two separate clusters (a cluster for tissue and a cluster for background). Specifically, the first stage employs the K-means algorithm, taking into account the colour (i.e. three intensity values ranging from 0 to 255) of each pixel for grouping. For both clusters the method initially defines a colour centroid (a center point of intensity values), in order to compare it with the colour of each pixel. During K-means execution, an iterative procedure assigns each pixel of the image either to tissue cluster or to background cluster, based upon the minimum colour distance with the centroids. In each iteration of the algorithm, the centroids are reconsidered according to the color of the members (pixels) of the cluster. The iteration stops when the color centroids are stabilized for two consecutive iterations. At the end of the algorithm execution, tissue pixels have been identified and the tissue area is calculated.

Stage 2: Once the tissue region has been identified, we attempt to detect all white regions in the core. Image processing techniques focusing on the detection of circular white regions within tissue, are used. Initially, a thresholding method converts the image into binary (0 or 1 pixel values) and then morphological operations employ a mask, with a specific shape and size to operate on that image. In our case, a circular mask is selected in order to recognise lipid droplets, eliminating all other structures. However, due to size variation between lipid droplets, an iterative procedure is utilised; in each iteration, the size of the circle into the mask is increased to match all the droplet sizes. The result

of the above procedure is the generation of a binary image, where pixels with value “1” belong to white regions in the core, while pixels with value “0” belong to ‘normal tissue’.

Stage 3: The methodology is finalised with the verification of lipid droplets in the set of white regions already detected in stage 2. During stage 2, several artefacts may be detected, such as vessels, gaps between cells, fragmented tissue, etc. Machine learning based on unsupervised hierarchical clustering approach is employed for the verification stage. The concept is the separation of white regions according to their size and roundness, in a) small-round, b) big-round, c) small-non-round, and d) big-non-round. Each of these groups may require different further analysis, due to the diverse characteristics of findings. Practically, the whole region dataset is clustered into two clusters of regions, and then each of them is further clustered into two sub-clusters. Hence, four sub-clusters of white regions have been extracted, which are assigned into a), b), c) and d) cases. Small-non-round regions are directly rejected, while round small are verified as lipid droplets. Big-non-round regions should be further examined due to the existence of accumulating fat areas. Finally, the evaluation of the method reveals that big round regions rarely exist. The area of steatosis is computed from the verified lipid droplets. The edges of verified lipid droplets are marked as green (Figure 1). The whole area of steatosis, divided by the whole tissue area, is computed as the fat percentage (fat%) in the core (Figure 1).

9.3.2 Image analysis for fibrosis

The proposed methodology to quantify fibrosis has already been validated in patients with chronic hepatitis C infection[435]. Briefly, it provides a fully automated image analysis of liver biopsies to extract Collagen Proportional Area (CPA). This is based upon clustering, classification algorithms, and machine learning in order to avoid manual tuning parameters and to provide tissue region characterisation. Regions on the image that have been characterized as vessels, muscles, capsule, or structural collagen are excluded from the computation of CPA. Results are given as Collagen Proportional Area (CPA), i.e. the percentage of collagen within the core.

SUPPLEMENTARY RESULTS

10 Supplementary Results

10.1.1 SLC38A4 rs11183620 association with baseline demography and assessment variables

Table 10.1: Baseline characteristics of cases with severe alcoholic hepatitis, by rs11183620 genotype

Characteristics	*SLC38A4* rs11183620 Genotype			Significance (p)
	AA (n = 236)	AG (n = 424)	GG (n = 200)	
Baseline demographics				
Age (years)	49 (41 – 56)	49 (42 – 55)	49 (42 – 57)	0.55
Male gender	150 (62%)	290 (66%)	124 (60%)	0.27
Alcohol consumption (units/week)	140 (85 – 210)	126 (82 – 210)	126 (84 – 210)	0.94
Overt hepatic encephalopathy	68 (28%)	109 (25%)	48 (23%)	0.48
Baseline laboratory variables				
White cell count ($x10^6/mm^3$)	8.0 (5.9 – 12.1)	9.1 (6.4 – 12.1)	9.1 (5.9 – 13.2)	0.32
Bilirubin (µmol/l)	270 (177 – 414)	269 (167 – 412)	274 (150 – 420)	0.64
Albumin (g/l)	25 (21 – 29)	25 (21 – 29)	26 (22 – 30)	0.42
Aspartate transaminase (IU/l)	118 (85 – 154)	130 (90 – 178)	118 (82 – 163)	0.38
Alkaline phosphatase (IU/l)	187 (139 – 245)	169 (128 – 229)	159 (129 – 209)	0.12
International normalised ratio	1.7 (1.5 – 2.0)	1.7 (1.5 – 2.0)	1.7 (1.6 – 2.0)	0.30
Urea (mmol/l)	3.3 (2.1 – 5.1)	3.1 (2.2 – 5.2)	3.4 (2.3 – 5.6)	0.59
Creatinine (µmol/l)	64 (52 – 84)	64 (52 – 82)		0.79
Prognostic scores				
DF	56 (43 – 74)	55 (44 – 73)	54 (43 – 74)	0.82
MELD	20 (17 – 24)	20 (17 – 24)	20 (16 – 24)	0.98
GAHS	9 (7 – 9)	8 (7 – 9)	8 (7 – 9)	0.12

Data expressed as median (IQR) or as number (%)

Abbreviations: DF: Discriminant function; MELD: Model for End-Stage Liver Disease; GAHS: The Glasgow Alcoholic Hepatitis Score

10.1.2 SLC38A4 rs11183620 genotype and histological appearances

Table 10.2 Histological features of alcoholic steatohepatitis, by rs11183620 genotype

SLC38A4 rs11183620 genotype	AA	AG	GG	Significance (p)
Primary analysis	**n=27**	**n=62**	**n=26**	
Severe inflammation	7 (26%)	13 (21%)	4 (15%)	0.45
Severe hepatocyte ballooning	17 (63%)	34 (55%)	14 (54%)	0.60
Megamitochondria	14 (52%)	26 (42%)	13 (50%)	0.45
Mallory-Denk bodies	17 (63%)	37 (60%)	15 (58%)	0.82
Sensitivity analysis	**n=36**	**n=66**	**n=27**	
Severe inflammation	11 (31%)	13 (20%)	5 (19%)	0.07
Severe hepatocyte ballooning	23 (64%)	37 (56%)	14 (52%)	0.35
Megamitochondria	16 (44%)	27 (41%)	12 (44%)	0.61
Mallory-Denk bodies	24 (67%)	39 (59%)	15 (56%)	0.37

Data are shown as n (%)

10.1.3 Impact of rs11183620 genotype on short-term survival and treatment response

Table 10.3 Ninety-day mortality in cases with severe alcoholic hepatitis, by treatment allocation and rs11183620 genotype

Treatment allocation	Cases (n)	Overall deaths (n: %)	Deaths, by rs11183620 genotype (n: %)		
			AA (n=236)	AG (n=424)	GG (n=200)
Prednisolone	435	103 (23.7%)	30 (25.9%)	51 (24.4%)	22 (22%)
No prednisolone	425	110 (25.9%)	29 (24.2%)	57 (26.5%)	24 (24%)
Total	**860**	**213 (24.8%)**	**59 (25%)**	**108 (25.5%)**	**46 (23%)**

10.1.4 Impact of rs11183620 genotype on recovery of liver function

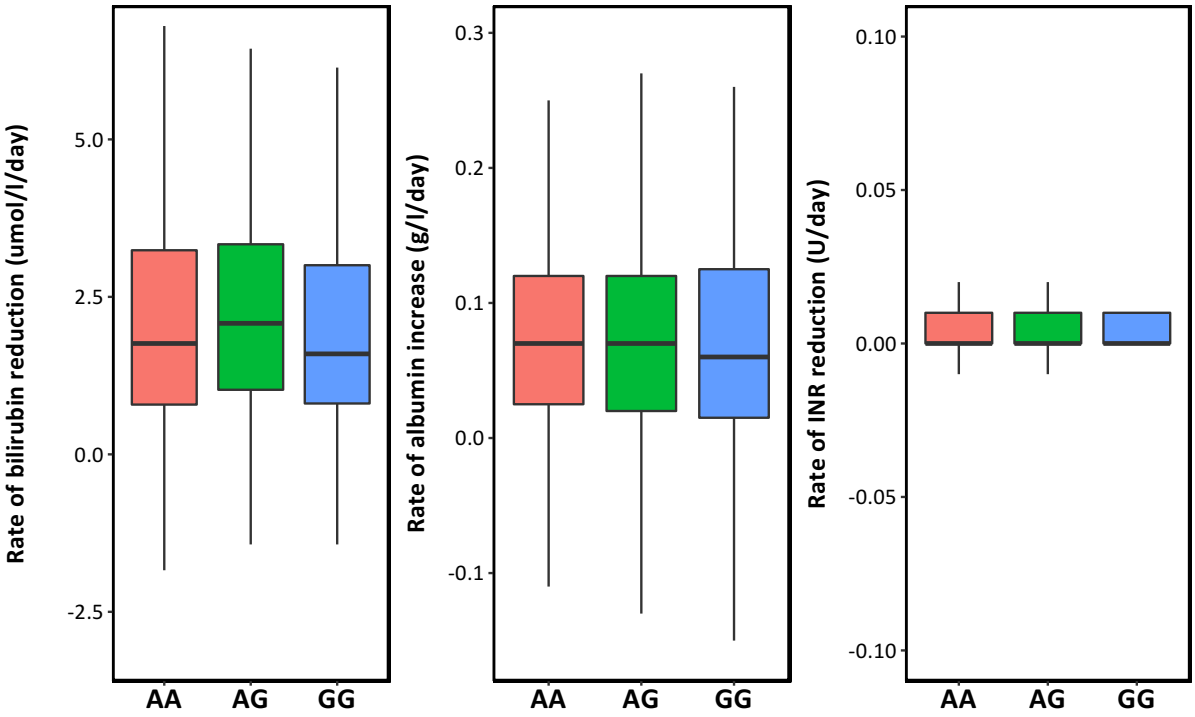

Figure 10.1 Rate of recovery in biomarkers of liver function over the 90 days since the start of treatment in patients with severe alcoholic hepatitis, by *SLC38A4* rs11183620 genotype

Data are displayed as the median (solid bar), interquartile range (box) and 95% confidence interval (whiskers).

APPENDICES

University of Southampton Clinical Trials Unit

Patient Initials: | | | | Patient Trial ID: | | | | - | | | | |

If no middle initial insert '-'

SCREENING VISIT FORM

Date of Visit: d d m m m y y y y

Initial Admission Date: d d m m m y y y y

(including any stay in another hospital for this episode)

Informed Consent

Date ICF signed d d m m m y y y y

Registration

Please REGISTER Patient with TENALEA

Patient Trial ID assigned | | | | - | | | | |

Month and **Year** of Birth: m m m y y y y

Gender: Male ☐ Female ☐ Unknown ☐

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

SCREENING VISIT FORM

Marital Status

Single, never married ☐

Married and living with *(husband/wife)* ☐

Civil partner in a legally recognised civil partnership ☐

Married and separated from *(husband/wife)* ☐

Divorced ☐

Widowed ☐

Formerly in a legally recognised civil partnership and separated from civil partner ☐

Formerly in a legally recognised civil partnership and civil partnership is now legally dissolved ☐

A surviving civil partner (*partner has since died*) ☐

Other, please specify _____

Ethnicity

White ☐

Mixed ☐

Asian or Asian British ☐

Black or Black British ☐

Chinese ☐

Other ethnic group ☐

Not stated ☐

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Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

SCREENING VISIT FORM

Current (or most recent) Employment

Working full-time..... ☐

Working part-time..... ☐

Unemployed..... ☐

Student (incl. pupil at school/college, those in training)..... ☐

Looking after family / carer..... ☐

Long-term sick or disabled..... ☐

Retired from paid work..... ☐

Not in paid work for some other reason..... ☐

Other, please specify _____

Housing

Detached house or bungalow..... ☐

Semi-detached house or bungalow .. ☐

Terrace house .. ☐

Flat or maisonette..... ☐

Room/rooms..... ☐

None .. . ☐

Other, please specify _____

Postcode of patient's usual residence: ☐☐☐☐ ☐☐☐ or no fixed abode ☐

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: [ ][ ][ ] Patient Trial ID: [ ][ ][ ] - [ ][ ][ ][ ]

If no middle initial insert '-'

SCREENING VISIT FORM

Age when Finished Continuous Full-time School/College Education

Not yet finished	☐
Never went to school	☐
14 or under	☐
15	☐
16	☐
17	☐
18	☐
19 or over	☐

Alcohol Consumption (1 unit = 8g alcohol)

In past 2 months, what was the maximum number of units consumed in a week? _____ units / week

Medical History

Medical History Form completed Yes ☐ No ☐

Vital Signs & Physical Examination

Height (cm) ☐ Weight (kg) ☐ Temp (°C) ☐ Pulse (bpm) ☐

Systolic/Diastolic BP (mmHg) ☐ / ☐

Physical Examination completed Yes ☐ No ☐

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Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

SCREENING VISIT FORM

Hepatic Encephalopathy

None ☐ Grade I ☐ Grade II ☐ Grade III ☐ Grade IV ☐

Pregnancy Test

Positive ☐ Negative ☐ Not Applicable ☐

Discriminant Function (DF)

Serum Total Bilirubin (µmol/L) ☐☐☐

Prothrombin Time (patient) (secs) ☐☐☐

Prothrombin Time (control) (secs) ☐☐☐

Discriminant Function ☐☐☐

DF = 4.6 x (Prothrombin time ($PT_{PATIENT}$ – $PT_{CONTROL}$) + Serum Bilirubin (µmol/l) / 17.1

Laboratory Analyses - Serum Chemistry (Enter **ND** if the assessment was 'Not Done')

Creatinine	_____ umol/L	Urea	_____ mmol/L
AST (SGOT)	_____ U/L	ALP	_____ U/L
ALT (SGPT)	_____ U/L	Bilirubin	_____ µmol/L
Sodium	_____ mmol/L	Amylase	_____ U/L
Inorganic Phosphate	_____ mmol/L	Potassium	_____ mmol/L
Glucose	_____ mmol/L	Calcium	_____ mmol/L
Albumin	_____ g/L	Total Protein	_____ g/L

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
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Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

SCREENING VISIT FORM

Laboratory Analyses – Haematology (Enter **ND** if the assessment was 'Not Done')

Hb _____ g/L WBC _____ x 10^9/L INR _____

Platelets _____ x 10^9/L Neutrophils_____ x 10^9/L

Patients with evidence of sepsis, gastrointestinal bleeding or renal failure, may be treated for up to 7 days and if stable, the patients can then be re-screened for eligibility. Treatment can continue for more than 7 days if they are stable. Patients who are not stable after 7 days of treatment will not be eligible for the study.

Evidence of Gastrointestinal Bleed: Yes ☐ No ☐

If Yes Variceal ☐ Non-variceal ☐ Unknown ☐

Resolved/stable after 7 days Yes ☐ No ☐

Evidence of Sepsis: Yes ☐ No ☐

If Yes Site(s) _____

Was an organism identified? Yes ☐ No ☐

If Yes Organism _____

Resolved/stable after 7 days Yes ☐ No ☐

Evidence of Renal Failure (serum creatinine greater than 500µmol/L or requiring renal support): Yes ☐ No ☐

Resolved/stable after 7 days Yes ☐ No ☐

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: [ ][ ][ ] Patient Trial ID: [ ][ ][ ] - [ ][ ][ ][ ]

If no middle initial insert '-'

SCREENING VISIT FORM

Investigations

Hepatitis B result	Negative	☐	Positive	☐		
Hepatitis C result	Negative	☐	Positive	☐		
Chest X-Ray*	Done	☐	Not Done	☐		
Liver Ultrasound*	Done	☐	Not Done	☐		
Blood Cultures*	Done	☐	Not Done	☐	Not Applicable	☐
Ascitic Tap	Done	☐	Not Done	☐	Not Applicable	☐
MSU Dipstick	Negative	☐	Positive**	☐	Not Done	☐

* To be performed between admission and randomisation
** Please culture urine sample and complete sepsis section

WHO Performance Status

WHO Performance Status [ ]

Definitions:

0 Asymptomatic (Fully active, able to carry on all pre-disease activities without restriction)

1 Symptomatic but completely ambulatory (Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature. For example, light housework, office work)

2 Symptomatic, <50% in bed during the day (Ambulatory and capable of all self care but unable to carry out any work activities. Up and about more than 50% of waking hours)

3 Symptomatic, >50% in bed, but not bedbound (Capable of only limited self-care, confined to bed or chair 50% or more of waking hours)

4 Bedbound (Completely disabled. Cannot carry out any self-care. Totally confined to bed or chair)

5 Death

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

SCREENING VISIT FORM

Adverse Events (AEs)

Has the patient experienced an unexpected adverse event since the consent?

Yes ☐ No ☐

If **Yes**, please record on the **Adverse Event Form**. Please ensure all medications given are recorded on the **Concomitant Medication Form**.

N.B. All unexpected Adverse Events/Serious Adverse Events should be recorded / reported for up to 4 weeks after the last dose of IMP. **All** SUSARs should be recorded & reported as usual.

Patient Eligibility

Does the patient meet ALL inclusion /exclusion criteria to enter the trial?

Yes ☐ No ☐

Signed: _____ **Date of completion:** _____ /_____ /_____
dd mmm yyyy

Print Name: _____
(Authorised person – only those entered on Site Delegation Log and approved by UoSCTU)

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

University of Southampton Clinical Trials Unit

Patient Initials: | | | Patient Trial ID: | | | - | | | |

If no middle initial insert '-'

BASELINE VISIT FORM

Date of Visit: d d m m m y y y y

Vital Signs

Temp (°C) |

Pulse (bpm) |

Systolic/Diastolic BP (mm/Hg) | / |

Hepatic Encephalopathy:

None | Grade I | Grade II | Grade III | Grade IV |

Laboratory Analyses - Serum Chemistry (Required **if more than 48 hours** since screening bloods) (Enter **ND** if the assessment was 'Not Done')

Creatinine	_____ umol/L	Urea	_____ mmol/L
AST (SGOT)	_____ U/L	ALP	_____ U/L
ALT (SGPT)	_____ U/L	Bilirubin	_____ µmol/L
Sodium	_____ mmol/L	Potassium	_____ mmol/L
Inorganic Phosphate	_____ mmol/L	Calcium	_____ mmol/L
Glucose	_____ mmol/L	Total Protein	_____ g/L
Albumin	_____ g/L		

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: | | | | Patient Trial ID: | | | | - | | | | |

If no middle initial insert '-'

BASELINE VISIT FORM

Laboratory Analyses – Haematology (Required **if more than 48 hours** since screening bloods) (Enter **ND** if the assessment was 'Not Done')

Hb _____ g/L WBC _____ x 10^9/L INR _____

Platelets _____ x 10^9/L Neutrophils_____ x 10^9/L

Discriminant Function (DF)

Serum Total Bilirubin (µmol/L) | | | |

Prothrombin Time (patient) (secs) | | | |

Prothrombin Time (control) (secs) | | | |

Discriminant Function | | | |

DF = 4.6 x (Prothrombin time ($PT_{PATIENT}$ – $PT_{CONTROL}$) + Serum Bilirubin (µmol/l) / 17.1

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

BASELINE VISIT FORM

Patients with evidence of sepsis, gastrointestinal bleeding or renal failure, may be treated for up to 7 days and if stable, the patients can then be re-screened for eligibility. Treatment can continue for more than 7 days if they are stable. Patients who are not stable after 7 days of treatment will not be eligible for the study.

Evidence of Gastrointestinal Bleed: Yes ☐ No ☐

If Yes Variceal ☐ Non-variceal ☐ Unknown ☐

Resolved/stable after 7 days Yes ☐ No ☐

Evidence of Sepsis: Yes ☐ No ☐

If Yes Site(s) _____

Was an organism identified? Yes ☐ No ☐

If Yes Organism _____

Resolved/stable after 7 days Yes ☐ No ☐

Evidence of Renal Failure (serum creatinine greater than 500µmol/L or requiring renal support): Yes ☐ No ☐

Resolved/stable after 7 days Yes ☐ No ☐

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Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

BASELINE VISIT FORM

WHO Performance Status

WHO Performance Status ☐

Definitions:

0 Asymptomatic (Fully active, able to carry on all pre-disease activities without restriction)

1 Symptomatic but completely ambulatory (Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature. For example, light housework, office work)

2 Symptomatic, <50% in bed during the day (Ambulatory and capable of all self care but unable to carry out any work activities. Up and about more than 50% of waking hours)

3 Symptomatic, >50% in bed, but not bedbound (Capable of only limited self-care, confined to bed or chair 50% or more of waking hours)

4 Bedbound (Completely disabled. Cannot carry out any self-care. Totally confined to bed or chair)

5 Death

AUDIT

AUDIT completed by patient Yes ☐ No ☐

SADQ

SADQ completed by patient Yes ☐ No ☐

EDTA Sample

5ml blood sample in EDTA tube taken for DNA analysis

Yes ☐ No ☐ Consent not given ☐

Serum Sample

10ml serum sample collected and prepared

Yes ☐ No ☐ Consent not given ☐

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Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

BASELINE VISIT FORM

Adverse Events (AEs)

Has the patient experienced an unexpected adverse event since the last assessment?

Yes ☐ No ☐

If **Yes**, please record on the **Adverse Event Form**. Please ensure <u>all</u> medications given are recorded on the **Concomitant Medication Form**.

N.B. All unexpected Adverse Events/Serious Adverse Events should be recorded / reported for up to 4 weeks after the last dose of IMP. **All** SUSARs should be recorded & reported as usual.

Randomisation

Date of Randomization: ☐☐ ☐☐☐ ☐☐☐☐ (d d m m m y y y y)

Risk*: High ☐

Intermediate ☐

Please RANDOMISE patient with TENALEA

Patient is randomised to patient pack number ☐☐☐☐

*(High risk is defined as either sepsis or history of GI bleeding in the previous 7 days or creatinine > 150µmol/L or any combination of the these; Intermediate risk is defined as no sepsis and no history of GI bleeding in the previous 7 days and creatinine <= 150µmol/L)

Signed: _____ **Date of completion:** _____ /_____/_____
dd mmm yyyy

Print Name: _____
(Authorised person – only those entered on Site Delegation Log and approved by UoSCTU)

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

ON TREATMENT FORM

CRF for: DAY 7 ☐ **DAY 14** ☐ **DAY 21** ☐ **DAY 28** ☐

Date of Visit: d d m m m y y y y

Treatment Start Date: d d m m m y y y y

DAY 7 ONLY

Has the patient had a treatment break of >24 hours between Day 1 and Day 7?

Yes ☐ No ☐

DAY 28 ONLY

Medication

How many capsules are left in Bottle A ☐☐ Bottle B ☐☐

Liver Transplant

Has the patient had a liver transplant since study treatment started?

Yes ☐ No ☐

If yes, please give date: d d m m m y y y y

Vital Signs

Temp (°C) ☐

Pulse (bpm) ☐

Systolic/Diastolic BP (mm/Hg) ☐ / ☐

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
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Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

ON TREATMENT FORM

Hepatic Encephalopathy

None ☐ Grade I ☐ Grade II ☐ Grade III ☐ Grade IV ☐

Laboratory Analyses - Serum (Enter **ND** if the assessment was 'Not Done')

Creatinine	_____ umol/L	Urea	_____ mmol/L
AST (SGOT)	_____ U/L	ALP	_____ U/L
ALT (SGPT)	_____ U/L	Bilirubin	_____ µmol/L
Sodium	_____ mmol/L	Potassium	_____ mmol/L
Inorganic Phosphate	_____ mmol/L	Calcium	_____ mmol/L
Glucose	_____ mmol/L	Total Protein	_____ g/L
Albumin	_____ g/L		

Laboratory Analyses – Haematology (Enter **ND** if the assessment was 'Not Done')

Hb	_____ g/L	WBC	_____ x 10^9/L
Platelets	_____ x 10^9/L	Neutrophils	_____ x 10^9/L
Prothrombin Time (patient)	_____ secs	Prothrombin Time (control)	_____ secs
INR	_____		

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

ON TREATMENT FORM

Gastrointestinal Bleed since the last visit: Yes ☐ No ☐

If Yes Variceal ☐ Non-variceal ☐ Unknown ☐

Now Resolved Yes ☐ No ☐

Sepsis since the last visit: Yes ☐ No ☐

If Yes Site(s) _____

Was an organism identified? Yes ☐ No ☐

If Yes Organism _____

Now Resolved Yes ☐ No ☐

Renal Failure (serum creatinine greater than 500µmol/L or requiring renal support)

since the last visit: Yes ☐ No ☐

Now Resolved Yes ☐ No ☐

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

ON TREATMENT FORM

WHO Performance Status

WHO Performance Status ☐

Definitions:

0	Asymptomatic (Fully active, able to carry on all pre-disease activities without restriction)
1	Symptomatic but completely ambulatory (Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature. For example, light housework, office work)
2	Symptomatic, <50% in bed during the day (Ambulatory and capable of all self care but unable to carry out any work activities. Up and about more than 50% of waking hours)
3	Symptomatic, >50% in bed, but not bedbound (Capable of only limited self-care, confined to bed or chair 50% or more of waking hours)
4	Bedbound (Completely disabled. Cannot carry out any self-care. Totally confined to bed or chair)
5	Death

Adverse Events (AEs)

Has the patient experienced an unexpected adverse event since the last assessment?

Yes ☐ No ☐

If **Yes**, please record on the **Adverse Event Form**. Please ensure all medications given are recorded on the **Concomitant Medication Form**.

N.B. All unexpected Adverse Events/Serious Adverse Events should be recorded / reported for up to 4 weeks after the last dose of IMP. **All** SUSARs should be recorded & reported as usual.

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
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Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

ON TREATMENT FORM

Premature End of Treatment

Follow– up should continue, per protocol for those patients who permanently stop treatment during the treatment period.

Has this patient permanently stopped treatment? Yes ☐ No ☐

If yes, Treatment Stop Date: d d m m m y y y y

Reason treatment has been stopped for this patient:

_____

_____

Comments – Please record reasons for any missing data

_____

_____

_____

_____

Signed: _____ **Date of completion:** _____/_____/_____
dd mmm yyyy

Print Name: _____
(Authorised person – only those entered on Site Delegation Log and approved by UoSCTU)

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STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: | | | | Patient Trial ID: | | | | - | | | | |

If no middle initial insert '-'

DAY 90 VISIT FORM

Date of Visit: d d m m m y y y y

Alcohol Consumption (Since the last assessment)

Abstinent: ☐

Reduced drinking to below safety limits: ☐

Reduced drinking but above safety limits: ☐

Not reduced: (i.e. still drinking as much or more than when presented) ☐

Vital Signs & Physical Examination

Weight (kg) ☐ Temp (°C) ☐ Pulse (bpm) ☐

Systolic/Diastolic BP (mmHg) ☐ / ☐

Physical Examination completed Yes ☐ No ☐

Hepatic Encephalopathy

None ☐ Grade I ☐ Grade II ☐ Grade III ☐ Grade IV ☐

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

DAY 90 VISIT FORM

Laboratory Analyses - Serum Chemistry (Enter **ND** if the assessment was 'Not Done')

Creatinine	_____ umol/L	Urea	_____ mmol/L
AST (SGOT)	_____ U/L	ALP	_____ U/L
ALT (SGPT)	_____ U/L	Bilirubin	_____ µmol/L
Sodium	_____ mmol/L	Potassium	_____ mmol/L
Inorganic Phosphate	_____ mmol/L	Calcium	_____ mmol/L
Glucose	_____ mmol/L	Total Protein	_____ g/L
Albumin	_____ g/L		

Laboratory Analyses – Haematology (Enter **ND** if the assessment was 'Not Done')

Hb	_____ g/L	WBC	_____ x 10^9/L
Platelets	_____ x 10^9/L	Neutrophils	_____ x 10^9/L
Prothrombin Time (patient)	_____ secs	Prothrombin Time (control)	_____ secs
INR	_____		

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
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Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

DAY 90 VISIT FORM

Gastrointestinal Bleed since the last visit: Yes ☐ No ☐

If Yes Variceal ☐ Non-variceal ☐ Unknown ☐

Now Resolved Yes ☐ No ☐

Sepsis since the last visit: Yes ☐ No ☐

If Yes Site(s) _____

Was an organism identified? Yes ☐ No ☐

If Yes Organism _____

Now Resolved Yes ☐ No ☐

Renal Failure (serum creatinine greater than 500µmol/L or requiring renal support)

since the last visit: Yes ☐ No ☐

Now Resolved Yes ☐ No ☐

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

DAY 90 VISIT FORM

WHO Performance Status

WHO Performance Status ☐

Definitions:

0 Asymptomatic (Fully active, able to carry on all pre-disease activities without restriction)

1 Symptomatic but completely ambulatory (Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature. For example, light housework, office work)

2 Symptomatic, <50% in bed during the day (Ambulatory and capable of all self care but unable to carry out any work activities. Up and about more than 50% of waking hours)

3 Symptomatic, >50% in bed, but not bedbound (Capable of only limited self-care, confined to bed or chair 50% or more of waking hours)

4 Bedbound (Completely disabled. Cannot carry out any self-care. Totally confined to bed or chair)

5 Death

SF36 completed: Yes ☐ No ☐

EQ-5D completed: Yes ☐ No ☐

Use of medical services completed: Yes ☐ No ☐

Attendance at alcohol counselling services

Has the patient attended 1 or more sessions? Yes ☐ No ☐

If yes, is the patient still attending sessions? Yes ☐ No ☐

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STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

DAY 90 VISIT FORM

Is the patient taking any of the following medications?

Acamprosate	Yes ☐	No ☐
Disulfiram	Yes ☐	No ☐
Baclofen	Yes ☐	No ☐
Other medication for alcohol dependence	Yes ☐	No ☐

If **yes**, please complete the **Concomitant Medication Form**.

Liver Transplant

Has the patient had a liver transplant since study treatment started?

Yes ☐ No ☐

If yes, please give date: d d m m m y y y y

Adverse Events (AEs)

Has the patient experienced an unexpected adverse event since the last assessment?

Yes ☐ No ☐

If **Yes** please record all adverse events on the **Adverse Event Form**.

N.B. All unexpected Adverse Events/Serious Adverse Events should be recorded/reported for up to 4 weeks after the last dose of IMP. **All** SUSARs should be recorded & reported as usual.

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Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

DAY 90 VISIT FORM

Concomitant Medications

Please ensure all medications given are recorded on the **Concomitant Medication Form (including any steroids or pentoxifylline given post-Day 28).**

Comments – please record any reasons for any missing data

_____

_____

_____

_____

_____

_____

Signed: _____ **Date of completion:** _____ /_____/_____
dd mmm yyyy

Print Name: _____
(Authorised person – only those entered on Site Delegation Log and approved by UoSCTU)

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: | | | | Patient Trial ID: | | | | - | | | | |

If no middle initial insert '-'

1 YEAR VISIT FORM

Date of Visit: d d m m m y y y y

Marital Status

- Single, never married ☐
- Married and living with *(husband/wife)* ☐
- Civil partner in a legally recognised civil partnership ☐
- Married and separated from *(husband/wife)* ☐
- Divorced ☐
- Widowed ☐
- Formerly in a legally recognised civil partnership and separated from civil partner ☐
- Formerly in a legally recognised civil partnership and civil partnership is now legally dissolved ☐
- A surviving civil partner (*partner has since died*) ☐
- Other, please specify _____

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

1 YEAR VISIT FORM

Current (or most recent) Employment

Working full-time..... ☐

Working part-time..... ☐

Unemployed..... ☐

Student (incl. pupil at school/college, those in training)..... ☐

Looking after family / carer..... ☐

Long-term sick or disabled..... ☐

Retired from paid work..... ☐

Not in paid work for some other reason..... ☐

Other, please specify _____

Housing

Detached house or bungalow..... ☐

Semi-detached house or bungalow .. ☐

Terrace house .. ☐

Flat or maisonette..... ☐

Room/rooms..... ☐

None .. ☐

Other, please specify _____

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STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

1 YEAR VISIT FORM

Alcohol Consumption (Since the last assessment)

Abstinent: ☐

Reduced drinking to below safety limits: ☐

Reduced drinking but above safety limits: ☐

Not reduced: (i.e. still drinking as much or more than when presented) ☐

Medical History

Medical History Form completed: Yes ☐ No ☐

Vital Signs & Physical Examination

Weight (kg) ☐ Temp (°C) ☐ Pulse (bpm) ☐

Systolic/Diastolic BP (mmHg) ☐ / ☐

Physical Examination completed Yes ☐ No ☐

Hepatic Encephalopathy

None ☐ Grade I ☐ Grade II ☐ Grade III ☐ Grade IV ☐

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

1 YEAR VISIT FORM

Laboratory Analyses - Serum Chemistry (Enter **ND** if the assessment was 'Not Done')

Creatinine	_____ umol/L	Urea	_____ mmol/L
AST (SGOT)	_____ U/L	ALP	_____ U/L
ALT (SGPT)	_____ U/L	Bilirubin	_____ µmol/L
Sodium	_____ mmol/L	Potassium	_____ mmol/L
Inorganic Phosphate	_____ mmol/L	Calcium	_____ mmol/L
Glucose	_____ mmol/L	Total Protein	_____ g/L
Albumin	_____ g/L		

Laboratory Analyses – Haematology (Enter **ND** if the assessment was 'Not Done')

Hb	_____ g/L	WBC	_____ x 10^9/L
Platelets	_____ x 10^9/L	Neutrophils	_____ x 10^9/L
Prothrombin Time (patient)	_____ secs	Prothrombin Time (control)	_____ secs
INR	_____		

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

1 YEAR VISIT FORM

Gastrointestinal Bleed since the last visit: Yes ☐ No ☐

If Yes Variceal ☐ Non-variceal ☐ Unknown ☐

Now Resolved Yes ☐ No ☐

Sepsis since the last visit: Yes ☐ No ☐

If Yes Site(s) _____

Was an organism identified? Yes ☐ No ☐

If Yes Organism _____

Now Resolved Yes ☐ No ☐

Renal Failure (serum creatinine greater than 500µmol/L or requiring renal support)

since the last visit: Yes ☐ No ☐

Now Resolved Yes ☐ No ☐

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: [ ][ ][ ] Patient Trial ID: [ ][ ][ ] - [ ][ ][ ][ ]

If no middle initial insert '-'

1 YEAR VISIT FORM

WHO Performance Status

WHO Performance Status [ ]

Definitions:

0 Asymptomatic (Fully active, able to carry on all pre-disease activities without restriction)

1 Symptomatic but completely ambulatory (Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature. For example, light housework, office work)

2 Symptomatic, <50% in bed during the day (Ambulatory and capable of all self care but unable to carry out any work activities. Up and about more than 50% of waking hours)

3 Symptomatic, >50% in bed, but not bedbound (Capable of only limited self-care, confined to bed or chair 50% or more of waking hours)

4 Bedbound (Completely disabled. Cannot carry out any self-care. Totally confined to bed or chair)

5 Death

SF36 completed: Yes [ ] No [ ]

EQ-5D completed: Yes [ ] No [ ]

Use of medical services completed: Yes [ ] No [ ]

Attendance at alcohol counselling services

Has the patient attended 1 or more sessions? Yes [ ] No [ ]

If yes, is the patient still attending sessions? Yes [ ] No [ ]

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: [ ][ ][ ] Patient Trial ID: [ ][ ][ ] - [ ][ ][ ][ ]

If no middle initial insert '-'

1 YEAR VISIT FORM

Is the patient taking any of the following medications?

Acamprosate	Yes ☐	No ☐
Disulfiram	Yes ☐	No ☐
Baclofen	Yes ☐	No ☐
Other medication for alcohol dependence	Yes ☐	No ☐

If **yes**, please complete the **Concomitant Medication Form**.

Liver Transplant

Has the patient had a liver transplant since study treatment started?

Yes ☐ No ☐

If yes, please give date: [d][d] [m][m][m] [y][y][y][y]

Adverse Events (AEs)

Please document any **changes** to adverse events that existed at the last assessment on the **Adverse Event Form**.

Concomitant Medications

Please ensure all medications given are recorded on the **Concomitant Medication Form (<u>including</u> any steroids or pentoxifylline given post-Day 28).**

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Patient Initials: ☐☐☐ Patient Trial ID: ☐☐☐ - ☐☐☐☐

If no middle initial insert '-'

1 YEAR VISIT FORM

Comments – Please record reasons for any missing data

_____

_____

_____

Signed: _____ **Date of completion:** _____ /_____/_____
dd mmm yyyy

Print Name: _____
(Authorised person – only those entered on Site Delegation Log and approved by UoSCTU)

Please retain a copy of the CRF and send the original, within 2 weeks of the visit, to:
STOPAH Data Officer, University of Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

3) Bays H, Cohen DE, Chalasani NP, Harrison SA; The National Lipid Association's Statin Safety Task Force. An assessment by the Statin Liver Safety Task Force: 2014 update. J Clin Lipidol 2014;8(3 Suppl):S47-S57.

View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep.29841
Potential conflict of interest: Nothing to report.

People Who Survive an Episode of Severe Alcoholic Hepatitis Should Be Advised to Maintain Total Abstinence From Alcohol

TO THE EDITOR:

We read with interest the article published in HEPATOLOGY by Louvet et al.[1] highlighting factors influencing outcomes in people with severe alcoholic hepatitis. They found that beyond 6 months, alcohol relapse, defined as consumption of ≥30 g/day, was an independent predictor of mortality with a dose-related effect on the hazard ratio (HR). The effect of drinking behavior on outcome has also been examined in data collected in the Steroids and Pentoxyfylline for Severe Alcoholic Hepatitis (STOPAH) trial.[2] Patients were classified, in the original published analysis, as abstinent or drinking. A return to alcohol consumption at day 90 was associated with a significantly higher mortality at day 450 than abstinence (HR, 2.77; 95% confidence interval [CI], 1.79-4.29; $P < 0.00001$).[2] We have re-examined these data in an attempt to replicate the dose-

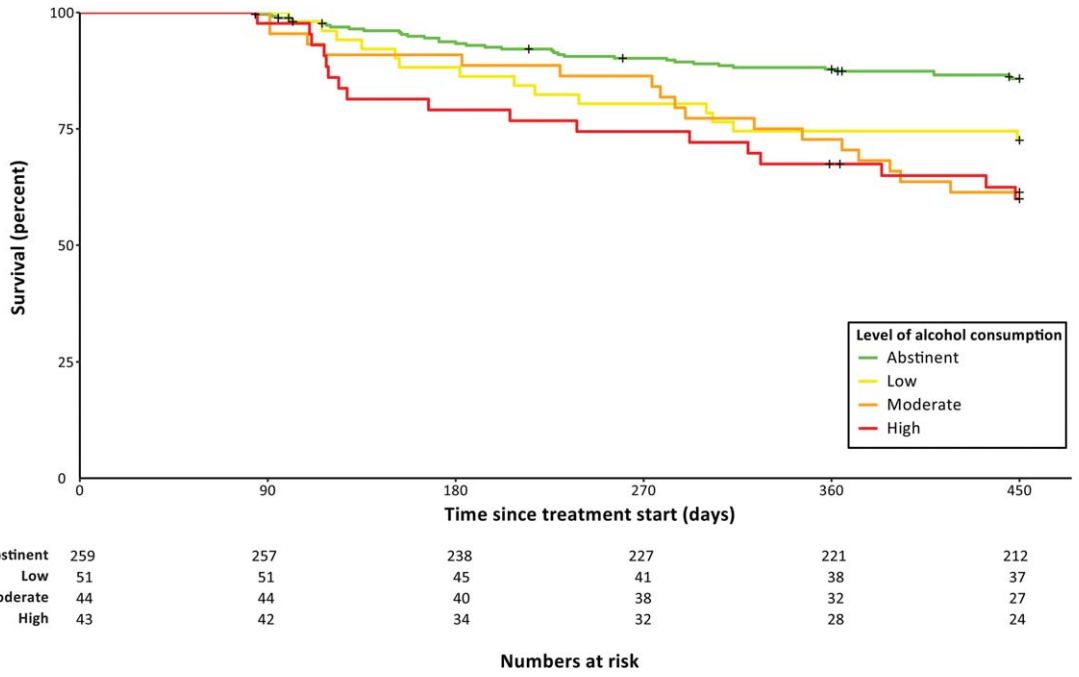

FIG. 1. Survival in patients with severe alcoholic hepatitis alive at 90 days, by subsequent drinking behavior. Survival times, and mortality endpoints, were calculated with respect to the treatment start date or, if not recorded, the date of randomization; cases were censored at the time of liver transplantation, the limit of follow-up or day 450, whichever occurred first. Compared with abstinence, a clear dose-dependent increase in the risk of mortality at day 450 is observed with low (HR, 2.09; 95% CI, 1.13-3.88; $P = 0.02$), moderate (HR, 3.00; 95% CI, 1.69-5.35; $P < 0.001$) and high-level alcohol consumption (HR, 3.31; 95% CI, 1.86-5.90; $P < 0.001$).

dependent effect of drinking on mortality observed by Louvet et al.[1]

Participants enrolled in the STOPAH trial were treated for 28 days with prednisolone, pentoxifylline, both, or placebo.[3] Patients categorized their drinking behavior at day 90 as: (1) abstinent or (2) drinking daily at low levels (men, ≤24 g; women, ≤16 g); (3) moderate levels (men, >24 but ≤60 g; women, >16 but ≤40 g); or (4) high levels (men, >60 g; women, >40 g). The association between drinking behavior and survival was examined using Cox proportional hazards regression analysis.

Data on drinking behavior were available in 397 patients; of these, 84 (9.7%) had died by day 450. A total of 138 (35%) had returned to drinking; the distribution within the three drinking categories was reasonably even. There was a clear dose-dependent increase in the HR as drinking levels increased *viz*: low, 2.09 (95% CI, 1.13-3.88; $P = 0.02$), moderate, 3.00 (95% CI, 1.69-5.35; $P < 0.001$) and high, 3.31 (95% CI, 1.86-5.90; $P < 0.001$) (Fig. 1).

Thus, while Louvet et al.[1] found a dose-related effect of drinking on the HR for death above a threshold of 30g/day, we have shown that a return to drinking, *at any level*, confers a dose-related increase in the risk of death. Our use of sex-specific drinking thresholds, based on our previous finding that sex is an independent risk factor for mortality in people with severe alcoholic hepatitis who return to drinking,[2] contrasts with the French group's use of a generic drinking threshold; this may explain the difference in our findings.

Although alcohol relapse has a significantly detrimental effect on outcome, people who survive an episode of severe alcoholic hepatitis and subsequently attain and maintain abstinence from alcohol still exhibit an appreciable mortality. We have shown previously that homozygosity for rs738409:G in *PNPLA3* is an independent risk factor for medium-term mortality in this population.[2] Both this genetic variant and sex should be added to the risk factors identified by Louvet et al. as determinants of outcome in people with severe alcoholic hepatitis.[1]

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View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep.29825
Potential conflict of interest: Nothing to report.
Supported by NIHR HTA grant 08/14/44; University College London (Impact PhD fellowship award MJW); Medical Research Council (UK) (grant no.: MR/M003132/1); and Imperial College BRC program.
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New Biomarkers for Drug-Induced Liver Injury

TO THE EDITOR:

As a researcher developing biomarkers for drug-induced liver injury (DILI), the new HEPATOLOGY paper by Church et al. was of great interest.[1] This important publication presents data regarding biomarkers that were measured in healthy subjects and patients with DILI.

Church et al. state in their abstract that "glutamate dehydrogenase (GLDH) appears to be more useful

Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jhep.2017.11.038.

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Reply to: "The *PNPLA3* SNP rs738409:G allele is associated with increased liver disease-associated mortality but reduced overall mortality in a population-based cohort"

To the Editor:

We thank Meffert and co-workers[1] for their interest in our study and for providing data which support our finding of an association between carriage of rs738409:G in patatin-like phospholipase domain-containing 3 (*PNPLA3*) and the risk of liver-associated mortality, at least in men.[2] The authors evaluated the association of rs738409:G with mortality in adults participating in a population-based health study in Pomerania. The included population of 4,081 was sub-classified by sex and by the absence/presence of hepatic steatosis on ultrasound. Participants were censored at death or when lost to follow-up with the length of follow-up defined as birth to censorship. The median follow-up period was 11.3 (interquartile range: 10.6–11.8) years, though this is difficult to equate with the definition of the follow-up period provided. In men, carriage of rs738409:G was associated with a fourfold increase in the hazard of liver-disease-related mortality; there were too few events in women for analysis. These data corroborate not only our findings that carriage of rs738409:G is a negative risk factor for survival,[2] but also the reported association with a reduction in survival in people listed for liver transplantation[3] and in those with cirrhosis and hepatocellular carcinoma.[4] The authors also showed that, in men, carriage of rs738409:G was associated with a decrease in the risk of death from coronary artery disease; there was no such effect in women.[1] Liu and colleagues[5] recently reported in an exome-wide association study in >300,000 individuals that carriage of rs738409:G was associated with a lower risk of coronary artery disease,

Keywords: Alcohol dependence; Alcoholic hepatitis; Alcohol-related cirrhosis; Genetic polymorphism; PNPLA3; Risk allele; Survival; Mortality; Liver-related; Sex-variant interaction.

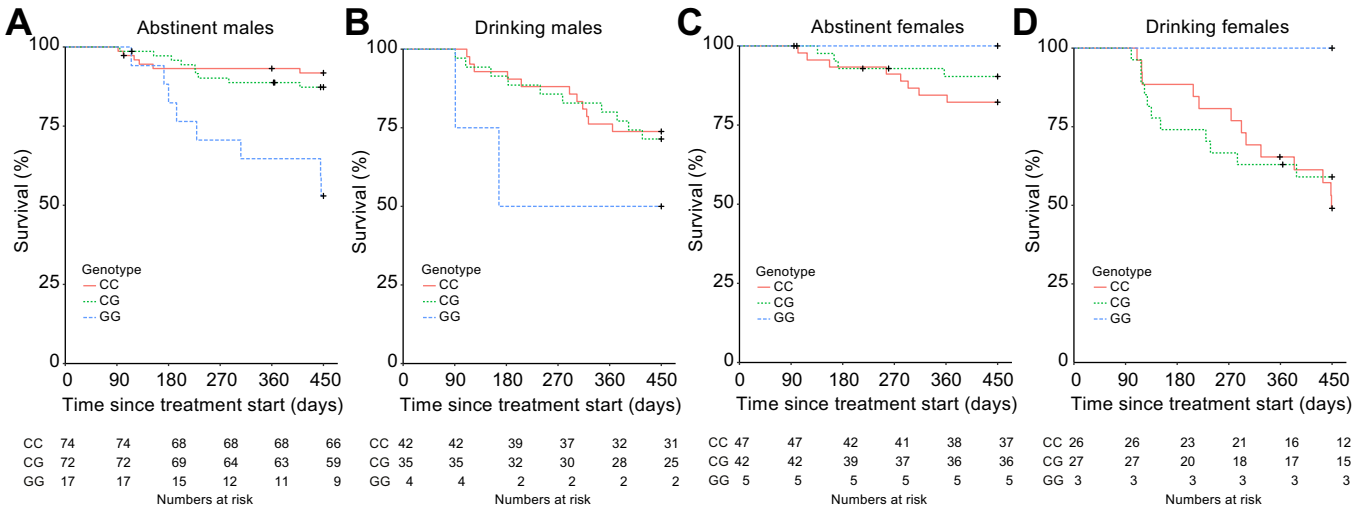

Fig. 1. Medium-term survival in cases with severe alcoholic hepatitis surviving at least 90 days, by gender and drinking status. (A) In male patients who maintain abstinence rs738409:G is associated with an apparent reduction in survival over the 90–450 day period. (B) The same pattern is seen in male patients who return to drinking. (C, D) In female patients the pattern appears to be reversed. (This figure appears in colour on the web.)

corroborating this finding, although sex-specific data were not provided.

The main point of interest in the data provided by Meffert and co-workers[1] is the apparent sex-related differences in disease-specific mortality associated with carriage of rs738409:G, which needs to be confirmed. Consequently we re-analysed our study data to test for the presence of interactions between sex and the rs738409:G allele and medium-term mortality (90 to 450 days after initial presentation). The study populations and data processing methodology were as described in the original publication of these data.[2,6] Information on deaths within the study period was collected via the study reporting forms while information on deaths outwith the study period were obtained from the NHS Information Centre Data Linkage Service. Cox regression analysis was used to examine for associations between survival, rs738409:G, sex and a return to drinking with incorporation of a multiplicative interaction term for rs738409:G and sex.

Eighty-two (20.7%) of the 397 patients included in the analysis died during the follow-up period. Information on the cause of death was only available in 60 (73%); the deaths in 47 (78%) were classified as definitely liver-related; two were definitely not liver-related, whilst the remaining 11 deaths were not classifiable, as such. There was a highly significant multiplicative interaction between rs738409 genotype and sex in relation to medium-term mortality (hazard ratio [HR] 0.30; 95% CI 0.14–0.62, $p = 0.001$), which was independent of the return to drinking (HR 2.91; 95% CI 1.88–4.50; $p < 0.001$). Of particular note was the sex-specific difference in the survival in homozygous carriers of rs738409:G; thus, all eight female homozygotes survived to day 450 compared with only ten (48%) of their 21 male counterparts (Fig. 1).

The comparative survival advantage in women with alcohol-related cirrhosis is well-documented.[7–10] However, its occurrence is unexplained, although differences in body composition which result in relative preservation of lean muscle mass in women may play a significant role.[11,12] The findings reported by Meffert *et al.*[1] and confirmed in the reanalysis of our study data suggest that the sex-related differences in the risk of liver-related deaths may relate, at least in part, to a sex-variant interaction with rs738409:G in *PNPLA3*. Sex-variant interactions have previously been described in the field of cardiovascular medicine[13] and are worthy of further exploration in the field of liver medicine.

Financial support

NIHR HTA grant 08/14/44. University College London (Impact PhD fellowship award MJW). Medical Research Council (UK) – Grant number MR/M003132/1. Imperial College BRC programme.

Conflict of interest

The authors have no conflicts of interest to declare.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contribution

SRA performed genotyping, statistical analyses and with MYM drafted and revised the manuscript. MJW performed genotyping. AQ, MYM and MRT recruited participants. AQ and MRT critically appraised and revised the manuscript.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jhep.2017.12.005.

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Detecting microvascular invasion in HCC with contrast-enhanced MRI: Is it a good idea?

To the Editor:

We have read with interest the paper "Preoperative gadoxetic acid-enhanced MRI for predicting microvascular invasion in patients with single hepatocellular carcinoma" by Drs. S. Lee *et al.* published in a recent issue of the *Journal of Hepatology*.[1] This study clearly described the feasibility of contrast-enhanced MRI by using gadoxetic acid to detect microvascular invasion (mVI) in a surgical cohort of patients with hepatocellular carcinoma (HCC) ≤5 cm in diameter. Although their findings highlight the role of contrast-enhanced MRI for small HCC, a few concerns may need attention to justify its usefulness in clinical practice.

Consistent with several independent studies,[2,3] mVI has been shown to be a major risk factor for tumor recurrence in patients with HCC undergoing surgical treatment. Preoperative MRI could be useful in detecting the extent of tumor involvement. However, its accuracy is often uncertain, and the confirmation of mVI can only be demonstrated from the serially slice-cut resected specimen. This is especially important when the prevalence rate of mVI was reported to reach 40.5% for tumors ≤2 cm and 49.6% for tumors 2.1–4 cm in diameter, respectively, in a surgical series of 322 patients.[2] Notably, the reported prevalence rate of mVI in Lee *et al.'s* series is much lower (around 20% for tumor ≤2 cm and 30% for tumor 2.1–4 cm).[1] Underestimation of mVI might lower the threshold for post-operative follow-up in detecting early tumor recurrence.

The potentially high rate of mVI in small HCC is further supported by an earlier study that looked at the explant livers in patients with HCC undergoing liver transplantation.[4] Small satellite nodules were often found in the explants through serially examining the slice-cut specimen, thus greatly limiting imaging studies' ability to detect multi-centric lesions. These findings suggest that the current preoperative imaging studies are usually not very reliable for minute lesions, such as mVI or tiny (<0.5 cm) tumor nodules.

Therefore, for those who receive curative resection, the identification of mVI should be best demonstrated from multiple pathological specimens. For those who undergo palliative therapy, such as transarterial chemoembolization when tumor biopsy is often not available, the clinical significance of mVI might not be as paramount as in the surgical case. In summary, mVI represents a distinct tumor behavior and may serve as a crucial prognostic marker for HCC. The current preoperative MRI is mandatory, but may not be adequate or efficient in terms of detecting the presence of mVI. To avoid possible underestimation of mVI, a thorough examination of the surgical specimen remains the standard method of confirmation.

Keywords: Vascular invasion; Hepatocellular carcinoma.

Financial support

This study was supported by the grant from the Center of Excellence for Cancer Research at Taipei Veterans General Hospital (MOHW106-TDU-B-211-144-003), Taiwan, and the grants from Taipei Veterans General Hospital (VN106-11, V106C-021,V107A-008), Taipei, Taiwan.

JOURNAL OF HEPATOLOGY

Universal screening of acute medical admissions for excess alcohol consumption: What's the misuse?

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See Article, pages 559–567

Alcohol misuse is frequently identified amongst patients presenting to Emergency Departments. Additionally, "covert" alcohol excess may be identified in cases where admission is not obviously related to alcohol or its sequelae. In this issue, Westwood and colleagues examine the feasibility of screening acute medical admissions for alcohol use disorders with a retrospective, observational cohort study encompassing more than 50,000 admissions over a 3-year period. Screening was completed in >90% of hospital admissions. Patients at "high" and "increasing" risk of alcohol related harm, the minority, were identified using a modification of the Paddington Alcohol Test and further assessed by an Alcohol Specialist Nurse Service (ASNS) using the Alcohol Use Disorders Identification Test (AUDIT). In their 1968 paper, 'Principles and Practice of Screening for Disease', Wilson and Jungner described principles central to the effective detection of early disease; the study by Westwood and colleagues can be considered with respect to these criteria (Table 1).[1]

Certainly, alcohol misuse is an important health problem. Around 200 different diseases, including a significant proportion of cancers, are wholly or partly attributable to alcohol.[2] Globally nearly 6% of all deaths may be attributed to alcohol, rising to almost half for cirrhosis-related deaths.[3,4] ALD is the most common aetiology in emergency presentations with decompensated cirrhosis. The healthcare and economic costs associated with alcohol misuse are estimated at £3.5bn and £21bn per annum, respectively, in England alone.[5] Alcohol related liver, pancreas or brain damage all have an early phase that can be latent or symptomatic, satisfying Wilson and Jungner's second criterion. Consequently, treatment of alcohol misuse could both reduce costs and arrest the development of end-organ damage, avoiding future hospital admissions, morbidity and mortality.

In this study, AUDIT scores were indicative of dependence in 68% and 80% of intermediate and higher risk individuals respectively. This demonstrates the suitability of the modified electronic Paddington Alcohol Test (mePAT) as a screening tool for alcohol use disorders. Individual susceptibility to end-organ damage from excess alcohol is highly variable and modulated by genetic and environmental co-factors. Thus, the mePAT cannot meet Wilson and Jungner's third criterion regarding end-organ damage. Ultimately, to address the burden of diseases such as ALD, specific studies are required to assess the practicability of identifying and treating patients with early end-organ damage, secondary to alcohol, during hospital attendance.

Rates of engagement with healthcare services amongst patients with alcohol use disorders are known to be suboptimal.[6] A large proportion of patients with ALD are not engaged with clinical services until they develop advanced disease. Indeed, in those eventually diagnosed with ALD, prior hospital attendance implies prior opportunity to detect and manage alcohol misuse: in almost half of patients who died during their index admission for liver disease, an opportunity to intervene on an earlier admission was identified.[7] This is further highlighted in the current study – higher risk drinkers attended hospital more frequently in the preceding three years. Notably, the cohort of higher risk drinkers who did not undergo further assessment was characterised by more frequent emergency department attendances, a short duration of admission and higher likelihood of self-discharge, potentially reflecting entrenched behaviour. In contrast, patients who were admitted for reasons unrelated to alcohol misuse, but were at "increasing risk" of alcohol related harm, may represent those suitable for the screening test, in whom intervention may be more effective.

Completion of screening using the mePAT tool in 91% of admissions indicates its acceptability for use in the acute medical setting. The cost of implementation also appears acceptable: there will be an increased workload for admitting nursing staff, but this is minimised by electronic integration into standard workflows. By its nature, the method also allows for a continuing process of case identification.

However, significant uncertainty persists regarding treatment of alcohol use disorders and the recognition of when to treat, criterion 6. Westwood and colleagues were unable to demonstrate

Keywords: Alcohol; Emergency; Screening; Healthcare.
Received 23 May 2017; received in revised form 2 June 2017; accepted 7 June 2017
☆ DOI of original article: http://dx.doi.org/10.1016/j.jhep.2017.04.017.
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Table 1. An assessment of the current study in relation to the principle of screening for early disease outlined by Wilson and Jungner.[1]

Criterion	Satisfied for alcohol use disorder	Satisfied for alcohol-related liver disease
1. Importance of health problem	Yes	Yes
2. Latent or early symptomatic phase with an understood natural history	Yes	Yes
3. Suitable and acceptable test	Yes	Partially
4. Acceptable cost of testing	Yes	Unclear in the absence of a defined test
5. Continuing process of case detection	Yes	No
6. Treatment of disease and recognition of when to treat	Yes	Partially
7. Capacity for diagnosis and treatment	No	No

that assessment by an alcohol specialist nurse was associated with a significantly greater reduction in maximum daily alcohol consumption or the risk of a subsequent hospital admission. However, this was not the primary aim of the study. In addition, there is a well-recognised under-treatment of alcohol dependence syndromes, which raises significant questions regarding our ability to offer effective treatment to individuals identified via this screening process. Changes in drinking behaviour such as the increasing prevalence of binge drinking in Western countries, particularly the United Kingdom, pose a particular challenge. Binge drinkers disclose a higher risk of presentation to emergency units[8,9] and are typically younger (18–43 years old)[10] – presenting an opportunity to intervene at, or prior to, the early stages of end-organ damage. However, therapeutic tools to effectively engage with this cohort are lacking. Brief interventions, whilst effective in other groups, do not demonstrate any sustained benefit in this group.[11–13] The present study enrolled patients irrespective of drinking patterns. However, it raises the question of whether screening for alcohol misuse, followed by more intensive assessment and intervention, delivered by an ASNS may be effective in combatting recalcitrant binge drinking in young individuals. The fact that these individuals apparently do not consider themselves unwell and may rate their health at or above the level of the general population may explain their apparent resistance to intervention.[8,9]

This study also raises important questions about healthcare capacity. An estimated 1,000 patients per year were identified by screening, of which half were referred to inpatient services. If replicated elsewhere this workload would likely overwhelm the capacity of most alcohol services. Furthermore, the proportion of people with alcohol use disorders receiving treatment appears to be low – across Europe data indicates that fewer than 10% of individuals with an alcohol-use disorder receive treatment.[14,15] Recent data from the United Kingdom indicates that only a third of individuals with probable dependence had consulted a doctor regarding their potential alcohol use disorder and a comparative minority, around 6%, were receiving substance misuse medication or counselling.[16] The issue is not limited to alcohol use disorders however. In a large European study only 26% of patients diagnosed with a mental health disorder within a 12-month period had consulted formal mental health services within the same timeframe.[14] This under-treatment is likely multifactorial – reflecting inadequate service provision and resources in combination with stigmatisation, marginalisation and difficulties engaging with healthcare services. Indeed, higher AUDIT scores have been associated with an increasing prevalence of requests for treatment being denied.[16]

Even with a dramatic increase in resources it seems unlikely that this unmet demand for treatment can be met. In this context, it is worth considering how additional assessments may be made to more accurately define the potential benefit from intervention. This may entail defining individuals (i) most likely to engage based upon previous behaviour, (ii) with an increased risk of end-organ damage, potentially defined by known genetic risk loci such as the variant rs738409 in *PNPLA3* or (iii) evidence of end-organ damage either clinically or assessed by non-invasive methods.

Alcohol misuse is an immensely important health issue, with known opportunities and effective methods for intervention. Westwood and colleagues describe an acceptable, effective, feasible and sustainable method for screening acute medical admissions for evidence of alcohol use disorders. However, ensuring the required tools and resources are available for diagnosis presents significant challenges for therapeutic application, especially in the context of healthcare systems already operating at the limit of their resources. Before significant healthcare resource can be invested to address these challenges, a richer evidence base is required.

Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Editorial

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Research Article

JOURNAL OF HEPATOLOGY

Homozygosity for rs738409:G in *PNPLA3* is associated with increased mortality following an episode of severe alcoholic hepatitis

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See Editorial, pages 12–14

Background & Aims: Carriage of rs738409:G in *PNPLA3* is associated with an increased risk of developing alcohol-related cirrhosis and has a significant negative effect on survival. Short-term mortality in patients with severe alcoholic hepatitis is high; drinking behaviour is a major determinant of outcome in survivors. The aim of this study was to determine whether carriage of rs738409:G has an additional detrimental effect on survival in this patient group.

Methods: Genotyping was undertaken in 898 cases with severe alcoholic hepatitis, recruited through the UK Steroids or Pentoxifylline for Alcoholic Hepatitis (STOPAH) trial, and 1188 White British/Irish alcohol dependent controls with no liver injury, recruited via University College London. Subsequent drinking behaviour was classified, in cases surviving ≥90 days, as abstinent or drinking. The relationship between rs738409 genotype, drinking behaviour and survival was explored.

Results: The frequency of rs738409:G was significantly higher in cases than controls (29.5% *vs.* 18.9%; $p = 2.15 \times 10^{-15}$; odds ratio 1.80 [95% confidence interval (CI) 1.55–2.08]). Case-mortality at days 28, 90 and 450 was 16%, 25% and 41% respectively. There was no association between rs738409:G and 28-day mortality. Mortality in the 90 to 450-day period was higher in survivors who subsequently resumed drinking (hazard ratio [HR] 2.77, 95% CI 1.79–4.29; p <0.0001) and in individuals homozygous for rs738409:G (HR 1.69, 95% CI 1.02–2.81, $p = 0.04$).

Conclusion: Homozygosity for rs738409:G in *PNPLA3* confers significant additional risk of medium-term mortality in patients with severe alcoholic hepatitis. Rs738409 genotype may be taken into account when considering treatment options for these patients.

Lay summary: Individuals misusing alcohol who carry a particular variant of the gene *PNPLA3* are more at risk of developing severe alcoholic hepatitis, a condition with a poor chance of survival. The longer-term outcome in people with this condition who survive the initial illness is strongly influenced by their ability to remain abstinent from alcohol. However, carriers of this gene variant are less likely to survive even if they are able to stop drinking completely. Knowing if someone carries this gene variant could influence the way in which they are managed.

Clinical trial numbers: EudraCT reference number: 2009-013897-42; ISRCTN reference number: ISRCTN88782125.

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Introduction

Cirrhosis is a major cause of global mortality, accounting for around one million deaths per annum.[1] Alcohol misuse is the leading cause of cirrhosis in the Western world and is implicated in almost half of cirrhosis-related deaths.[2]

Alcohol produces a spectrum of liver injury ranging from hepatic steatosis to cirrhosis and hepatocellular carcinoma. Only 15 to 20% of individuals who chronically misuse alcohol develop cirrhosis;[3,4] approximately 15% of these individuals will eventually develop hepatocellular carcinoma.[5,6] The development of alcohol-related liver injury and its evolution to cirrhosis is generally asymptomatic, with the majority of individuals presenting incidentally. Symptomatic presentation is associated with hepatic decompensation in patients with established cirrhosis or, much less frequently, severe alcoholic hepatitis.

The clinical syndrome of alcoholic hepatitis is typified by the recent onset of jaundice and other features of liver failure in the context of active, chronic and heavy alcohol consumption. The severity of the liver injury is conventionally defined by Maddrey's discriminant function (DF), a calculation based on the serum bilirubin concentration and prothrombin time;[7] a DF ≥32 indicates severe disease and carries an adverse prognosis, with mortality rates of 15 to 30% in the first month[8–11] and upwards of 50% within a year of presentation.[9,11–13]

Poor short-term prognosis, in severe alcoholic hepatitis, is associated with high serum bilirubin and creatinine concentra-

Keywords: Alcohol dependence; Hepatitis, alcoholic; Liver cirrhosis, alcoholic; Genetic polymorphism, *PNPLA3*; Genotype; Prednisolone; Prognostic scores; Risk allele; Survival.

Received 1 March 2016; received in revised form 11 January 2017; accepted 13 January 2017; available online 2 February 2017

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tions, significant prolongation of the prothrombin time, hypoalbuminaemia, hepatic encephalopathy, and ascites.[7,11,14] The long-term prognosis is influenced by several factors including sex, disease severity at presentation, the presence of/evolution to cirrhosis and subsequent drinking behaviour.[9,12,13,15,16]

The role of prednisolone in the management of alcoholic hepatitis remains controversial. The 2008 Cochrane meta-analysis reported that corticosteroids significantly reduce 28-day mortality in patients with a DF ≥32 or hepatic encephalopathy.[17] These findings were later endorsed by an analysis of individual patient data from five randomized clinical trials.[10] The Steroids or Pentoxifylline for Alcoholic Hepatitis (STOPAH) trial[11] did not demonstrate a significant reduction in 28-day mortality with prednisolone treatment. Nevertheless, a recent systematic review and network meta-analysis, incorporating the STOPAH data, reported a significant reduction in short-term mortality in patients treated with prednisolone.[18] Treatment has not, however, been reported to reduce medium- or long-term mortality.[11,16]

The role of genetic polymorphisms in determining liver disease risk and outcome has received considerable attention in recent years. A common single nucleotide polymorphism (SNP), rs738409; C>G in the gene patatin-like phospholipase domain containing protein 3 (*PNPLA3*) results in substitution of an isoleucine residue for methionine at position 148 of the protein (Ile148Met; I148M). There is considerable evidence that carriage of the risk allele, rs738409:G, plays an important role in determining the risk of developing alcohol-related cirrhosis from individual studies,[19–22] a meta-analysis[23] and, most recently, a genome-wide association study.[24] In addition, rs738409:G has been shown to be a significant risk factor for the development of hepatocellular carcinoma in patients with established cirrhosis both in individual studies,[25–29] and in a meta-analysis based on individual patient data.[30] Furthermore there is growing evidence that rs738409:G influences several other important aspects of alcohol-related liver disease; thus, carriage of the G allele is associated with earlier development of cirrhosis, independently of the age of onset of at-risk alcohol consumption;[31] more rapid progression towards decompensated disease;[32] a reduction in transplantation-free survival;[32] and, poorer outcomes following development of hepatocellular carcinoma.[33]

Although the frequency of rs738409:G was reported as significantly increased in patients with severe alcoholic hepatitis in one small study,[34] it is not known whether carriage of this allele otherwise influences the course of the disease or its outcome. The availability of DNA from many of the participants in the STOPAH trial[11] provided an opportunity to explore the role of this variant in disease progression and outcome in this patient population.

The aims of the present study were:

1. To identify variables associated with short-term (<28 days) survival in patients with severe alcoholic hepatitis, looking specifically at the effect of carriage of rs738409:G in *PNPLA3* and the response to treatment;
2. To identify variables associated with medium-term (90 to 450 days) survival in this population, looking specifically at the effect of rs738409:G in *PNPLA3*;

Patients and methods

Study population

Cases

Patients with severe alcoholic hepatitis were recruited as per the STOPAH trial protocol.[35] DNA samples and matched clinical data were available for 898 of the 1103 enrolled patients (81.4%). All had a history of long-standing alcohol misuse; compatible clinical, laboratory and/or liver biopsy features of alcoholic hepatitis; no other identified cause for their liver disease; and a DF ≥32. All were British; 860 (95.8%) identified themselves as White; three (0.3%) as Black or Black British; 23 (2.6%) as Asian or Asian British; five (0.6%) as of mixed origin; and seven (0.8%) as 'other' or not stated.

Patients were randomized to treatment with prednisolone or pentoxifylline for 28 days using a double blind, double dummy design.[35] Randomization was block designed and stratified by geographical region and dichotomous risk status; the presence of sepsis, gastrointestinal bleeding or renal failure prior to randomization defined high-risk.

Individuals who survived the initial hospitalisation were further evaluated at 90 days and at one year to ascertain clinical status particularly in relation to their self-reported alcohol use. Patients were consented for follow-up via the NHS Information Centre Data Linkage service ensuring reliable capture of mortality data.

Ethical approval was granted for this study by the Wales Research Ethics Committee (REC 09/MRE09/59). The study was conducted according to the Declaration of Helsinki (Hong Kong Amendment) and Good Clinical Practice (European guidelines). All participants, or their legally appointed representatives, provided written informed consent.

Controls

Controls with a background of alcohol dependence but with no evidence of liver injury (n = 1188) were recruited via the University College London Consortium. The majority had been drinking hazardously for over 15 years and were actively drinking at the time of enrolment. In approximately one-third of participants, the absence of significant alcohol-related liver injury was confirmed on liver biopsy. The remainder had no historical, clinical or radiological features suggestive of significant liver injury either at presentation or during prolonged follow-up. People with more than one grandparent of white European Caucasian origin were excluded---so the maximum allowed was one. None of the individuals was related.

United Kingdom National Health Service Multicentre Research Ethics Committee approval was granted for this study (MREC/03/11/090). This was ratified by the local ethics committees associated with the individual participating centres. All participants provided written informed consent.

PNPLA3 genotyping

Genotyping for rs738409 in *PNPLA3* was performed using the K-Biosciences Competitive Allele Specific PCR (LGC Genomics, Hoddesdon, UK) platform with amplification and detection undertaken using a LightCycler® 480 real-time PCR system (Roche Molecular Diagnostics, Burgess Hill, UK). Genotype calling was performed automatically using proprietary software with minor manual editing of genotype calls. Approximately 12% of the samples, randomly selected *a priori*, were genotyped in duplicate to ensure consistent genotype calling.

Data processing and statistical analyses

Routinely collected demographic and laboratory data were used to calculate prognostic scores *viz*: the model for end-stage liver disease (MELD),[36]; the Glasgow alcoholic hepatitis score (GAHS),[37] and the Lille score.[38]

Patients self-categorised their current drinking behaviour at day 90 and at 1 year as (i) abstinent; (ii) drinking at low levels: men ≤24 g/day; women: ≤16 g/day; (iii) drinking at moderate levels: men >24 but ≤60 g/day; women >16 but ≤40 g/day; (iv) drinking at high levels: men >60 g/day; women >40 g/day. For the purposes of statistical analysis patients were classified as either abstinent (i) or drinking (ii-iv). However, in view of the relatively high incidence of missing data on drinking behaviour at the day 90 and 1-year time points, additional sensitivity analyses were undertaken based on the following:

1. A reclassification of the drinking behaviour at day 90 in light of additional information gathered at 1 year, where available.
2. The assumption that individuals in whom information on drinking behaviour was not available at day 90, for any reason, had returned to drinking.

Tests for primary allelic associations, missingness and deviation from Hardy-Weinberg equilibrium, were performed using PLINK v1.9.[39,40] Samples with conflicting calls were excluded from further analysis.

The influence of genotype on patient characteristics at presentation, including prognostic scores, was tested using Kruskall-Wallis or Chi-square tests across all three groups.

The STOPAH trial showed no beneficial effect of pentoxifylline on outcome in cases with severe alcoholic hepatitis but a modest benefit from use of prednisolone.[11] Thus, treatment effects were examined dichotomously *viz.*

Table 1. Genotype frequencies and association analysis of rs738409 in cases with severe alcoholic hepatitis and controls with alcohol dependence but no liver injury.

SNP	Cases (n = 867)		Controls (n = 1175)		Cases *vs.* controls	
	Genotype count CC/CG/GG	MAF (%)	Genotype count CC/CG/GG	MAF (%)	*p* value	OR (95% CI)
rs738409	425/372/70	29.5	772/362/41	18.9	2.15×10^{-15}	1.80 (1.55–2.08)

SNP, single nuclear polymorphism; MAF, minor allele frequency; OR, odds ratio; CI, confidence interval.
Data were analyzed using logistic regression performed in PLINK v 1.9.[39,40]

treatment with prednisolone (cases treated with prednisolone plus placebo and prednisolone plus pentoxifylline: n = 429) or no treatment with prednisolone (cases treated with pentoxifylline plus placebo or placebo plus placebo: n = 438).

Survival times, and mortality endpoints, were calculated with respect to the treatment start date or, if not recorded, the date of randomization. A data cut-off of 450 days was applied because the large variation in follow-up times engendered: (i) a risk of informative censorship; (ii) a risk of disproportionate censorship between genotypic groups; and, (iii) the likely impact of additional factors such as a delayed return to drinking and the development of co-morbid disease on longer-term survival. Thus, cases were censored at the time of liver transplantation, the limit of follow-up or day 450, whichever occurred first.

Cox proportional hazards models were used to test for associations and interactions between explanatory variables and survival. Where significant interactions were found, univariate and multivariable analyses were undertaken in relevant population subgroups to better understand the main effects of the covariates on outcome. Tests for genotypic association were performed using three models of inheritance *viz*: additive (CC [0]. CG [1] and GG [2]; p_{ADD}), recessive (CC + CG *vs.* GG; p_{REC}) and dominant (CC *vs.* CG + GG; p_{DOM}); the model showing the greatest statistical significance was used in subsequent multivariable analyses. Separate models were fitted for clinically relevant features and biochemical parameters. Variables demonstrating marginal statistical significance ($p < 0.1$) in univariate analysis were included in multivariable analyses. These models were fitted by backward elimination with a cut-off of $p = 0.05$. Where a composite variable and its constituents were both associated with outcome, only the most significantly associated was incorporated into the multivariable analyses in order to reduce co-linearity.

Statistical analyses were performed using SPSS version 22 (IBM, Armonk, USA). Survival curves were plotted in R[41] using the packages ggplot2, survival, gridExtra, reshape and plyr.

For further details regarding the materials used, please refer to the CTAT table.

Results

Genotyping accuracy

The overall genotyping rate was 98%. Genotypes were successfully called in 867 (97%) of 898 case samples and in 1175 (99%) of the 1188 control samples. Two samples (<0.05% of total) demonstrated conflicting genotypes and were excluded. The marker followed Hardy-Weinberg equilibrium in both case and control populations ($p > 0.05$).

PNPLA3 allelic association analysis

A significant increase in the frequency of rs738409:G was observed in cases compared with controls (allelic $p = 2.15 \times 10^{-15}$, odds ratio [OR] 1.80: 95% confidence intervals [CI] 1.55–2.08) (Table 1).

PNPLA3 genotypic association with baseline demography and assessment variables

There were no significant differences in age, sex distribution, alcohol consumption, or the majority of the clinical or laboratory variables at baseline in relation to rs738409 genotype (Table S1).

Survival data

Survival data were available for all 867 genotyped cases; the median (range) duration of follow-up was 844 (352–1452) days.

Overall 52 cases (6.2%) were censored because their duration of follow-up was too short; two patients (0.2%) underwent orthotopic liver transplantation at day 215 and day 359 post-enrolment while 360 (41.5%) died during the follow-up period; the mortality rates at days 28, 90 and 450 were, 15% (131/864), 25% (216/861) and 44% (360/813) respectively.

Impact of genotype on treatment response and short-term survival

One-hundred and thirty-one (15.0%) of the 867 cases with severe alcoholic hepatitis had died by day 28 while a further three were lost to follow-up. There was no significant relationship between 28-day mortality and rs738409 genotype ($p_{ADD} = 0.95$, $p_{DOM} = 0.88$, $p_{REC} = 0.64$; Fig. 1, Table 2). Treatment with prednisolone was associated with a decreased risk of mortality compared with placebo (hazard ratio [HR] = 0.67; 95% CI 0.48–0.95, $p = 0.03$). No significant interaction was detected between rs738409 genotype and prednisolone treatment in relation to 28-day mortality.

Cox proportional hazards regression analysis identified randomization risk, treatment with prednisolone, age, the presence of overt hepatic encephalopathy, total white blood cell and neutrophil counts, blood urea, international normalised ratio (INR), and the serum bilirubin and creatinine concentrations as significantly associated with 28-day mortality (Table 3).

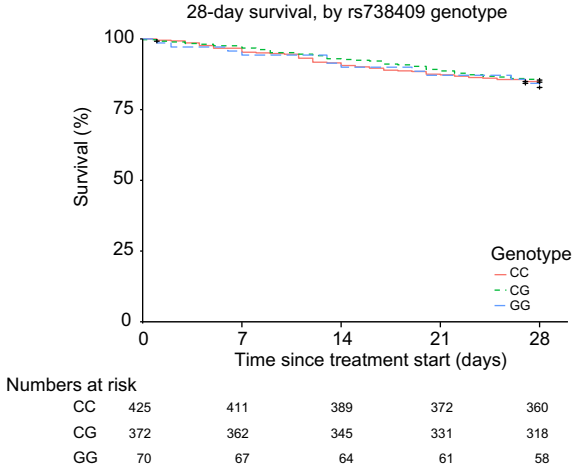

Fig. 1. Twenty-eight day survival in cases with severe alcoholic hepatitis stratified by rs738409 genotype. There was no impact of rs738409 genotype on short-term survival. (This figure appears in colour on the web.)

Table 2. Twenty-eight-day mortality in cases with severe alcoholic hepatitis, by treatment allocation and rs738409 genotype.

Treatment allocation	Cases (n)	Overall deaths (n: %)	Deaths, by rs734809 genotype (n: %)		
			CC	CG	GG
Prednisolone	429	53 (12.4%)	25 (11.8%)	22 (12.3%)	6 (15.4%)
No prednisolone	438	78 (17.8%)	40 (18.7%)	32 (16.6%)	6 (19.4%)
Total	867	131 (15.1%)	65 (15.3%)	54 (14.5%)	12 (17.1%)

Table 3. Variables associated with 28-day mortality in cases with severe alcoholic hepatitis.

Variable	Univariate			Multivariable		
	HR	95% CI	*p* value	HR	95% CI	*p* value
Age	1.05	1.04–1.07	<0.001	1.04	1.02–1.07	<0.001
Sex	0.88	0.74–1.06	0.19			
Alcohol consumption[§]	1.00	0.99–1.00	0.10			
Overt hepatic encephalopathy	2.85	2.02–4.02	<0.001	2.46	1.55–3.90	<0.001
White cell count[*] ($\times 10^6$/mm^3)	1.08	1.06–1.11	<0.001			
Neutrophils ($\times 10^6$/mm^3)	1.09	1.06–1.12	<0.001	1.06	1.02–1.09	0.001
Bilirubin (μmol/L)	1.003	1.002–1.005	<0.001	1.001	1.000–1.003	0.09
Aspartate transaminase (IU/L)[§]	1.002	1.000–1.005	0.09			
Alkaline phosphatase (IU/L)	0.999	0.997–1.001	0.45			
Albumin (g/L)	0.99	0.97–1.02	0.67			
Urea (mmol/L)	1.09	1.07–1.12	<0.001	1.11	1.07–1.15	<0.001
Creatinine (μmol/L)[§]	1.01	1.008–1.013	<0.001			
International normalised ratio	1.21	1.06–1.38	0.004	1.27	1.06–1.51	0.009
Randomization risk[§]	1.51	1.26–1.81	<0.001			
rs738409:G homozygosity[§]	1.15	0.64–2.09	0.64			
Prednisolone	0.67	0.48–0.95	0.03	0.59	0.37–0.93	0.02

HR, hazard ratio; CI, confidence intervals.
Analyses were undertaken using Cox proportional hazards models.
[*] Variable not entered into the Cox multivariable analysis due to co-linearity (more significantly associated constituent part of the variable exists).
[§] Variable excluded from the Cox multivariable analysis by backward elimination due to lack of significant independent association.

Multivariable Cox regression analyses, incorporating the variables associated on univariate analysis (p <0.1), together with a term for homozygosity for rs738409:G, confirmed significant, independent associations with 28-day survival for many of the variables identified in univariate analysis, including prednisolone treatment; homozygosity for rs738409:G was not independently associated (Table 3).

Impact of genotype on prognostic scoring systems

There were no differences in the distributions of the prognostic scores calculated at baseline or the Lille score at day 7 in relation to rs738409 genotype (Table 4). All four of the commonly used scoring systems were significantly associated with 28-day mortality. The Lille score had the highest predictive accuracy (Table S2). No statistically significant interactions were found between any of the scoring systems and rs738409 genotype in relation to 28-day mortality.

Impact of genotype and drinking behaviour on medium-term survival

There was no impact of rs738409 genotype on 90-day survival. However, in the cohort of patients surviving beyond this timepoint, homozygosity for rs738409:G was associated with a significant increase in mortality at day 450 (GG: 34.7% (17/49); CG: 21.8% (53/243); CC: 25.1% (74/295); p_{REC} = 0.04; [HR_{REC} 1.69, 95% CI 1.02–2.81]; p_{ADD} = 0.62; p_{DOM} = 0.67) (Fig. 2A).

Information on drinking behaviour post hospital discharge was available in 397 (46%) of the 867 cases with severe alcoholic hepatitis at day 90 and in 174 (20.1%) at 1 year. Reported abstinence rates were 65% and 57% respectively. Significant differences in survival to day 450 were observed in relation to drinking behaviour recorded at day 90 (Fig. 2B); mortality in those who were drinking was 35.3% (47/133) *vs.* 14.3% (35/244) in those classified as abstinent (HR 2.77, 95% CI 1.79–4.29; p <0.00001). This association was robust to the incorporation of the additional data on drinking behaviour collected at 1 year. This approach may be prone to bias due to potential conditioning on the future; however the association retained significance in additional sensitivity analysis where all cases with missing data at day 90 were assumed to have resumed drinking (Table S3).

The association between rs738409 homozygosity and 450-day survival was independent of a return to drinking (Table S4). Statistically significant interactions were identified between drinking behaviour and both serum bilirubin concentrations (p = 0.004) and neutrophil count (p = 0.002) at day 90 in relation to medium-term survival. Interactions between drinking behaviour and homozygosity for rs738409:G (p = 0.1) and the INR at day 90 (p = 0.09) were not significant. In view of these interactions, factors influencing medium-term survival were examined separately in groups defined by drinking status.

In cases reporting drinking at day 90, homozygosity for rs738409:G had no significant effect on survival; mortality rates were around 30% in all three genotypic groups over the 90 to 450 day period (Fig. 2C). This lack of effect was confirmed on

Table 4. Prognostic scores in cases with severe alcoholic hepatitis, by rs738409 genotype.

Prognostic scoring system	*PNPLA3* rs738409 genotype			*p* value
	CC (n = 425)	CG (n = 372)	GG (n = 70)	
Baseline DF [7]	62 ± 29	62 ± 25	64 ± 27	0.50
Baseline MELD [36]	21 ± 6	21 ± 6	21 ± 7	0.53
Baseline GAHS [37]	8 ± 1	8 ± 1	8 ± 1	0.41
	CC (n = 292)	**CG (n = 246)**	**GG (n = 37)**	
Lille* [38]	0.46 ± 0.3	0.49 ± 0.3	0.43 ± 0.3	0.55
Lille responders (<0.45)	158 (54.1%)	119 (48.3%)	23 (62.2%)	0.19

Comparisons were made using Kruskall-Wallis or Chi-square tests.
Data expressed as mean ± SD or as number (%) *n = 575.
DF, discriminant function calculated as 4.6 × (patient prothrombin time [s] – control prothrombin time [s]) + (serum bilirubin [μmol/l]/17.1): scores >32 indicate severe disease; MELD, model for end-stage liver disease: scores range from 6 to 40, with higher scores indicating worse prognosis; GWAS, the Glasgow alcoholic hepatitis score: ranges from 5 to 12, with higher scores indicating worse prognosis; Lille: composite scoring system incorporating age, serum albumin and bilirubin levels at baseline and 7 days after the start of treatment. A score of >0.45, 7 days after initiation of treatment predicts an adverse outcome; patients with a score of <0.45 on day 7 are classified as responders.

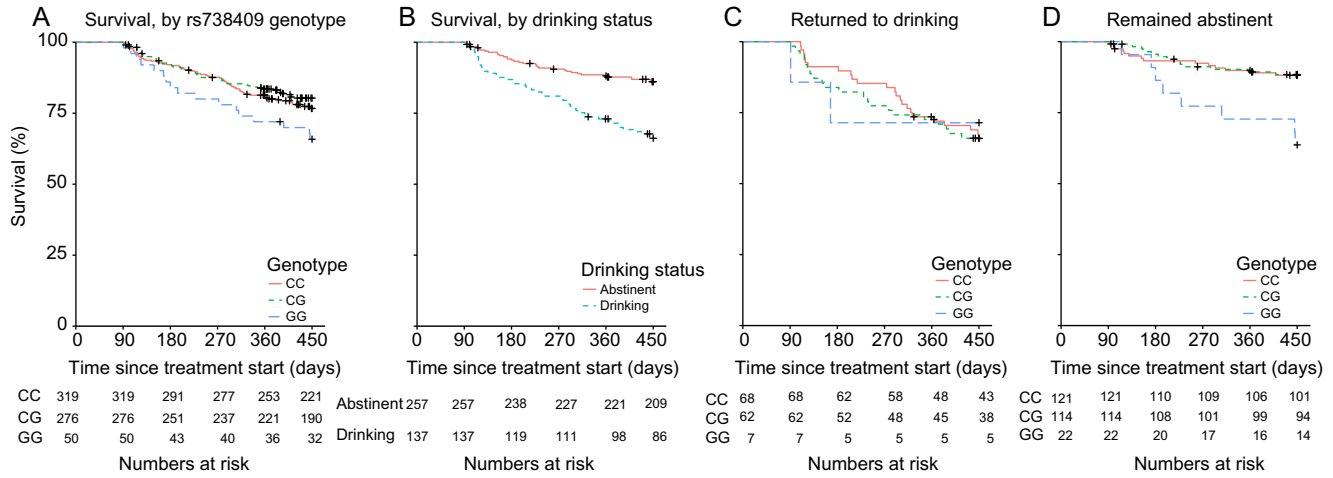

Fig. 2. Medium-term survival in cases with severe alcoholic hepatitis surviving at least 90 days. (A) Mortality was increased in cases homozygous for rs738409:G (GG: 34.7%; CG: 21.8%; CC: 25.1%; HR 1.69, 95% CI 1.02–2.81%; p_{REC} = 0.04); (B) Patients reporting alcohol consumption at day 90 have increased mortality at day 450 compared to those reporting abstinence (35.3% *vs.* 14.3%; HR 2.77, 95% CI 1.79–4.29; p <0.00001); (C) In cases who resumed drinking outcome was not affected by genotype; (D) In cases who attained abstinence, survival was reduced in rs738409:G homozygotes (GG: 36.4%; CG 12.1%; CC 12.2%; HR 3.40, 95% CI 1.54–7.49, p_{REC} = 0.002). (This figure appears in colour on the web.)

multivariable regression (Table 5). However, in cases reporting abstinence at day 90 homozygosity for rs738409:G was associated with a significantly higher mortality during the follow-up period (GG: 36.4% (8/22); CG 12.1% (13/107); CC 12.2% (14/115); HR 3.40, 95% CI 1.54–7.49, p = 0.002) (Fig. 2D). Cox multivariable regression analysis confirmed that homozygosity for rs738409:G was significantly and independently associated with reduced survival in this group (HR 2.56, 95% CI 1.03–6.34, p = 0.04) (Table 6).

These differences were maintained when drinking behaviour was further refined based on the data collected at 1 year. Analyses undertaken assuming that the patients in whom 90-day data were missing had resumed drinking confirmed the significant independent associations with 450-day survival for both homozygosity for rs738409:G and drinking behaviour; they also revealed a significant interaction between these two variables (Table S5).

Discussion

The variant rs738409:G in *PNPLA3* has been consistently associated with the risk of developing alcohol-related cirrhosis and has also been implicated in more rapid disease progression and the risk of developing hepatocellular carcinoma.[22,28–31] Severe alcoholic hepatitis has considerable associated mortality[8,9,11–13] but apart from one small series, published in abstract form,[32] which identified rs738409:G as a risk factor for developing severe alcoholic hepatitis, the potential impact of this genetic polymorphism on disease presentation, progression and outcome has not been evaluated. The results of the present study have helped clarify these associations.

First: this study identifies rs738409:G as a risk factor for the development of severe alcoholic hepatitis. Many of the included cases had co-existing alcohol-related cirrhosis and a high proportion of the remainder are likely to develop cirrhosis over time. This finding is not, therefore, surprising but given the size and appropriateness of the case and control populations it provides robust confirmation of the results of the previous much smaller study.[32]

Second: there is no evidence that rs738409 genotype plays a role in determining the onset timing, mode of presentation or severity of alcoholic hepatitis. Thus, the age, sex distribution, the quantity of alcohol consumed, the duration of alcohol misuse

Table 5. Variables associated with 450-day mortality in cases with severe alcoholic hepatitis who resumed alcohol consumption.

Variable	Univariate			Multivariable		
	HR	95% CI	*p* value	HR	95% CI	*p* value
Age	1.04	1.00–1.07	0.04			
Sex	1.68	0.95–2.98	0.08	2.02	1.05–3.90	0.04
Overt hepatic encephalopathy	2.34	1.12–4.90	0.02			
White cell count* ($\times 10^6$/mm^3)	1.07	1.00–1.13	0.04			
Neutrophils§ ($\times 10^6$/mm^3)	1.09	1.02–1.17	0.01			
Bilirubin (μmol/L)	1.004	1.002–1.006	<0.001	1.005	1.002–1.007	<0.001
Aspartate transaminase (IU/L)†	1.01	1.001–1.011	0.01			
Alkaline phosphatase (IU/L)	1.002	1.000–1.004	0.03	1.002	1.000–1.005	0.03
Albumin (g/L)	0.94	0.90–0.99	0.01			
Urea (mmol/L)	1.22	1.10–1.35	<0.001	1.23	1.10–1.38	<0.001
Creatinine§ (μmol/L)	1.02	1.01–1.03	0.005			
International normalised ratio	1.00	0.81–1.24	0.98			
Randomization risk	0.71	0.42–1.18	0.19			
rs738409 homozygosity§	0.88	0.21–3.63	0.86			
Prednisolone	0.75	0.42–1.33	0.32			

HR, hazard ratio; CI, confidence Intervals.
Analyses were undertaken using Cox proportional hazards models.
* Variable not entered into the Cox multivariable analysis due to co-linearity (more significantly associated constituent part of the variable exists).
§ Variable excluded from the Cox multivariable analysis by backward elimination due to lack of significant independent association.
† Variable not entered into the Cox multivariable analysis due to significant missing information (>10%).

Table 6. Variables associated with 450-day mortality in cases with severe alcoholic hepatitis who maintained abstinence from alcohol.

Variable	Univariate			Multivariable		
	HR	95% CI	*p* value	HR	95% CI	*p* value
Age§	1.06	1.03–1.10	0.001			
Sex	0.91	0.45–1.83	0.79			
Overt hepatic encephalopathy	2.11	0.81–5.46	0.13			
White cell count* ($\times 10^6$/mm^3)	1.25	1.13–1.38	<0.001			
Neutrophils ($\times 10^6$/mm^3)	1.33	1.19–1.49	<0.001	1.22	1.06–1.41	0.005
Bilirubin (μmol/L)	1.01	1.01–1.02	<0.001	1.007	1.002–1.012	0.006
Aspartate transaminase (IU/L)	1.01	0.99–1.03	0.17			
Alkaline phosphatase§ (IU/L)	1.006	1.001–1.010	0.02			
Albumin (g/L)	0.90	0.86–0.94	<0.001	0.92	0.88–0.97	0.002
Urea§ (mmol/L)	1.25	1.14–1.37	<0.001	1.15	1.03–1.29	0.02
Creatinine§ (μmol/L)	1.01	1.005–1.023	0.003			
International normalised ratio	1.23	1.10–1.39	0.001	1.24	1.08–1.42	0.003
Randomization risk§	1.36	0.94–1.96	0.1			
rs738409 homozygosity	3.40	1.54–7.49	0.002	2.56	1.03–6.34	0.04
Prednisolone	1.29	0.66–2.52	0.46			

HR, hazard ratio; CI, confidence intervals.
Analyses were undertaken using Cox proportional hazards models.
* Variable not entered into the Cox multivariable analysis due to co-linearity (more significantly associated constituent part of the variable exists).
§ Variable excluded from the Cox multivariable analysis by backward elimination due to lack of significant independent association.

and disease severity, assessed using the available scoring systems, were similar in all subgroups defined by genotype.

Third: there is no evidence that the rs738409 genotype is associated with short-term mortality in patients with severe alcoholic hepatitis, nor does it interact with the severity of liver disease, prednisolone treatment or early improvement in liver function, as measured by the Lille score.

Fourth: the study provides clear evidence supporting the primacy of drinking behaviour as a determinant of medium-term outcome in patients with severe alcoholic hepatitis who survive the initial illness.[15,16] Individuals who maintain abstinence have a significantly lower mortality rate than individuals who resume drinking, at any level. Resumption of alcohol consumption also appears to influence the relative associations of several variables with survival, particularly neutrophil count and serum bilirubin concentrations.

Fifth: rs738409 genotype influences medium-term survival. Thus, in the entire population surviving beyond day 90, taken as a whole, mortality was significantly higher in individuals homozygous for the G allele. Sensitivity analyses, conducted on the assumption of resumed drinking where data were missing, showed that the independent associations of both drinking behaviour and homozygosity for rs738409:G with survival were robust. This relationship may not be entirely straight forward as there is evidence of an interaction between these two variables, albeit only significant on the sensitivity analysis. Thus, while there was no difference in mortality, by genotype, in individuals who continued to drink, abstinence from alcohol

was associated with improved survival in heterozygote carriers of rs738409:G or non-carriers but not in patients homozygous for rs738409:G. This suggests that the effect of rs738409 genotype on survival outcome is subservient to drinking behaviour in those continuing to drink.

This study has a number of limitations *viz.*: (i) The information on drinking behaviour was based on self-reported estimates of alcohol intake collected on day 90 and was only available for 46% of the cases; information on drinking behaviour was only available in 21% of survivors at 1 year. Sensitivity analyses were conducted to evaluate the potential effect on outcome of adjustment of drinking status based on 1-year data and on the assumption of resumed drinking in those in whom the data were missing. The results of the subsequent analyses show clear differentiation in the direction expected and hence confirm the robustness of our findings. (ii) A small proportion of cases were of non-British ancestry (n = 38, 4.2%). There are ethnic differences in the frequency of rs738409:G but its association with an increased risk of developing alcohol-related liver disease is consistent across ethnic groups. Thus, inclusion of these individuals in the analyses is unlikely to have confounded the results to any appreciable degree. (iii) Survival data were captured using the NHS database of registered deaths but registration is often delayed, and deaths occurring outside the UK are not registered; thus the number of deaths may have been underestimated. (iv) Data on the number of cases undergoing liver transplantation were only captured for the duration of the STOPAH trial, although it is likely that the number of participants transplanted beyond this immediate time-point would have been small. (v) Although the number of cases was large the number of individuals homozygous for rs738409:G was relatively small and this may have limited the power.

In conclusion: individuals with severe alcoholic hepatitis who survive the acute event and are homozygous for rs738409:G in *PNPLA3* would appear to be at increased risk of mortality in the medium-term, even if they attain and maintain abstinence from alcohol. Genotyping rs738409 in *PNPLA3* will identify these individuals and the results could be taken into account in clinical decision-making, potentially allowing these particularly vulnerable individuals to be considered early for liver transplantation or novel therapies. The need to employ measures to assist patients with severe alcoholic hepatitis to attain and maintain abstinence is highlighted again in this study as of critical importance.

Financial support

NIHR HTA grant 08/14/44. University College London (Impact PhD fellowship award MJW). Medical Research Council (UK) – Grant number MR/M003132/1. Imperial College BRC programme.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contributions

SRA performed genotyping, statistical analyses and drafted and revised the manuscript. MJW performed genotyping and revised the manuscript. AM recruited participants and revised the manuscript. MYM conceptualised the idea, recruited participants and revised the manuscript. MRT recruited participants and revised the manuscript.

Acknowledgements

We would like to thank everyone who took part in the study. The UCL samples were collected with the support of the National Institute for Health Research (NIHR) Mental Health Research Network. The STOPAH samples were collected with the support of the NIHR and STOPAH Investigators Consortium.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2017.01.018.

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Gastroenterology 2017;152:1068–1077

CLINICAL—LIVER

In Patients With Severe Alcoholic Hepatitis, Prednisolone Increases Susceptibility to Infection and Infection-Related Mortality, and Is Associated With High Circulating Levels of Bacterial DNA

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See editorial on page 938.

BACKGROUND & AIMS: Infections are common in patients with severe alcoholic hepatitis (SAH), but little information is available on how to predict their development or their effects on patients. Prednisolone is advocated for treatment of SAH, but can increase susceptibility to infection. We compared the effects of infection on clinical outcomes of patients treated with and without prednisolone, and identified risk factors for development of infection in SAH. **METHODS:** We analyzed data from 1092 patients enrolled in a double-blind placebo-controlled trial to evaluate the efficacy of treatment with prednisolone (40 mg daily) or pentoxifylline (400 mg 3 times each day) in patients with SAH. The 2 × 2 factorial design led to 547 patients receiving prednisolone; 546 were treated with pentoxifylline. The trial was conducted in the United Kingdom from January 2011 through February 2014. Data on development of infection were collected at evaluations performed at screening, baseline, weekly during admission, on discharge, and after 90 days. Patients were diagnosed with infection based on published clinical and microbiologic criteria. Risk factors for development of infection and effects on 90-day mortality were evaluated separately in patients treated with prednisolone (n = 547) and patients not treated with prednisolone (n = 545) using logistic regression. Pretreatment blood levels of bacterial DNA (bDNA) were measured in 731 patients. **RESULTS:** Of the 1092 patients in the study, 135 had an infection at baseline, 251 developed infections during treatment, and 89 patients developed an infection after treatment. There was no association between pentoxifylline therapy and the risk of serious infection ($P = .084$), infection during treatment ($P = .20$), or infection after treatment ($P = .27$). Infections classified as serious were more frequent in patients treated with prednisolone (odds ratio [OR], 1.27; 95% confidence interval [CI], 1.27−2.92; $P = .002$). There was no association between prednisolone therapy and infection during treatment (OR, 1.04; 95% CI, 0.78−1.37; $P = .80$). However, a higher proportion (10%) of patients receiving prednisolone developed an infection after treatment than of patients not given prednisolone (6%) (OR, 1.70; 95% CI, 1.07−2.69; $P = .024$). Development of infection was associated with increased 90-day mortality in patients with SAH treated with prednisolone, independent of model for end-stage liver disease or Lille score (OR, 2.46; 95% CI, 1.41−4.30; $P = .002$). High circulating bDNA predicted infection that developed within 7 days of prednisolone therapy, independent of Model for End-Stage Liver Disease and white blood cell count (OR, 4.68; 95% CI, 1.80−12.17; $P = .001$). In patients who did not receive prednisolone, infection was not independently associated with 90-day mortality (OR, 0.94; 95% CI, 0.54−1.62; $P = .82$) or levels of bDNA (OR, 0.83; 95% CI, 0.39−1.75; $P = .62$). **CONCLUSIONS:** Patients with SAH given prednisolone are at greater risk for developing serious infections and infections after treatment than patients not given prednisolone, which may offset its therapeutic benefit. Level of circulating bDNA before treatment could identify patients at high risk of infection if given prednisolone; these data could be used to select therapies for patients with SAH. EudraCT no: 2009-013897-42; Current Controlled Trials no: ISRCTN88782125.

Keywords: STOPAH Trial; MELD; *E coli*; Steroid.

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Severe alcoholic hepatitis (SAH) is a clinical syndrome characterized by the recent onset of jaundice and liver failure after prolonged, heavy alcohol misuse. Severe cases are defined by the Maddrey's discriminant function (DF), a calculation utilizing the serum bilirubin and prothrombin time. Where DF is ≥32, ninety-day mortality is 30%–40%; below this threshold spontaneous survival is >95%.[1–3] In common with other forms of liver failure, SAH is associated with increased susceptibility to infection. In the context of SAH, it has been reported that 13%–25% of patients have an infection at presentation and a similar proportion develop an infection during treatment.[3,4]

Current guidelines recommend the use of prednisolone, a corticosteroid with broad anti-inflammatory and immunosuppressive actions for the management of SAH, although few studies have shown benefit beyond 28 days.[5–7] In the Steroids or Pentoxyfilline for Alcoholic Hepatitis (STOPAH) trial, prednisolone almost doubled the risk of infections reported as serious adverse events (13% vs 7%, which was significant at the $P = .002$ level). However, the relationships between prednisolone and liver function, infection, and mortality remain contentious.[4,8]

The aim of this study was to characterize the incidence and impact of infection in SAH using the data from the large cohort of patients recruited to the multicenter STOPAH trial. In addition, this study evaluates pretreatment circulating levels of 16S ribosomal bacterial DNA (bDNA) as a predictor of the subsequent development of infection in patients treated with and without prednisolone by random double-blind allocation.

Materials and Methods

Study Population

Patients were recruited in accordance with the STOPAH trial protocol.[9] All had a history of alcohol misuse; compatible clinical, laboratory, and/or liver biopsy features of alcoholic hepatitis; no other identified causes of liver disease; and DF ≥32. Infections, if present, were treated and controlled with antibiotics before enrolment. All participants, or their legally appointed representative, provided written informed consent.

The trial was approved by the Multicenter Research Ethics Committee (reference 09/MRE09/59) and conducted in accordance with the Medicines for Human Use (Clinical Trials) Regulations 2004 (2006 amendment); the European Union Clinical Trials Directive (Directive 2001/20/EC) guidelines; the principles of the International Conference on Harmonization Good Clinical Practice and under the oversight of University of Southampton Clinical Trials Unit. All participants, or their legally appointed representative, provided written informed consent. All authors had access to the study data and have reviewed and approved the final manuscript.

Group Allocation

STOPAH utilized a double-blind, double-dummy, 2 × 2 factorial design.[9] Patients were randomized to treatment with 40 mg prednisolone once a day or 400 mg pentoxyfilline 3 times a day, neither, or both. There was no mortality benefit from pentoxyfilline, but a possible 28-day mortality benefit from prednisolone.[10] The effect of prednisolone on infection was examined by comparing 2 groups: prednisolone (n = 547) and no-prednisolone–treated patients (n = 545).

Mortality Data

Data regarding date and cause of death were collected during the follow-up period. Patients were also consented for follow-up via the National Health Service Information Centre Data Linkage service, ensuring that if they were lost to follow-up and died, this information could be captured. Mortality at 90 days was analyzed in order to capture the occurrence and impact of all infections occurring during or after the treatment period.

Periods of Infection and Antibiotic Treatment

Clinical data regarding the development of infection were collected at trial visits that occurred at screening, baseline, weekly during admission, on discharge and at 90 days. Data regarding the development of infection submitted in reports of serious adverse events (SAEs) were also incorporated. The diagnosis of infection was made prospectively by treating physicians who were blind to treatment allocation with or without prednisolone. Diagnosis was guided by criteria for infection in the setting of liver disease outlined by Bajaj et al.[11]

Baseline infections were defined as those that occurred between admission and the start of therapy. Active antibiotic treatment at the start of trial therapy was defined as intravenous antibiotics commenced and continued within 5 days prior to treatment start date. Incident infections were defined as those that occurred after the start of treatment—these were further broken down into 3 categories relevant to the clinical management of these patients:

1. Day 7 infections occurred within the first 7 days of therapy (aligned with liver function data available at 7 days from which Lille score was calculated);
2. On-treatment infections within the study treatment period (28 days);
3. Post-treatment infections occurring in the day 28 to day 90 follow-up period;

Bacterial DNA Measurement

An EDTA blood sample was taken from patients at enrolment. DNA extraction was performed on 400 μL blood using Qiagen (Hilden, Germany) QIAamp DNA Mini kits under aseptic

*Authors share co-first authorship.

Abbreviations used in this paper: bDNA, bacterial DNA; CI, confidence interval; ^{hi}bDNA, bacterial DNA >18 pg/mL; DF, discriminant function; MELD, Model for End-Stage Liver Disease; OR, odds ratio; PCR, polymerase chain reaction; SAE, serious adverse event; SAH, severe alcoholic hepatitis; STOPAH, Steroids or Pentoxifylline for Alcoholic Hepatitis.

Most current article

0016-5085
http://dx.doi.org/10.1053/j.gastro.2016.12.019

conditions. The quantity of 16S ribosomal bDNA was determined and measured by real-time polymerase chain reaction (PCR). There are no established cut-off values that define positive from negative bDNA values. In this study, bDNA level that had 80% specificity for predicting the subsequent development of infection in prednisolone-treated patients within 7 days (18.5 pg/mL) was considered a high bDNA level (^{hi}bDNA) for subsequent modeling analyses.

The PCR methodology was adapted from that reported previously.[12] Briefly, primers directed against the V7−V9 variable region of the 16S gene (forward: RW01; 5′->3′ sequence AACTGGAGGAAGGTGGGGAT, reverse: DG74.R; 5′->3′ sequence AGGAGGTGATCCAACCGCA) were combined with a custom fluorescent probe (6-FAM- TACAAGGCCCGGGAACGTATTCACCG-TAMRA; Life Technologies, Carlsbad, CA) at final concentrations of 0.5 μM and 0.25 μM, respectively. This was combined with 10 μL Taqman Gene Expression mix (Applied Biosciences, Foster City, CA), 4 μL extracted DNA and PCR-grade water, to give a final reaction volume of 20 μL. PCR was performed on a StepOne Plus PCR machine (Applied Biosciences) with hot-start activation (2 minutes at 50°C, 10 minutes at 95°C) and 40 reaction cycles (15 seconds at 95°C, 30 seconds at 60°C and 60 seconds at 72°C to collect fluorescence). Serial 10-fold dilutions of *Escherichia coli* DNA (0.08 ng/μL to 0.000008 ng/μL) and a negative control were run to generate a standard curve. Standards and samples were run in triplicate. Any sample displaying a positive signal at or below the level of the negative control was considered negative. Any triplicate group with readings >1 copy cycle apart was considered unreliable and discarded; otherwise, the mean reading was calculated. Standard curves were generated and concentrations interpolated in Prism, version 7.0 (GraphPad, La Jolla, CA). bDNA levels are given as picograms bDNA per milliliter of whole blood from which it was extracted.

Statistical Analysis

Statistical analyses were conducted in SPSS, version 23 (IBM, Armonk, NY) and survival curves were drawn using R (Vienna, Austria). Comparisons between groups were tested using either Mann−Whitney U test for nonparametrically distributed continuous variables or χ^2 test for proportions. Associations between explanatory variables and end points were tested using logistic regression. Early improvement in liver function was defined as Lille score <.45.[13]

In light of previously published data regarding the relationship between prednisolone and early improvement in liver function, infection, and mortality,[4] we tested, a priori, for an interaction between these factors and the end points under consideration by logistic regression.

Previous studies have confirmed that infection and mortality, if present, are positively associated.[4,8] In view of this, and the biologic implausibility that infection could be associated with reduced mortality, a one-tailed test of association between bDNA and 90-day mortality in prednisolone-treated patients was performed. Secondary outcomes were tested post hoc and are not corrected for multiple testing because they are exploratory. For analyses that modeled the expected 90-day mortality in patients with high bDNA treated with or without prednisolone, matching was performed using the FUZZY extension within SPSS, specifying tolerance of 2 pg/mL bDNA.

Results

Population Characteristics

Data regarding infection were available in 1092 of 1103 (99%) of patients randomized in the STOPAH trial; baseline characteristics are presented in Table 1.

Baseline Infection

Infection at baseline occurred in 12% (135 of 1092) of patients (Supplementary Table 1). Chest infections were the single largest category, accounting for 34% (42 of 125) of infections that specified a site of origin (Supplementary Table 2). Positive microbiological cultures were reported in 56 of 135 (41%) patients. *E coli* was the most commonly isolated organism (12 of 40 [30%]; Supplementary Table 3).

Between admission and initiation of trial therapy, 492 of 1092 (45%) patients were prescribed an antibiotic. Of those patients, 293 (60%) continued to receive antibiotic therapy into the treatment period.

Overall, there was no statistically significant association between baseline infection and mortality at 90 days (31% vs 26%; odds ratio [OR], 1.31; 95% confidence interval [CI], 0.88−1.94; $P = .18$; Figure 1*A*). In patients with baseline infection who did not receive prednisolone, active antibiotic therapy when starting treatment had no impact on mortality (30% vs 32%; $P = .81$; Figure 1*B*). However, in those who received prednisolone, there was a significant reduction in 90-day mortality associated with continued antibiotic therapy when compared with those patients in whom antibiotic therapy was stopped before initiating prednisolone (13% vs 52%; OR, 0.13; 95% CI, 0.038−0.47; $P = .002$; Figure 1*C*).

Incident Infection

On-treatment infections were diagnosed in 251 patients (23%) and post-treatment infections were seen in 89 patients (Supplementary Table 1). The most common site of infection in both cases was chest (37% [110 of 301] and 39% [40 of 102], respectively). On-treatment infection was significantly associated with recurrent post-treatment infection risk (OR, 1.93; 95% CI, 1.21−3.06; $P = .005$).

Taken together positive cultures were reported in 147 of 372 episodes of incident infection (40%). *E coli* was the most frequently cultured organism (33 of 133 [25%]; Supplementary Table 3). In patients developing incident infection, median time to develop the infection was 13 days after the start of treatment.

Univariable factors associated with the development of incident infection are given in Table 2. On multivariable analysis, an independent effect was demonstrated for peripheral white cell count (OR, 1.04; 95% CI, 1.02−1.07; $P = .002$) and age (OR, 1.02; 95% CI, 1.00−1.03; $P = .01$). Baseline DF and Model for End-Stage Liver Disease (MELD) scores were both strongly associated with the subsequent risk of developing an infection ($P = .002$ and $P < .001$, respectively; Table 2).

Treatment and Infection Risk

Serious infections (SAEs), on-treatment infections, and post-treatment infections were considered separately when

Table 1. Baseline Characteristics of Study Population

Variable	All patients	Baseline infection only (n = 94)	Baseline and incident infection (n = 41)	Incident infection only (n = 268)	Never infected (n = 689)
Age, *y*	48.8 (41.9–56.3)	49.5 (41.9–54.7)	47.1 (41.1–56.9)	50.3 (42.6–58.8)	48.3 (41.8–55.8)
Sex, male, n (%)	685 (62.7)	60 (63.8)	26 (63.4)	159 (59.3)	440 (63.9)
Alcohol consumption, *U/wk*	132 (84–210)	125 (80–197)	184 (96–249)	120 (80–199)	128 (84–210)
Prednisolone, n (%)	547 (50)	44 (47)	20 (49)	144 (54)	339 (49)
Systolic blood pressure, *mm Hg*	110 (102–120)	112 (105–121)	113 (100–126)	110 (100–120)	110 (102–120)
Diastolic blood pressure, *mm Hg*	90 (60–74)	69 (60–77)	66 (58–77)	65 (60–73)	68 (60–75)
Pulse, *beats/min*	90 (80–98)	82 (88–98)	95 (77–102)	91 (80–100)	89 (80–98)
Temperature, *°C*	36.8 (36.5–37.1)	36.8 (36.6–37.1)	36.8 (36.4–37.3)	36.8 (36.5–37.1)	36.8 (36.5–37.1)
Hemoglobin, *g/L*	107 (94–120)	102 (90–114)	100 (88–118)	105 (94–120)	108 (95–121)
Total white cell count, $\times 10^3$ *per* mm^3	9.00 (6.23–12.6)	9.90 (6.68–14.4)	10.6 (7.05–16.1)	10.1 (7.1–13.7)	8.20 (6.00–11.9)
Neutrophils, $\times 10^3$ *per* mm^3	6.2 (4.1–9.8)	7.2 (4.2–11.6)	6.9 (5.4–13.3)	7.3 (4.9–11.0)	5.7 (3.9–9.0)
International normalized ratio	1.80 (1.56–2.09)	1.91 (1.60–2.32)	1.74 (1.58–2.00)	1.82 (1.60–2.12)	1.70 (1.51–2.00)
Albumin, g/*L*	25 (21–29)	26 (22–31)	25 (18–31)	24 (20–28)	25 (21–29)
Bilirubin, *mg/dL*	16.1 (10.1–24.4)	14.7 (9.47–24.4)	18.6 (9.6–25.7)	16.7 (10.6–25.1)	15.9 (9.90–24.0)
Alanine transaminase, *IU/L*	43 (30–61)	38 (27–51)	39 (31–61)	44 (28–64_	43 (31–62)
Aspartate transaminase, *IU/L*	124 (87–169)	125 (89–148)	120 (90–164)	122 (87–178)	125 (87–171)
Sodium, *mmol/L*	134 (130–136)	134 (131–138)	134 (130–137)	133 (130–136)	134 (130–137)
Urea, *mmol/L*	3.3 (2.2–5.2)	3.5 (2.4–6.7)	4.3 (2.6–7.1)	3.6 (2.2–5.4)	3.1 (2.2–4.9)
Creatinine, *mg/dL*	0.72 (0.60–0.97)	0.72 (0.59–0.99)	0.75 (0.62–1.03)	0.76 (0.60–1.06)	0.72 (0.60–0.92)
Discriminant function[a]	55.4 (43.1–73.7)	62.1 (46.6–86.7)	56.9 (47.0–68.4)	60.6 (45.5–82.0)	53.4 (42.1–69.8)
Model for End-Stage Liver Disease[b]	23.4 (21.0–26.4)	24.4 (21.7–28.6)	24.3 (21.7–27.0)	24.2 (21.4–28.1)	22.9 (20.8–25.7)

NOTE. Groupings are based on the entire study population, with subgroups of when the infection was diagnosed relative to the start of treatment. Baseline infection was defined as those that occurred between admission and the start of therapy. Incident infections were those that occurred after initiation of therapy. Data are presented median (interquartile range) unless otherwise indicated.

[a]Discriminant function = $4.6 \times (PT_{Patient} - PT_{Control}$ [seconds]) + bilirubin [mg/dL].

[b]Model for End-Stage Liver Disease = $3.78 \times$ ln[serum bilirubin (mg/dL)] + $11.2 \times$ ln[INR] + $9.57 \times$ ln[serum creatinine (mg/dL)] + 6.43.

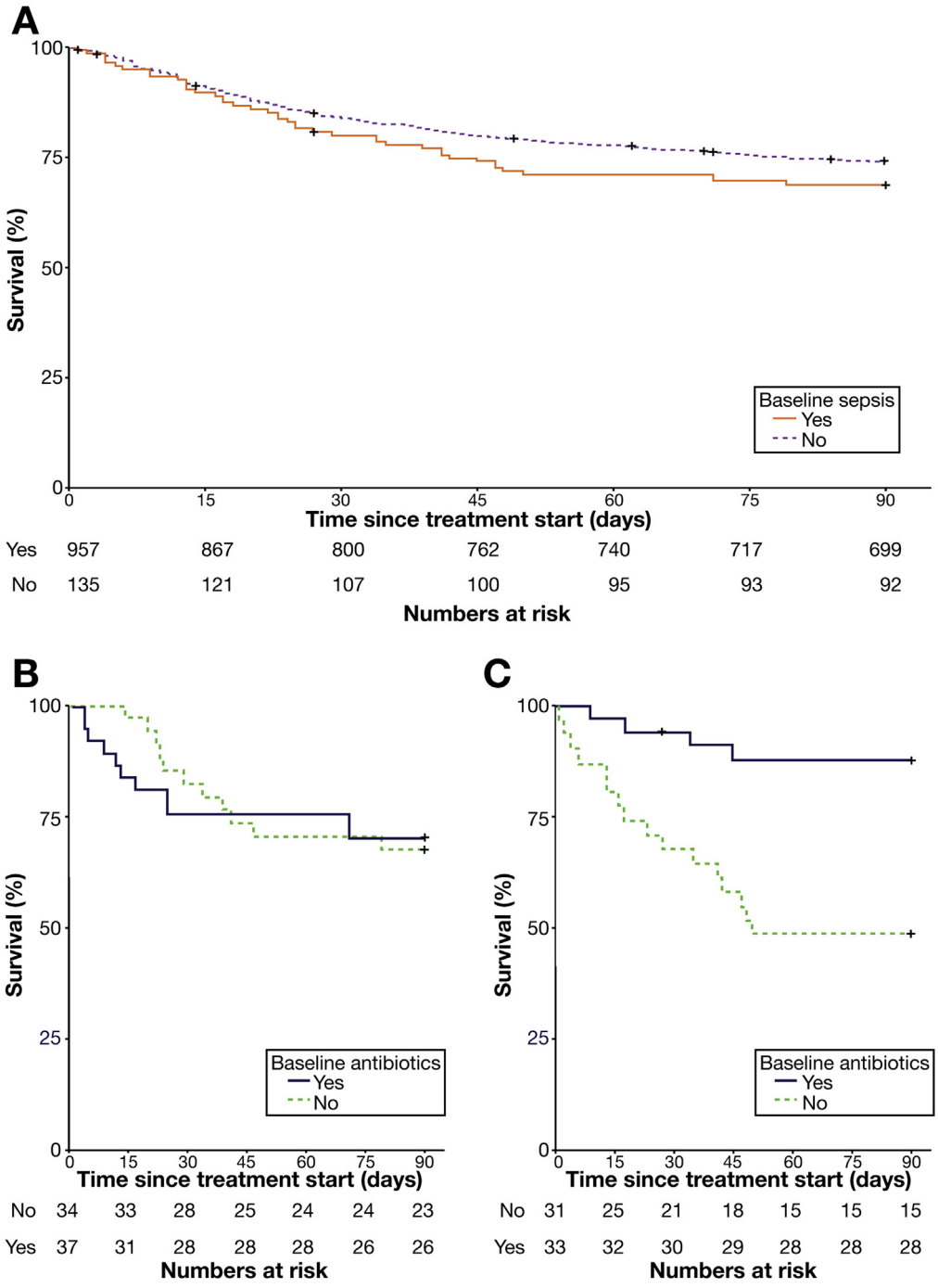

Figure 1. Prescription of antibiotics significantly modulates the impact of baseline infection on 90-day mortality in prednisolone-treated patients. In all patients, no statistically significant impact of baseline sepsis on mortality is seen (*A*). In patients who present with infection and do not receive prednisolone, continuation of antibiotics alongside treatment for AH does not impact upon mortality (*B*), however, in patients who receive prednisolone concurrent antibiotic therapy significantly reduces mortality (*C*).

testing for associations with treatment, in light of published findings that prednisolone increases the risk of serious and late infections in particular.[3,14]

Pentoxyfilline

There was no association between pentoxyfilline therapy and the risk of serious (SAE), on-treatment, or post-treatment infections (OR, 0.70; 95% CI, 0.46−1.05; $P = .084$; OR, 0.83; 95% CI, 0.63−1.10; $P = .20$; and OR, 0.78; 95% CI, 0.50−1.21; $P = .27$, respectively).

Prednisolone

Infections classified as serious (SAEs) were more frequent in patients treated with prednisolone (OR, 1.27; 95% CI, 1.27−2.92; $P = .002$).[3] There was no association between prednisolone therapy and on-treatment infection (OR, 1.04; 95% CI, 0.78−1.37; $P = .80$). However, prednisolone was associated with an increased risk of developing post-treatment infection (56 of 547 [10%] vs 33 of 545 [6%]; OR, 1.70; 95% CI, 1.07−2.69; $P = .024$).

In addition, there were significant interactions between prednisolone and Lille response in relation to both 90-day

Table 2. Associations Between Baseline Characteristics and the Development of Incident Infection

Variable	Univariable		Multivariable	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Demographics				
Age, *y*	1.01 (1.00−1.03)	.055	1.02 (1.00−1.03)	.013
Sex, *male*	1.18 (0.90−1.55)	.220	—	—
Alcohol consumption, *U/wk*	1.00 (0.99−1.00)	.522	—	—
Observations				
Systolic blood pressure, *mm Hg*	1.00 (0.99−1.01)	.909	—	—
Diastolic blood pressure, *mm Hg*	0.99 (0.98−1.00)	.098	0.99 (0.98−1.00)	.177
Pulse, *beats/min*	1.01 (0.99−1.02)	.077	1.01 (1.00−1.02)	.056
Temperature, *°C*	1.06 (0.82−1.39)	.656	—	—
Hematology and biochemistry				
Hemoglobin, *g/L*	0.99 (0.99−1.00)	.221	—	—
Total WBC, $\times 10^3$ *per* mm^3	1.05 (1.03−1.08)	<.001	1.04 (1.02−1.07)	.002
Neutrophils, $\times 10^3$ *per* mm^3	1.06 (1.03−1.08)	<.001	—	—
INR	1.45 (1.12−1.89)	.005	1.31 (0.99−1.73)	.058
Albumin, *g/L*	0.98 (0.95−0.99)	.031	0.98 (0.96−1.00)	.092
Bilirubin, *mg/dL*	1.01 (0.99−1.03)	.065	1.00 (0.99−1.02)	.648
Alanine transaminase, *IU/L*	1.00 (0.99−1.00)	.841	—	—
Aspartate transaminase, *IU/L*	0.99 (0.99−1.00)	.485	—	—
Sodium, *mmol/L*	0.98 (0.95−1.00)	.062	0.99 (0.97−1.02)	.695
Urea, *mmol/L*	1.03 (0.99−1.06)	.102	—	—
Creatinine, *mg/dL*	1.38 (1.09−1.75)	.009	1.20 (0.91−1.58)	.203
Clinical scores				
Discriminant function[a]	1.01 (1.00−1.01)	.002	—	—
MELD[b]	1.06 (1.03−1.09)	<.001	—	—

NOTE. Variables showing a trend to significance on univariable analysis ($P < .10$) were entered into multivariable analysis.
INR, international normalized ratio; MELD, Model for End-Stage Liver Disease; WBC, white blood cell count.
[a]Discriminant function = 4.6 × ($PT_{Patient}$-$PT_{Control}$ [seconds]) + bilirubin [mg/dL].
[b]MELD = 3.78 × ln[serum bilirubin (mg/dL)] + 11.2 × ln[INR] + 9.57 × ln[serum creatinine (mg/dL)] + 6.43

mortality ($P = .00017$) and infection ($P = .045$). Consequently, prednisolone and no-prednisolone groups were considered separately for statistical analyses other than comparisons between treatment arms.

Development of an incident infection was significantly associated with mortality in prednisolone-treated patients (prednisolone: 39% vs 22%; OR, 2.27; 95% CI, 1.52−3.38; $P < .0001$), but was not in the patients who did not receive prednisolone (31% vs 24%; OR, 1.36; 95% CI, 0.89−2.08; $P = .15$).

Multivariable analysis incorporating terms reflecting development of infection, baseline severity of liver disease (MELD), presence of encephalopathy, and response to treatment (Lille score <0.45) was performed. In prednisolone-treated patients an independent effect of infection on 90-day mortality was seen (OR, 2.46; 95% CI, 1.41−4.30; $P = .002$) (Table 3).

Alcohol and Infection Risk

Recidivism after the episode of SAH was recorded at 90 days. Importantly, there was no association between prednisolone treatment and a return to alcohol drinking ($P = .95$). Further detail is provided in Supplementary Results.

Table 3. Multivariable Analysis Examining the Effect of Incident Infection on Mortality by Logistic Regression, After Adjusting Liver Function (Model for End-Stage Liver Disease), Encephalopathy, and Treatment Response (Lille Response)

Variable	Prednisolone		No prednisolone	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Infection	2.46 (1.41−4.30)	.002	.94 (.54−1.62)	.82
MELD	1.08 (1.02−1.15)	.012	1.12 (1.06−1.20)	<.001
Encephalopathy	1.83 (1.02−3.28)	.042	2.19 (1.24−3.84)	.007
Lille response	.36 (.21−.64)	<.001	.29 (.16−.50)	<.001

NOTE. Results are given for both prednisolone-treated and no-prednisolone groups.
MELD, Model For End-Stage Liver Disease.

Infection and Early Improvement in Liver Function

Failure to demonstrate an early improvement in liver function (Lille score >.45) was associated with an increased risk of infection in prednisolone-treated patients (52% vs 29%; OR, 2.70; 95% CI, 1.69−4.32; $P = .00003$), but not in patients treated without prednisolone (34% vs 29%; OR, 1.28; 95% CI, 0.82−1.98; $P = .28$).

Day 7 infections, developing before calculation of the Lille score at day 7, were associated with a significantly increased risk of Lille nonresponse in prednisolone-treated patients (OR, 2.82; 95% CI 1.48−5.26; $P = .002$), but not in patients treated without prednisolone (OR, 1.28; 95% CI, 0.70−2.34; $P = .43$). Accordingly, prednisolone treatment was associated with a significant increase in 90-day mortality in patients who developed infection within 7 days (59% vs 38%; OR, 2.34; 95% CI, 1.12−4.88; $P = .023$) (Figure 2).

Utility of Bacterial DNA Level to Predict Infection and Mortality

Whole blood samples were available for bDNA analysis in 68% (740 of 1092) of patients included in the clinical data analysis. Further detail regarding characteristics of patients from whom bDNA results were not available is provided in Supplementary Results.

Ninety percent of SAH patients (661 of 731) had detectable bDNA from whole blood samples. However, there was no correlation between age or alcohol consumption and bDNA ($r_s < -.01$, $P = .97$ and $r_s = -.05$, $P = .21$). There was also no correlation between baseline bDNA and baseline liver function as described by MELD, DF, or Glasgow Alcoholic Hepatitis Score ($r_s = .04$, $P = .25$; $r_s = .04$, $P = .25$; and $r_s = .04$, $P = .32$, respectively). Clinical characteristics of patients are presented in Supplementary Table 4 by day 7 infection status.

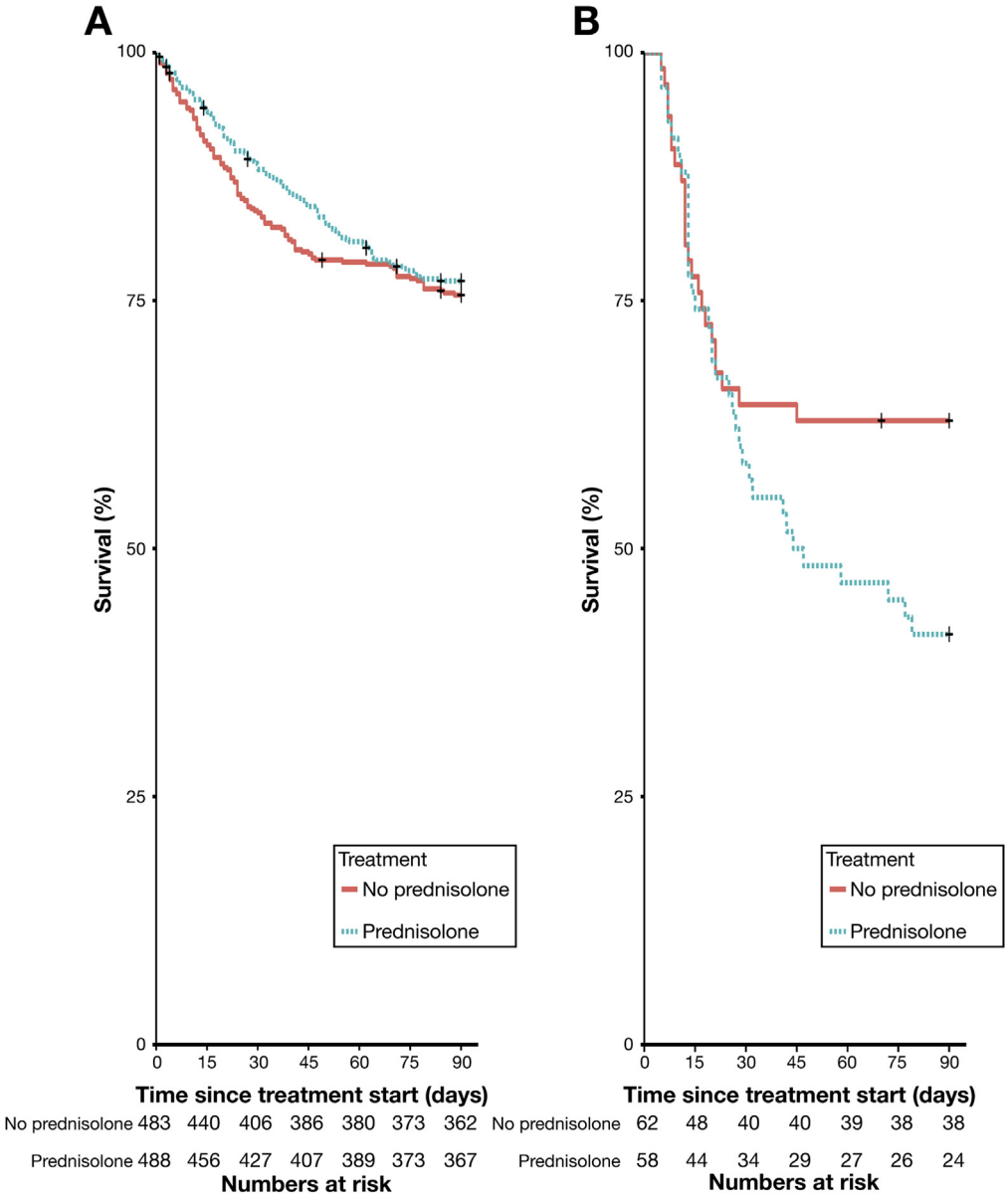

Figure 2. Early-onset infection leads to excess mortality in patients treated with prednisolone. In patients who do not develop infection within the first 7 days, there is a nonsustained improvement in mortality at 28 days (*A*). However, in patients who have early onset of infection, treatment with prednisolone is associated with a dramatic increase in mortality (*B*).

Because antibiotic therapy before sampling is likely to reduce bDNA levels, we sought and found an interaction between bDNA and intravenous antibiotic therapy in the prediction of day 7 infection ($P = .02$). Patients who had been treated with intravenous antibiotics within 5 days before sampling were therefore excluded (195 patients, leaving 536 patients available for further analysis). Patients were further divided by treatment with prednisolone (prednisolone, n = 265; no prednisolone n = 271) in line with previous analyses.

There was a striking association between bDNA and development of infection within 7 days in patients treated with prednisolone (developed infection vs did not develop infection: 20.9 vs 8.3 pg/mL [median values]; $P = .004$). Area under receiver operating characteristic curve for prednisolone-treated patients was .704 (95% CI .58−.83; $P = .0032$). By way of comparison, the area under the receiver operating characteristic for white blood cell count to predict infection within 7 days was .577, but this was not statistically significant ($P = .265$; Supplementary Table 5). bDNA level was not associated with day 7 infection in patients treated without prednisolone (developed infection vs did not develop infection: 12.7 vs 12.3 pg/mL; $P = .95$).

A cut-off of 18.5 pg/mL bDNA was 80% specific for prediction of infection within 7 days. This cut-off was used to define a high level of bDNA (^{hi}bDNA). ^{hi}bDNA was associated with increased risk of infection by day 7 in prednisolone-treated patients (OR, 4.48; 95% CI, 1.70−11.81; $P = .002$). This association remained significant after multivariable analysis that controlled for confounding factors of MELD and white blood cell count (Table 4). In contrast, ^{hi}bDNA was not associated with the development of day 7 infection in either univariable or multivariable analysis for patients treated without prednisolone (Table 4).

All patients were considered for survival analyses (n = 731). bDNA level before treatment correlated with Lille score ($r_s = .16$; $P = .0006$), irrespective of antibiotic treatment ($r_s = .27$, $P = .003$ for antibiotic treated patients and $r_s = .12$, $P = .02$ for patients not treated with antibiotics within 5 days before sampling). In addition, bDNA level was higher for patients who died by 90 days compared with those who survived to 90 days (11.2 vs 9.3 pg/mL; $P = .04$). ^{hi}bDNA was associated with 90-day mortality (OR, 1.39; 95% CI, .98−2.0; 29% vs 23% 90-day mortality in patients with ^{hi}bDNA vs patients without ^{hi}bDNA; $P = .03$).

Finally, the strategy of using ^{hi}bDNA to exclude use of prednisolone was modeled by matching ^{hi}bDNA patients in the prednisolone-treated group with patients in the no-prednisolone group. This would estimate the likely mortality at 90 days if these patients had not received prednisolone. In patients with ^{hi}bDNA, avoidance of prednisolone treatment was associated with a reduction in 90-day mortality (17% vs 29%; OR, 1.96; 95% CI, .84−4.3; $P = .05$) (Figure 3).

Table 4. Multivariable Logistic Regression Analysis Incorporating Bacterial DNA, Model for End-Stage Liver Disease, and White Blood Cell Count for Prediction of Day 7 Infection in Patients Treated With and Without Prednisolone

	Prednisolone		No prednisolone	
Variable	OR (95% CI)	*P* value	OR (95% CI)	*P* value
^{hi}bDNA	4.68 (1.80−12.17)	.001	0.83 (0.39−1.75)	.62
MELD	1.08 (0.99−1.17)	.097	1.07 (0.99−1.15)	.08
WBC	1.06 (0.97−1.16)	.187	1.07 (0.99−1.15)	.07

MELD, Model for End-Stage Liver Disease; WBC, white blood cell count.

Discussion

Our analysis of 1092 patients with SAH confirms that infection is highly prevalent, with 12% having infection at baseline and 23% of SAH patients developing infection on treatment. Prednisolone is associated with a significant increase in the risk of serious infections.[3] Furthermore, these data indicate that prednisolone therapy appears to confer an excess risk of post-treatment infections, irrespective of severity. Cabre et al[14] also described an increased rate of late infections in patients treated with prednisolone compared to those treated with enteral nutrition. This phenomenon might partly explain why early improvements in liver function attributable to prednisolone did not translate into a sustained survival benefit.

Although there was no overall association between presentation with infection and mortality, these data suggest that baseline infection might not be entirely benign. Decisions regarding continuation of antibiotic therapy are important when patients are to receive prednisolone. The current study suggests that continued antibiotic therapy in patients with baseline infection confers a survival advantage.

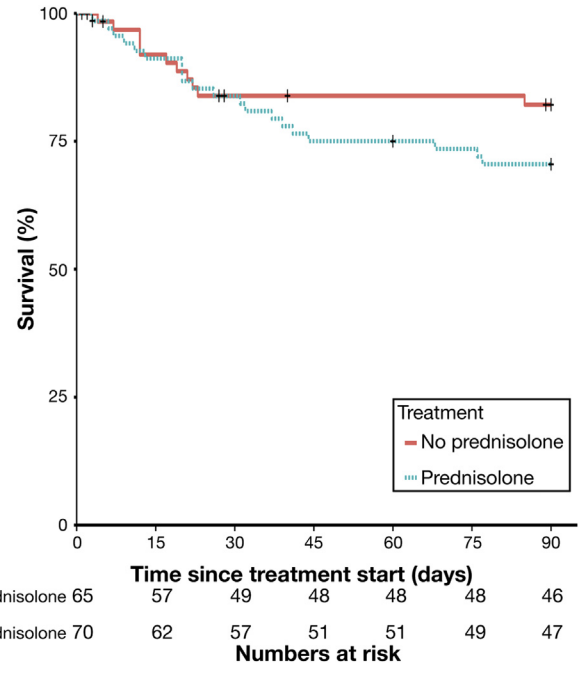

Figure 3. Comparison of survival curves to 90 days in patients with matched and high bDNA levels who were treated with prednisolone vs no prednisolone.

The impact of infection on 90-day mortality is critically modulated by prednisolone. In patients treated with prednisolone, infection exerts an independent effect on mortality by 90 days. When prednisolone is not used, the effect of infection on 90-day mortality is secondary to baseline liver impairment and early improvement in liver function. In other words, patients who are not treated with prednisolone but who have poor liver function are more likely to develop infection and die within 90 days. Further, we show that development of infection before calculation of the Lille score at day 7 is associated with classification as a Lille nonresponder; this timing raises the possibility that early infection might modulate Lille score. In patients who developed infection within the first 7 days, prednisolone dramatically increased the risk of mortality at 90 days.

Concerns about infectious complications have restricted use of prednisolone. As a result, strategies that aim to first test for benefit from prednisolone before continued use have gained support. One approach is to use the Lille model after 7 days of prednisolone therapy to determine whether corticosteroids should be continued or not. However, in a trial of patients who were Lille nonresponders after 7 days of corticosteroid therapy, there was no survival benefit associated with withdrawal of prednisolone and replacement with pentoxyfilline compared with patients who were treated for the full 28 days with prednisolone.[15] We speculate that 7 days of prednisolone therapy may be enough to impair host immunity to allow development of serious infection, and that discontinuation of steroids after 7 days may be unable to reverse the damage.

Consequently, the ability of pretreatment bDNA levels to predict the development of infection in patients who were uninfected at the time of sampling and who subsequently receive prednisolone is of interest. This strategy differs from previous studies in which investigators sought to differentiate SAH patients with infection *at the time of presentation* from those without,[16] and is the first attempt to evaluate bDNA in the context of corticosteroid immunosuppression.[17] While the area under the receiver operating characteristic for bDNA to predict the subsequent development of infection was modest in the current study, bDNA was nonetheless superior to white blood cell count in this regard. Also of interest is the observation that bDNA was not predictive of infection when patients were not subsequently treated with prednisolone: only when the immune system had been modulated by prednisolone *and* when the circulating bacterial load was high was there a heightened risk of developing infection. The ability of bDNA to predict infection before alternative immunosuppressive agents are used is an enticing prospect that warrants dedicated testing.

bDNA level may also be regarded as a target for therapy before initiation of immunosuppression. Where culture results are unavailable but bDNA levels are high, a possible paradigm could be to repeat microbiological screening and treat with broad-spectrum antibiotics until bDNA has returned to normal levels. Randomly allocated empirical broad-spectrum antibiotic therapy in SAH is the subject of ongoing clinical trials.[18,19]

The translocation of bacterial products from gut to portal vein in heavy alcohol drinkers has been proposed as a mechanism of hepatic injury and cause of hepatic inflammation in SAH.[20] Indeed, >90% of SAH patients had detectable bDNA levels in the current study, which is substantially higher than rates seen in healthy controls, patients with suspected bloodstream infections, and patients with other forms of decompensated liver disease.[17,21,22] The higher rate of bacteremia seen in these SAH patients might represent extensive bacterial translocation[23] or defective leukocyte clearance,[24–26] or both. Bacterial translocation has been implicated in the pathogenesis of SAH.[20,27] However, in the current study, while circulating bDNA predicted the development of infection, it did not correlate with markers of baseline liver function such as MELD, DF, or Glasgow Alcoholic Hepatitis Score.

In common with other studies in the field, the central limitation of this study is the lack of a gold standard to diagnose infection. In our data, only a minority of infections (40% of incident infections) yielded an organism on microbiological culture; most were diagnosed clinically. Clinical diagnosis of infection will be sensitive but may lack specificity, with physicians unable to differentiate inflammatory responses driven by underlying alcoholic hepatitis, from infection. However, in this regard, we highlight the contrasting outcomes of patients diagnosed with infection in this study in relation to the double-blind allocation of prednisolone. The association between randomly allocated prednisolone therapy and poor outcomes for this subset of patients suggests that they had a condition exacerbated by immunosuppression, which is very likely to have been infection.

No treatment was shown to reduce 90-day mortality for SAH in the STOPAH study.[3] In the current retrospective analysis, a reduction in 90-day mortality was estimated by using pretreatment bDNA level to guide prescription of prednisolone and was of borderline statistical significance. Larger prospective randomized studies are needed to definitely report whether bDNA-guided therapy can impact on mortality, in SAH, and perhaps in other acute inflammatory conditions where immunosuppression is required.

In summary, these data show that infections are frequent in SAH, but are only independently associated with mortality when patients receive prednisolone. These infections may be predicted by measuring levels of circulating bacterial DNA, raising the possibility that such infections, and consequent mortality, could be avoided by bDNA-stratified prednisolone prescribing for SAH patients.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dxdoi.org/10.1053/j.gastro.2016.12.019.

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Received August 23, 2016. Accepted December 3, 2016.

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Acknowledgments
The authors thank the STOPAH trial management group, the National Institute for Health Research Clinical Research Network, the Imperial College Biomedical Research Centre and Southampton Clinical Trials Unit.

Conflicts of interest
These authors disclose the following: Mark R. Thursz receives lecture fees and consulting fees from Gilead, Bristol-Myers Squibb, AbbVie, and Abbott. Michael Allison receives consulting fees from Norgine. The remaining authors disclose no conflicts.

Funding
This study was supported by National Institute for Health Research (NIHR) Health Technology Assessment, Wellcome Trust (WT100566MA), Medical Research Council (MR/M003132/1) and the NIHR Imperial Biomedical Research Centre (BRC).

Supplementary Results

Alcohol and Infection Risk

Drinking data was collected at the day-90 visit time point. Data regarding drinking behavior are incomplete and available for 478 patients surviving to complete visit at 90 days. For the purposes of subsequent analysis, drinking behavior is considered dichotomously as abstinent or drinking.

There is no association between prednisolone treatment and a return to drinking ($P = .95$) in the cohort of patients with available data. No association is revealed when analysis is restricted to those completing treatment (survival to day 28) or surviving a minimum of 90 days after the start of treatment.

No association is revealed between the reported maximum level of alcohol consumption at baseline and the development of a serious infection (SAE) ($P = .89$), on treatment ($P = .11$), or post-treatment ($P = .20$) infection. There was a trend toward significance for an increased risk of post-treatment infection associated with a return to drinking (OR, 1.68; 95% CI, 0.97−2.91; $P = .07$). This result should, however, be interpreted with caution. It is highly probable that patients who had returned to drinking and developed an infection were more likely to attend follow-up than those who had returned to drinking but experienced no infection. Follow-up visits may, for example, have been completed opportunistically when patients who had returned to drinking had attended hospital for assessment and treatment of their infective complication. Consequently, the sample of patients who returned to drinking might well be biased in favor of those who had developed an infection.

Smoking and Infection Risk

Smoking data were available on 986 subjects. A minority of patients reported never smoking (n = 306 [31%]), while the remainder were split between active (n = 447 [41%]) and former smokers (n = 233 [21%]).

No statistically significant effect of smoking status (never, current, former) was found on the occurrence of chest infections, including when adjusted for prednisolone treatment (Supplementary Table 6).

Bacterial DNA Analyses

Whole blood samples were available for bDNA analysis in 68% (740 of 1092) of patients included in the clinical data analysis. There was insufficient sample available from the remaining patients (352 of 1092 [32%]) who participated in the STOPAH study. In an additional 9 patients, the discrepancy between PCR replicates was >1 copy cycle and the result deemed invalid. A bDNA result was therefore not available in 361 of 1092 patients (33%). Incident infection in patients for whom bDNA was measured was higher than in patients for whom bDNA was not measured (228 of 731 [31%] vs 81 of 362 [22%]), although the mortality rate within the population in whom bDNA was measured was similar to the mortality rate for those in whom bDNA was not measured (29% [106 of 361]) vs 25% [180 of 731]).

Supplementary Table 1. Development of Infection and Death in Each of the Study Periods: at Baseline, on Treatment and Post-Treatment

			Incident infection			
	Baseline infection		On treatment		Post-treatment	
Variable treatment arm	Prednisolone (n = 547)	No prednisolone (n = 545)	Prednisolone (n = 547)	No prednisolone (n = 545)	Prednisolone (n = 463)	No prednisolone (n = 447)
Deaths	NA	NA	76 (14)	98 (18)	145 (31)	141 (32)
Patients developing an infection	64 (12)	71 (13)	127 (23)	124 (23)	56 (12)	33 (7)
Patients developing infection at >1 site	2 (0.4)	4 (0.7)	8 (2)	10 (2)	6 (1)	4 (0.9)
Patients developing >1 infection	0	0	16 (3)	12 (2)	0	0

NOTE. Values are n (%). Subgroups of patients developing more than 1 infection or at more than 1 site are shown.
NA, not applicable.

Supplementary Table 2. Full Breakdown of Infections by Site and Time of Occurrence in Relation to Presentation and Treatment

		Incident infection	
Site of infection	Baseline infection (n = 141)	On treatment (n = 301)	Post-treatment (n = 102)
Respiratory, n (%)	42 (33.6)	110 (36.5)	40 (39.2)
Lower respiratory tract, n	41	108	39
Upper respiratory tract, n	1	2	1
SBP and bacteremia, n (%)	28 (22.4)	75 (24.9)	23 (22.5)
SBP, n	16	47	13
Bacteremia, n	8	28	10
Urinary, n (%)	24 (19.2)	31 (10.3)	10 (9.8)
Other, n (%)	12 (9.6)	46 (15.3)	10 (9.8)
Biliary, n	1	0	0
Intra-abdominal, n	1	6	2
Deep tissue, n	1	4	1
Gastrointestinal, n	3	9	3
Skin and soft tissue, n	6	25	2
Orodontal, n	0	2	0
ENT, n	0	0	2
Unknown, n (%)	19 (15.2)	36 (12.0)	18 (17.6)
Missing data	14	3	1

ENT, ear, nose, and throat; SBP, spontaneous bacterial peritonitis.

Supplementary Table 3. Full Breakdown of Organisms Isolated From Patients Presenting With or Developing Infection, by Relation in Terms of Timing to the Treatment Period

Organism	Baseline	Incident infection	
		On treatment	Post-treatment
Gram-negative bacilli, n (%)	23 (57.5)	45 (42.9)	19 (67.9)
Campylobacter spp, n	0	1	
Coliforms (NOS), n	5	4	1
Enterobacter spp, n	0	1	
Enterobacter cloacae, n	1	1	
Escherichia coli, n	12	25	8
Fusobacterium nucleatum, n	0	0	1
Klebsiella spp, n	1	0	1
Klebsiella pneumoniae, n	2	7	4
Pseudomonas spp, n	0	2	
Serratia marcescens, n	0	1	1
Unknown, n	2	3	3
Gram negative coccus, n (%)	0	2 (1.9)	0
Moraxella catarrhalis, n	0	1	
Unknown, n	0	1	
Gram positive bacilli, n (%)	2 (5)	7 (6.7)	3 (10.7)
Clostridium difficile, n	2	6	3
Unknown, n	0	1	
Gram positive coccus, n (%)	11 (27.5)	44 (41.9)	5 (17.9)
Enterococcus spp, n	2	9	1
Enterococcus faecalis, n	1	1	
Enterococcus faecium, n	0	2	
Gemella spp, n	0	1	
Micrococcus spp, n	0	1	
Staphylococcus spp, n	2	6	1
Staphylococcus aureus, n	1	7	3
Staphylococcus epidermidis, n	0	1	
Staphylococcus warneri, n	0	1	
Streptococcus spp, n	2	9	
Streptococcus anginosus, n	1	0	
Streptococcus gordonii, n	1	0	
Streptococcus mitis, n	0	1	
Streptococcus pneumoniae, n	1	1	
Streptococcus viridans, n	0	1	
Unknown, n	0	3	
Mixed NOS, n (%)	2 (5)	2 (1.9)	0
Fungus, n (%)	2 (5)	4 (3.8)	1 (3.6)
Candida spp, n	1	0	
Candida albicans, n	0	4	1
Unknown, n	1	0	
Viral, n (%)	0	1 (1.0)	0
Norovirus, n	0	1	

NOS, not otherwise specified.

Supplementary Table 4. Baseline Characteristics of Bacterial DNA Cohort, by Day 7 Infection Status

Variable	No early-onset infection (n = 638)	Early-onset infection (n = 93)
Age, *y*	49 (42–57)	50 (41–58)
Sex, *male*, n (%)	415 (57)	54 (58)
Alcohol consumption, *U/wk*	126 (81–206)	140 (98–213)
Prednisolone, n (%)	326 (51)	40 (43)
Systolic blood pressure, *mm Hg*	111 (103–121)	110 (100–123)
Diastolic blood pressure, *mm Hg*	68 (60–75)	65 (60–72)
Pulse, *beats/min*	90 (80–98)	92 (80–101)
Temperature, *°C*	36.8 (36.5–37.1)	36.8 (36.5–37.0)
Haemoglobin, *g/L*	108 (95–121)	100 (90–114)
White blood cell count, $\times 10^3$ *per* mm^3	8.7 (6.0–12.3)	10.3 (7.3–14.7)
Neutrophils, $\times 10^3$ *per* mm^3	5.9 (4.0–9.3)	7.9 (5.4–12.0)
International normalised ratio	1.7 (1.5–2.0)	1.9 (1.6–2.2)
Albumin, *g/dL*	24 (21–29)	25 (21–29)
Bilirubin, *mg/dL*	16.5 (10.0–24.3)	17.3 (11.7–24.6)
Alanine transaminase, *IU/L*	44 (31–64)	41 (26–66)
Aspartate transaminase, *IU/L*	128 (90–176)	120 (86–155)
Sodium, *mmol/L*	134 (130–137)	133 (129–136)
Urea, *mmol/L*	3.2 (2.2–5.1)	3.7 (2.3–5.6)
Creatinine, mg/dL	0.72 (0.60–0.95)	0.74 (0.58–1.09)
Discriminant function	54 (42–71)	61 (46–81)
Model for End-Stage Liver Disease	23 (21–26)	24 (22–29)
Pretreatment antibiotics,[a] n (%)	156/638 (24)	39/93 (42)
bDNA, *pg/mL*	9.2 (3.2–23.8)	12.3 (5.6–39.4)

NOTE. Data are median (interquartile range) unless other indicated.
MELD, Model for End-Stage Liver Disease.
[a]Pretreatment antibiotics defined as intravenous antibiotics commenced within 5 days prior to starting trial therapy.

Supplementary Table 5. Area Under Receiver Operating Curve Comparison Between Bacterial DNA and White Blood Cell Count

Parameter	AUROC	*P* value	95% CI
bDNA	0.704	.003	.57–.83
White blood cell count	0.577	.265	.44–.72

AUROC, area under receiver operating curve.

Supplementary Table 6. Effect of Smoking on Risk of Developing Incident Infection

Term	OR (95% CI)	*P* value
Smoking (never)	Reference	.14
Smoking (current)	.68 (.46–1.01)	.06
Smoking (prior)	.91 (.58–1.41)	.67
Prednisolone	1.23 (.88–1.73)	.23