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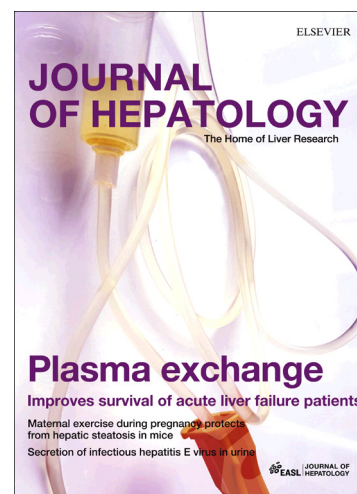
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Homozygosity for rs738409:G in *PNPLA3* is associated with increased

mortality following an episode of severe alcoholic hepatitis

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List of abbreviations: PNPLA3: patatin-like phospholipase domain containing protein 3; STOPAH: Steroids or Pentoxifylline for Alcoholic Hepatitis; DF: Discriminant Function MELD: Model for End-stage Liver Disease; INR: International Normalised Ratio; GAHS: Glasgow Alcoholic Hepatitis Score; HR: Hazard ratio; CI: Confidence Intervals; SNP: single nucleotide polymorphism

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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.

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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.

Abstract

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 1,188 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=2.15×10^{-15}; OR 1.80 [95%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 period day 90-450 was higher in survivors who subsequently resumed drinking (Hazard Ratio [HR] 2.77, 95% Confidence Interval [CI] 1.79–4.29; p<0.0001) and 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 in these patients.

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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 responsible for nearly 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, significant prolongation of the prothrombin time, hepatic encephalopathy, hypoalbuminaemia and ascites [7,11,14]. The long-term prognosis is influenced by several factors including gender, 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

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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 controlled trials [18]. 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 [19]. 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 (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 risk for the development of alcohol-related cirrhosis from individual studies [20-23], a meta-analysis [24] and, most recently, a genome-wide association study [25]. In addition, rs738409:G has been shown to be a significant risk factor for the development of hepatocellular carcinoma in patients with established cirrhosis in individual studies [26-30], and in a meta-analysis based on individual patient data [31]. 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 [32]; more rapid progression towards decompensated disease [33]; a reduction in transplantation-free survival [33] and poorer outcomes following development of hepatocellular carcinoma [34].

Although the frequency of rs738409:G was reported to be significantly increased in patients with severe alcoholic hepatitis in one small study [35], 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:

(i) 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;

(ii) 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

Cases with severe alcoholic hepatitis were recruited as per the STOPAH trial protocol [36]. DNA samples and matched clinical data were available for 898 of 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 causes for their liver disease; and a DF $\geq$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.

Patients were randomized to treatment with prednisolone or pentoxifylline for 28 days using a double blind, double dummy design [36]. Randomization was block designed and stratified by geographical region and dichotomous risk status with the presence of sepsis, gastrointestinal bleeding or renal failure prior to randomization defining 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 ongoing follow-up and 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=1,188) 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. 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.

United Kingdom National Health Service Multicentre Research Ethics Committee approval was granted for this study (MREC/03/11/090). This was ratified by local ethics committees associated with 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 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), [37]; the Glasgow Alcoholic Hepatitis Score (GAHS), [38] and the Lille score [39].

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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. For 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 upon the following:

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

2. 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 [40, 41]. 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.* 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 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.

Cox proportional hazards models were used to test associations between explanatory variables and survival and interactions between explanatory variables in relation to 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 (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 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. 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.

Statistical analyses were performed using SPSS version 22 (IBM, Armonk, USA). Survival curves were plotted in R [42] using the packages ggplot2, survival, gridExtra, reshape and plyr.

Results

Genotyping accuracy

The overall genotyping rate was 98%. Genotypes were successfully called in 867 (97%) of 898 case samples and in 1,175 (99%) of the 1,188 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 association with baseline demography and assessment variables

There were no significant differences in age, gender distribution, alcohol consumption, or the majority of the clinical or laboratory variables at baseline in relation to rs738409 genotype (Supplementary Table 1).

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 days 215 and 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.

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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; Figure 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 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 as significantly associated with 28-day mortality (Table 3).

Multivariable Cox regression 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 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 (Supplementary Table 2). No statistically significant interactions were found between any of the scoring systems and rs738409 genotype in relation to 28-day mortality.

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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 time-point, 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) **(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 one 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 were 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 remained in additional sensitivity analysis where all cases with missing data at day 90 were assumed to have resumed drinking (Supplementary Table 3).

The association between rs738409 homozygosity and 450-day survival was independent of a return to drinking (Supplementary Table 4). 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=0.1) and the INR at day 90 (p=0.09) did not reach statistical significance. 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 statistically significant effect on survival; mortality rates were around 30% in all three genotypic groups

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over the 90 to 450 day period (**Fig 2C**). This lack of effect was confirmed on 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 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 (Supplementary Table 5).

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Discussion

The variant rs738409:G in *PNPLA3* has been consistently associated with the risk of developing alcohol-related cirrhosis, and has been implicated in more rapid disease progression and the risk of developing hepatocellular carcinoma [23,29, 30-32]. Severe alcoholic hepatitis has considerable associated mortality [8, 9, 11-13] but apart from one small series, published in abstract form [33], 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 a previous much smaller study.

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, gender distribution, the quantity of alcohol consumed, the duration of alcohol misuse 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 in relation to short-term mortality.

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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 significantly lower mortality 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.

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 with drinking behaviour, albeit only significant on sensitivity analysis in this population. 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 the effect of rs738409 genotype is subservient to drinking behaviour in those who continue 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 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 our findings are robust. (ii) A small

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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 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 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 as of critical importance.

Acknowledgements

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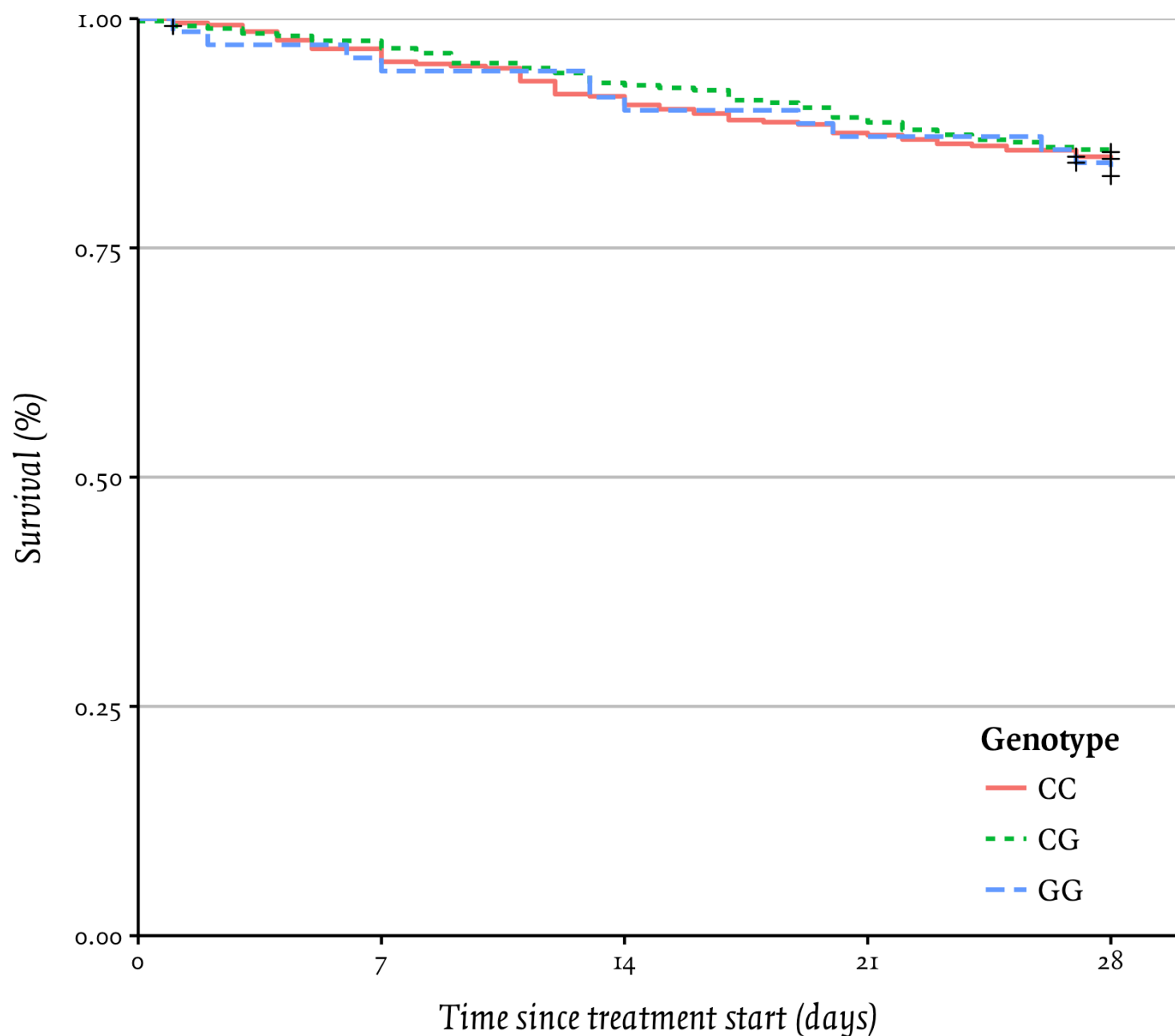
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Legends to figures

Fig. 1. Twenty-eight day survival in cases with severe alcoholic hepatitis stratified by rs738409 genotype. There is no impact of rs738409 genotype on short-term survival.

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 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).

Twenty-eight day survival, by rs738409 genotype

Survival (%)

1.00

0.75

0.50

0.25

0.00

Genotype
— CC
--- CG
-- GG

0   7   14   21   28

Time since treatment start (days)

CC	425	411	389	372	360
CG	372	362	345	331	318
GG	70	67	64	61	58

Numbers at risk
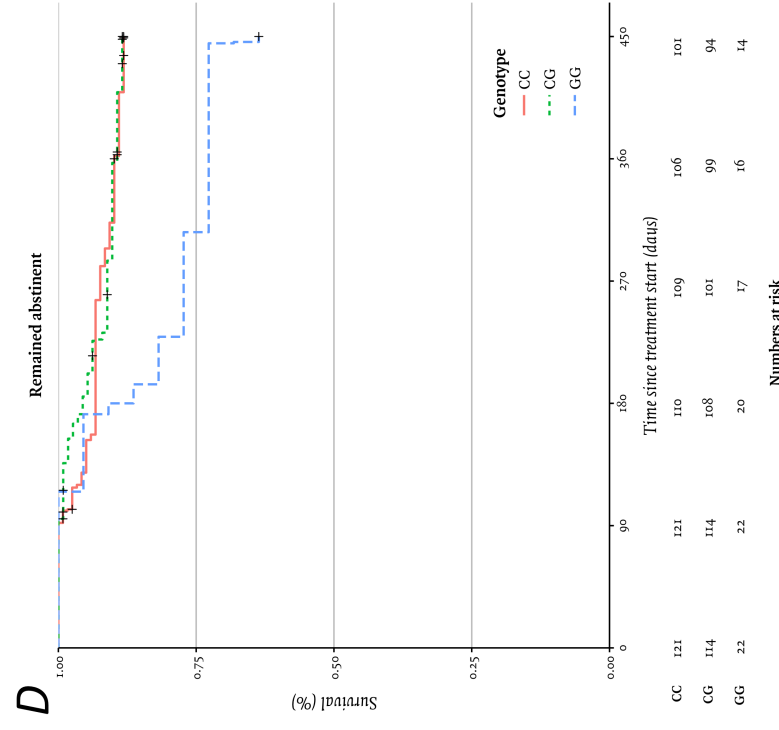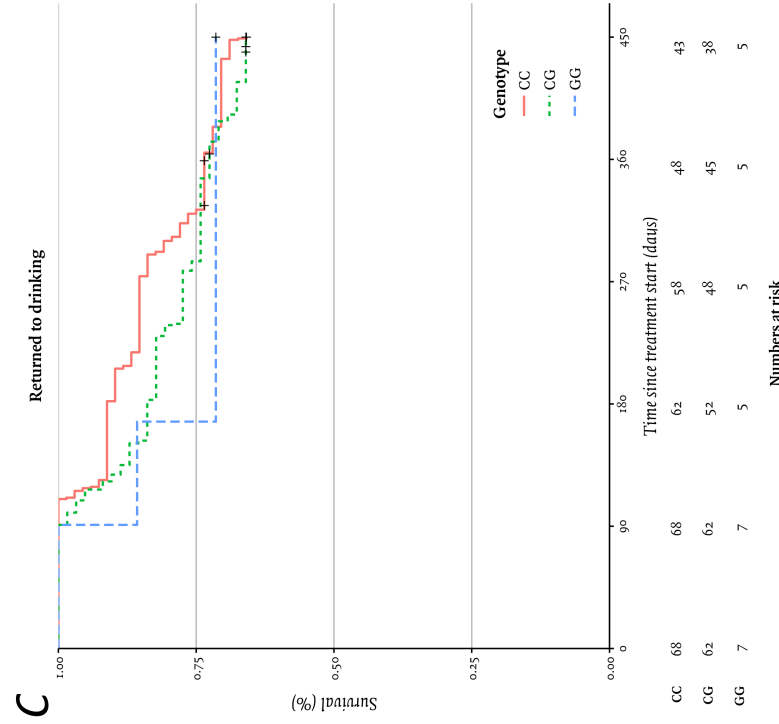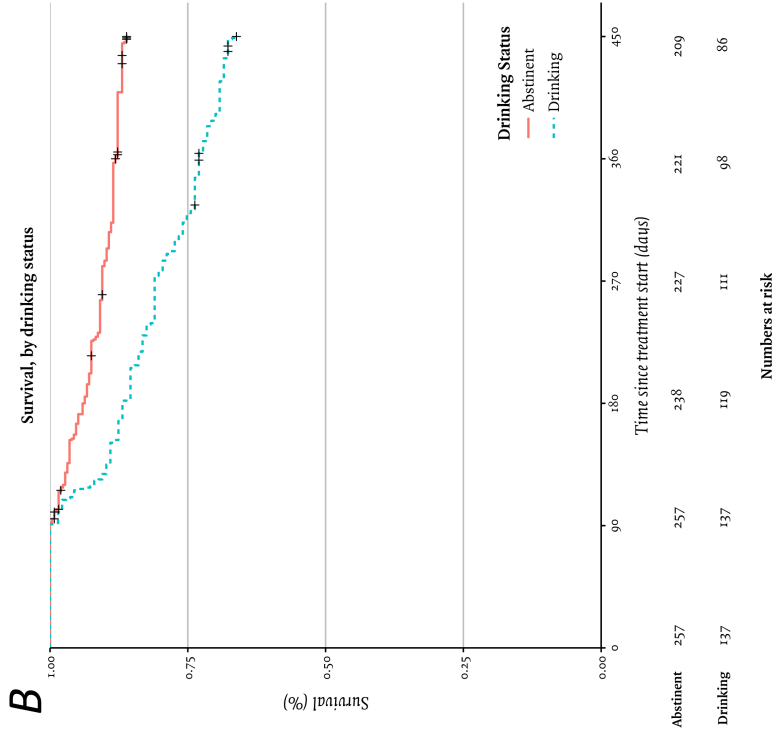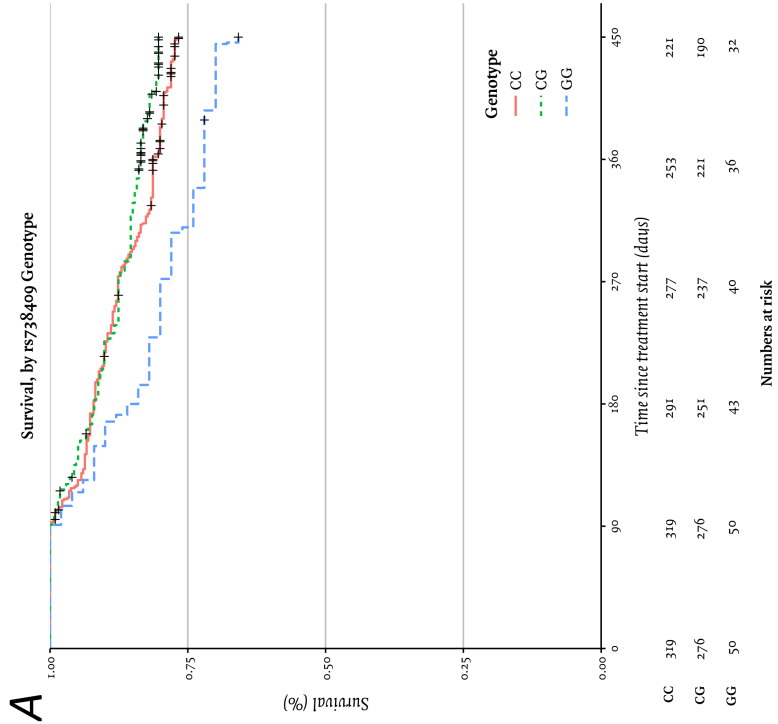
A Survival, by rs738409 Genotype

Survival (%)

Genotype
— CC
--- CG
--- GG

Time since treatment start (days)

Numbers at risk

CC	319	319	291	277	253	221
CG	276	276	251	237	221	190
GG	50	50	43	40	36	32

B Survival, by drinking status

Survival (%)

Drinking Status
— Abstinent
--- Drinking

Time since treatment start (days)

Numbers at risk

Abstinent	257	257	238	227	221	209
Drinking	137	137	119	111	98	86

C Returned to drinking

Survival (%)

Genotype
— CC
--- CG
--- GG

Time since treatment start (days)

Numbers at risk

CC	68	68	62	58	48	43
CG	62	62	52	48	45	38
GG	7	7	5	5	5	5

D Remained abstinent

Survival (%)

Genotype
— CC
--- CG
--- GG

Time since treatment start (days)

Numbers at risk

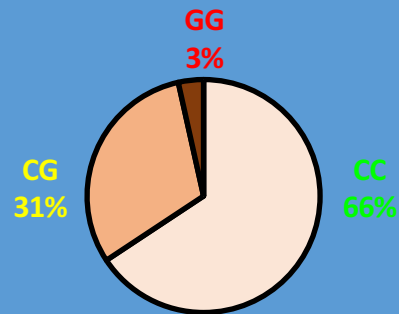|							
CC	121	121	110	109	106	101
CG	114	114	108	101	99	94
GG	22	22	20	17	16	14

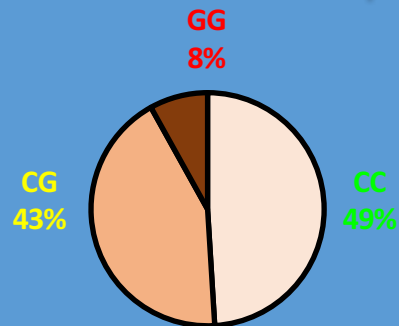**Homozygosity for rs738409:G in *PNPLA3* is associated with increased mortality in patients with severe alcoholic hepatitis**
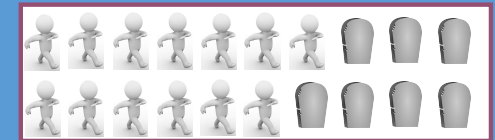
Table 1. Genotype frequencies and association analysis of rs738409 in cases with severe alcoholic hepatitis and controls with alcohol dependence but no liver injury

SNP	Cases (n=867)			Controls (n= 1175)			Cases *vs.* controls	
	Genotype count CC/CG/GG	MAF (%)		Genotype count CC/CG/GG	MAF (%)		Significance (p)	OR (95% CI)
rs738409	425/372/70	29.5		772/362/41	18.9		2.15×10^{-15}	1.80 (1.55-2.08)

Abbreviations: SNP: single nuclear polymorphism; MAF: Minor Allele Frequency; OR: Odds ratio; CI: Confidence Interval

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	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
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
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: HR: Hazard Ratio; CI: Confidence Intervals
* Variable not entered into the Cox multivariable analysis due to co-linearity (more significantly associated constituent part of variable exists)
§ Variable excluded from the Cox multivariable analysis by backward elimination due to lack of significant independent association

Table 4: Prognostic scores in cases with severe alcoholic hepatitis, by rs738409 genotype

Prognostic scoring system	*PNPLA3* rs738409 Genotype			Significance (p)
	CC (n = 425)	CG (n = 372)	GG (n = 70)	
Baseline DF[7]	62±29	62±25	64±27	0.50
Baseline MELD[37]	21±6	21±6	21±7	0.53
Baseline GAHS[38]	8±1	8±1	8±1	0.41
	CC (n = 292)	CG (n = 246)	GG (n = 37)	
Lille*[39]	0.46±0.3	0.49±0.3	0.43±0.3	0.55
Lille response (<0.45)	158 (54.1%)	119 (48.3%)	23 (62.2%)	0.19

Data expressed as mean ± 1SD or as number (%) *n=575

Abbreviations:
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.

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Table 5: Variables associated with 450-day mortality 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* ($\times 10^6$/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			
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 (more significantly associated constituent part of variable exists)
§ Variable excluded from the multivariable analysis by backward elimination due to lack of significant independent association
†Variable not entered into 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	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
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
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 multivariable analysis due to significant co-correlation with another variable
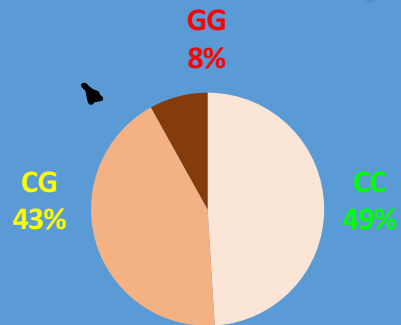
§Variable excluded from the multivariable analysis by backward elimination due to lack of significant association

Homozygosity for rs738409:G in *PNPLA3* is associated with increased mortality in patients with severe alcoholic hepatitis
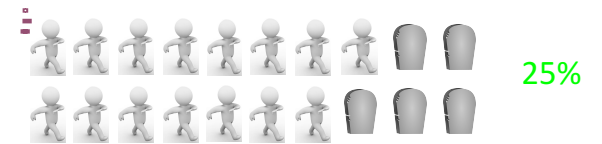
Heavy drinkers without liver disease

GG
3%

CG
31%

CC
66%

Patients with alcoholic hepatitis

GG
8%

CG
43%

CC
49%
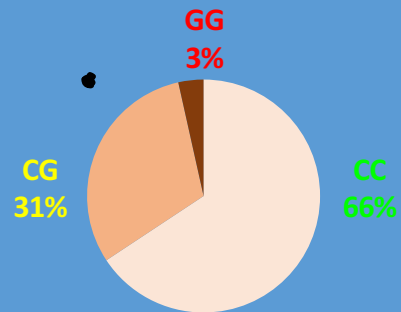
Abstinent

Mortality 14.3%

Returned to drinking

Mortality 35.3%

Mortality

25%

CG
22%

GG
35%