Research Article
Cirrhosis and Liver Failure

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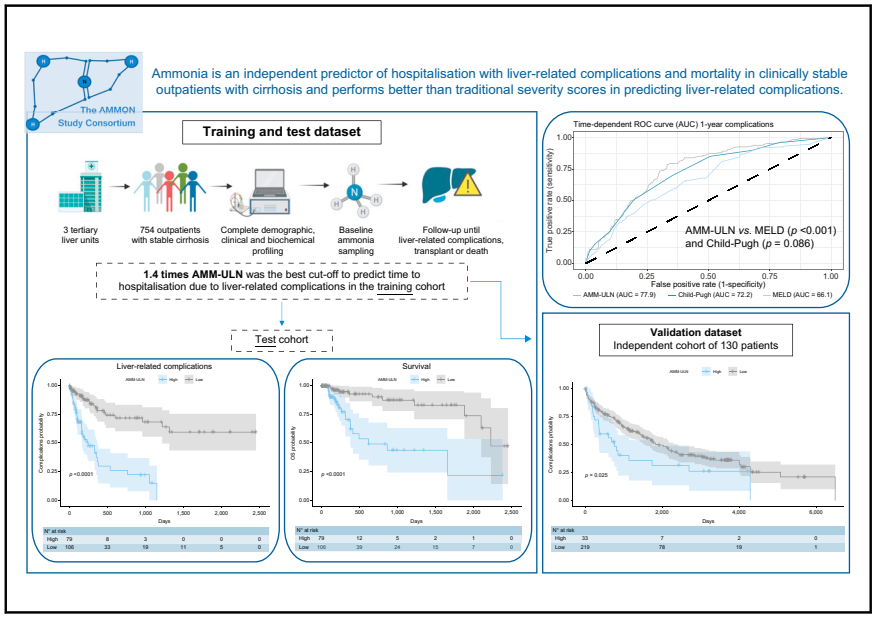
Graphical abstract



Highlights

- Ammonia is an independent predictor of both hospitalisation with liver-related complications and mortality in stable outpatients with cirrhosis.
- Ammonia performs better than traditional severity scores in predicting liver-related complications.
- A cut-off level of 1.4x the upper limit of normal ammonia defines the risk of both hospitalisation with liver-related complications and mortality.
- Ammonia is a key variable for the prediction of liver-related complications in a derivation cohort and upon external validation.

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Lay summary

We conducted a prospective cohort study evaluating the association of blood ammonia levels with the risk of adverse outcomes in 754 patients with stable cirrhosis across 3 independent liver units. We found that ammonia is a key determinant that helps to predict which patients will be hospitalised, develop liver-related complications and die; this was confirmed in an independent cohort of patients.

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Plasma ammonia levels predict hospitalisation with liver-related complications and mortality in clinically stable outpatients with cirrhosis

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Background & Aims: Hyperammonaemia is central in the pathogenesis of hepatic encephalopathy. It also has pleiotropic deleterious effects on several organ systems, such as immune function, sarcopenia, energy metabolism and portal hypertension. This study was performed to test the hypothesis that severity of hyperammonaemia is a risk factor for liver-related complications in clinically stable outpatients with cirrhosis.

Methods: We studied 754 clinically stable outpatients with cirrhosis from 3 independent liver units. Baseline ammonia levels were corrected to the upper limit of normal (AMM-ULN) for the reference laboratory. The primary endpoint was hospitalisation with liver-related complications (a composite endpoint of bacterial infection, variceal bleeding, overt hepatic encephalopathy, or new onset or worsening of ascites). Multivariable competing risk frailty analyses using fast unified random forests were performed to predict complications and mortality. External validation was carried out using prospective data from 130 patients with cirrhosis in an independent tertiary liver centre.

Results: Overall, 260 (35%) patients were hospitalised with liver-related complications. On multivariable analysis, AMM-ULN was an independent predictor of both liver-related complications (hazard ratio 2.13; 95% CI 1.89–2.40; p <0.001) and mortality (hazard ratio 1.45; 95% CI 1.20–1.76; p <0.001). The AUROC of AMM-ULN was 77.9% for 1-year liver-related complications, which is higher than traditional severity scores. Statistical differences in survival were found between high and low levels of AMM-ULN both for complications and mortality (p <0.001) using 1.4 as the optimal cut-off from the training set. AMM-ULN remained a key variable for the prediction of complications within the random forests model in the derivation cohort and upon external validation.

Conclusion: Ammonia is an independent predictor of hospitalisation with liver-related complications and mortality in clinically stable outpatients with cirrhosis and performs better than traditional prognostic scores in predicting complications.

Lay summary: We conducted a prospective cohort study evaluating the association of blood ammonia levels with the risk of adverse outcomes in 754 patients with stable cirrhosis across 3 independent liver units. We found that ammonia is a key determinant that helps to predict which patients will be hospitalised, develop liver-related complications and die; this was confirmed in an independent cohort of patients.

Introduction

The Child-Pugh (CP) score and the model for end-stage liver disease (MELD) score are the most utilised non-invasive tools for prediction of survival in patients with cirrhosis but are limited by interobserver subjectivity and their initial derivations in predicting survival after surgery and transjugular intrahepatic portosystemic shunt, respectively.[1,2] Furthermore, the composite features of the MELD score (bilirubin, albumin, international normalised ratio [INR] and creatinine) reflect incomplete facets of the pathophysiology of cirrhotic portal hypertension that are restricted to liver synthetic dysfunction and renal insufficiency. Predictive prognostic models for the development of liver-related complications in patients with stable compensated cirrhosis are also limited. Endoscopic surveillance for varices is useful in determining the risk of variceal bleeding, neuropsychiatric tests provide insight into the risk of overt hepatic

Keywords: Cirrhosis; Ammonia; Liver-related complications; Hepatic encephalopathy; Variceal bleeding; Ascites; Bacterial infection.
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encephalopathy (HE) and detection of clinically significant portal hypertension using hepatic venous pressure gradient (HVPG) provides insights into the risk of development of liver-related complications.[3–5] However, measurement of HVPG is expensive, invasive, requires specialist equipment, and is not routinely available. A simple, cost-effective, and widely available tool to define the risk of future liver-related complications remains a key area of unmet need.

Ammonia has been long established as a gut-derived neurotoxin whose impaired metabolism in chronic liver disease plays a pivotal role in the development of HE.[6] Whilst hyperammonaemia is directly associated with cerebral oedema and raised intracranial pressure in acute liver failure, which is a critical and often fatal phenomenon,[7] the role of hyperammonaemia in HE complicating cirrhosis is less well defined and its relevance in routine clinical practice remains controversial.

Ammonia has been implicated in the pathogenesis of other liver-related complications such as liver cell injury, immune dysfunction, sarcopenia and portal hypertension.[8] Recently, ammonia levels have been shown to be an independent predictor of mortality in patients with acute decompensation or acute-on-chronic liver failure irrespective of the severity of HE, suggesting that it may be a useful biomarker for predicting other liver-related complications.[9–12]

We hypothesised that hyperammonaemia is a risk factor for the development of not only HE but also other liver-related complications and consequent mortality in clinically stable outpatients with cirrhosis. In this study, our primary aim was to determine whether ammonia levels define the risk of subsequent hospitalisation with liver-related complications such as bacterial infection, variceal bleeding, overt HE and ascites. The secondary aims were to determine whether ammonia levels were associated with mortality and the individual liver-related complications. We also sought to determine a threshold value of ammonia that defines the risk of complications and mortality and, to develop a prognostic model for the prediction of these events in stable outpatients with cirrhosis.

Patients and methods

Study design and patient selection

The AMMON consortium was created to determine the role of ammonia in the pathogenesis and treatment of complications of cirrhosis. This analysis is part of the ongoing studies within the Consortium. This is the first study of this programme and evaluates data from 4 independent liver units in Europe: King's College Hospital (KCH), London, United Kingdom (UK), Hospital Clínico Universitario de Valencia (HCUV), Spain, Royal Free Hospital (RFH), London, UK and Virgen del Rocio University Hospital (VRUH), Seville, Spain.

A prospective observational cohort study of clinically stable outpatients with cirrhosis was conducted across these sites. Inclusion criteria were patients aged ≥18 years with cirrhosis based on histological criteria, characteristic radiological findings and/or typical clinical presentation. Patients with both compensated and decompensated cirrhosis were included in the study, however hospital inpatients at the time of assessment and patients hospitalised with an acute decompensation within the previous 4 weeks were excluded. Baseline ammonia levels were measured in all patients at the beginning of the study. Specific characteristics of patients comprising the training and test and validation cohorts were:

- Training and test cohort
  - Cohort 1 – KCH, n = 447: patients sequentially assessed for liver transplantation. Arterial ammonia levels were measured.
  - Cohort 2 - HCUV, n = 156: consecutive patients reviewed without previous episodes of overt HE or hepatocellular carcinoma (HCC). Venous ammonia levels were measured.
  - Cohort 3 - RFH, n = 151: consecutive patients without previous episodes of overt HE or HCC, not candidates for liver transplantation. Venous ammonia levels were measured.
- Validation cohort: consecutive outpatients from VRUH (n = 130). Venous ammonia levels were measured.

Patients were followed up until liver transplantation, death, or study closure. Clinical datasets came from studies approved by ethical review boards at each study site.

Variables

Ammonia was measured in each hospital either for routine clinical purposes or as part of other studies addressing the role of ammonia in the pathogenesis of complications of cirrhosis at the time of outpatient clinical evaluation. All centres used standard operating procedures for ammonia measurement that involved collection of the sample in cooled EDTA tubes, rapid sample transport to the laboratory on ice and spectrophotometric assays. There was a requirement to standardise ammonia levels to correct for differences in phlebotomy and laboratory handling protocols between centres; we transformed the crude ammonia measurement to a calibrated ammonia level (AMM-ULN) using the formula: AMM-ULN = serum ammonia (μmol/L)/reference laboratory upper limit of normal for ammonia (μmol/L). In this manner, we were able to express the ratio of the patient ammonia level corrected to a normal population measured with the same local test system and test conditions.

The following clinical and demographic information were collected at baseline: age, sex, anthropometric data, aetiology of cirrhosis, comorbidities, co-prescribed medications, previous liver-related complications suggesting decompensated disease, laboratory parameters and disease severity assessed by MELD, MELD-Na and CP scores. The presence of portal hypertension was determined using its surrogates; this included presence of varices, evidence of portosystemic collaterals, splenomegaly and/or use of beta-blockers.

The primary endpoint of the study was hospitalisation due to liver-related complications which represented a composite endpoint of bacterial infection, variceal bleeding, overt HE and new onset, or worsening ascites; more details on the definitions of liver-related complications are available in the supplementary materials. Secondary endpoints included overall survival (OS), type of complication and both liver-related complications and survival at 3, 6, and 12 months and 5 years.

Statistical analyses

Continuous demographic, clinical and laboratory variables were analysed for normality using the Shapiro-Wilk test. Normally distributed data were reported as mean (SD) and non-normally distributed data were reported as median (IQR). Comparisons between hospitals were performed using ANOVA or Kruskal-Wallis tests for normally and non-normally distributed data,

respectively. Categorical data were reported as n (%) and comparisons analysed by Chi-squared (χ^2) test. All correlations were performed using Pearson's correlation coefficient except for ordinal variables where Kendall's tau was used.

Multivariable competing risk frailty analysis was performed, considering liver transplantation as a competing risk, to identify factors independently associated with complications and mortality using Fine-Gray subdistribution hazard modelling. Only original variables (not CP or MELD score) were included in the multivariable model to avoid multicollinearity. A competing risk cause-specific Cox regression model was applied to study the effect of original variables in each liver-related complication. Time-dependent receiver-operating characteristic (ROC) curves were constructed and compared considering the competing risk within the model.[13]

Data was randomly split into training (75%) and test (25%) sets and the optimal cut-off for AMM-ULN was calculated using maximally selected rank statistics for time to development of liver-related complications (Fig. S1). This value was then used in the test sample to construct Kaplan Meier curves for time to development of liver-related complications and OS. Differences in survival were assessed using log-rank tests. AMM-ULN was subsequently evaluated in an independent external validation set.

Fast unified random forests with 500 trees, using log-rank splitting criteria with bootstrapping and cross-validation, were

Table 1. Description of patient characteristics in the 3 prospective hospital cohorts.

Parameter	Total (n = 754)	RFH (n = 151)	HCUV (n = 156)	KCH (n = 447)	*p* value
Demographic					
Age (mean, SD)	56 (11)	57 (11)	63 (9)	54 (11)	<0.001
Sex (no. male, %)	498 (66)	80 (53)	125 (80)	293 (66)	<0.001
Disease aetiology, n (%)					
ALD	300 (40)	73 (48)	69 (44)	158 (35)	<0.001
NAFLD	106 (14)	3 (2)	14 (9)	89 (20)	
Viral hepatitis	182 (24)	60 (40)	58 (37)	64 (14)	
Autoimmune liver disease	110 (15)	9 (6)	4 (3)	97 (22)	
Other	56 (7)	6 (4)	11 (7)	39 (9)	
Comorbidities, n (%)					
Diabetes mellitus	263 (35)	55 (36)	55 (35)	153 (34)	0.875
Co-prescribed medications, n (%)					
Non-selective β-blockers	355 (47)	104 (69)	58 (37)	193 (43)	<0.001
Lactulose	266 (35)	–	55 (35)	211 (47)	<0.001
Rifaximin	176 (23)	–	1 (0.6)	175 (39)	<0.001
Laboratory parameters mean (SD)					
Albumin (g/dl)	3.3 (0.7)	2.9 (0.6)	3.9 (0.6)	3.3 (0.6)	<0.001
Bilirubin (mg/dl)	3.4 (4.4)	4.9 (5.7)	1.3 (1.2)	3.5 (4.4)	<0.001
Creatinine (mg/dl)	0.9 (0.5)	1.0 (0.6)	0.9 (0.3)	0.9 (0.4)	0.001
INR	1.5 (0.4)	1.4 (0.3)	1.2 (0.3)	1.6 (0.5)	<0.001
WBC ($x10^9$/L)	5.3 (2.7)	6.6 (3.8)	5.9 (2.2)	4.7 (2.3)	<0.001
Platelets ($x10^9$/L)	115 (71)	–	123 (60)	112 (75)	0.126
Sodium (mmol/L)	136 (5)	135 (5)	139 (3)	136 (5)	<0.001
Ammonia level (μmol/L)	63 (40)	61 (17)	29 (29)	75 (42)	<0.001
AMM-ULN	1.4 (0.8)	1.5 (0.4)	0.9 (0.9)	1.5 (0.8)	<0.001
Disease severity, n (%)					
Portal hypertension	708 (94)	148 (98)	127 (81)	433 (97)	<0.001
Hepatocellular carcinoma	79 (10)	0	0	79 (18)	<0.001
Decompensated cirrhosis	484 (64)	127 (84)	25 (16)	332 (74)	<0.001
Child-Pugh score, mean (SD)	8 (2)	9 (2)	6 (1)	9 (2)	<0.001
Child-Pugh group					
A	197 (26)	16 (11)	106 (69)	75 (17)	<0.001
B	346 (46)	83 (55)	42(28)	221 (50)	
C	207 (28)	52 (34)	5 (3)	150 (34)	
MELD Score (mean, SD)	14 (6)	14 (7)	10 (4)	15 (6)	<0.001
MELD-Na Score (mean, SD)	15 (7)	17 (7)	9 (3)	17 (6)	
Outcome, n (%)					
Transplant	359 (48)	0 (0)	3 (2)	355 (79)	<0.001
Development of liver-related complication	260 (35)	73 (48)	53 (34)	134 (30)	<0.001
Type of complication, n (%)					
Ascites	56 (7)	16 (11)	16 (10)	24 (5)	<0.001
Variceal bleeding	28 (4)	9 (6)	10 (7)	9 (2)	
Infection	123 (16)	30 (20)	15 (10)	78 (17)	
Hepatic encephalopathy	52 (7)	16 (11)	12 (8)	24 (5)	
Mortality, n (%)	120 (16)	29 (19)	40 (26)	51 (11)	<0.001
Follow-up (days; median, range)	223 (2,451)	232 (422)	1,385 (2,418)	138 (1,341)	<0.001

Continuous variables are presented as mean (SD), categorical variables are presented as n (%). Comparisons were performed between patient cohorts using ANOVA and Kruskal Wallis tests for normally and non-normally distributed data, respectively and Chi-squared for categorical data. p <0.05 was considered statistically significant.
ALD, alcohol-related liver disease; AMM-ULN, ammonia level corrected to the upper limit of normal; HCUV, Hospital Clínico Universitario de Valencia; INR, international normalized ratio; KCH, King's College Hospital; MELD, model for end-stage liver disease score; NAFLD, non-alcoholic fatty liver disease; RFH, Royal Free Hospital; WBC, white blood cell count.

used to predict mortality and complications in the combined dataset. Fourteen different models were included using different subsets of variables; the external validation set was used to test model performance of all 14 models. Performance in risk prediction for each model was evaluated using Brier score comparisons. Variable importance (VIMP) was extracted using subsampling to calculate confidence intervals and standard errors.

All analyses were performed with R (version 4.0.2, R Core, 2021) with the cut-off for statistical significance set at 0.05. Maxstat package[14] was used for maximally selected rank statistics; Survival[15] and Survminer[16] for Kaplan Meier curves and log-rank statistic calculation; timeROC[13] package for time-dependent ROC curves and AUC comparison; randomForestSRC package[17] for the random forest analyses; and pec package[18] for Brier score model comparison.

Results

Patient characteristics

A total of 754 patients were included (66% males; mean 56 years) in the training and test cohort. Patient characteristics from KCH, HCUV, RFH, and the combined dataset are summarised in Table 1. Alcohol and viral hepatitis were the main aetiologies in RFH and HCUV, while non-alcoholic fatty liver disease (NAFLD) was more prevalent in KCH. Severity of liver disease was higher (with higher CP and MELD scores) in the RFH and KCH cohorts than in the HCUV cohort. Median follow-up of the total cohort was 223 days (range: 2–2,453). Overall, 35% of patients developed a liver-related complication during follow-up, with infections (16%) representing the most frequent type of complication. Amongst 120 patients who died in the study, 36% (n = 43) died outside the tertiary centre with cause of death not known; amongst the remainder, 38% (n = 29) died of complications of cirrhosis or acute-on-chronic liver failure, 28% (n = 22) of infection, 10% (n = 8) of HCC, 12% (n = 9) of other malignancy and 12% (n = 9) died with other non-liver-related conditions.

AMM-ULN demonstrated differential association with disease aetiology (p = 0.002) and was most marked in patients with NAFLD cirrhosis (mean: 1.6, SD: 0.8). Furthermore, ammonia levels were higher in patients who had diabetes (1.5 *vs.* 1.3, p <0.001). AMM-ULN was predictably higher in patients with more advanced stages of cirrhosis (0.9, 1.5 and 1.6 in CP groups A, B and C, respectively and 1.0, 1.4 and 1.6 in MELD score groups ≤9, 9–12 and ≥12, respectively p <0.001) (Table S1). AMM-ULN also correlated with the MELD-Na score (r = 0.25, p <0.001), and individual markers of liver function – bilirubin (r = 0.083, p = 0.023), albumin (r = -0.322, p <0.001) and INR (r = 0.172, p <0.001) – as well as platelets (r = -0.209, p <0.001) and creatinine (r = 0.120, p = 0.001). Anthropometric data were not available at every centre, however within the KCH cohort no correlation was found between nutrition or muscle status and AMM-ULN (p >0.05).

AMM-ULN as a prognostic biomarker for the prediction of complications and mortality

AMM-ULN was an independent predictor of hospitalisation with liver-related complications on univariable analysis in each of the 3 cohorts (KCH: hazard ratio [HR] 2.43, 95% CI 2.17–2.72, p <0.001; HCUV: HR 1.65, 95% CI 1.39–1.97, p <0.001; and RFH: HR 5.13, 95% CI 3.06–8.59, p <0.001) and in the univariable (HR 2.21, 95% CI 1.96–2.49, p <0.001) and multivariable (HR = 2.13, 95% CI 1.89–2.40, p <0.001) analyses of the combined dataset. Other significant risk factors for hospitalisation with liver-related complications were diabetes (HR 1.53, 95% CI 1.12–1.94, p = 0.003) and INR (HR 1.77, 95% CI 1.39–2.27, p = 0.001) (Table 2). Subgroup analyses were performed within the KCH cohort to address the impact of nutrition and muscle status on the risk of hospitalisation with liver-related complications. A stepwise procedure including AMM-ULN and each anthropometric parameter showed the best model included only AMM-ULN (Table S2).

AMM-ULN was also independently associated with the risk of hospitalisation with the individual liver-related complications; overt HE (HR 2.19, 95% CI 2.10–2.29, p <0.001), variceal bleeding (HR 1.93, 95% CI 1.73–2.16, p <0.001), ascites (HR 1.76, 95% CI 1.35–2.30, p <0.001) and bacterial infection (HR 2.35, 95% CI 1.93–2.87, p <0.001) (Table 3). The AUROC for AMM-ULN was 77.9% for 1-year complications; higher than the MELD score (66.1%, p <0.001) but not significantly higher than the CP score (72.2%, p = 0.062), Fig. 1A. The AUROCs of AMM-ULN for hospitalisation due to liver-related complications at 3 and 6 months and, at 5 years were 73.2%, 74.9% and 74.4%, respectively (Fig. S2A–C).

AMM-ULN was an independent predictor of mortality in each independent hospital (KCH: HR 1.83, 95% CI 1.61–2.08, p <0.001; HCUV: HR 1.40, 95% CI 1.08–1.81, p = 0.011 and RFH: HR 4.89, 95% CI 2.28–10.48, p <0.001) and the combined cohort (HR 1.73, 95% CI 1.48–2.02, p <0.001). Furthermore, it was an independent risk factor for mortality in the multivariable frailty competing risk model (Table S3). The AUROC of AMM-ULN, 70.5%, was not significantly higher than CP (p = 0.803) and MELD scores (p = 0.073) for 1-year survival (Fig. 1B).

Identifying the optimal cut-off of AMM-ULN to predict hospitalisation due to liver-related complications and mortality

Maximally selected rank statistics determined AMM-ULN >1.4 as the best cut-off to predict time to hospitalisation due to liver-related complications in the training cohort. Applying this to the test set, statistical differences in survival were found between high and low levels of AMM-ULN for liver-related complications (log-rank p <0.001) and mortality (log-rank p <0.001) (Fig. 2). Patients in the high-risk group were more likely to have diabetes (42% *vs.* 30%, p = 0.001), be receiving lactulose (42% *vs.* 30%, p <0.001) and rifaximin (33% *vs.* 16%, p <0.001) and have more advanced liver disease (MELD-Na 17 *vs.* 14, p <0.001; CP score 9 *vs.* 8, p <0.001) (Table S4). Patients in the high-risk group were more likely to require hospitalisation due to liver-related complications (58% *vs.* 17%, p <0.001), including bacterial infections (28% *vs.* 8%, p <0.001), and patients had a higher mortality (23% *vs.* 10%, p <0.001) over the course of the study follow-up.

Prognostic model to predict hospitalisation due to liver-related complications

Cox regression and random forest models were developed and evaluated using bootstrap cross-validation. The integrated Brier score showed that the best model to predict liver-related complications was the random forest model with original variables;

Table 2. Univariable and multivariable model for liver-related complications.

	Univariable model			Multivariable model		
Parameter	**HR**	**95% CI**	***p* value**	**HR**	**95% CI**	***p* value**
Demographic						
Age	1.005	0.99–1.02	0.39			
Sex	0.96	0.74–1.25	0.79			
Disease aetiology						
ALD	Ref.	Ref.	Ref.			
NAFLD	1.45	1.01–2.08	0.46			
Viral hepatitis	0.67	0.48–0.93	0.02			
Autoimmune liver disease	0.79	0.53–1.21	0.29			
Other	1.17	0.73–1.88	0.5			
Comorbidities						
Diabetes mellitus	1.75	1.37–2.24	<0.001	1.53	1.12–1.94	0.005
Co-prescribed medications						
Non-selective β-blockers	1.23	0.96–1.59	0.1			
Lactulose	1.88	1.44–2.45	<0.001			
Rifaximin	1.59	1.21–2.09	<0.001			
Laboratory parameters						
Albumin	0.56	0.45–0.69	<0.001			
Bilirubin	1.03	1.01–1.05	0.004			
Creatinine	1.42	1.16–1.73	<0.001			
INR	1.86	1.46–2.36	<0.001	1.77	1.39–2.27	<0.001
WBC	0.99	0.94–1.04	0.68			
Platelets	0.995	0.992–0.997	<0.001			
Sodium	0.97	0.95–0.99	0.015	0.97	0.94–1.00	0.070
AMM-ULN	2.21	1.96–2.49	<0.001	2.13	1.89–2.40	<0.001
Disease severity						
Child-Pugh class						
A	Ref.	Ref.	Ref.			
B	2.46	1.65–3.67	<0.001			
C	4.21	2.72–6.49	<0.001			
MELD score	1.05	1.03–1.07	<0.001			
MELD-Na	1.06	1.04–1.07	<0.001			
Decompensated cirrhosis	3.01	1.88–5.09	<0.001	1.47	0.94–2.3	0.090

Competing risk frailty analysis was performed considering liver transplantation as a competing risk to identify factors independently associated with complications and mortality using Fine-Gray subdistribution hazard modelling. A backward-forward stepwise procedure was conducted for variable selection using Akaike's information criterion.

ALD, alcohol-related liver disease; AMM-ULN, ammonia level corrected to the upper limit of normal; HR, hazard ratio; INR, international normalized ratio; MELD, model for end-stage liver disease score; NAFLD, non-alcoholic fatty liver disease; WBC, white blood cell count.

further models with a reduced number of variables had similar margins of prediction error (Fig. 3). The error rate for the selected model according to the number of trees and subsampled forests to estimate standard errors and confidence intervals for VIMP is described in Fig. 4. Variables that were retained in the model according to their VIMP to predict liver-related complications were AMM-ULN, sodium, creatinine, albumin, bilirubin, and INR with AMM-ULN identified as the single most important variable for predicting hospitalisation due to liver-related complications. The addition of a history of previous liver decompensation to the prognostic model did not increase its predictive ability (Fig. S3).

External cohort validation of AMM-ULN as a predictor of hospitalisation due to liver-related complications

AMM-ULN remained a risk factor for hospitalisation due to liver-related complications in an independent validation cohort of 130 patients (HR 1.56; 95% CI 1.18–2.05; p = 0.002); comparative clinical data from the derivation and validation sets is summarised (Table S5). Kaplan-Meier analysis with log-rank test using the AMM-ULN cut-off of 1.4 again demonstrated significant differences in predicting liver-related complications, p = 0.025 (Fig. 5). Application of the Cox regression and random forest models from our derivation sets to the validation cohort further demonstrated the optimal performance of random forest with original variables and the central importance of AMM-ULN as a prediction variable within the most accurate models (Fig. S4).

Discussion

In this study, we sought to consider hyperammonaemia outside of the context of HE and to define its role as a prognostic biomarker in patients with stable cirrhosis. For the first time, we have demonstrated that ammonia is an independent predictor of hospitalisation due to liver-related complications and mortality in clinically stable outpatients with cirrhosis. Multivariable analysis revealed that the component variables of traditional scoring systems, bilirubin, creatinine, albumin, INR, and sodium, remained significant in the prediction of liver-related complications. However, AMM-ULN was independently associated with adverse outcomes and carried greater weight than any other prognostic variable. Although the cohorts in the AMMON study were collected in 4 tertiary liver centres and encompassed patients followed up with different clinical characteristics, we observed concordance in the ability of AMM-ULN to independently predict both hospitalisation due to liver-related complications and survival. Furthermore, AMM-ULN performed better than traditional severity scores in predicting hospitalisation due to liver-related complications at 6 months, 12 months, and 5

Table 3. Results of multivariable cause-specific competing risk frailty models.

	Multivariable model		
	HR	95% CI	*p* value
Ascites			
Diabetes	0.99	0.44–2.20	0.985
Bilirubin	1.03	0.99–1.06	0.106
Albumin	0.97	0.50–1.90	0.937
INR	1.77	1.57–1.99	<0.001
AMM-ULN	1.76	1.35–2.30	<0.001
Hepatic encephalopathy			
Diabetes	1.49	1.01–2.20	0.047
Bilirubin	1.05	1.03–1.08	<0.001
Albumin	0.77	0.30–2.01	0.600
INR	1.32	1.15–1.52	<0.001
AMM-ULN	2.19	2.10–2.29	<0.001
Variceal bleeding			
Diabetes	0.84	0.53–1.32	0.43
Bilirubin	1.12	1.05–1.19	<0.001
Albumin	0.34	0.25–0.46	<0.001
INR	0.28	0.14–0.55	<0.001
AMM-ULN	1.93	1.73–2.16	<0.001
Infection			
Diabetes	2.05	1.72–2.45	<0.001
Bilirubin	1.04	1.01–1.19	0.015
Albumin	0.56	0.38–0.83	0.004
INR	2.67	2.16–3.31	<0.001
AMM-ULN	2.35	1.93–2.87	<0.001

Results of multivariable cause-specific competing risk frailty models demonstrating variables independently associated with the risk of developing individual complications of liver disease. Competing risk frailty analysis was performed considering liver transplantation as a competing risk to identify factors independently associated with complications and mortality using Fine-Gray subdistribution hazard modelling. A backward-forward stepwise procedure was conducted for variable selection using Akaike's information criterion. AMM-ULN was independently associated with each individual liver-related complication on multivariable analysis. p <0.05 was considered statistically significant.
AMM-ULN, ammonia level corrected to the upper limit of normal; INR, international normalized ratio.

years. Our data demonstrate that AMM-ULN >1.4 correlates with a significantly higher risk of hospitalisation due to liver-related complications (58% *vs.* 17%, p <0.001) and mortality (23% *vs.* 10%, p <0.001). Contrary to expectation, prior decompensation did not predict hospitalisation with liver-related complications; moreover, addition of prior decompensation to the prognostic model did not increase its predictive ability – this could be because of its interaction with ammonia in defining outcomes.

We derived a predictive model using random forest plots that demonstrated superiority to established predictive models such as the MELD and CP scores in addition to models derived from Cox regression within our dataset; AMM-ULN was the parameter that carried the highest VIMP in this model. On external validation we verified that ammonia was again associated with risk of hospitalisation due to liver-related complications and remains a highly important variable in the random forest models with the best predictive accuracy. Taken together, these data provide compelling evidence for the potential role of ammonia to risk stratify outpatients with stable cirrhosis.

Hyperammonaemia is classically described in the context of HE. It crosses the blood-brain barrier and exerts several toxic effects leading to astrocytic swelling, neuroinflammation, cell signalling and alterations in neurotransmission.[19,20] Additionally, ammonia has multisystem deleterious effects that may contribute to the emergence of late-stage cirrhotic complications.[8] In this study, we have shown that hyperammonaemia is independently associated not only with the development of HE, but also other decompensating events such as bacterial infections, variceal bleeding, and ascites. Ammonia can directly induce hepatocyte cell death, thereby contributing to the progression of liver injury, fibrosis, and portal hypertension through the activation of hepatic stellate cells.[21,22] Hyperammonaemia also propagates circulating innate immune dysfunction in the context of cirrhosis.[23] Commensurate with this observation, and in the setting of significant portal hypertension, we report increased rates of bacterial infections in patients with AMM-ULN >1.4. Sarcopenia is associated with mortality in cirrhosis and is bidirectionally linked with hyperammonaemia;[24] progressive sarcopenia leads to loss of a compensatory route of extrahepatic ammonia detoxification through glutamine synthetase whilst ammonia has been shown to promote skeletal muscle loss via multiple mechanisms.[25] However, in our study, it was neither associated with AMM-ULN nor was it a significant variable

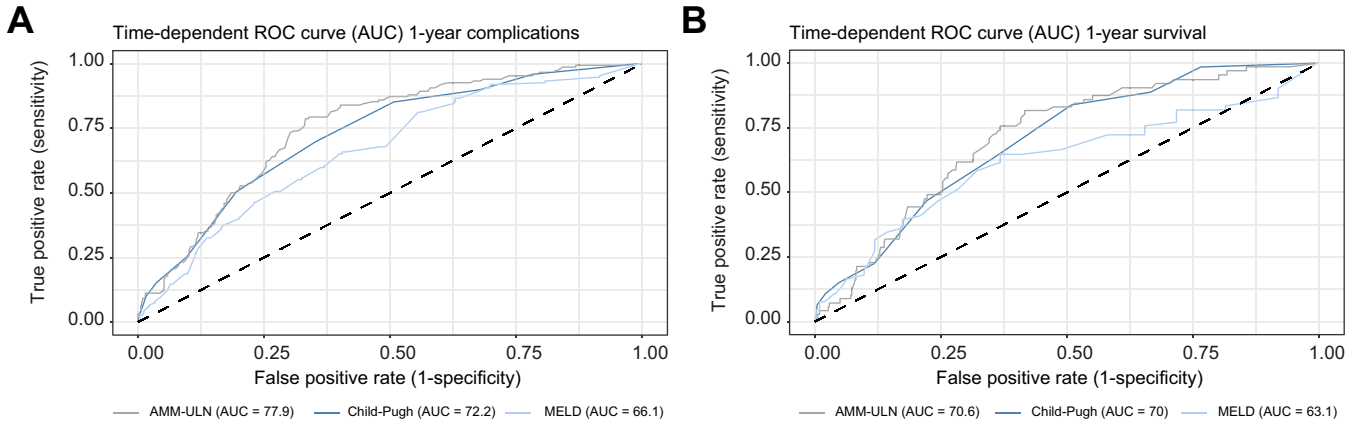

Fig. 1. Time-dependent AUROC curve. Comparison of AMM-ULN against Child-Pugh and MELD score for predicting (A) hospitalisation due to liver-related complications, and (B) mortality at 1 year. p <0.05 was considered statistically significant. AMM-ULN demonstrates improved predictive performance (77.9%) for the development of liver-related complications when compared against the MELD score (66.1%, p <0.001), and showed a tendency to perform better than the Child-Pugh score (72.2%, p = 0.086). AMM-ULN was not significantly better than the MELD (p = 0.073) and Child-Pugh (p = 0.803) scores for the prediction of 1-year mortality. AMM-ULN, ammonia level corrected to the upper limit of normal; MELD, model for end-stage liver disease.

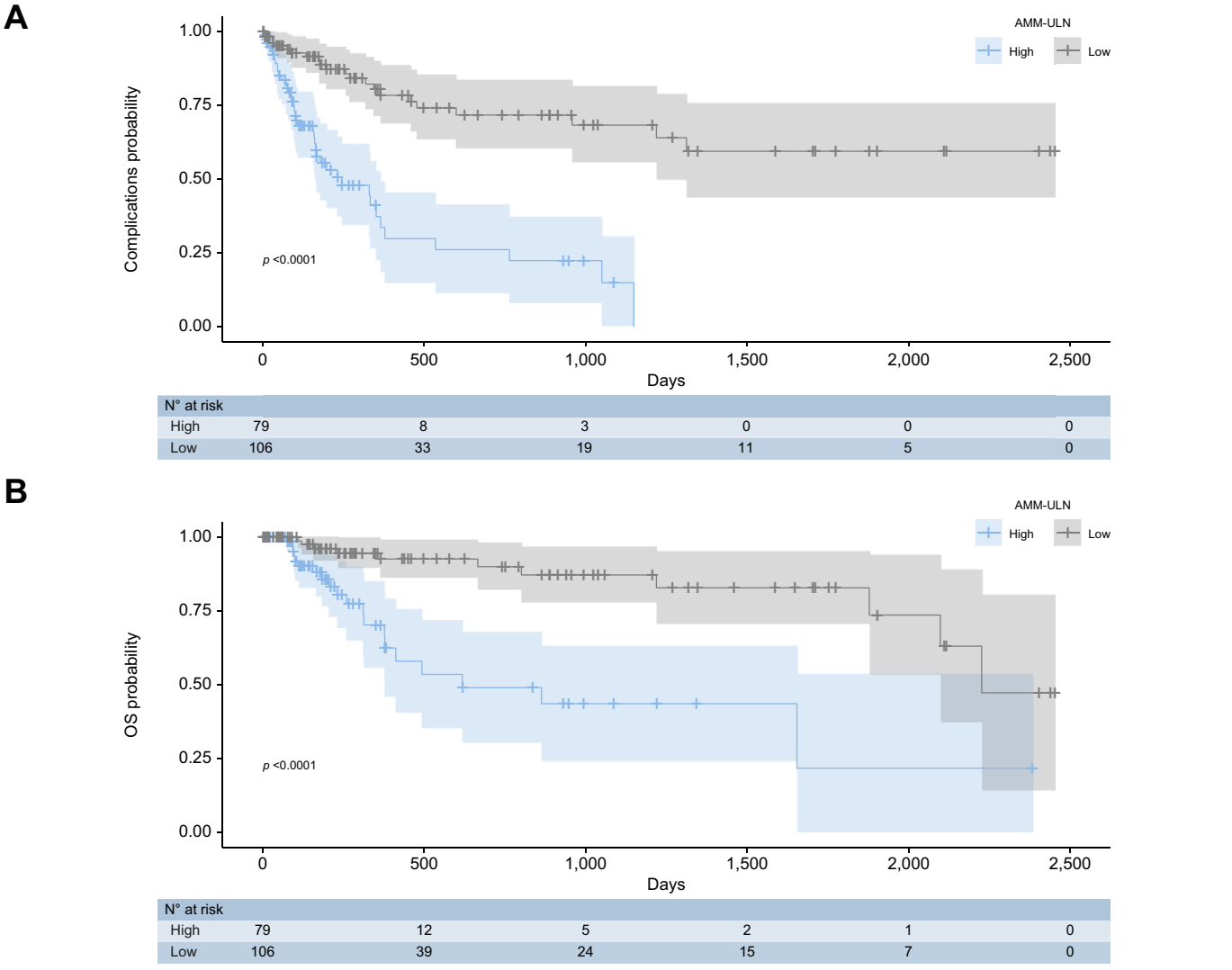

Fig. 2. Kaplan Meier plots of hospitalisation due to liver-related complications and mortality. Kaplan Meier plots demonstrating cumulative probability of the development of (A) liver-related complications (composite endpoint of sepsis, variceal bleeding, overt hepatic encephalopathy, acute onset, or worsening ascites), and (B) OS during follow-up. Patients with AMM-ULN ≥1.4 were allocated to the high-risk group. Differences in median overall survival were assessed by log-rank test. p <0.05 was considered statistically significant. AMM-ULN, ammonia level corrected to the upper limit of normal; OS, overall survival.

predicting the development of liver-related complications in the KCH cohort, where this relationship could be evaluated.

Hyperammonaemia in cirrhosis may arise as a consequence of impaired liver synthetic function, portosystemic shunting, sarcopenia (with reduced extrahepatic ammonia clearance), renal dysfunction and intestinal dysbiosis in cirrhosis.[26] Thus, hyperammonaemia reflects complex, multiorgan interactions describing any combination of hepatic, renal, neurological, immunological and skeletal muscle functions that become dysregulated in cirrhosis and therefore represents an important biomarker of physiological reserve.[8] In this study, ammonia levels were higher in patients with NAFLD and patients with diabetes. The underlying mechanism of this association is unknown but may be due to the negative impact of liver steatosis on hepatic expression and activity of urea cycle enzymes.[27,28] The intestinal microbiome is highly dysregulated in both NAFLD and cirrhosis, which may contribute to excess ammonia production directly or indirectly by modulating the activity of intestinal glutaminase.[29]

One area that has hampered widespread usage of ammonia measurement is the inter-laboratory differences in protocols of measurement and the reflected differences in the range and distribution of measurements. Here, AMM-ULN performed better than crude ammonia levels in predicting both complications and mortality (Akaike information criterion = 3,119 *vs.* 3,131 and 1,348 *vs.* 1,359, respectively). Furthermore, this study includes patients with both arterial (KCH) and venous (RFH, HCUV, VRUH) sampling of ammonia with the potential for arteriovenous differences in ammonia measurements secondary to muscle metabolism.[30] Despite this, AMM-ULN levels remained strongly predictive of hospitalisation due to liver-related complications and mortality in stable cirrhosis. We propose that using AMM-ULN may harmonise ammonia levels being reported widely in the literature, which are often affected by local laboratory practices, in a manner akin to reporting of the international normalised ratio corrected to laboratory prothrombin time controls[31] and severity grading of

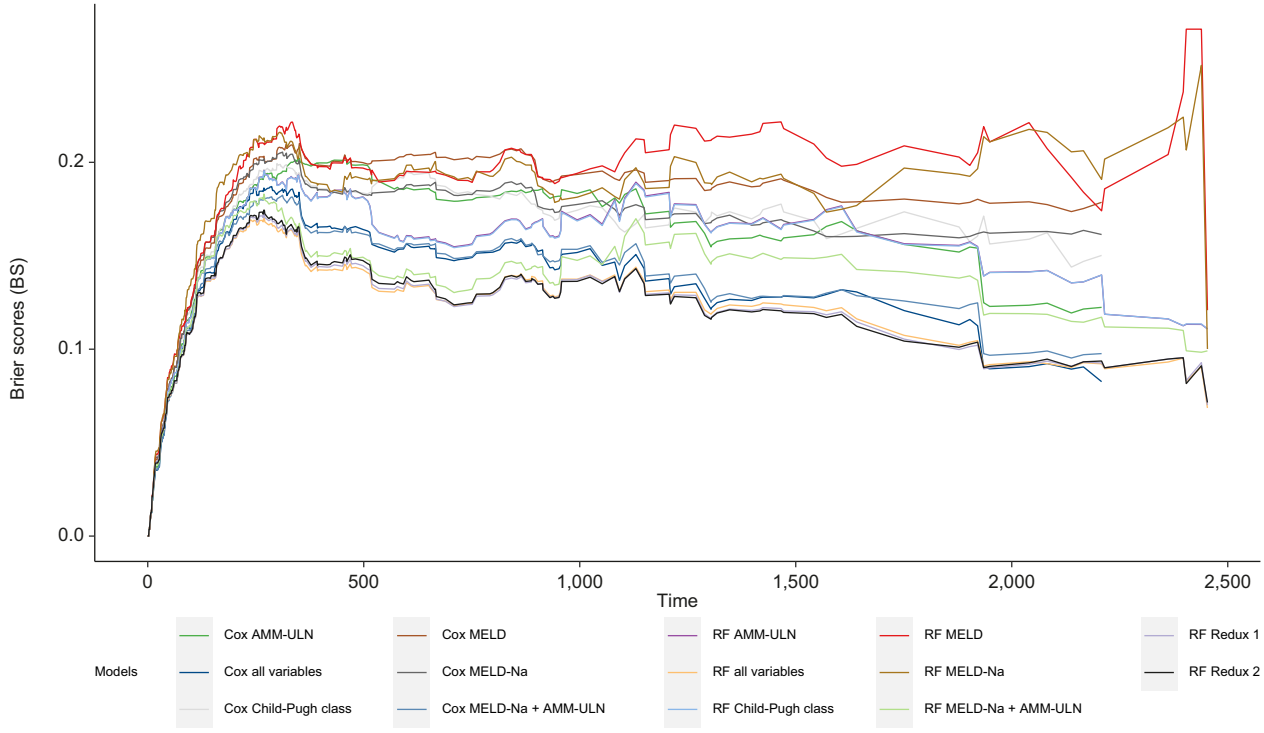

Fig. 3. Integrated Brier score to evaluate model performance using bootstrap cross-validation to predict hospitalisation due to liver-related complications. The integrated Brier score calculates the predictive error over time with larger values of the integrated Brier score indicating worse performance of the predictive model. Fourteen models from our training set were compared; these were developed using Cox regression and RF modelling with input variables restricted to AMM-ULN, CP class, MELD score, MELD-Na score, or all variables (age, sex, aetiology of liver disease, diabetes, bilirubin, albumin, creatinine, INR, and sodium). Cox AMM-ULN and RF AMM-ULN performed better than their respective comparative models based on CP and MELD scores. The optimal predictive model was a RF model incorporating all variables. A further 2 models with reduced variables (RF Redux 1: AMM-ULN, INR, bilirubin, albumin, creatinine, sodium, and diabetes; RF Redux 2: AMM-ULN, INR, bilirubin, albumin, creatinine, and sodium) maintained an excellent predictive performance. AMM-ULN, ammonia level corrected to the upper limit of normal; CP, Child-Pugh; INR, international normalized ratio; MELD, model for end-stage liver disease; RF, random forest; ULN, upper limit of normal. (This figure appears in color on the web.)

drug-induced liver injury classified as a ratio of the ULN of alanine aminotransferase and aspartate aminotransferase.[32]

The results of this study should be interpreted considering its limitations. First, the study patient cohorts were derived from 3 separate centres and included patients with different severities of cirrhosis. Despite this, concordant results were observed with respect to the independent association of ammonia with complications and mortality in cirrhosis across the centres that were

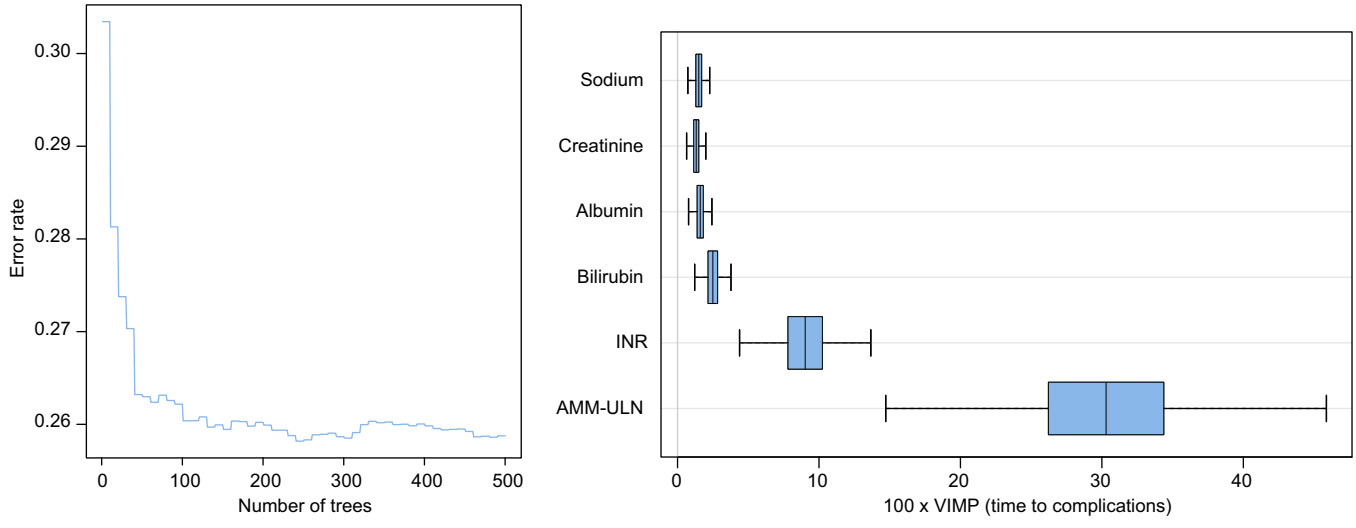

Fig. 4. Prognostic model using random forest to predict hospitalisation due to liver-related complications. Error rate for the selected random forest model according to number of trees (left panel) and subsampled forests to estimate standard errors and confidence intervals for VIMP (right panel). AMM-ULN represented the variable with the highest VIMP within the predictive model. AMM-ULN, ammonia level corrected to the upper limit of normal; INR, international normalized ratio; VIMP, variable importance.

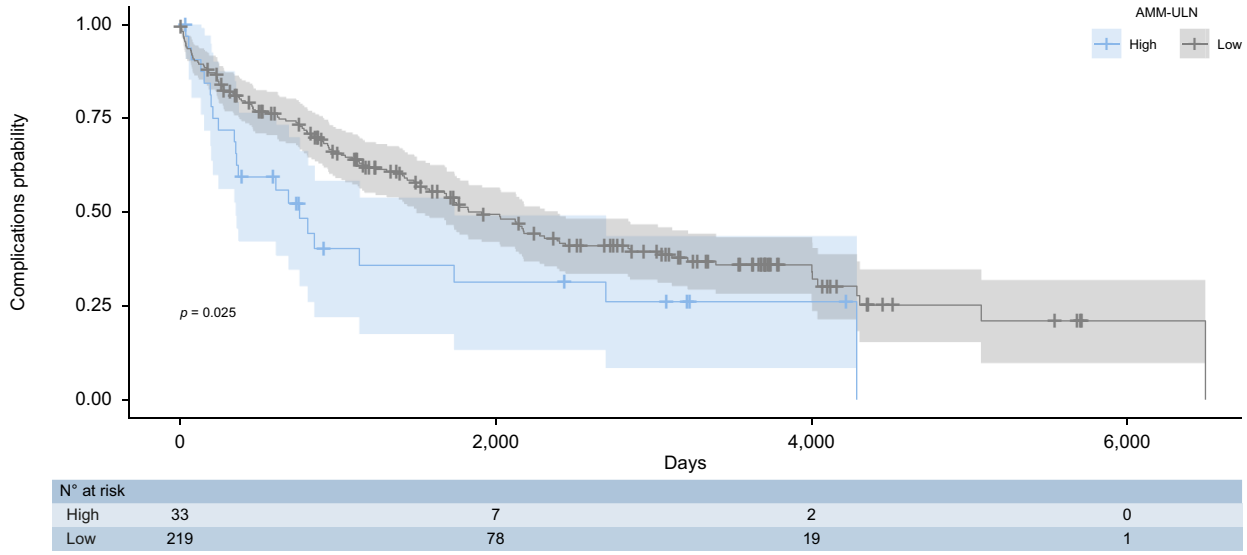

Fig. 5. Kaplan Meier plot of hospitalisation due to liver-related complications in the validation cohort. Kaplan Meier plot demonstrating cumulative probability of hospitalisation due to liver-related complications in the validation cohort (n = 130). p <0.05 was considered statistically significant. AMM-ULN ≥1.4 retained the ability to discriminate patients at high risk of cirrhotic complications, p = 0.025. Differences in median overall survival were assessed by log-rank test. AMM-ULN, ammonia level corrected to the upper limit of normal.

further validated in an independent external cohort. Second, this was an observational study using data collected either for routine clinical use or as part of other studies and the timing and indications for single-timepoint ammonia measurement were not protocolised, which may have introduced an element of selection bias for patients included in the study. However, each centre is a tertiary liver unit and ammonia measurements are performed using standard operating procedures with a high degree of fidelity. Whilst most patients had evidence of clinically significant portal hypertension, data was not collected on the presence and impact of portal-systemic shunts which represents an area for further investigation. Finally, whether reduction of ammonia levels is associated with better prognosis was not evaluated in this group of patients. Therefore, the results of our study do not allow conclusions on whether AMM-ULN is simply a biomarker or indeed a therapeutic target. Future studies will be needed to address this question.

In summary, these data highlight the importance of serum ammonia levels in predicting hospitalisation due to liver-related complications and survival in clinically stable cirrhotic outpatients. AMM-ULN performs better than established prognostic models in cirrhosis and represents an important biomarker in predicting adverse outcomes, stratifying individualised patient risk. With further validation, the results presented here could be adopted in clinical practice readily given the widespread availability, relative ease, and low cost of measuring AMM-ULN.

Abbreviations

AMM-ULN, ammonia level corrected to the upper limit of normal; AUROC, area under the receiver-operating curve; HCC, hepatocellular carcinoma; HCUV, Hospital Clínico Universitario de Valencia; HE, hepatic encephalopathy; INR, international normalised ratio; KCH, King's College Hospital; MELD, model for end stage liver disease; MELD-Na, MELD-sodium; NAFLD, non-alcoholic fatty liver disease; RFH, Royal Free Hospital; VIMP, variable importance.

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Conflicts of interest

Rajiv Jalan is the inventor of OPA, which has been patented by UCL and licensed to Mallinckrodt Pharma. He is also the founder of Yaqrit Discovery, a spin out company from University College London, Hepyx Limited and Cyberliver. He had research collaborations with Yaqrit Discovery. The other authors have no conflicts of interest to declare.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Statistical analyses were performed by JA C-A. The manuscript was prepared and written by THT and MPB and revised by RJ and DLS and M C-L, D E-G and CM. JA, GA, AC, YS, KLT, JAK and MR-G contributed to data collection. All authors have reviewed and approved the final submitted manuscript.

Data availability statement

Data are available upon request to the corresponding author.

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Supplementary data

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References

[1] Kamath PS, Wiesner RH, Malinchoc M, Kremers W, Therneau TM, Kosberg CL, et al. A model to predict survival in patients with end-stage liver disease. Hepatology 2001;33:464–470.
[2] Pugh RN, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. Br J Surg 1973;60:646–649.
[3] Formentin C, Zarantonello L, Mangini C, Frigo AC, Montagnese S, Merkel C. Clinical, neuropsychological and neurophysiological indices and predictors of hepatic encephalopathy (HE). Liver Int 2021;41:1070–1082.
[4] Brocchi E, Caletti G, Brambilla G, La Mantia L, Lupinacci G, Pisano G, et al. North Italian Endoscopic Club for the Study and Treatment of Esophageal Varices. Prediction of the first variceal hemorrhage in patients with cirrhosis of the liver and esophageal varices. A prospective multicenter study. N Engl J Med 1988;319:983–989.
[5] Ripoll C, Groszmann R, Garcia-Tsao G, Grace N, Burroughs A, Planas R, et al. Hepatic venous pressure gradient predicts clinical decompensation in patients with compensated cirrhosis. Gastroenterology 2007;133:481–488.
[6] Sherlock S, Summerskill WHJ, White L, Phear E. Portal-systemic encephalopathy neurological complications of liver disease. Lancet 1954;264:453–457.
[7] Jalan R, Olde Damink SW, Hayes PC, Deutz NE, Lee A. Pathogenesis of intracranial hypertension in acute liver failure: inflammation, ammonia and cerebral blood flow. J Hepatol 2004;41:613–620.
[8] Tapper EB, Jiang ZG, Patwardhan VR. Refining the ammonia hypothesis: a physiology-driven approach to the treatment of hepatic encephalopathy. Mayo Clin Proc 2015;90:646–658.
[9] Hu C, Huang K, Zhao L, Zhang F, Wu Z, Li L. Serum ammonia is a strong prognostic factor for patients with acute-on-chronic liver failure. Sci Rep 2020;10:16970.
[10] Patwardhan VR, Jiang ZG, Risech-Neiman Y, Piatkowski G, Afdhal NH, Mukamal K, et al. Serum ammonia is associated with transplant-free survival in hospitalized patients with acutely decompensated cirrhosis [corrected]. J Clin Gastroenterol 2016;50:345–350.
[11] Verma N, Dhiman RK, Choudhury A, Taneja S, Duseja A, Singh V, et al. Dynamic assessments of hepatic encephalopathy and ammonia levels predict mortality in acute-on-chronic liver failure. Hepatol Int 2021;15:970–982.
[12] Sawhney R, Holland-Fischer P, Rosselli M, Mookerjee RP, Agarwal B, Jalan R. Role of ammonia, inflammation, and cerebral oxygenation in brain dysfunction of acute-on-chronic liver failure patients. Liver Transpl 2016;22:732–742.
[13] Blanche P, Dartigues JF, Jacqmin-Gadda H. Estimating and comparing time-dependent areas under receiver operating characteristic curves for censored event times with competing risks. Stat Med 2013;32:5381–5397.
[14] Hothorn T. Maximally selected rank statistics with several p-value approximations. Version 0.7-25. https://cran.r-project.org/web/packages/maxstat/index.html. 2017.
[15] Terry M. Therneau PMG. A package for survival analysis in R. R package version 3.2-13, https://CRAN.R-project.org/package=survival. 2021.
[16] Kassambara A KM, Biecek P, Fabian S. Drawing survival curves using 'ggplot2'. https://cran.r-project.org/web/packages/survminer/index.html. 2021.
[17] Ishwaran HKU. Fast unified random forests for survival, regression, and classification (RF-SRC). R package version 3.0.0. https://cran.r-project.org/package=randomForestSRC. 2022.
[18] Mogensen UB, Ishwaran H, Gerds TA. Evaluating random forests for survival analysis using prediction error curves. J Stat Softw 2012;50:1–23.
[19] Felipo V, Butterworth RF. Neurobiology of ammonia. Prog Neurobiol 2002;67:259–279.
[20] Albrecht J, Norenberg MD. Glutamine: a Trojan horse in ammonia neurotoxicity. Hepatology 2006;44:788–794.
[21] Jia B, Yu ZJ, Duan ZF, Lü XQ, Li JJ, Liu XR, et al. Hyperammonaemia induces hepatic injury with alteration of gene expression profiles. Liver Int 2014;34:748–758.
[22] Jalan R, De Chiara F, Balasubramaniyan V, Andreola F, Khetan V, Malago M, et al. Ammonia produces pathological changes in human hepatic stellate cells and is a target for therapy of portal hypertension. J Hepatol 2016;64:823–833.
[23] Shawcross DL, Wright GA, Stadlbauer V, Hodges SJ, Davies NA, Wheeler-Jones C, et al. Ammonia impairs neutrophil phagocytic function in liver disease. Hepatology 2008;48:1202–1212.
[24] Kim G, Kang SH, Kim MY, Baik SK. Prognostic value of sarcopenia in patients with liver cirrhosis: a systematic review and meta-analysis. PLoS One 2017;12:e0186990.
[25] Wright G, Noiret L, Olde Damink SW, Jalan R. Interorgan ammonia metabolism in liver failure: the basis of current and future therapies. Liver Int 2011;31:163–175.
[26] Tranah TH, Paolino A, Shawcross DL. Pathophysiological mechanisms of hepatic encephalopathy. Clin Liver Dis (Hoboken) 2015;5:59–63.
[27] De Chiara F, Thomsen KL, Habtesion A, Jones H, Davies N, Gracia-Sancho J, et al. Ammonia scavenging prevents progression of fibrosis in experimental nonalcoholic fatty liver disease. Hepatology 2020;71:874–892.
[28] Eriksen PL, Vilstrup H, Rigbolt K, Suppli MP, Sørensen M, Heebøll S, et al. Non-alcoholic fatty liver disease alters expression of genes governing hepatic nitrogen conversion. Liver Int 2019;39:2094–2101.
[29] Chen Z, Ruan J, Li D, Wang M, Han Z, Qiu W, et al. The role of intestinal bacteria and gut–brain axis in hepatic encephalopathy. Front Cell Infect Microbiol 2021:10.
[30] Olde Damink SW, Jalan R, Redhead DN, Hayes PC, Deutz NE, Soeters PB. Interorgan ammonia and amino acid metabolism in metabolically stable patients with cirrhosis and a TIPSS. Hepatology 2002;36:1163–1171.
[31] Poller L. International Normalized Ratios (INR): the first 20 years. J Thromb Haemost 2004;2:849–860.
[32] Aithal GP, Watkins PB, Andrade RJ, Larrey D, Molokhia M, Takikawa H, et al. Case definition and phenotype standardization in drug-induced liver injury. Clin Pharmacol Ther 2011;89:806–815.