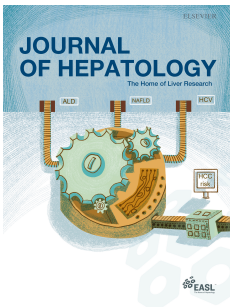

Plasma ammonia levels predict hospitalisation with liver-related complications and mortality in clinically stable outpatients with cirrhosis

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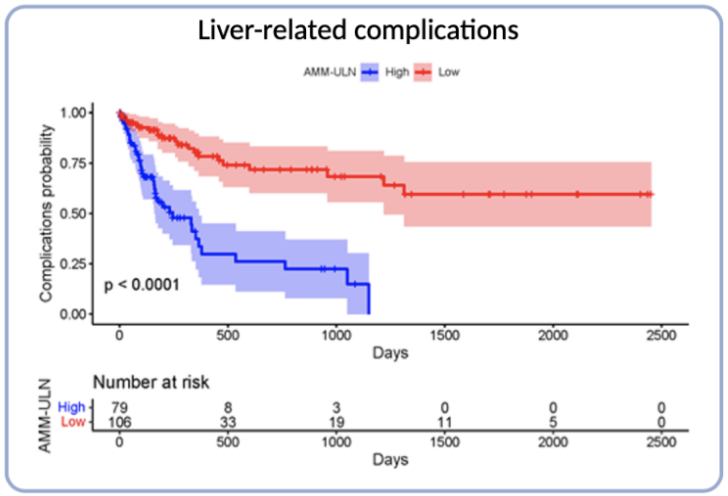
Ammonia is an independent predictor of hospitalization with liver-related complications and mortality in clinically stable outpatients with cirrhosis and performs better than traditional severity scores in predicting liver-related complications.

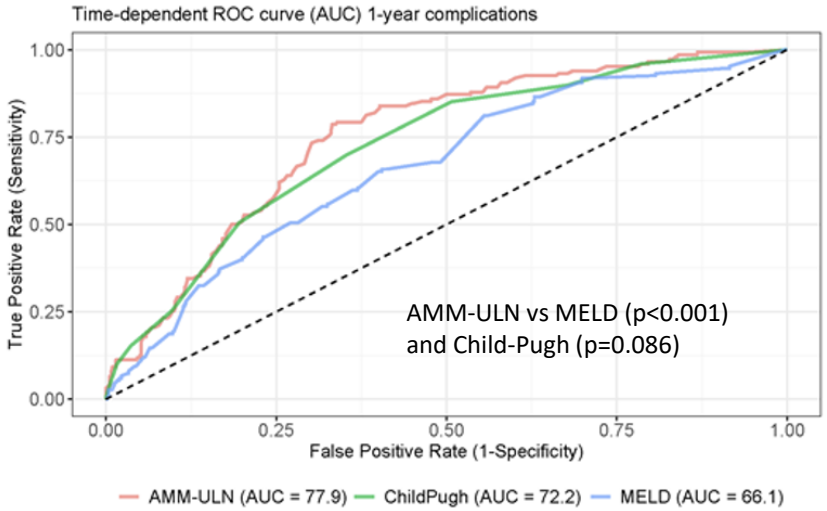
The AMMON Study Consortium

Training and Test Dataset

1.4 times AMM-ULN was the best cut-off to predict time to hospitalization due to liver-related complications in the training cohort

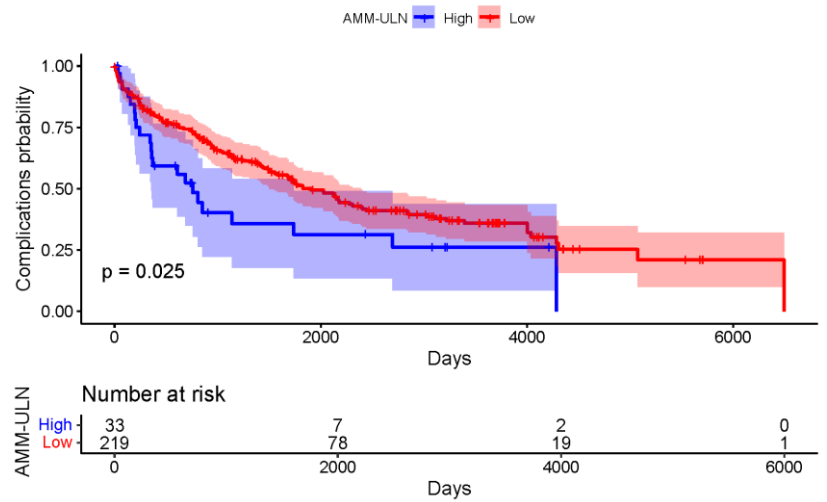
Test cohort





Validation Dataset

independent cohort of 130 patients



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

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Abstract: 275 words. **Manuscript:** 5298 words, 3 Tables, 5 Figures, 34 Pages, 32 References. **Supplementary materials:** 5 Tables, 4 Figures, 17 Pages, 4 References

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

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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.

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Data availability statement: Data is available upon request to the corresponding author.

Abbreviations

ACLF, acute-on-chronic liver failure; ALD, alcohol-related liver disease; ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AMM-ULN, ammonia level corrected to the upper limit of normal; AUROC, area under the receiver operating curve; CI, confidence interval; HCUV, Hospital Clínico Universitario de Valencia; HE, hepatic encephalopathy; INR, international normalised ratio; IQR, interquartile range; KCH, King's College Hospital; MELD, model for end stage liver disease; MELD-Na, MELD-sodium score; NAFLD, non-alcoholic fatty liver disease; r, Pearson rank correlation coefficient; RFH, Royal Free Hospital; SD, standard deviation; TIPS, transjugular intrahepatic portosystemic shunt; VB, variceal bleeding; VIMP, variable importance; WBC, white blood cell count.

KEY-WORDS

Cirrhosis

Ammonia

Liver-related complications

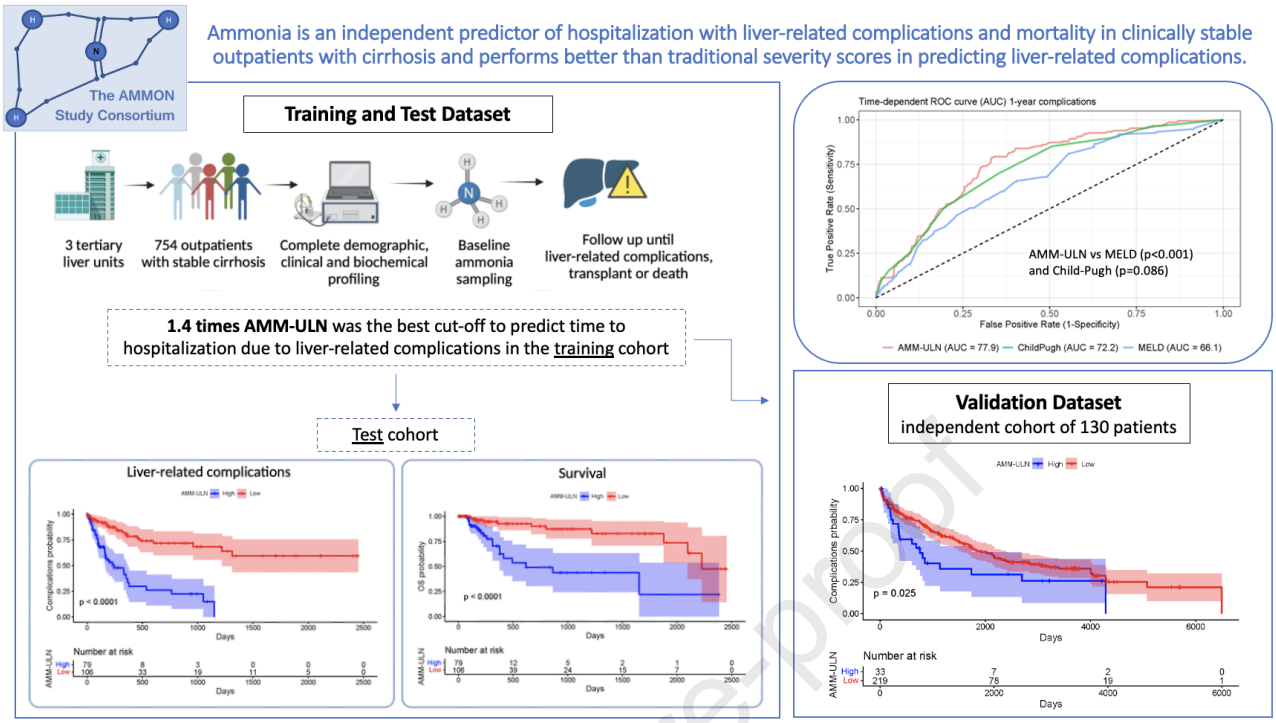
Hepatic encephalopathy

Variceal bleeding

Ascites

Bacterial infection

GRAPHICAL ABSTRACT



HIGHLIGHTS

- Ammonia is an independent predictor of both hospitalisation with liver-related complications and mortality in clinically stable outpatients with cirrhosis.
- Ammonia performs better than traditional severity scores in predicting liver-related complications.
- A cut-off level of 1.4 times 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.

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.

ABSTRACT

Background and Aims: Hyperammonaemia is central in the pathogenesis of hepatic encephalopathy, but also has pleiotropic deleterious effects on several organ systems, impacting on 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 collected data from 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 analysis and fast unified random forest were performed to predict complications and mortality. External validation was carried out using prospective data from 130 cirrhotic patients 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 (HR=2.13; 95%CI=1.89-2.40; p<0.001) and mortality (HR=1.45; 95%CI=1.20-1.76; p<0.001). AUROC of AMM-ULN was 77.9% for 1-year complications, 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.

INTRODUCTION

The Child-Pugh-Turcotte (CP) score and the model for end-stage liver disease (MELD) score are the most utilised non-invasive tools for prediction of survival in cirrhotic patients but are limited by interobserver subjectivity and their initial derivations in predicting survival after surgery and transjugular intrahepatic portosystemic shunt (TIPS), 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 stable compensated cirrhosis patients are also limited. Endoscopic surveillance for varices is useful in determining the risk of variceal bleeding, neuropsychiatric tests provide insight into risk of overt hepatic 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 with impaired metabolism in chronic liver disease that 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 utility 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 (ACLF) 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.

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 four 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, 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 and standard deviation (SD) and non-normally distributed data were reported as median and interquartile range (IQR). Comparisons between hospitals were performed using One-way Analysis of Variance (ANOVA) or Kruskal-Wallis tests for normally and non-normally distributed data, respectively. Categorical data were reported as number and percentage (%) 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 competing risk 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 (Supplementary Figure 1). This value was then used in the test sample to construct Kaplan Meier curves for time to development of liver-related complications and overall survival (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 for survival with 500 trees using log-rank as splitting criteria was used to predict mortality and complications using bootstrapping and cross-validation 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 fourteen models. Performance in risk prediction for each model was evaluated using Brier score. 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 NAFLD was more prevalent in KCH. Severity of liver disease was higher in RFH and KCH compared to HCUV patients with higher CP and MELD scores. Median follow-up of the total cohort was 223 days (range: 2–2453). 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 ACLF, 28% (n=22) of infection, 10% (n=8) of HCC, 12% (n=9) or 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 versus 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) (Supplementary Table 1). 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), 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 three cohorts (KCH: 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 (Supplementary table 2).

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 area under the receiver operating characteristic (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), Figure 1a. AUROC of AMM-ULN for hospitalisation due to liver-related complications at 3- and 6-months and, at 5-years was 73.2%, 74.9% and 74.4%, respectively (Supplementary Figure 2a-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 (Supplementary Table 3). 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 (Figure 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) (Figure 2). Patients in the high-risk group were more likely to have diabetes (42% versus 30%, p=0.001), be receiving lactulose (42% versus 30%, p<0.001) and rifaximin (33% versus 16%, p<0.001) and have more advanced liver disease (MELD-Na 17 versus 14, p<0.001; CP score 9 versus 8, p<0.001) (Supplementary Table 4). Patients in the high-risk group were more likely to require hospitalisation due to liver-related complications (58% versus 17%, p<0.001), including bacterial infections (28% versus 8%, p<0.001), and patients had a higher mortality (23% versus 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; further models with a reduced number of variables had similar margins of prediction error (Figure 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 variable importance (VIMP) is described in Figure 4. Variables that were kept 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. Addition of previous decompensation to the prognostic model did not increase its predictive ability (Supplementary Figure 3).

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 (Supplementary Table 5). Kaplan-Meier analysis with log-rank test using AMM-ULN cut-off of 1.4 again demonstrated significant differences in predicting liver-related complications, p=0.025 (Figure 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 (Supplementary Figure 4).

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 four 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 years. Our data demonstrate that AMM-ULN >1.4 correlates with a significantly higher risk of hospitalisation due to liver-related complications (58% versus 17%, $p<0.001$) and mortality (23% versus 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. The reason for this is not clear but likely to represent potential interaction with ammonia in defining the 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 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 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 extra-hepatic ammonia clearance), renal dysfunction and intestinal dysbiosis incirrhosis.[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 (AIC) = 3119 vs 3131 and 1348 vs 1359, 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 drug-induced liver injury classified as a ratio of the upper limit of normal of alanine aminotransferase (ALT) and aspartate aminotransferase (AST).[32]

The results of this study should be interpreted considering its limitations. First, the study patient cohorts were derived from three 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 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.

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Table 1: Description of patient characteristics in the three 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					
Portal hypertension [n (%)]	708 (94)	148 (98)	127 (81)	433 (97)	<0.001
Hepatocellular carcinoma [n (%)]	79 (10)	0	0	79 (18)	<0.001
Decompensated cirrhosis [n (%)]	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 [n (%)] - A - B 7 compensated - C	 197 (26) 346 (46) 207 (28)	 16 (11) 83 (55) 52 (34)	 106 (69) 42(28) 5 (3)	 75 (17) 221 (50) 150 (34)	<0.001
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					
Transplant [n (%)]	359 (48)	0 (0)	3 (2)	355 (79)	<0.001
Development of liver-related complication [n (%)]	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 (2451)	232 (422)	1385 (2418)	138 (1341)	<0.001

Continuous variables are presented as mean with standard deviation (SD), categorical variables are presented as absolute and relative frequencies [n (%)]. Comparisons were performed between patient cohorts using ANOVA and Kruskal Wallis tests for normally and non-normally distributed data, respectively and Chi-Square χ^2 for categorical data. $p < 0.05$ was considered statistically significant. **Abbreviations:** ALD, alcohol-related liver disease; NAFLD, non-alcoholic fatty liver disease;; WBC, white blood cell count; MELD, model for end-stage liver disease score.

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

Parameter	Univariable model			Multivariable model		
	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 - B - C	 Ref. 2.46 4.21	 Ref. 1.65-3.67 2.72-6.49	 Ref. <0.001 <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. Backward-forward stepwise procedure was conducted for variable selection was performed using Akaike's Information Criterion. **Abbreviations:** HR, hazard ratio; CI, confidence interval; WBC, white blood cells; INR, international normalised ratio

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. Backward-forward stepwise procedure was conducted for variable selection was performed 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. **Abbreviations:** HR, hazard ratio; CI, confidence interval.

FIGURE LEGENDS

Figure 1: Time-dependent AUROC curve. Comparison of ammonia level corrected to the upper limit of normal (AMM-ULN) against Child-Pugh and model for end stage liver disease (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 be higher than the CP score (72.2%, $p=0.086$). AMM-ULN was not significantly higher than MELD ($p=0.073$) and CP ($p=0.803$) score for the prediction of 1-year mortality.

Figure 2: Kaplan Meier plots of hospitalisation due to liver-related complication 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) overall survival (OS) during follow-up. Patients with ammonia level corrected to the upper limit of normal (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.

Figure 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 random forest (RF) modelling with input variables restricted to ammonia level corrected to the upper limit of normal (AMM-ULN), Child Pugh (CP) class, model for end stage liver

disease (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 two 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.

Figure 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 variable importance (VIMP) (right panel). Ammonia level corrected to the upper limit of normal (AMM-ULN) represented the variable with the highest VIMP within the predictive model.

Figure 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. Ammonia level corrected to the upper limit of normal (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.

Figure 1: Time-dependent AUROC curve.

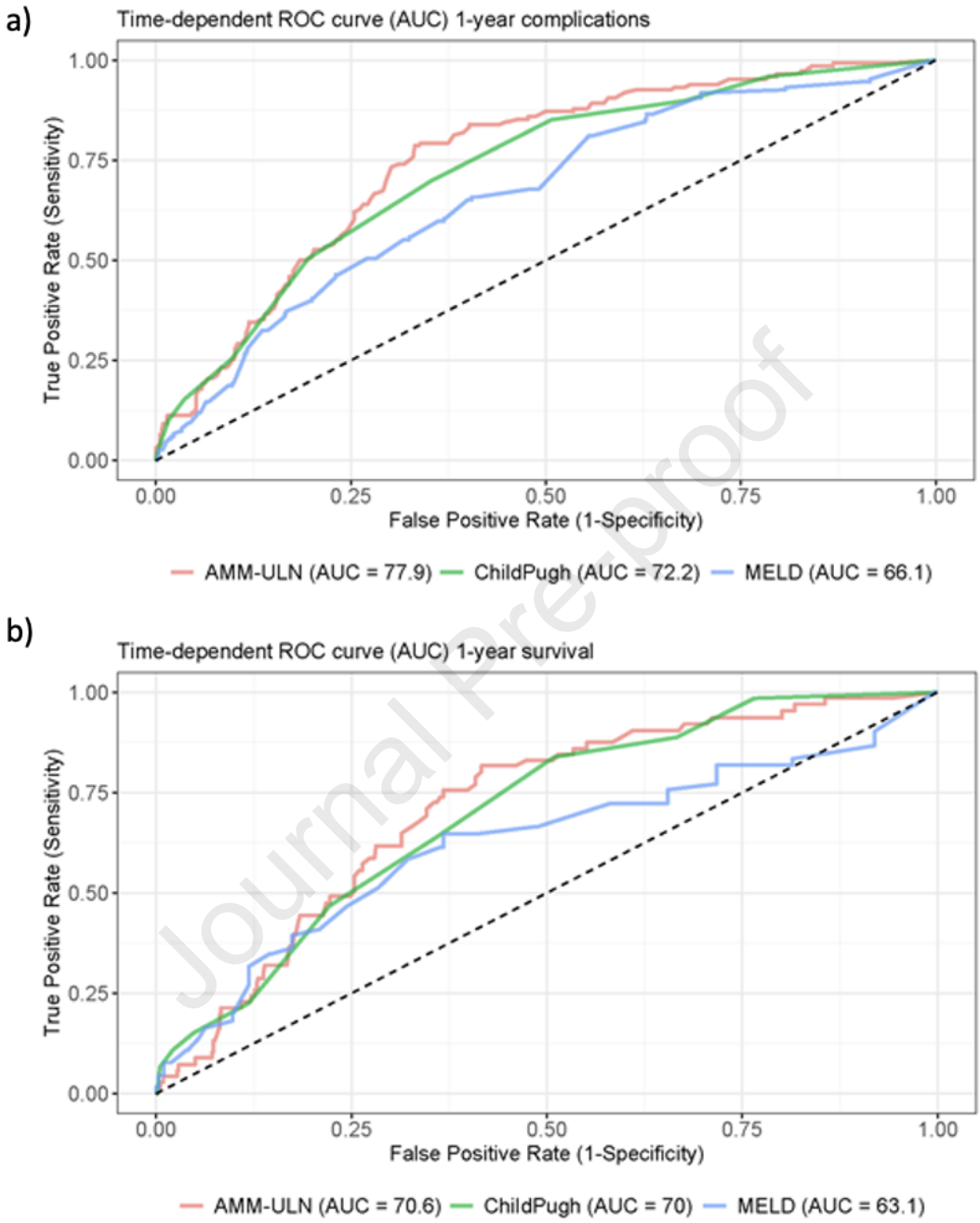


Figure 2: Kaplan Meier plots of hospitalisation due to liver-related complication and mortality.

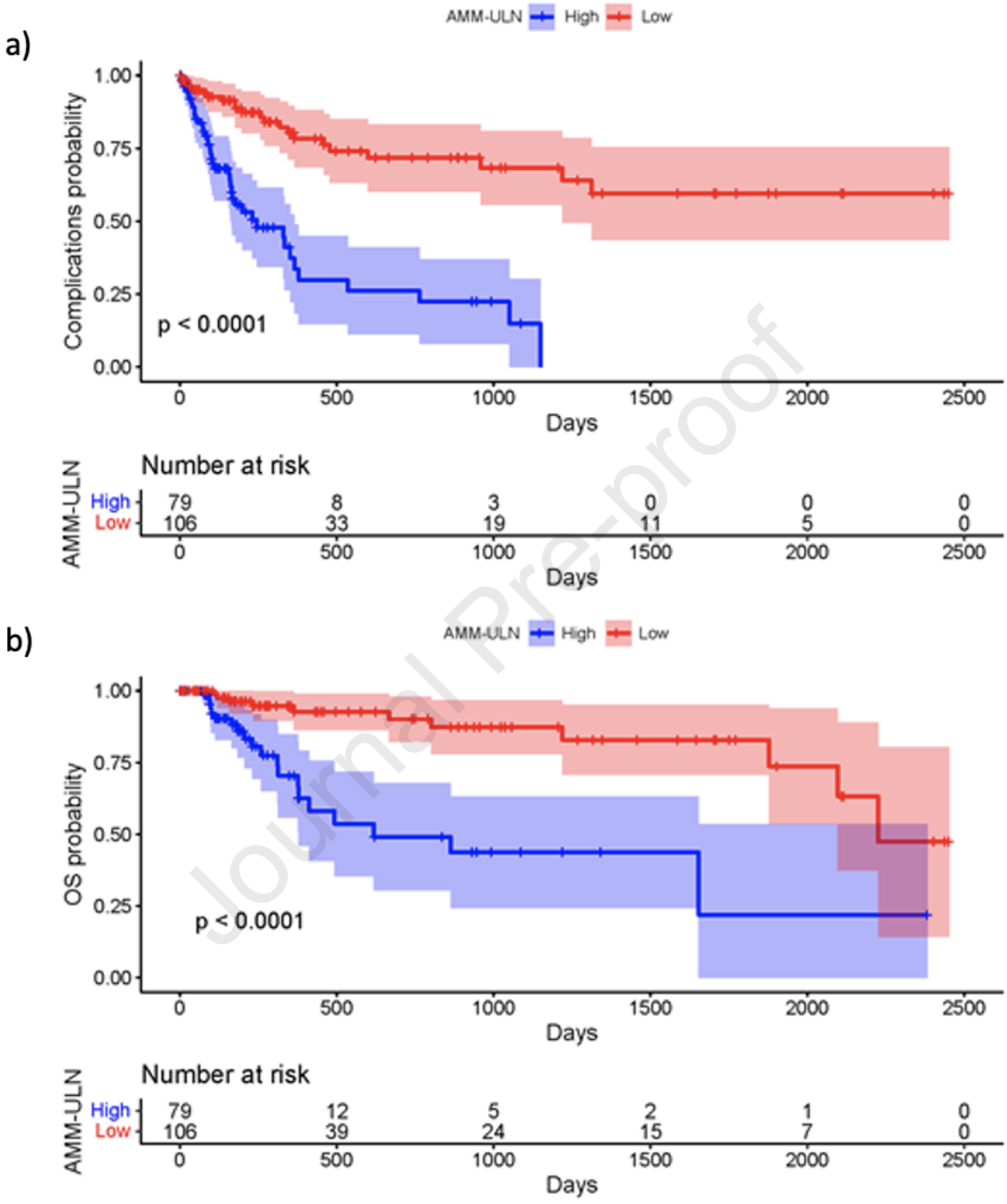


Figure 3: Integrated Brier score to evaluate model performance using bootstrap cross-validation to predict hospitalisation due to liver-related complications

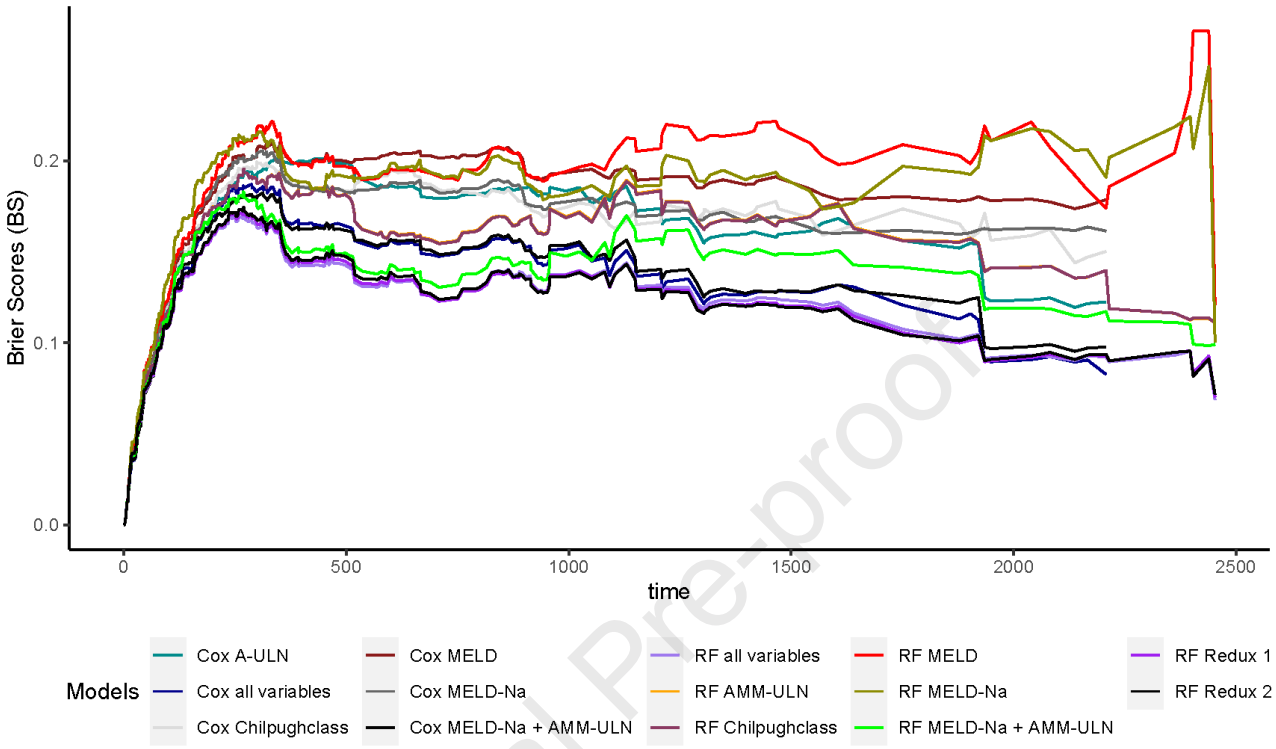


Figure 4: Prognostic model using random forest to predict hospitalisation due to liver-related complications.

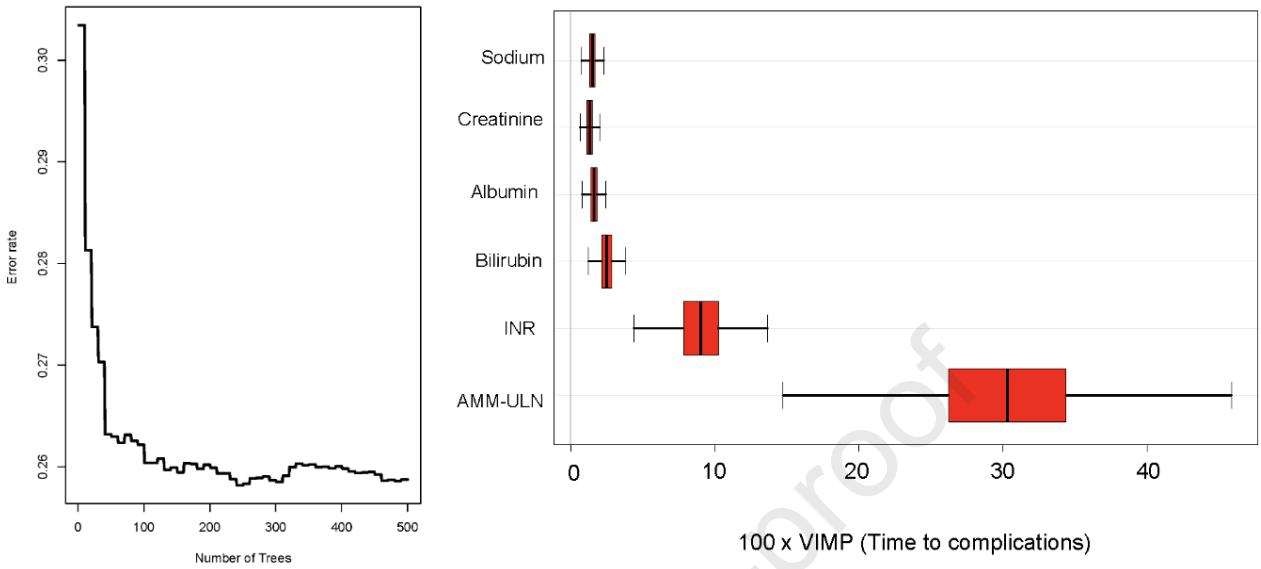
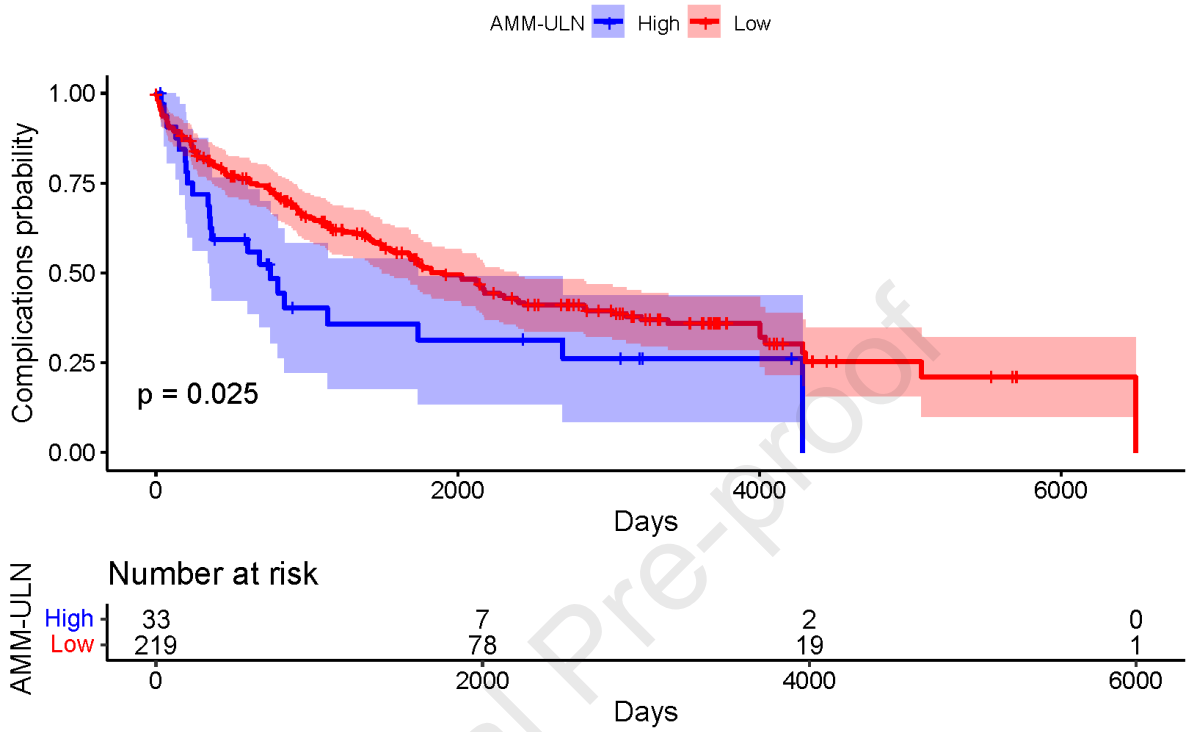


Figure 5: Kaplan Meier plot of hospitalisation due to liver-related complications in the validation cohort



HIGHLIGHTS

- Ammonia is an independent predictor of both hospitalisation with liver-related complications and mortality in clinically stable outpatients with cirrhosis.
- Ammonia performs better than traditional severity scores in predicting liver-related complications.
- A cut-off level of 1.4 times 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.