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In Severe Alcoholic Hepatitis, Serum Keratin-18 Fragments Are Diagnostic, Prognostic, and Theragnostic Biomarkers

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INTRODUCTION: **Up to 40% of patients with severe alcoholic hepatitis (AH) die within 6 months of presentation, making prompt diagnosis and appropriate treatment essential. We determined the associations between serum keratin-18 (K18) and histological features, prognosis, and differential response to prednisolone in patients with severe AH.**

METHODS: **Total (K18-M65) and caspase-cleaved K18 (K18-M30) were quantified in pretreatment sera from 824 patients enrolled in the Steroids or Pentoxifylline for Alcoholic Hepatitis trial (87 with suitable histological samples) and disease controls.**

RESULTS: **K18 fragments were markedly elevated in severe AH and strongly predicted steatohepatitis (alcoholic steatohepatitis) on biopsy (area under receiver operating characteristics: 0.787 and 0.807). Application of published thresholds to predict alcoholic steatohepatitis would have rendered biopsy unnecessary in 84% of all AH cases. K18-M30 and M65 were associated with 90-day mortality, independent of age and Model for End-stage Liver Disease score in untreated patients. The association for K18-M65 was independent of both age and Model for End-stage Liver Disease in prednisolone-treated patients. Modelling of the effect of prednisolone on 90-day mortality as a function of pretreatment serum K18 levels indicated benefit in those with high serum levels of K18-M30. At low pretreatment serum K18 levels, prednisolone was potentially harmful. A threshold of K18-M30 5 kIU/L predicted therapeutic benefit from prednisolone above this level (odds ratio: 0.433, 95% confidence interval: 0.19–0.95, $P = 0.0398$), but not below (odds ratio: 1.271, 95% confidence interval: 0.88–1.84, $P = 0.199$). Restricting prednisolone usage to the former group would have reduced exposure by 87%.**

DISCUSSION: **In a large cohort of patients with severe AH, serum K18 strongly correlated with histological severity, independently associated with 90-day mortality, and predicted response to prednisolone therapy. Quantification of serum K18 levels could assist in clinical decision-making.**

SUPPLEMENTARY MATERIAL accompanies this paper at http://links.lww.com/AJG/B649, http://links.lww.com/AJG/B650, http://links.lww.com/AJG/B651, http://links.lww.com/AJG/B652, http://links.lww.com/AJG/B653, http://links.lww.com/AJG/B654, and http://links.lww.com/AJG/B655

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INTRODUCTION

Alcoholic hepatitis (AH) is a clinical syndrome characterized by the rapid onset of jaundice and liver failure in patients with active chronic, heavy alcohol misuse (1). Up to 40% of patients with severe AH, defined as a Maddrey's discriminant function (DF) > 32 (2), die within 6 months of presentation, making prompt diagnosis and appropriate treatment essential (3). A number of algorithms have been used to predict outcomes in severe AH including DF (2), the Glasgow AH score (4), and the Age-Bilirubin-INR-Creatinine score (5); all of these have modest performance characteristics in assessing short-term mortality (6). The Model for End-stage Liver Disease (MELD) score has been used with varying

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cutoff values between studies but performs best when used as a continuous variable in individual patients (6). Notably, the correlation between these scoring systems and the histopathological features of alcoholic steatohepatitis (ASH) remains poor (7). The latter include hepatocyte ballooning, neutrophil infiltration, and Mallory-Denk bodies. A histologic scoring system has been developed that independently correlates with 90-day mortality (8). However, a combination of expense, practicality, and availability often means that it is not feasible to perform liver biopsy (9).

Acute hepatocellular injury in AH results in necrosis and apoptosis. This is likely secondary to increased gastrointestinal permeability with translocation of bacterial products into the portal and systemic circulations with an intense inflammatory reaction resulting from both processes (10). These acute events take place in a setting of pre-existing chronic liver disease with a variable amount of liver fibrosis and consequently decreased hepatocellular reserve. Correspondingly, of the commonly analyzed histological features, the presence of bridging liver fibrosis constitutes the strongest negative prognostic marker, whereas severe neutrophil infiltration and occurrence of megamitochondria are signs associated with more favorable outcomes (8). Previous data indicate that marked neutrophilic infiltration confers a better prognosis in prednisolone-treated patients (11), suggesting that those with the most severe acute, inflammatory disease might benefit most from immunosuppressive treatments.

Hepatocellular injury markers are routinely used in the diagnosis of severe AH. Aspartate transaminase (AST) levels above 50 IU/L, and AST:alanine transaminase (ALT) ratios >1.5 are considered diagnostic for AH, although the levels are generally below 400 IU/L (9). Keratin-18 (K18) is an abundant, cytoplasmic protein expressed in glandular and single-layered epithelia (12,13). Serum levels of K18 fragments have been widely used as markers of hepatocellular death (14,15). The M65 antibody-detected protein reflects total cell death, whereas the M30 antibody-detected fragment is generated when K18 is cleaved during apoptosis (14). K18 fragment serum have been shown to be markedly elevated in the serum of patients with AH, and their ability to distinguish them from healthy controls and individuals with alcoholic cirrhosis indicates diagnostic utility (7,16). Moreover, in multiple liver disorders such as alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD), or chronic hepatitis C infection (17–19), K18 serum fragments mirrored the extent of histological disease activity.

Corticosteroids are the first-line treatment option in severe AH (1). However, their use only confers a minor improvement in the 28-day mortality (20–22). Furthermore, steroids carry a risk of severe adverse events, particularly they predispose to the development of infection (23,24). Consequently, careful patient selection is required to ensure those treated derive benefit, and others are not unduly exposed to the risk of infection, especially when liver transplantation remains a potential treatment option (25).

We hypothesized that the degree of acute hepatocellular injury and inflammation might determine the prognosis of patients with AH and identify individuals who will benefit from corticosteroid therapy. To test this hypothesis, we determined the relationship between serum K18 fragments and histological features, outcome, and response to prednisolone in participants recruited to the Steroids or Pentoxifylline for Alcoholic Hepatitis (STOPAH) clinical trial (ISRCTN:88782125).

METHODS

Study populations

Cases with severe AH were recruited via the STOPAH trial as per the trial protocol (26,27) (see Supplemental Information, Supplementary Digital Content 7, http://links.lww.com/AJG/B655). Patients were randomized to treatment with prednisolone or pentoxifylline for 28 days using a double-blind, double-dummy factorial 2 × 2 design. Outcome data were collected for mortality at 28 and 90 days. Ethical approval was granted for this study by the Wales Research Ethics Committee (REC 09/MRE09/59). Patients with alcoholic cirrhosis recruited to the prospective longitudinal Compensated Cirrhosis Cohort in Nottingham study (Ref 10/H0403/10; approved by East Midlands Nottingham 1 ethics committee) were used as an additional control group (see Supplemental Information, Supplementary Digital Content 7, http://links.lww.com/AJG/B655). The study was conducted according to the Declaration of Helsinki (Hong Kong Amendment) and Good Clinical Practice (European guidelines). All participants, or their legally-appointed representatives, provided written informed consent.

Measurement of laboratory parameters

Clinical data were recorded at the baseline visit. Pretreatment serum samples were available from 866 patients. Sufficient serum was available for the quantification of K18-M65 and K18-M30 by ELISA (VLVbio, Stockholm, Sweden) in 824 patients (95%). Estimation was performed according to the manufacturer's instruction; samples with high values outside the standard curve were reassessed after dilution in "Buffer A." AST was determined in the serum from patients where clinical data were unavailable, as detailed in Supplemental Information (see Supplementary Digital Content 7, http://links.lww.com/AJG/B655).

Histological analyses

Two experienced histopathologists (R.G. and A.Q.), blinded to patient treatment and outcomes, independently assessed the histological features of each biopsy using the AH histological scoring system (8), and fibrosis was graded using the Laennec system (28) (see Supplementary Table 1, Supplementary Digital Content 7, http://links.lww.com/AJG/B655). The presence or absence of Mallory-Denk bodies and megamitochondria was also recorded.

Data processing and statistical analysis

MELD (29) and DF prognostic scores were derived in accordance with their original descriptions (30). Statistical analyses were conducted in R (R Foundation, Vienna, Austria) using "DescTools," "dplyr," "qwraps2," "pROC," "OptimalCutpoints," "ggplot2," "survival," and "psych." Specific statistical tests used for baseline data analysis are detailed in Supplemental Information (see Supplementary Digital Content 7, http://links.lww.com/AJG/B655).

Mortality associations

To provide independent populations for mortality association testing, the cohort was split into exploratory and validation groups based on the trial's random assignment to receive prednisolone. This approach was chosen to (i) leverage the original randomization structure that incorporated factors including baseline risk and recruiting center and (ii) demonstrate validity of associations with and without prednisolone treatment. Several studies and meta-analyses have failed to demonstrate an effect of pentoxifylline on 28-day or subsequent mortality either alone or in combination with prednisolone; consequently, pentoxifylline treatment was not incorporated into statistical modelling. Ninety-day mortality was considered the primary outcome with death at 28 days used in secondary analyses. Logistic regression

Table 1. Baseline demographics of populations based on biopsy status

Variable median (interquartile range) or "n" (%)	Biopsy cohort (n = 87)	Nonbiopsy cohort (n = 737)
Age (yr)	47.0 (42.5–53.8)	49.1 (41.9–56.6)
Sex (male)	60 (69%)	460 (62%)
Alcohol (units/wk)	120 (84–196)	132 (84–210)
Encephalopathy		
Grade 0	61 (72%)	522 (74%)
Grade 1	21 (25%)	123 (17%)
Grade 2	2 (2%)	42 (6%)
Grade 3	1 (1%)	16 (2%)
Mortality at 90 d	15 (17%)	179 (24%)
Hemoglobin (g/L)	106 (95–118)	107 (94–120)
White cell count ($\times 10^6$/mm^3)	9.5 (6.2–12.3)	8.5 (6.1–12.3)
Neutrophils ($\times 10^6$/mm^3)	6.5 (3.9–9.6)	6.0 (4.1–9.6)
Bilirubin (μmol/L)	319 (192–452)	258 (161–401)
ALT (IU/L)	42 (29–53)	42 (30–61)
AST (IU/L)	120 (85–153)	115 (85–158)
Albumin (g/L)	26 (22–30)	25 (21–29)
Urea (mmol/L)	3.6 (2.5–6.5)	3.2 (2.2–5.1)
Creatinine (μmol/L)	69 (58–102)	63 (51–82)
INR	1.7 (1.5–2.0)	1.8 (1.6–2.1)
DF	56 (41–77)	55 (43–74)
MELD	24 (21–28)	23 (21–26)
K18-M30 (kIU/L)	1.98 (0.91–3.65)	1.81 (0.97–3.35)
K18-M65 (kIU/L)	4.87 (2.54–7.44)	4.36 (2.53–6.74)
K18-M30:M65 ratio	0.43 (0.36–0.57)	0.45 (0.39–0.53)

ALT, alanine transaminase; AST, aspartate transaminase; DF, Maddrey's discriminant function; INR, international normalised ratio; K18, keratin-18; MELD, Model for End-stage Liver Disease.

was used to test for associations with either 28- or 90-day mortality. Multivariate models were specified including the MELD score and age as covariates. Area Under the Receiver Operating Characteristics (AUROC) analysis was used to assess predictive performance. Models combining the MELD and serum biomarkers were generated using logistic regression analysis and leave one of 10-fold cross-validation in the exploratory cohort to avoid over-optimism; the model generated was subsequently tested in the validation cohort.

Interaction testing

To detect any influence of K18 on prednisolone efficacy, we performed interaction testing in the entire cohort (see Supplemental Information, Supplementary Digital Content 7, http://links.lww.com/AJG/B655). To explore the implied relationship between prednisolone, K18, and mortality, the adjusted interaction models were used to generate 90-day mortality risks with, and without, prednisolone across a range of K18 values. Age and MELD were set at the mean values for the overall data set. Plots were generated estimating the effect of prednisolone across the range of K18 values, candidate cutoffs were selected based on maximal divergence of the confidence intervals (CIs) of prednisolone effect in those above and below the selected level. Survival curves were generated to illustrate the differences in outcome between populations defined by any cutoff and prednisolone treatment status. The same methodology was applied to detect interactions between the Lille score and prednisolone in relation to mortality.

Infection associations

Prednisolone administration has previously been associated with an excess of infection, including in this cohort. Severe infection was defined as previously described (24,27). The association between prednisolone and severe infection was tested using logistic regression. Analyses were additionally adjusted for age and MELD score.

RESULTS

Composition of the study cohort and serum K18 levels

The 824 individuals from the STOPAH cohort included in this study had similar baseline characteristics (Table 1) to those previously described for the full trial cohort (27). ALT levels were within the normal range or only slightly increased, whereas AST was typically twice or 3 times higher than the upper limit of normal (31). Serum K18 fragments were successfully quantified in 814/824 cases (98.8%). Serum K18-M30 values ranged from 125 to 82,660 IU; the median value was 1812.78, at least 7 times higher than the values typically seen in healthy individuals. K18-M65 values ranged from 296 to 71,852 IU/L, with a median of 4,381, i.e., >10 times higher than the 95% percentile defined for the normal population (32).

In the control group of patients with alcoholic cirrhosis including compensated (n = 76) and decompensated cirrhosis (n = 10), K18-M30 levels ranged from 31 to 989 IU/L and K18-M65 ranged 24 to 1968 (see Supplementary Table 2, Supplementary Digital Content 7, http://links.lww.com/AJG/B655). The ratio of K18-M30 to K18-M65 was calculated and ranged from 0.1 to 1.55; the median was 0.45. In view of their magnitude, values are reported as kIU/L (1 kIU/L = 1,000 IU/L).

Histological analyses

In total, 87 liver biopsy samples from patients with serum K18 measurements were eligible for histological analyses. In 79 cases (91%), there was a definite histological diagnosis of ASH. The biopsy cohort demonstrated similar baseline characteristics to the overall cohort though with somewhat lower 90-day mortality (Table 1).

Increasing Laennec fibrosis stage was associated with decreasing serum K18-M30 ($P = 0.00523$) and K18-M65 ($P = 0.00207$) (Figure 1a, b). K18-M30 and K18-M65 levels were significantly greater in cases with severe inflammation on biopsy (both $P < 0.0001$) and marked hepatocyte ballooning (M30: $P = 0.000431$, M65: $P = 0.000675$; Figure 1c–f). Cases where Mallory-Denk body formation was observed also disclosed significantly higher K18-M30 (median: 2.82 kIU/L [Interquartile range (IQR): 1.15–4.54] *vs* 1.04 kIU/L [0.80–1.77], $P = 0.000149$) and K18-M65 (5.98 kIU/L [3.86–8.36] *vs* 2.77 kIU/L [2.00–4.87], $P < 0.0001$; see

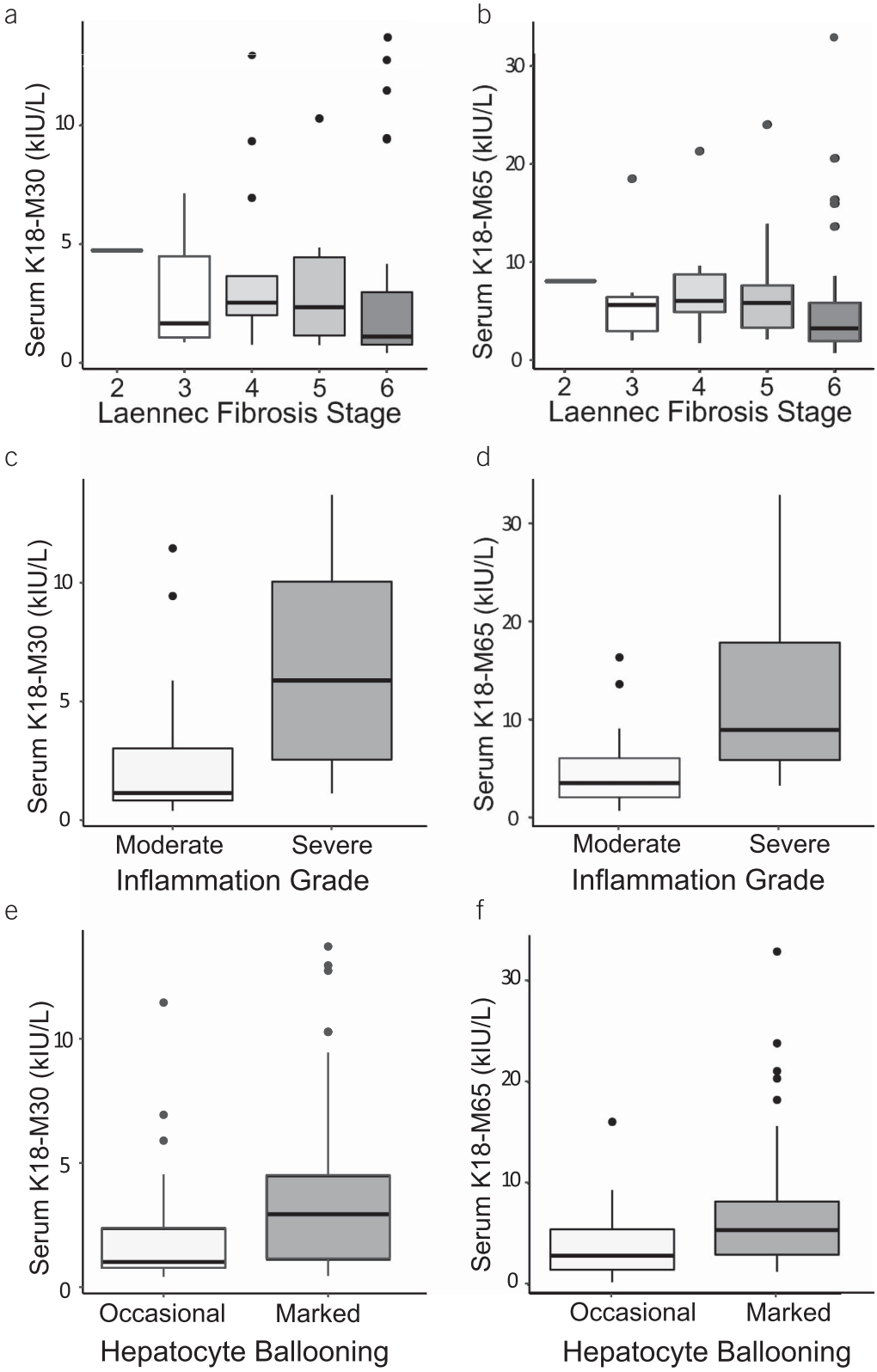

Figure 1. Levels of serum K18-M30 and K18-M65 in patients with different histological Laenec fibrosis grades (**a**, **b**), inflammation severities (**c**, **d**), and hepatocyte ballooning severities (**e**, **f**) according to the alcoholic hepatitis histological scoring system. Data are displayed as the median (solid bar), interquartile range (box), and 95% confidence interval (whiskers). K18, keratin-18.

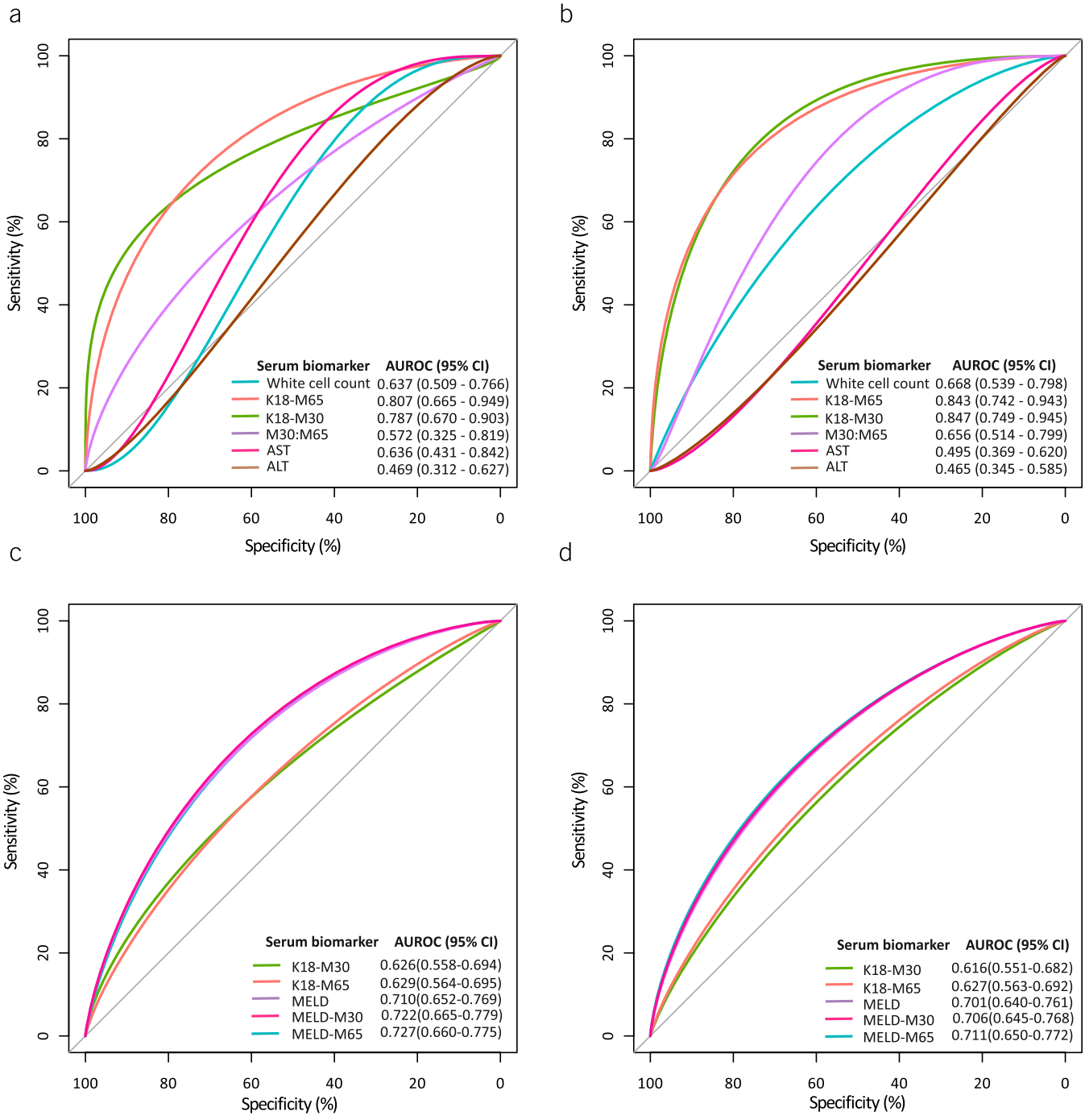

Figure 2. Receiver operating characteristic (ROC) curves for the confirmation of histological alcoholic steatohepatitis (ASH) in patients with clinically diagnosed alcoholic hepatitis (AH) (a) and the presence of severe inflammation in patients with clinically diagnosed AH and ASH on biopsy (b). ROC curves for the prediction of 90-day mortality by serum keratin 18 fragments alone or combination with the Model for End-stage Liver Disease score in patients with clinically diagnosed AH and treated without (c) or with (d) prednisolone. ALT, alanine transaminase; AST, aspartate transaminase; CI, confidence interval; K18, keratin-18; MELD, Model for End-stage Liver Disease.

Supplementary Figure 1a, b, Supplementary Digital Content 1, http://links.lww.com/AJG/B649).

The M30:M65 ratio was significantly higher in cases with severe inflammation (0.49 [0.41–0.63] *vs* 0.42 [0.35–0.54], $P = 0.0400$; see Supplementary Figure 2b, Supplementary Digital Content 2, http://links.lww.com/AJG/B650). However, it did not differ significantly in groups defined by Laennec fibrosis stage ($P = 0.510$), the presence of marked hepatocyte ballooning (0.42 [0.38–0.59] *vs* 0.46 [0.35–0.55], $P = 0.800$), or the presence of Mallory-Denk bodies (0.46 [0.39–0.59] *vs* 0.41 [0.34–0.56], $P = 0.111$; see Supplementary Figure 2a, c, Supplementary Digital Content 2, http://links.lww.com/AJG/B650 and Supplementary Figure 1c, Supplementary Digital Content 1, http://links.lww.com/AJG/B649).

Table 2. Logistic regression analyses for serum K18 fragments in relation to 90-day mortality

	Exploratory (− prednisolone)			Validation (+ prednisolone)		
	Univariate analysis					
	OR[a]	95% CI	*P*	OR[a]	95% CI	*P*
K18-M30 (kIU/L)	1.098	1.037–1.167	0.0016	1.080	1.023–1.151	0.0126
K18-M65 (kIU/L)	1.060	1.020–1.102	0.0032	1.063	1.025–1.107	0.0019
M30:M65 ratio	6.354	1.682–24.46	0.0064	1.104	0.223–4.897	0.8989
	Exploratory (− prednisolone)			Validation (+ prednisolone)		
	Multivariate analysis for K18-M30					
	OR[a]	95% CI	*P*	OR[a]	95% CI	*P*
Age (yr)	1.038	1.013–1.065	0.0036	1.097	1.065–1.131	<0.001
MELD (points)	1.202	1.132–1.283	<0.001	1.166	1.106–1.233	<0.001
K18-M30 (kIU/L)	1.080	1.020–1.147	0.0092	1.059	1.000–1.137	0.0962
	Exploratory (− prednisolone)			Validation (+ prednisolone)		
	Multivariate analysis for K18-M65					
	OR[a]	95% CI	*P*	OR[a]	95% CI	*P*
Age (yr)	1.039	1.014–1.066	0.0025	1.097	1.066–1.132	<0.001
MELD (points)	1.199	1.129–1.280	<0.001	1.162	1.102–1.229	<0.001
K18-M65 (kIU/L)	1.044	1.000–1.090	0.0438	1.0000	1.008–1.100	0.0278

CI, confidence interval; K18, keratin-18; MELD, Model for End-stage Liver Disease; OR, odds ratio.
[a]ORs are quoted per 1 unit increase in the predictor variable. For K18-M30 and -M65, the ORs are per 1 kIU/L increase in serum concentration; for age, it is per additional year and MELD score per whole point increase. For K18 M30:M65 ratios, the OR is quoted per whole number increase.

None of the K18-M30 (1.14 kIU/L [0.89–3.73] *vs* 2.32 kIU/L [1.08–3.64], $P = 0.331$), K18-M65 (3.48 kIU/L [2.10–7.04] *vs* 5.11 kIU/L [3.15–7.76], $P = 0.250$), or M30:M65 ratios (0.40 [0.35–0.53] *vs* 0.47 [0.39–0.59], $P = 0.141$) differed significantly between cases defined by the presence or absence of mega-mitochondria (see Supplementary Figure 1d–f, Supplementary Digital Content 1, http://links.lww.com/AJG/B649).

Circulating K18 fragments as diagnostic markers

The utility of serum K18 fragments to predict definite histological ASH and the presence of severe inflammation was evaluated. Comparison was made with the white cell count and serum AST and ALT values as traditional markers of disease activity.

The AUROC for K18-M65 for the prediction of ASH was 0.807 (95% CI: 0.665–0.949) with K18-M30 demonstrating a similar predictive capacity (0.787 [95% CI: 0.670–0.903]). All of the K18-M30:M65 ratios, white cell count, and serum ALT and AST demonstrated moderate-to-poor predictive capacity (Figure 2a).

In the 79 cases with definitive ASH analyzed, 60 (76%) had moderate inflammation with the remainder classified as severe. The AUROC for K18-M30 was 0.847 (95% CI: 0.749–0.945), whereas for K18-M65, it was 0.843 (95% CI: 0.742–0.943). None of the K18 M30:M65 ratios, white cell count, and serum AST or ALT demonstrated reasonable performance in the prediction of severe inflammation (Figure 2b).

Evaluation of previously reported diagnostic thresholds

Previous studies have reported a serum K18-M65 >2 kIU/L as having a high positive predictive value for the presence of ASH on biopsy in patients with clinically suspected AH (7). In the biopsy cohort included in histological analyses, K18-M65 >2 kIU/L had a sensitivity and specificity of 85% and 50%, respectively, for the presence of ASH. Accordingly, the positive predicted value (PPV) was 94%, whereas the negative predictive value (NPV) was 25%. Conversely, the serum K18-M65 levels <0.65 kIU/L were reported to have a high NPV for the presence of steatohepatitis. The lowest serum K18-M65 level in this cohort was 0.708 kIU/L; thus, the previously published lower cutoff was not assessed.

When considered in the context of the entire cohort, 679/814 (83.4%) patients had a serum K18-M65 value >2 kIU/L, whereas only 2 individuals (0.25%) disclosed a serum K18 level <0.65 kIU/L. Consequently, using a serum K18-M65 <2 kIU/L but >0.65 kIU/L to define patients meeting clinical criteria for severe AH but requiring confirmatory liver biopsy would achieve an 84% reduction in the numbers requiring biopsy.

In the control compensated (n = 76) and decompensated (n = 10) alcoholic cirrhosis cohorts, none had K18-M65 >2 kIU/L; levels were <0.65 kIU/L in 79/84; median was 0.119 kIU/L (IQR: 0.024–0.213) in the compensated and 0.134 kIU/L (IQR: 0.042–0.225) in decompensated groups (see Supplementary Table 2, Supplementary Digital Content 7, http://links.lww.com/AJG/B655).

Clinical correlates of serum K18 fragments

Serum K18 fragments were not correlated with age (M30: rho = 0.011, q = 0.78; M65: rho = 0.023, $P = 0.64$, see Supplementary Figure 3, Supplementary Digital Content 3, http://links.lww.com/AJG/B651). Weak positive correlations were noted with baseline scores of liver dysfunction (MELD—M30: rho = 0.21, q < 0.01; M65: rho = 0.24, q < 0.01; DF—M30: rho = 0.11, q < 0.01; M65: rho = 0.11, q < 0.01) and the Lille score (see Supplementary

Table 3. Logistic regression interaction analyses for serum K18-M30 and K18-M65 with prednisolone in relation to 90-day mortality

Model A: interaction between prednisolone and K18-M30			
	OR[a]	95% CI	*P*
Prednisolone	1.498	0.842–2.703	0.174
K18-M30 (kIU/L)	1.305	1.132–1.537	0.0006
K18-M30^2	1.000	1.000–1.000	0.0281
Age (yr)	1.066	1.046–1.087	<0.0001
MELD	1.171	1.125–1.221	<0.0001
Prednisolone:K18-M30	0.814	0.675–0.965	0.0237
Prednisolone:K18-M30^2	1.000	1.000–1.000	0.0397
Model B: interaction between prednisolone and K18-M65			
	OR	95% CI	*P*
Prednisolone	1.806	0.812–4.138	0.154
K18-M65 (kIU/L)	1.286	1.114–1.501	0.0009
K18-M65^2	1.000	1.000–1.000	0.0068
Age (yr)	1.065	1.045–1.085	<0.0001
MELD	1.168	1.122–1.218	<0.0001
Prednisolone:K18-M65	0.830	0.698–0.978	0.0300
Prednisolone:K18-M65^2	1.000	1.000–1.000	0.0160

CI, confidence interval; K18, keratin-18; MELD, Model for End-stage Liver Disease; OR, odds ratio.
[a]ORs are quoted per 1 unit increase in the predictor variable. For K18-M30 and -M65, the ORs are per 1 kIU/L increase in serum concentration; for age, it is per additional year and MELD score per whole point increase.

Figures 3 and 4, Supplementary Digital Content 3 and 4, http://links.lww.com/AJG/B651 and http://links.lww.com/AJG/B652). A modest correlation with the serum AST level was noted for both the M30 (rho = 0.22, q < 0.01) and M65 fragments (rho = 0.27, q < 0.01). Stronger correlations were seen with the circulating total white cell count (M30: rho = 0.40, q < 0.01; M65: rho = 0.41, q < 0.01) and the neutrophil count (M30: rho = 0.43, q < 0.01; M65: rho = 0.45, q < 0.01). By contrast, the M30:M65 ratio did not show strong correlations with any baseline clinicodemographic variables (see Supplementary Figure 3, Supplementary Digital Content 3, http://links.lww.com/AJG/B651).

Analysis of prognostic associations of K18 fragments

The association between serum K18 fragments and 90-day mortality was examined initially in prednisolone-untreated patients (n = 417). The population treated with prednisolone (n = 407) was used to validate these findings. The 2 cohorts were well matched for size, baseline demographics, including exposure to pentoxifylline, and outcomes (see Supplementary Table 3, Supplementary Digital Content 7, http://links.lww.com/AJG/B649).

Serum K18-M30 (odds ratio [OR]: 1.098, 95% CI: 1.037–1.167, *P* = 0.0016), K18-M65 (OR: 1.060, 95% CI: 1.020–1.102, *P* = 0.0032) and the M30:M65 ratios (OR: 6.35, 95% CI: 1.68–24.5, *P* = 0.0064) were all significantly associated with 90-day mortality in prednisolone-untreated patients. In multivariate analyses, both the K18-M30 and K18-M65 demonstrated an association with mortality, independent of age and MELD score (Table 2). This pattern of associations was also replicated for 28-day mortality (see Supplementary Table 4, Supplementary Digital Content 4, http://links.lww.com/AJG/B649).

The associations of K18-M30 (OR: 1.080, 95% CI: 1.023–1.151, *P* = 0.0126) and K18-M65 (OR: 1.063, 95% CI: 1.025–1.107, *P* = 0.0019) with 90-day mortality were replicated in prednisolone-treated patients (Table 2). The M30:M65 ratio was not associated with 90-day mortality in the validation cohort (OR: 1.104, 95% CI: 0.223–4.897, *P* = 0.899). When adjusted for age and MELD score, K18-M65 retained independent significance. Again, the same pattern of associations was observed for 28-day mortality (see Supplementary Table 4, Supplementary Digital Content 4, http://links.lww.com/AJG/B649).

Prediction of mortality

In relation to 90-day mortality, the AUROC for K18-M30 was 0.626 (95% CI: 0.558–0.694) in the exploratory cohort and 0.616 (95% CI: 0.551–0.682) in the validation cohort (Figure 2c, d). Values for serum K18-M65 were similar with AUROCs of 0.629 (95% CI: 0.564–0.695) and 0.627 (95% CI: 0.563–0.692) in the exploratory and validation cohorts, respectively. By comparison, the equivalent AUROCs for the MELD score were 0.710 (95% CI: 0.652–0.769) and 0.701 (95% CI: 0.640–0.761) in the 2 cohorts. Scores combining the MELD score and either K18-M30 or K18-M65 demonstrated an incremental increase in the AUROC in the exploratory cohort (MELD-M30: 0.722 [95% CI: 0.665–0.779]; MELD-M65: 0.727 [95% CI: 0.660–0.775]) (Figure 2c). A similar effect was seen in the validation population (MELD-M30: 0.706 [95% CI: 0.645–0.768]; MELD-M65: 0.711 [95% CI: 0.650–0.772] (Figure 2d).

For 28-day mortality, the analyses demonstrated similar results with AUROCs ranging from 0.634 to 0.677 for K18 fragments alone (see Supplementary Figure 5, Supplementary Digital Content 5, http://links.lww.com/AJG/B653) compared with 0.718–0.791 for the MELD score. In both the exploratory and validation cohorts, the combination of MELD and K18 fragments yielded a modest numerical improvement in the AUROC.

Interaction testing with prednisolone

In models assessing a multiplicative interaction, together with lower order effects, between either K18-M30 or K18-M65 and prednisolone, the interaction terms were not significant (*P* = 0.704 and *P* = 0.925, respectively). Incorporation of a quadratic interaction between prednisolone and K18 revealed significant interactions for both K18-M30 and K18-M65. Likelihood ratios testing indicated a significant improvement in fit with incorporation of the interaction terms (K18-M30: *P* = 0.00437; K18-M65: *P* = 0.00179). The interaction terms remained significant when the models were additionally adjusted for age and MELD (Table 3). No significant interaction was noted between the M30:M65 ratio and prednisolone either when modelled as a multiplicative or curvilinear effect (see Supplementary Table 5, Supplementary Digital Content 5, http://links.lww.com/AJG/B649, data not shown). Evaluation of predicted 90-day mortality risks as a function of serum K18 levels in patients treated with and without prednisolone demonstrated that at the lowest serum K18 levels, prednisolone was associated with a trend toward an increased risk of death. At higher serum K18-M30 and M65 levels, prednisolone therapy was associated with a reduced 90-day mortality risk (Figure 3a, b). Examination of prednisolone effect in subgroups defined by serial K18 fragment cutoffs indicated no

Table 4. Baseline demographics of populations defined by serum K18-M30 above or below 5 kIU/L

Variable median (interquartile range) or "n" (%)	Low K18-M30[a] (n = 710)	High K18-M30[a] (n = 104)
Age (yr)	48.8 (41.8–56.1)	49.5 (43–57.1)
Sex (male)	449 (63%)	65 (62%)
Alcohol (units/wk)	130 (85–208)	140 (84–210)
Pentoxifylline	355 (50%)	54 (52%)
Encephalopathy		
Grade 0	499 (73%)	76 (78%)
Grade 1	129 (19%)	14 (14%)
Grade 2	38 (6%)	5 (5%)
Grade 3	15 (2%)	2 (2%)
90-day mortality	145 (20%)	46 (44%)
Haemoglobin (g/L)	107 (94–120)	103 (93–119)
White cell count ($\times 10^6$/mm^3)	8.2 (5.9–11.7)	12.6 (8.8–14.8)
Neutrophils ($\times 10^6$/mm^3)	5.7 (3.9–9.9)	9.8 (6.1–12.1)
Bilirubin (μmol/L)	260 (158–403)	320 (205–420)
ALT (IU/L)	41 (29–61)	46 (30–61)
AST (IU/L)	114 (85–154)	141 (107–177)
Albumin (g/L)	25 (21–29)	26 (22–30)
Urea (mmol/L)	3.1 (2.1–5.0)	4.7 (2.9–8.0)
Creatinine (μmol/L)	63 (52–80)	74 (55–101)
INR	1.8 (1.6–2.0)	1.8 (1.5–2.1)
DF	55 (43–73)	59 (46–77)
MELD	23 (21–26)	24 (22–28)
K18-M30 (kIU/L)	1.42 (0.89–2.65)	9.27 (6.91–12.2)
K18-M65 (kIU/L)	3.82 (2.34–5.82)	14.2 (9.85–20.7)
K18-M30:M65 ratio	0.43 (0.37–0.51)	0.66 (0.55–0.79)

ALT, alanine transaminase; AST, aspartate transaminase; DF, Maddrey's discriminant function; INR, International Normalised Ratio; K18, keratin-18; MELD, Model for End-stage Liver Disease.
[a]High K18-M30 defined as serum K18-M30 >5 kIU/L.

convincing separation in the CIs for K18-M65 but an apparent divergence for K18-M30 at approximately 5 kIU/L (Figure 3c, d). Accordingly, prednisolone was associated with improved 90-day survival in those with serum K18-M30 >5 kIU/L, but not those below this threshold (Figure 3e, f). Overall, 104/814 (12.8%) of individuals displayed a serum K18-M30 >5 kIU/L. Examination of baseline demographics in groups defined by this K18-M30 cutoff demonstrated a tendency to higher white cell and neutrophil counts and greater serum bilirubin and AST concentrations in the high K18-M30 group (Table 4).

Comparison of high K18-M30 with Lille score as markers of prednisolone response

The interaction terms were not significant in models assessing either multiplicative or curvilinear interactions between the Lille score and prednisolone therapy, irrespective of adjustment for age and baseline MELD score (see Supplementary Table 6, Supplementary Digital Content 6, http://links.lww.com/AJG/B649, data not shown). Consequently, prednisolone was not associated with 90-day mortality in either Lille responders or nonresponders (see Supplementary Figure 6a, b, Supplementary Digital Content 6, http://links.lww.com/AJG/B654).

Sequential evaluation of K18-M30 >5 kIU/L, and Lille response to evaluate prednisolone efficacy was investigated. In individuals with a low serum K18-M30 (<5 kIU/L), there was no benefit from prednisolone in either Lille responders (OR: 1.357, 95% CI: 0.631–2.981, $P = 0.437$) or nonresponders (OR: 1.223, 95% CI: 0.651–2.283, $P = 0.529$), consistent with a lack of effect in the entire group (see Supplementary Figure 6e, f, Supplementary Digital Content 6, http://links.lww.com/AJG/B654). In the cohort of patients with K18-M30 >5 kIU/L, prednisolone was associated with significant survival benefit in Lille responders (OR: 0.143, 95% CI: 0.0185–0.7313, $P = 0.0306$), but not nonresponders (OR: 1.444, 95% CI: 0.295–8.164, $P = 0.655$, see Supplementary Figures 6c, d, Supplementary Digital Content 6, http://links.lww.com/AJG/B654).

Influence of high K18-M30 on prednisolone-related infection risk

In line with studies of the entire population, severe infections were more frequent in prednisolone-treated patients in this subset of the STOPAH cohort (prednisolone: 48/407, 11.8% *vs* no prednisolone: 29/417, 7.0%; OR: 1.789, 95% CI: 1.111–2.928, $P = 0.0182$). Although a greater proportion of patients with K18-M30 >5 kIU/L experienced a severe infection (>5 kIU/L 13/104, 12.5% *vs* <5 kIU/L 63/710, 8.9%), there was no overall association between K18-M30 and the risk of severe infection (OR: 1.015, 95% CI: 0.965–1.055, $P = 0.471$). No interaction was found between serum K18-M30 and prednisolone in relation to the development of severe infection ($P = 0.764$). Accordingly, there was a greater prevalence of severe infections in prednisolone-treated patients in both the high (>5 kIU/L, 7/48 [14.6%] *vs* 6/56 [10.7%]) and low K18-M30 populations (<5 kIU/L, 40/353 [11.3%] *vs* 23/357 [6.4%]). All of these associations were robust to adjustment for age and baseline MELD score.

DISCUSSION

AH is challenging to diagnose; a definitive diagnosis requires characteristic clinical and laboratory features with histological confirmation of steatohepatitis on liver biopsy (9). Given that, readily assayed serum biomarkers that aid diagnosis and prognostication would significantly aid in clinical decision-making. Serum markers of hepatocellular death, particularly K18 fragments have shown promise as diagnostic markers in those with alcohol-related liver disease of varying severity (7,33). This study, conducted in a large number of patients with severe AH who participated in the STOPAH trial, helps to clarify a number of issues: (i) serum K18 fragments are dramatically elevated in severe AH, (ii) serum K18-M30 and M65 levels are strongly associated with the presence of steatohepatitis and severity of inflammation on biopsy, (iii) previously published thresholds for diagnosis performed well in this study—a K18-M65 level >2,000 IU/L had a 94% PPV for the presence of steatohepatitis on biopsy, (iv) K18 fragment levels also predict the presence of severe inflammation and are associated with outcome, independent of the severity of liver injury at presentation, and (v) serum K18-M30 is a likely theragnostic biomarker.

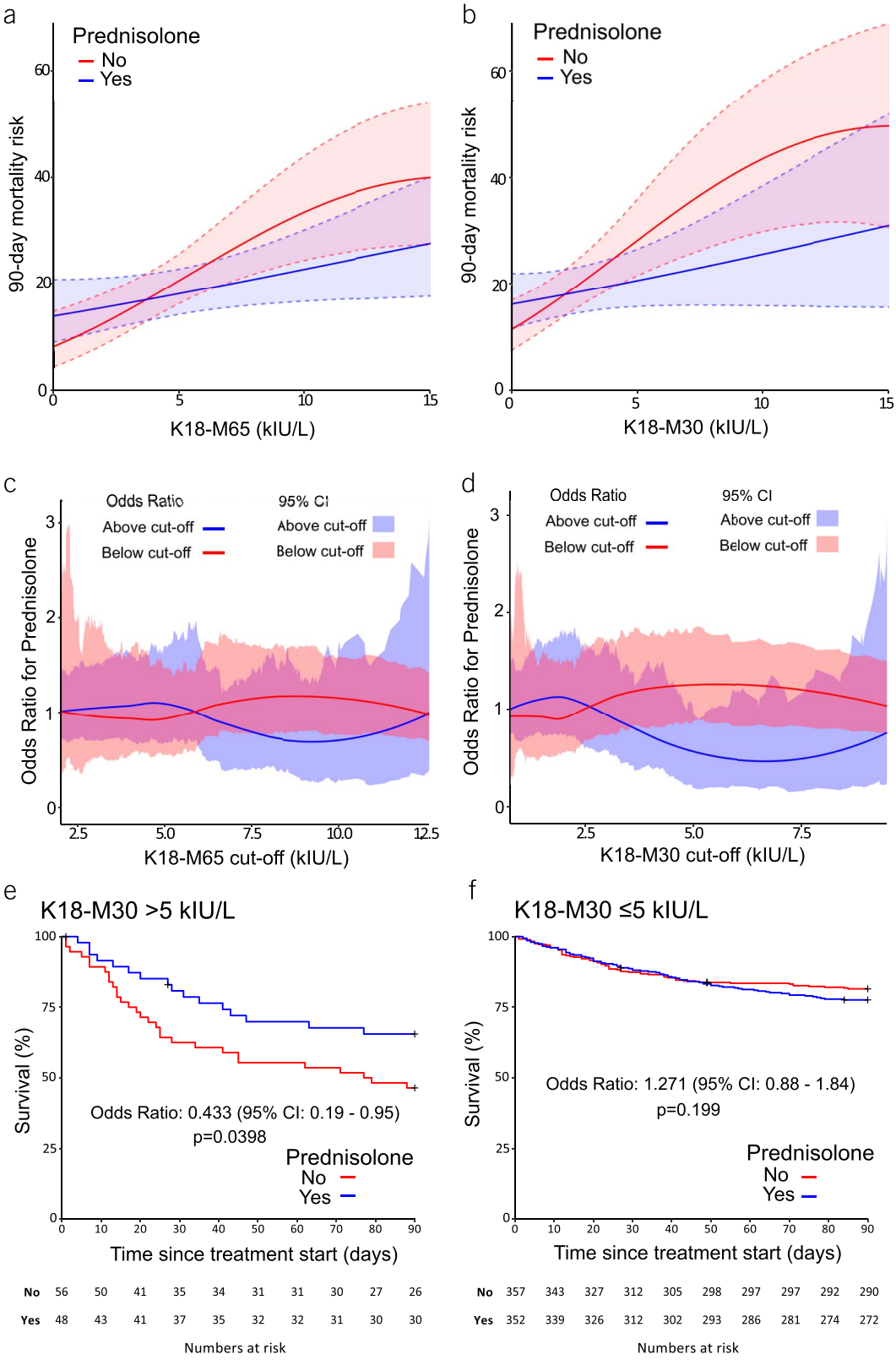

Figure 3. Plots illustrating predicted 90-day mortality risk in patients treated with and without prednisolone implied by the interaction models for (**a**) serum K18-M65 and (**b**) serum K18-M30, values adjusted for Model for End-stage Liver Disease score and age. Estimates for prednisolone efficacy when dichotomizing the population across a range of (**c**) K18-M65 and (**d**) K18-M30 values. Kaplan-Meier survival functions illustrating the effect of prednisolone treatment in populations defined by a serum K18-M30 above (**e**) or below (**f**) 5 kIU/L. Data are displayed as estimated mortality risk (solid line) with 95% confidence intervals (dashed line and shaded area) with (blue) and without (red) prednisolone therapy. CI, confidence interval; K18, keratin-18.

Evaluation of the relationship between K18 fragments and prednisolone in relation to 90-day mortality indicates a curvilinear interaction with benefit restricted to those with higher serum K18 levels and potential harm at the lower end of the spectrum (Figure 3c, d). In this cohort, pretreatment serum K18-M30 exceeding 5 kIU/L defined a subgroup within which prednisolone conferred a 90-day survival benefit (Figure 3e). Below this threshold, no therapeutic benefit was seen; however, an increased risk of severe infection remained indicating potential harm in the absence of therapeutic benefit (Figure 3f). These data are consistent with historical reports of harm associated with steroid therapy in patients with decompensated cirrhosis (34). Our data also suggest that K18-M30-guided prednisolone administration may be further refined through application of the Lille score with survival benefit apparently limited to responders. However, after case exclusion because of missing data and subdivision based on Lille response, the groups under evaluation in these analyses are small and the introduction of bias is likely. Thus, the results of these analyses should be confirmed in future studies.

In this cohort, K18-M30 and M65 values were markedly increased and often ≥10-fold higher than levels previously reported in healthy individuals (32,35). Importantly, K18-M30 and M65 levels reported here substantially exceeded values reported in several chronic liver diseases including alcoholic cirrhosis both in its compensated and decompensated state and were comparable with those observed in severe acute-on-chronic liver failure (19,35,36) and acute liver failure (37,38).

Serum K18 levels seem to be particularly elevated by alcohol exposure and the resulting liver injury. Accordingly, Macdonald et al. (36) detected significantly higher serum CK fragment levels in cirrhotics who had consumed alcohol in the preceding 3 months compared with those who had not. This observation is particularly interesting, given that alterations of the keratin network with formation of Mallory-Denk bodies and so-called "empty cells" devoid of any keratin staining are characteristic features of ASH (14,39). Notably, keratins constitute stress-inducible genes and their hepatic expression rises with severity of inflammation/injury (40).

The study has a number of strengths. Recruitment of patients via a prospective multicentre trial permitted inclusion of a large number of patients with predetermined inclusion criteria. Consequently, exploration and validation cohorts had a similar case mix, and allocation to prednisolone therapy was entirely random, minimizing systematic bias. The key biomarkers were estimated in the overwhelming majority of the patient cohort. The AST:ALT ratio was >2 in most participants, in line with its proposed use as a diagnostic criterion for AH (31). The availability of systematically evaluated liver biopsies allowed us to demonstrate that serum K18 fragment levels are helpful in predicting both the presence of ASH and severity of inflammation, greatly exceeding that of the serum AST and ALT that are widely considered to be markers of disease activity. This corroborates recently published data (7,33), and shows that K18 fragments constitute attractive noninvasive diagnostic markers of ASH. In contrast to the published studies that enrolled patients with varying severities of alcohol-related liver disease and comparatively small numbers of patients with severe AH, all patients included here met the diagnostic criteria for severe AH. Our finding that serum K18 fragments retain diagnostic, prognostic, and potentially theragnostic associations in this more homogenous population with more severe disease underscores their relationship to disease activity and clinical utility. Application of previously defined thresholds to triage patients to biopsy in this population would result in a dramatic 84% reduction in the numbers requiring biopsy. Furthermore, this biomarker test can be completed within 24 hours of sampling.

In line with the observed correlation of M30 and M65 with the histological severity of ASH, we detected an association between both biomarkers and 90-day mortality in both exploratory and validation cohorts. This is consistent with their prognostic abilities in individuals with acute liver failure, compensated and decompensated liver cirrhosis, and alcohol-related liver disease (7,16,19,33,36–38). The somewhat limited prognostic accuracy of both biomarkers is likely because of the fact that they predominantly reflect only acute injury and do not fully account for the extent of underlying liver fibrosis, which is also of prognostic importance (8). Because of this unique feature, K18-M30 and K18-M65 should be combined with markers reflecting cirrhosis-associated liver failure. In that respect, the predictive power of K18-M30 and K18-M65 was independent of the MELD score, and combination of these parameters resulted in a modest increase in the AUROC values.

Given that prednisolone constitutes the only routine treatment option in AH, we were particularly interested to test whether the assessed markers of hepatocellular death predict the usefulness of this treatment. Notably, prednisolone administration was beneficial in individuals with K18-M30 >5 kIU/L consistent with our observation that this cutoff is highly specific for patients with AH with severe inflammation. Application of this cutoff in clinical practice could restrict prednisolone usage to around 13% of patients—maximizing benefit while dramatically reducing the risk of steroid-related complications.

This study has limitations. Importantly, the use of a trial population means that findings from this study cannot be readily generalized to individuals who do not match this patient population. In particular, some individuals with the most profound organ failure were excluded from the STOPAH trial (26). Interaction modelling indicates a potential window for prednisolone benefit; however, large CIs and a fall in the predicted mortality risk for untreated individuals at the higher extremes of serum K18 levels likely reflects a combination of the limits of the parametric model and poor calibration at these levels. Our search for a therapeutic cutoff using the same data in which the interaction models were fitted means that the value generated is likely over-fitted and requires validation in a further cohort. Evaluation of the temporal evolution of serum K18-M30, as a marker of prednisolone response in treated patients or marker of deterioration in untreated patients, would provide additional support for use of K18-M30 as a theragnostic marker and warrants investigation in future studies.

In conclusion, our data indicate the potential applications of K18-M30 and K18-M65 as biomarkers in severe AH. The ability to predict the presence of steatohepatitis on biopsy seen here and the validation of findings from previous studies indicates that K18-M65 should be considered for adoption into clinical practice as a diagnostic adjunct. In addition, K18 has prognostic utility, independent of the MELD score. Finally, K18 may act as a theragnostic biomarker, guiding triage to prednisolone therapy though validation in an independent cohort is desirable. Considering the large groups of patients involved and use of independent exploratory and validation cohorts, serum K18 estimation should be adopted into routine clinical practice because it identifies patients with severe AH, especially those who benefit from steroid therapy, without the need to perform a liver biopsy.

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CONFLICTS OF INTEREST

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Study Highlights

WHAT IS KNOWN

- ✓ Severe AH is associated with significant mortality.
- ✓ Definite diagnosis requires liver biopsy.
- ✓ Prednisolone treatment is associated with complications.
- ✓ Robust diagnostic, prognostic, and theragnostic biomarkers are lacking.
- ✓ K18 fragments are promising diagnostic and prognostic markers in liver disease.
- ✓ Conclusive data are needed for application in severe AH.

WHAT IS NEW HERE

- ✓ In patients with the clinical syndrome of severe AH.
- ✓ Serum K18 fragments predict ASH and severe inflammation on biopsy.
- ✓ Serum K18-M65 predicts 90-day mortality, independent of MELD score.
- ✓ Prednisolone may only benefit where K18-M30 exceeds 5 kIU/L.
- ✓ K18 measurement could reduce biopsy rates and prednisolone exposure.

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