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ORIGINAL RESEARCH

Plasma thrombomodulin as a candidate biomarker for the diagnosis and prognosis of HBV-related acute-on-chronic liver failure

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Background and Aim: Hepatitis B virus-related acute-on-chronic liver failure (HBV-ACLF) is a complicated syndrome with high short-term mortality. Effective biomarkers are required for its early diagnosis and prognosis. This study aimed to determine the diagnostic and prognostic value of thrombomodulin (TM) in patients with HBV-ACLF.

Methods: The expression of TM during disease progression was evaluated through transcriptomics analysis. The plasma TM concentrations of 393 subjects with HBV-ACLF (n=213), acute-on-chronic hepatic dysfunction (ACHD, n=50), liver cirrhosis (LC, n=50) or chronic hepatitis B (CHB, n=50), and normal controls (NC, n=30) from a prospective multicenter cohort, were measured to verify the diagnostic and prognostic significance of plasma TM for HBV-ACLF patients by enzyme-linked immunosorbent assay (ELISA).

Results: TM mRNA was highly expressed in the HBV-ACLF group compared with the ACHD group (AUROC=0.710). High expression of TM predicted poor prognosis for HBV-ACLF patients at 28/90 days (AUROCs=0.823/0.788). Functional analysis showed that TM was significantly associated with complement activation and the inflammatory signaling pathway. External validation confirmed its high diagnostic accuracy for HBV-ACLF patients (AUROC=0.796). Plasma TM concentrations were correlated with organ failure, including coagulation and kidney failure. Plasma TM concentrations showed a potential prognostic value for 28-day mortality rates (AUROC=0.702). Risk stratification specifically identified HBV-ACLF patients with a high risk of death as having a plasma TM concentration of ≥8.4 ng/mL.

Conclusion: This study reveals that the plasma TM can be a candidate biomarker for early diagnosis and prognosis of HBV-ACLF, and might play a vital role in coagulation and inflammation.

Keywords: thrombomodulin, HBV-ACLF, biomarker, organ failure

Introduction

Acute-on-chronic liver failure (ACLF) is generally regarded as a complex clinical syndrome characterized by acute decompensation of liver function and multiple organ failure in patients suffering from chronic liver disease.[1] The pathologic features and etiology of ACLF patients differ with regional distribution.[2] Our previous study showed that the clinical characteristics of patients with hepatitis B virus-related ACLF (HBV-ACLF) were significantly different from those of alcoholic hepatitis-related ACLF patients in Western countries.[3] HBV-ACLF may progress fast, with a short-term mortality rate of 50% ~ 90%, even with the most intensive treatment.[3,4] Therefore, to guide concise clinical

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decisions, to improve management, and to minimize futile and expensive care, insight into effective biomarkers for the early diagnosis and prognosis of patients with HBV-ACLF is imperative.

Thrombomodulin (TM), a transmembrane glycoprotein receptor, is specifically expressed on endothelial cells' surface to bind thrombin and activate protein C. This facilitates the degradation of coagulation factors Va and VIIIa, reducing thrombin production.[5] Soluble TM is a reliable biomarker for indicating endothelial cell injury.[6] Liver sinusoidal endothelial cells (LSECs) participate in liver angiogenesis. During the progression of liver disease, soluble TM protein is released into blood due to the injury of these vascular cells.[7] Several studies have shown elevated concentrations of soluble TM across a range of diverse liver diseases, including acute liver damage, chronic hepatitis and cirrhosis.[8–10] Recently, the clinical significance of TM as a biomarker in liver disease has also attracted more attention. For example, Wei et al reported that high plasma TM concentrations could indicate poor prognosis in decompensated cirrhosis.[11] To the best of our knowledge, no previous research has been conducted to investigate the relationship between TM and HBV-ACLF. Therefore, this study aims to evaluate the diagnostic and prognostic value of TM measurements in HBV-ACLF patients.

Materials and Methods

Study Design

Gene expression analysis and functional enrichment analysis of transcriptome sequencing data studied in our early stage were carried out to identify the expression and function of TM gene and its potential prognostic value for short-term mortality during HBV-ACLF progression.[12] Next, plasma TM concentrations were externally measured in subjects from a prospective multicenter Chinese Group on the Study of Severe Hepatitis B (COSSH) study cohort between January 2019 and December 2021 and assessed by enzyme-linked immunosorbent assay (ELISA).

Patients

Between January 2019 and December 2021, 213 patients with HBV-ACLF were randomly stratified according to the prevalence rate of HBV-ACLF grade in COSSH study. Fifty inpatients with HBV-related acute-on-chronic hepatic dysfunction (ACHD), 50 outpatients with liver cirrhosis (LC) who had chronic HBV infection, 50 outpatients with chronic hepatitis B (CHB), and 30 eligible normal controls (NCs) in the First Affiliated Hospital of Zhejiang University were also included. Blood was sampled from patients and controls within 24 hours after enrolment. Laboratory, demographic, clinical, and follow-up data (28/90 days after sample collection) for all subjects were obtained from case reports and electronic data collection systems. Standard treatment principles were strictly applied, including antiviral therapy with nucleoside analogs, routine regimen administration for patients with hepatic encephalopathy (HE), ascites, and microbial infections, oxygen support for patients with respiratory dysfunction, and renal replacement therapy for patients with hepatorenal syndrome, as mentioned earlier.[3]

Definitions

HBV-ACLF was diagnosed based on the COSSH-ACLF criteria.[3] COSSH-ACLF was graded into the following three groups: ACLF-1, ACLF-2, and ACLF-3.[3] ACHD was defined as pre-ACLF patients who did not meet the ACLF diagnostic criteria, as mentioned in our previous study.[12] LC was defined as patients with stable compensatory cirrhosis, whose diagnosis was based on endoscopy, pathology, radiology, clinical signs, or laboratory examination, as described previously.[12] CHB was defined on the basis of the 2009 American Association for the Study of Liver Diseases (AASLD) guidelines.[13] NCs were participants of normal medical examination. Organ failure was diagnosed as follows: kidney failure, serum creatinine concentration of ≥2 mg/dl; liver failure, serum total bilirubin (TB) concentration of ≥12 mg/dl; coagulation failure, international normalized ratio (INR) ≥2.5; circulation failure, use of vasopressor drugs; cerebral failure, HE grades III or IV; respiratory failure, SpO2/FiO2 ≤214 or PaO_2/FiO_2 ≤200, or use of mechanical ventilation not due to HE.[14]

Transcriptomic Data

We obtained the peripheral blood mononuclear cell (PBMC) transcriptomic data of patients with HBV-ACLF (n=20), ACHD (n=10), LC (n=10) and CHB (n=10) and NCs (n=15) from our earlier study, which could be found in the Sequence Read Archive database (accession number PRJNA548207).[12]

Functional Enrichment Analysis

The pathway enrichment score was quantified on a single-sample basis, and the gene set variation analysis (GSVA) was carried out by using the R package GSVA.[15] The Gene Ontology (GO) biological processes and blood transcriptional modules (BTM)[16] were used as functional annotations for GSVA. A P value of less than 0.05 indicated that the result was statistically significant.

Method for ELISA

Plasma TM concentrations were assayed by ELISA using commercially available kits (ab46508, ABCAM, Cambridge, UK) according to the instructions provided by the manufacturer.

Scoring Systems

Formulas for calculating the scoring systems are listed below: Model for End-stage Liver Disease score (MELDs) = 3.78 × ln[TB (mg/dL)] + 11.2 × ln(INR) + 9.6 × ln[creatinine (mg/dL)] + 6.43;[17] MELD-Na score = MELD - Na - (0.025 × MELD × (140 - Na)) + 140, and the concentration of serum sodium was between 125 and 140 mmol/L;[18] COSSH-ACLF II score = 1.649 × ln(INR) + 0.576 × ln[serum urea (mmol/L)] + 0.457 × hepatic encephalopathy score (HE grade: 0/1, 1–2/2 and 3–4/3) + 0.425 × ln[neutrophil (10^9/L)] + 0.396 × ln[TB (μmol/L)] + 0.033 × age.[19]

Statistical Analysis

Student's *t*-tests (for data distributed normally) and Mann–Whitney *U*-tests (for data distributed non-normally) were used to analyze continuous variables, while the χ^2 test and Fisher's exact test were applied for categorical variables, as appropriate. The normality assumption was assessed by the Kolmogorov–Smirnov test. The results are presented as the means ± standard deviations (SD), medians with interquartile ranges (IQRs) or numbers (percentages), unless indicated otherwise. Pearson's correlation coefficients were calculated for the correlation analysis. Area under the receiver operating characteristic curve (AUROC) values were calculated, and Delong's test was applied for its comparison. To stratify HBV-ACLF patients into low-risk and high-risk of death groups, X-tile software (version 3.6.1) was utilized to determine the optimal cut-off value of the plasma TM concentration.[20] The thresholds were defined as the value that yielded the largest χ^2 value in the Mantel–Cox test. The Kaplan–Meier method was used for survival analysis, and the Log rank test was used to determine the significant differences between the low-risk and high-risk groups. Statistical analyses were performed using IBM SPSS Statistics (version 27.0), GraphPad Prism software (version 8.0) and R software (version 4.0.3).

Results

Analysis of TM Expression and Functional Enrichment

In order to identify the expression of TM during the progression of HBV-ACLF, we analyzed the mRNA expression level between the HBV-ACLF group and other groups based on PBMC transcriptomics data. The clinical characteristics of all subjects could be found in our previous article.[12] The results showed an increase in TM expression during the progression of disease from NC/CHB/LC to HBV-ACLF (Figure 1A), and the AUROC of TM expression for discriminating ACLF from ACHD was 0.710 (95% confidence interval (CI): 0.519–0.902) (Figure 1B). Meanwhile, we compared TM expression between the ACLF-deceased (ACLF-D) group and the ACLF-surviving (ACLF-S) group to determine the potential prognostic value of TM. Higher expression of TM was observed in the ACLF-D group than in the ACLF-S group at 28 days ($P<0.05$) (Figure 1C). ROC analysis showed that the prognostic accuracy of TM expression for predicting 28-day mortality rates was 0.823 (95% CI: 0.631–1.000) (Figure 1D). Similar results could be found at 90 days (Figure 1E), with

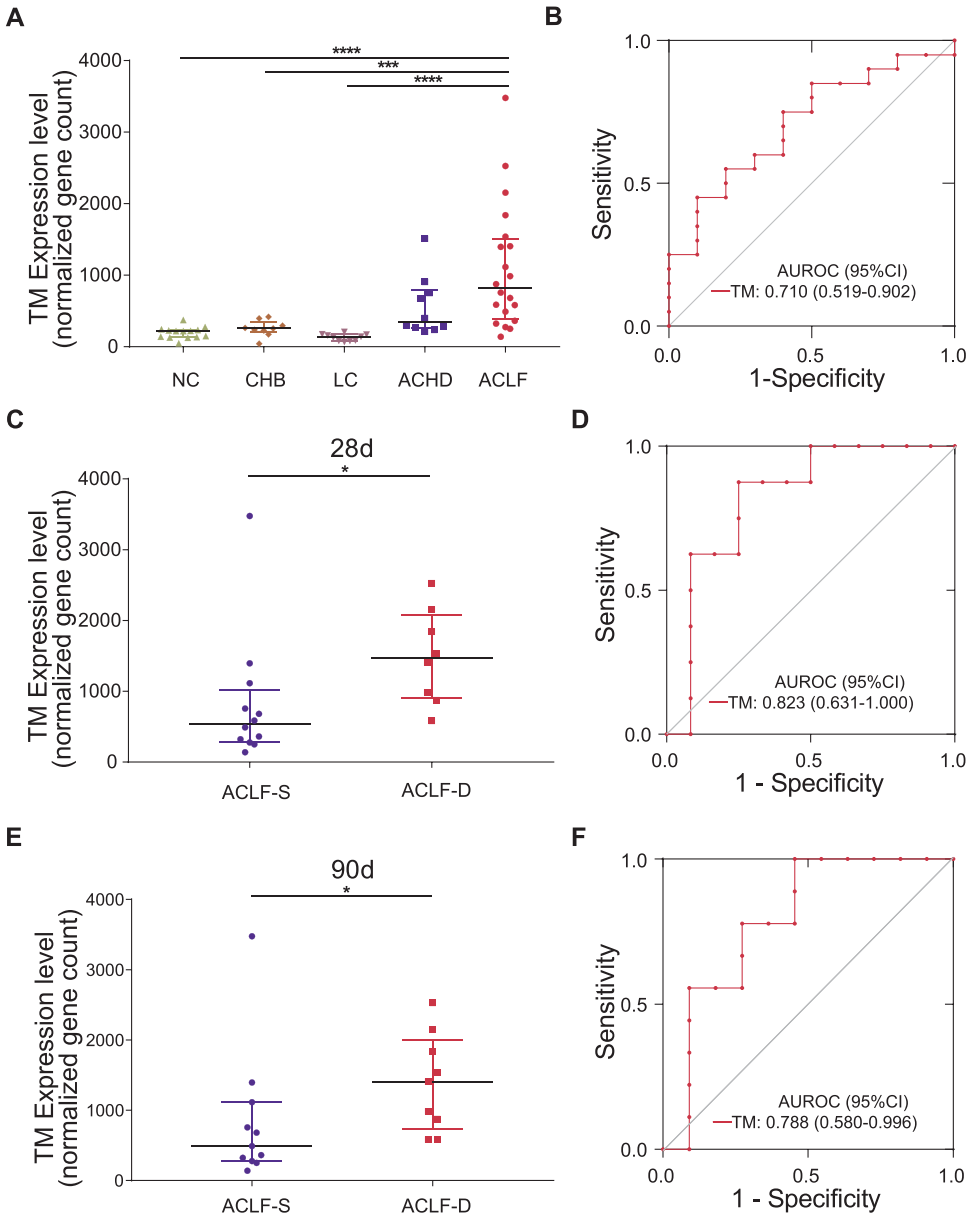

Figure 1 PBMC TM expression analysis. (**A**) PBMC TM expression levels in patients at different disease stages. (**B**) ROC curve of PBMC TM expression levels for distinguishing HBV-ACLF from ACHD. (**C**) PBMC TM expression levels in HBV-ACLF patients with different outcomes at 28 days. (**D**) ROC curve of PBMC TM expression levels for predicting the 28-day mortality rates of HBV-ACLF patients. (**E**) PBMC TM expression levels in HBV-ACLF patients with different outcomes at 90 days. (**F**) ROC curve of PBMC TM expression levels for predicting the 90-day mortality rates of HBV-ACLF patients.

Abbreviations: PBMC, peripheral blood mononuclear cell; TM, thrombomodulin; HBV-ACLF, hepatitis B virus-related acute-on-chronic liver failure; ACHD, acute-on-chronic hepatic dysfunction; LC, liver cirrhosis; CHB, chronic hepatitis B; NC, normal controls; AUROC, area under the receiver operating characteristic; ACLF-D, ACLF-deceased; ACLF-S, ACLF-surviving.

a predictive AUROC value of 0.788 (95% CI: 0.580–0.996) (Figure 1F). Further functional enrichment analysis showed that TM expression was significantly associated with several key biological processes, including complement activation, regulation of the inflammatory signaling pathways and cellular response to oxidative stress and external stimuli (Figure 2), which indicated that TM may play a vital role in the immune and inflammatory response in HBV-ACLF progression.

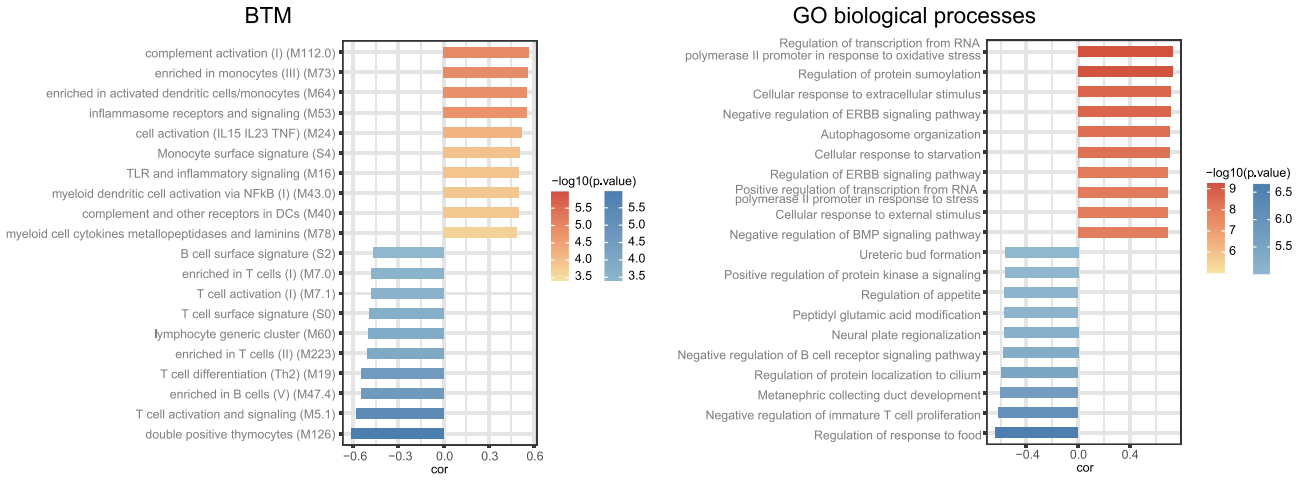

Figure 2 Functional analysis of TM. Histogram showing the Pearson correlation coefficients for the relationships between TM mRNA expression levels and BTMs (left) and GO biological processes (right). The terms that are positively or negatively correlated are shown in red or blue, respectively. The top 20 terms with high correlation coefficients are displayed.

The Use of Plasma TM as a Diagnostic Biomarker for HBV-ACLF

To confirm the diagnostic value of TM for HBV-ACLF, the relationship between plasma TM concentrations and HBV-ACLF progression was further investigated by ELISA. The clinical characteristics of all subjects in the ELISA group are listed in Table 1. Compared with the other groups, the baseline laboratory indicators in the HBV-ACLF group were significantly higher, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), TB, prothrombin time (PT), INR, etc. Baseline plasma TM concentrations were compared among the groups and were found to be markedly higher in the HBV-ACLF group than in the ACHD, LC, CHB and NC groups (8.9 [6.0–12.9] vs 3.2 [1.9–9.0], 1.5 [0.9–2.1], 1.5 [1.0–2.2] and 1.3 [0.9–2.4] ng/mL, respectively; P<0.0001 for all comparisons) (Figure 3A). The AUROC of

Table 1 Clinical Characteristics of the Patients in the ELISA Group

Characteristics	HBV-ACLF (n=213)	ACHD (n=50)	LC (n=50)	CHB (n=50)	NC (n=30)
Age (years)	50 [38, 58]	48 [44, 55]	47 [42, 53]	46 [41, 51]*	37 [32, 45]**
Male (no.)	83.1% (177)	78.0% (39)	64.0% (32)**	58.0% (29)***	80.0% (24)
MAP (mmHg)	83.7 [75.5, 93.8]	85.3 [77.4, 96.9]	/	/	/
Cirrhosis (no.)	59.2% (126)	74.0% (47)*	100.0% (50)***	0***	0***
Complication					
Hepatic encephalopathy	13.1% (28)	2.0% (1)*	/	/	/
Gastrointestinal bleeding	4.2% (9)	30.0% (15)***	/	/	/
Ascites	61.0% (130)	44.0% (22)***	/	/	/
Bacterial infection	26.3% (56)	20.0% (10)	/	/	/
HBV DNA level (IU/mL)		***	***	***	***
≤ 2×10^2	16% (34)	52.0% (26)	94.0% (47)	66.0% (33)	100% (30)
2×10^2–2×10^6	60.1% (128)	34.0% (17)	6.0% (3)	26.0% (13)	0
> 2×10^6	23.9% (51)	14.0% (7)	0	8.0% (4)	0
Laboratory data					
ALT (U/L)	191.0 [83.5, 371.5]	36.5 [23.0, 187.0]***	23.5 [16.0, 38.0]***	23.0 [16.0, 32.3]***	20.0 [14.8, 36.3]***
AST (U/L)	122.0 [80.0, 224.5]	51.5 [30.0, 161.0]***	22.0 [19.0, 28.0]***	21.5 [18.0, 28.0]***	22.0 [18.0, 22.5]***
ALB (g/L)	30.6±3.8	30.3±4.5	46.9±2.4***	46.0±3.2***	44.0±1.3***
TB (μmol/l)	354.9 [284.3, 428.0]	39.5 [23.8, 143.0]***	11.4 [8.7, 15.5]***	11.6 [8.9, 15.3]***	14.3 [9.2, 17.1]***
GGT (U/L)	70.0 [49.0, 109.0]	52.0 [21.5, 103.0]*	24.0 [14.8, 37.0]***	15.0 [12.0, 19.3]***	23.0 [16.0, 35.5]***
Cr (μmol/L)	61.0 [52.0, 74.5]	70.5 [62.0, 80.0]***	69.5 [61.8, 79.0]**	73.5 [63.8, 88.0]***	74.0 [62.3, 79.0]**
Serum urea (mmol/L)	4.0 [2.9, 5.6]	4.4 [3.5, 5.8]	4.5 [4.1, 5.6]*	4.6 [4.1, 5.5]**	4.9 [4.4, 5.4]**

(*Continued*)

Table 1 (Continued).

Characteristics	HBV-ACLF (n=213)	ACHD (n=50)	LC (n=50)	CHB (n=50)	NC (n=30)
Sodium (mmol/L)	138.0 [136.0, 140.0]	140.0 [138.0, 142.0]***	140.0 [141.0, 143.0]***	141.0 [140.0, 142.0]***	/
WBC (10^9/L)	6.9 [5.4, 9.1]	4.0 [2.4, 5.1]***	4.4 [3.6, 6.4]***	5.1 [4.1, 5.9]***	5.6 [5.1, 5.9]**
Neutrophil count (10^9/L)	4.6 [3.3, 6.7]	2.1 [1.1, 3.2]***	2.5 [2.2, 3.8]***	3.0 [2.4, 3.4]***	3.1 [2.6, 3.5]***
PLT (10^9/L)	94.0 [65.5, 130.0]	61.0 [45.0, 138.3]	165.0 [123.8, 213.8]***	181.5 [152.0, 219.0]***	212.0 [199.3, 259.3]***
HGB (g/L)	119.3±21.8	106.3±27.9**	145.8±17.6***	149.8±13.6***	149.8±3.6***
FIB (g/L)	1.3 [1.0, 1.6]	1.5 [1.4, 1.9]**	1.3 [1.0, 1.6]***	2.4 [2.2, 2.6]***	/
PT (s)	21.7 [19.5, 26.7]	15.8 [14.6, 17.5]***	21.7 [19.5, 26.7]***	11.5 [11.1, 12.1]***	/
D dimer (μg/L)	2157 [1088, 3882]	818.5 [438.8, 3290.8]**	/	/	/
INR	1.9 [1.7, 2.4]	1.4 [1.3, 1.5]***	1.9 [1.7, 2.4]***	1.0 [1.0, 1.1]***	/
TM (ng/mL)	8.9 [6.0, 12.9]	3.2 [1.9, 7.0]***	1.5 [0.9, 2.1]***	1.5 [1.0, 2.2]***	1.3 [0.9, 2.4]***
Organ failure (No.)					
Liver	97.7% (208)	/	/	/	/
Coagulation	23.5% (50)	/	/	/	/
Kidney	2.8% (6)	/	/	/	/
Cerebral	6.1% (13)	/	/	/	/
Lung	0.9% (2)	/	/	/	/
Circulation	0.9% (2)	/	/	/	/
Severity score					
MELDs	22.0 [20.0, 24.9]	/	/	/	/
MELD-Nas	22.9 [21.0, 26.1]	/	/	/	/
COSSH-ACLF IIs	7.0 [6.5, 7.6]	/	/	/	/

Notes: Categorical variables are expressed as % (n); continuous variables are expressed as either the mean ± SD or median (Q1-Q3). (Student's *t* test, Mann–Whitney *U*-test, χ^2 test or Fisher's exact test). *P value (<0.05), **P value (<0.01) and ***P value (<0.001) for comparisons between groups.
Abbreviations: HBV-ACLF, hepatitis B virus-related acute-on-chronic liver failure; ACHD, acute-on-chronic hepatic dysfunction; LC, liver cirrhosis; CHB, chronic hepatitis B; NC, normal controls; MAP, mean arterial pressure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALB, albumin; TB, total bilirubin; GGT, gamma glutamyl transferase; Cr, creatinine; WBC, white blood cell count; PLT, platelet count; HGB, haemoglobin; FIB, fibrinogen; PT, prothrombin time; INR, international normalized ratio; TM, thrombomodulin; MELDs, model for end-stage liver disease score; MELD-Nas, MELD-sodium score; COSSH-ACLF IIs, Chinese Group on the Study of Severe Hepatitis B-ACLF II score.

plasma TM concentrations to discriminate ACLF from ACHD was 0.796 (95% CI: 0.719–0.873) (Figure 3B). These results showed that plasma TM could be used as a diagnostic marker for HBV-ACLF, which confirmed the reliability of the results from the transcriptomic analysis.

To further investigate the relationship between plasma TM concentrations and HBV-ACLF, we assessed the correlation between plasma TM concentrations and the laboratory indicators (Table 2). Positive correlations were observed between

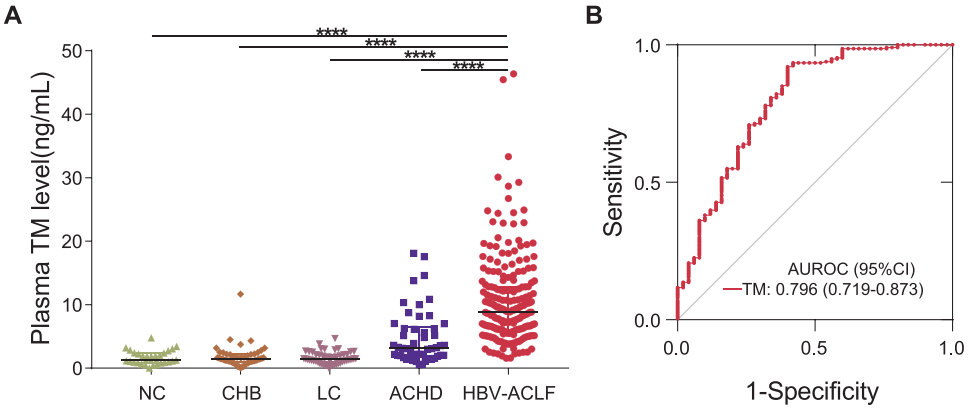

Figure 3 The use of plasma TM concentrations for diagnosing HBV-ACLF at admission. (**A**) Plasma TM concentrations in patients at different disease stages. (**B**) ROC curve of plasma TM concentrations for distinguishing HBV-ACLF from ACHD. ****P < 0.0001.
Abbreviations: TM, thrombomodulin; HBV-ACLF, hepatitis B virus-related acute-on-chronic liver failure; ACHD, acute-on-chronic hepatic dysfunction; LC, liver cirrhosis; CHB, chronic hepatitis B; NC, normal controls; AUROC, area under the receiver operating characteristic.

Table 2 Associations of Clinical Parameters and Prognostic Scoring Systems with Plasma TM Concentration in Patients with HBV-ACLF

Variable	Regression Coefficient	P
Laboratory data		
ALT (U/L)	−0.067	0.334
AST (U/L)	0.001	0.991
ALB (g/L)	−0.237	<0.001
TB (μmol/L)	0.117	0.088
GGT (U/L)	−0.239	<0.001
Cr (μmol/L)	0.261	<0.001
Serum urea (mmol/L)	0.477	<0.001
Sodium (mmol/L)	−0.126	0.066
WBC (10^9/L)	0.102	0.140
Neutrophil count (10^9/L)	0.040	0.558
HGB (g/L)	−0.164	0.017
PLT (10^9/L)	−0.165	0.016
FIB (g/L)	−0.180	0.009
PT (s)	0.225	<0.001
D dimer (μg/L)	0.339	<0.001
INR	0.227	<0.001
Severity score		
MELDs	0.411	<0.001
MELD-Nas	0.406	<0.001
COSSH-ACLF IIs	0.411	<0.001

Note: Data are P-value (r-value).

Abbreviations: TM, thrombomodulin; HBV-ACLF, hepatitis B virus-related acute-on-chronic liver failure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALB, albumin; TB, total bilirubin; GGT, glutamyl transferase; Cr, creatinine; WBC, white blood cell count; PLT, platelet count; HGB, hemoglobin; FIB, fibrinogen; PT, prothrombin time; INR, international normalized ratio; MELDs, model for end-stage liver disease score; MELD-Nas, MELD-sodium score; COSSH-ACLF IIs, Chinese Group on the Study of Severe Hepatitis B-ACLF II score.

plasma TM concentrations and creatinine (Cr) (r=0.261, P<0.001), serum urea (r=0.477, P<0.001), PT (r=0.225, P<0.001), D dimer (r=0.339, P<0.001) and INR (r=0.227, P<0.001). Meanwhile, we found that plasma TM concentrations were inversely correlated with albumin (ALB) (r =−0.237, P<0.001), gamma-glutamyl transferase (GGT)(r =−0.239, P<0.001), hemoglobin (HGB) (r =−0.164, P=0.017), platelet count (PLT) (r =−0.165, P=0.016) and fibrinogen (FIB) (r =−0.180, P=0.009) concentrations. Moreover, plasma TM concentrations were significantly increased in patients with ACLF-2/3 (P<0.001) (Figure 4A) and patients with higher COSSH-ACLF II scores (7.4~8.4 vs <7.4, P<0.001; >8.4 vs <7.4, P<0.0001) (Figure 4B). Plasma TM concentrations were also positively correlated with coagulation failure (P<0.001) (Figure 4C) and kidney failure (P<0.05) (Figure 4D), but not significantly associated with cerebral failure (Figure 4E). These results indicated that plasma TM, as a diagnostic biomarker for HBV-ACLF, could reflect coagulation status, inflammation, and disease severity.

The Use of Plasma TM for Prognosis and Risk Stratification

We further explored the prognostic value of plasma TM for short-term mortality rates in HBV-ACLF patients. The clinical characteristics of patients in the ACLF-D and ACLF-S groups within 28 days are shown in detail in Table 3. Among both groups, males dominated (73.4 vs 87.2%, respectively). ACLF-D patients were older (55 [48, 60] vs 47 [37, 57] years, P<0.001). The laboratory indicators, including TB (390.6 ± 119.2 vs 344.9 ± 116.7 μmol/L, P=0.004), WBC

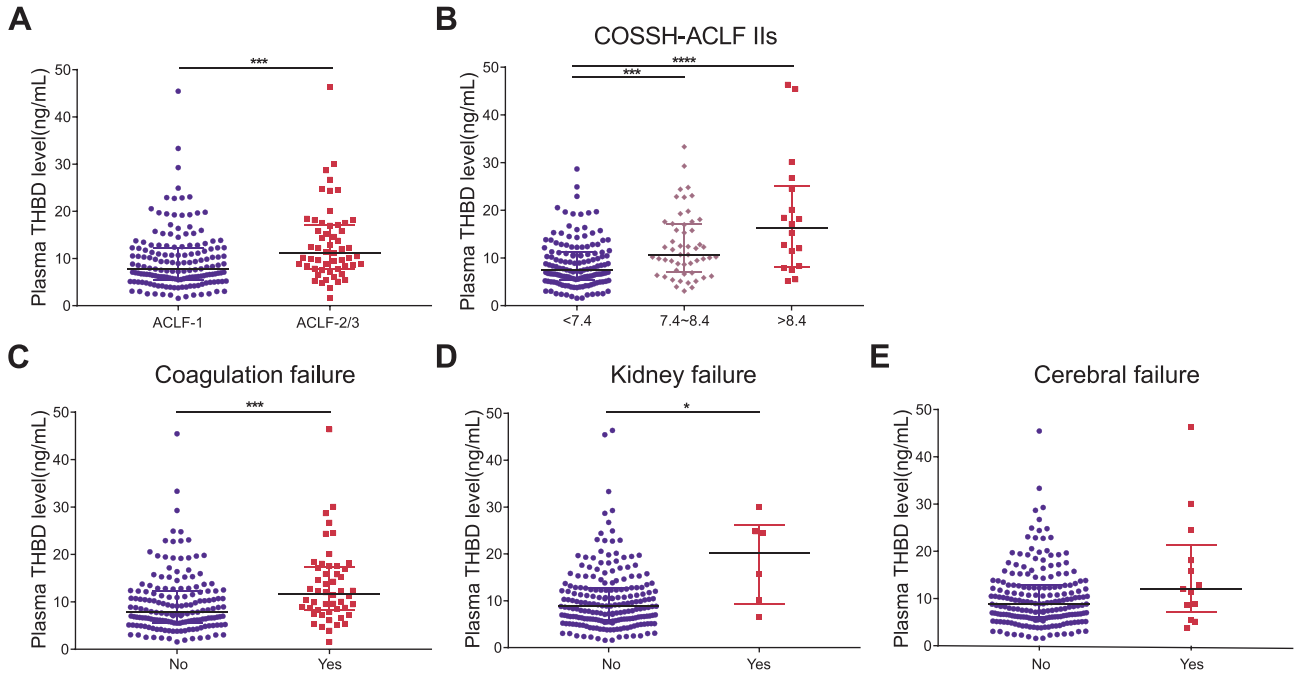

Figure 4 Plasma TM concentrations are related to the severity of HBV-ACLF. (**A**) Plasma TM concentrations in ACLF-1 and ACLF-2/3 patients. (**B**) Plasma TM concentrations in patients stratified by COSSH-ACLF IIs. (**C**) Plasma TM concentrations and coagulation failure. (**D**) Plasma TM concentrations and kidney failure. (**E**) Plasma TM concentrations and cerebral failure. *P < 0.5; ***P < 0.001; ****P < 0.0001.
Abbreviations: TM, thrombomodulin; ACLF, acute-on-chronic liver failure; COSSH-ACLF IIs, Chinese Group on the Study of Severe Hepatitis B-ACLF II score.

(7.7 [6.1, 9.5] vs 6.6 [4.9, 8.8] 10^9/L, P=0.016) and INR (2.5 [2.1, 3.2] vs 1.8 [1.6, 2.1], P<0.001), were significantly worse in ACLF-D patients than in ACLF-S patients. The ACLF-D group had a much higher frequency of organ failure than the ACLF-S group, including coagulation failure (51.6% vs 11.4%, P<0.001), kidney failure (6.3% vs 1.3%, P=0.047), cerebral failure (17.2% vs 1.3%, P<0.001) and lung failure (3.1% vs 0, P=0.030). The MELD, MELD-Na, and COSSH-ACLF II scores for patients in the ACLF-D group were 24.7 [21.5, 28.7], 25.9 [22.4, 29.7] and 8.0 [7.3, 8.4],

Table 3 Characteristics of Patients in ACLF-D and ACLF-S Group at 28 Days

Characteristics	ACLF-D (n=64)	ACLF-S (n=149)	P value
Age (years)	55 [48, 60]	47 [37, 57]	<0.001
Male (no.)	73.4% (47)	87.2% (130)	0.014
MAP (mmHg)	84.1 [76.8, 96.6]	83.0 [75.0, 92.2]	0.300
Cirrhosis (no.)	67.2% (43)	55.7% (83)	0.118
Complication			
Hepatic encephalopathy	32.8% (21)	4.7% (7)	<0.001
Gastrointestinal bleeding	7.8% (5)	2.7% (4)	0.088
Ascites	67.2% (43)	58.4% (87)	0.227
Bacterial infection	29.7% (19)	24.8% (37)	0.461
HBV DNA level (IU/mL)			0.070
≤ 2×10^2	10.9% (7)	18.9% (27)	
2×10^2–2×10^6	59.4% (38)	61.7% (92)	
> 2×10^6	32.8% (21)	20.1% (30)	
Laboratory data			
ALT (U/L)	214.0 [91.8, 551.0]	188.0 [81.0, 335.5]	0.159
AST (U/L)	145.0 [76.0, 312.8]	118.0 [80.5, 196.5]	0.104
ALB (g/L)	29.9 [28.0, 32.5]	30.9 [28.2, 33.6]	0.160
TB (μmol/L)	390.6±119.2	344.9±116.7	0.004

(*Continued*)

Table 3 (Continued).

Characteristics	ACLF-D (n=64)	ACLF-S (n=149)	P value
GGT (U/L)	61.5 [38.8, 98.0]	77.0 [51.0, 116.5]	0.136
Cr (μmol/L)	58.5 [52.0, 75.5]	62.0 [53.0, 74.5]	0.394
Serum urea (mmol/L)	4.6 [3.1, 6.7]	4.3 [2.8, 5.0]	0.032
Sodium (mmol/L)	138.0 [136.0, 140.8]	138.0 [136.0, 140.0]	0.626
WBC (10^9/L)	7.7 [6.1, 9.5]	6.6 [4.9, 8.8]	0.016
Neutrophil count (10^9/L)	5.7 [4, 7.4]	4.4 [2.9, 6.4]	0.001
PLT (10^9/L)	91.0 [65.5, 118.0]	97.0 [65.5, 134.0]	0.194
HGB (g/L)	116.7±22.6	120.4±21.4	0.266
FIB (g/L)	1.1 [0.9, 1.4]	1.4 [1.1, 1.7]	<0.001
PT (s)	27.4 [22.7, 3.4]	21.0 [18.6, 23.4]	<0.001
D dimer (μg/L)	3184 [2122, 5266]	1742 [751, 3469]	<0.001
INR	2.5 [2.1, 3.2]	1.8 [1.6, 2.1]	<0.001
TM (ng/mL)	11.7 [8.5, 18.1]	7.5 [5.4, 11.9]	<0.001
Organ failure (No.)			
Liver	96.9% (62)	98.0% (146)	0.623
Coagulation	51.6% (33)	11.4% (17)	<0.001
Kidney	6.3% (4)	1.3% (2)	0.047
Cerebral	17.2% (11)	1.3% (2)	<0.001
Lung	3.1% (2)	0	0.030
Circulation	1.6% (1)	0.7% (1)	0.536
Severity score			
MELDs	24.7 [21.5, 28.7]	21.3 [19.5, 23.3]	<0.001
MELD-Nas	25.9 [22.4, 29.7]	22.2 [20.6, 24.5]	<0.001
COSSH-ACLF IIs	8.0 [7.3, 8.4]	6.8 [6.3, 7.2]	<0.001

Notes: Categorical variables are expressed as % (n); continuous variables are expressed as either the mean ± SD or median (Q1-Q3). (Student's *t*-test, Mann–Whitney *U*-test, $\chi 2$ test or Fisher's exact test). P-values for comparisons between patients in ACLF-D and ACLF-S group.

Abbreviations: ACLF-D, ACLF-deceased; ACLF-S, ACLF-survived; MAP, mean arterial pressure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALB, albumin; TB, total bilirubin; GGT, glutamyl transferase; Cr, creatinine; WBC, white blood cell count; PLT, platelet count; HGB, hemoglobin; FIB, fibrinogen; PT, prothrombin time; INR, international normalized ratio; TM, thrombomodulin; MELDs, model for end-stage liver disease score; MELD-Nas, MELD-sodium score; COSSH-ACLF IIs, Chinese Group on the Study of Severe Hepatitis B-ACLF II score.

respectively, which were notably higher than those for patients in the ACLF-S group (all P<0.001). Correlation analysis revealed that plasma TM concentrations correlated positively with diverse prognostic scores (MELD: r=0.411, P<0.001; MELD-Na: r=0.406, P<0.001; COSSH-ACLF II: r=0.411, P<0.001) (Table 2).

The ELISA results showed a significantly higher concentration of plasma TM in the ACLF-D group than that in the ACLF-S group (11.7 [8.5, 18.1] vs 7.5 [5.4, 11.9] ng/mL, P<0.0001) at 28 days (Figure 5A), and similar results were shown at 90 days (Figure 5B). ROC analysis showed that the prognostic accuracy of plasma TM concentrations for predicting 28-/90-day mortality rates was 0.702/0.670, which was comparable to that of two commonly used clinical scores (MELD, AUROC=0.724/0.735, P=0.631/0.132; MELD-Na, AUROC=0.711/0.723, P=0.855/0.227) but poorer than COSSH-ACLF IIs (AUROC=0.874/0.861, P=0.001/<0.0001) (Figure 5C and D). The addition of plasma TM concentrations improved the prognostic effectiveness of MELD-Na (AUROC: 0.729 vs 0.723, P=0.036) for 90-day mortality rates but not that of MELD-Na (AUROC: 0.744 vs 0.711, P=0.161) for 28-day mortality rates or that of COSSH-ACLF IIs (AUROC: 0.878 vs 0.874, P=0.571/0.863 vs 0.861, P=0.649) or MELD (AUROC: 0.752 vs 0.724, P=0.222/0.750 vs 0.735, P=0.292) for 28-/90-day mortality rates (Supplementary Figure 1).

The risk classification of plasma TM concentrations was generated using X-tile plots for ease of clinical application. According to the optimal cut-off values for plasma TM concentrations (<8.4 ng/mL for the low-risk group and ≥8.4 ng/mL for the high-risk group), HBV-ACLF patients were categorized into two distinct groups with significantly varying mortality

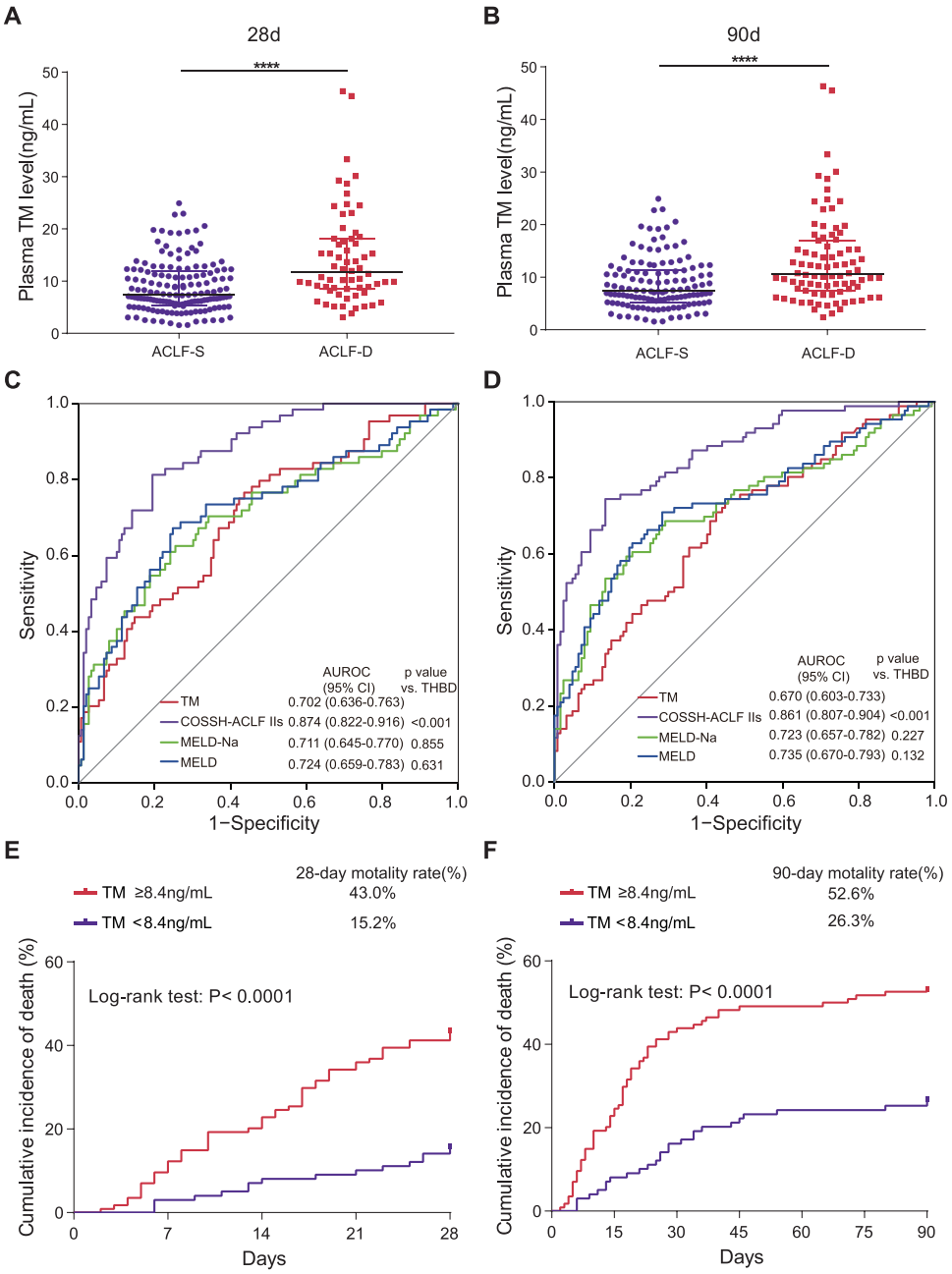

Figure 5 The use of plasma TM concentrations for predicting the 28-day and 90-day mortality rates of HBV-ACLF patients. (**A**) Plasma TM concentrations in HBV-ACLF patients with different outcomes at 28 days. (**B**) Plasma TM concentrations in HBV-ACLF patients with different outcomes at 90 days. (**C**) ROC curve of plasma TM concentrations for predicting the 28-day mortality rates of HBV-ACLF patients compared to other clinical scores. (**D**) ROC curve of plasma TM concentrations for predicting the 90-day mortality rates of HBV-ACLF patients compared to other clinical scores. (**E**) Cumulative incidence of death (high-/low-risk: plasma TM concentrations ≥8.4/<8.4 ng/mL) in patients with HBV-ACLF in 28 days. (**F**) Cumulative incidence of death in patients with HBV-ACLF in 90 days. ****P < 0.0001.

Abbreviations: TM, thrombomodulin; ACLF-D, ACLF-deceased; ACLF-S, ACLF-surviving; COSSH-ACLF IIs, Chinese Group on the Study of Severe Hepatitis B-ACLF II score; MELD, Model for End-stage Liver Disease; MELD-Na, MELD-sodium; AUROC, area under the receiver operating characteristic.

rates. Among those with high plasma concentrations, 43% and 52.6% of patients died within 28 and 90 days, respectively (Figure 5E and F). The low-risk and high-risk groups showed a significant difference in Kaplan-Meier survival analysis (P<0.0001 at 28/90 days). The results above indicated a potential prognostic significance of plasma TM concentrations for HBV-ACLF patients.

Discussion

HBV-ACLF is a lethal syndrome with a high short-term mortality rate and is characterized by hepatic and extrahepatic organ failure. Effective biomarkers for the early diagnosis of patients with HBV-ACLF and prognostication of their mortality are needed. In our work, we investigated the relationship between plasma TM concentrations and HBV-ACLF and found that plasma TM can serve as a candidate biomarker for the diagnosis and prognosis of HBV-ACLF.

The criteria developed to diagnose ACLF are commonly based on the assessment of multiple organ failure and several predictors.[3,14,21] An easy-to-use and reliable diagnostic biomarker can thus be useful for the early identification of HBV-ACLF. A TM mRNA expression analysis based on PBMC transcriptome data revealed its diagnostic and prognostic value for HBV-ACLF at the gene level. TM is an effective anticoagulant that binds to thrombin and then inactivates it to initiate an anticoagulant cascade. The thrombin-thrombomodulin complex activates protein C, a key mediator in the inactivation of coagulant factors Va and VIIIa, further reducing thrombin production during anticoagulation.[22] In our study, plasma TM concentrations in patients with HBV-ACLF were found to be much higher than those in normal controls and those in patients in the CHB, LC, and ACHD groups, and likewise showed a high diagnostic value for HBV-ACLF in the ROC analysis. Furthermore, plasma TM concentrations were correlated with multiple organ failure in HBV-ACLF, including coagulation and kidney failure, suggesting that higher plasma TM concentrations could indicate severer HBV-ACLF. Some coagulation indicators were found to be correlated with plasma TM concentrations, such as PT, D dimer and INR, thus reflecting its important role in coagulation dysfunction. Since plasma TM is metabolized mainly by the liver and kidney, renal dysfunction can also result in an increase in plasma TM concentrations,[11] which may explain the positive correlation between Cr, serum urea, and plasma TM concentrations. Systemic inflammation is a major factor in determining the development, course, and prognosis of ACLF.[23] Our previous study found that immune-metabolism disorder plays a key role during the development of HBV-ACLF.[12] The functional analysis in our study showed that plasma TM concentrations were significantly associated with complement activation and regulation of the inflammatory signaling pathway. In vivo and in vitro studies have found that TM is released from endothelial cells in the form of soluble molecules by the concerted action of TNF-alpha and neutrophils in systemic or local inflammatory diseases.[24] Although elevated TM can be seen in both ACLF and ACHD group, it elevated more significantly in the former. Thus, we deduced that TM elevates because of liver function decline during decompensation, along with specific immune/inflammation mechanisms in HBV-ACLF progression. The role of TM in the immune response to ACLF necessitates further exploration.

A subset of patients with HBV-ACLF recover after intensive therapy, while others progress rapidly and die in the short term. There have been many scoring systems commonly used to predict mortality rates in patients with HBV-ACLF, including COSSH-ACLF II, COSSH-ACLF, MELD and MELD-Na.[3,19,21] As a single molecule, the performance of plasma TM concentrations for predicting HBV-ACLF outcomes has not been reported. In this study, we compared the prognostic ability of plasma TM concentrations with various prognostic scores, confirming that plasma TM is a potential biomarker for predicting the 28-/90-day mortality rates for HBV-ACLF patients. The phenomenon that high concentrations of soluble TM are associated with higher mortality rates has been reported in various diseases. A prospective study found that high plasma soluble TM concentrations indicated poor prognosis of decompensated liver cirrhosis.[11] Analysis from a multicenter randomized controlled trial revealed that TM was associated with increased mortality rates and organ failure in children with acute respiratory failure who were ventilated mechanically.[25] One possible mechanism for the correlation between elevated plasma TM concentrations and poor clinical outcome is intravascular thrombosis, resulting in microcirculatory disturbance and multiorgan failure.[26] We speculated that inflammation in HBV-ACLF patients causes injury to endothelial cells, with reduced TM protein expression in the membrane. Depletion of endothelial cell membrane-bound TM and relatively reduced anticoagulant effects of its soluble form[27] lead to intravascular clotting and secondary hyperfibrinolysis, which contributes to an increased risk of bleeding and multiple organ failure. Previous studies have found that recombinant TM participates in protection against vascular injury and reversal of disseminated intravascular coagulation (DIC) by replacing the depletion of local TM.[28–30] The detailed mechanism of TM in HBV-ACLF should be clarified in future studies. Further risk stratification analysis indicated that patients with concentrations of ≥8.4 ng/mL plasma TM have a higher risk of death and require more careful treatment to reduce that risk. This information may be helpful to better monitor the progression of HBV-ACLF, ultimately improving its short-term survival rates in clinical practice.

Our study has a few limitations. First, the number of participants included in this study is not large enough to alleviate inherent bias among these populations. More subjects should be enrolled to further clarify the role of TM in HBV-ACLF patients. Second, the diagnostic and prognostic value of TM for ACLF of other etiologies, such as chronic alcoholic hepatitis in Western populations, is not elucidated in our work.

Conclusions

Taken together, plasma TM could serve as a candidate biomarker for the diagnosis and prognosis of HBV-ACLF, as well as being crucial for the coagulation and inflammation of this disease.

Abbreviations

ACLF, acute-on-chronic liver failure; ACLF-D, ACLF-deceased; ACLF-S, ACLF survivors; ACHD, acute-on-chronic hepatic dysfunction; AUROC, area under the receiver operating characteristic; CHB, chronic hepatitis B; CI, confidence interval; COSSH, Chinese Group on the Study of Severe Hepatitis B; COSSH-ACLF IIs, COSSH-ACLF II score; ELISA, enzyme-linked immunosorbent assay; GSVA, gene set variation analysis; HBV, hepatitis B virus; HBV-ACLF, hepatitis B virus-related acute-on-chronic liver failure; HE, hepatic encephalopathy; INR, international normalized ratio; LC, liver cirrhosis; MELD, Model for End-stage Liver Disease; MELD-Na, MELD-sodium; NCs, normal controls; PBMC, peripheral blood mononuclear cell; PLT, platelet count; PT, prothrombin time; TB, total bilirubin; TM, thrombomodulin.

Data Sharing Statement

All data relevant to the study are included in the article. The datasets for transcriptome sequencing are available in the Sequence Read Archive database (accession number: PRJNA548207).

Ethics Approval and Informed Consent

Written informed consent of all subjects or their legal surrogates participating in this prospective study was obtained, and the research protocol was approved by the Clinical Research Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine. The study was performed in accordance with the Declaration of Helsinki.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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