International Journal of General Medicine Dovepress

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Alteration of Serum MLKL Levels and Their Association with Severity and Clinical Outcomes in Human Severe Traumatic Brain Injury: A Prospective Cohort Study

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Background: Mixed lineage kinase domain-like protein (MLKL), which modulates necroptosis, has been implicated in pathophysiological processes following acute brain injury. Here, serum MLKL was quantified to determine its prognostic significance in severe traumatic brain injury (sTBI).

Methods: This prospective cohort study enrolled 155 patients with sTBI and 155 healthy volunteers. The severity metrics included the Glasgow Coma Scale (GCS) score and Rotterdam computed tomography (CT) classification. The extended Glasgow outcome scale (GOSE) at posttraumatic 180 days was considered as a prognostic parameter, with a score of 1–4 as indicating poor prognosis. Univariate and subsequent multivariate analyses were used for independent factorial investigation.

Results: Compared to controls, patients displayed profoundly elevated serum MLKL levels. In the framework of restricted cubic spline analysis, serum MLKL levels were linearly correlated with the likelihood of mortality, overall survival, and poor prognosis. Serum MLKL levels were not only independently correlated with GCS, Rotterdam CT scores and GOSE scores, but were also independently predictive of death, overall survival, and poor prognosis. Subgroup analysis showed that serum MLKL levels exhibited negligible interactions with age, sex, hypertension, diabetes, smoking habits, and alcohol consumption to distinguish the possibility of death, overall survival, and poor prognosis. Within the context of receiver operating characteristic curve analysis, serum MLKL levels had strong discrimination effectiveness for death and poor prognosis and, in contrast to GCS and Rotterdam CT scores, were considered to have equivalent predictive ability.

Conclusion: Extreme elevation of serum MLKL levels is intimately related to trauma severity, death, and neurological outcomes, suggesting that serum MLKL may act as a potential predictor for facilitating severity stratification and prognosis prediction of sTBI.

Keywords: traumatic brain injury, mixed lineage kinase domain-like protein, severity, outcome, death, overall survival, biomarkers

Introduction

Traumatic brain injury (TBI) is a singular traumatic entity that damages brain tissue from an external force that mechanistically encompasses primary and secondary brain injuries.[1] Primary brain injury is an irreversible direct injury which is irreversible in essence.[2] Secondary brain injury is very complex in pathophysiological processes, which has been up to date substantiated to involve inflammatory activation, mitochondrial impairment, oxidative disturbance, neuronal death, brain edema formation and blood-brain barrier damage.[3] Clinical parameters such as the Glasgow coma scale (GCS) score and radiological indicators such as Rotterdam computed tomography (CT) grading are considered acceptable for severity stratification and outcome prediction in the management of TBI.[4,5] As an upgraded

Received: 15 August 2024
Accepted: 28 October 2024
Published: 6 November 2024

version of the Glasgow Outcome Scale (GOS), the extended GOS (GOSE) is of great clinical value for appraising neurological outcomes in neurological field.[6] The easy obtainability of fluid samples has necessitated the exploration of biomarkers with respect to their prognostic implications in TBI in previous decades.[7–9]

Necroptosis is classified as a type of recently discovered programmed necrosis, which is characterized by cell swelling, plasma membrane disruption, subsequent cell leakage, and immune system activation;[10,11] These intracellular and extracellular changes have indicated that necroptosis can confer strong proinflammatory effects in inflammatory and immune diseases such as Alzheimer's disease, non-alcoholic fatty liver disease, and acute lung injury.[12–14] Necroptosis is a new type of necrosis with inevitable involvement in molecular mechanisms following acute brain injury.[15,16] Mixed lineage kinase domain-like protein (MLKL), which acts as a crucial regulator of necroptotic cell death, participates in inflammation-related illnesses, such as ulcerative colitis, sepsis, and cancers.[17–19] MLKL can be localized to astrocytes and neurons, and its expression is markedly enhanced following experimental intracerebral hemorrhage.[20–22] Alternatively, in vivo and in vitro experiments using the gene-knock technique or MLKL-inhibitory agents have revealed that MLKL may have detrimental effects in acute brain injury, as demonstrated by reduced neuroinflammation, decreased neuronal death percentage, improved neurological deficit, reduced lesion size, decreased brain water content rate, and attenuated blood-brain barrier permeability following oxygen glucose deprivation, intracerebral hemorrhage, or ischemic stroke.[22–24] Overall, MLKL may have prognostic significance in acute brain injury. A cohort of patients was selected to evaluate the value of serum MLKL as a prognostic biomarker for severe TBI (sTBI).

Materials and Methods

Study Design and Plan, Recruitment Process of Participants and Ethical Permission

This study, which was conducted from January 2021 to July 2023 at Shengzhou People's Hospital, was implemented in two parts. One was a cross-sectional study in which serum MLKL levels were measured to uncover the evolution between patients with sTBI and healthy controls. Another study included a prospective cohort of patients with sTBI to explore the prognostic performance of serum MLKL. The study plan and eligibility criteria for the patients and controls are shown in Figure 1. This study was conducted in compliance with the ethical principles of the Declaration of Helsinki and its amendments. The study protocol was approved by the Institutional Review Committee of Shengzhou People's Hospital (No. 2021-039-01). The participants agreed to participate in the current study, and the legal proxies of patients and controls provided signed informed consent forms beforehand.

Basic Data Collection and Outcome Evaluation

Recorded demographic data included age and sex. The registered adverse habits included tobacco smoking and alcohol consumption. The two inquired chronic diseases were hypertension and diabetes mellitus. Traumatic causes can be categorized into two types: traffic accidents and others. Recordings regarding non-invasive arterial blood pressure were acquired upon arrival at the emergency center. Admission time was defined as the interval from trauma to hospital admission and blood collection time was defined as the period between trauma and blood sampling. Based on the hemorrhagic locations identified by the head CT scan, five types of traumatic bleeding were identified: intracerebral, epidural, subdural, subarachnoid, and intraventricular cavities. Brain contusions and pneumocephalus were observed simultaneously. CT readings were affirmed by two doctors and if the results were inconsistent, the third doctor was inquired. Post-resuscitation GCS and Rotterdam CT scale scores were considered the respective clinical and radiological severity assessment parameters. Surgical treatment, emergency operation within post-traumatic twenty-four hours, decompressive craniectomy and seizure onset were recorded. Patients were followed-up via telephone or outpatient visits. A GOSE score of 1–4 at one-hundred and eighty days following trauma signifies poor prognosis.[25]

Blood Obtainment, Sample Preparation and Immune Test

Peripheral venous blood was drawn from the median cubital vein of the patients upon admission and from that of the controls at their entrance to the study. Admission blood glucose levels and blood leucocyte counts were detected using the conventional methods. And for measurements of serum MLKL, blood specimens were promptly laid in 5 mL gel-

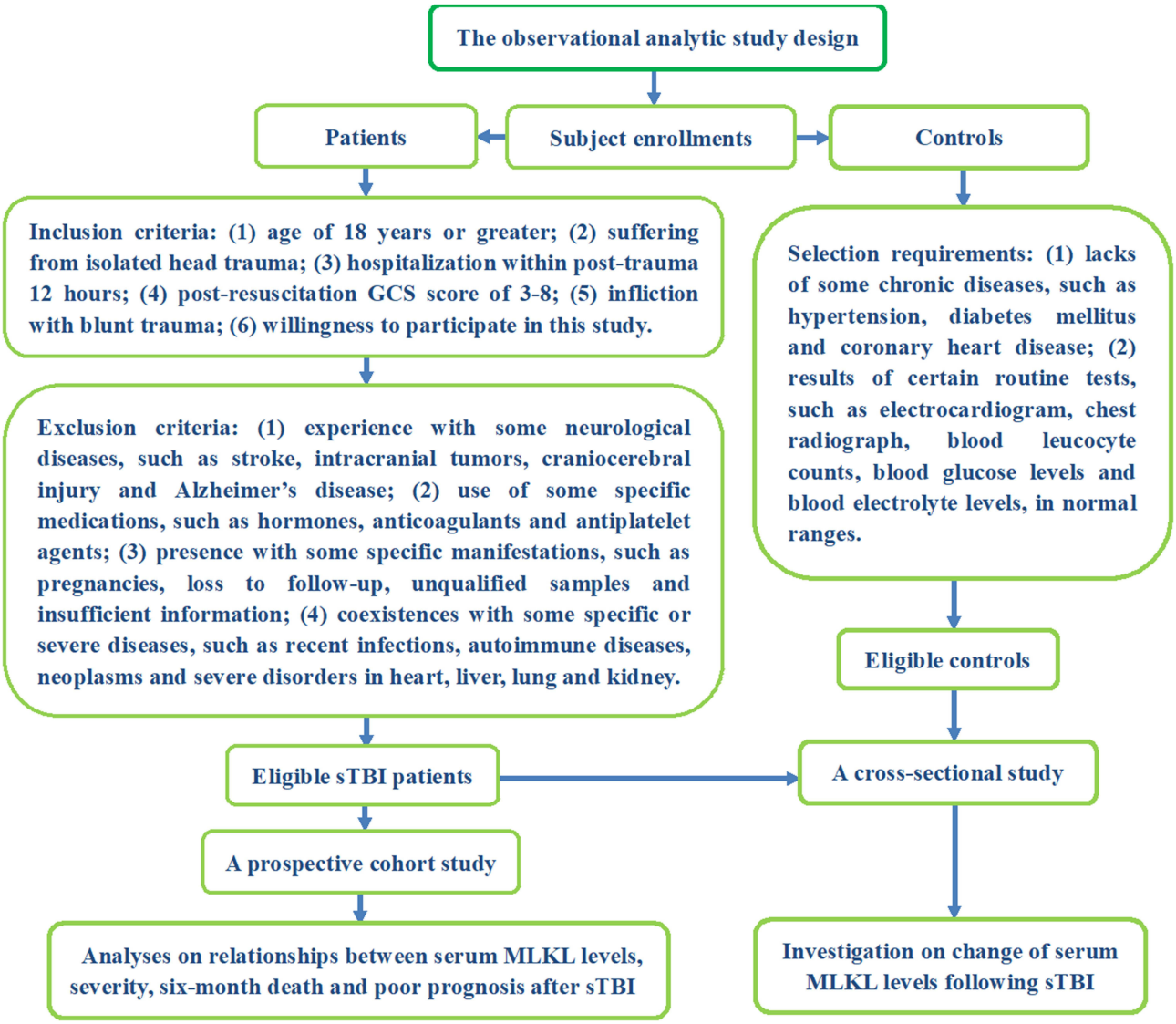

Figure 1 Study plan diagram for clinical investigation in severe traumatic brain injury. The purpose of this study was to determine whether serum mixed-lineage kinase domain-like protein levels are altered and can be considered a prognostic biomarker of severe traumatic brain injury.
Abbreviations: GCS, Glasgow coma scale; sTBI, severe traumatic brain injury; MLKL, mixed lineage kinase domain-like protein.

containing biochemistry tubes, centrifuged after coagulation, and then the supernatant was transferred into Eppendorf tubes and stored at −80°C until testing as needed. To prevent MLKL from decomposing, the serum MLKL levels were measured in batches. A set of samples, which were acquired within three months, were melted once the completion of the three-month collection to detect serum MLKL levels using the sandwich enzyme-linked immunosorbent assay kit (Catalogue number, BES6097K; SHANGHAI BOSEN BIOLOGICAL TECHNOLOGY CO., LTD., China). Its detection range spanned from 31.2 to 2000 pg/mL, with respective inter-assay and intra-assay precision coefficients of variation below 10% and 8%. All detections were performed in duplicate by the same proficient technical staff. The average values of the dual measurements were used for ultimate assessment.

Statistical Analysis

In this study, there was an application of the SPSS statistical package version 20.0 (SPSS Inc., Chicago, Illinois, USA) for statistical processing. Categorical variables are presented as counts (percentage). Continuous data were analyzed for judging normality using the Shapiro–Wilk test or Kolmogorov–Smirnov test as deemed suitable Normally-distributed

continuous data are shown as means (standard deviations, SDs) and non-normally distributed continuous data are presented as medians (percentiles 25th-75th). Because serum GOSE score and serum MLKL levels were non-normally distributed continuous variables, their respective bivariate correlations were assessed via the Spearman test. Two-group comparisons of data were done using Pearson's Chi-square test for categorical variables, independent *t*-test for normally-distributed continuous variables, Mann–Whitney *U*-test for non-normally distributed continuous variables, and Log rank test for overall survival time. Binary logistic regression analysis was applied for investigating independent predictors of death and poor prognosis, multivariate linear regression analysis was employed for determining independently correlative factors of GOSE scores and serum MLKL levels, and Cox's proportional hazard analysis was deployed for revealing independent predictors of overall survival. Hosmer–Lemeshow test was in utilization for assessing model fit of binary logistic regression analysis. As abnormal cisterns, midline shift > 5 mm, subarachnoid hemorrhage and epidural hematoma constitute the Rotterdam CT assessment system, these variables were not integrated into the multivariate model. Receiver operating the curve analysis was applied using MedCalc statistical software version 17.4 (MedCalc Software, Mariakerke, Belgium), and a sufficient sample size was calculated. The variance inflation factor (VIF) was produced using R software (version 3.5.1; https://www.r-project.org), and restricted cubic spline (RCS) and subgroup analyses were performed. Statistical significance was set at $p < 0.05$.

Results

Participant Selection and Characteristics

An initial consecutive enrollment of 209 patients with sTBI was fulfilled, thereafter fifty-four cases were eliminated from this study owing to the exclusion criteria in Figure 1, and an accumulative of 155 patients were finally retained. The baseline features of all patients are displayed in Table 1. Additionally, 155 controls were selected according to the eligibility criteria in

Table 1 Patient Basic Characteristics and Correlative Analyses of Serum Mixed Lineage Kinase Domain-Like Protein Levels Following Severe Traumatic Brain Injury

	All Patients	Spearman Test	
		ρ	*P* value
Male	88 (56.8%)	−0.136	0.092
Age (years)	40.2±11.8	0.052	0.522
Cigarette smoking	45 (29.0%)	0.094	0.245
Alcohol consumption	49 (31.6%)	0.144	0.073
Hypertension	34 (21.9%)	0.157	0.050
Diabetes mellitus	21 (13.6%)	0.140	0.082
Admission time (h)	4.6 (3.4–5.8)	−0.055	0.498
Blood-collection time (h)	5.6 (4.4–7.4)	−0.100	0.215
Traffic accident	74 (47.7%)	0.077	0.399
GCS scores	5 (4–7)	−0.617	<0.001
Systolic arterial pressure (mmHg)	125.9 ± 21.4	0.026	0.750
Diastolic arterial pressure (mmHg)	74.2 ± 12.3	0.019	0.816
Rotterdam CT scores	4 (4–5)	0.609	<0.001
Abnormal cisterns	120 (77.4%)	0.285	<0.001
Midline shift > 5 mm	95 (61.3%)	0.314	<0.001
Epidural hematoma	80 (51.6%)	0.033	0.682
Subdural hematoma	90 (58.1%)	0.056	0.488
Subarachnoid hemorrhage	103 (66.5%)	0.176	0.028
Intraventricular hemorrhage	18 (11.6%)	0.014	0.868
Intracerebral hematoma	81 (52.3%)	0.029	0.719

(*Continued*)

Table 1 (Continued).

	All Patients	Spearman Test	
		ρ	*P* value
Brain contusion	89 (57.1%)	0.153	0.058
Pneumocephalus	55 (35.5%)	−0.028	0.726
Operation within 24 hours	66 (42.6%)	0.279	<0.001
Surgical treatment	83 (53.6%)	0.090	0.266
Decompressive craniectomy	25 (16.1%)	0.060	0.461
Seizure	42 (27.1%)	0.035	0.668
Blood glucose levels (mmol/l)	8.2 (6.3–11.6)	0.282	<0.001
Blood leucocyte count ($\times 10^9$/l)	8.2 (6.6–10.4)	0.045	0.578

Abbreviations: CT, computed tomography; GCS, Glasgow coma scale.

Figure 1. The control group was aged from 18 to 68 years (mean, 41.6 years; SD, 13.5 years), including 84 males and 71 females, as well as 43 tobacco smokers and 44 alcohol users. The mean age, sex percentage, and proportion of alcohol consumers and cigarette smokers were not significantly different between patients and controls (all P>0.05).

Alteration of Serum MLKL Levels and Its Relevance to Severity Following sTBI

In contrast to controls, patients had extremely elevated serum MLKL levels (P<0.001; Figure 2). Spearman test was performed, in which serum MLKL levels were regarded as a dependent factor. As shown in Table 1, serum MLKL levels were significantly correlated with GCS scores (P<0.001) and positively correlated with Rotterdam CT scores (P<0.001), abnormal cisterns (P<0.001), midline shift > 5 mm (P<0.001), subarachnoid hemorrhage (P<0.05), surgery within 24 h (P<0.001), and blood glucose levels (P<0.001). Subsequently, GCS scores, Rotterdam CT classification, operation within 24 h and blood glucose levels were entered into the multiple-variable model. It was demonstrated that serum MLKL levels were independently correlated with GCS score [beta, −0.256; 95% confidence interval (CI], −0.404–0.108; VIF, 1.606; P=0.008) and Rotterdam CT score (beta, 0.636; 95% CI; 0.398–0.875; VIF, 1.448; P=0.001).

Serum MLKL Levels and Post-Traumatic 180-Day Death

A collective of Thirty-six patients were deceased within 180 days following sTBI, indicating that post-trauma 180-day mortality was 23.2% (36/155) in this host of patients with sTBI. As shown in Figure 3, there was a linear correlation between serum MLKL levels and likelihood of death (P for nonlinear >0.05). The serum MLKL levels were significantly higher in the dying group than in the alive group (P>0.001; Table 2). Also, the dead, relative to the alive, had

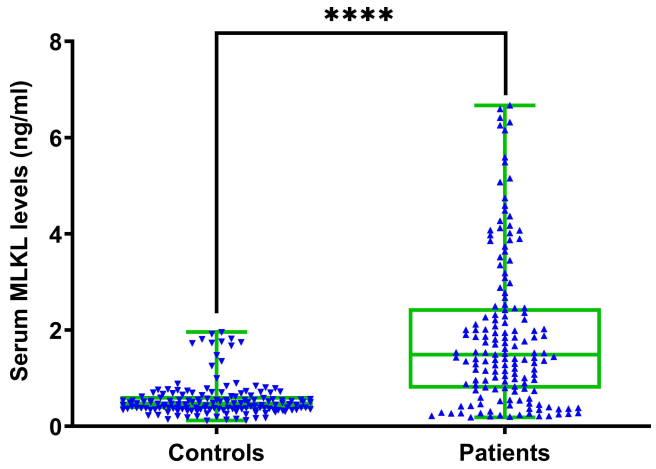

Figure 2 Alteration of serum mixed lineage kinase domain-like protein levels after severe traumatic brain injury. Serum mixed-lineage kinase domain-like protein levels were markedly higher in patients with severe traumatic brain injury than in the controls (****P<0.001).
Abbreviation: MLKL, mixed lineage kinase domain-like protein.

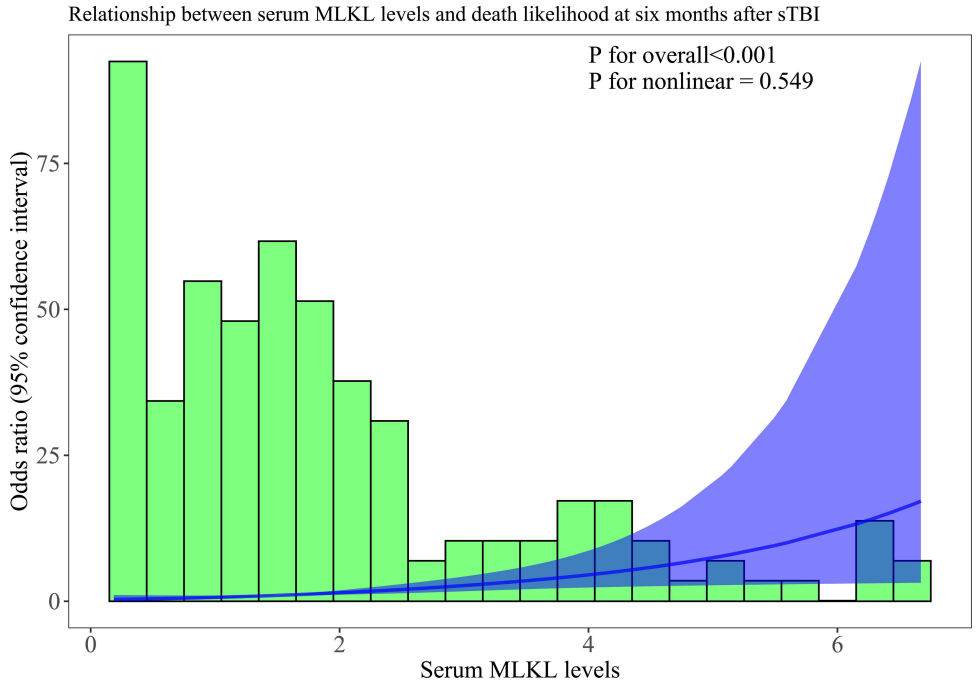

Figure 3 Relationship between serum mixed lineage kinase domain-like protein levels and death risk following severe traumatic brain injury. Under restricted cubic spline, serum mixed-lineage kinase domain-like protein levels were linearly correlated with the risk of death following severe traumatic brain injury (P for nonlinear >0.05).
Abbreviations: sTBI, severe traumatic brain injury; MLKL, mixed-lineage kinase domain-like protein.

significantly lower GCS scores, displayed substantially higher Rotterdam CT scores and blood glucose levels, and exhibited dramatically elevated proportions of abnormal cisterns, midline shift above 5 mm, subarachnoid hemorrhage and operation with 24 hours (all P<0.05; Table 2). Thus, serum MLKL levels, GCS scores, Rotterdam CT scores, blood glucose levels and operation within 24 h were included in the binary logistic regression model. Of note, the retained independent predictors of death were serum MLKL level [odds ratio (OR], 1.477; 95% CI, 1.018–1.882; VIF, 1.712;

Table 2 Factors Pertaining to 180-Day Death and Overall Survival Following Severe Traumatic Brain Injury

	180-Day Death			180-Day Overall Survival	
	The Deceased	The Alive	*P* value	HR (95% CI)	*P* value
Male	22 (61.1%)	66 (55.5%)	0.549	1.201 (0.614–2.347)	0.592
Age (years)	41.1±12.3	40.0±11.7	0.587	1.007 (0.979–1.034)	0.642
Cigarette smoking	12 (33.3%)	33 (27.7%)	0.516	1.169 (0.584–2.337)	0.659
Alcohol consumption	16 (44.4%)	33 (27.7%)	0.059	1.780 (0.922–3.436)	0.086
Hypertension	12 (33.3%)	22 (18.5%)	0.059	1.814 (0.907–3.630)	0.092
Diabetes mellitus	8 (22.2%)	13 (10.9%)	0.098	1.947 (0.887–4.273)	0.097
Admission time (h)	4.3 (2.9–5.2)	4.6 (3.5–5.9)	0.358	0.957 (0.829–1.105)	0.547
Blood-collection time (h)	5.1 (4.4–6.8)	5.6 (4.5–7.5)	0.261	0.935 (0.815–1.072)	0.334
Traffic accident	22 (61.1%)	52 (43.7%)	0.067	1.802 (0.922–3.522)	0.085
GCS scores	4 (3–5)	6 (4–7)	<0.001	0.443 (0.324–0.604)	<0.001
Systolic arterial pressure (mmHg)	122.6±21.0	126.9±21.5	0.287	0.992 (0.977–1.008)	0.312
Diastolic arterial pressure (mmHg)	73.0±12.6	74.6±12.2	0.488	0.992 (0.966–1.018)	0.543
Rotterdam CT scores	5 (4–6)	4 (4–5)	<0.001	2.737 (1.895–3.955)	<0.001
Abnormal cisterns	36 (100.0%)	84 (70.6%)	<0.001	32.037 (1.325–774.779)	0.033
Midline shift > 5 mm	34 (94.4%)	61 (51.3%)	<0.001	12.742 (3.059–53.064)	<0.001
Epidural hematoma	20 (55.6%)	60 (50.4%)	0.589	1.140 (0.591–2.200)	0.696
Subdural hematoma	20 (55.6%)	70 (58.8%)	0.728	0.938 (0.486–1.811)	0.850
Subarachnoid hemorrhage	30 (83.3%)	73 (61.3%)	0.014	2.888 (1.202–6.940)	0.018

(*Continued*)

Table 2 (Continued).

	180-Day Death			180-Day Overall Survival	
	The Deceased	The Alive	*P* value	HR (95% CI)	*P* value
Intraventricular hemorrhage	4 (11.1%)	14 (11.8%)	1.000	0.936 (0.331–2.647)	0.900
Intracerebral hematoma	22 (61.1%)	59 (50.0%)	0.225	1.487 (0.761–2.907)	0.246
Brain contusion	20 (55.6%)	69 (58.0%)	0.796	0.918 (0.476–1.771)	0.798
Pneumocephalus	14 (38.9%)	41 (34.5%)	0.626	1.201 (0.615–2.348)	0.597
Operation within 24 hours	22 (61.1%)	44 (37.0%)	0.010	2.317 (1.185–4.530)	0.014
Surgical treatment	22 (61.1%)	61 (51.3%)	0.299	1.403 (0.718–2.742)	0.322
Decompressive craniectomy	7 (19.4%)	8 (15.1%)	0.537	1.363 (0.597–3.111)	0.463
Seizure	14 (38.9%)	28 (23.5%)	0.069	1.861 (0.952–3.637)	0.069
Blood glucose levels (mmol/l)	9.4 (7.7–16.7)	8.0 (6.0–10.8)	0.002	1.140 (1.063–1.221)	<0.001
Blood leucocyte count ($\times 10^9$/l)	8.3 (6.9–11.7)	8.2 (6.5–10.3)	0.939	1.023 (0.894–1.170)	0.739
Serum MLKL levels (ng/mL)	2.4 (1.4–4.5)	1.3 (0.5–2.1)	<0.001	1.775 (1.469–2.145)	<0.001

Notes: Count (proportion), median (lower-upper quartiles) and mean ± standard deviation were presented for representing different types of data. As applicable, the Pearson's Chi-square test, Mann–Whitney *U*-test or independent *t* test was employed for data comparison between two groups. Hazard ratio was yielded using univariate Cox's proportional hazard regression analysis.
Abbreviations: CT, computed tomography; GCS, Glasgow coma scale; MLKL, mixed lineage kinase domain-like protein; HR, hazard ratio; 95% CI, 95% confidence interval.

P=0.015), GCS score (OR, 0.533; 95% CI, 0.338–0.843; VIF, 1.623; P=0.007), and Rotterdam CT score (OR, 2.357; 95% CI, 1.310–4.241; VIF, 1.700; P=0.004). The Hosmer–Lemeshow test showed that the model fit was satisfactory (P=0.273). There were negligible interactions between serum MLKL levels and other usual parameters such as age, sex, and smoking (all P interaction >0.05; Figure 4). As shown in Figure 5, the serum MLKL levels significantly discriminated the possibility of death. Moreover, its discrimination efficiency was equivalent to that of the GCS and Rotterdam CT scores (both P>0.05; Figure 6).

As shown in Figure 7, a linear relationship between serum MLKL levels and the probability of 180-day overall survival was proven by applying RCS (P for nonlinear >0.05). Patients were classified into four subgroups at a based on the median, lower, and upper quartiles of serum MLKL levels. Notably, overall survival time was dramatically shortened with increasing serum MLKL levels after sTBI (P<0.001; Figure 8). Using univariate Cox's proportional hazards regression analysis, the factors, which were pronouncedly associated with 180-day overall survival, were abnormal cisterns, midline shift above 5 mm, subarachnoid hemorrhage, Rotterdam CT scores, serum MLKL levels, GCS scores, blood glucose levels and operation within 24 hours (all P<0.05; Table 2). Hence, Rotterdam CT scores, serum MLKL levels, GCS scores, blood glucose levels and operation within 24 hours were included in the multivariate model. Noteworthily, serum MLKL levels [hazard ratio (HR), 1.387; 95% CI, 1.010–1.640; VIF, 1.487; P=0.012], GCS scores (OR, 0.586; 95% CI, 0.413–0.831; VIF, 1.265; P=0.003) and Rotterdam CT scores (OR, 1.609; 95% CI, 1.033–2.506; VIF, 1.417; P=0.005) independently forecasted overall survival. As shown in Figure 9, serum MLKL levels nonsignificantly interacted with age, sex, hypertension, diabetes mellitus, smoking, or drinking (all P interaction >0.05).

Serum MLKL Levels and 180-Day Neurological Outcome After sTBI

At 180 days after sTBI, GOSE scores varied from 1 to 8 (median, 5; percentiles 25^{th}–75^{th}: 2–6). Using the Spearman test, GOSE scores were intimately related to diabetes mellitus, GCS scores, Rotterdam CT scores, abnormal cisterns, midline > 5 mm, subarachnoid hemorrhage, serum MLKL levels, operation within twenty-four hours, blood glucose levels, and blood leukocyte counts (all P<0.05; Table 3). Therefore, diabetes mellitus, GCS scores, Rotterdam CT scores, serum MLKL levels, operation within twenty-four hours, blood glucose levels, and blood leukocyte counts were included in the multivariate model. Finally, GOSE scores were independently correlated with GCS scores (beta, 0.435; 95% CI; 0.219–0.651; VIF, 1.742; P=0.001), Rotterdam CT scores (beta, −0.972; 95% CI; −1.345–0.598; VIF, 1.817; P=0.005), and serum MLKL levels (beta, −0.300; 95% CI; −0.531–0.069; VIF, 1.914; P=0.011).

At 180 days following sTBI, a total of 36, 17, 8, 10, 31, 25, 16, and 12 cases had GOSE scores from 1 to 8 respectively, and 71 patients had poor prognosis (GOSE scores of 1–4). Figure 10 shows linear correlation between

Subgroup	Total	OR(95% CI)	P	P interaction
Age				0.919
≥ 60 years	24	1.540 (0.640-3.702)	0.335	
< 60 years	131	1.412 (1.025-1.945)	0.035	
Gender				0.069
Male	88	1.626 (1.087-2.432)	0.018	
Female	67	1.534 (0.877-2.683)	0.134	
Hypertension				0.131
Yes	34	1.664(0.851-3.254)	0.137	
No	121	1.564 (1.145-2.136)	0.005	
Diabetes mellitus				0.122
Yes	21	7.381 (0.847-64.298)	0.072	
No	134	1.607 (1.207-2.141)	0.001	
Cigarette smoking				0.231
Yes	45	1.706 (0.796-3.656)	0.174	
No	110	1.380 (1.057-4.940)	0.031	
Alcohol drinking				0.121
Yes	49	1.767 (0.961-3.249)	0.067	
No	106	1.446 (1.022-2.049)	0.038	

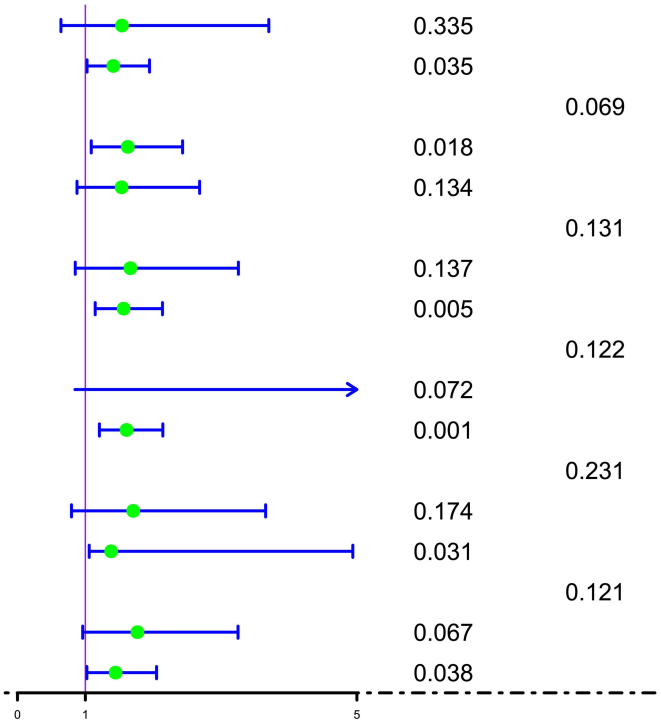

Figure 4 Subgroup analysis delineating interactions of serum mixed lineage kinase domain-like protein levels with other conventional factors in death prognosis assessment after severe traumatic brain injury. No significant interactions existed between serum mixed lineage kinase domain-like protein levels and age, sex, diabetes, hypertension, alcohol consumption, or tobacco smoking for death prediction (all P interaction >0.05).
Abbreviations: OR, odds ratio; 95% CI, 95% confidence interval.

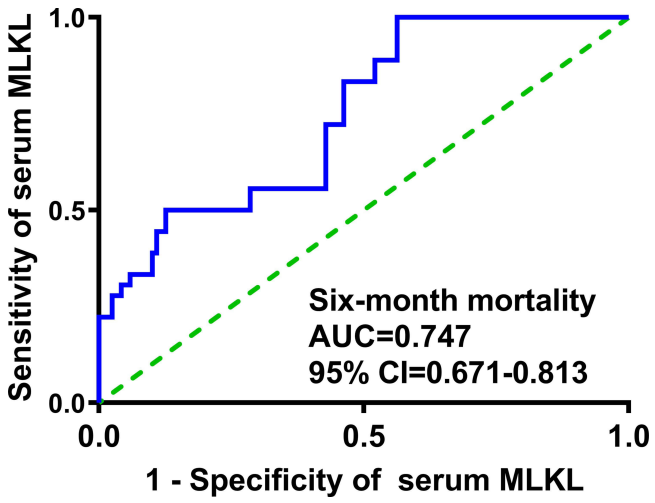

Figure 5 Receiver operating characteristic curve demonstrating discrimination efficacy of serum mixed lineage kinase domain-like protein levels for death risk after severe traumatic brain injury. The probability of death after severe traumatic brain injury could be effectively distinguished based on serum mixed-lineage kinase domain-like protein levels.
Abbreviations: AUC, area under curve; 95% CI, 95% confidence interval; MLKL, mixed lineage kinase domain-like protein.

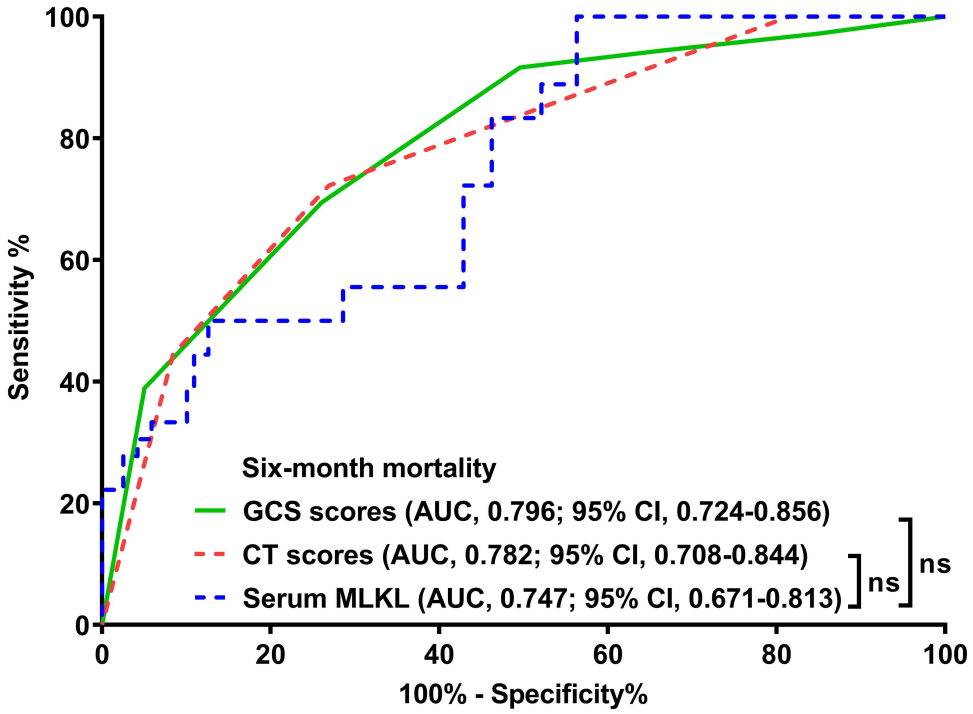

Figure 6 Comparisons of death prediction ability of serum mixed lineage kinase domain-like protein levels with other variables in human severe traumatic brain injury. Under receiver operating characteristic curve, discrimination of the possibility of death after severe traumatic brain injury could be similarly efficiently completed by serum mixed-lineage kinase domain-like protein levels, along with Glasgow coma scale scores and Rotterdam computed tomography scores (both P>0.05).
Abbreviations: AUC, area under curve; 95% CI, 95% confidence interval; MLKL, mixed lineage kinase domain-like protein; GCS, Glasgow Coma Scale; CT, computed tomography; ns, non-significant.

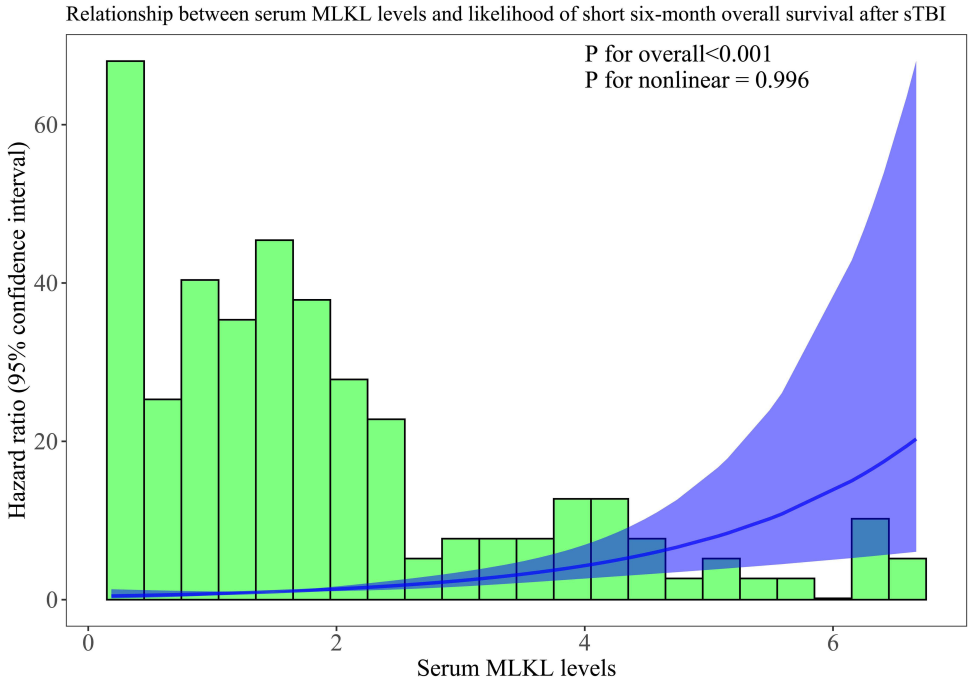

Figure 7 Serum mixed lineage kinase domain-like protein levels and likelihood of lowered overall survival after severe traumatic brain injury. Under restricted cubic spline, serum mixed-lineage kinase domain-like protein levels were linearly related to the probability of reduced overall survival after severe traumatic brain injury (P for nonlinear >0.05).
Abbreviations: MLKL, mixed-lineage kinase domain-like protein; sTBI, severe traumatic brain injury.

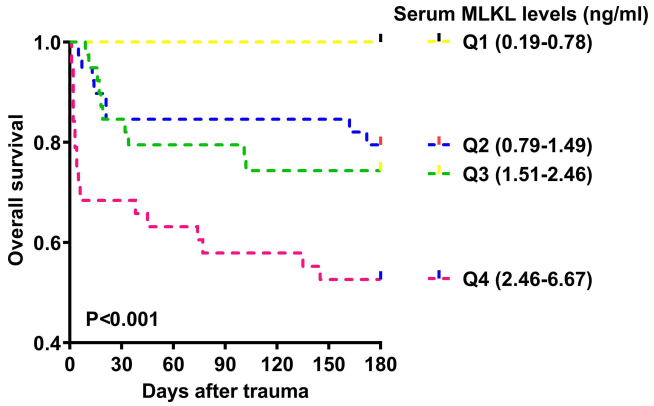

Figure 8 Survival curve reflecting relationship between serum mixed lineage kinase domain-like protein levels and overall survival after human severe traumatic brain injury. Patients were divided into four groups based on the median, lower quartile, and upper quartile values of serum mixed-lineage kinase domain-like protein levels, named as Q1, Q2, Q3 and Q4 successively. With increasing levels of serum mixed-lineage kinase domain-like proteins, overall survival was significantly reduced after severe traumatic brain injury (P<0.001).
Abbreviation: MLKL, mixed lineage kinase domain-like protein.

Subgroup	Total	HR(95% CI)	P	P interaction
Age				0.636
≥ 60 years	24	1.412 (0.656-3.309)	0.378	
< 60 years	131	1.445 (1.165-1.793)	0.007	
Gender				0.372
Male	88	1.465 (1.054-2.035)	0.023	
Female	67	1.648 (1.168-2.326)	0.004	
Hypertension				0.415
Yes	34	1.484 (0.947-2.324)	0.085	
No	121	1.422 (1.096-1.845)	0.008	
Diabetes mellitus				0.159
Yes	21	1.153 (0.887-1.500)	0.287	
No	134	3.477 (1.482-8.156)	0.004	
Cigarette smoking				0.303
Yes	45	1.750 (0.994-3.080)	0.052	
No	110	1.378 (1.052-1.805)	0.022	
Alcohol drinking				0.513
Yes	49	1.207 (0.857-1.700)	0.282	
No	106	1.523 (1.046-2.217)	0.028	

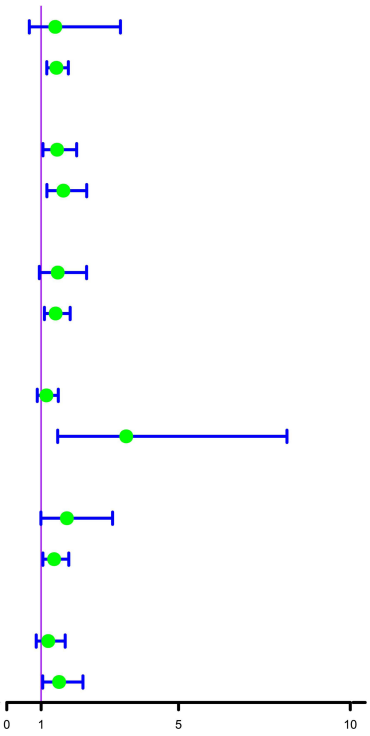

Figure 9 Verification of interaction between serum mixed lineage kinase domain-like protein levels and other common variables for predicting overall survival after severe traumatic brain injury. By employing subgroup analysis, no obvious interactions were observed for serum mixed-lineage kinase domain-like protein levels relevant to age, sex, diabetes, hypertension, alcohol use, and tobacco smoking in predicting overall survival following severe traumatic brain injury (all P interaction >0.05).
Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.

serum MLKL levels and the risk of poor prognosis after sTBI (P for nonlinear >0.05). In addition, there were remarkable differences in terms of diabetes mellitus, GCS scores, Rotterdam CT scores, abnormal cisterns, midline > 5 mm, subarachnoid hemorrhage, serum MLKL levels, operation within twenty-four hours, blood glucose levels, and blood leukocyte counts between patients with poor prognosis and those without (all P<0.05; Table 3). Consequently, incorporation of diabetes mellitus, GCS scores, Rotterdam CT scores, serum MLKL levels, operation within twenty-four hours,

Table 3 Parameters Relevant to Extended Glasgow Outcome Scale Scores and Poor Prognosis at 180 Days After Severe Traumatic Brain Injury

	Spearman Test		GOSE Scores		
	ρ	*P* value	1–4	5–8	*P* value
Male	0.029	0.718	39 (54.9%)	49 (58.3%)	0.670
Age (years)	−0.023	0.778	40.7 ± 12.1	39.7 ± 11.6	0.611
Cigarette smoking	−0.123	0.126	23 (32.4%)	22 (26.2%)	0.397
Alcohol consumption	−0.085	0.295	23 (32.4%)	26 (31.0%)	0.847
Hypertension	−0.105	0.195	16 (22.5%)	18 (21.4%)	0.868
Diabetes mellitus	−0.221	0.006	15 (21.1%)	6 (7.1%)	0.011
Admission time (h)	0.072	0.371	4.4 (2.9–5.3)	4.6 (3.6–5.9)	0.207
Blood-collection time (h)	0.113	0.162	5.2 (4.3–7.2)	5.6 (4.7–7.6)	0.116
Traffic accident	−0.155	0.054	37 (51.2%)	37 (44.1%)	0.317
GCS scores	0.601	<0.001	4 (4–5)	6 (5–7)	<0.001
Systolic arterial pressure (mmHg)	0.147	0.068	123.4 ± 21.1	128.0 ± 21.7	0.185
Diastolic arterial pressure (mmHg)	0.059	0.469	74.2 ± 13.2	74.2 ± 11.6	0.966
Rotterdam CT scores	−0.651	<0.001	5 (4–6)	4 (4–4)	<0.001
Abnormal cisterns	−0.345	<0.001	66 (93.0%)	54 (64.3%)	<0.001
Midline shift > 5 mm	−0.527	<0.001	60 (84.5%)	35 (41.7%)	<0.001
Epidural hematoma	−0.076	0.350	42 (59.2%)	38 (45.2%)	0.084
Subdural hematoma	−0.036	0.660	45 (63.4%)	45 (53.6%)	0.218
Subarachnoid hemorrhage	−0.288	<0.001	55 (77.5%)	48 (57.1%)	0.008
Intraventricular hemorrhage	0.037	0.652	8 (11.3%)	10 (11.9%)	0.902
Intracerebral hematoma	−0.151	0.060	43 (60.6%)	38 (45.2%)	0.057
Brain contusion	−0.137	0.090	45 (63.4%)	44 (52.4%)	0.168
Pneumocephalus	0.025	0.758	22 (31.0%)	33 (39.3%)	0.282
Operation within 24 hours	−0.280	<0.001	38 (53.5%)	28 (33.3%)	0.011
Surgical treatment	−0.089	0.270	40 (56.3%)	43 (51.2%)	0.522
Decompressive craniectomy	−0.075	0.351	14 (19.7%)	11 (13.1%)	0.264
Seizure	−0.091	0.258	22 (31.0%)	20 (23.8%)	0.317
Blood glucose levels (mmol/l)	−0.201	0.012	9.3 (7.3–13.6)	8.0 (5.6–9.9)	0.004
Blood leucocyte count ($\times 10^9$/l)	−0.207	0.010	9.0 (6.9–11.8)	8.0 (6.4–10.0)	0.039
Serum MLKL levels (ng/mL)	−0.604	<0.001	2.0 (1.4–3.5)	0.9 (0.4–1.9)	<0.001

Notes: Count (proportion), median (lower-upper quartiles) and mean ± standard deviation were reported for manifesting different types of data. As applicable, the Pearson's Chi-square test, Mann–Whitney *U*-test or independent *t* test was adopted for intergroup comparison of data.

Abbreviations: CT, computed tomography; GCS, Glasgow coma scale; MLKL, mixed lineage kinase domain-like protein; GOSE, extended Glasgow outcome scale.

blood glucose levels, and blood leukocyte counts into the multivariate model led to the finding that serum MLKL levels (OR, 1.712; 95% CI, 1.230–3.372; VIF, 1.712; P=0.012), GCS scores (OR, 0.490; 95% CI, 0.340–0.706; VIF, 1.615; P=0.005), and Rotterdam CT scores (OR, 3.541; 95% CI, 1.906–9.580; VIF, 1.719; P=0.009) independently predicted poor clinical outcomes after sTBI. Satisfactory model stability was obtained by applying the Hosmer–Lemeshow test (P=0.392). Substantial interactions were not observed between serum MLKL levels and age, sex, diabetes, etc. (all P interaction >0.05; Figure 11). Additionally, the risk of poor prognosis was effectively distinguished by the serum MLKL levels (Figure 12). Furthermore, its predictive ability was in the range of the GCS and Rotterdam CT scores (both P>0.05; Figure 13).

Discussion

MLKL, which participates in necroptosis, may function as a detrimental factor in acute brain injury.[22–24] In this study, serum MLKL was explored to identify its implications as a prognostic biochemical marker at the 180-day mark of sTBI. Subsequently, several intriguing results were obtained. First, relative to individuals without health conditions, serum

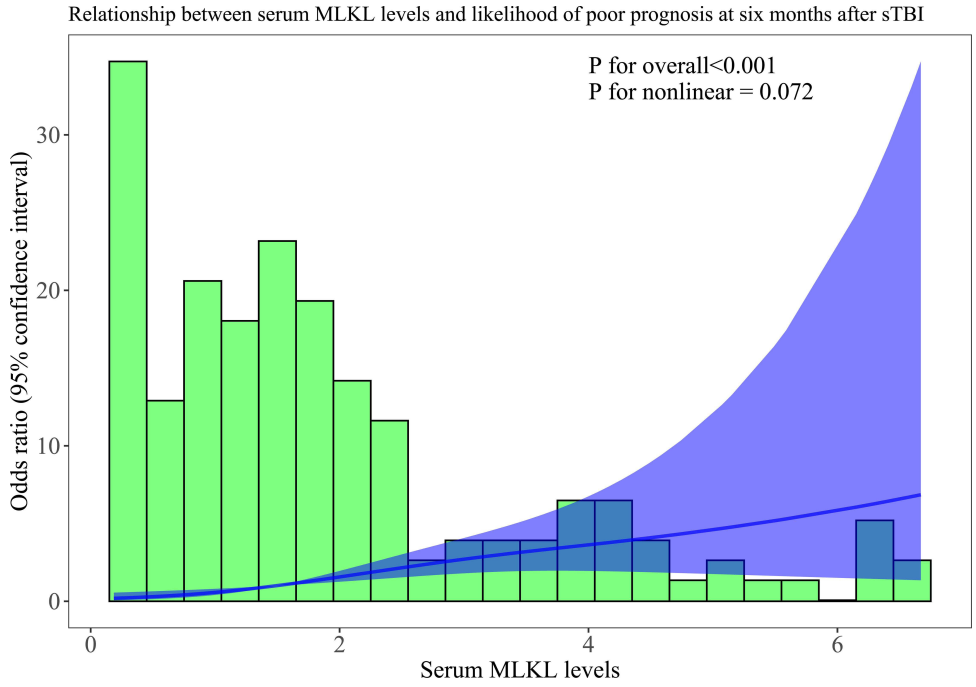

Figure 10 Serum mixed lineage kinase domain-like protein levels and likelihood of poor prognosis following severe traumatic brain injury. Within framework of restricted cubic spline, serum mixed-lineage kinase domain-like protein levels showed a linear relationship with poor prognosis in severe traumatic brain injury (P for nonlinear >0.05).
Abbreviations: sTBI, severe traumatic brain injury; MLKL, mixed-lineage kinase domain-like protein.

MLKL levels displayed a notable elevation after sTBI; second, within the framework of restricted cubic spline assessment, serum MLKL levels displayed a linear relation to two conventional severity indicators (namely, GCS scores and Rotterdam CT scores), a frequently-used prognostic parameter (ie, GOSE scores), and the likelihoods of suffering from death and decreased overall survival and experiencing a poor prognosis after sTBI; third, both GCS scores and Rotterdam CT scores appeared as the two independently correlative variables of serum MLKL levels following sTBI; fourth, serum MLKL emerged as an independently correlated factor of GOSE scores and also as an independent predictor of death, overall survival and poor prognosis; fifth, as for predicting death, overall survival and poor prognosis, serum MLKL levels exhibited negligible interactions with several common variables, such as age, gender, smoking habits, etc. by employing segmented analysis; last, in the context of ROC curve, serum MLKL levels efficaciously distinguished the likelihoods of death and poor prognosis. The above evidence is offered to be highly supportive of the conception that increased levels of serum MLKL could accurately signify traumatic severity and have a high discrimination efficiency for risk of death and poor neurological outcomes, and therefore serum AIM2 may stands out as an encouraging prognostic indicator for sTBI.

Generally, peripheral blood is readily available. In this study, serum was obtained from a cohort of patients with sTBI and from a group of individuals without healthy conditions. Serum MLKL levels were higher in the patients than in the controls. The origin of MLKL in the blood may be derived from the central nervous system, since all MLKL could be abundantly generated from brain tissues after acute brain injury[22–24] and therefore, it could be transferred to the blood pool via the damaged blood-brain barrier after TBI.[26] However, the systemic inflammatory response is an accepted phenomenon that frequently occurs following sTBI.[27] MLKL can be secreted from several types of peripheral cells such as neutrophils and macrophages.[28,29] Thus, MLKL may be secreted from the peripheral cells in response to systemic inflammation. In the future, the determination of mRNA levels in peripheral cells may offer valuable information for deciphering this puzzle.

Exploration of severity indicators is a crucial step in clinical work for the management of sTBI, owing to the importance of severity assessment, risk stratification, and prognosis prediction. Although GCS scoring and Rotterdam CT scaling are firmly believed to be the two most common clinical and radiological severity assessment systems,[4,5]

Subgroup	Total	OR(95% CI)	P	P interaction
Age				0.593
≥ 60 years	24	1.633 (0.616-4.331)	0.324	
< 60 years	131	1.697 (1.252-2.298)	0.001	
Gender				0.292
Male	88	1.868 (1.023-3.411)	0.042	
Female	67	1.140 (0.798-1.629)	0.471	
Hypertension				0.079
Yes	34	1.179 (0.594-2.343)	0.638	
No	121	1.788 (1.262-2.534)	0.003	
Diabetes mellitus				0.143
Yes	21	7.868 (0.685-90.344)	0.098	
No	134	1.631 (1.229-2.166)	0.001	
Cigarette smoking				0.273
Yes	45	1.136 (0.780-1.653)	0.506	
No	110	2.076 (1.177-3.660)	0.012	
Alcohol drinking				0.644
Yes	49	1.484 (0.867-2.542)	0.152	
No	106	1.683 (1.174-2.414)	0.005	

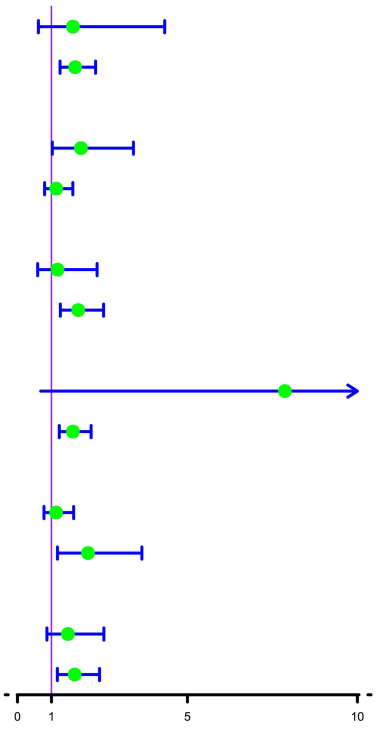

Figure 11 Interactive relation of serum mixed lineage kinase domain-like protein levels to other parameters for predicting poor prognosis in patients with severe traumatic brain injury. Using subgroup analysis, serum mixed lineage kinase domain-like protein levels did not significantly interact with other variables, such as age, sex, hypertension, diabetes, alcohol consumption, and tobacco use, in predicting poor prognosis after severe traumatic brain injury (all P interaction >0.05).
Abbreviations: OR, odds ratio; 95% CI, 95% confidence interval.

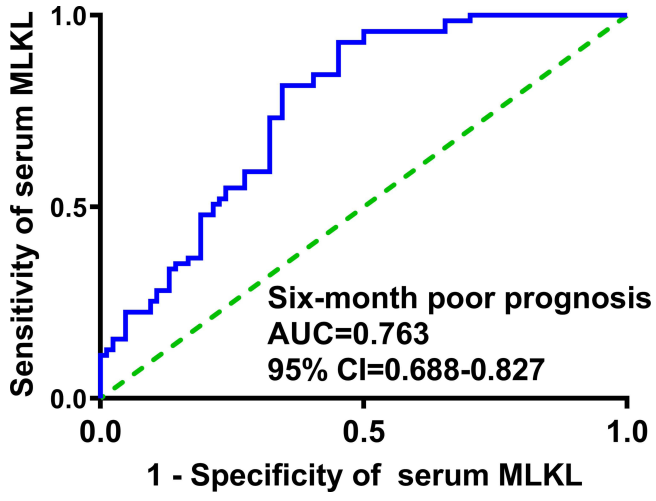

Figure 12 Determination of the predictive capability of serum mixed lineage kinase domain-like protein levels for poor prognosis after severe traumatic brain injury. Using receiver operating characteristic curve analysis, serum mixed-lineage kinase domain-like protein levels showed a significant discriminatory ability for poor prognosis following severe traumatic brain injury.
Abbreviations: AUC, area under curve; 95% CI, 95% confidence interval; MLKL, mixed lineage kinase domain-like protein.

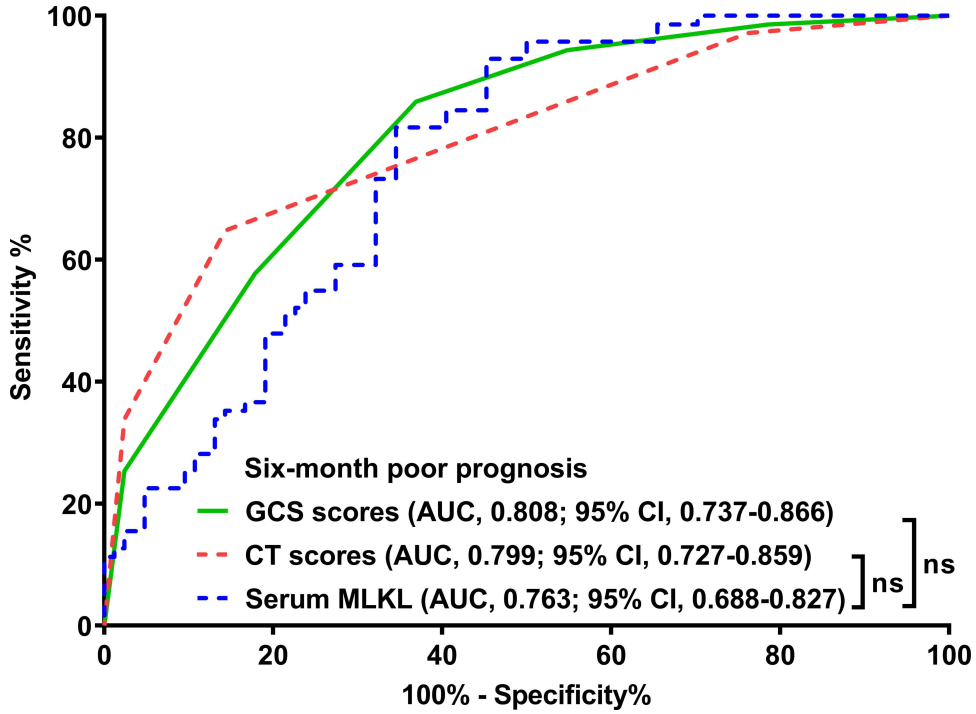

Figure 13 Distinctions in terms of prognostic predictive capabilities between serum mixed lineage kinase domain-like protein levels and other variables after severe traumatic brain injury. Under receiver operating characteristic curve, serum mixed lineage kinase domain-like protein levels after severe traumatic brain injury showed a non-significantly different prognostic predictive ability, in contrast to the Glasgow coma scale scores and Rotterdam computed tomography scores (both P>0.05).
Abbreviations: AUC, area under curve; 95% CI, 95% confidence interval; MLKL, mixed lineage kinase domain-like protein; GCS, Glasgow Coma Scale; CT, computed tomography; ns, nonsignificant.

circulating biomarkers have been extensively studied with respect to their prognostic importance in sTBI.[7–9] By employing a series of multivariate statistical metrics, serum MLKL was identified as an independent parameter because of significant correlations with GCS, Rotterdam CT, and GOSE scores, as well as due to substantial associations with death, overall survival, and poor prognosis at 180 days after sTBI. These results may have been highly supported the presumption that serum MLKL may be a useful biomarker for assessing trauma severity and forecasting clinical outcomes in patients with sTBI. To make the conclusions statistically rigorous from the perspective of evidence-based medicine, multiple RCS and subgroup analyses were performed. No notable interactions with several conventional variables, such as age, sex, smoking habits, alcohol consumption, hypertension, and diabetes were found. All linear correlations with GCS, Rotterdam CT, and GOSE scores, as well as death, overall survival, and poor prognosis, were revealed. Notably, serum MLKL levels effectively demonstrated discrimination efficiency for death and poor prognosis in this cohort of sTBI patients. Overall, an interesting notion may be formed that serum MLKL may be substantialized as an encouraging biochemical biomarker of prognosis after sTBI.

There are two limitations. First, only isolated head trauma has been included in this study, meaning that, polytrauma may be existent, but moderate-severe injury in other aspects have been excluded. Thus, serum MLKL may be a good prognostic biomarker of reflecting isolated sTBI. However, cerebrospinal fluid MLKL measurement or combination of serum MLKL with other indicators from surrounding organs may hopefully provide more accurate predictive ability in non-isolated sTBI. Second, these patients were statistically enough for clinical analysis, but any conclusions should be validated in future larger cohort study.

Conclusions

A dramatic enhancement of serum MLKL levels following sTBI is independently relevant to severity, mortality, overall survival, GOSE scores, and poor prognosis at the 180-day mark following sTBI. In summary, serum MLKL may emerge as a biochemical predictor that could be of great importance in predicting the clinical outcomes of sTBI.

Data Sharing Statement

The datasets generated and/or analyzed during the current study are not publicly available because they are personal data, but are available from the corresponding author upon reasonable request.

Acknowledgments

We gratefully thank all study participants, their relatives, and the staff at the recruitment centers for their invaluable contributions.

Funding

The authors received no financial support for the research.

Disclosure

The authors declared no potential conflicts of interest in this work.

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