

Evaluation of Serum NLRC4 as a Potential Prognostic Biochemical Marker in Humans with Severe Traumatic Brain Injury: A Prospective Cohort Study

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Objective: Involvement of NLR CARD domain containing 4 (NLRC4) in neuroinflammation has been demonstrated. The aim of this study was to ascertain the prognostic role of serum NLRC4 in severe traumatic brain injury (sTBI).

Methods: In this prospective cohort study including 140 sTBI patients and 140 controls, serum NLRC4 levels were quantified. Follow-up time was 180 days after trauma and poor prognosis was designated as extended Glasgow outcome scale (GOSE) scores of 1–4. Severity correlations and prognosis associations were determined under multivariate models.

Results: Enhanced serum NLRC4 levels after sTBI, in comparison to controls (median, 0.8 ng/mL versus 0.1 ng/mL; $P < 0.001$), were independently correlated with Glasgow coma scale (GCS) scores (β , -0.091 ; 95% confidence interval (CI), -0.161 – 0.021 ; $P = 0.011$), Rotterdam computed tomography (CT) scores (β , 0.136 ; 95% CI, 0.024 – 0.248 ; $P = 0.018$), serum C-reactive protein levels (β , 0.016 ; 95% CI, 0.002 – 0.030 ; $P = 0.025$) and 180-day GOSE scores (β , -0.906 ; 95% CI, -1.632 – 0.180 ; $P = 0.015$); and were independently predictive of 180-day death (odds ratio, 4.307 ; 95% CI, 1.706 – 10.879 ; $P = 0.014$), overall survival (hazard ratio, 2.360 ; 95% CI, 1.118 – 4.981 ; $P = 0.040$) and poor prognosis (odds ratio, 6.705 ; 95% CI, 2.889 – 15.561 ; $P = 0.016$). Under receiver operating characteristic curve, combination of serum NLRC4 levels, GCS scores and Rotterdam CT scores had significantly higher death predictive ability than Rotterdam CT scores ($P = 0.040$), but not than GCS scores ($P = 0.070$); and exhibited substantially higher predictive capability for poor prognosis than Rotterdam CT scores ($P < 0.001$) and GCS scores alone ($P = 0.023$).

Conclusion: There is a dramatical elevation of serum NLRC4 levels after sTBI, which has strong correlation with severity and inflammation, and is significantly associated with long-term death and poor outcome, substantiating serum NLRC4 as an inflammatory, prognostic biomarker in sTBI.

Keywords: traumatic brain injury, nucleotide-binding domain leucine-rich repeat-containing protein family caspase activation and recruitment domain-containing protein 4, severity, prognosis, biomarkers, inflammation

Introduction

Severe traumatic brain injury (sTBI) is a lethal neurosurgical disease in humans.¹ Following external force, the two key pathophysiological processes, namely primary brain injury and secondary brain injury, are closely relevant to neurological dysfunction and even death after sTBI.² In response to primary brain insult, secondary injury occurs, which triggers a series of cascading signaling events that potentiate the formation of sterile neuroinflammation, aggravation of brain edema, disruption of blood–brain barrier and development of neuronal injury.³ Besides treatments, severity assessment and prognostic prediction are of rather importance in clinical managements of sTBI.⁴ Conventionally, clinical and radiological scales, such as the Glasgow coma scale (GCS) and Rotterdam computed tomography (CT) scale, have

been frequently utilized in routine work of sTBI.⁵ Intriguingly, biochemical biomarkers have recently emerged as an indispensable tool in decision-making of sTBI.^{6,7}

Neuroinflammatory response accompanies sTBI.⁸ Inflammasome, an intracellular multi-protein complex, harbors inflammatory potentials and is involved in secondary brain injury after trauma.^{8,9} The nucleotide-binding and oligomerization domain-like receptor (NLR) family responds to innate immunity by forming inflammasomes.¹⁰ Following activation, NLR CARD domain containing 4 (NLRC4) inflammasomes facilitate conversion of precursor caspase-1 to cleaved caspase-1, whereby precursor interleukin-1 β and interleukin-18 are converted into biologically active mature pro-inflammatory cytokines, thereby finally triggering inflammatory cascade.¹¹ NLRC4 expressions by microglia and astrocyte mediated sterile inflammasome activation in central nervous system.¹² NLRC4 has been widely expressed in animal injured brain tissues after hemorrhagic or ischemic stroke.^{13,14} Clearly, NLRC4 inflammasome is implicated in neuroinflammation, and therefore contributes to increased cerebral edema, blood–brain barrier disruption and neuronal injury. In addition, its inhibition could markedly relieve acute brain injury, subsequently improving neurological function of experimental rats.^{13,14} Overall, NLRC4 may represent a potential biomarker for reflecting acute brain injury. Herein, we intended to investigate relationship between serum NLRC4 levels and trauma severity plus clinical outcome after human sTBI.

Materials and Methods

Study Design, Ethical Considerations and Participant Enrollment

In this prospective cohort study implemented between July 2017 and December 2020 at the First People's Hospital of Jiande City, we consecutively recruited two groups of subjects, including controls and head trauma patients. The current study was performed in agreement with the ethical guidelines of the Declaration of Helsinki. The approval for the protocol of this study was acquired from the Institutional Review Committee at the First People's Hospital of Jiande City (NO. JDYY2017023). The informed written consent to participate in this study was given by patients' proxies or controls themselves.

Inclusion criteria for head trauma patients were that (1) blunt trauma; (2) age equal to or greater than 18 years; (3) post-resuscitation GCS scores of 3–8; (4) hospital admission within 12 hours since trauma and (5) injury severity score <9 in non-cranial aspects. Exclusion criteria for head trauma patients were that (1) previous or coexisting neurological diseases, for instance, stroke, intracranial tumors and multiple sclerosis; and (2) other specific conditions or diseases, for example, recent infections, autoimmune diseases, malignancies and severe hepatic, renal, pulmonary or cardiac diseases.

Inclusion criteria for controls were that (1) age of 18 years or greater; and (2) Normal results in some routine blood tests, including blood glucose levels, blood leucocyte count, blood platelet count, blood hemoglobin levels and blood albumin levels. Exclusion criteria for controls were (1) previous chronic diseases, eg, hypertension, diabetes mellitus and coronary heart disease; and (2) other specific conditions, such as use of immunosuppressants or dexamethasone, recent infections and pregnancies.

Data Collection, Outcome Assessment and Immune Analysis

Some conventional information should be collected, including age, gender, body mass index, cigarette smoking, alcohol drinking and admission time since trauma. Other specific diseases or conditions should be inquired, such as hypertension, diabetes mellitus, hyperlipidemia, medications (use of antihypertensive drugs, hypoglycemic drugs or insulin, and statins) and traumatic causes (automobile/motorcycle, fall/jump or others). We recorded some baseline vital signs, mainly including systolic and diastolic arterial blood pressure. Mean arterial blood pressure was calculated using the following equation: mean arterial blood pressure = diastolic arterial blood pressure + ((systolic arterial blood pressure–diastolic arterial blood pressure)/3). Clinical severity was under assessment using GCS. Positive radiologic characteristics included abnormal cisterns, midline shift, epidural hematoma, subdural hematoma, subarachnoid hemorrhage, intraventricular hemorrhage, intracerebral hematoma, brain contusion and pneumocephalus. Rotterdam CT scores were calculated based on the preceding positive radiological parameters. Follow-up time for patients was 180 days. Clinical outcome was

evaluated via 8-grade Extended Glasgow Outcome Scale (GOSE). GOSE scores of 1–4 was referred to as a poor prognosis.¹⁵

Blood samples of participants were acquired and then blood was centrifugated. Obtained serum samples were preserved at -80°C until further analyses. Every 3 months, a batch of samples were melted and, using a commercially available Enzyme-Linked Immunosorbent Assay kit (SinoGeneclon Biotech, Hangzhou, China), serum NLRC4 levels were in duplicate quantified by the same technician, who was blinded to clinical data. Two measurements were averaged for ultimate clinical investigation.

Statistical Analysis

The MedCalc statistical software version 17.4 (MedCalc Software, Mariakerke, Belgium) was applied for receiver-operating characteristic (ROC) curve analysis and the SPSS 20.0 statistical package (SPSS Inc., Chicago, Illinois, USA) was in use for other data analyses. There are three types of variables, namely, qualitative data, normally and non-normally distributed quantitative variables, which were presented as frequencies (proportions), means (standard deviations, SDs) and medians (percentiles 25th-75th), respectively. In order to complete the data comparison between two groups, the used statistical methods included the Pearson's Chi-square test, Fisher's exact test, independent *t*-test and Mann-Whitney test. The Kruskal-Wallis *H*-test was done for data comparison among multiple groups. Baseline characteristics were compared between survivors and non-survivors as well as between patients with poor prognosis and those with good prognosis, and then the variables with substantial difference were enforced into the binary logistic regression model, thereby determining the independent predictors of death and poor prognosis. The Spearman test was done for bivariate correlative assessment, and afterwards the significantly correlated variables were incorporated into the multivariate linear regression model to identify independent factors of serum NLRC4 levels and GOSE scores. Patients were dichotomized based on the median value of serum NLRC4 levels, and the Log rank test was performed for comparing 180-day overall survival time between two groups. The univariate Cox's proportional hazard analysis was implemented to screen significant variables, and such variables were subsequently entered into the multivariable model, wherein independent predictors were ascertained. ROC curves were established to assess the predictive performance of some designated parameters for mortality and poor prognosis. In consideration of this, the Youden index method was fulfilled to discern their optimal cut-off values. Differences with *P*-values <0.05 were deemed as statistical significance.

Results

Participant Selection and Characteristics

In this clinical survey, we at first enrolled 177 patients with head trauma, who fitted the inclusion criteria in the above-mentioned section, and then 37 patients were removed from the final analysis because of previous or coexisting neurological diseases (15 cases), other specific conditions or diseases (17 cases), loss to follow-up (1 case), incomplete clinical materials (1 case), unavailable blood samplings (2 cases) and refusal to participation (1 case). In summary, a total of 140 adults with blunt and isolated sTBI obtained an ultimate evaluation. Also, there were an aggregate of 140 healthy individuals as controls. Controls, 78 being males and 62 being females, comprised of 46 alcohol drinkers and 48 cigarette smokers and were aged from 18 to 79 years (mean, 40.7 years; SD, 13.9 years), with body mass index ranging from 18.3 to 34.7 kg/m^2 (mean, 24.1 kg/m^2 ; SD, 3.9 kg/m^2). Age, gender, body mass index, alcohol drinking, cigarette smoking and other parameters of patients are listed in Table 1. By statistical analysis, it was revealed that proportions of males, smokers and drinkers, as well as mean age and body mass index were not significantly different between controls and patients (all $P > 0.05$).

Serum NLRC4 Levels After sTBI and Its Correlative Factors

There was a significant elevation of serum NLRC4 levels in patients, in comparison to controls ($P < 0.001$; Figure 1). In Table 1, there were eight variables in close correlation with serum NLRC4 levels using spearman test, which were GCS scores, Rotterdam CT scores, unreactive pupils, abnormal cisterns, midline shift 5 mm, serum C-reactive protein levels, blood glucose levels and blood leucocyte count (all $P < 0.05$). Subsequently, the multivariate linear regression model was configured, wherein

Table 1 Baseline Characteristics of Head Trauma Patients and Factors in Relation to Serum NLRC4 Levels After Head Trauma

	All Patients	ρ	P value
Gender (male/female)	74/66	-0.084	0.322
Age (y)	38.8±18.0	0.035	0.679
Body mass index (kg/m ²)	24.0±3.3	0.106	0.214
Current cigarette smoking	44 (31.4%)	0.071	0.406
Current alcohol drinking	45 (32.1%)	0.033	0.698
Hypertension	12 (8.6%)	0.125	0.141
Diabetes mellitus	8 (5.7%)	-0.065	0.447
Hyperlipidemia	19 (13.6%)	0.028	0.746
Previous use of antihypertensive drugs	8 (5.7%)	0.053	0.538
Previous use of hypoglycemic drugs or insulin	5 (3.6%)	0.018	0.836
Previous use of statins	11 (7.9%)	0.077	0.365
Admission time since trauma (h)	4.4 (1.8–6.5)	-0.107	0.210
Blood-collection time since trauma (h)	5.4 (2.8–8.1)	-0.118	0.165
Traumatic causes (automobile/motorcycle: fall/jump: others)	73/56/11	0.105	0.219
Glasgow coma scale scores	5 (4–6)	-0.608	<0.001
Rotterdam scores	4 (4–5)	0.589	<0.001
Unreactive pupils	88 (62.9%)	0.256	0.002
Surgery in the first 24 hours	80 (57.1%)	-0.043	0.612
Epileptic seizure	20 (14.3%)	-0.011	0.896
Systolic arterial pressure (mmHg)	123.4±25.2	-0.027	0.748
Diastolic arterial pressure (mmHg)	74.6±16.2	-0.008	0.921
Mean arterial pressure (mmHg)	91.1±18.1	-0.015	0.864
Epidural hematoma	67 (47.9%)	0.055	0.519
Subdural hematoma	74 (52.9%)	0.089	0.298
Subarachnoid hemorrhage	88 (62.9%)	0.102	0.232
Intraventricular hemorrhage	14 (10.0%)	0.027	0.756
Intracerebral hematoma	75 (53.6%)	0.077	0.368
Brain contusion	77 (55.0%)	0.119	0.162
Pneumocephalus	58 (41.4%)	-0.064	0.453
Abnormal cisterns	101 (72.1%)	0.208	0.014
Midline shift > 5 mm	88 (62.9%)	0.230	0.006
Serum C-reactive protein levels (mg/l)	14.6 (10.2–17.9)	0.420	<0.001
Blood glucose levels (mmol/l)	9.9 (8.3–11.5)	0.382	<0.001
Blood leucocyte count (×10 ⁹ /l)	7.1 (5.0–9.8)	0.240	0.004

Notes: Data were summarized as mean ± standard deviation, median (25th–75th percentiles) or count (percentage) as appropriate. Bivariate correlations were analyzed using Spearman test and correlations were reported as ρ values.

the aforementioned eight significantly correlated factors were forced. And, it was found that serum NLRC4 levels were independently correlated with GCS scores (β , -0.091; 95% CI, -0.161–0.021; $P = 0.011$), Rotterdam CT scores (β , 0.136; 95% CI, 0.024–0.248; $P = 0.018$) and serum C-reactive protein levels (β , 0.016; 95% CI, 0.002–0.030; $P = 0.025$). Also, its strong relations to GCS scores ($P < 0.001$; [Figure 2A](#)), Rotterdam CT scores ($P < 0.001$; [Figure 2B](#)) and serum C-reactive protein levels ($P < 0.001$; [Figure 2C](#)) were depicted in a diagram. When GCS scores and Rotterdam CT scores were considered as the two categorical variables, it was revealed that serum NLRC4 levels, which were significantly decreased in order of GCS scores from 3 to 8 ($P < 0.001$; [Figure 3A](#)), were substantially increased in order of Rotterdam CT scores from 3 to 6 ($P < 0.001$; [Figure 3B](#)).

Serum NLRC4 Levels and 180-Day Death After Head Trauma

There were 33 non-survivors (23.6%) within 180 days after head trauma. The dead had substantially higher percentages of alcohol drinking, unreactive pupils, epidural hematoma, subdural hematoma, abnormal cisterns and midline shift above 5 mm

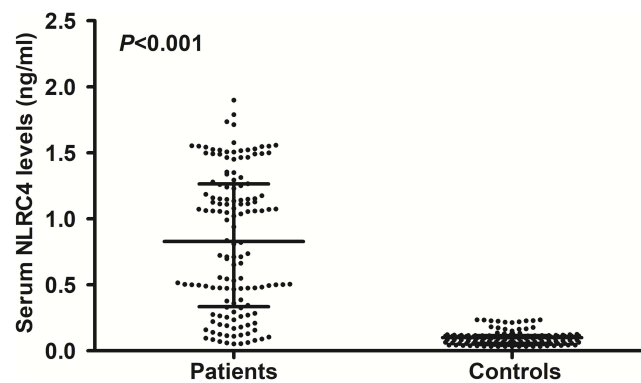


Figure 1 Serum NLR4 levels after severe traumatic brain injury. Serum NLR4 levels were substantially elevated after severe traumatic brain injury, in comparison to controls ($P < 0.001$).

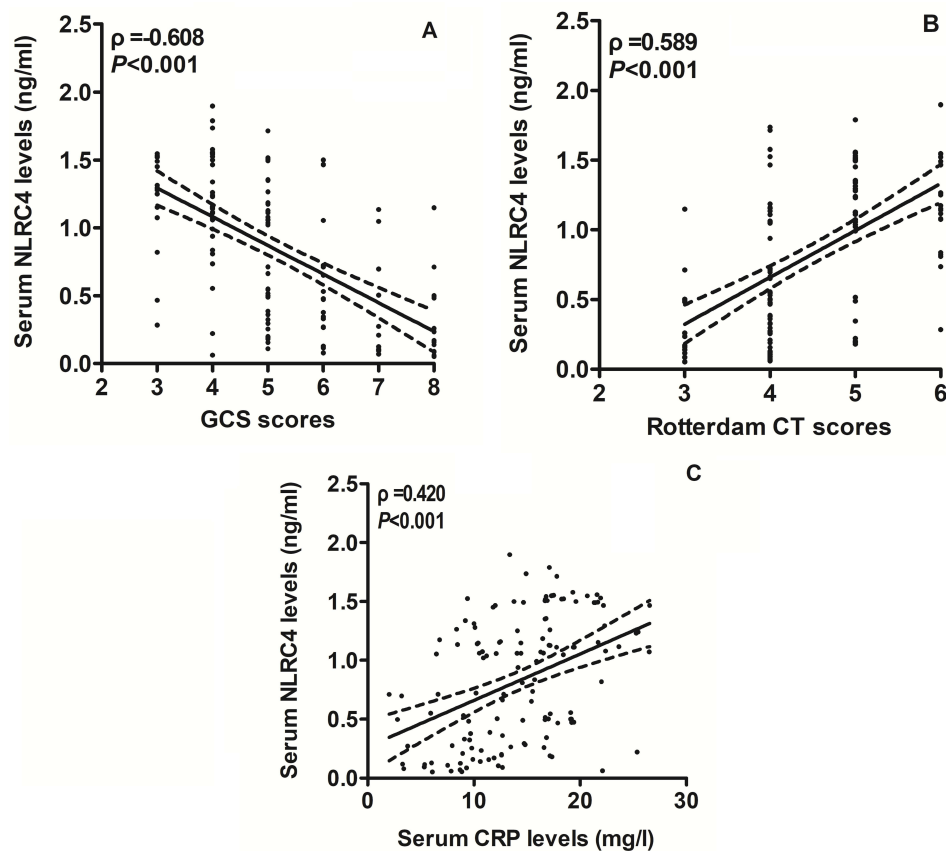


Figure 2 Correlative analysis of serum NLR4 levels following severe traumatic brain injury. **(A)** Correlation of serum NLR4 levels with Glasgow coma scale scores after severe traumatic brain injury. Serum NLR4 levels were significantly correlated with Glasgow coma scale scores after severe traumatic brain injury ($P < 0.001$). **(B)** Correlation of serum NLR4 levels with Rotterdam computed tomography scores after severe traumatic brain injury. Serum NLR4 levels were significantly correlated with Rotterdam computed tomography scores after severe traumatic brain injury ($P < 0.001$). **(C)** Correlation of serum NLR4 levels with serum C-reactive protein levels after severe traumatic brain injury. Serum NLR4 levels were significantly correlated with serum C-reactive protein levels after severe traumatic brain injury ($P < 0.001$). **Abbreviations:** GCS, Glasgow coma scale; CT, computed tomography; CRP, C-reactive protein.

than the alive (all $P < 0.05$; Table 2); and, in comparison to survivors, GCS scores were significantly declined and Rotterdam CT scores, serum C-reactive protein levels, blood leucocyte count, blood glucose levels and serum NLR4 levels were substantially increased (all $P < 0.05$; Table 2). The above significant variables were entered into the binary logistic regression model and then, it was confirmed that GCS scores, Rotterdam CT scores and serum NLR4 levels were independently predictive of 180-day death with odds ratio values of 0.355 (95% CI, 0.192–0.659; $P = 0.001$), 2.789 (95% CI, 1.351–5.755; $P = 0.006$) and 4.307 (95% CI,

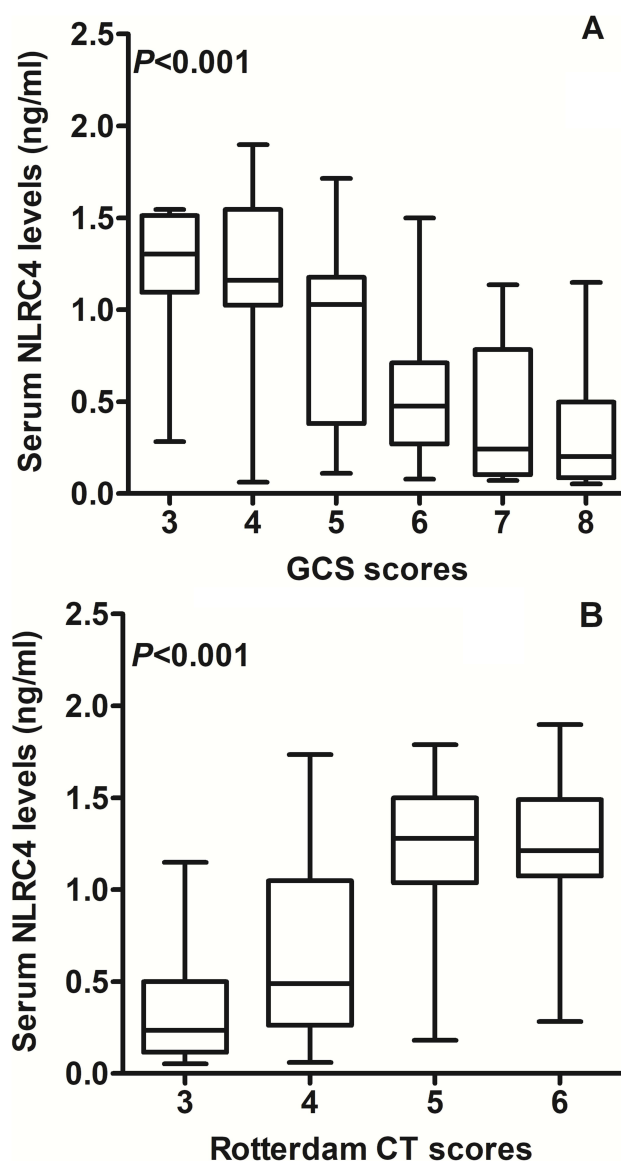


Figure 3 Serum NLRC4 levels among severe traumatic brain injury subgroups according to clinical and radiological severity scales. **(A)** Serum NLRC4 levels among subgroups with different Glasgow outcome scale scores after severe traumatic brain injury. Serum NLRC4 levels were significantly decreased in order of Glasgow outcome scale scores from 3 to 8 ($P < 0.001$). **(B)** Serum NLRC4 levels among subgroups with different Rotterdam computerized tomography scores after severe traumatic brain injury. Serum NLRC4 levels were significantly increased in order of Rotterdam computerized tomography scores from 3 to 6 ($P < 0.001$).

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

1.706–10.879; $P = 0.014$). Under ROC curve, serum NLRC4 levels substantially discriminated patients at risk of 180-day death (Figure 4A). Additionally, combination of serum NLRC4 levels, GCS scores and Rotterdam CT scores had insignificantly higher AUC than GCS scores ($P = 0.070$; Figure 4B), while displayed substantially higher AUC than Rotterdam CT scores ($P = 0.040$; Figure 4B).

Serum NLRC4 Levels and 180-Day Overall Survival After Head Trauma

This cohort of sTBI patients had the mean 180-day overall survival time at 145.8 days (95% CI, 135.0–156.6 days). Patients were divided into two subgroups in accordance with the median value of serum NLRC4 levels (ie, 0.83 ng/mL). In Figure 5, mean 180-day overall survival time was markedly shorter in patients with serum NLRC4 levels >0.83 ng/mL than in other remainders ($P < 0.01$). Additionally, variables, which were significantly associated with 180-day overall

Table 2 Factors in Relation to 180-Day Mortality After Head Trauma

	Non-Survivors	Survivors	P value
Gender (male/female)	19/14	55/52	0.535
Age (y)	43.7±18.0	37.2±17.8	0.070
Body mass index (kg/m ²)	24.2±3.2	23.9±3.4	0.743
Current cigarette smoking	14 (42.4%)	30 (28.0%)	0.120
Current alcohol drinking	16 (48.5%)	29 (27.1%)	0.021
Hypertension	2 (6.1%)	10 (9.4%)	0.732
Diabetes mellitus	3 (9.1%)	5 (4.7%)	0.392
Hyperlipidemia	5 (15.2%)	14 (13.0%)	0.762
Previous use of antihypertensive drugs	1 (3.0%)	7 (6.5%)	0.680
Previous use of hypoglycemic drugs or insulin	2 (6.1%)	3 (2.8%)	0.337
Previous use of statins	3 (9.1%)	8 (7.5%)	0.721
Admission time since trauma (h)	3.6 (2.1–6.0)	4.6 (1.8–6.5)	0.700
Blood-collection time since trauma (h)	4.6 (2.7–7.6)	5.7 (2.8–8.3)	0.551
Traumatic causes			0.600
Automobile/motorcycle	16	57	
Fall/jump	13	43	
Others	4	7	
Glasgow coma scale scores	4 (3–4)	5 (5–6)	<0.001
Rotterdam scores	5 (5–6)	4 (4–5)	<0.001
Unreactive pupils	28 (84.9%)	60 (56.1%)	0.003
Surgery in the first 24 hours	22 (66.7%)	58 (54.2%)	0.206
Epileptic seizure	6 (18.2%)	14 (13.1%)	0.569
Systolic arterial pressure (mmHg)	129.1±23.8	121.6±25.5	0.136
Diastolic arterial pressure (mmHg)	77.6±16.3	73.7±16.1	0.229
Mean arterial pressure (mmHg)	95.9±16.7	89.6±18.4	0.081
Epidural hematoma	23 (69.7%)	44 (41.1%)	0.004
Subdural hematoma	23 (69.7%)	51 (47.7%)	0.027
Subarachnoid hemorrhage	25 (75.8%)	63 (58.9%)	0.079
Intraventricular hemorrhage	5 (15.2%)	9 (8.4%)	0.318
Intracerebral hematoma	20 (60.6%)	55 (51.4%)	0.354
Brain contusion	20 (60.6%)	57 (53.3%)	0.459
Pneumocephalus	14 (42.4%)	44 (41.1%)	0.894
Abnormal cisterns	31 (93.9%)	70 (65.4%)	0.001
Midline shift > 5 mm	27 (81.8%)	61 (57.0%)	0.010
Serum C-reactive protein levels (mg/l)	17.5 (15.7–21.8)	12.6 (9.6–17.0)	<0.001
Blood glucose levels (mmol/l)	11.4 (10.1–13.9)	9.7 (7.9–10.9)	0.001
Blood leucocyte count (×10 ⁹ /l)	12.0 (4.5–13.8)	6.3 (5.1–8.2)	0.001
Serum NLRC4 levels (ng/mL)	1.3 (0.8–1.5)	0.6 (0.3–1.1)	<0.001

Notes: Data were presented as mean ± standard deviation, median (25th–75th percentiles) or count (percentage) and were compared by the t-test, Mann–Whitney U-test, Pearson chi-square test or Fisher's exact test.

survival time using univariate Cox's proportional hazard analysis, were alcohol drinking, GCS scores, Rotterdam CT scores, unreactive pupils, epidural hematoma, subdural hematoma, abnormal cisterns, midline shift above 5 mm, serum C-reactive protein levels, blood leucocyte count, blood glucose levels and serum NLRC4 levels (all $P < 0.05$; Table 3). Next, the aforementioned significant variables were incorporated into the multivariable model and afterwards, it was confirmed that GCS scores (hazard ratio, 0.592; 95% CI, 0.360–0.973; $P = 0.013$), Rotterdam CT scores (hazard ratio, 2.283; 95% CI, 1.186–4.393; $P = 0.025$) and serum NLRC4 levels (hazard ratio, 2.360; 95% CI, 1.118–4.981; $P = 0.040$) had independent association with 180-day overall survival after head trauma.

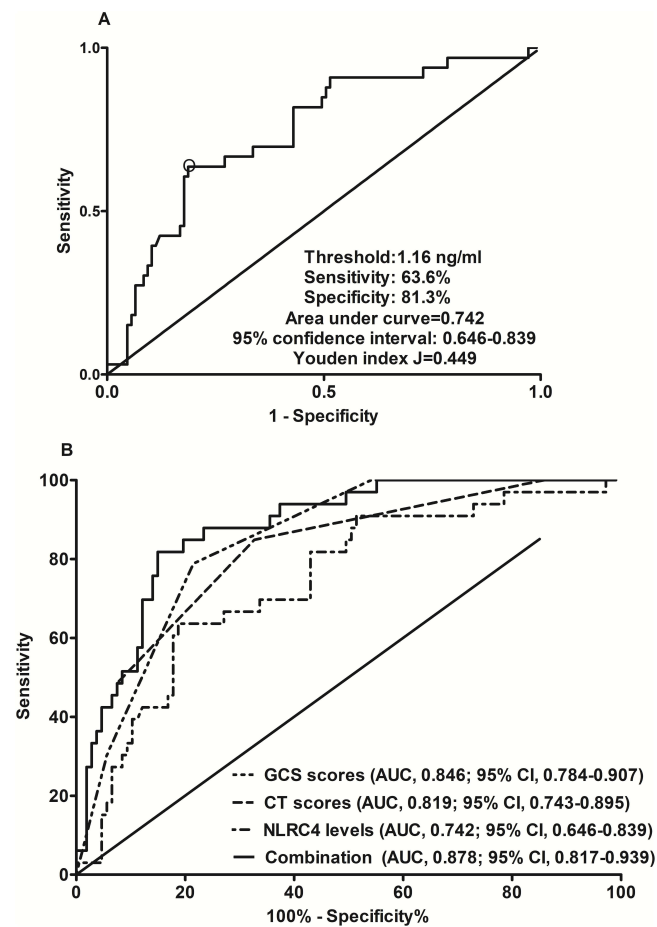


Figure 4 Serum NLRC4 levels with respect to discriminatory ability for risk of 180-day death after severe traumatic brain injury. **(A)** Predictive value of serum NLRC4 levels for 180-day death after severe traumatic brain injury. Serum NLRC4 levels efficiently distinguished patients at risk of 180-day death following severe traumatic brain injury ($P < 0.001$). **(B)** Differences in 180-day death predictive ability between serum NLRC4 levels and other variables among severe traumatic brain injury patients. Combination of serum NLRC4 levels, Glasgow coma scale scores and Rotterdam computerized tomography scores had insignificantly higher area under curve than Glasgow coma scale scores ($P > 0.05$), while displayed substantially higher area under curve than Rotterdam computerized tomography scores ($P < 0.05$).

Abbreviations: GCS, Glasgow coma scale; CT, computed tomography; AUC, area under curve; 95% CI, 95% confidence interval.

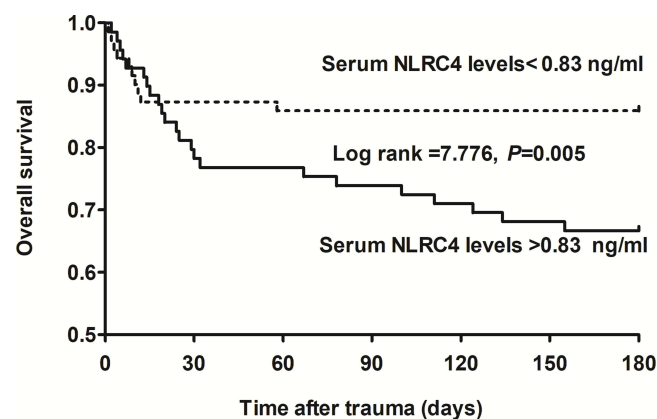


Figure 5 Difference in 180-day overall survival time according to serum NLRC4 levels after severe traumatic brain injury. Based on median value of serum NLRC4 levels (namely, 0.83 ng/mL), patients were dichotomized into two subgroups. Patients with serum NLRC4 levels > 0.83 ng/mL exhibited markedly shorter 180-day overall survival time than other remainders ($P < 0.01$).

Table 3 Factors in Relation to 180-Day Overall Survival After Head Trauma

	Hazard Ratio	95% Confidence Interval	P value
Gender (male/female)	1.212	0.608–2.417	0.585
Age (y)	1.015	0.998–1.033	0.088
Body mass index (kg/m ²)	1.016	0.918–1.124	0.763
Current cigarette smoking	1.583	0.793–3.157	0.192
Current alcohol drinking	2.114	1.068–4.185	0.032
Hypertension	0.682	0.163–2.851	0.600
Diabetes mellitus	1.968	0.600–6.449	0.264
Hyperlipidemia	1.274	0.492–3.299	0.619
Previous use of antihypertensive drugs	0.465	0.064–3.403	0.451
Previous use of hypoglycemic drugs or insulin	1.928	0.461–8.062	0.369
Previous use of statins	1.299	0.396–4.258	0.665
Admission time since trauma (h)	0.972	0.873–1.081	0.596
Blood-collection time since trauma (h)	0.964	0.873–1.064	0.465
Traumatic causes			
Automobile/motorcycle	Reference		
Fall/jump	0.537	0.179–1.608	0.267
Others	0.588	0.192–1.804	0.353
Glasgow coma scale scores	0.359	0.250–0.516	<0.001
Rotterdam cores	3.663	2.352–5.706	<0.001
Unreactive pupils	3.719	1.436–9.636	0.007
Surgery in the first 24 hours	1.542	0.748–3.181	0.241
Epileptic seizure	1.515	0.625–3.670	0.358
Systolic arterial pressure (mmHg)	1.011	0.997–1.025	0.115
Diastolic arterial pressure (mmHg)	1.014	0.992–1.037	0.215
Mean arterial pressure (mmHg)	1.018	0.998–1.039	0.073
Epidural hematoma	2.788	1.326–5.861	0.007
Subdural hematoma	2.224	1.058–4.674	0.035
Subarachnoid hemorrhage	2.006	0.905–4.448	0.087
Intraventricular hemorrhage	1.712	0.661–4.436	0.268
Intracerebral hematoma	1.362	0.677–2.738	0.386
Brain contusion	1.281	0.637–2.575	0.487
Pneumocephalus	1.092	0.547–2.178	0.803
Abnormal cisterns	6.723	1.608–28.102	0.009
Midline shift > 5 mm	3.028	1.250–7.337	0.014
Serum C-reactive protein levels (mg/l)	1.189	1.108–1.275	<0.001
Blood glucose levels (mmol/l)	1.132	1.048–1.222	0.002
Blood leucocyte count ($\times 10^9/l$)	1.301	1.175–1.440	<0.001
Serum NLRC4 levels (ng/mL)	3.946	1.881–8.279	<0.001

Notes: Associations were shown as hazard ratios (95% confidence intervals) using univariate Cox's proportional hazard regression model.

Serum NLRC4 Levels and 180-Day GOSE Scores After Head Trauma

In [Table 4](#), GOSE scores were in intimate correlation with cigarette smoking, GCS scores, Rotterdam CT scores, unreactive pupils, epidural hematoma, brain contusion, abnormal cisterns, midline shift more than 5 mm, serum C-reactive protein levels, blood glucose levels and serum NLRC levels (all $P < 0.05$; [Table 4](#)). The preceding factors were forced into the multivariate model and afterwards, it was verified that GCS scores (β , 0.504; 95% CI, 0.203–0.805; $P = 0.001$), Rotterdam CT scores (β , -0.907; 95% CI, -1.391–0.423; $P = 0.005$) and serum NLRC4 levels (β , -0.906; 95% CI, -1.632–0.180; $P = 0.015$) were independently correlative with 180-day GOSE scores after head trauma. Also, [Figure 6A](#) shows a tight relationship between GOSE scores and serum NLRC4 levels ($P < 0.001$). When GOSE was

Table 4 Factors in Relation to Extended Glasgow Outcome Scale Scores at 180 Days After Head Trauma

	ρ	P value
Gender (male/female)	-0.099	0.244
Age (y)	-0.154	0.068
Body mass index (kg/m ²)	-0.144	0.090
Current cigarette smoking	-0.205	0.015
Current alcohol drinking	-0.092	0.280
Hypertension	-0.071	0.406
Diabetes mellitus	-0.133	0.117
Hyperlipidemia	0.017	0.844
Previous use of antihypertensive drugs	0.039	0.644
Previous use of hypoglycemic drugs or insulin	-0.134	0.115
Previous use of statins	-0.067	0.434
Admission time since trauma (h)	-0.041	0.629
Blood-collection time since trauma (h)	-0.012	0.886
Traumatic causes	-0.009	0.918
Glasgow coma scale scores	0.664	<0.001
Rotterdam scores	-0.624	<0.001
Unreactive pupils	-0.316	<0.001
Surgery in the first 24 hours	-0.162	0.056
Epileptic seizure	-0.106	0.213
Systolic arterial pressure (mmHg)	-0.138	0.104
Diastolic arterial pressure (mmHg)	-0.062	0.468
Mean arterial pressure (mmHg)	-0.140	0.098
Epidural hematoma	-0.331	<0.001
Subdural hematoma	-0.145	0.088
Subarachnoid hemorrhage	-0.117	0.169
Intraventricular hemorrhage	-0.010	0.908
Intracerebral hematoma	-0.130	0.127
Brain contusion	-0.229	0.007
Pneumocephalus	0.130	0.125
Abnormal cisterns	-0.258	0.002
Midline shift > 5 mm	-0.354	<0.001
Serum C-reactive protein levels (mg/l)	-0.379	<0.001
Blood glucose levels (mmol/l)	-0.388	<0.001
Blood leucocyte count ($\times 10^9/l$)	-0.166	0.050
Serum NLRC4 levels (ng/mL)	-0.507	<0.001

Note: Bivariate correlations were assessed by Spearman test.

considered as the categorical variable, it was proved that serum NLRC4 levels were markedly reduced in order of GOSE scores from 1 to 8 ($P < 0.001$; [Figure 6B](#)).

Serum NLRC4 Levels and 180-Day Poor Outcome After Head Trauma

An aggregate of 67 patients (47.9%) suffered from post-traumatic poor 180-day outcome. Just as depicted in [Table 5](#), poor-outcome patients, in comparison to other remainders, tended to show markedly elevated proportions of unreactive pupils, epidural hematoma, brain contusion, abnormal cisterns and midline shift >5 mm, were prone to have significantly decreased GCS scores, and were likely to exhibited significantly increased Rotterdam CT scores, blood glucose levels, serum C-reactive protein levels and serum NLRC4 levels (all $P < 0.05$). Moreover, the binary logistic regression model was constructed, where the above-mentioned variables were enforced, and it was confirmed that GCS scores (odds ratio, 0.371; 95% CI, 0.209–0.689; $P = 0.001$), Rotterdam CT scores (odds ratio, 3.596; 95% CI, 1.646–7.857; $P = 0.003$) and serum NLRC4 levels (odds ratio, 6.705; 95% CI, 2.889–15.561; $P = 0.016$) were the three independent predictors for

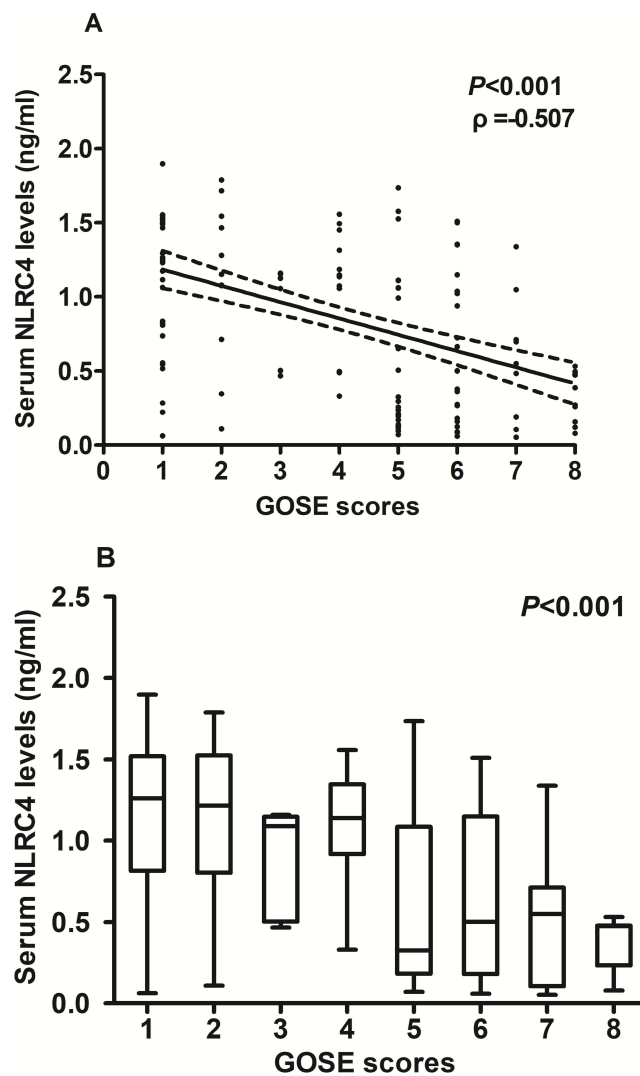


Figure 6 Relationship between serum NLR4 levels and 180-day extended Glasgow outcome scale scores after severe traumatic brain injury. **(A)** Correlation of serum NLR4 levels with 180-day extended Glasgow outcome scale scores after severe traumatic brain injury. Serum NLR4 levels were in close correlation with 180-day extended Glasgow outcome scale scores after severe traumatic brain injury ($P < 0.001$). **(B)** Difference in serum NLR4 levels among subgroups with different 180-day extended Glasgow outcome scale scores after severe traumatic brain injury. Serum NLR4 levels were dramatically declined in order of 180-day extended Glasgow outcome scale scores from 1 to 8 ($P < 0.001$).

Abbreviation: GOSE, Extended Glasgow outcome scale.

poor 180-day outcome after head trauma. Using ROC curve, serum NLR4 levels pronouncedly distinguished patients with the development of 180-day poor outcome (Figure 7A). In addition, serum NLR4 levels combined with GCS scores and Rotterdam CT scores exhibited substantially higher AUC than Rotterdam CT scores ($P < 0.001$; Figure 7B) and GCS scores alone ($P = 0.023$; Figure 7B).

Table 5 Factors in Relation to 180-Day Poor Outcome After Head Trauma

	Poor Outcome	Good Outcome	P value
Gender (male/female)	38/29	36/37	0.381
Age (y)	39.2±17.3	38.3±18.7	0.774
Body mass index (kg/m ²)	24.5±3.5	23.6±3.1	0.114
Current cigarette smoking	26 (38.8%)	18 (24.7%)	0.072
Current alcohol drinking	23 (34.3%)	22 (30.1%)	0.596

(Continued)

Table 5 (Continued).

	Poor Outcome	Good Outcome	P value
Hypertension	7 (10.5%)	5 (6.9%)	0.447
Diabetes mellitus	6 (9.0%)	2 (2.7%)	0.152
Hyperlipidemia	9 (13.4%)	10 (13.7%)	0.963
Previous use of antihypertensive drugs	5 (7.5%)	3 (4.1%)	0.480
Previous use of hypoglycemic drugs or insulin	4 (6.0%)	1 (1.4%)	0.193
Previous use of statins	7 (10.5%)	4 (5.5%)	0.275
Admission time since trauma (h)	4.4 (2.1–7.1)	4.3 (1.6–6.2)	0.390
Blood-collection time since trauma (h)	5.3 (3.1–8.2)	5.5 (2.5–7.9)	0.657
Traumatic causes			0.226
Automobile/motorcycle	33	40	
Fall/jump	26	30	
Others	8	3	
Glasgow coma scale scores	4 (4–5)	6 (5–7)	<0.001
Rotterdam scores	5 (4–6)	4 (4–4)	<0.001
Unreactive pupils	50 (74.6%)	38 (52.1%)	0.006
Surgery in the first 24 hours	42 (62.7%)	38 (52.1%)	0.204
Epileptic seizure	12 (17.9%)	8 (11.0%)	0.240
Systolic arterial pressure (mmHg)	125.3±26.8	121.6±23.7	0.376
Diastolic arterial pressure (mmHg)	74.8±18.0	74.4±14.5	0.887
Mean arterial pressure (mmHg)	92.2±19.6	90.1±16.8	0.491
Epidural hematoma	40 (59.7%)	27 (37.0%)	0.007
Subdural hematoma	39 (58.2%)	35 (48.0%)	0.224
Subarachnoid hemorrhage	46 (68.7%)	42 (57.5%)	0.174
Intraventricular hemorrhage	7 (10.5%)	7 (9.6%)	0.866
Intracerebral hematoma	41 (61.2%)	34 (46.6%)	0.083
Brain contusion	45 (67.2%)	32 (43.8%)	0.006
Pneumocephalus	23 (34.3%)	35 (48.0%)	0.102
Abnormal cisterns	57 (85.1%)	44 (60.3%)	0.001
Midline shift > 5 mm	52 (77.6%)	36 (49.3%)	0.001
Serum C-reactive protein levels (mg/l)	16.8 (12.7–21.0)	12.3 (9.2–16.7)	<0.001
Blood glucose levels (mmol/l)	11.0 (9.8–13.5)	9.3 (7.3–9.9)	<0.001
Blood leucocyte count ($\times 10^9/l$)	8.0 (4.5–12.1)	7.0 (5.1–8.3)	0.323
Serum NLRC4 levels (ng/mL)	1.2 (0.8–1.5)	0.5 (0.2–1.0)	<0.001

Notes: Data were presented as mean \pm standard deviation, median (25th–75th percentiles) or count (percentage) and were compared by the *t*-test, Mann–Whitney *U*-test, Pearson chi-square test or Fisher's exact test.

Discussion

To the best of our knowledge, there is a paucity of data available regarding NLRC4 levels in peripheral blood of subjects with acute brain injury. We found that (1) elevated serum NLRC4 levels after sTBI were independently correlated with serum C-reactive protein levels, GCS scores, Rotterdam CT scores and GOSE scores at 180 days after head trauma, were independently predictive of 180-day death and poor prognosis, as well as were independently associated with 180-day overall survival following sTBI; and (2) When combined with GCS scores and Rotterdam CT scores, serum NLRC4 levels showed efficient additive effect on prognostic prediction ability reflected by AUC. Hence, it is deduced that it may be of interest to measure serum NLRC4 levels for assessing trauma severity and predicting clinical outcome of patients with sTBI.

Neuroinflammation is a very well-known process, which, although complicated, is involved in pathophysiological mechanisms of secondary brain injury after TBI.⁸ Inflammasomes, which are recognized as large multimolecular complexes, play essential effects in activation of the proteolytic enzyme caspase-1.¹⁶ In other words, inflammasomes are implicated in pyroptosis via regulating the proteolytic maturation of interleukin-1 β and interleukin-18.¹⁷ Besides

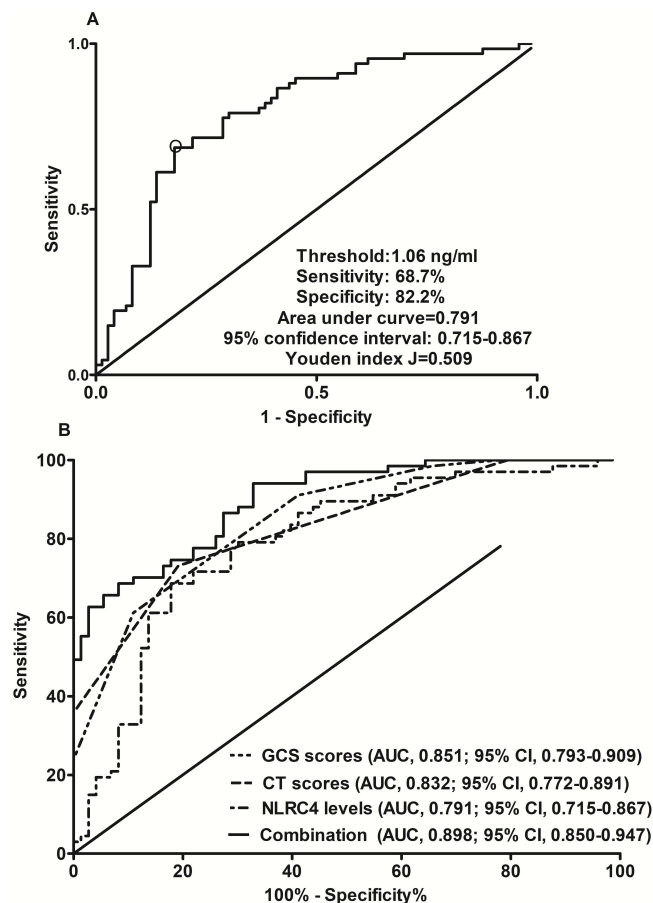


Figure 7 Serum NLRC4 levels concerning distinguishable capability for risk of 180-day poor outcome after severe traumatic brain injury. **(A)** Predictive ability of serum NLRC4 levels for 180-day poor prognosis after severe traumatic brain injury. Serum NLRC4 levels substantially distinguished patients with development of 180-day poor prognosis following severe traumatic brain injury ($P<0.001$). **(B)** Differences in 180-day prognostic predictive ability between serum NLRC4 levels and other parameters among severe traumatic brain injury patients. Serum NLRC4 levels combined with Glasgow coma scale scores and Rotterdam computerized tomography scores exhibited profoundly higher area under curve than Rotterdam computerized tomography scores and Glasgow coma scale scores alone (both $P<0.05$).

Abbreviations: GCS, Glasgow coma scale; CT, computed tomography; AUC, area under curve; 95% CI, 95% confidence interval.

pathogens (pathogen-associated molecular patterns), endogenous danger signals (danger-associated molecular patterns) derived from damaged or dying cells can lead to activation of inflammasomes, thereby triggering pathological inflammation in sterile inflammatory conditions or diseases, such as TBI, and finally inducing neurologic dysfunction.¹⁸ In summary, inflammasomes may harbor noxious potential in acute brain injury via participating in inflammatory processes.

NLRC4 is a very important component of inflammasome.¹⁹ NLRC4 was highly expressed in brain tissues of mice after intracerebral hemorrhage in a time-dependent manner, with peaking at post-hemorrhage 24 h.²⁰ In addition, the highest expression of rat NLRC4 in ischemia area was revealed at 4h of transient cerebral ischemia/reperfusion.²¹ Moreover, NLRC4 was dominantly located in neurons after animal cerebral ischemia²² and in glial cells under lysophosphatidylcholine, a molecule associated with neurodegeneration and demyelination.¹² Clearly, NLRC4 was highly expressed in brain tissues of TBI mice.²³ The current study showed a significant elevation of serum NLRC4 levels after human sTBI, in comparison to controls. Taken together, it is inferred that NLRC4 in peripheral blood of sTBI patients may be at least partially derived from traumatized brain tissues.

NLRC4 inflammasome was activated in microglia and astrocytes subjected to lysophosphatidylcholine.¹² Involvement of NLRC4 in neuroinflammation of rats after hemorrhagic stroke was confirmed because of its strong ability to increase accumulation of microglia and the release of interleukin-1 β and interleukin-18, induce neuronal death, disrupt blood–brain barrier, and aggravate brain edema.¹³ In contrast, knocking down NLRC4 using siRNA exerted neuroprotective effects in experimental intracerebral hemorrhage.²⁰ In addition, similar results were found in

ischemic stroke using siRNA knockdown of NLRC4.¹⁴ In the current study, serum NLRC4 levels were independently correlated with serum C-reactive protein levels. NLRC4 may act as an inflammatory protein to participate in the neuroinflammation. Moreover, targeting NLRC4 may be considered as another approach to lessen acute brain injury.

It is unclear regarding the relationship between serum NLRC4 levels and illness severity in addition to clinical outcome after sTBI. In this study, both GCS and Rotterdam CT classification were considered as the severity indicators. Interestingly, an independent correlation was found between serum NLRC4 levels and GCS scores, as well as between serum NLRC4 levels and Rotterdam CT scores. In addition, GOSE was regarded as the continuous and categorical variables. 180-day overall survival, death and poor prognosis were selected as the three prognostic parameters. Using multivariate analysis, serum NLRC4 levels had independent correlation with GOSE at 180 days after head trauma; and serum NLRC4 retained as an independent predictor of 180-day death, overall survival and poor prognosis. Of note, serum NLRC4 levels combined with GCS scores and Rotterdam CT scores exhibited substantially higher prognostic predictive capability than Rotterdam CT scores and GCS scores alone. In summary, such data were strongly supportive of the notion that serum NLRC4 may represent a promising prognostic biochemical biomarker of human sTBI.

There are several strengths and weaknesses in this study. The strengths are that (1) there was the follow-up time of 180 days after head trauma, thereby aiding in analysis of long-term prognosis after sTBI; and (2) all correlations or associations of serum NLRC4 levels with severity and prognosis following trauma were verified using multivariate analyses. The weaknesses are that (1) serum NLRC4 levels were only measured at a time point after head trauma and therefore, dynamic change of its levels is not discerned; and (2) using a medium sample size of patients, we investigated relationships between serum NLRC4 levels and trauma severity plus long-term prognosis of sTBI patients, and subsequently the conclusions need to be validated in a larger cohort study.

Conclusions

To the best of our knowledge, this is a first series for quantifying serum NLRC4 levels after human sTBI. Our main findings are that (1) serum NLRC4 levels are in independent relation to serum C-reactive protein levels, GCS scores, Rotterdam CT scores, 180-day GOSE scores, death, overall survival and poor prognosis; and (2) When combined with GCS scores and Rotterdam CT scores, serum NLRC4 levels show efficient additive effect on prognostic prediction ability reflected by AUC. In a word, serum NLRC4 may serve as an inflammatory predictive factor of clinical outcome in human sTBI.

Abbreviations

sTBI, severe traumatic brain injury; GCS, Glasgow coma scale; ROC, receiver operating characteristic; AUC, area under curve; OR, odds ratio; HR, hazard ratio; 95% CI, 95% confidence interval; GOSE, extended Glasgow Outcome Scale; NLR, nucleotide-binding and oligomerization domain-like receptor; NLRC4, NLR CARD domain containing 4.

Data Sharing Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Ethics Statement

This study was carried out in compliance with the tenets of the Declaration of Helsinki and the protocol conformed to the ethical standards of the First People's Hospital of Jiande City on human subjects. The study protocol was approved by the Human Investigation Committee at the First People's Hospital of Jiande City (NO. JDYY2017023). We acquired written informed consent to participate in the current study from legal representatives of patients or controls themselves.

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Disclosure

The authors declare that there were no conflicts of interest.

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