Original Article

Diagnostic and Predictive Levels of Calcium-binding Protein A8 and Tumor Necrosis Factor Receptor-associated Factor 6 in Sepsis-associated Encephalopathy: A Prospective Observational Study

Li-Na Zhang[1], Xiao-Hong Wang[2], Long Wu[1], Li Huang[1], Chun-Guang Zhao[1], Qian-Yi Peng[1], Yu-Hang Ai[1]

[1]Department of Critical Care Medicine, Xiangya Hospital, Central South University, Changsha, Hunan 410008, China
[2]Department of Anesthesiology and Critical Care Medicine, Hunan Provincial Maternal and Child Health Care Hospital, Changsha, Hunan 410008, China

Li-Na Zhang and Xiao-Hong Wang contributed equally to this work.

Abstract

Background: Despite its high prevalence, morbidity, and mortality, sepsis-associated encephalopathy (SAE) is still poorly understood. The aim of this prospective and observational study was to investigate the clinical significance of calcium-binding protein A8 (S100A8) in serum and tumor necrosis factor receptor-associated factor 6 (TRAF6) in peripheral blood mononuclear cells (PBMCs) in diagnosing SAE and predicting its prognosis.

Methods: Data of septic patients were collected within 24 h after Intensive Care Unit admission from July 2014 to March 2015. Healthy medical personnel served as the control group. SAE was defined as cerebral dysfunction in the presence of sepsis that fulfilled the exclusion criteria. The biochemical indicators, Glasgow Coma Scale, Acute Physiology and Chronic Health Evaluation score II, TRAF6 in PBMC, serum S100A8, S100β, and neuron-specific enolase were evaluated in SAE patients afresh. TRAF6 and S100A8 were also measured in the control group.

Results: Of the 57 enrolled patients, 29 were diagnosed with SAE. The S100A8 and TRAF6 concentrations in SAE patients were both significantly higher than that in no-encephalopathy (NE) patients, and higher in NE than that in controls (3.74 ± 3.13 vs. 1.08 ± 0.75 vs. 0.37 ± 0.14 ng/ml, $P < 0.01$; 3.18 ± 1.55 vs. 1.02 ± 0.63 vs. 0.47 ± 0.10, $P < 0.01$). S100A8 levels of 1.93 ng/ml were diagnostic of SAE with 92.90% specificity and 69.00% sensitivity in the receiver operating characteristic (ROC) curve, and the area under the curve was 0.86 (95% confidence interval [*CI*]: 0.76–0.95). TRAF6-relative levels of 1.44 were diagnostic of SAE with 85.70% specificity and 86.20% sensitivity, and the area under the curve was 0.94 (95% *CI:* 0.88–0.99). In addition, S100A8 levels of 2.41 ng/ml predicted 28-day mortality of SAE with 90.00% specificity and 73.70% sensitivity in the ROC curve, and the area under the curve was 0.88. TRAF6 relative levels of 2.94 predicted 28-day mortality of SAE with 80.00% specificity and 68.40% sensitivity, and the area under the curve was 0.77. Compared with TRAF6, the specificity of serum S100A8 in diagnosing SAE and predicting mortality was higher, although the sensitivity was low. In contrast, the TRAF6 had higher sensitivity for diagnosis.

Conclusions: Peripheral blood levels of S100A8 and TRAF6 in SAE patients were elevated and might be related to the severity of SAE and predict the outcome of SAE. The efficacy and specificity of S100A8 for SAE diagnosis were superior, despite its weak sensitivity. S100A8 might be a better biomarker for diagnosis of SAE and predicting prognosis.

Key words: Biomarker; Calcium-binding Protein A8; Sepsis-associated Encephalopathy; Tumor Necrosis Factor Receptor-associated Factor 6

Introduction

Sepsis often leads to multiple organ dysfunction including acute respiratory distress syndrome and acute renal and liver injury, which is also the main cause of increased mortality in these patients.[1] However, the brain injury in sepsis, known as sepsis-associated encephalopathy (SAE), is often ignored. SAE is a diffuse brain dysfunction secondary to infection

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.4103/0366-6999.185860

Address for correspondence: Prof. Yu-Hang Ai, Department of Critical Care Medicine, Xiangya Hospital, Central South University, No. 87, Xiangya Road, Changsha, Hunan 410008, China
E-Mail: ayhicu1978@126.com

Received: 15-02-2016 **Edited by:** Peng Lyu
How to cite this article: Zhang LN, Wang XH, Wu L, Huang L, Zhao CG, Peng QY, Ai YH. Diagnostic and Predictive Levels of Calcium-binding Protein A8 and Tumor Necrosis Factor Receptor-associated Factor 6 in Sepsis-associated Encephalopathy: A Prospective Observational Study. Chin Med J 2016;129:1674-81.

in the body without overt central nervous system infection. Symptoms of SAE vary from delirium to coma occur at any time in the disease process, even earlier than the common clinical symptoms of sepsis.[2,3] Despite its high prevalence, morbidity, and mortality, SAE is still poorly understood. In addition, survivors of SAE might display prolonged cognitive dysfunction.[4]

The diagnosis of SAE is one of exclusions, usually depends on case history and clinical symptoms.[5,6] Secondary screening methods such as electroencephalograph,[7] somatosensory-evoked potentials,[8] bispectral index,[9] and transcranial Doppler monitoring of cerebral blood flow[10] enable the clinical diagnosis of SAE. Biomarkers such as neuron-specific enolase (NSE) and calcium-binding protein β (S100β) facilitate rapid and cost-effective diagnosis of SAE.[11,12] However, early measurement of serum S100β levels in SAE patients cannot determine the loss of consciousness.[13] A study has shown that even elevated levels of serum S100β show no relation to the severity of SAE.[14] Therefore, it is essential to investigate new biomarkers for diagnosis of SAE.

It is well established that overwhelming inflammation is a fundamental component of sepsis pathophysiology.[15] Calcium-binding protein A8 (S100A8), also called calgranulin A, and migration inhibitory factor-related protein 8 was shown to mediate the recognition of damage-associated molecular patterns.[16,17] Similar to pathogen-associated molecular patterns,[18] it can be combined with pattern recognition receptors, in immune response. Studies have shown that S100A8 plays an important role in tumorigenesis and inflammation.[19] Extracellular protein S100A8 mainly functions by combining with toll-like receptor 4 (TLR4) on the cell membrane in intracellular signaling pathways. On ligand binding to TLR4, an adaptor molecule myeloid differentiation factor 88 (MyD88) is recruited to the TLR complex as a dimer. The MyD88 recruits interleukin-1 receptor (IL-1R)-associated kinase 1, and tumor necrosis factor receptor-associated factor 6 (TRAF6), resulting in the activation of nuclear factor-kappa B (NF-κB) and production of inflammatory cytokines.[20] In the wild-type mice, the S100A8/A9 messenger RNA (mRNA) expression was obviously higher in the early reperfusion during cerebral ischemia-reperfusion injury.[21] TRAF 6 is a member of TRAFs superfamily. TRAFs are a type of membrane adapter proteins, with conserved genetic structure. Further, it is a major signal transduction factor mediating the interaction between TNFR superfamily and the IL-1R/TLR superfamily.[22]

We investigated the changes in gene expression profile of SAE rats via monitoring the brain electrical physiology in a cecum ligation perforation SAE rat model and found that S100A8 expression level increased significantly. As a key molecule in multiple pathways, TRAF6 expression was increased significantly in brain ischemia-reperfusion injury in the rat model[23] and was also related to microglial activation.[24] However, most previous studies showed that S100A8 or TRAF6 increased during sepsis or brain ischemia-reperfusion injury but not in SAE. S100A8 and TRAF6 expressions are upregulated in numerous cell types by oxidative stress and by specific cytokines and growth factors.[25] However, the expression of S100A8 and TRAF6 in SAE pathological conditions remains unknown. Therefore, we collected data to study the clinical significance of S100A8 and TRAF6 in diagnosing SAE and determining its prognosis.

Methods

Study design and enrollment

The study was conducted after approved by the local ethics committee of Xiangya Hospital and with the patients' informed consent. In this prospective and observational study, we selected 57 patients from the central Intensive Care Unit (ICU) diagnosed with sepsis. Selection was based on the criteria of the 2001 international guidelines for management of severe sepsis and septic shock,[26] from July 2014 to March 2015 for the study. The SAE was defined as cerebral dysfunction in the presence of sepsis and in the absence of any of the exclusion criteria. Exclusion criteria included: age <18 years, pregnancy, congenital brain dysplasia, primary brain injury (traumatic brain injury [TBI], intracranial infection, cerebral infarction, cerebral hemorrhage, and epilepsy), and secondary encephalopathy (hepatic encephalopathy, pulmonary encephalopathy, uremic encephalopathy, after cardiopulmonary resuscitation). All patients were screened daily with the Confusion Assessment Method for the ICU (CAM-ICU) scores for their consciousness level. Those without any of the exclusion criteria but with CAM-ICU positive represented the SAE group, and the negative was the NE group. Moreover, ten volunteer doctors served as the control group, for TRAF6 and S100A8 detection. In patients with sedated analgesia, we performed spontaneous awakening trials daily to assess the consciousness. The longest evaluation time after stopping sedation in spontaneous awakening trials was 24 h. If the patients were not awake within 24 h, they were diagnosed as SAE.

Baseline clinical demographics were collected within 24 h after ICU admission or starting with the diagnosis of SAE, including age, gender, previous health status, presence of shock, infection, Acute Physiology and Chronic Health Evaluation (APACHE) II scores, and Glasgow Coma Scale (GCS). Laboratory data included routine blood measurements, liver and renal function, arterial blood gas analysis, procalcitonin, C-reactive protein, NSE, S100β, and IL-6. Peripheral blood was drawn to measure S100 A8, TRAF6, IL-1β, and tumor necrosis factor-α (TNF-α). Finally, hospital and ICU stay, bacteriological categories, and 28-day mortality were also recorded in all the enrolled patients.

Peripheral blood mononuclear cells isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of septic patients and volunteer

doctors by Ficoll gradient centrifugation (Histopaque-1077, Sigma-Aldrich, USA) at room temperature. Anticoagulated blood was layered onto Histopaque-1077 without disturbing the layers and then centrifuged at 400 ×*g* for exactly 30 min at room temperature. The plasma and cells between the plasma and the Percoll gradients were then collected, washed, and resuspended in 0.1 mol/L phosphate buffer saline (PBS), stored at −80°C until use.

Western blotting

The total protein from PBMCs was obtained with RIPA Lysis Buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% tritonx-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfonate [SDS]) containing an inhibitor cocktail (100 mmol/L Phenylmethanesulfonyl fluoride [PMSF], 10 mmol/L leupeptin, and 2.1 mg/nl aprotinin). The protein concentration was measured using the bicinchoninic acid Protein Quantitation Kit (Beyotime, Shanghai, China) and 30 μg of denatured proteins from each lysate was subjected to SDS-polyacrylamide gel electrophoresis on criterion tris-HCL 8% precast gels and then transferred onto polyvinylidene fluoride membrane (Millipore, Massachusetts, USA). After blocking with 5% skim milk for 2 h at room temperature, the membrane was sequentially incubated with primary antibodies anti-TRAF6 (1:2000, Abcam, Cambridgeshire, UK) and reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) (1:1000; Beyotime, Shanghai, China) overnight at 4°C and washed three times for 10 min each with tris-buffered saline combined with Tween-20 (TBST). Subsequent incubation with monoclonal horseradish peroxidase-conjugated antibody was performed for 1h at room temperature in 5% skim milk and then washed three times with TBST. The images were acquired using an ECL (Millipore, Massachusetts, USA) Western blotting detection system (Bio-Rad, California, USA) and quantified by Image Lab 4.1 software (Bio-Rad, California, USA). GAPDH was used as a loading control. The intensity of bands of interest was normalized to the signal intensity of the control.

Enzyme-linked immunosorbent assay

Concentrations of S100A8 in the plasma were determined by human enzyme-linked immunosorbent assay (ELISA) kit (Cusabio, Wuhan, China) according to the manufacturer's protocol. IL-1β and TNF-α levels were determined using human ELISA kit (MultiSciences, Hangzhou, China) according to manufacturer's instructions.

Statistical analysis

Data were statistically analyzed and expressed as a mean ± standard deviations (SD). Means were analyzed by one-way analysis of variance (ANOVA) followed by the *t*-test for quantitative data or Chi-square test for qualitative data. Receiver operating characteristic (ROC) curves for the ability of S100A8, TRAF6, S100β, and NSE to diagnose SAE and predict the 28-day mortality, respectively, were carried out, and the cutoff points were obtained from the curves for the highest sum of sensitivity and specificity. $P < 0.05$ was considered significant differences. The statistical analyses were performed using SPSS version 18.0 (International Business Machine, Chicago, IL, USA) and GraphPad Prism version 5.0 (GraphPad Software Inc., CA, USA) for Windows.

Results

We enrolled 59 septic patients and 10 volunteers, and 29 patients were diagnosed with SAE with 50.88% morbidity. The median day of admission with SAE was the 3rd day. The incidence of shock and APACHE II scores in the SAE group were higher than those in the NE group, which was in contrast to the GCSs ($P < 0.01$). The abdominal cavity was the most common infection site, followed by urinary tract infections. Gram-negative bacteria were the most common organisms. SAE patients had a higher 28-day mortality rate than the NE patients (17.85% vs. 65.52%, $P < 0.01$). The pH value, arterial blood lactate (lac), alanine transaminase (ALT), and S100β were higher in SAE patients than in NEs. NSE values in SAE were higher than in the NE group, but the difference was not statistically significant. The detailed demographic and clinical features are summarized in Table 1.

The S100A8 level in SAE patients was significantly higher than in NE (3.74 ± 3.13 vs. 1.08 ± 0.75, $P < 0.01$), and NE was higher than in control (1.08 ± 0.75 vs. 0.37 ± 0.14, $P < 0.01$). The TRAF6 level in SAE patients was significantly higher than in NE (3.18 ± 1.55 vs. 1.02 ± 0.63, $P < 0.01$), and higher in NE than in control (1.02 ± 0.63 vs. 0.47 ± 0.10, $P < 0.01$). SAE patients had higher IL-1β, TNF-α, and IL-6 levels than the NE patients (1384.39 ± 775.84 vs. 163.99 ± 102.95; 1422.08 ± 500.93 vs. 491.26 ± 423.59; 1548.98 ± 552.34 vs. 923.82 ± 131.73, $P < 0.05$), and NE patients had higher IL-1β and TNF-α than the controls (163.99 ± 102.95 vs. 9.61 ± 5.97; 491.26 ± 423.59 vs. 64.85 ± 42.88, $P < 0.01$) [Figure 1]. SAE patients who died within 28 days had higher S100A8, TRAF6, IL-1β, and TNF-α level than those who survived (4.54 ± 3.45 vs. 2.21 ± 1.61, 3.64 ± 1.58 vs. 2.29 ± 1.05, 1593.52 ± 749.48 vs. 987.03 ± 693.87, and 1589.91 ± 440.67 vs. 1103.19 ± 468.85, $P < 0.01$).

ROC curves evaluating the ability of S100A8, TRAF6, S100β, and NSE to diagnose SAE and predict the 28-day mortality, respectively, are fully displayed in Figure 2. S100A8 levels of 1.93 ng/ml were diagnostic of SAE with 92.90% specificity and 69.00% sensitivity. The area under the curve was 0.86 (95% *CI*: 0.76–0.95). TRAF6 relative levels of 1.44 were diagnostic of SAE with 87.50% specificity and 86.20% sensitivity, and the area under the curve was 0.94 (95% *CI*: 0.88–0.99). S100β levels of 0.28 μg/L were diagnostic of SAE with 68.80% specificity and 83.30% sensitivity, with an area under the curve of 0.79 (95% *CI*: 0.65–0.94). NSE levels of 0.79 ng/ml were diagnostic of SAE with 87.50% specificity and 58.30% sensitivity, and the area under the curve was 0.79 (95% *CI*: 0.65–0.93). S100A8 levels 2.41 ng/ml were predictive of 28-day mortality of SAE with 90.00% specificity and 73.70% sensitivity, and the area under the curve was 0.88 (95% *CI*: 0.76–1.00).TRAF6 relative levels of 2.94

Table 1: Demographics and clinical characteristics of study groups

Items	NE (n = 28)	SAE (n = 29)	Statistical value	P
Age (years)	56.21 ± 12.85	55.55 ± 12.72	0.20*	0.85
Gender (male/female), n	13/15	20/9	2.97†	0.09
Hospital stay time (days)	14.75 ± 9.19	12.45 ± 9.72	0.92*	0.36
ICU stay time (days)	4.71 ± 5.86	5.17 ± 4.74	−0.33*	0.75
Shock (yes/no), n	12/16	23/6	7.99†	0.01
GCSs	15 ± 0.00	12.72 ± 1.31	9.38*	0.00
APACHE II scores	12.04 ± 3.93	20.79 ± 9.07	−4.76*	0.00
28-day mortality, % (n)	17.86 (5/28)	65.52 (19/29)	13.27†	0.00
pH	7.38 ± 0.06	7.28 ± 0.14	3.24*	0.02
Lactate (mmol/L)	1.79 ± 0.21	4.37 ± 0.68	−3.99*	0.00
WBC ($\times 10^9$/L)	13.71 ± 8.71	17.76 ± 11.96	−1.46*	0.15
Platelet ($\times 10^9$/L)	133.43 ± 82.13	104.66 ± 72.88	1.40*	0.17
PCT (mg/L)	55.92 ± 11.45	73.87 ± 13.20	−1.02*	0.31
CRP (mg/L)	128.52 ± 38.08	84.87 ± 17.65	1.00*	0.32
ALT (U/L)	38.15 ± 8.66	250.15 ± 99.43	−2.12*	0.04
Serum creatinine (mmol/L)	175.70 ± 33.76	231.78 ± 31.90	−1.21*	0.23
S100β (μg/L)	0.61 ± 0.26	2.50 ± 0.49	−3.41*	0.00
NSE (ng/ml)	13.16 ± 1.43	43.92 ± 14.66	−1.71*	0.10
Infection sites, n			8.26†	0.31
Lung	3	5		
Abdominal cavity	10	10		
Urinary system	13	8		
Skin or soft tissue	2	1		
Blood	0	5		
Bacteriological categories, n			2.58†	0.63
Gram-negative bacteria	8	7		
Gram-positive bacteria	3	6		
Fungi	1	2		
Mixed bacteria	4	6		

Values are presented as a mean ± SD or n/N. *t value; †χ^2 value. The incidence of shock and APACHE II scores in the SAE group were higher than in the NE group, which was in contrast to the GCSs (P<0.01). SAE patients had a higher 28-day mortality rate than the NE patients (17.85% vs. 65.52%, P<0.01). The pH value, lactate, ALT, and S100β were higher in SAE patients than in NEs. NSE values in SAE were higher than that in the NE group, but the difference was not statistically significant. The abdominal cavity was the most common infection site, followed by urinary tract infections, Gram-negative bacteria were the most common organisms, however, the difference was not statistically significant. NEs: No-encephalopathies; SAE: Sepsis-associated encephalopathy; ICU: Intensive Care Unit; GCS: Glasgow Coma Scale; APACHE: Acute Physiology and Chronic Health Evaluation score; ALT: Alanine transaminase; WBC: White blood cell; PCT: Procalcitonin; CRP: C-reactive protein; S100β: Calcium-binding protein β; NSE: Neuron-specific enolase; SD: Standard deviation.

were predictive of 28-day mortality in SAE with 80.00% specificity and 68.40% sensitivity, and the area under the curve was 0.77 (95% *CI*: 0.60–0.95). S100β levels 1.22 μg/L predicted the 28-day mortality of SAE with 77.80% specificity and 73.30% sensitivity, and the area under the curve was 0.80 (95% *CI*: 0.62–0.98). NSE levels 27.02 ng/ml were predictive of 28-day mortality in SAE with 88.90% specificity and 60.00% sensitivity, and the area under the curve was 0.75 (95% *CI*: 0.55–0.95).

Discussion

Sepsis is associated with substantial long-term morbidity and is a common and rapidly growing public health concern in older people. Although the mortality rate has decreased significantly in the past 2 years, the majority of septic patients die within 5 years.[27,28] SAE is one of the major causes of death or disability.[5] Compared with previous studies, our study results show an incidence rate of 50.88% and a mortality rate of 65.52% in SAE patients. Previous studies suggested that APACHE II score, ALT, aspartate aminotransferase, pH value, lactic acidosis, and other indicators were risk factors for SAE and death.[29,30] APACHE II scoring system is widely recognized as a nonspecific indicator of the severity of disease and is used to evaluate the severity and prognosis of SAE.[29] The study showed that APACHE II scores for patients with SAE were higher than in NE patients, and SAE deaths were also higher than in survivors of APACHE II assessment, indicating that the SAE was related to the severity of disease. We also found that the percentage of shock and arterial blood lactate levels was higher in SAE than in NE group. Previous studies have shown that SAE pathogenesis was closely related to brain perfusion and cerebral blood flow.[30,31] Therefore, we speculated that early improvement in circulation and brain perfusion might reduce the incidence of SAE and improve the clinical outcomes in SAE.

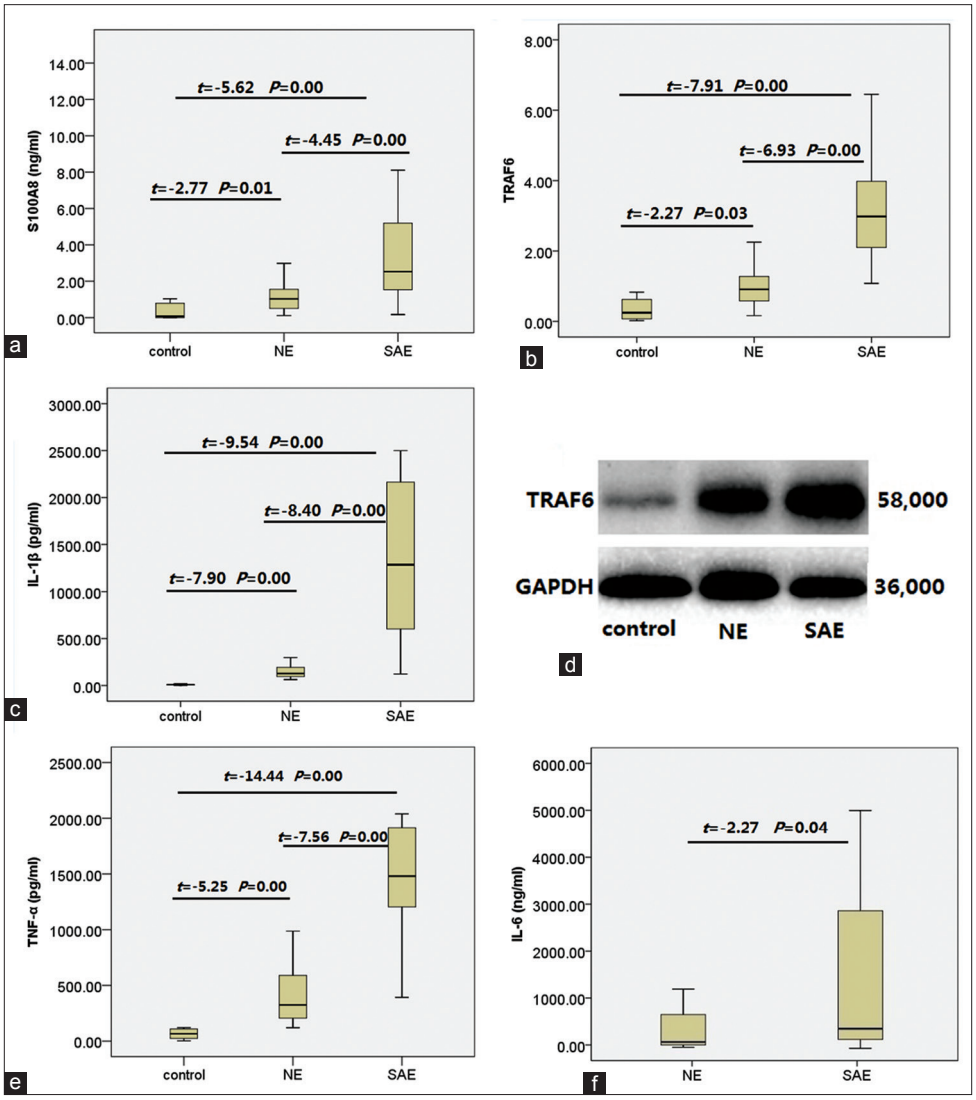

Figure 1: The S100A8 level in SAE patients was significantly higher than that in NE (3.74 ± 3.13 vs. 1.08 ± 0.75, $P < 0.01$), and NE was higher than that in control (1.08 ± 0.75 vs. 0.37 ± 0.15, $P < 0.01$) (a). The TRAF6 level in SAE patients was significantly higher than that in NE (3.18 ± 1.55 vs. 1.02 ± 0.63, $P < 0.01$), and higher in NE than that in control (1.02 ± 0.63 vs. 0.47 ± 0.10, $P < 0.01$) (b and d). SAE patients had higher IL-1β, TNF-α, and IL-6 levels than the NE patients (1384.39 ± 775.84 vs. 163.99 ± 102.95; 1422.08 ± 500.93 vs. 491.26 ± 423.59; 1548.98 ± 552.34 vs. 923.82 ± 131.73, $P < 0.05$), and NE patients had higher IL-1β and TNF-α than the controls (163.99 ± 102.95 vs. 9.61 ± 5.97; 491.26 ± 423.59 vs. 64.85 ± 42.88, $P < 0.01$) (c, e and f). NE: No-encephalopathy; SAE: Sepsis-associated encephalopathy; S100A8: Calcium-binding protein A8; TRAF6: Tumor necrosis factor receptor-associated factor 6; IL-1β: Interleukin-1β; GAPDH: Glyceraldehyde-phosphate dehydrogenase; TNF-α: Tumor necrosis factor α; IL-6: Interleukin-6.

We found that the peripheral blood S100A8 levels in SAE patients were higher than those in NE patients and higher in NE patients than in healthy individuals. The mechanism of increase in peripheral blood S100A8 concentration remains unknown. S100 protein family is a group of structurally and functionally homologous calcium-binding proteins. Studies have shown that serum S100 protein concentrations represented biochemical markers of brain injury.[32] Previous studies found that the S100A8/A9 mRNA expression was significantly increased in brain tissue in the early stages of cerebral ischemia-reperfusion injury in wild-type mice.[21] Another study reported that S100A8 mRNA levels in the hippocampus of patients with Alzheimer's disease were twice the levels of normal individuals.[33] What is more, S100A8 expression was induced approximately 30-fold in cultured human microglia following chronic treatment with oligomeric Aβ1-42.[34] Using immunohistochemical analysis, S100A8, S100A9, S100A12, and S100β levels were shown to be associated with vascular structures in brains.[35] The totality of evidence supports a key role for S100A8 in neuroinflammation and microangiopathy. Therefore, S100A8 is likely to mediate SAE through activated microglia or vascular endothelial cells. However, the mechanisms leading to a progressive abundance of circulating free S100A8 during the development of SAE are currently unknown.

We also analyzed the value of S100β and NSE for the diagnosis and prognosis of SAE. The results suggest that despite the lower diagnostic sensitivity, S100A8 showed the highest specificity, and higher sensitivity and specificity in

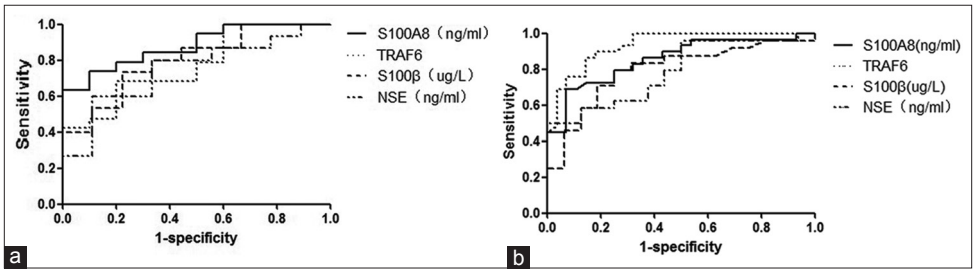

Figure 2: Receiver operating characteristic curves regarding the ability of S100A8, TRAF6, S100β, and NSE to diagnose SAE (a) and predict the 28-day mortality (b). S100A8 levels of 1.93 ng/ml were diagnostic of SAE with 92.90% specificity and 69.0% sensitivity. The area under the curve was 0.86 (95% *CI:* 0.76–0.95). TRAF6 relative levels of 1.44 were diagnostic of SAE with 87.50% specificity and 86.20% sensitivity, and the area under the curve was 0.94 (95% *CI*: 0.88–0.99). S100β levels of 0.28 μg/L were diagnostic of SAE with 68.8% specificity and 83.3% sensitivity, with an area under the curve of 0.79 (95% *CI*: 0.65–0.94). NSE levels of 0.79 ng/ml were diagnostic of SAE with 87.50% specificity and 58.30% sensitivity, and the area under the curve was 0.79 (95% *CI:* 0.65–0.93). S100A8 levels 2.41 ng/ml were predictive of 28-day mortality of SAE with 90% specificity and 73.70% sensitivity, and the area under the curve was 0.88 (95% *CI:* 0.76–1.00). TRAF6 relative levels of 2.94 were predictive of 28-day mortality in SAE with 80.00% specificity and 68.40% sensitivity, and the area under the curve was 0.77 (95% *CI*: 0.60–0.95). S100β levels 1.22 μg/L predicted the 28-day mortality of SAE with 77.80% specificity and 73.30% sensitivity, and the area under the curve was 0.80 (95% *CI*: 0.62–0.98). NSE levels 27.02 ng/ml were predictive of 28-day mortality in SAE with 88.90% specificity and 60.00% sensitivity, and the area under the curve was 0.75 (95% *CI*: 0.55–0.95). S100A8: Calcium-binding protein A8; TRAF6: Tumor necrosis factor receptor-associated factor 6; S100β: Calcium-binding protein β; NSE: Neuron-specific enolase; *CI*: Confidence interval; SAE: Sepsis-associated encephalopathy.

predicting SAE prognosis than S100β and NSE. Compared with S100β, S100A8 levels reflect the degree of SAE accurately as they are mainly expressed in microglia of brain tissue while S100β is found in astrocytes. Microglial cells constitute the first line of defense in the central immune response while astrocytes play a major neurotrophic role.

TRAF6 is a key factor in inflammation and apoptosis. It is also closely related to brain injury.[36,37] Chen *et al*.[23] developed a mouse model of TBI to show that TRAF6 expression levels were significantly increased in the first 7 days after TBI modeling. In addition, TRAF6 and caspase 3 expressions were significantly increased in the injured ipsilateral brain compared with the opposite side. Peripheral TRAF6 expression was increased in SAE in our study, translocated from brain to the peripheral blood through the blood-brain barrier or expressed peripherally. In summary, our findings suggest that TRAF6 might be involved in early inflammatory damage in SAE. The ROC for the ability of TRAF6 to diagnose SAE showed a large area under the curve and high sensitivity compared with S100β, indicating optimal diagnostic ability.

Increased expression of S100A8 and TRAF6 provides a basis for the mechanism of SAE. We also tested serum inflammatory markers such as TNF-α, IL-1β, and IL-6, and found elevated expression in SAE patients than in NE patients, suggesting a positive feedback loop between S100A8, TRAF6, and inflammatory factors in sepsis and SAE. LPS from bacteria is recognized by the surface receptor TLR4, activating MyD88-dependent and independent pathways. IRAKs and TRAF6 are recruited to MyD88 and subsequently activate a complex of transforming growth factor-activated kinase 1 (TAK1) and TAK1-binding proteins resulting in phosphorylation of inhibitor of NF-κB and nuclear translocation of NF-κB. Simultaneously, TAK1 activates mitogen-activated protein kinase cascades leading to the activation of activator protein 1, resulting in a large number of cytokines.[38] The role of TLR4 in inflammation is unrelated to the exogenous pathogenic composition. TLR4 promoted the cascade of inflammatory reactions in the body including the heart, lungs, and brain.[39,40] TRAF6 deletion leads to defective TLR4 signaling, inhibition of NF-κB activation, and reduced cytokine production.[41] Downregulation of TLR4 and TRAF6 expression plays a protective role in ischemic stroke.[42] In this study, we found that peripheral blood S100A8 and TRAF6 were elevated similar to cytokines such as IL-1β, TNF-α, and IL-6 in SAE. Combined with the experimental results and related studies, we speculate that as an endogenous ligand, S100A8 in conjunction with TLR4 and then via TRAF6-mediated NF-κB signaling plays an important role during early inflammatory injury of SAE. However, the mechanism of S100A8 via other pathways or downstream molecules in the inflammatory response remains to be explored.

The study limitations are related to the use of peripheral blood indicators indirectly reflecting encephalopathy. Our study is only an observational study without intervention, which failed to confirm the interaction between S100A8 with TRAF6. The sample size in this experiment was another limitation. In addition, sedation might induce delirium, and we cannot exclude the effect of sedation. Further, we only studied patients during the ICU stay, excluding patients with sepsis outside ICU and afflicted with SAE. Further, our indicators were not dynamic. Finally, shock, APACHE II, GCSs, and hospital mortality suggested severe SAE. Probably, the severity of sepsis represents a risk factor for ICU delirium and a confounding factor for the relationship between SAE and S100A8 or TRAF6 levels, which requires additional investigations.

In conclusion, peripheral blood levels of S100A8 and TRAF6 in SAE patients were elevated and might be related to the severity of SAE and predict the outcome of SAE. The efficacy and specificity of S100A8 for SAE diagnosis were

superior, despite its weak sensitivity. S100A8 is a better biomarker for diagnosis of SAE and predicting prognosis.

Financial support and sponsorship

This work was supported by the National Natural Science Foundation of China (No. 81401099).

Conflicts of interest

There are no conflicts of interest.

References

1. de Montmollin E, Annane D. Year in review 2010: Critical care-Multiple organ dysfunction and sepsis. Crit Care 2011;15:236. doi: 10.1186/cc10359.
2. Jacob A, Brorson JR, Alexander JJ. Septic encephalopathy: Inflammation in man and mouse. Neurochem Int 2011;58:472-6. doi: 10.1016/j.neuint.2011.01.004.
3. Zampieri FG, Park M, Machado FS, Azevedo LC. Sepsis-associated encephalopathy: Not just delirium. Clinics (Sao Paulo) 2011;66:1825-31. doi: 10.1590/S1807-59322011001000024.
4. Widmann CN, Heneka MT. Long-term cerebral consequences of sepsis. Lancet Neurol 2014;13:630-6. doi: 10.1016/S1474-4422(14)70017-1.
5. Gofton TE, Young GB. Sepsis-associated encephalopathy. Nat Rev Neurol 2012;8:557-66. doi: 10.1038/nrneurol.2012.183.
6. Cotena S, Piazza O. Sepsis-associated encephalopathy. Transl Med UniSa 2012;2:20.
7. Jaber S, Chanques G, Altairac C, Sebbane M, Vergne C, Perrigault PF, *et al. A* prospective study of agitation in a medical-surgical ICU: Incidence, risk factors, and outcomes. Chest 2005;128:2749-57. doi: 10.1378/chest.128.4.2749.
8. Sharshar T, Annane D, de la Grandmaison GL, Brouland JP, Hopkinson NS, Françoise G. The neuropathology of septic shock. Brain Pathol 2004;14:21-33. doi: 10.1111/j.1750-3639.2004.tb00494.x.
9. Sharshar T, Carlier R, Bernard F, Guidoux C, Brouland JP, Nardi O, *et al.* Brain lesions in septic shock: A magnetic resonance imaging study. Intensive Care Med 2007;33:798-806. doi: 10.1007/s00134-007-0598-y.
10. Pierrakos C, Attou R, Decorte L, Kolyviras A, Malinverni S, Gottignies P, *et al.* Transcranial Doppler to assess sepsis-associated encephalopathy in critically ill patients. BMC Anesthesiol 2014;14:45. doi: 10.1186/1471-2253-14-45.
11. Iacobone E, Bailly-Salin J, Polito A, Friedman D, Stevens RD, Sharshar T. Sepsis-associated encephalopathy and its differential diagnosis. Crit Care Med 2009;37 10 Suppl:S331-6. doi: 10.1097/CCM.0b013e3181b6ed58.
12. Panni JK, Panni MK. Changes in S100B levels rather than absolute values may be a better marker of severity of septic encephalopathy. Br J Anaesth 2008;100:419-20. doi: 10.1093/bja/aen009.
13. Karnatovskaia LV, Festic E. Sepsis: A review for the neurohospitalist. Neurohospitalist 2012;2:144-53. doi: 10.1177/1941874412453338.
14. Piazza O, Russo E, Cotena S, Esposito G, Tufano R. Elevated S100B levels do not correlate with the severity of encephalopathy during sepsis. Br J Anaesth 2007;99:518-21. doi: 10.1093/bja/aem201.
15. Michels M, Danielski LG, Dal-Pizzol F, Petronilho F. Neuroinflammation: Microglial activation during sepsis. Curr Neurovasc Res 2014;11:262-70. doi: 10.1016/s0952-7915(96)80098-7.
16. Seong SY, Matzinger P. Hydrophobicity: An ancient damage-associated molecular pattern that initiates innate immune responses. Nat Rev Immunol 2004;4:469-78. doi: 10.1038/nri1372.
17. Jin S, Park CO, Shin JU, Noh JY, Lee YS, Lee NR, *et al.* DAMP molecules S100A9 and S100A8 activated by IL-17A and house-dust mites are increased in atopic dermatitis. Exp Dermatol 2014;23:938-41. doi: 10.1111/exd.12563.
18. Hoffmann JA, Reichhart JM, Hetru C. Innate immunity in higher insects. Curr Opin Immunol 1996;8:8-13. doi: 10.1016/s0952-7915(96)80098-7.
19. Gebhardt C, Németh J, Angel P, Hess J. S100A8 and S100A9 in inflammation and cancer. Biochem Pharmacol 2006;72:1622-31. doi: 10.1016/j.bcp. 2006.05.017.
20. Muroi M, Tanamoto K. TRAF6 distinctively mediates MyD88- and IRAK-1-induced activation of NF-kappaB. J Leukoc Biol 2008;83:702-7. doi: 10.1189/jlb.0907629.
21. Ziegler G, Prinz V, Albrecht MW, Harhausen D, Khojasteh U, Nacken W, *et al.* Mrp-8 and -14 mediate CNS injury in focal cerebral ischemia. Biochim Biophys Acta 2009;1792:1198-204. doi: 10.1016/j.bbadis.2009.10.003.
22. Lee NK, Lee SY. Modulation of life and death by the tumor necrosis factor receptor-associated factors (TRAFs). J Biochem Mol Biol 2002;35:61-6. doi: 10.5483/BMBRep.2002.35.1.061.
23. Chen J, Wu X, Shao B, Zhao W, Shi W, Zhang S, *et al*. Increased expression of TNF receptor-associated factor 6 after rat traumatic brain injury. Cell Mol Neurobiol 2011;31:269-75. doi: 10.1007/s10571-010-9617-6.
24. Yao L, Kan EM, Kaur C, Dheen ST, Hao A, Lu J, *et al.* Notch-1 signaling regulates microglia activation via NF-κB pathway after hypoxic exposure *in vivo* and *in vitro*. PLoS One 2013;8:e78439. doi: 10.1371/journal.pone.0078439.
25. Hsu K, Champaiboon C, Guenther BD, Sorenson BS, Khammanivong A, Ross KF, *et al*. Anti-infective protective properties of S100 calgranulins. Antiinflamm Antiallergy Agents Med Chem 2009;8:290-305. doi: 10.2174/187152309789838975.
26. Levy MM, Fink MP, Marshall JC, Abraham E, Angus D, Cook D, *et al.* 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. Crit Care Med 2003;31:1250-6. doi: 10.1097/01.CCM.0000050454.01978.3B.
27. Iwashyna TJ, Cooke CR, Wunsch H, Kahn JM. Population burden of long-term survivorship after severe sepsis in older Americans. J Am Geriatr Soc 2012;60:1070-7. doi: 10.1111/j.1532-5415.2012.03989.x.
28. Stevenson EK, Rubenstein AR, Radin GT, Wiener RS, Walkey AJ. Two decades of mortality trends among patients with severe sepsis: A comparative meta-analysis. Crit Care Med 2014;42:625-31. doi: 10.1097/CCM.0000000000000026.
29. Eidelman LA, Putterman D, Putterman C, Sprung CL. The spectrum of septic encephalopathy. Definitions, etiologies, and mortalities. JAMA 1996;275:470-3. doi: 10.1001/jama.1996.03530300054040.
30. Terborg C, Schummer W, Albrecht M, Reinhart K, Weiller C, Röther J. Dysfunction of vasomotor reactivity in severe sepsis and septic shock. Intensive Care Med 2001;27:1231-4. doi: 10.1007/s001340101005.
31. Rosengarten B, Hecht M, Auch D, Ghofrani HA, Schermuly RT, Grimminger F, *et al.* Microcirculatory dysfunction in the brain precedes changes in evoked potentials in endotoxin-induced sepsis syndrome in rats. Cerebrovasc Dis 2007;23:140-7. doi: 10.1159/000097051.
32. Schiavi P, Laccarino C, Servadei F. The value of the calcium binding protein S100 in the management of patients with traumatic brain injury. Acta Biomed 2012;83:5-20.
33. Lue LF, Kuo YM, Beach T, Walker DG. Microglia activation and anti-inflammatory regulation in Alzheimer's disease. Mol Neurobiol 2010;41:115-28. doi: 10.1007/s12035-010-8106-8.
34. Walker DG, Link J, Lue LF, Dalsing-Hernandez JE, Boyes BE. Gene expression changes by amyloid beta peptide-stimulated human postmortem brain microglia identify activation of multiple inflammatory processes. J Leukoc Biol 2006;79:596-610. doi: 10.1189/jlb.0705377.
35. Shepherd CE, Goyette J, Utter V, Rahimi F, Yang Z, Geczy CL, *et al.* Inflammatory S100A9 and S100A12 proteins in Alzheimer's disease. Neurobiol Aging 2006;27:1554-63. doi: 10.1016/j.neurobiolaging.2005.09.033.
36. Bruneau S, Datta D, Flaxenburg JA, Pal S, Briscoe DM. TRAF6 inhibits proangiogenic signals in endothelial cells and regulates the expression of vascular endothelial growth factor. Biochem Biophys Res Commun 2012;419:66-71. doi: 10.1016/j.bbrc.2012.01.128.
37. Liu S, Lutz J, Chang J, Liu D, Heemann U, Baumann M. TRAF6 knockdown promotes survival and inhibits inflammatory response

to lipopolysaccharides in rat primary renal proximal tubule cells. Acta Physiol (Oxf) 2010;199:339-46. doi: 10.1111/j.1748-1716.2010.02097.x.
38. Lu SM, Yu CJ, Liu YH, Dong HQ, Zhang X, Zhang SS, *et al.* S100A8 contributes to postoperative cognitive dysfunction in mice undergoing tibial fracture surgery by activating the TLR4/MyD88 pathway. Brain Behav Immun 2015;44:221-34. doi: 10.1016/j.bbi.2014.10.011.
39. Caso JR, Pradillo JM, Hurtado O, Lorenzo P, Moro MA, Lizasoain I. Toll-like receptor 4 is involved in brain damage and inflammation after experimental stroke. Circulation 2007;115:1599-608. doi: 10.1161/CIRCULATIONAHA.106.603431.
40. Oyama J, Blais C Jr., Liu X, Pu M, Kobzik L, Kelly RA, *et al.* Reduced myocardial ischemia-reperfusion injury in toll-like receptor 4-deficient mice. Circulation 2004;109:784-9. doi: 10.1161/01.CIR.0000112575.66565.84.
41. Lutgens E, Lievens D, Beckers L, Wijnands E, Soehnlein O, Zernecke A, *et al.* Deficient CD40-TRAF6 signaling in leukocytes prevents atherosclerosis by skewing the immune response toward an antiinflammatory profile. J Exp Med 2010;207:391-404. doi: 10.1084/jem.20091293.
42. Zhang J, Fu B, Zhang X, Chen L, Zhang L, Zhao X, *et al.* Neuroprotective effect of bicyclol in rat ischemic stroke: down-regulates TLR4, TLR9, TRAF6, NF-κB, MMP-9 and up-regulates claudin-5 expression. Brain Res 2013;1528:80-8. doi: 10.1016/j.brainres.2013.06.032.