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ORIGINAL RESEARCH

Prognostic Relevance of ACSL4 as a Serological Marker and Its Mediating Roles in Acute Supratentorial Intracerebral Hemorrhage: An Observational Analytical Study

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Background: Acyl-CoA synthetase long-chain family member 4 (ACSL4) is an index of ferroptosis. Here, serum ACSL4 levels were examined to determine their prognostic significance and mediating roles in patients with acute intracerebral hemorrhage (ICH).

Methods: In this observational analytical study, serum ACSL4 levels were measured at admission in 317 patients with supratentorial intraparenchymal hemorrhage and at study entry in 100 controls. The National Institutes of Health Stroke Scale (NIHSS) score and hematoma volume were used as the severity metrics. Poor prognosis was designated as modified Rankin Scale (mRS) 3–6 at six months following ICH. Stroke-associated pneumonia (SAP) was defined as an acute lower airway infection within a week post-ICH. The outcome variables of interest were SAP and poor prognosis. Multifactorial means were implemented for severity and outcome analyses.

Results: Patients had significantly higher serum ACSL4 levels than controls. Serum ACSL4 levels remained linearly connected with NIHSS scores, hematoma volume, SAP, mRS scores, and poor prognosis under restricted cubic spline and kept substantially associated with them even after adjusting for other confounding factors. The independent associations with poor prognosis and SAP were robust via sensitivity analysis, and exhibited no interactions with age, sex, tobacco smoking, and others through subgroup analysis. Regarding the predictive ability for poor prognosis and SAP under the receiver operating characteristic curve, serum ACSL4 levels were statistically comparable to the NIHSS scores and hematoma volume. Using mediation analysis, serum ACSL4 levels partially mediated the associations of NIHSS scores and hematoma volume with poor prognosis and SAP, and SAP in part mediated the relationship between serum ACSL4 levels and mRS scores plus poor prognosis.

Conclusion: Enhanced serum ACSL4 levels post-ICH are significantly linked to ICH severity and clinical outcomes, and serum ACSL4 may partially interpret outcome associations, therefore extrapolating serum ACSL4 as a prognostic adjunct of ICH.

Keywords: Acyl-CoA synthetase long chain family member 4, intracerebral hemorrhage, prognosis, stroke-associated pneumonia, severity, biomarkers

Introduction

Spontaneous intracerebral hemorrhage (ICH) is a ubiquitous cerebrovascular event that frequently threatens human health and disturbs the economy and society.[1] ICH, generally referred to as the primary type, results from rupture of the cerebrovascular wall, which is pathologically attributed to atherosclerosis or amyloidosis.[2] Hematoma, once entering the intraparenchymal structure, directly deforms neurons and glia, and simultaneously ignites a succession of molecular chain reactions, involving inflammatory and oxidative processes, mitochondrial impairments, amino acid excitotoxicity, and so forth, thereby breaking the blood–brain barrier, exacerbating cerebral edema, and eliciting subsequent neuronal demises.[3] In clinical practice, the National Institutes of Health Stroke Scale (NIHSS) score and hematoma volume are two selectable metrics for severity appraisal and outcome prediction.[4] The ability of stroke patients to perform daily living is usually assessed using the modified Rankin Scale (mRS).[5] Stroke-associated pneumonia (SAP), a modifiable

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complication of ICH, is frequently encountered in clinical work and predisposes patients to poor prognosis.[6] Accordingly, it is equally important to discriminate the risk of both SAP and poor prognosis of ICH.[7,8] It is fully convinced that, apart for clinical and radiological indicators, biochemical markers are rather feasible tools to forecast clinical outcomes of ICH and therefore their related studies have been being emphasized in the neurological field.[9–11]

Ferroptosis is lately recognized as a form of cell death.[12] In contrast to other forms of cell death, such as apoptosis, necrosis, and autophagy, ferroptosis is marked by lipid peroxidation, which relies on iron.[13] The accumulation of reactive oxygen species is a driver of ferroptosis, which manifests as oxidative injury to cellular membranes primarily as a result of iron overload and excessive lipid peroxidation.[14] Ferroptosis is a crucial pathophysiological process associated with neuronal injury, secondary to acute brain injury.[15] Repression of ferroptosis signaling can profoundly diminish acute brain injury.[16] Acyl-CoA synthetase long-chain family member 4 (ACSL4) is a ferroptosis-related protein that participates in lipid metabolism by increasing lipid peroxidation.[17] ACSL4 is enriched in neurons and glial cells, and its activation can overwhelmingly aggravate brain injury.[18] Based on these experimental data, it is suggested that ACSL4 may be an appealing target for therapeutic interventions in acute brain injury. A recent clinical study of patients with post-ischemic stroke lower limb neurological sequelae showed that serum ACSL4 levels were pronouncedly higher in cases with poor recovery of motor function than in the other remainders.[19] Consistently, serum ACSL4 levels were reported to be independently associated with post-operative 30-day poor prognosis in patients with spontaneous subarachnoid hemorrhage patients who underwent clipping surgery to secure aneurysms.[20] Hence, serum ACSL4 may be a biomarker of brain injury. However, there is a paucity of data regarding circulating ACSL4 levels in ICH. In this study, serum ACSL4 levels were measured to determine their prognostic significance and their mediating role in acute ICH.

Materials and Methods

Subject Enrollments and Ethical Approval

This observational analytical study was conducted between January 2022 and January 2025 at the Quzhou Affiliated Hospital of Wenzhou Medical University (Quzhou, China). Two groups of subjects, patients with ICH and controls, were recruited in compliance with the established criteria, as shown in Figure 1. This clinical epidemiological investigation entailed two segments, namely, the cross-sectional study and the prospective cohort study. The first was to discern alterations in the serum-based ACSL4 levels after ICH. The second was used to ascertain the prognostic implications of serum-based ACSL4 levels and delineate the mediating effects of ACSL4 levels in ICH. This study was performed in accordance with the Declaration of Helsinki and its subsequent guidelines. The study protocol was approved by the Ethics Committee of the Quzhou Affiliated Hospital of Wenzhou Medical University (Quzhou, China) (approval number: 2022-002). Statutory proxies of the patients, together with the controls themselves, were informed of the study proposal and independently provided written informed consent.

Collections of Baseline Data and Scorings

Two conventional demographic variables (age and sex), two common lifestyle habits (cigarette smoking and alcohol consumption), three types of stroke-related chronic diseases (hypertension, diabetes mellitus, and dyslipidemia), and three frequently used medications (statins, anticoagulants, and antiplatelet agents) were assessed. Admission systolic arterial blood pressure and diastolic arterial blood pressure were noninvasively gauged. We recorded time from onset of stroke symptoms to hospital admission and duration from ictus of stroke symptoms to sampling. At admission, the patients underwent an initial assessment of neurological function using the NIHSS.[21] Vomiting was considered as a concomitant symptom. Dysphagia is a neurological dysfunction diagnosed using the swallowing test. Hematomas were of two types according to their location: superficial and deep. We determined whether the superficial hemorrhage and deep hematoma extended into the subarachnoid space and intraventricular cavity, respectively. Hematoma amount was estimated by computing the hematoma volume using the following formula: $0.5 \times a \times b \times c$.[22] SAP was diagnosed as an acute lower airway infection within a week post-ICH.[23] In the form of structured telephone interviews, neurological conditions were appraised by adopting the seven-point mRS scoring at 6 months after ICH, and scores of 3–6 were termed as poor prognosis.[24]

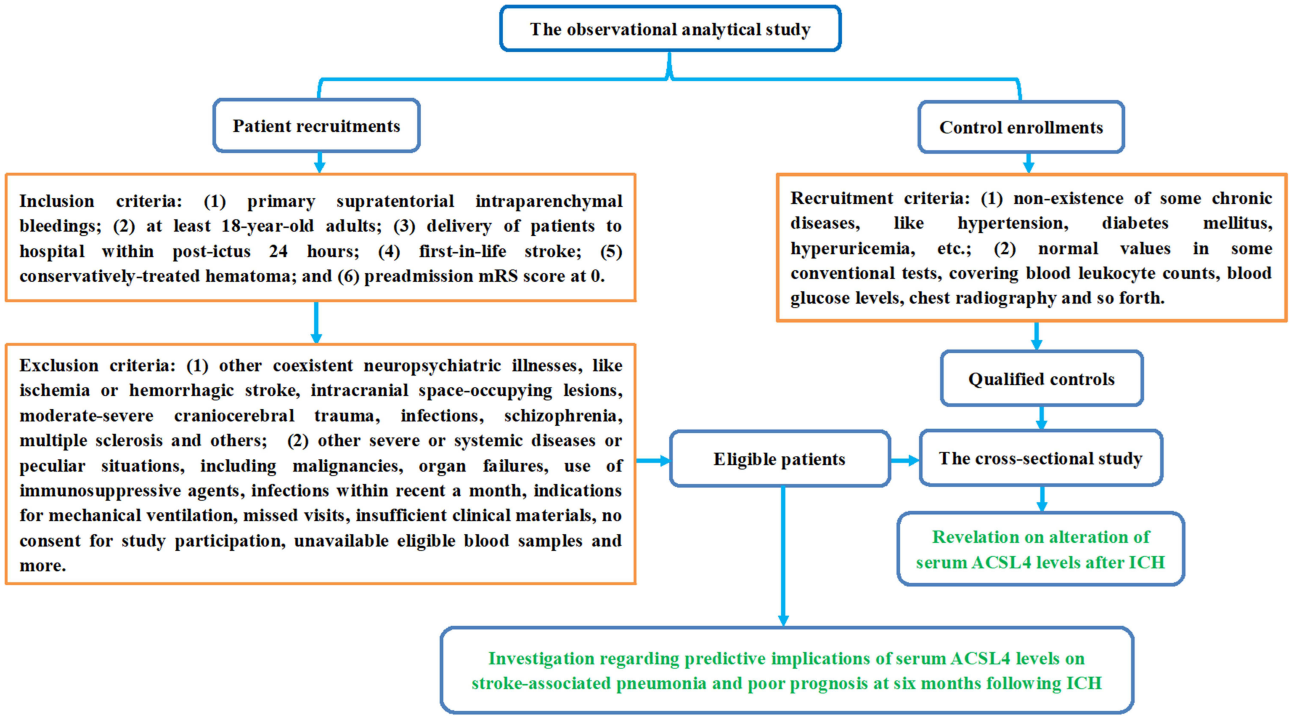

Figure 1 Study design and subject enrollments. Controls and patients with intracerebral hemorrhage were recruited to comply with pre-established requirements. Observational analytical studies were classified into prospective and cross-sectional sub-studies. The objective of this study was to observe the variations in serum ACSL4 levels and their prognostic implications in intracerebral hemorrhage.
Abbreviations: ACSL4, acyl-CoA synthetase long-chain family member 4; ICH, intracerebral hemorrhage.

Blood Samplings and Measurements

Venous blood specimens were obtained via antecubital venipuncture at admission of all patients and at study entry of controls. The acquired blood samples were rapidly deposited inside gel pre-stored tubes, awaiting coagulation. Centrifugation was performed at 2000 × g for 10 minutes, so as to separate cells and debris. The supernatants were aspirated and transferred to Eppendorf tubes for preservation at –80°C. To prevent ACSL4 from decomposing, samples were melted for quantification within 3 months of collection. By adopting the enzyme-linked immunosorbent assay kit (Catalogue number: EH6088; Wuhan Fine Biotech Co., Ltd., China), serum ACSL4 levels were in duplicate measured by the professional staff in a blinded fashion following the kit manual. The two measured values were converted to average values for further statistical analysis. As for this kit, sensitivity was 18.75 pg/mL, detection range was from 31.25 pg/mL to 2000 pg/mL, and both intra- and inter-assay coefficients of variation were less than 10%.

Statistical Analysis

The software packages, which were used for compiling graphs and analyzing data, encompassed SPSS 23.0 (SPSS Inc., Chicago, IL), R 3.5.1 (www.r-project.org), GraphPad Prism 7.01 (GraphPad Software, Inc., San Diego, CA) and MedCalc 20 (MedCalc Software, Ltd., Ostend, Belgium). The data were categorized as categorical and numerical variables. The former were summarized as frequencies (percentages); the latter as medians (lower–upper quartiles) on account of the non-normality distribution of all numerical variables via demonstration by the Kolmogorov–Smirnov test in this study. The Chi-square test or Fisher's exact test was performed for intergroup comparisons of categorical variables, and the Mann–Whitney *U*-test and Kruskal–Wallis test were performed to clarify differences in numerical variables between two groups and among multiple groups separately. The Spearman's rank correlation test was used to determine the correlation between the two factors. To discover indices that were independently related to serum ACSL4 levels, mRS scores, SAP, and poor prognosis, univariate analyses were scheduled to screen those significantly correlated or different parameters ($p < 0.05$), and the selected variables were incorporated into the multivariate linear regression

model or binary logistic regression model, as deemed appropriate. The correlations and associations were presented as beta (β) and odds ratio (OR) values, respectively, accompanied by 95% confidence intervals (CIs). Here, the restricted cubic spline (RCS) analysis was performed to assess the linearity relationship in order to assure the rationality of utilization of the linearity model in the process of statistical analyses;[25] sensitivity analyses, including E-value computation and adjustments for other factors in regression models, were conducted to verify the robustness of the results from the binary regression models;[26] The Hosmer-Lemeshow test was performed, and the Brier score was computed to prove goodness of fit of regression models; variance inflation factor (VIF) value was generated to determine multicollinearity in regression models, wherein a VIF value of more than 10 generally signifies the presence of multicollinearity;[27] subgroup analysis, followed by the likelihood ratio test, was implemented to demonstrate whether other conventional factors, such as age, gender, hypertension, etc, moderated associations of serum ACSL4 levels with outcome variables. The area under the receiver operating characteristic (ROC) curve (AUC) was used to assess discrimination efficiency, and the Z-test was performed to compare the AUCs. By aid of R 3.5.1 (www.r-project.org), mediating analysis was performed to confirm whether serum ACSL4 levels mediated the association between severity metrics and clinical outcomes, and whether SAP mediated the relationship between serum ACSL4 levels and poor prognosis or mRS scores. Based on the following parameters: type 1 error (alpha) = 0.05, test power (1-beta) = 0.95, and effect size = 0.8, sample size was estimated for conducting comparison of serum ACSL4 levels; and its estimation accuracy was validated in help of the priori power analysis supplied by the G*Power 3.1.9.4 software (Heinrich-Heine Universität Düsseldorf, Germany).[28] For all analyses, two-sided p-values < 0.05 indicated significant differences.

Results

Study Subjects

An initial evaluation was performed of 403 patients with ICH who met the prespecified inclusion criteria. Then, 86 cases were eliminated from the study cohort due to other coexisting neuropsychiatric illnesses (37 cases) and other severe or systemic diseases or peculiar situations (49 cases), subsequently leading to the entry of 317 patients into the final clinical analyses. The baseline patient characteristics are shown in Table 1. In addition, 100 controls were enrolled for comparison, aged from 44 to 78 years (median, 66 years; 25th–75th percentiles, 55–71 years), comprising 61 males and 39 females, and encompassing 36 tobacco smokers and 35 alcohol drinkers. The preceding four parameters displayed no significant differences between controls and patients (all $p > 0.05$).

Table 1 Baseline Features and Bivariate Correlation Analyses of Patients with Acute Intracerebral Hemorrhage

Variables	All Patients	Serum ACSL4 Levels		mRS Scores	
		ρ	P values	ρ	P values
Age (years)	63 (55–72)	0.072	0.202	0.168	0.003
Gender (male/female)	188/129	0.012	0.836	−0.105	0.063
Cigarette smoking	112 (35.3%)	0.106	0.061	−0.016	0.771
Alcohol drinking	120 (37.9%)	0.080	0.153	−0.054	0.339
Hypertension	211 (66.6%)	0.007	0.904	−0.047	0.406
Diabetes mellitus	57 (18.0%)	0.122	0.030	0.080	0.157
Dyslipidemia	115 (36.3%)	−0.004	0.944	0.003	0.953
Previous statin use	86 (27.1%)	0.051	0.364	0.090	0.111
Previous anticoagulant use	23 (7.3%)	0.021	0.716	−0.043	0.441
Previous antiplatelet use	43 (13.6%)	−0.029	0.602	−0.015	0.795
Admission time (h)	7.3 (4.6–12.3)	0.020	0.718	−0.097	0.086
Sampling time (h)	7.9 (5.3–12.9)	0.030	0.600	−0.086	0.125
Dysphasia	70 (22.1%)	0.256	<0.001	0.343	<0.001

(*Continued*)

Table 1 (Continued).

Variables	All Patients	Serum ACSL4 Levels		mRS Scores	
		ρ	P values	*ρ*	P values
Vomiting	85 (26.8%)	0.172	0.002	0.248	<0.001
Systolic arterial pressure (mmHg)	131 (120–145)	0.006	0.917	0.062	0.273
Diastolic arterial pressure (mmHg)	85 (78–94)	0.005	0.933	0.044	0.435
Hemorrhagic locations (superficial/deep)	86/231	−0.001	0.982	−0.017	0.758
Intraventricular expansion of hematoma	46 (14.5%)	0.197	<0.001	0.248	<0.001
Subarachnoidal expansion of hematoma	11 (3.5%)	−0.010	0.859	0.056	0.317
Stroke-associated pneumonia	80 (25.2%)	–	–	0.217	<0.001
NIHSS scores	9 (6–13)	0.628	<0.001	0.607	<0.001
Hematoma volume (mL)	14 (11–23)	0.614	<0.001	0.595	<0.001
Blood leucocyte count ($\times 10^9$/l)	7.6 (5.7–10.5)	0.104	0.065	0.140	0.012
Blood glucose levels (mmol/l)	10.3 (8.6–13.0)	0.167	0.003	0.190	0.001
Serum ACSL4 levels (ng/mL)	32.0 (26.4–45.5)	–	–	0.577	<0.001

Notes: Data were presented as frequencies (proportions), means ± standard deviations or medians (percentiles 25^{th}–75^{th}) as deemed applicable. Correlations were reported as *ρ* values by the Spearman test.
Abbreviations: NIHSS, National Institutes of Health Stroke Scale; mRS, modified Rankin Scale; ACSL4, Acyl-CoA synthetase long chain family member 4.

Alteration of Serum ACSL4 Levels and Its Relation to ICH Severity

In contrast to the controls, serum ACSL4 levels were significantly higher in the patient group ($p < 0.001$; Figure 2). Serum ACSL4 levels in patients were linearly related to NIHSS scores (p for nonlinear > 0.05; Supplementary Figure 1A) and hematoma volume (p for nonlinear > 0.05; Supplementary Figure 1B), and were strongly positively correlated with NIHSS scores ($p < 0.001$; Supplementary Figure 2A) and hematoma volume ($p < 0.001$; Supplementary Figure 2B). Patients were divided into four subgroups according to the NIHSS score or hematoma volume. Higher NIHSS scores or hematoma volumes were associated with higher serum ACSL4 levels (both $p < 0.001$; Supplementary Figure 3A and B). Moreover, other variables such as diabetes mellitus, dysphasia, vomiting, intraventricular expansion of hematoma, and blood glucose

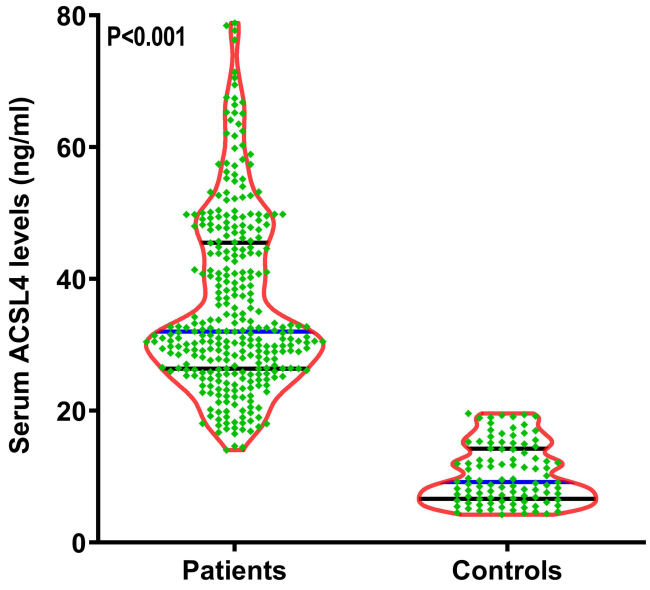

Figure 2 Alteration of serum ACSL4 levels post-intracerebral hemorrhage. Patients with acute intracerebral hemorrhage had substantially higher serum ACSL4 levels than the controls ($p < 0.001$).
Abbreviation: ACSL4, acyl-CoA synthetase long chain family member 4.

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levels were highly correlated with serum ACSL4 levels (all $p < 0.05$; Table 1). These factors, together with hematoma volume and NIHSS scores, were added to the multivariate model, and NIHSS scores (β = 1.149; 95% CI = 0.778–1.519; VIF = 2.610; $p = 0.001$) and hematoma volume (β = 0.362; 95% CI = 0.156–0.569; VIF = 2.647; $p = 0.003$) were independently correlated with serum ACSL4 level.

Serum ACSL4 Levels and Post-ICH 6-Month Neurological Conditions

Six-month mRS scores were linearly correlated with serum ACSL4 levels (p nonlinear > 0.05; Supplementary Figure 4) and positively correlated with these levels ($p < 0.001$; Supplementary Figure 5A). Moreover, patients were grouped into seven groups based on mRS; therefore, serum ACSL4 levels gradually increased in the order of scores from 0 to 6 ($p < 0.001$; Supplementary Figure 5B). In addition, serum ACSL4 levels, age, dysphagia, vomiting, intraventricular enlargement of hematoma, SAP, NIHSS scores, hematoma amount, blood leukocyte counts, and blood glucose levels were significantly associated with the mRS scores (all $p < 0.05$; Table 1). All of these factors were forced into the multifactorial model, and it was confirmed that serum ACSL4 levels (β = 0.045; 95% CI = 0.032–0.059; VIF = 2.050; $p = 0.001$), SAP (β = 0.053; 95% CI = 0.141–0.919; VIF = 1.215; $p = 0.008$), NIHSS score (β = 0.122; 95% CI = 0.077–0.168; VIF = 2.925; $p = 0.001$), and hematoma volume (β = 0.021; 95% CI = 0.008–0.034; VIF = 1.070; $p = 0.002$) remained independently correlated with mRS scores.

Serum ACSL4 levels were elevated in patients with poor prognosis compared to those with good prognosis ($p < 0.001$; Figure 3A). Alternatively, serum ACSL4 levels efficaciously anticipated the emergence of poor prognosis, and the Youden approach was employed to determine the optimal cutoff value of serum ACSL4 levels to distinguish risk of poor prognosis with medium-high sensitivity and specificity (Figure 3B). With the help of RCS, serum ACSL4 levels showed a linear relationship with the probability of a poor prognosis (p nonlinear > 0.05; Figure 4). Substantially different variables between patients with poor prognosis and those without such an event included age, diabetes mellitus, dysphagia, vomiting, intraventricular extension of hematoma, SAP, NIHSS scores, hematoma volume, blood glucose levels, and serum ACSL4 levels (all $p < 0.05$; Table 2). After integration of the aforementioned parameters into a multifactorial model, serum ACSL4 levels (OR = 1.075; 95% CI = 1.040–1.111; VIF = 2.125; $p = 0.008$), SAP (OR = 4.031; 95% CI = 1.943–8.367; VIF = 2.106; $p = 0.011$), NIHSS score (OR = 1.219; 95% CI = 1.122–1.323; VIF = 2.451; $p = 0.001$), and hematoma volume (OR = 1.116; 95% CI = 1.076–1.157; VIF = 1.782; $p = 0.001$) were independent predictors of poor prognosis. Using the Hosmer-Lemeshow test, the p-value was 0.398 and the Brier score was 0.216. E value for the sensitivity appraisal equaled to 1.3529 (95% CI, 1.2440–1.4567). Even after adjusting for demographic data, lifestyle habits, chronic diseases, medications, and time parameters, serum ACSL4 levels were highly associated with poor prognosis following ICH (all $p < 0.05$; Supplementary Table 1). This association was not moderated by age, sex, alcohol consumption, or other factors (all $p > 0.05$; Supplementary Table 2). In Figure 5, serum ACSL4 levels were in possession of analogous AUC, as compared to NIHSS scores and hematoma volumes, in terms of prognostic prediction of ICH (both $p > 0.05$). In addition, serum ACSL4 levels partially mediated the association between poor prognosis and NIHSS score (Supplementary Figure 6A) and hematoma volume (Supplementary Figure 6B).

Serum ACSL4 Levels and Post-ICH SAP

Patients with SAP had significantly enhanced serum ACSL4 levels compared with those without SAP ($p < 0.001$; Figure 6A). Additionally, SAP was effectively forecasted by serum ACSL4 levels and the appropriate threshold value of serum ACSL4 levels was selected by the Youden approach, for the sake of predicting SAP of ICH patients (Figure 6B). With the aid of RCS, a linear correlation was observed between serum ACSL4 levels and the likelihood of SAP (p nonlinear > 0.05; Figure 7). Marked disparate components between patients with SAP and those without were inclusive of age, dysphagia, vomiting, intraventricular extension of hematoma, NIHSS scores, hematoma volume, blood glucose levels, blood leukocyte counts, and serum ACSL4 levels (all $p < 0.05$; Table 2). After the preceding factors were entered into the multivariable model, the independent predictors of SAP included serum ACSL4 levels (OR = 1.041; 95% CI = 1.012–1.070; VIF = 1.954; $p = 0.005$), NIHSS score (OR = 1.208; 95% CI = 1.123–1.300; VIF = 2.005; $p = 0.001$), and hematoma volume (OR = 1.100; 95% CI = 1.059–1.142; VIF = 2.310; $p = 0.002$). In the Hosmer-Lemeshow test, the p value reached 0.327, and the Brier score was 0.229. For sensitivity analysis, E value reached 1.2476 (95% CI, 1.1222–1.3437). After correcting for age, sex, and tobacco

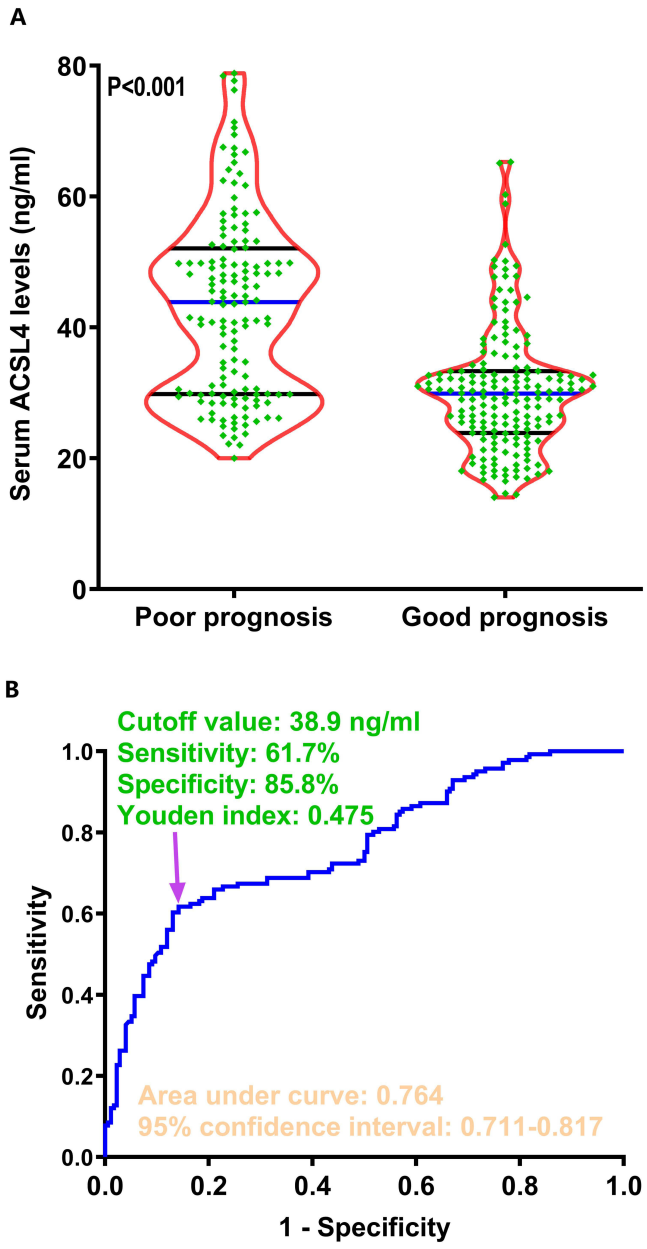

Figure 3 Serum ACSL4 levels by neurological outcome 6 months following acute intracerebral hemorrhage. In the context of the Mann–Whitney *U*-test, patients with poor prognosis displayed profoundly elevated serum ACSL4 levels ($p < 0.001$; (**A**). In the receiver operating characteristic curve analysis, serum ACSL4 levels showed a significant predictive ability for poor prognosis (**B**). Violet arrow points to cutoff value.
Abbreviation: ACSL4, acyl-CoA synthetase long chain family member 4.

smoking, a strong association was still observed between serum ACSL4 levels and SAP after ICH (all $p < 0.05$; Supplementary Table 1). Furthermore, such a link was negligibly moderated by age, sex, alcohol consumption, and so on (all p interactions > 0.05; Supplementary Table 2). As shown in Figure 8, with respect to SAP anticipation, the AUC of serum ACSL4 levels resembled those of the NIHSS scores and hematoma volumes (both $p > 0.05$). Concurrently, serum ACSL4 levels partially mediated the relationship between SAP and the NIHSS score (Supplementary Figure 7A) and hematoma volume (Supplementary Figure 7B). SAP also partially mediated the relationship between serum ACSL4 levels and mRS scores (Supplementary Figure 8A) as well as between serum ACSL4 levels and poor prognosis (Supplementary Figure 8B).

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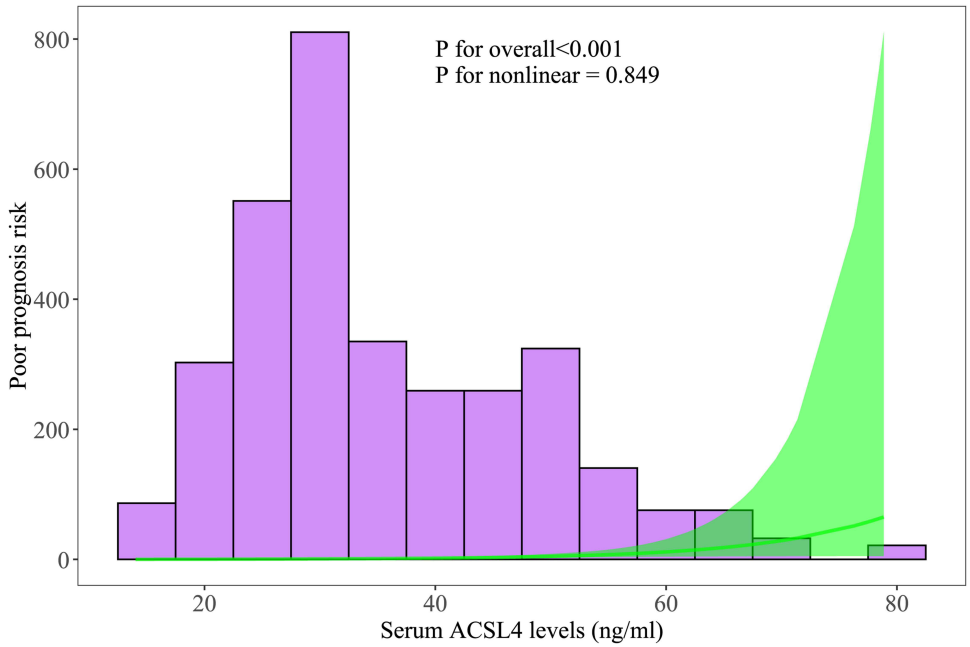

Figure 4 Linearity relationship between serum ACSL4 levels and possibility of poor prognosis at 6-months after intracerebral hemorrhage. Under a restricted cubic spline, serum ACSL4 levels were linearly related to the risk of poor prognosis after intracerebral hemorrhage (*p* for nonlinearity > 0.05).
Abbreviation: ACSL4, acyl-CoA synthetase long chain family member 4.

Discussion

To the best of our knowledge, this is the first investigational study to measure serum ACSL4 levels and to determine their prognostic relevance in human ICH. This study prompted us to obtain the following results: First, serum ACSL4 levels tended to increase significantly with ICH happening. Second, serum ACSL4 levels, under RCS, were linearly correlated

Table 2 Factors in Relevance to Stroke-Associated Pneumonia and Six-month Poor Prognosis After Acute Intracerebral Hemorrhage

Variables	Six-Month Neurological Outcome			Stroke-Associated Pneumonia		
	Poor Prognosis	Good Prognosis	P values	Presence	Absence	P values
Age (years)	64 (55–73)	62 (55–71)	0.034	68 (60–73)	62 (55–71)	0.028
Gender (male/female)	78/63	110/66	0.196	51/29	137/100	0.349
Cigarette smoking	49 (34.8%)	63 (35.8%)	0.847	33 (41.3%)	79 (33.3%)	0.200
Alcohol drinking	51 (36.2%)	69 (39.2%)	0.580	37 (46.3%)	83 (35.0%)	0.073
Hypertension	87 (61.7%)	124 (70.5%)	0.101	54 (67.5%)	157 (66.2%)	0.837
Diabetes mellitus	33 (23.4%)	24 (13.6%)	0.024	16 (20.0%)	41 (17.3%)	0.587
Dyslipidemia	50 (35.5%)	65 (36.9%)	0.787	29 (36.3%)	86 (36.3%)	0.995
Previous statin use	44 (31.2%)	42 (23.9%)	0.144	18 (22.5%)	68 (28.7%)	0.281
Previous anticoagulant use	10 (7.1%)	13 (7.4%)	0.920	6 (7.5%)	17 (7.2%)	0.922
Previous antiplatelet use	16 (11.3%)	27 (15.3%)	0.302	16 (20.0%)	27 (11.4%)	0.052
Admission time (h)	6.9 (4.8–10.2)	7.9 (4.3–12.8)	0.056	7.9 (5.4–12.5)	6.9 (4.4–11.9)	0.075
Sampling time (h)	7.4 (5.5–11.1)	8.4 (5.1–13.5)	0.113	8.8 (6.5–13.6)	7.5 (5.3–12.7)	0.054
Dysphasia	50 (35.5%)	20 (11.4%)	<0.001	34 (42.5%)	36 (15.2%)	<0.001
Vomiting	52 (36.9%)	33 (18.8%)	<0.001	37 (46.3%)	48 (20.3%)	<0.001
Systolic arterial pressure (mmHg)	132 (121–144)	131 (120–149)	0.804	131 (125–144)	132 (119–150)	0.657
Diastolic arterial pressure (mmHg)	85 (78–92)	85 (78–94)	0.934	85 (81–92)	85 (77–95)	0.954
Hemorrhagic locations (superficial/deep)	35/106	51/125	0.408	15/65	71/166	0.051
Intraventricular expansion of hematoma	32 (22.7%)	14 (8.0%)	<0.001	30 (37.5%)	16 (6.8%)	<0.001

(*Continued*)

Table 2 (Continued).

Variables	Six-Month Neurological Outcome			Stroke-Associated Pneumonia		
	Poor Prognosis	Good Prognosis	P values	Presence	Absence	P values
Subarachnoidal expansion of hematoma	5 (3.5%)	6 (3.4%)	0.947	3 (3.8%)	8 (3.4%)	0.555
Stroke-associated pneumonia	47 (33.3%)	33 (18.8%)	0.003	–	–	–
NIHSS scores	12 (9–15)	7 (5–10)	<0.001	13 (9–15)	8 (5–11)	<0.001
Hematoma volume (mL)	21 (14–26)	11 (8–17)	<0.001	23 (17–29)	12 (10–19)	<0.001
Blood leucocyte count ($\times 10^9$/l)	8.6 (5.4–11.0)	6.9 (5.9–9.2)	0.073	9.1 (5.8–11.8)	7.2 (5.7–9.7)	0.029
Blood glucose levels (mmol/l)	10.8 (8.8–15.3)	9.8 (8.5–11.5)	<0.001	11.1 (9.1–15.0)	10.0 (8.6–12.4)	0.037
Serum ACSL4 levels (ng/mL)	43.9 (29.9–52.0)	29.9 (23.9–33.3)	<0.001	47.3 (31.8–54.9)	30.5 (25.4–39.4)	<0.001

Notes: Data were reported as count (percentage), mean ± standard deviation or median (upper-lower quartiles) as designated appropriate. The Chi-square test, Fisher exact test, Student's *t*-test or Mann–Whitney test was used for data analyses.
Abbreviations: NIHSS, National Institutes of Health Stroke Scale; ACSL4, Acyl-CoA synthetase long chain family member 4.

with both NIHSS score and hematoma volume, as well as, as demonstrated by multivariate approach, were independently related to them. Third, the results of the regression analysis showed that serum ACSL4 levels were independently associated with poor prognosis and SAP and were robust with the aid of sensitivity, linearity, interaction, and multi-collinearity analyses. Finally, the spectrum of mediating effects of serum ACSL4 levels was confirmed in relation to ICH severity and clinical outcomes. Collectively, serum ACSL4 levels may be an acceptable alternative biomarker for severity appraisal and outcome prediction in ICH.

ICH is the leading cause of death among stroke patients.[29] Pathophysiological processes, mainly oxidative stress, inflammatory response, excitotoxicity, and apoptosis, contribute to brain injury secondary to ICH.[30] Lipid peroxidation

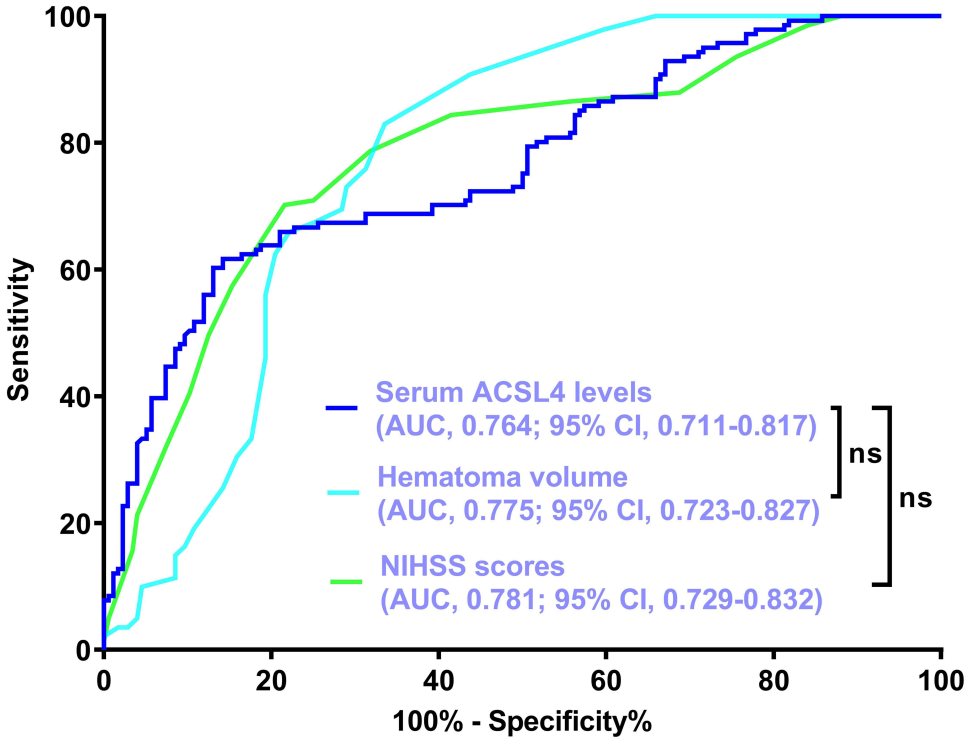

Figure 5 Capability of serum ACSL4 levels and other severity indices for forecasting poor prognosis following intracerebral hemorrhage. By plotting the receiver operating characteristic curve, the predictive ability of serum ACSL4 levels for poor prognosis was equivalent to that of NIHSS scores and hematoma volume in intracerebral hemorrhage (both $p > 0.05$).
Abbreviations: NIHSS, National Institutes of Health Stroke Scale; ACSL4, acyl-CoA synthetase long-chain family member 4; AUC, area under the curve; 95% CI, 95% confidence interval; ns, non-significant.

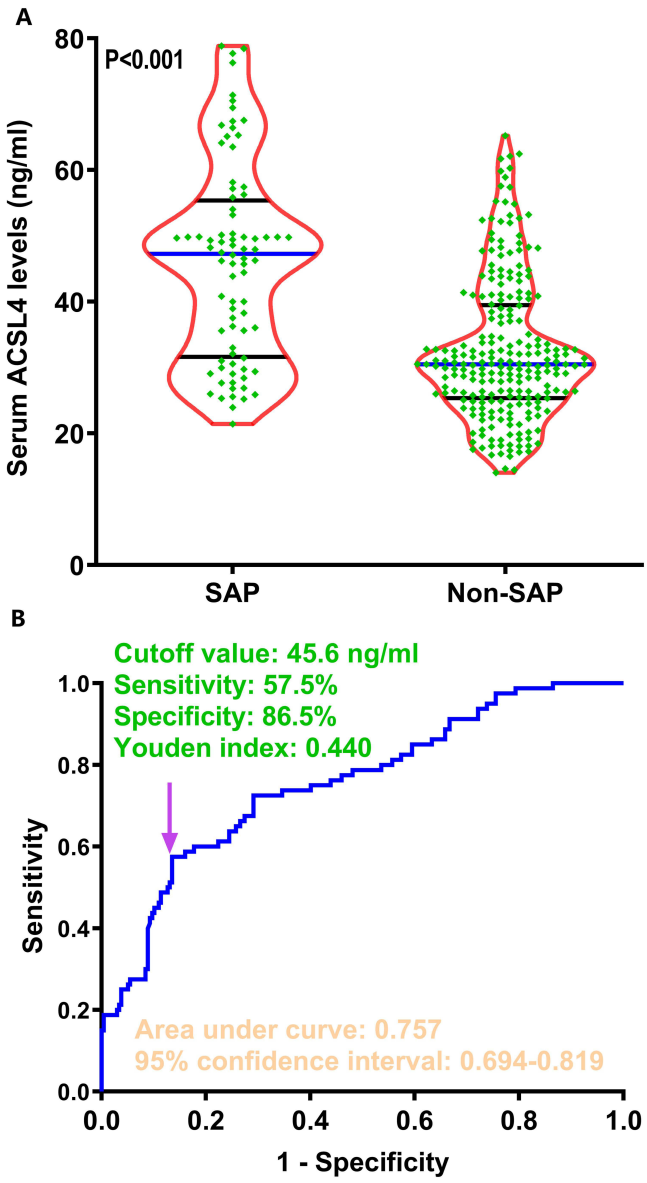

Figure 6 Serum ACSL4 levels by stroke-associated pneumonia following intracerebral hemorrhage. Serum ACSL4 levels were markedly higher in patients with stroke-associated pneumonia than in those without ($p < 0.001$; (**A**). Stroke-associated pneumonia is effectively induced by serum ACSL4 levels in intracerebral hemorrhage patients (**B**). Violet arrow points to cutoff value.

Abbreviations: SAP, stroke-associated pneumonia; ACSL4, acyl-CoA synthetase long-chain family member 4.

typifies ferroptosis in ICH as well.[31,32] ACSL4 is a pivotal enzyme involved in ferroptosis lipid peroxidation.[33] Polyunsaturated fatty acids are abundant in brain tissues, thereby facilitating the involvement of ACSCL4 in brain injury.[34] In fact, ACSL4 expression by neurons and glial cells is enhanced in response to acute stimuli, and ACSL4 can elicit neuroinflammation and ferroptosis in response to harmful insults, leading to the notion that ACSL4 may be a potential therapeutic target for acute brain injury diseases.[34–37] In the current study, serum ACSL4 levels were significantly higher in patients with ICH than in the controls. In conjunction with the abundance of ACSL4 in brain tissues,[34–37] ACSL4 may be released into the peripheral system from the central nervous system via a disrupted blood–brain barrier, thereby causing an elevation in circulating levels of ACSL4 subsequent to acute ICH. Nevertheless, ACSL4 can be expressed by other peripheral cells such as macrophages, endothelial cells, and alveolar cells.[38–40] Systemic injury, which is represented by the systemic inflammatory response syndrome, is ubiquitous in ICH.[41,42] Accordingly, it is possible that ACSL4 is secreted by the peripheral cells. However, it is necessary to measure ACSL4 mRNA expression to determine the origin of ACSL4.

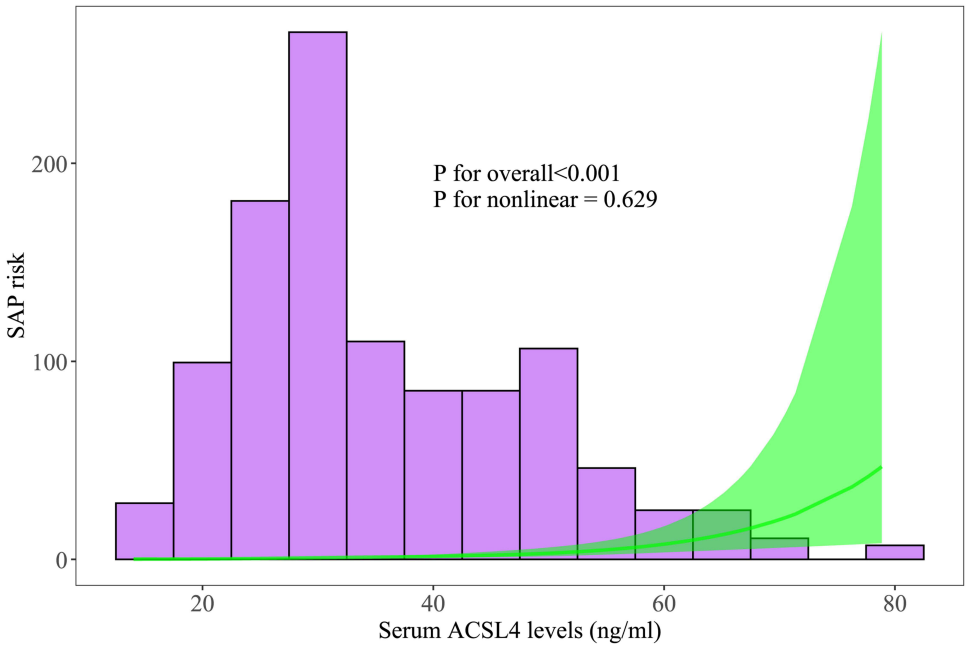

Figure 7 Linearity relationship between serum ACSL4 levels and likelihood of stroke-associated pneumonia after intracerebral hemorrhage. In the context of restricted cubic spline analysis, serum ACSL4 levels were linearly correlated with the risk of stroke-associated pneumonia following intracerebral hemorrhage (*p* for nonlinear > 0.05).
Abbreviations: SAP, stroke-associated pneumonia; ACSL4, acyl-CoA synthetase long-chain family member 4.

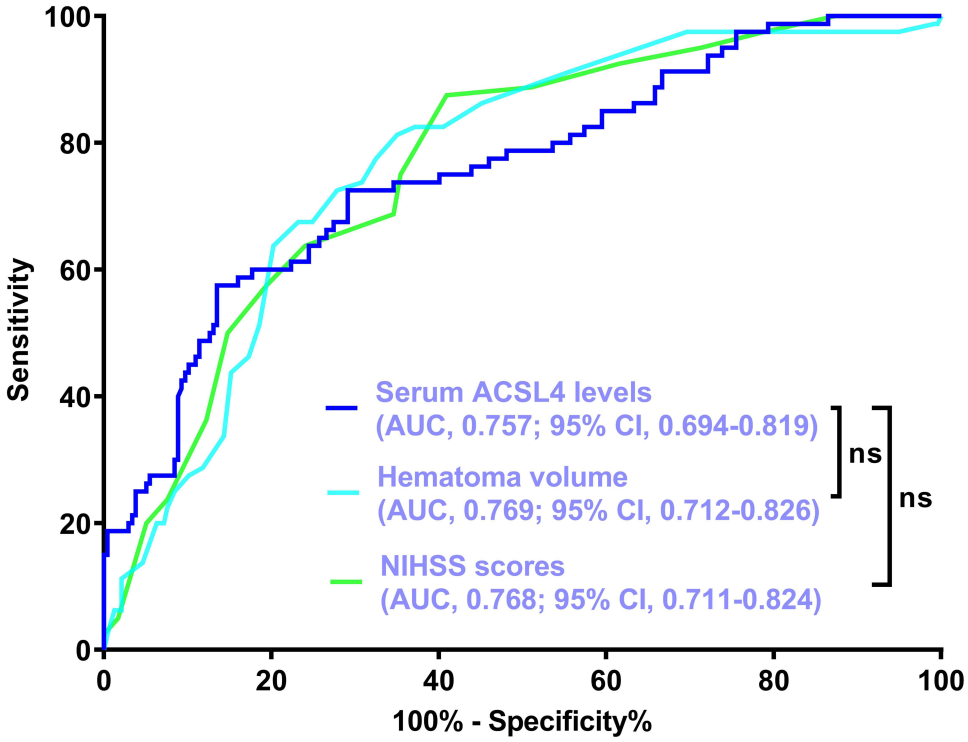

Figure 8 Capability of serum ACSL4 levels and other severity metrics for anticipating stroke-associated pneumonia after intracerebral hemorrhage. By drawing a receiver operating characteristic curve, the predictive ability of serum ACSL4 levels for stroke-associated pneumonia was analogous to that of the NIHSS scores and hematoma volume after intracerebral hemorrhage (both $p > 0.05$).
Abbreviations: SAP, stroke-associated pneumonia; NIHSS, National Institutes of Health Stroke Scale; ACSL4, acyl-CoA synthetase long-chain family member 4; AUC, area under the curve; 95% CI, 95% confidence interval; ns, non-significant.

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Ferroptosis is believed to participate in brain damage and lung injury, and, consequently, ACSL4 is involved in pathophysiological processes.[16,43,44] Its detailed mechanisms include iron overload, excessive lipid peroxidation, redundant oxidative stress, worse inflammatory response, superfluous excitotoxicity, and increased apoptosis, resulting in brain or lung edema, and even cellular demise.[16,43,44] In this study, serum ACSL4 partially explained the association of NIHSS score and hematoma volume with SAP and poor prognosis, and SAP had a partial interpretation of the relationship between serum ACSL4 and poor prognosis, indicating that ACSL4 may be implicated in secondary brain injury following ICH, and also provides insight into the notion that serum ACSL4 may be a potential prognostic biomarker of ICH.

In 99 patients experiencing lower limb neurological sequelae following ischemic stroke, poorer recovery of motor function was more likely to be observed in those with higher serum ACSL4 levels.[19] Consistently, serum ACSL4 levels in 210 patients were independently associated with poor 30-day postoperative prognosis following aneurysmal subarachnoid hemorrhage.[20] Compared with the above two clinical studies, our study had the following characteristics: (1) the sample size, to some extent, was enlarged to 317 patients; (2) the follow-up duration was lengthened to 6 months following stroke; (3) the severity correlation was verified; and (4) another interesting outcome variable, SAP, was supplemented. In this study, serum ACSL4 levels in patients with ICH were independently correlated with NIHSS scores and hematoma volume, and serum ACSL4 was an independent predictor of SAP and poor prognosis at 6-months following ICH. Furthermore, sensitivity analysis, multicollinearity analysis, subgroup analysis, linearity correlation analysis, and regression analysis showed that results were robust. In addition, serum ACSL4 had a comparable predictive capability for SAP and poor prognosis as opposed to NIHSS scores and hematoma volume in this cohort of patients with ICH. Taken together, serum ACSL4 level may emerge as an appealing prognostic metric for ICH.

This study has several limitations. First, although a medium sample size of 317 patients was analyzed, the subsequent generalization of serum ACSL4 as a prognostic biomarker of ICH necessitates the implementation of a larger and multicenter cohort study. Second, patients with supratentorial intraparenchymal hemorrhage were enrolled in this clinical study. However, whether the prognostic role of serum ACSL4 is suitable for ICH at other sites should be further analyzed. Third, serum ACSL4 levels at a median time of 7.9 hours following ICH were measured in the current study, while its dynamic evolution is uninvestigated; future studies are needed to measure serum ACSL4 levels at different time points to compare their prognostic predictive ability in the scenario of ICH and thereby to determine an optimal time point. Fourth, in terms of statistical analysis, mediation analyses should be interpreted cautiously in consideration of the observational design; moreover, although sensitivity analysis was leveraged in this study, potential residual confounding should be acknowledged. Finally, given that clinical studies can not provide some mechanistic information; so, it is necessary to conduct animal and cell experiments to clarify specific mechanisms of ACSL4's involvement in secondary brain injury after ICH and SAP.

Conclusions

Serum ACSL4 levels sharply increase in response to acute ICH. Serum ACSL4 levels, which are closely related to NIHSS scores coupled with hematoma volume, are independently predictive of SAP and poor neurological conditions 6 months post-ICH, together with acceptable predictive effectiveness. Moreover, serum ACSL4 levels could partially navigate the links between bleeding intensity, SAP, and worse neurological status. Presumably, serum ACSL4 levels may be utilized as adjunctive biomarkers for severity appraisal and prognosis prediction of ICH.

Data Sharing Statement

The datasets generated and/or analyzed during the current study are not publicly available because of the inclusion of personal data but can be obtained from the corresponding author upon reasonable request.

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Disclosure

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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