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The Role of Fibrinogen in Mediating NGAL-Associated Neuronal Damage in Acute Ischemic Stroke: A Moderated Mediation Analysis

Nan Zhao[1,*], Yi Chen[1,*], Zhongjiao Lu[1,*], Lu Han[1], Yaying Song[1], Jie Ding[1], Desheng Zhu[1], Yangtai Guan[1,2]

[1]Department of Neurology, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, 200127, People's Republic of China; [2]Department of Neurology, Punan Hospital, Pudong New District, Shanghai, 200125, People's Republic of China

*These authors contributed equally to this work

Correspondence: Yangtai Guan; Desheng Zhu, Department of Neurology, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, No. 160, Pujian Road, Shanghai, 200127, People's Republic of China, Tel +86-13386271865; +86-13564719779, Fax +86-21-68383482, Email guanyangtai@renji.com; deshengzhu2008@sina.com

Background: Both neutrophil gelatinase-associated lipocalin (NGAL) and fibrinogen are involved in the inflammation in acute ischemic stroke (AIS), but the interaction among them is unknown. Clarifying this issue will contribute to a better understanding of the mechanisms of injury in AIS. This study aimed to explore the association between NGAL, fibrinogen and neuronal damage in AIS.

Methods: This study is a cross-section study. One hundred ninety-six successively hospitalized AIS patients in Renji Hospital in China between January 1, 2023, and May 31, 2023, were included. Circulating NGAL and fibrinogen were measured, and neuron-specific enolase (NSE) was detected to evaluate central neuronal damage. All data were analyzed by linear curve fitting analysis, multiple linear regression analysis, and moderated mediation analysis, respectively.

Results: There are linear relationships between log2-transformed NGAL, NSE, and fibrinogen, respectively. The β (95%) for the positive association between log2-transformed NGAL and NSE was 2.24 (1.15–3.32, $p < 0.01$), between log2-transformed NGAL and fibrinogen was 0.37 (0.19–0.56, $p < 0.01$), and between fibrinogen and NSE was 1.16 (0.48–1.85, $p < 0.01$) adjusting for potential confounders. These associations remained consistent in sensitivity analysis and hierarchical analysis. Increased fibrinogen significantly ($p < 0.01$) mediated 14.28% of log2-transformed NGAL-associated increased NSE risk.

Conclusion: The NGAL levels were associated with NSE, and the NGAL-associated neuronal damage might be partially mediated by fibrinogen, suggesting that the inflammatory response among NGAL, fibrinogen, and NSE should be intervened to reduce neuronal damage after ischemic stroke. Further animal experiments are needed to clarify their specific mechanisms and precise relationships.

Keywords: acute ischemic stroke, NGAL, fibrinogen, neuron-specific enolase, inflammation, mediation analysis

Introduction

Acute ischemic stroke (AIS) is characterized by high incidence, high risk of recurrence, and high mortality and disability rates worldwide, with a recurrence rate of up to 25–30% even with antiplatelet and statin drugs for secondary prevention.[1,2] After cerebral infarction, rapid hypoxic damage occurs in the brain tissue in the core area of infarction, followed by neuroinflammation, and sequential tissue degeneration in the surrounding area.[3] Subsequently, some pro-inflammatory cytokines enter the blood circulation via destructed BBB, causing inflammation in the vascular endothelium throughout the body.[4] The systemic inflammation after stroke has become a more feasible target for therapeutic intervention.[5,6]

Like many other inflammation-related proteins, the level of neutrophil gelatinase-associated lipid carrier protein (NGAL) in the plasma changes after AIS. NGAL is originally discovered in activated neutrophils. It is a small-molecular -weight secreted protein that mediates inflammation in the acute phase.[6] Studies have shown that NGAL in brain tissue

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and plasma increases after AIS, and can participate in the pro-inflammatory activation of glial cells and aggravate nerve injury.[7] Clinical studies have shown that higher plasma NGAL levels are associated with worse disease prognosis.[8] In preclinical studies, reducing NGAL levels can help middle cerebral artery occlusion (MCAO) mice achieve better prognosis.[9] NGAL can not only be produced by glial cells in central nervous system (CNS) but also from the peripheral circulation.[9,10] Activated neutrophils in peripheral circulation might play a role in the increase of NGAL after AIS.

Another protein associated with activated neutrophils in peripheral circulation is fibrinogen. After AIS, pro-inflammatory signals, such as immune mediators, can recruit peripheral circulating leukocytes, which have a potentially critical role in the process of neuronal injury after stroke.[11,12] Fibrinogen not only affects blood viscosity and platelet activation but also participates in the pathophysiological process of inflammation and neutrophil activation.[13,14] Both circulating leukocytes and fibrinogen can infiltrate into ischemic lesions through the damaged BBB to exacerbate the inflammatory response after stroke.[15] Clinical studies have found that the levels of circulating white blood cells and fibrinogen in AIS patients are higher than those in the normal population. Similarly, increased plasma fibrinogen levels have also been observed in rat models with middle cerebral artery occlusion.[16] Epidemiological studies have shown that fibrinogen levels gradually increase in the first 24 hours after cerebral infarction, and elevated fibrinogen levels at admission are associated with poor prognosis in patients with AIS.[17–19]

Different from fibrinogen, NSE, which is a reliable nerve injury marker,[20,21] can flow from ischemic brain lesions into the peripheral circulation through the damaged BBB after AIS. Considering the different sources of NGAL, fibrinogen, and NSE, clarifying the relationship and mechanism of the three after AIS may help to unravel the link between CNS injury and systemic inflammation after AIS, identify potential inflammatory regulatory targets, and provide more ways to reduce inflammation response and neuronal damage after ischemic stroke. However, the interaction among circulating NGAL, fibrinogen, and NSE after ischemic stroke is unclear. Thus, the present study constructs a moderated mediation model aimed to examine whether NGAL is associated with neuronal damage and mediated by fibrinogen in AIS.

Subjects and Methods

Ethics

In accordance with the Declaration of Helsinki, this study was approved by the ethics committee of Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital, Shanghai, China. All study subjects or their immediate family members (Patients with consciousness disorder or dysarthria after AIS) provided informed consent prior to sample collection.

Design

The study was performed in a cross-sectional design aimed to explore the association between NGAL and NSE and moderation by fibrinogen in AIS patients. Consecutive AIS patients were enrolled in this study from Renji Hospital in China during January 1, 2023, and May 31, 2023. In the hospital's Stroke Registry Database, patient data were recorded.

Study Subjects

AIS patients were diagnosed according to the World Health Organization criteria.[22] The inclusion criteria were as follows: (1) AIS onset within 24 hours, (2) clinical symptoms and signs can be evaluated, (3) confirmed by brain imaging [computed tomography (CT) or magnetic resonance imaging (MRI)], and (4) aged 18 years or older.

The following exclusion criteria were employed: (1) transient ischemic attack, (2) intracerebral hemorrhage, (3) malignancies, (4) primary thrombocytopenia, megaloblastic anemia, post-splenectomy, leukemia, giant platelet syndrome, and aplastic anemia, (5) cardiac valvulopathy and acute myocardial infarction, and (6) clinical and laboratory data were not available for analysis, including unintegrated patient data.

Clinical Characteristics and Laboratory Data

All patients' medical records and sample data were kept in our hospital as described in our previous study.[15] The baseline data for demographic characteristics, medical history (ischemic stroke, hypertension, diabetes, atrial fibrillation, cardiac

insufficiency, and pneumonia), and drugs used before admission (antiplatelet drugs, anticoagulant drugs, lipid lowering drugs, antidiabetic drugs, and antihypertensive drugs) were collected in detail by interview with patients and their family members upon admission.

Fasting venous blood samples were obtained within 1 hour after admission and before administration of therapy, including intravenous recombinant tissue type plasminogen activator (rt-PA) and any angioplasty procedure in the emergency room. These blood samples are collected continuously based on the patient's visit to the hospital. Blood sample was collected into an EDTA-containing vacuum tube to assess levels of fibrinogen, which were measured with a commercially available fibrinogen kit (semi-automatic coagulation instrument) purchased from the Biotechnology Co., Ltd (Shanghai, China). The intra-assay and inter-assay coefficients of variation were 2.3% and 5.34%, respectively, while the detection limits ranged from 0.39 to 25.0 g/L for fibrinogen, and the normal reference range of fibrinogen ranged between 2 g/L and 4 g/L. Levels of NSE were assessed using commercially available quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kits purchased from R&D Systems (Shanghai, China). The intra-assay and inter-assay coefficients of variation were b = 3% and b = 7%, respectively. The minimum effective detection limit was 0.229 ng/mL, and the detection values ranged from 0.625 to 40 ng/mL for NSE, and the normal NSE value in our laboratory was <16.3 mg/L.

Plasma samples anticoagulated with EDTA were taken for NGAL detection, and NGAL levels were detected using the commercially available "Neutrophil Gelatinase-Associated Lipocalin Detection Kit (Latex-enhanced Immunoturbidimetry)" [Shanghai Machinery Injection 20192400099] purchased from Reigncom Biotech (Shanghai, China). The linear detection range of NGAL was 50–5000 ng/mL, the normal reference range was <180 ng/mL, the coefficient of variation within the assay was ≤7.0%, and the coefficient of variation between assays was ≤10.0%.

Fasting blood samples were collected by venipunctures to measure routine blood examination (neutrophil count and platelet count), serum biochemical indexes [levels of alkaline phosphatase (ALP), direct bilirubin, albumin/globulin (A/G) ratio, blood urea nitrogen (BUN), creatinine, uric acid, the estimated glomerular filtration rate calculated using the modification of diet in renal disease' equation (eGFR-MDRD), fasting blood sugar, BNP, homocysteine, cystatin C, and interleukin 6 (IL-6)], blood lipids [levels of low-density lipoprotein cholesterol (LDL-C), and small and dense low-density lipoprotein (sdLDL)]. All determinations were performed by laboratory technicians blinded to all clinical data.

Groups

All included AIS patients were grouped by two criteria. First, AIS patients were grouped by clinical normal reference values of NGAL, NSE, and fibrinogen in baseline characteristic analysis. High NGAL and high NSE were identified when their value was greater than or equal to 180 ng/mL and 16.3 ng/mL, respectively. High fibrinogen was identified when its value was greater than 4 g/L. In addition, AIS patients were categorized into the T1 (low), T2 (middle), and T3 (high) groups according to the log2 transformed NGAL and fibrinogen tertile levels, respectively, in descriptive analysis and multivariate linear regression analysis. Second, AIS patients were organized according to the normal reference values of laboratory indicators in the hierarchical analysis, such as eGFR-MDRD (≥90 mL/min). While dates of albumin/globulin ratio, BUN, BNP, creatinine, cystatin C, direct bilirubin, homocysteine, IL-6, and sdLDL were categorized into low and high groups by dichotomization according to statistical method.

Statistical Analysis

The characteristics of study participants at baseline are presented by NGAL level. Categorical variables expressed as n (%) were analyzed using x^2 and Fisher's exact tests. Continuous variables were presented as means with standard deviations (SD) for normal distribution data, which were analyzed by *t* tests, and they were expressed as median (interquartile range, IQR) for abnormal distribution data, which were analyzed by Mann–Whitney *U*-tests. NGAL levels were abnormal distribution and log2-transformed for analyses. The association among log2 transformed NGAL, fibrinogen, and NSE were assessed by linear curve fitting analyses (generalized additive models) and multivariate linear regression analysis. Age and gender were included in the multivariate models as conventional adjustment factors, and baseline variables considered clinically relevant to NGAL and NSE or that showed a univariate relationship with high fibrinogen or high NSE were selected into a multivariate linear regression model. Multicollinearity was tested using

Variance Inflation Factor (VIF), and a VIF less than 10 was indicative of no collinearity. Both non-adjusted and adjusted models were used, and stratified analyses and interaction testing were performed. Sensitivity analyses using linear regression models were performed among patients with non-cardiac insufficiency, non-pneumonia, and eGFR-MDRD≥90. This study examined the proportion of mediation through fibrinogen in the associations of log2 transformed NGAL levels and NSE using the Process SPSS macro tool based on the mediation method recommended by Hayes. Statistical analyses were performed using the Statistical Package for the Social Sciences Software (SPSS) (version 24.0, Chicago, IL, USA) and R (version 3.6.3). The statistical significance level was set at a two-tailed *p*-value of <0.05.[23]

Results

Baseline Characteristics

At the time of the final survey on May 31, 2023, a total of 270 consecutive AIS candidates were recruited for the study. Among these AIS candidates, patients who had met any exclusion criteria were excluded (n = 22), and patients who had missing data related to fibrinogen, NGAL, and NSE were also excluded from the eligible candidates for the study (n = 35). Those with implausible values of NGAL (<20 ng/mL) (n = 6) and those with unreliable values of fibrinogen (<0.5 g/L) (n = 11) were also excluded from the eligible candidates. As a result, a total of 196 AIS subjects were included in the final analyses. There were 118 patients (60.2%) with anterior circulation infarction and 81 patients (41.33%) with large-artery atherosclerosis in this study. According to the clinically normal range of NGAL, 196 AIS subjects were categorized into normal (n = 116) and high (80) NGAL groups. A flowchart of the study is shown in Figure 1.

Among the 196 studies of AIS subjects, women accounted for 31.12% (n = 61) and men for 68.88% (n = 135). The age of the enrolled subjects ranged from 39 to 91 yr (women, 39–91 yr; men, 41–91 yr) with a mean age of 68.42 ± 10.64 yr (women, 70.11 ± 11.23 yr; men, 67.67 ± 10.32 yr). The disease duration before admission ranged from 1.0 to 28 days

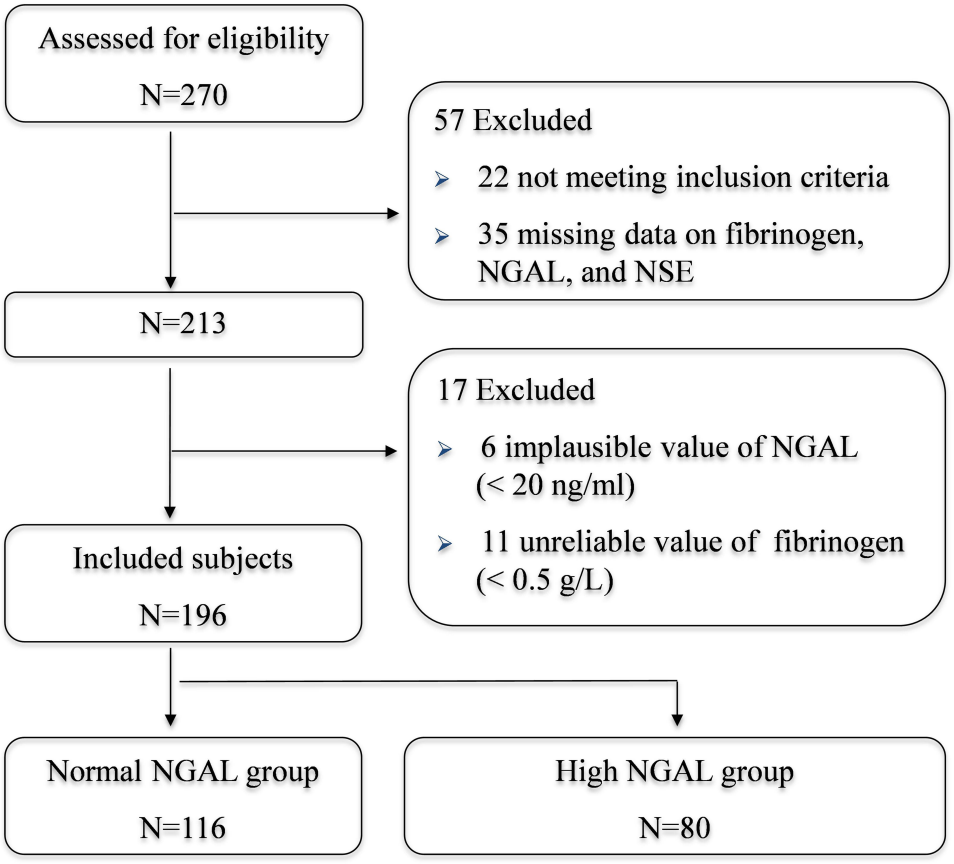

Figure 1 A flowchart of the study.

with a median and interquartile range of 4.0 (3.0–7.0) days. The NSE ranged from 6.22 to 29.50 ng/mL, with a mean level of 14.13 ± 4.46 ng/mL. The fibrinogen ranged from 1.66 to 7.40 g/L, with a mean level of 3.37 ± 0.96 g/L. The baseline characteristics of the included patients are shown in Table 1 and Supplement Tables 1, and 2.

The Relationship Between log2 Transformed NGAL and NSE

Between the normal and high NGAL groups, the mean level of NSE was higher in the high NGAL group than that in the normal NGAL group (15.89 ± 4.63 ng/mL vs 12.91 ± 3.93 ng/mL, $p < 0.001$). In all included AIS patients, the mean serum NSE levels

Table 1 Baseline Characteristics of Participants by NGAL Level

Index	Normal NGAL group (N=116)	High NGAL group (N=80)	Standardize diff	*P*-value
Basic information				
Sex (men) (%)	79 (68.10)	56 (70.00)	0.04 (−0.24, 0.33)	0.778
Age (years)	67.28±10.02	70.09±11.34	0.26 (−0.02, 0.55)	0.070
Disease duration (days)	4.00 (3.00–6.25)	4.00 (3.00–7.25)	0.06 (−0.22, 0.35)	0.411
NIHSS	2.00 (1.00–4.00)	5.00 (2.00–9.25)	0.91 (0.61, 1.21)	<0.001
OCSP-TACI+PACI (%)	72 (62.07)	46 (57.50)	0.09 (−0.19, 0.38)	0.521
TOAST-LAA (%)	50 (43.10)	31 (38.75)	0.09 (−0.20, 0.37)	0.543
Medical history				
Ischemic stroke (%)	43 (37.07)	29 (36.25)	0.02 (−0.27, 0.30)	0.907
Hypertension (%)	84 (72.41)	53 (66.25)	0.13 (−0.15, 0.42)	0.355
Diabetes (%)	51 (43.97)	25 (31.25)	0.26 (−0.02, 0.55)	0.073
Atrial fibrillation (%)	14 (12.07)	17 (21.25)	0.25 (−0.04, 0.53)	0.083
Cardiac insufficiency (%)	4 (3.45)	13 (16.25)	0.44 (0.15, 0.73)	0.002
Pneumonia (%)	14 (12.07)	23 (28.75)	0.42 (0.14, 0.71)	0.003
Laboratory findings				
Neutrophil count (10^9/L)	3.51±1.16	5.22±1.90	1.09 (0.79, 1.40)	<0.001
Platelet (10^9/L)	217.00±63.60	230.96±60.34	0.23 (−0.06, 0.51)	0.125
ALP (U/L)	75.88±19.43	83.15±22.03	0.35 (0.06, 0.64)	0.016
Direct bilirubin (μmol/l)	4.48±1.96	4.77±2.31	0.13 (−0.15, 0.42)	0.350
A/G ratio	1.67±0.29	1.54±0.31	0.43 (0.14, 0.72)	0.003
BUN (mmol/L)	5.03±1.33	5.57±2.04	0.31 (0.03, 0.60)	0.026
Creatinine (μmol/L)	65.00 (54.00–76.00)	68.00 (56.75–80.50)	0.34 (0.05, 0.63)	0.012
Uric acid (μmol/L)	320.67±76.93	314.35±88.13	0.08 (−0.21, 0.36)	0.595
EGFR-MDRD (mL/min)	105.47±30.47	94.54±34.66	0.33 (0.05, 0.62)	0.021
Fasting glucose (mmol/L)	6.31±2.04	6.11±2.40	0.09 (−0.20, 0.37)	0.537
BNP (pg/mL)	38.50 (19.50–70.50)	60.00 (24.00–140.50)	0.019	0.019

(*Continued*)

Table 1 (Continued).

Index	Normal NGAL group (N=116)	High NGAL group (N=80)	Standardize diff	*P*-value
LDL-C (mmol/L)	2.82±0.98	2.63±0.97	0.20 (−0.09, 0.49)	0.171
SdLDL (mmol/L)	0.56 (0.39–0.81)	0.41 (0.30–0.65)	0.33 (0.05, 0.62)	0.007
Hemocyanin (μmol/L)	12.25 (10.17–14.88)	14.05 (11.00–18.35)	0.41 (0.12, 0.70)	0.007
Cystatin C (mg/L)	1.09±0.21	1.31±0.38	0.71 (0.42, 1.01)	<0.001
IL-6 (pg/mL)	5.54 (4.00–8.16)	9.20 (5.89–22.39)	0.60 (0.30, 0.89)	<0.001
Fibrinogen (g/L)	3.04±0.72	3.85±1.08	0.89 (0.59, 1.19)	<0.001
NGAL (ng/mL)	133.09±27.68	279.75±105.54	1.90 (1.56, 2.24)	<0.001
Log2 transformed NGAL (ng/mL)	7.02±0.32	8.04±0.48	2.52 (2.14, 2.90)	<0.001
NSE (ng/mL)	12.91±3.93	15.89±4.63	0.69 (0.40, 0.99)	<0.001
Medication use before admission				
Antiplatelet drugs (%)	110 (94.83)	70 (87.50)	0.26 (−0.03, 0.55)	0.066
Anticoagulant drugs (%)	16 (13.79)	24 (30.00)	0.40 (0.11, 0.69)	0.006
Lipid lowering drugs (%)	114 (98.28)	79 (98.75)	0.04 (−0.25, 0.32)	0.790
Antidiabetic drugs (%)	48 (41.38)	28 (35.00)	0.13 (−0.15, 0.42)	0.368
Antihypertensive drugs (%)	75 (64.66)	54 (67.50)	0.06 (−0.22, 0.35)	0.680

were 12.54 ± 3.91 ng/mL, 13.88 ± 4.34 ng/mL, and 15.83 ± 4.53 ng/mL in the low (T1), middle (T2), and high (T3) log2 transformed NGAL tertiles for all patients (Figure 2A), respectively, and there was a significant difference among the three groups ($p < 0.01$). The Pearson's correlation coefficient (95%) for the relationship between log2 transformed NGALs and NSE was 0.376 (0.249–0.491, $p < 0.001$) in all AIS patients. Smoothed plots (Figure 2B) showed linear relationships between log2 transformed NGAL and NSE after adjusting for sex, age, creatinine, cystatin C, eGFR-MDRD, sdLDL, IL-6, and BNP ($F = 16.424$, $p < 0.001$).

In multiple linear regression analyses of the relationship between log2 transformed NGAL and NSE, baseline variables that were considered relevant to NSE and log2 transformed NGAL by difference analysis or previous references or that showed a univariate relationship with NSE were selected into multivariate linear regression model (Supplement Table 3). As a result, sex, age, creatinine, cystatin C, eGFR-MDRD, sdLDL, IL-6, and BNP were regarded as confounding factors related to NSE. In multiple linear regression analysis, the β (95% CI) for the relationship between log2 transformed NGAL and NSE were 2.64 (1.73–3.56, $p < 0.001$) and 2.24 (1.15–3.32, $p < 0.001$) in all patients before and after adjusting for sex, age, creatinine, cystatin C, eGFR-MDRD, sdLDL, IL-6, and BNP. After adjusted confounder factors, the β (95% CI) for the relationship between log2 transformed NGAL and NSE showed a grade increase according to the log2 transformed NGAL tertile by trend analysis ($p < 0.001$) (Table 2), which showed a statistical significance. Both sensitivity analysis and hierarchical analysis (according to sex, age, creatinine, cystatin C, eGFR-MDRD, sdLDL, IL-6, and BNP) also showed that the association between log2 transformed NGAL and NSE was statistically significant (Figure 2C, Supplement Tables 4 and 5).

The Relationship Between log2 Transformed NGAL and Fibrinogen

Between the normal and high NGAL groups, the mean level of fibrinogen was higher in the high NGAL group than that in the normal NGAL group (3.85 ± 1.08 ng/mL vs 3.04 ± 0.72 ng/mL, $p < 0.001$). In all included AIS patients, the mean

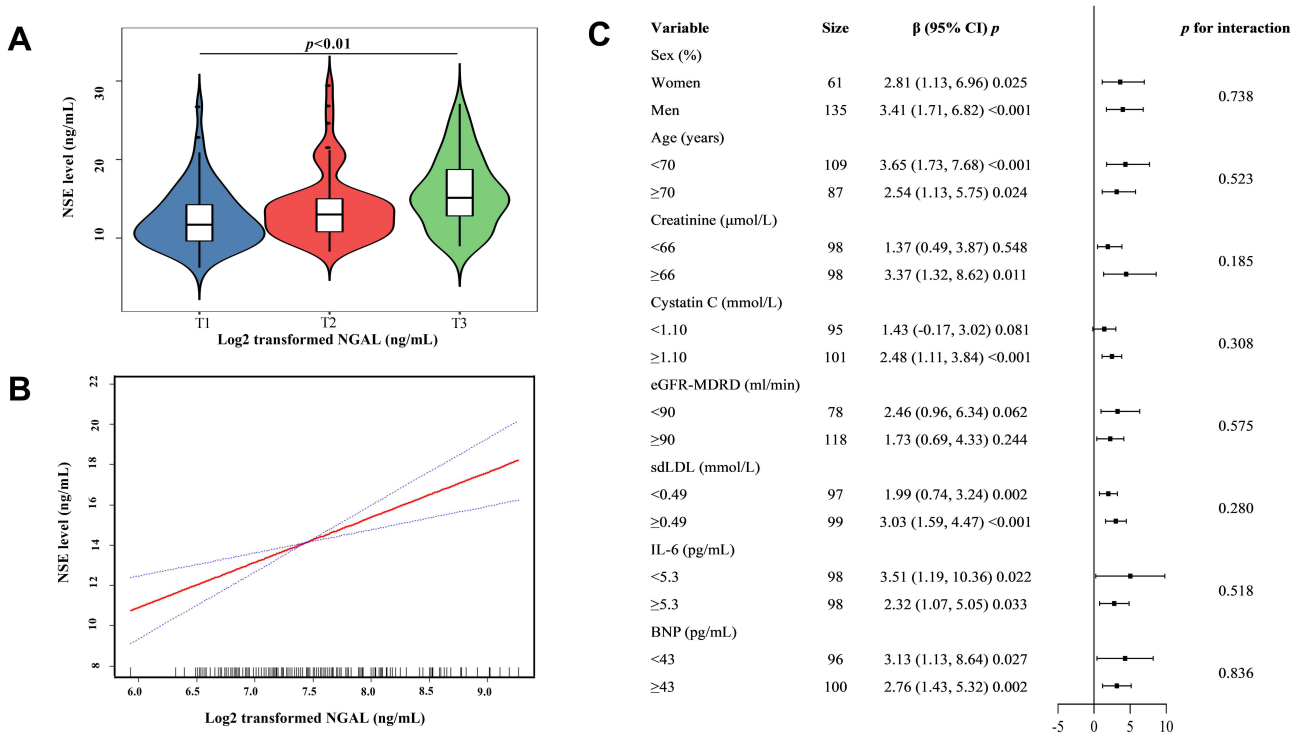

Figure 2 The relationship between NGAL and NSE. (**A**) The mean serum NSE levels showed a grade increase according to the log2 transformed NGAL tertile's levels in all AIS patients ($p < 0.01$). (**B**) A linear relationship between log2 transformed NGAL and NSE after adjusting for confounder factors. Solid lines represent the fitting curve and dotted lines represent the corresponding 95% CI. (**C**) Hierarchical analysis on the relationship of log2 transformed NGAL and NSE.

serum fibrinogen levels were 3.07 ± 0.78 ng/mL, 3.09 ± 0.74 ng/mL, and 3.93 ± 1.08 ng/mL in the low (T1), middle (T2), and high (T3) log2 transformed NGAL tertiles for all patients (Figure 3A), respectively, and there was a significant difference among the three groups ($p < 0.01$). The Pearson's correlation coefficient (95%) for the relationship between log2 transformed NGALs and fibrinogen was 0.406 (0.282–0.517, $p < 0.001$) in all AIS patients. Smoothed plots (Figure 3B) showed linear relationships between log2 transformed NGAL and fibrinogen after adjusting for sex, age, direct bilirubin, albumin/globulin ratio, BUN, cardiac insufficiency, and anticoagulant drugs ($F = 7.755$, $p < 0.001$).

In multiple linear regression analyses of the relationship between log2 transformed NGAL and fibrinogen, baseline variables that were considered relevant to fibrinogen and log2 transformed NGAL by difference analysis or previous

Table 2 Multivariate Logistic Regression for Effects of log2 Transformed NGAL on NSE

Variable	Model 1 (unadjusted)		Model 2 (adjusted)	
	N	β (95% CI) p	N	β (95% CI) p
Log2 transformed NGAL (total) (continuous)	196	2.64 (1.73, 3.56) <0.001	196	2.24 (1.15, 3.32) <0.001
Bisection				
Low (5.93–7.38)	96	Ref	96	Ref
High (7.39–9.27)	100	2.86 (1.67, 4.04) <0.001	100	2.24 (0.99, 3.49) <0.001
p for trend		3.89 (2.27, 5.50) <0.001		3.05 (1.35, 4.75) <0.001
Trisection				
Tertile 1 (5.93–7.08)	63	Ref	63	Ref
Tertile 2 (7.10–7.59)	65	1.34 (−0.14, 2.82) 0.077	65	1.38 (−0.11, 2.87) 0.071
Tertile 3 (7.61–9.27)	68	3.29 (1.82, 4.75) <0.001	68	2.35 (0.77, 3.93) 0.004
p for trend		2.77 (1.55, 3.99) <0.001		1.96 (0.64, 3.29) 0.004

Note: Model 1: unadjusted, Model 2: adjusted for sex, age, creatinine, cystatin C, eGFR-MDRD, sdLDL, IL-6, and BNP.

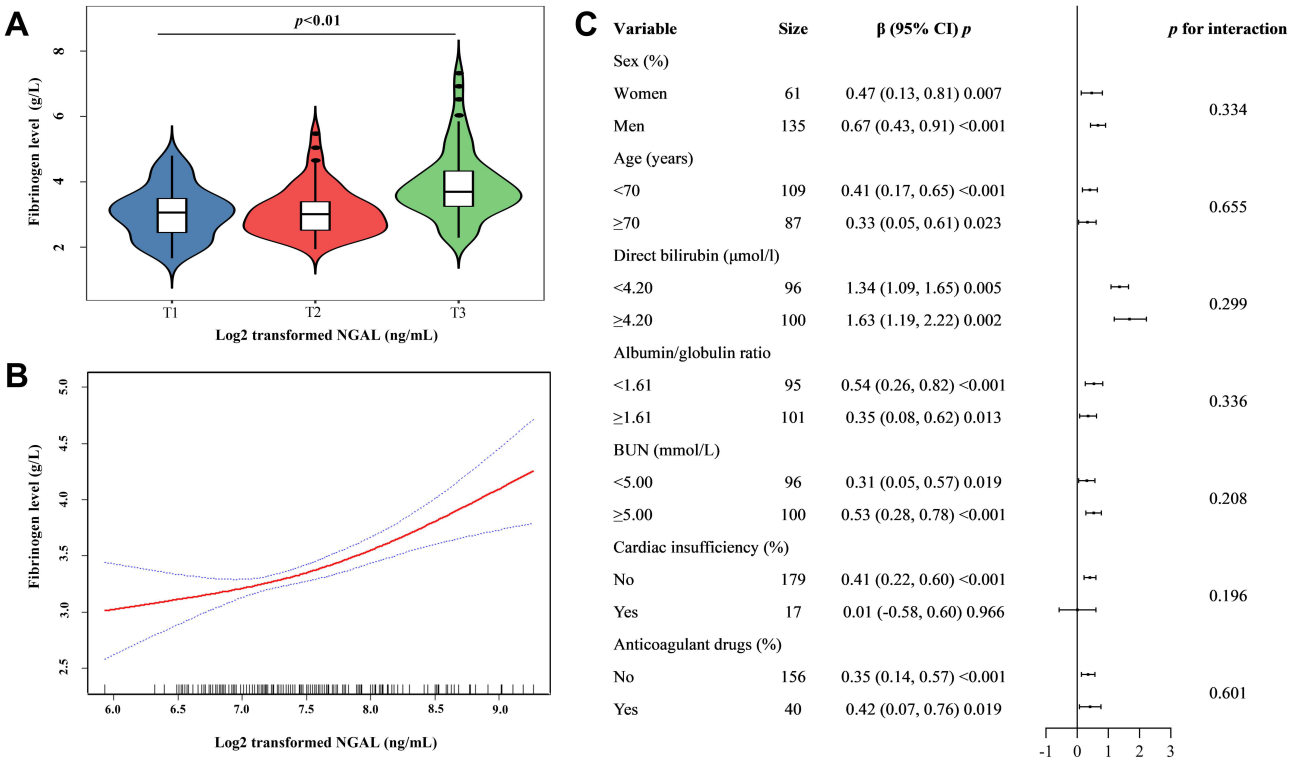

Figure 3 The relationship between NGAL and fibrinogen. (**A**) The mean serum fibrinogen levels showed a grade increase according to the log2 transformed NGAL tertile's levels in all AIS patients ($p < 0.01$). (**B**) A linear relationship between log2 transformed NGAL and fibrinogen after adjusting for confounder factors. Solid lines represent the fitting curve and dotted lines represent the corresponding 95% CI. (**C**) Hierarchical analysis on the relationship of log2 transformed NGAL and fibrinogen.

references or that showed a univariate relationship with fibrinogen were selected into multivariate linear regression model (Supplement Table 6). As a result, sex, age, direct bilirubin, albumin/globulin ratio, BUN, cardiac insufficiency, and anticoagulant drugs were regarded as confounding factors related to fibrinogen. In multiple linear regression analysis, the β (95% CI) for the relationship between log2 transformed NGAL and fibrinogen were 0.62 (0.42–0.82, $p < 0.001$) and 0.37 (0.19–0.56), $p < 0.001$) in all patients before and after adjusting for sex, age, direct bilirubin, albumin/globulin ratio, BUN, cardiac insufficiency, and anticoagulant drugs. After adjusted confounder factors, the β (95% CI) for the relationship between log2 transformed NGAL and fibrinogen showed a grade increase according to the log2 transformed NGAL tertiles by trend analysis ($p < 0.001$) (Table 3), which showed a statistical significance. Both sensitivity analysis and

Table 3 Multivariate Logistic Regression for Effects of log2 Transformed NGAL on Fibrinogen

Variable	Model 1 (unadjusted)		Model 2 (adjusted)	
	N	β (95% CI) *p*	N	β (95% CI) *p*
Log2 transformed NGAL (total) (continuous)	196	0.62 (0.42, 0.82) <0.001	196	0.37 (0.19, 0.56) <0.001
Bisection				
Low (5.93–7.38)	96	Ref	96	Ref
High (7.39–9.27)	100	0.65 (0.39, 0.90) <0.001	100	0.39 (0.16, 0.63) <0.001
p for trend		0.88 (0.53, 1.23) <0.001		0.54 (0.22, 0.86) <0.001
Trisection				
Tertile 1 (5.93–7.08)	63	Ref	63	Ref
Tertile 2 (7.10–7.59)	65	0.02 (−0.28, 0.33) 0.882	65	−0.13 (−0.41, 0.15) 0.356
Tertile 3 (7.61–9.27)	68	0.86 (0.56, 1.17) <0.001	68	0.54 (0.26, 0.82) <0.001
p for trend		0.75 (0.49, 1.01) <0.001		0.48 (0.25, 0.72) <0.001

Note: Model 1: unadjusted, Model 2: adjusted for sex, age, direct bilirubin, albumin/globulin ratio, BUN, cardiac insufficiency, and anticoagulant drugs.

hierarchical analysis (according to sex, age, direct bilirubin, albumin/globulin ratio, BUN, cardiac insufficiency, and anticoagulant drugs) also showed that the association between log2 transformed NGAL and fibrinogen was statistically significant (Figure 3C, Supplement Tables 7 and 8).

The Relationship Between Fibrinogen and NSE

Between the normal and high fibrinogen groups, the mean level of NSE was higher in the high fibrinogen group than that in the normal fibrinogen group (NSE: 16.54 ± 4.88 ng/mL vs 13.54 ± 4.17 ng/mL, $p < 0.001$). In all included AIS patients, the mean serum NSE levels were 12.83 ± 3.75 ng/mL, 13.59 ± 4.02 ng/mL, and 15.92 ± 4.98 ng/mL in the low (T1), middle (T2), and high (T3) fibrinogen tertiles for all patients (Figure 4A), respectively, and there was a significant difference among the three groups ($p < 0.01$). The Pearson's correlation coefficient (95%) for the relationship between fibrinogen and NSE was 0.329 (0.198–0.448, $p < 0.001$) in all AIS patients. Smoothed plots (Figure 4B) showed linear relationships between fibrinogen and NSE after adjusting for sex, age, direct bilirubin, creatinine, cystatin C, eGFR-MDRD, sdLDL, homocysteine, IL-6, BNP, and antiplatelet drugs (F = 11.180, $p < 0.001$).

In multiple linear regression analyses of the relationship between fibrinogen and NSE, baseline variables that were considered relevant to NSE and fibrinogen by difference analysis or previous references or that showed a univariate relationship with NSE were selected into multivariate linear regression model (Supplement Table 9). As a result, sex, age, direct bilirubin, creatinine, cystatin C, eGFR-MDRD, sdLDL, homocysteine, IL-6, BNP, and antiplatelet drugs were regarded as confounding factors related to NSE. In multiple linear regression analysis, the β (95% CI) for the relationship between fibrinogen and NSE were 1.52 (0.90–2.13, $p < 0.001$) and 1.16 (0.48–1.85, $p < 0.001$) in all patients before and after adjusting for sex, age, direct bilirubin, creatinine, cystatin C, eGFR-MDRD, sdLDL, homocysteine, IL-6, BNP, and antiplatelet drugs. After adjusted confounder factors, the β (95% CI) for the relationship between fibrinogen and NSE showed a grade increase according to the fibrinogen tertile by trend analysis ($p < 0.001$) (Table 4), which showed a statistical significance. Both sensitivity analysis and hierarchical analysis (according to sex, age, direct bilirubin, creatinine, cystatin C, eGFR-MDRD, sdLDL, homocysteine, IL-6, BNP, and

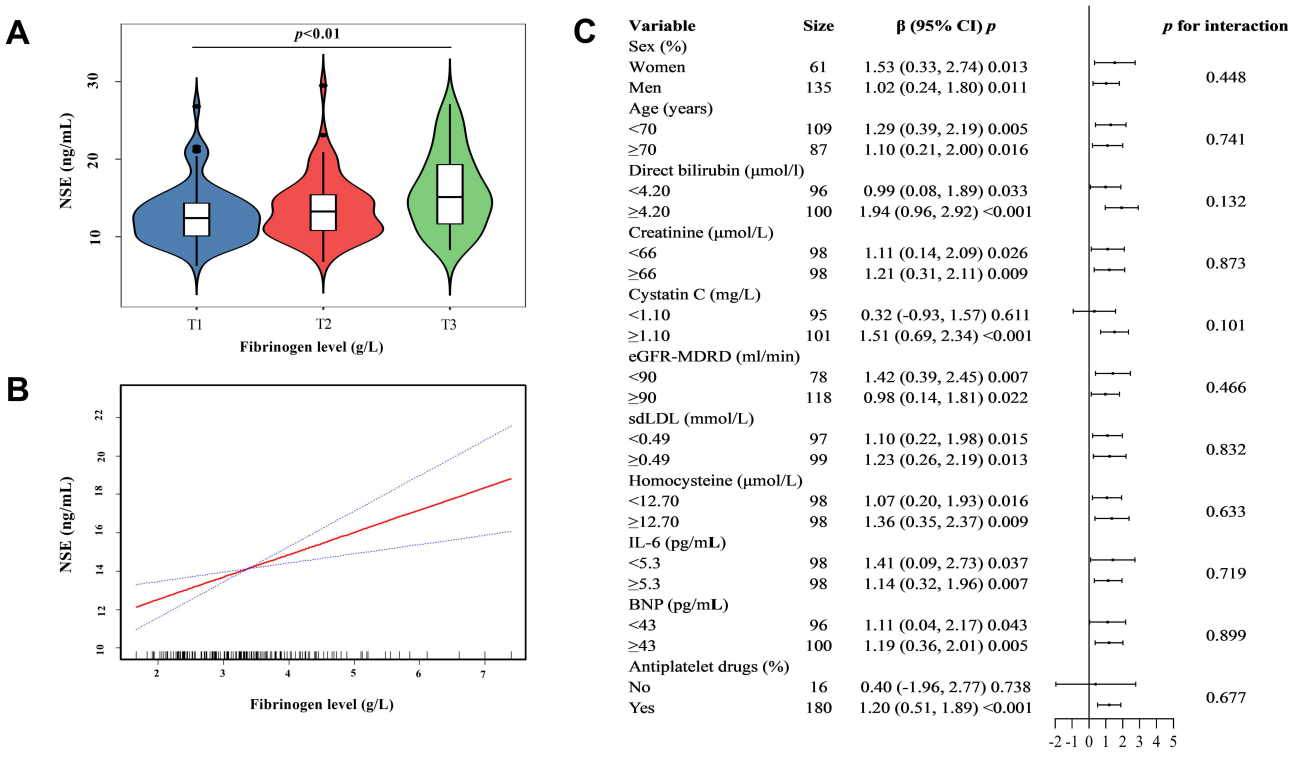

Figure 4 The relationship between fibrinogen and NSE. (**A**) The mean serum NSE levels showed a grade increase according to the fibrinogen tertile's levels in all AIS patients ($p < 0.01$). (**B**) A linear relationship between fibrinogen and NSE after adjusting for confounder factors. Solid lines represent the fitting curve and dotted lines represent the corresponding 95% CI. (**C**) Hierarchical analysis on the relationship of fibrinogen and NSE.

Table 4 Multivariate Logistic Regression for Effects of Fibrinogen on NSE

Variable	Model 1 (unadjusted)		Model 2 (adjusted)	
	N	β (95% CI) *p*	N	β (95% CI) *p*
Fibrinogen (increased 1 g/L)	196	1.52 (0.90, 2.13) <0.001	196	1.16 (0.48, 1.85) <0.001
Bisection				
Low (1.66–3.24)	96	ref	96	ref
High (3.25–7.40)	100	2.05 (0.83, 3.27) <0.001	100	2.04 (0.81, 3.27) 0.001
p for trend		1.74 (0.70, 2.77) <0.001		1.73 (0.68, 2.77) <0.01
Trisection				
Tertile 1 (1.66–2.88)	64	ref	64	ref
Tertile 2 (2.89–3.54)	66	0.76 (−0.71, 2.24) 0.311	66	0.59 (−0.82, 2.00) 0.413
Tertile 3 (3.57–7.40)	66	3.09 (1.62, 4.57) <0.001	66	2.08 (0.51, 3.64) 0.010
p for trend		1.75 (0.92, 2.57) <0.001		1.14 (0.27, 2.01) 0.011

Note: Model 1: unadjusted, Model 2: adjusted for sex, age, direct bilirubin, creatinine, cystatin C, eGFR-MDRD, sdLDL, homocysteine, IL-6, BNP, and antiplatelet drugs.

antiplatelet drugs) also showed that the association between fibrinogen and NSE was statistically significant (Figure 4C, Supplement Tables 10 and 11).

The Effect of log2 Transformed NGAL on NSE Mediated by Fibrinogen

According to the results of the multiple linear regression analysis, the relationships between log2 transformed NGAL, fibrinogen, and NSE meet the requirements of the moderated mediation model test.

Table 5 shows the total and direct effect between log2 transformed NGAL and NSE, and a significant partial mediated effect by fibrinogen was observed on the relationships between log2 transformed NGAL levels and NSE (Figure 5A) (Supplement Tables 12 and 13). Increased fibrinogen levels significantly mediated 14.28% of the log2 transformed NGAL-associated elevated NSE risk. The interaction effect was not significantly observed between log2 transformed NGAL and fibrinogen on NSE ($p = 0.679$). The simple slope analysis demonstrated the mediated but not moderating role of fibrinogen in the association between log2 transformed NGAL and NSE (Figure 5B). This may mean that initially NGAL activates fibrinogen, followed by fibrinogen acts on NSE, while fibrinogen does not enhance or weaken the effect of NGAL on NSE during an inflammatory response. Thus, these findings indicate that the association between log2 transformed NGAL and NSE was partially mediated by the fibrinogen.

Discussion

This study found that in AIS patients, NGAL levels are independently associated with NSE, and this positive effect is evident in all subgroups that are carefully adjusted. Further mediation analysis shows the mediated role of fibrinogen in

Table 5 Mediated Effects by Fibrinogen on the Associations of log2 Transformed NGAL Levels with NSE

Categories	β (95% CI)	*P*-value
Mediation: Fibrinogen levels		
Total effects (95% CI)	2.135 (1.055–3.216)	<0.001
Direct effects (95% CI)	1.830 (0.743–2.917)	0.001
Mediated effects (95% CI)	0.305 (0.025–0.610)	<0.001
Proportion mediated by fibrinogen 14.28%		

Note: Adjusted for sex, age, direct bilirubin, creatinine, cystatin C, eGFR-MDRD, sdLDL, homocysteine, IL-6, BNP, and antiplatelet drugs.

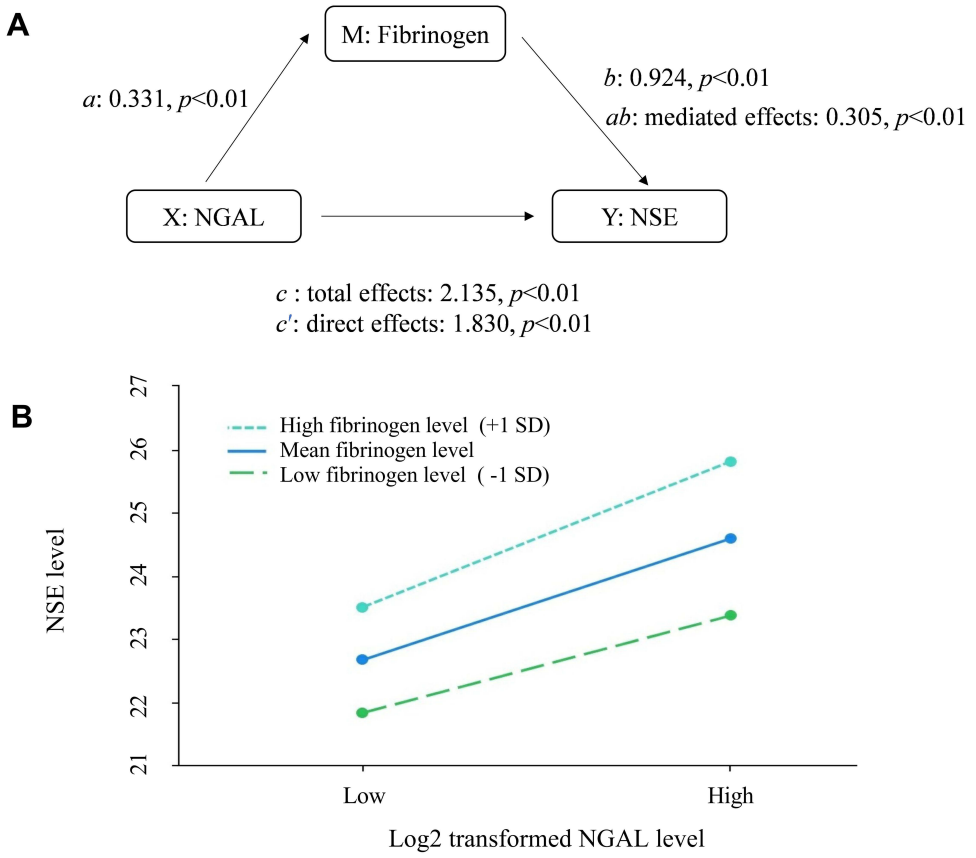

Figure 5 The mediation model-the effect of log2 transformed NGAL on NSE is mediated by fibrinogen. **(A)** Interaction indexes, *a* refers to the direct effect of log2 transformed NGAL on fibrinogen, *b* refers the direct effect of fibrinogen on NSE, *ab* refers the indirect (mediated) effect of fibrinogen on NSE, *c* refers to the total effect of log2 transformed NGAL on NSE, and *c'* refer to the direct effect of log2 transformed NGAL on NSE after controlling the indirect (mediated) effect of fibrinogen. (**B**) The association between log2 transformed NGAL and NSE was significantly positive by low (β = 1.540 p < 0.05), mean (β = 1.923, p < 0.01), and high (β = 2.307, p < 0.01) fibrinogen groups.

the association between NGAL and NSE. NSE is a common marker of nerve injury. Therefore, these results suggest that serum NGAL is associated with AIS nerve injury, and fibrinogen may partially mediate NGAL-related neuronal injury in AIS patients. That means about 14.28% promoting function of nerve injury by NGAL is achieved by regulating fibrinogen.

As an inflammation-related protein, NGAL was first discovered in activated neutrophils and has been widely studied in tissues such as the heart, kidney, and brain, which are sensitive to blood perfusion.[10] NGAL can promote glial cells to produce pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 after hypoxic-ischemia.[9] Pro-inflammatory cytokines further trigger glial cell activation, aggravate inflammation and eventually lead to more nerve injury. Previous studies have suggested that a higher NGAL level is associated with a relatively poor clinical prognosis.[8] Also, NGAL can regulate metabolism and transmit signals to initiate protective mechanisms.[24] Interestingly, in previous studies, increased NGAL after cerebral infarction may be secreted by the activated glial cells in the central nervous system, or may be generated in the bone marrow, which is then gathered in brain lesions by inflammatory cytokines through blood circulation.[24] Considering the role of NGAL in metabolism and the correlation between blood lipids and stroke prognosis,[25] Lipid-related measures were included in this study. As shown in Table 1 and Supplementary Table 1, the Lipid-related measures, including LDL-C (mmol/L), sdLDL (mmol/L), and Lipid lowering drugs (%) were not confounding factors. The mechanisms of NGAL in ischemic brain lesions after have been investigated in a lot of studies. In order to alleviate the inflammatory damage better after cerebral infarction, more research should be done to further clarify the function and mechanism of NGAL in the blood circulation after cerebral infarction.

Our current results suggest that the level of NGAL in circulation is associated with the degree of nerve injury after AIS. Although there are various sources of NGAL, what is certain is that inhibition of NGAL expression can significantly reduce the nerve injury caused by cerebral infarction.[10] Previous studies have suggested that the expression of NGAL is STAT3 dependent, and treatment targeting the JAK/STAT3 pathway may alleviate nerve injury after AIS.[26,27] Moreover, a study in diabetes patients suggests that metformin treatment can reduce the level of NGAL.[28] It indicated a potential therapeutic drug for AIS. In the past, some studies found that NGAL and fibrinogen are respectively related to the occurrence and development of clinical events in cerebrovascular diseases.[29–31] However, few researchers have focused on the interaction and mechanism between NGAL and fibrinogen in circulation after AIS. Our current study suggests that there is some internal relationship between NGAL and fibrinogen in circulation after AIS. Patients with elevated NGAL are at risk of elevating fibrinogen after AIS, this elevation of fibrinogen may be caused by elevated NGAL, but the molecular mechanism remains unclear. Based on previous studies,[10,32] a reasonable assumption is that inflammatory responses, including neutrophil activation, may play an important role in the interaction between NGAL and fibrinogen in circulation after AIS.

The role played by fibrinogen in thrombosis has been widely studied.[33–35] Recent studies have proven that inflammation can stimulate the synthesis and secretion of fibrinogen, and fibrinogen is also involved in the process of inflammation.[36] Luyendyk et al found that fibrinogen plays an important role in intensive and chronic low-grade inflammation.[37] PKM2 in bone marrow cells regulates post ischemic inflammation of peripheral neutrophils after ischemic stroke by promoting STAT3 phosphorylation and affects fibrinogen level in the brain.[38] Additionally, fibrinogen can support neutrophil activation by interacting with the human adhesion glycoprotein αMβ2 integrin.[32]

NGAL is considered as a marker of neutrophil activation.[39] Considering STAT3's regulation on NGAL and combining with the results of this study, we hypothesized that after AIS, there may be a regulation pathway of STAT3-NGAL-fibrinogen that affects nerve injury. The specific molecular mechanisms could be a valuable research direction. Furthermore, some studies suggest that fibrinogen and neutrophil-to-lymphocyte ratio can serve as prognostic factors for predicting poor early response to intravenous thrombolysis. Our study indicates that fibrinogen can not only predict the prognosis of intravenous thrombolysis but can also be an early marker for predicting the degree of nerve injury after AIS. Regulating fibrinogen may help alleviate nerve injury after AIS.

Despite the fact that our study is novel, it has several limitations that should be considered. First, this is a single-center cross-sectional study. There may be biased due to the inclusion of subjects in centralized time and space. The creation of causal relationships shall be further verified in a multicenter, large-sample, prospective cohort study. Items such as thrombolysis were not included in the analysis in this study, which is a shortcoming of the study, which needs to be taken into consideration in subsequent studies. Second, the evidence for the association of NGAL, fibrinogen, and NSE comes from the literature and needs to be proved by relevant basic experiments. Despite these limitations, the data is considered to be impactful and reliable. We excluded participants with underlying medical conditions, which may affect fibrinogen values, such as malignancies and haematological disorders. The detection methods and operations used in this study have been validated by a large number of clinical samples and have been proven to be reliable.

Conclusion

In summary, our results found that elevated circulating NGAL were significantly related to the high levels of NSE and the NGAL-associated neuronal damage may be partially (14.28%) mediated by fibrinogen in AIS patients. The pathway between NGAL, fibrinogen, and NSE should be further studied and intervened to reduce nerve injury after ischemic stroke. This result is helpful to better understand the mechanism of nerve injury after stroke, and helps clinicians to better assess the nerve injury and prognosis of AIS through early laboratory results.

Human and Animal Rights Statement

This study was performed according to the principles of the Declaration of Helsinki and was approved by the ethics committee of Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital, Shanghai, China. We obtained informed consent from all study subjects or their immediate family members (Patients with consciousness disorder or dysarthria after AIS) prior to sample collection.

Data Sharing Statement

The datasets generated during the current study are available from the corresponding author (Desheng Zhu) upon reasonable request.

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Disclosure

The authors declare that they have no competing interests.

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