

Elevated Serum NOX2 Levels Contribute to Delayed Cerebral Ischemia and a Poor Prognosis After Aneurysmal Subarachnoid Hemorrhage: A Prospective Cohort Study

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Background: NADPH oxidase 2 (NOX2) is highly expressed in injured brain tissues. We determined serum NOX2 levels of aneurysmal subarachnoid hemorrhage (aSAH) patients and further investigated correlation of serum NOX2 levels with disease severity, delayed cerebral ischemia (DCI) plus prognosis after aSAH.

Methods: Serum NOX2 levels were measured in 123 aSAH patients and 123 healthy controls. World Federation of Neurological Surgeons scale (WFNS) score and modified Fisher (mFisher) score were utilized to assess disease severity. Modified Rankin scale (mRS) score was used to evaluate the clinical prognosis at 90 days after aSAH. Relations of serum NOX2 levels to DCI and 90-day poor prognosis (mRS score of 3–6) were analyzed using multivariate analysis. Receiver operating characteristic curve (ROC) was built to evaluate the prognostic predictive capability.

Results: Serum NOX2 levels in aSAH patients, compared with healthy controls, were significantly increased, and were independently correlated with WFNS score, mFisher score and post-stroke 90-day mRS score. Patients with poor prognosis or DCI had significantly higher serum NOX2 levels than other remainders, and serum NOX2 levels independently predicted 90-day poor prognosis and DCI. Serum NOX2 had high prognosis and DCI predictive abilities, and their areas under ROC curve were similar to those of WFNS score and mFisher score.

Conclusion: Serum NOX2 levels are significantly associated with hemorrhage severity, poor 90-day prognosis and DCI in aSAH patients. Hence, complement NOX2 may serve as a potential prognostic biomarker after aSAH.

Keywords: NADPH oxidase 2, biomarker, aneurysmal subarachnoid hemorrhage, disease severity, delayed cerebral ischemia, prognosis

Introduction

Spontaneous subarachnoid hemorrhage (SAH) is a common emergency in neurosurgical science. Aneurysmal SAH (aSAH) approximately accounts for 85% of spontaneous SAH.¹ aSAH is characterized by rapid onset, and high rates of disability and death. Delayed cerebral ischemia (DCI) is a serious complication after aSAH and can greatly increase the risk of poor prognosis in such patients.² Therefore, DCI prediction is of clinical benefit to aSAH management. During decades, biomarkers have been paid extensive attentions with respect to their aidance in severity assessment and prognostic prediction.

The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family includes 7 subtypes of oxidases, among which NADPH oxidase 2 (NOX2) is one of its main members and is most widely distributed in the central nervous

system.³ NOX2, as one of the main pathways for the production of reactive oxygen species (ROS) clusters in brain tissue,⁴ can reportedly promote inflammatory responses and oxidative stress, thereby disrupting the blood–brain barrier (BBB), exacerbating brain tissue edema and finally aggravating neuronal damage.^{5–8} NOX2 expression was significantly elevated in brain tissues of mice with traumatic brain injury (TBI) and SAH.^{5,9} Consistently, elevated expression of NOX2 was revealed in brain tissues from mice model of epileptic ictus or middle cerebral artery occlusion (MCAO).^{10,11} Also, NOX2 expression by astrocytes was significantly higher in TBI patients than in controls.¹² Overall, it is speculated that NOX2 may represent a potential biomarker of acute brain injury.

Materials and Methods

Participants

In this study, we prospectively and consecutively enrolled aSAH patients, who were admitted to Affiliated Hangzhou First People's Hospital, Zhejiang University School of Medicine (Hangzhou, China) from January 2018 to March 2021. We required (1) age between 18 and 80 years; (2) admission within 24 hours after symptom onset; (3) SAH confirmed by computerized tomography (CT) scan; (4) aneurysms diagnosed via CT angiography (CTA) or digital subtraction angiography (DSA); (5) securing aneurysm within 48 hours after admission. Further, we excluded patients with (1) history of neurologic diseases, such as SAH, spontaneous intracerebral hemorrhage, ischemic stroke, arteriovenous malformation, TBI, moyamoya's disease and intracranial tumors; (2) infection or operational procedures in recent a month; (3) aneurysmal rebleeding; (4) other specific diseases or conditions, eg, pregnancies, malignancies, uremia and cirrhosis; or (5) other conditions, ie, loss to follow-up, refusal to participation, unavailable samples and incomplete information. A group of healthy volunteers constituted controls. The protocol of this study was approved by the Institutional Review Board of Affiliated Hangzhou First People's Hospital, Zhejiang University School of Medicine (Opinion No: Medical Ethics Review No. 058-01). Owing to the serious conditions and poor consciousness of aSAH patients who were enrolled in this research, all informed consents of aSAH patients were signed by relatives of the patients. The use of relative consent vs patient consent was approved by the Institutional Review Board of Affiliated Hangzhou First People's Hospital, Zhejiang University School of Medicine. And the control themselves signed informed consent.

Variables

We collected information including age, gender, cigarette smoking, alcohol consumption, previous underlying diseases (including hypertension, diabetes mellitus and hyperlipidemia), admission time and blood-collection time. Modified Fisher (mFisher) score and World Federation of Neurological Surgeons (WFNS) scale were recorded to assess the hemorrhage severity. The location and size of intracranial aneurysm were determined according to CTA or DSA, and intraventricular hemorrhage and acute hydrocephalus were determined by CT scan. The following criteria were considered for DCI: 1) clinical deterioration (namely, a new focal deficit, decrease in concentration of consciousness, or both), and/or 2) a new infarct on head CT scan that was invisible at admission or immediately post-operatively, and cannot be attributed to other causes by means of clinical assessment, imaging of the brain, and appropriate laboratory studies.¹³ Surgical procedures for aneurysms included endovascular intervention or neurosurgical clipping. Patients were followed for 90 days and functional prognosis was assessed using a modified Rankin (mRS) score. And a poor prognosis was defined as mRS score of 3–6.¹⁴

Immune Analysis

Blood samples were collected from patients on admission and those of controls were obtained at entry into study. Laboratory indices, such as blood leukocyte count, blood platelet count, blood hemoglobin levels, blood glucose levels, were measured using the conventional methods. For the determination of NOX2 levels, acquired serum samples were stored at –80 °C until assayed. Serum NOX2 levels were quantified by commercially available enzyme linked immunosorbent assay (FANKEWEI, Qingdao, Shandong Province, China) according to the manufactures' instructions. All measurements were in duplicate done by the same technician who was blinded to the clinical data and two measurements were averaged for final analysis.

Statistical Analysis

All statistical analyses were performed using SPSS 26.0. Using GraphPad Prism version 9.0 (GraphPad Software Inc., La Jolla, CA, USA) and MedCalc 9.6.4.0 (MedCalc Software, Mariakerke, Belgium), the graphs were drawn. Categorical variables were expressed as frequencies (percentages), and continuous variables with normal and non-normal distribution were expressed as mean \pm standard deviation and median (upper and lower quartiles), respectively. χ^2 test or Fisher's Exact test was used to compare various variables between groups for qualitative data, and Mann-Whitney *U*-test or *t*-test was used for quantitative data. Spearman correlation coefficient was used to determine bivariate correlation. Linear regression model was established to determine the variables associated with serum NOX2 levels and modified Rankin score. Multivariate linear correlation analysis was performed to determine the variables independently associated with serum NOX2 levels and mRS score after adjusting for other confounding factors. Logistic regression model was built to investigate whether serum NOX2 levels were associated with DCI and 90-day poor prognosis. Variables with significant differences in univariate analysis were included in binary Logistic regression model to determine variables, which were independently associated with DCI and 90-day poor prognosis. Results were presented as odds ratios (ORs) and 95% confidence intervals (CIs). The receiver operating characteristic (ROC) curve was outlined to study the predictive value of serum NOX2 levels on DCI and 90-day poor prognosis in patients with aSAH, and the area under the curve (AUC) was estimated. Cutoff values were determined using the Youden J method, wherein sensitivity and specificity were generated. Differences of $P < 0.05$ were defined as statistically significant. In order to assess bivariate correlation, sample size should be at least 22. And for making intergroup comparison of categorical variables, least sample size should be 59. Thus, a total of 123 patients were investigated in the current study, and sample size was enough for clinical analysis.

Results

Patient Selection and Characteristics

This prospective cohort study included a total of 162 first-ever aSAH patients hospitalized within 24 hours after the onset of symptoms. Subsequently, 39 patients were excluded because of aneurysm rebleeding (5 cases), suspected pseudoaneurysm (4 cases), previous neurological diseases (7 cases), other special circumstances (11 cases), lost to follow-up (4 cases), refused to participation (3 cases), and unavailable blood samples (5 cases). Finally, a total of 123 patients were enrolled in the study (Figure 1). In addition, a total of 123 healthy volunteers were recruited as controls. There were no statistically significant differences in demographics between patients and controls (Table 1).

A total of 68 females and 55 males were included in the study with a female-to-male ratio of 1.24. Their age ranged from 30 to 78 years (mean, 54.5 years; standard deviation, 11.1 years), 25 cases (20.3%) were smokers, 32 cases (26.0%) were alcohol drinkers, 24 cases (19.5%) had hypertension, 15 cases (12.2%) had diabetes mellitus, and 21 cases (17.1%) suffered from hyperlipidemia. With respect to aSAH severity, WFNS score ranged from 1 to 5 (median, 3; upper-lower quartiles, 2–4), and mFisher score ranged from 1 to 4 (median, 2; upper-lower quartiles, 2–3). Aneurysmal characteristics included aneurysm shape (cystic, 102 cases; others, 21 cases), aneurysm location (anterior circulation, 97 cases; posterior circulation, 26 cases), and aneurysm diameter (≥ 10 mm, 50 cases; < 10 mm, 73 cases). There was surgical clipping in 50 cases (40.7%), and endovascular intervention in 73 cases (59.3%) for securing aneurysm. Complications included intraventricular bleeding in 21 cases (17.1%) and acute hydrocephalus in 19 cases (15.4%). An external ventricular drainage was done in 18 cases (14.6%). The range of time between onset of symptom and hospital admission was from 1.0 to 24.0 h (median, 7.0 h; upper-lower quartiles, 5.0–11.0 h), with their blood samples collected from 1.4 to 25.0 h (median, 7.5 h; upper-lower quartiles, 5.0–12.0 h). Arterial systolic blood pressure ranged from 95 to 185 mmHg (median, 149 mmHg; upper-lower quartiles, 133–161 mmHg), with arterial diastolic blood pressure from 53 to 119 mmHg (mean, 86.9 mmHg; standard deviation, 14.3 mmHg). In addition, blood glucose levels ranged from 4.0 to 20.8 mmol/L (median, 7.4 mmol/L; upper-lower quartiles, 5.6–9.7 mmol/L), blood potassium levels ranged from 2.84 to 5.36 mmol/L (mean, 3.79 mmol/L; standard deviation, 0.44 mmol/L) and blood leucocyte count ranged from 4.2 to $17.6 \times 10^9/L$ (mean, $9.1 \times 10^9/L$; standard deviation, $2.9 \times 10^9/L$). mRS score ranged from 0 to 6 (median, 2; upper-lower quartiles, 1–3). Ninety-day mRS score of aSAH patients was 0 in 29 patients, 1 in 23 patients, 2 in 24 patients, 3 in 19 patients, 4 in 15 patients, 5 in 7 patients, and 6 in 6 patients. In aggregate, 47 patients had a poor prognosis (mRS score of 3–6).

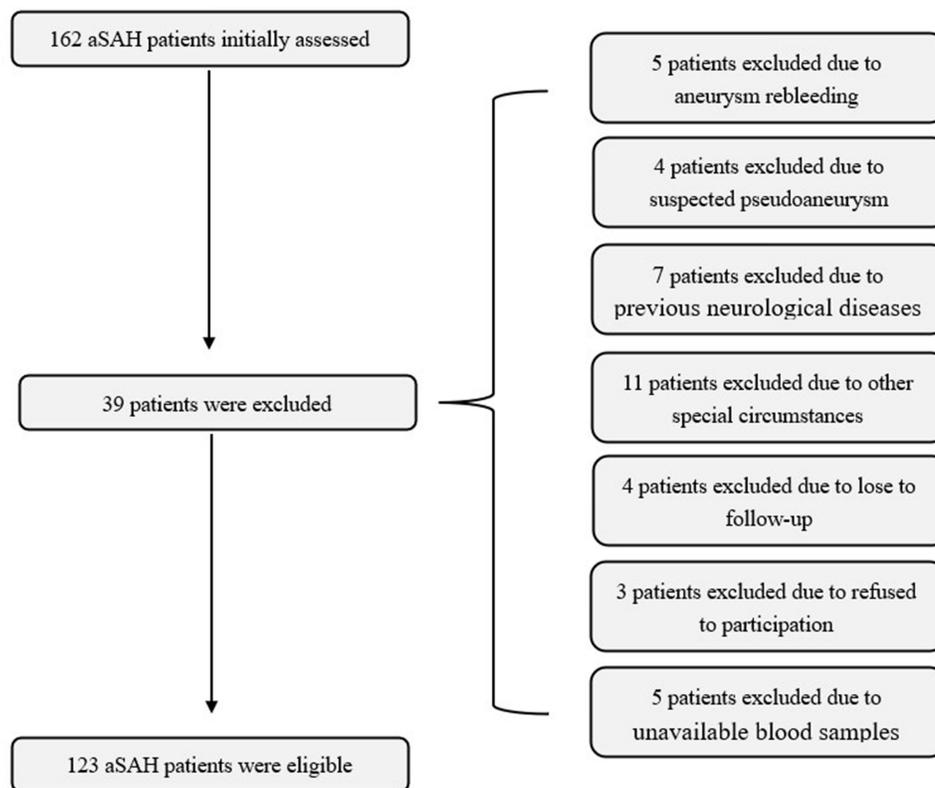


Figure 1 Flowing chart of selecting eligible patients with aneurysmal subarachnoid hemorrhage.

The Relationship Between Serum NOX2 Levels and Hemorrhage Severity After aSAH

In [Figure 2](#), serum NOX2 levels were significantly higher in patients than in controls (median, 2196.50 pg/mL vs 947.07 pg/mL). Using Spearman analysis, serum NOX2 levels were significantly correlated with WFNS score ($r=0.599$, $P<0.001$), mFisher score ($r=0.552$, $P<0.001$) and other variables, including intraventricular bleeding ($r=0.220$, $P=0.014$), acute hydrocephalus ($r=0.190$, $P=0.035$), blood glucose levels ($r=0.212$, $P=0.018$) and blood leukocyte count ($r=0.324$, $P<0.001$). When the above six variables were incorporated in a multivariate linear regression model, we found that WFNS score ($\beta=0.383$, $P<0.001$) and mFisher score ($\beta=0.252$, $P=0.019$) were independently associated with serum NOX2 levels ([Table 2](#)). All patients were dichotomized based on WFNS score or mFisher score, namely, WFNS scores 1–3 and 4–5, or mFisher scores 1–2 and 3–4. It was found that WFNS score and mFisher score, both of which were whether categorical or continuous variables, were significantly associated with serum NOX2 levels ([Figure 3](#)).

Table 1 Differences of Demographic Data and Vascular Risk Factors Between Initially Enrolled Patients and Finally Eligible Patients, as Well as Between Finally Eligible Patients and Controls

Variable	Initially Enrolled Patients (n=162)	Finally Eligible Patients (n=123)	P ₁ value	Controls (n=123)	P ₂ value
Gender (male/female)	73/89	55/68	0.954	57/66	0.798
Age (years)	54.8±11.0	54.5±11.1	0.851	56.0±10.7	0.310
Cigarette smoking	29 (17.9%)	25 (20.3%)	0.605	19 (15.4%)	0.318
Alcohol consumption	47 (29.0%)	32 (26.0%)	0.576	26 (21.1%)	0.367
Hypertension	38 (23.5%)	24 (19.5%)	0.424	0	<0.001
Diabetes mellitus	24 (14.8%)	15 (12.2%)	0.524	0	<0.001
Hyperlipidemia	27 (16.7%)	21 (17.1%)	0.925	0	<0.001

Notes: Quantitative data were reported as medians with upper and lower quartiles or the mean ± standard deviation as appropriate. Qualitative data were presented as counts (proportions). Intergroup comparisons of various variables were performed using the χ^2 test or Fisher's exact test for qualitative data, and Mann-Whitney *U*-test or *t*-test for quantitative data. P₁ indicated the differences of various variables between initially enrolled patients and finally eligible patients. P₂ indicated the differences of various variables between finally eligible patients and controls.

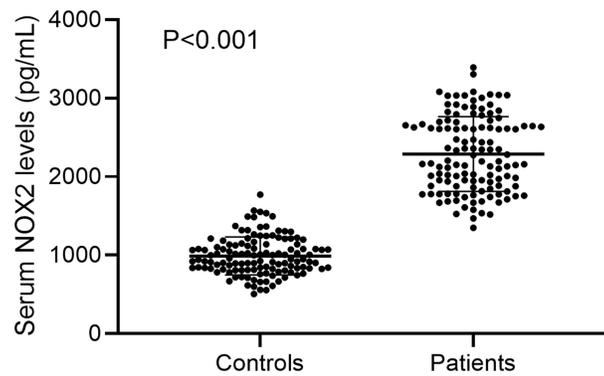


Figure 2 Boxplot illustrating serum NOX2 levels between patients with aneurysmal subarachnoid hemorrhage and healthy controls. Data were reported as median (upper-lower quartiles) and two groups were compared using the Mann–Whitney *U*-test.

Relationship Between Serum NOX2 Levels and mRS Score After aSAH

In [Figure 4](#), mRS score was highly correlated with serum NOX2 levels ($r=0.563$, $P<0.001$) and other variables, including WFNS score ($r=0.660$, $P<0.001$), mFisher score ($r=0.614$, $P<0.001$), blood leukocyte count ($r=0.324$, $P<0.001$), intraventricular bleeding ($r=0.394$, $P<0.001$), blood glucose level ($r=0.222$, $P=0.014$) and acute hydrocephalus ($r=0.267$, $P=0.003$) using Spearman analysis. In [Table 3](#), when those preceding variables were forced in a multivariate linear regression model, we found that WFNS score ($\beta=0.293$, $P=0.004$), mFisher score ($\beta=0.200$, $P=0.040$) and serum NOX2 level ($\beta=0.211$, $P=0.012$) were independently associated with mRS score.

Table 2 Correlation Between Serum NADPH Oxidase 2 Levels and Other Variables Using Spearman Correlation Coefficient and Linear Regression Analysis in Aneurysmal Subarachnoid Hemorrhage

Variable	r	P value	Univariate Analysis			Multivariate Analysis		
			t	Beta	P value	t	Beta	P value
Gender (male/female)	0.023	0.798	0.092	0.008	0.927	–		
Age (years)	0.147	0.104	1.291	0.117	0.199	–		
Cigarette smoking	0.114	0.209	1.438	0.190	0.153	–		
Alcohol consumption	–0.072	0.430	–0.818	–0.074	0.415	–		
Hypertension	–0.027	0.766	–0.388	–0.035	0.699	–		
Diabetes mellitus	0.043	0.634	0.597	0.054	0.554	–		
Hyperlipidemia	–0.060	0.512	–0.507	–0.046	0.613	–		
WFNS score	0.599	<0.001*	8.559	0.614	<0.001*	3.594	0.383	<0.001*
Modified Fisher score	0.552	<0.001*	7.811	0.579	<0.001*	2.373	0.252	0.019*
Aneurysmal position (posterior / anterior circulation)	–0.149	0.100	–1.474	–0.133	0.143	–		
Aneurysmal shape (cystic/others)	–0.015	0.867	0.086	0.008	0.931	–		
Aneurysmal diameter (<10 mm/≥10 mm)	0.065	0.478	0.780	0.071	0.437	–		
Intraventricular bleeding	0.220	0.014*	2.832	0.249	0.005*	–0.916	–0.075	0.361
Acute hydrocephalus	0.190	0.035*	2.374	0.211	0.019*	1.367	0.102	0.174
Admission time (h)	–0.113	0.214	–1.306	–0.118	0.194	–		
Blood-collection time (h)	–0.132	0.146	–1.473	–0.133	0.143	–		
Systolic arterial pressure (mmHg)	–0.059	0.515	–0.826	–0.075	0.410	–		
Diastolic arterial pressure (mmHg)	0.017	0.854	0.335	0.030	0.738	–		
Blood leukocyte count ($\times 10^9/L$)	0.324	<0.001*	3.652	0.315	<0.001*	1.545	0.114	0.125
Blood potassium concentrations (mmol/L)	0.007	0.939	0.157	0.014	0.875	–		
Blood glucose concentrations (mmol/L)	0.212	0.018*	2.579	0.228	0.011*	1.170	0.085	0.245

Notes: Correlations were done using Spearman correlation coefficient and Linear regression model in aneurysmal subarachnoid hemorrhage. The asterisk indicates statistical significance (* $P<0.05$).

Abbreviation: WFNS, World Federation of Neurological Societies Scale.

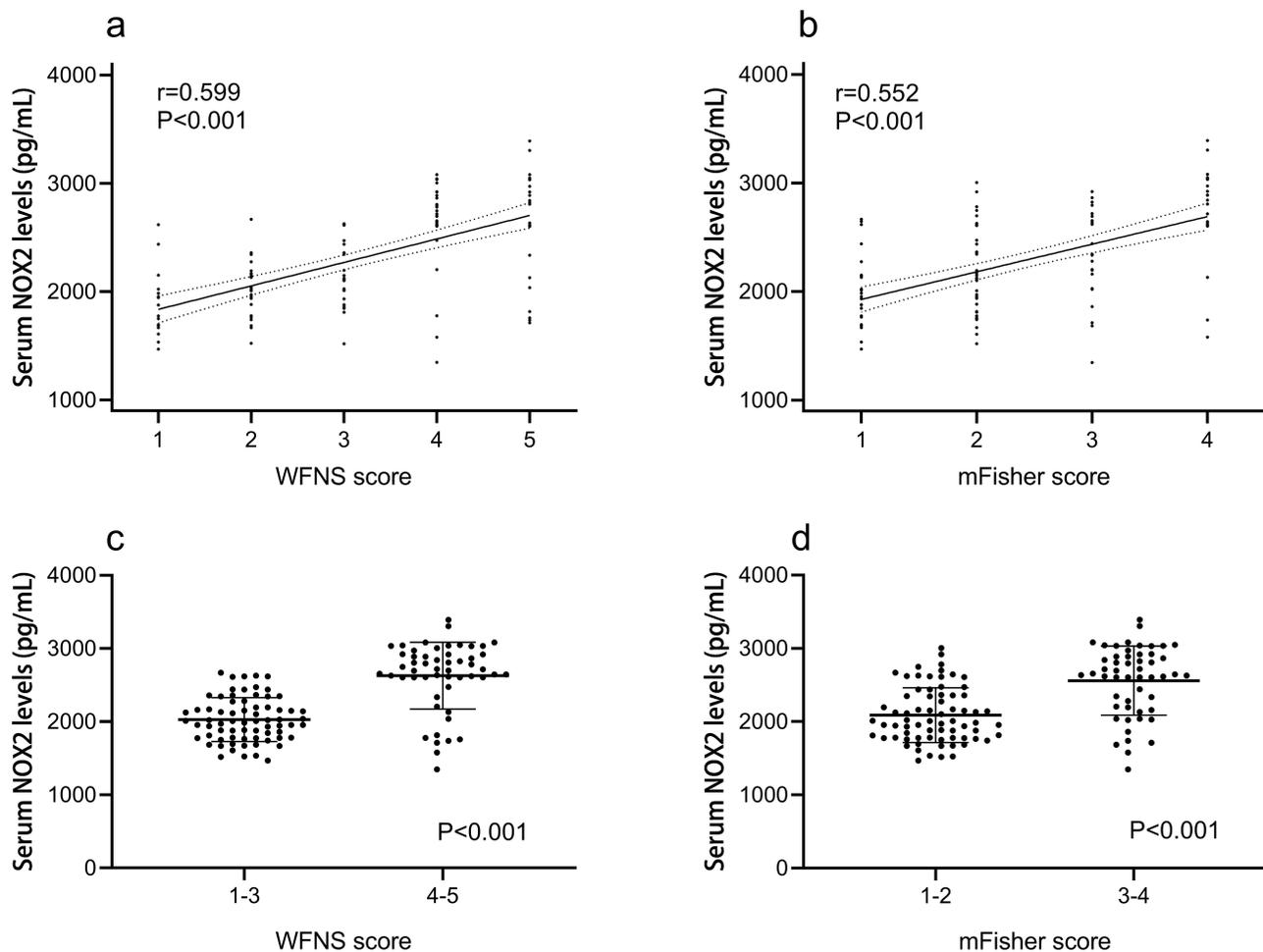


Figure 3 Boxplots and correlograms. (a) Correlograms illustrating the relationship between patients' serum NOX2 levels and WFNS score, using the Spearman correlation coefficient. (b) Correlograms illustrating the relationship between patients' serum NOX2 levels and mFisher score, using the Spearman correlation coefficient. (c) Boxplots illustrating serum NOX2 levels among subgroups based on WFNS score. Patients were divided into two groups in accordance with WFNS score, namely, 1–3 ($n=70$) and 4–5 ($n=53$). The intergroup comparison of data was done using Mann–Whitney U -test. (d) Boxplots illustrating serum NOX2 levels among subgroups based on mFisher score. Patients were divided into two groups in accordance with mFisher score, namely, 1–2 ($n=71$) and 3–4 ($n=52$). The intergroup comparison of data was done using Mann–Whitney U -test.

Relationship Between Serum NOX2 Levels and DCI

A total of 34 patients suffered from DCI. And serum NOX2 levels were significantly higher in patients with the development of DCI than in those who did not (Figure 5). In Figure 6, serum NOX2 levels effectively predicted the occurrence of DCI after aSAH (AUC, 0.730; 95% CI, 0.642–0.806) with a cut-off value of 2603.83 pg/mL being selected using Youden method. Serum NOX2 levels >2603.83 pg/mL discriminated patients at risk of DCI with sensitivity of 67.65% and specificity of 76.40% (maximum Youden index J , 0.4405). Interestingly, serum NOX2 levels had similar predictive ability, as compared to the mFisher score (AUC, 0.708; 95% CI, 0.619–0.786, $P=0.715$) and WFNS score (AUC, 0.776; 95% CI, 0.692–0.846; $P=0.467$). Subsequently, we constructed a combined binary logistic regression model using serum NOX2 levels, WFNS score and mFisher score. And it was found that the new combined model (AUC, 0.796, 95% CI 0.714–0.863) displayed significantly higher predictive ability for DCI than mFisher score ($P=0.028$) (Figure 7).

In Table 4, the proportion of patients with serum NOX2 levels >2603.83 pg/mL was significantly higher in DCI subgroup than in non-DCI subgroup ($\chi^2=20.779$, $P<0.001$). Alternatively, the WFNS score ($Z=-4.830$, $P<0.001$), mFisher score ($Z=-3.687$, $P<0.001$), blood leucocyte count ($Z=-2.161$, $P=0.031$), blood glucose levels ($Z=-2.033$, $P=0.042$), percentage of acute hydrocephalus ($\chi^2=7.016$, $P=0.008$) and percentage of intraventricular bleeding ($\chi^2=7.479$, $P=0.005$) were significantly increased in patients who experienced DCI, compared with those who did not (Table 4). Consistently, the above-mentioned associations were confirmed using univariate logistic regression model. Moreover, the

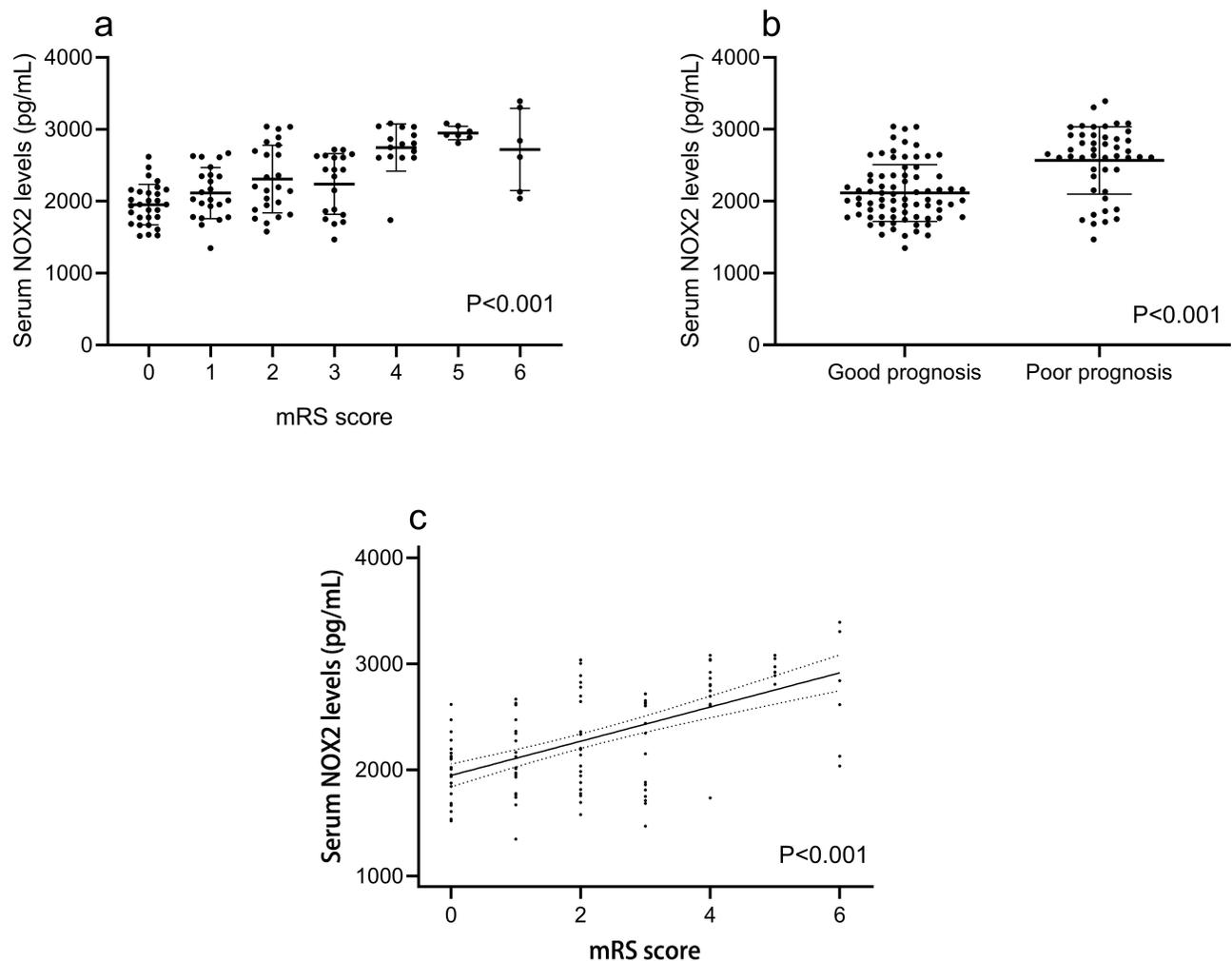


Figure 4 Boxplots and correlograms. (a) Correlograms illustrating the relationship between patients' serum NOX2 levels and modified Rankin scale, using the Spearman correlation coefficient. (b) Boxplots illustrating serum NOX2 levels among patients with different modified Rankin scale score. Patients were divided into seven groups in accordance with modified Rankin scale score. Data were compared among multiple groups using Kruskal–Wallis H-test. (c) Boxplots illustrating serum NOX2 levels between two groups named good prognosis and poor prognosis. A poor prognosis was defined as modified Rankin scale score of 3–6. The data of two groups were compared using Mann–Whitney *U*-test.

preceding significant variables in univariate analysis were entered in the multivariable logistic regression model and subsequently it was revealed WFNS score (OR=1.850; 95% CI, 1.061–3.226; $P=0.030$) and serum NOX2 levels >2603.83 pg/mL (OR=2.978; 95% CI, 1.008–8.803; $P=0.048$) emerged as the two independent predictors of DCI (Table 5).

Relationship Between Serum NOX2 Levels and 90-Day Functional Outcome After aSAH

A total of 47 patients had a poor prognosis (mRS score 3–6) at 90 days after aSAH. Interestingly, serum NOX2 levels were significantly higher in patients with poor prognosis than those with good prognosis ($P<0.001$). In addition to this, serum NOX2 levels substantially distinguished the occurrence of 90-day poor prognosis after aSAH (AUC, 0.760; 95% CI, 0.675–0.833). Furthermore, using Youden method, a cut-off value of 2364.86 pg/mL was chosen. And serum NOX2 levels >2364.86 pg/mL discriminated patients at risk of a poor prognosis at 90 days after stroke, with the sensitivity of 74.47%, and the specificity of 78.95% (maximum Youden index *J*, 0.5342). The predictive ability of serum NOX2 levels was similar to those of the mFisher score (AUC, 0.802; 95% CI, 0.721–0.869, $P=0.387$) and WFNS score (AUC, 0.785; 95% CI, 0.702–0.854, $P=0.590$) (Figure 8). Interestingly, AUC of serum NOX2 levels combined with WFNS score and

Table 3 Correlation Between Modified Rankin Scale and Other Variables Using Spearman Correlation Coefficient and Linear Regression Analysis in Aneurysmal Subarachnoid Hemorrhage

Variable	r	P value	Univariate Analysis			Multivariate Analysis		
			t	Beta	P value	t	Beta	P value
Gender (male/female)	0.020	0.823	0.187	0.017	0.852	–		
Age (years)	0.090	0.321	0.607	0.055	0.545	–		
Cigarette smoking	0.035	0.703	0.300	0.027	0.765	–		
Alcohol consumption	–0.053	0.562	–0.512	–0.046	0.610	–		
Hypertension	0.081	0.374	0.847	0.077	0.399	–		
Diabetes mellitus	0.014	0.876	0.221	0.020	0.825	–		
Hyperlipidemia	0.065	0.473	0.788	0.071	0.432	–		
WFNS score	0.660	<0.001*	9.930	0.670	<0.001*	2.943	0.293	0.004*
Modified Fisher score	0.614	<0.001*	9.208	0.642	<0.001*	2.076	0.200	0.040*
Aneurysmal position (posterior / anterior circulation)	–0.157	0.084	–1.680	–0.151	0.095	–		
Aneurysmal shape (cystic/others)	–0.013	0.889	–0.515	–0.047	0.608	–		
Aneurysmal diameter (<10 mm/≥10 mm)	0.053	0.562	0.761	0.069	0.448	–		
Surgery (clipping/ endovascular intervention)	0.035	0.704	0.551	0.050	0.582	–		
Intraventricular bleeding	0.394	<0.001*	5.057	0.418	<0.001*	1.430	0.104	0.155
Acute hydrocephalus	0.267	0.003*	3.408	0.296	0.001*	1.678	0.112	0.096
External ventricular drain	0.129	0.155	1.774	0.159	0.079	–		
Admission time (h)	–0.113	0.215	–1.304	–0.118	0.195	–		
Blood-collection time (h)	–0.124	0.173	–1.447	–0.130	0.150	–		
Systolic arterial pressure (mmHg)	–0.084	0.357	–0.862	–0.078	0.390	–		
Diastolic arterial pressure (mmHg)	0.045	0.623	0.199	0.018	0.842	–		
Blood leucocyte count ($\times 10^9/L$)	0.324	<0.001*	3.502	0.303	0.001*	0.808	0.053	0.421
Blood potassium concentrations (mmol/L)	–0.079	0.385	–0.799	–0.072	0.426	–		
Blood glucose concentrations (mmol/L)	0.222	0.014*	3.154	0.276	0.002*	1.253	0.081	0.213
Serum NOX2 levels (pg/mL)	0.563	<0.001*	8.071	0.592	<0.001*	2.567	0.211	0.012*

Notes: Correlations were done using Spearman correlation coefficient and Linear regression model in aneurysmal subarachnoid hemorrhage. The asterisk indicates statistical significance (* $P < 0.05$).

Abbreviations: WFNS, World Federation of Neurological Societies Scale; NOX2, NADPH oxidase 2.

mFisher score (AUC, 0.834, 95% CI 0.757–0.895) significantly exceeded that of WFNS score ($P = 0.383$) or serum NOX2 levels ($P = 0.363$) alone (Figure 9).

As compared to good prognosis patients, those with poor prognosis had significantly elevated percentage of serum NOX2 levels > 2364.86 pg/mL ($\chi^2 = 34.139$, $P < 0.001$). Alternatively, the WFNS score ($Z = -5.420$, $P < 0.001$), mFisher score ($Z = -5.836$, $P < 0.001$), blood white leucocyte count ($Z = -3.394$, $P = 0.001$), blood glucose levels ($Z = -2.788$,

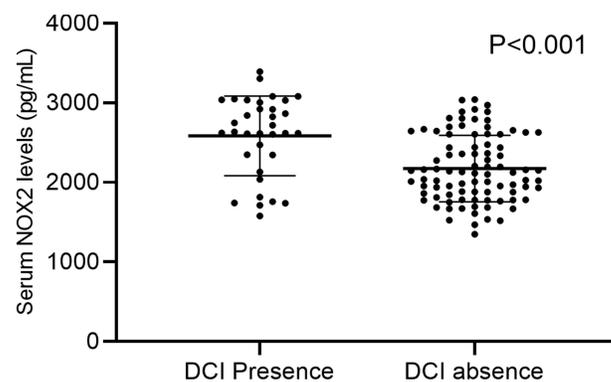


Figure 5 Boxplot illustrating serum NOX2 levels between patients with DCI and without DCI ($P < 0.001$). Data were reported as median (upper-lower quartiles) and two groups were compared using the Mann–Whitney U -test.

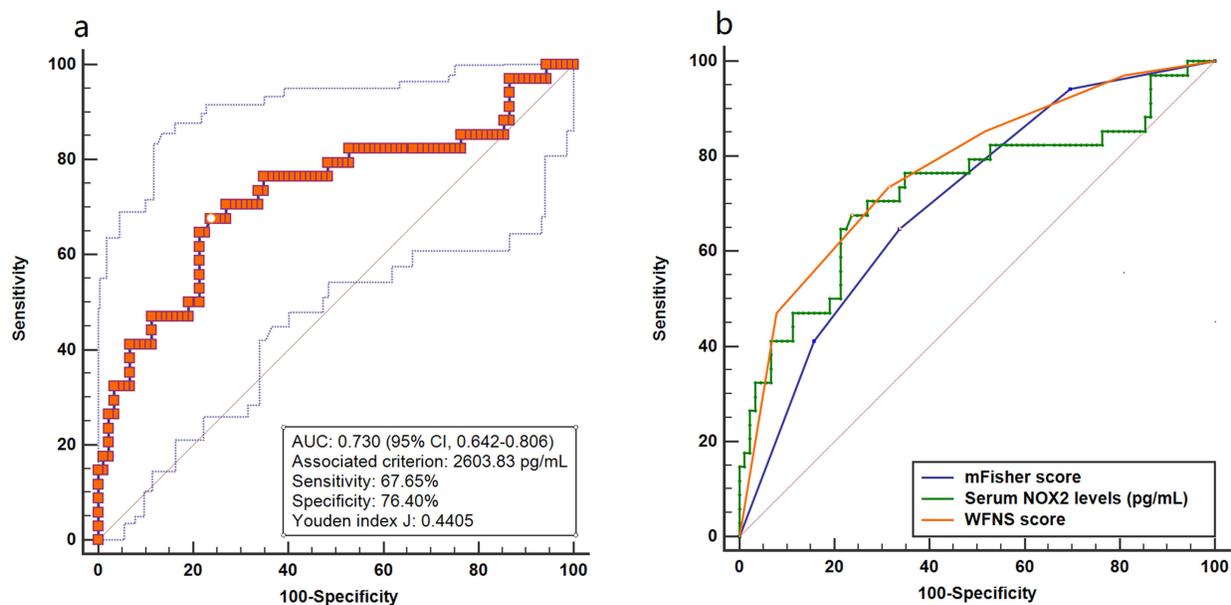


Figure 6 Receiver operating characteristic curve. (a) Receiver operating characteristic curve for serum NOX2 levels used to predict DCI after aneurysmal subarachnoid hemorrhage. (b) Receiver operating characteristic curve for serum NOX2 levels, WFNS score and mFisher score used to predict DCI after aneurysmal subarachnoid hemorrhage. The predictive ability of serum NOX2 levels was similar to those of WFNS score and mFisher score.

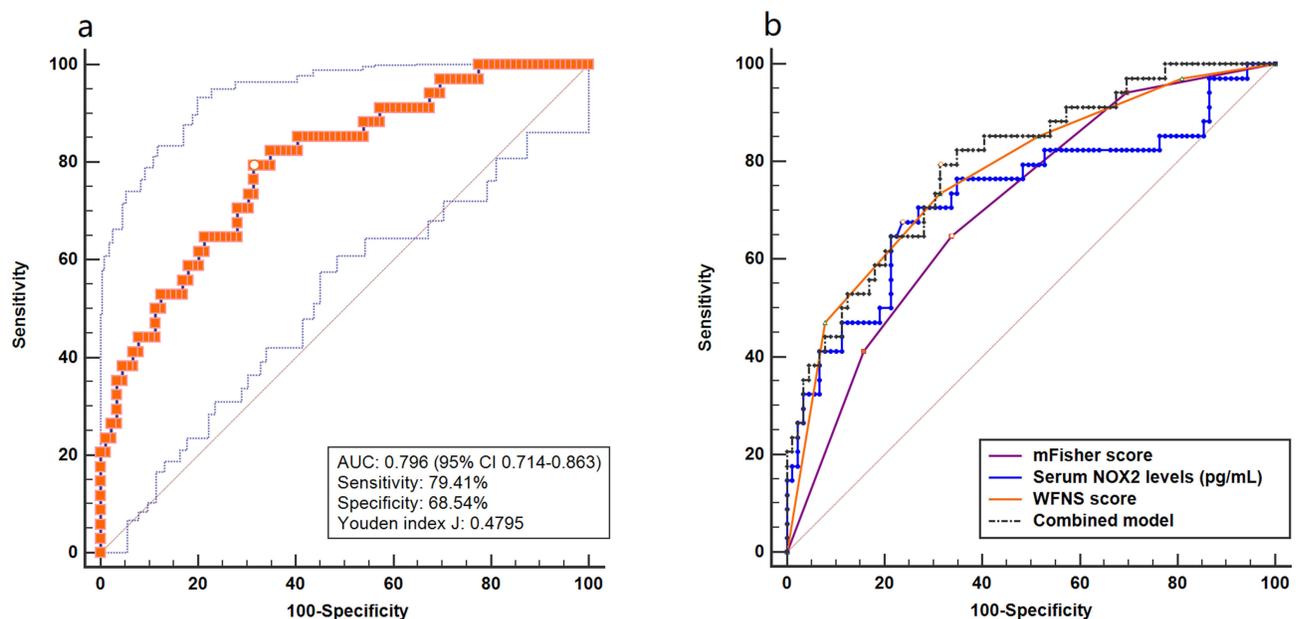


Figure 7 Receiver operating characteristic curve. (a) Receiver operating characteristic curve for the combined model used to predict DCI after aneurysmal subarachnoid hemorrhage. (b) Receiver operating characteristic curve for the combined model, serum NOX2 levels, WFNS score and mFisher score used to predict DCI after aneurysmal subarachnoid hemorrhage. The predictive ability of the combined model was similar to those of serum NOX2 levels and WFNS score, and had higher predictive ability compared with mFisher score.

$P=0.005$), percentage of acute hydrocephalus ($\chi^2=5.923$, $P=0.015$) and percentage of intraventricular bleeding ($\chi^2=8.685$, $P=0.003$) were significantly higher in patients with poor prognosis than in those with good prognosis (Table 6). The above-mentioned substantial associations were also demonstrated using univariate logistic regression analysis. Moreover, those significant variables were forced in the binary logistic regression model, and subsequently mFisher score (OR=2.088; 95% CI, 1.092–3.992; $P=0.026$) and serum NOX2 levels >2364.86 pg/mL (OR=5.133; 95% CI, 1.723–15.291; $P=0.003$) retained to be independently associated with poor prognosis at 90 days after stroke (Table 7).

Table 4 Factors Associated with Delayed Cerebral Ischemia in Aneurysmal Subarachnoid Hemorrhage

Variable	Delayed Cerebral Ischemia		Z	χ^2	P value
	Presence (n=34)	Absence (n=89)			
Gender (male/female)	18/16	37/52	–	0.000	0.995
Age (years)	55.5±11.2	54.1±11.1	–0.631	–	0.528
Cigarette smoking	8 (23.5%)	17 (19.1%)	–	0.298	0.585
Alcohol consumption	7 (20.6%)	25 (28.1%)	–	0.719	0.396
Hypertension	8 (23.5%)	16 (18.0%)	–	0.483	0.487
Diabetes mellitus	4 (11.8%)	11 (12.4%)	–	0.008	0.928
Hyperlipidemia	9 (26.5%)	12 (13.5%)	–	2.931	0.087
WFNS score	4 (3–5)	3 (2–4)	–4.830	–	<0.001*
Modified Fisher score	3 (2–4)	2 (1–3)	–3.687	–	<0.001*
Aneurysmal position (posterior / anterior circulation)	24/10	73/16	–	1.929	0.165
Aneurysmal shape (cystic/others)	29/5	73/16	–	0.186	0.666
Aneurysmal diameter (<10 mm/≥10 mm)	20/14	53/36	–	0.005	0.941
Surgery (clipping/ endovascular intervention)	16/18	34/55	–	0.800	0.371
Intraventricular bleeding	11 (32.4%)	10 (11.2%)	–	7.479	0.005*
Acute hydrocephalus	10 (29.4%)	9 (10.1%)	–	7.016	0.008*
External ventricular drain	7 (20.6%)	11 (12.4%)	–	1.333	0.248
Admission time (h)	7.0 (3.8–12.3)	7.0 (5.0–11.0)	–0.697	–	0.710
Blood-collection time (h)	7.5 (4.5–13.3)	7.5 (5.4–11.9)	–0.791	–	0.736
Systolic arterial pressure (mmHg)	147 (135–155)	150 (133–163)	–1.062	–	0.207
Diastolic arterial pressure (mmHg)	89.0±13.6	86.1±14.6	–0.010	–	0.311
Blood leucocyte count ($\times 10^9/L$)	9.9±3.1	8.6±2.6	–2.161	–	0.031*
Blood potassium concentrations (mmol/L)	3.76±0.53	3.81±0.40	–0.559	–	0.549
Blood glucose concentrations (mmol/L)	8.5 (6.0–14.6)	7.1 (5.3–8.8)	–2.033	–	0.042*
Serum NOX2 levels > 2603.83 pg/mL	23 (67.6%)	21 (23.6%)	–	20.779	<0.001*

Notes: Qualitative variables were presented as counts (percentages) and were compared for intergroup difference using chi-square test or Fisher exact test as appropriate. Quantitative variables were summarized as medians (Upper - lower quartiles) or the means \pm standard deviations as appropriate. Intergroup comparisons were done using unpaired Student *t*-test or Mann–Whitney *U*-test where appropriate. The asterisk indicates statistical significance (**P*<0.05).

Abbreviations: WFNS, World Federation of Neurological Societies Scale; NOX2, NADPH oxidase 2.

Table 5 Univariate and Multivariate Logistic Regression Analysis of Predictors for Delayed Cerebral Ischemia After Aneurysmal Subarachnoid Hemorrhage

Variable	Univariate Analysis		Multivariate Analysis	
	OR (95% CI)	P value	OR (95% CI)	P value
Gender (male/female)	0.632 (0.286–1.400)	0.258	–	–
Age (years)	1.012 (0.976–1.049)	0.524	–	–
Cigarette smoking	1.303 (0.503–3.378)	0.586	–	–
Alcohol consumption	0.664 (0.256–1.718)	0.398	–	–
Hypertension	0.712 (0.273–1.859)	0.488	–	–
Diabetes mellitus	0.945 (0.279–3.400)	0.928	–	–
Hyperlipidemia	2.310 (0.871–6.124)	0.092	–	–
WFNS score	2.407 (1.626–3.563)	<0.001*	1.850 (1.061–3.226)	0.030*
Modified Fisher score	2.081 (1.388–3.120)	<0.001*	0.825 (0.441–1.543)	0.547
Aneurysmal position (posterior / anterior circulation)	0.526 (0.211–1.313)	0.169	–	–
Aneurysmal shape (cystic/others)	1.271 (0.426–3.790)	0.667	–	–
Aneurysmal diameter (<10 mm/≥10 mm)	0.970 (0.434–2.167)	0.941	–	–
Surgery (clipping/ endovascular intervention)	0.695 (0.313–1.544)	0.372	–	–
Intraventricular bleeding	3.788 (1.426–10.009)	0.007*	1.100 (0.302–4.008)	0.885

(Continued)

Table 5 (Continued).

Variable	Univariate Analysis		Multivariate Analysis	
	OR (95% CI)	P value	OR (95% CI)	P value
Acute hydrocephalus	3.704 (1.350–10.164)	0.011*	3.067 (0.815–11.541)	0.097
External ventricular drain	1.838 (0.647–5.221)	0.253	–	–
Admission time (h)	1.000 (0.931–1.073)	0.991	–	–
Blood-collection time (h)	1.000 (0.933–1.071)	0.996	–	–
Systolic arterial pressure (mmHg)	0.989 (0.969–1.010)	0.314	–	–
Diastolic arterial pressure (mmHg)	1.015 (0.986–1.044)	0.311	–	–
Blood leucocyte count ($\times 10^9/L$)	1.186 (1.024–1.374)	0.023*	1.062 (0.903–1.250)	0.467
Blood potassium concentrations (mmol/L)	0.780 (0.311–1.956)	0.596	–	–
Blood glucose concentrations (mmol/L)	1.113 (1.015–1.222)	0.023*	1.052 (0.938–1.179)	0.386
Serum NOX2 levels > 2364.86 pg/mL	6.771 (2.839–16.149)	<0.001*	2.978 (1.008–8.803)	0.048*

Notes: Results were presented as odds ratio (95% confidence interval) using the univariate and multivariate logistic regression analysis. The asterisk indicates statistical significance (* $P < 0.05$).

Abbreviations: CI, confidence interval; WFNS, World Federation of Neurological Societies Scale; NOX2, NADPH oxidase 2.

Discussion

aSAH is a life-threatening critical illness in neurologic field, wherein DCI secondary to cerebral vasospasm is the most common and serious complication. Clinically, the WFNS score and mFisher score are very frequently used to assess disease severity and predict functional outcome and DCI occurrence after aSAH.¹⁵ During decades, researchers have focused on the prognostic role of circulating biomarkers in aSAH. In the current study, besides an interesting finding that there was a significantly higher serum NOX2 levels in aSAH patients than in healthy controls, we found that (1) serum NOX2 levels showed a significant positive correlation with mFisher score and WFNS score; (2) serum NOX2 levels were independently associated with DCI and 90-day poor prognosis after aSAH; (3) serum NOX2 levels had a significantly high predictive ability for DCI and poor 90-day prognosis, and its predictive ability was similar to those of mFisher score and WFNS score. In conclusion, elevated serum NOX2 levels were strongly correlated with hemorrhage severity and

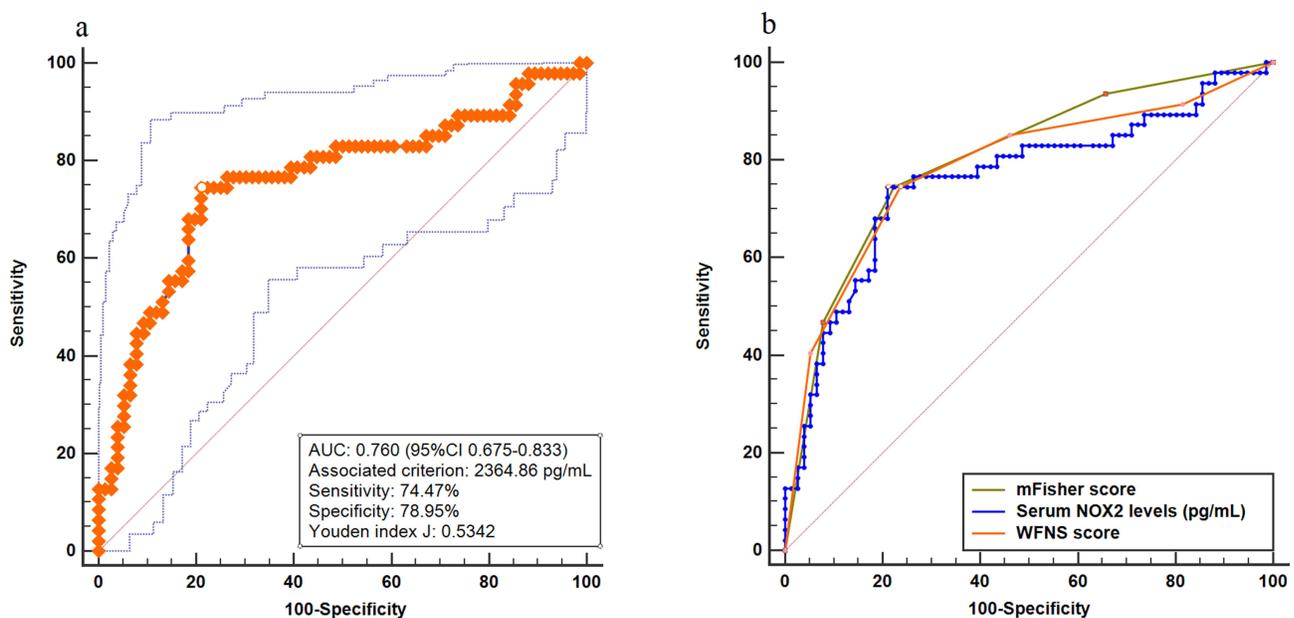


Figure 8 Receiver operating characteristic curve. (a) Receiver operating characteristic curve for serum NOX2 levels used to predict 90-day poor prognosis after aneurysmal subarachnoid hemorrhage. (b) Receiver operating characteristic curve for serum NOX2 levels, WFNS score and mFisher score used to predict 90-day poor prognosis after aneurysmal subarachnoid hemorrhage. The predictive ability of serum NOX2 levels was similar to those of WFNS score and mFisher score.

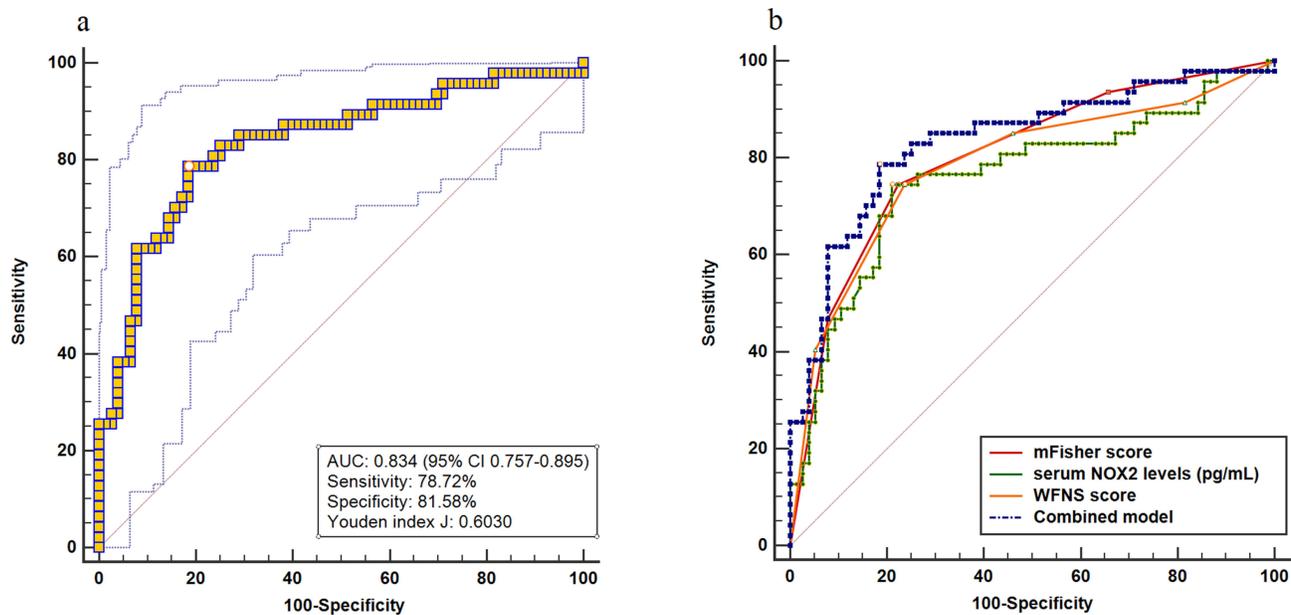


Figure 9 Receiver operating characteristic curve. (a) Receiver operating characteristic curve for the combined model used to predict 90-day poor prognosis after aneurysmal subarachnoid hemorrhage. (b) Receiver operating characteristic curve for the combined model, serum NOX2 levels, WFNS score and mFisher score used to predict 90-day poor prognosis after aneurysmal subarachnoid hemorrhage. The predictive ability of the combined model was similar to those of mFisher score and had higher predictive ability compared with serum NOX2 levels and WFNS score.

independently associated with functional outcome and DCI, suggesting that serum NOX2 may be a potential prognostic biomarker for aSAH. Compelling evidence has shown that inflammatory response and oxidative stress play important roles in disease progression of aSAH and are also associated with functional outcome and DCI. The pathophysiological process of DCI involves microcirculatory constriction, microthrombosis, cortical diffusion ischemia and delayed apoptosis, which result from neuroinflammation and oxidative damage after aSAH.^{16,17}

Table 6 Factors Associated with 90-Day Poor Prognosis in Aneurysmal Subarachnoid Hemorrhage

Variable	90-Day Poor Prognosis		Z	χ^2	P value
	Presence (n=47)	Absence (n=76)			
Gender (male/female)	21/26	34/42	–	1.286	0.257
Age (years)	55.2±12.0	54.0±10.6	–0.859	–	0.390
Cigarette smoking	10 (21.3%)	15 (19.7%)	–	0.043	0.837
Alcohol consumption	8 (17.0%)	24 (31.6%)	–	3.197	0.074
Hypertension	9 (19.1%)	15 (19.7%)	–	0.006	0.936
Diabetes mellitus	7 (14.9%)	8 (10.5%)	–	0.517	0.472
Hyperlipidemia	9 (19.1%)	12 (15.8%)	–	0.231	0.630
WFNS score	4 (3–5)	2 (2–3)	–5.420	–	<0.001*
Modified Fisher score	3 (2–4)	2 (1–2)	–5.836	–	<0.001*
Aneurysmal position (posterior / anterior circulation)	34/13	63/13	–	1.929	0.164
Aneurysmal shape (cystic/others)	38/9	64/12	–	0.186	0.630
Aneurysmal diameter (<10 mm/≥10 mm)	29/18	44/32	–	0.005	0.676
Surgery (clipping/ endovascular intervention)	19/28	31/45	–	0.800	0.968
Intraventricular bleeding	14 (29.8%)	7 (9.2%)	–	8.685	0.003*
Acute hydrocephalus	12 (25.5%)	7 (9.2%)	–	5.923	0.015*
External ventricular drain	8 (17.0%)	10 (13.2%)	–	0.347	0.556
Admission time (h)	7.0 (4.0–11.0)	7.0 (5.0–11.0)	–0.697	–	0.486

(Continued)

Table 6 (Continued).

Variable	90-Day Poor Prognosis		Z	χ^2	P value
	Presence (n=47)	Absence (n=76)			
Blood-collection time (h)	7.2 (4.5–12.0)	8.1 (5.2–12.0)	–0.791	–	0.429
Systolic arterial pressure (mmHg)	147 (129–157)	149 (137–163)	–1.062	–	0.288
Diastolic arterial pressure (mmHg)	86.6±14.4	87.1±14.4	–0.010	–	0.992
Blood leucocyte count ($\times 10^9/L$)	10.1±3.0	8.3±2.4	–3.394	–	0.001*
Blood potassium concentrations (mmol/L)	3.81±0.53	3.78±0.37	–0.034	–	0.973
Blood glucose concentrations (mmol/L)	8.20 (6.40–13.60)	7.10 (5.30–8.80)	–2.788	–	0.005*
Serum NOX2 levels > 2364.86 pg/mL	35 (74.5%)	16 (21.1%)	–	34.139	<0.001*

Notes: Qualitative variables were presented as counts (percentages) and were compared for intergroup difference using chi-square test or Fisher exact test as appropriate. Quantitative variables were summarized as medians (upper - lower quartiles) or the means \pm standard deviations as appropriate. Intergroup comparisons were done using unpaired Student t-test or Mann-Whitney U-test where appropriate. Modified Rankin scale of 3–6 was designated as poor prognosis. The asterisk indicates statistical significance (* $P < 0.05$).

Abbreviations: WFNS, World Federation of Neurological Societies Scale; NOX2, NADPH oxidase 2.

Table 7 Univariate and Multivariate Logistic Regression Analysis of Predictors for 90-Day Poor Prognosis After Aneurysmal Subarachnoid Hemorrhage

Variable	Univariate Analysis		Multivariate Analysis	
	OR (95% CI)	P value	OR (95% CI)	P value
Gender (male/female)	1.002 (0.482–2.083)	0.995	–	–
Age (years)	1.010 (0.977–1.044)	0.548	–	–
Cigarette smoking	1.099 (0.448–2.699)	0.837	–	–
Alcohol consumption	0.444 (0.180–1.095)	0.078	–	–
Hypertension	1.038 (0.414–2.606)	0.936	–	–
Diabetes mellitus	1.487 (0.502–4.411)	0.474	–	–
Hyperlipidemia	1.263 (0.487–3.276)	0.631	–	–
WFNS score	2.551 (1.768–3.681)	<0.001*	1.038 (0.603–1.787)	0.893
Modified Fisher score	3.557 (2.246–5.633)	<0.001*	2.088 (1.092–3.992)	0.026*
Aneurysmal position (posterior / anterior circulation)	0.540 (0.225–1.294)	0.167	–	–
Aneurysmal shape (cystic/others)	0.792 (0.305–2.053)	0.631	–	–
Aneurysmal diameter (<10 mm/≥10 mm)	1.172 (0.557–2.465)	0.676	–	–
Surgery (clipping/ endovascular intervention)	1.015 (0.484–2.129)	0.968	–	–
Intraventricular bleeding	4.182 (1.542–11.340)	0.005*	0.879 (0.196–3.931)	0.866
Acute hydrocephalus	3.380 (1.222–9.344)	0.019*	3.491 (0.824–14.796)	0.090
External ventricular drain	1.354 (0.493–3.719)	0.557	–	–
Admission time (h)	0.975 (0.912–1.042)	0.455	–	–
Blood-collection time (h)	0.972 (0.911–1.038)	0.396	–	–
Systolic arterial pressure (mmHg)	0.988 (0.970–1.008)	0.233	–	–
Diastolic arterial pressure (mmHg)	0.998 (0.972–1.023)	0.849	–	–
Blood leucocyte count ($\times 10^9/L$)	1.286 (1.109–1.491)	0.001*	1.166 (0.981–1.386)	0.082
Blood potassium concentrations (mmol/L)	1.174 (0.511–2.698)	0.705	–	–
Blood glucose concentrations (mmol/L)	1.140 (1.038–1.251)	0.006*	1.102 (0.972–1.250)	0.131
Serum NOX2 levels > 2364.86 pg/mL	10.937 (4.643–25.763)	<0.001*	5.133 (1.723–15.291)	0.003*

Notes: Results were presented as odds ratio (95% confidence interval) using the univariate and multivariate logistic regression analysis. Modified Rankin scale of 3–6 was designated as poor prognosis. The asterisk indicates statistical significance (* $P < 0.05$).

Abbreviations: CI, confidence interval; WFNS, World Federation of Neurological Societies Scale; NOX2, NADPH oxidase 2.

NOX2, as a major source of ROS, is mainly expressed in normal cerebral cortex and hippocampal neurons of animals. After experimental brain injury, including acute ischemic stroke, traumatic brain injury and aSAH, NOX2 levels in brain tissues were significantly elevated.¹⁸ In several clinical studies enrolling a small sample of patients with Nasu-Hakola

disease, TBI or aSAH, NOX2 expression by neuron and glia were significantly up-regulated in injured brain tissues.^{9,12,19,20} In peripheral blood of humans with brain injury, NOX2 levels were substantially elevated. Specifically, serum NOX2 levels were significantly higher in patients with neurodegenerative disease compared to healthy controls.²¹ Our study included more than 100 patients and found a significant elevation of serum NOX2 levels after aSAH. Clearly, blood–brain barrier is greatly disrupted after acute brain injury. Thus, it is deduced that NOX2 in the peripheral blood may be derived from the injured brain tissues. Nevertheless, in a controlled study of 22 patients with TBI, NOX2 expression was significantly elevated in blood leukocytes compared to controls.²⁰ Hence, it is possible that NOX2 in the peripheral blood of patients with acute brain injury may be partially released from peripheral blood cells.

Up to now, data have been supportive of the notion that NOX2 may exacerbate brain injury via aggravating oxidative stress.^{22,23} In an *in vitro* ischemic stroke model, NOX2 promoted the generation and accumulation of ROS in neurons, as well as the death of reoxidized neurons. In mice with acute cerebral ischemia, NOX2 knockout substantially reduced infarct size and lessened blood–brain barrier damage.²⁴ Similarly, NOX2 knockout mice showed a significant decrease in hematoma volume, brain edema, neurological deficits, and mortality after intracerebral hemorrhage.^{25,26} Also, application of NOX2 inhibitors to head-traumatized mice obviously resulted in a significant decrease in ROS expression of brain tissues, improved motor and cognitive function, and reduced damage volume.²⁷ Thus, NOX2 may exacerbate brain tissue and nerve damage after brain injury by producing excess ROS.

Since a previous clinical investigation showed that raised NOX2 expressions in brain tissues had a significant negative correlation with GCS scores and GOS scores of TBI patients,¹² no studies have analyzed the relationship between circulating NOX2 levels and severity plus prognosis of humans with acute brain injury. Using multivariate analysis, we found that serum NOX2 levels after aSAH were independently correlated with WFNS score, mFisher score, 90-day mRS scores; and independently discriminated patients at risk of DCI and poor prognosis (mRS score of 3–6). In addition, serum NOX2 level, WFNS score and mFisher score had similar predictive ability for DCI as well as poor prognosis. Intriguingly, serum NOX2 level combined with WFNS score and mFisher score showed significantly elevated prognostic ability, in comparison to WFNS score or mFisher score. In conclusion, serum NOX2 may be a potential biomarker to predict DCI and functional outcome, and assess disease severity after aSAH.

The current study has several limitations. First, although we enrolled 123 aSAH patients in this study, this was a single-center study, which is characterized by a small sample size and therefore, a larger cohort study is required to validate the current conclusions. Second, serum NOX2 levels were only tested at admission time. Hence, investigation of its dynamic change in future may be of clinical significance.

Conclusion

To the best of our knowledge, this is the first series for using multifactorial analysis to discern the relationship between serum NOX2 levels and hemorrhage severity, DCI, and long-term functional outcome after aSAH. In the current study, serum NOX2 levels are independently associated with WFNS score, mFisher score, DCI, and 90-day poor prognosis, suggesting that serum NOX2 levels may be highly correlated with hemorrhage severity, DCI, and long-term functional outcome. Thus, serum NOX2 may be a potential biomarker for assessing hemorrhage severity and predicting DCI and 90-day functional outcome after aSAH.

Abbreviations

aSAH, aneurysmal subarachnoid hemorrhage; WFNS, World Federation of Neurological Surgeons scale; mFisher, modified Fisher; mRS, Modified Rankin scale; CT, computerized tomography; ROC, receiver operating characteristic; AUC, area under curve; 95% CI, 95% confidence interval; ROS, reactive oxygen species.

Data Sharing Statement

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

Ethics Statement

This study was carried out in accordance with the ethical guidelines of the Helsinki Declaration, and its protocol was approved by the Institutional Review Committee of the Affiliated Hangzhou First People's Hospital, Zhejiang University School of Medicine (Opinion number: Medical Ethics Review No. (058)-01). Relatives of patients and controls themselves gave written informed consent to participate in this study.

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Disclosure

The authors declare that they have no competing interests.

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