BLOOD-BASED BIOMARKERS IN ACUTE ISCHEMIC STROKE AND HEMORRHAGIC STROKE

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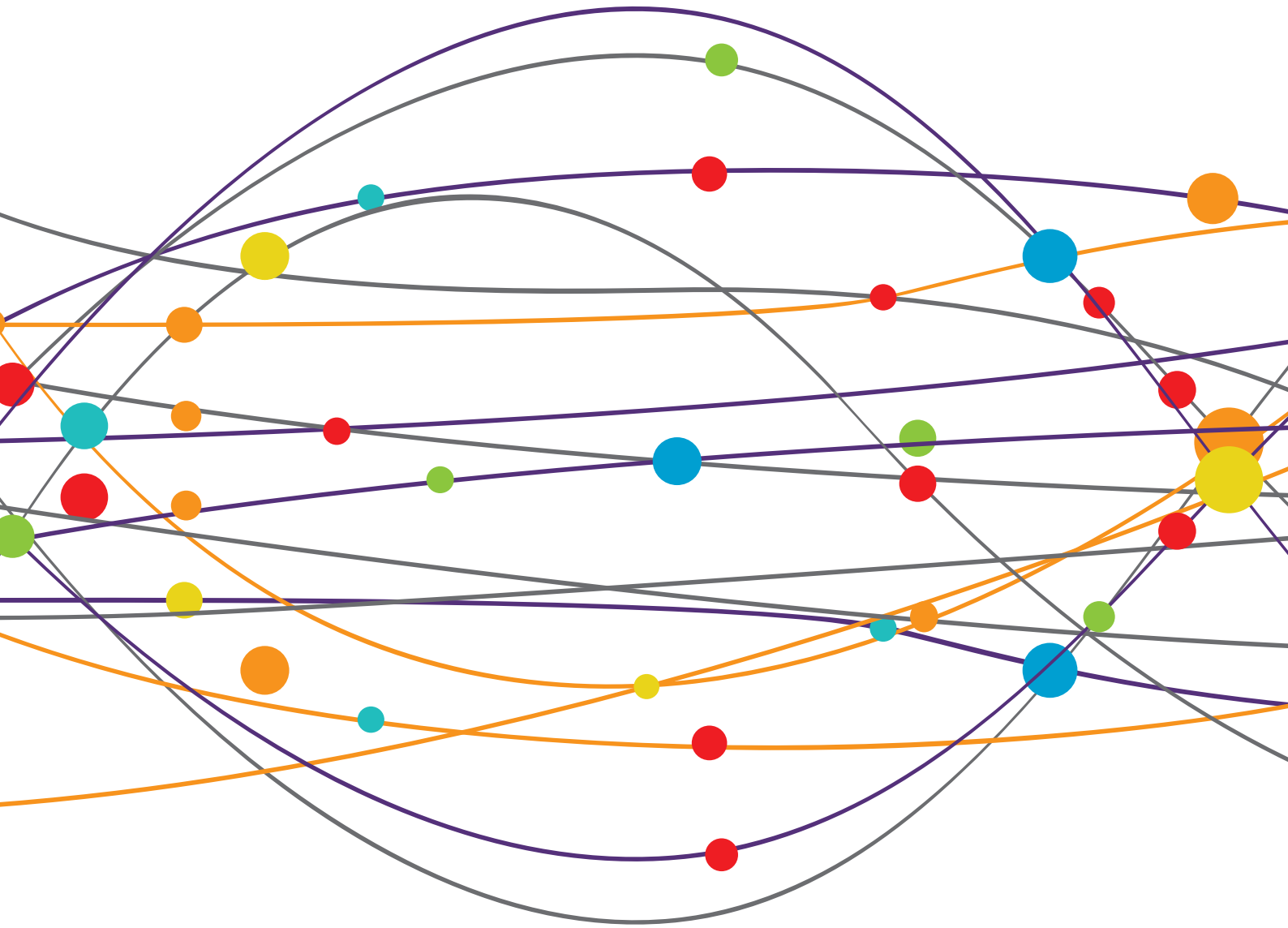

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BLOOD-BASED BIOMARKERS IN ACUTE ISCHEMIC STROKE AND HEMORRHAGIC STROKE

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EDITORIAL
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Editorial: Blood-Based Biomarkers in Acute Ischemic Stroke and Hemorrhagic Stroke

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Keywords: ischemic stroke (IS), hemorrhagic stroke, differential diagnosis, stroke etiology and classification, prognosis

Editorial on the Research Topic

Blood-Based Biomarkers in Acute Ischemic Stroke and Hemorrhagic Stroke

Stroke is one of the leading causes of death and disability worldwide (1). The application of acute treatment strategies is limited by several factors such as a narrow time window of reperfusion treatments in ischemic stroke as well as an incomplete understanding of biologic mechanisms of secondary brain damage; blood-based biomarkers might inform on local and systemic pathophysiological processes, assist in patient selection for treatments, and thus support clinical decision-making in the acute phase.

Emerging reperfusion therapies in acute ischemic stroke (AIS) as well as evolving strategies to reverse anticoagulation in hemorrhagic stroke require early differentiation of stroke type. Frequently used emergency imaging such as computed tomography (CT) distinguishes ischemic and hemorrhagic stroke and thereby opens the avenue for recanalization therapies if intracranial hematoma is absent. Nonetheless, such imaging techniques are typically unavailable in the prehospital setting; CT-scans are also not sensitive to show ischemic brain lesions in the hyperacute setting. Experimental investigations proposed microRNA (miRNA) and exosomes as well as metabolites as markers of cerebral ischemia that might support AIS diagnosis. However, translational data in humans are sparse.

Prognostication after stroke depends on the interplay between demographic factors (e.g., age, sex, ethnicity), stroke subtype, and stroke etiology, as well as clinical severity. Persisting disability following ischemic stroke is a result of neuronal death, network dysfunction, and synaptic loss; specific markers of neuronal damage showed superiority over others in predicting functional outcome after stroke (2, 3), whereas cardiac markers indicating comorbidities such as atrial fibrillation showed only utility in prediction of mortality (4). Moreover, a first ischemic stroke is associated with increased risk of further cerebrovascular and other events, calling for the unraveling of stroke etiology in order to select patient-dependent appropriate secondary preventive medication. The additional consideration of biological information through blood biomarkers might improve the prognostic assessment as well as etiologic work-up, as compared to routinely available information, mainly based on purely clinical and imaging information.

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Overall, there is a clear need for experimental as well as translational research on blood-based molecular and cellular biomarkers for differentiation of stroke and stroke mimics, stroke types (AIS vs. hemorrhagic stroke), to guide individual treatment decisions and provide information to patients and relatives. The same applies for the determination of stroke etiology and for the better understanding of secondary neuronal damage in order to develop new treatment paradigms within the hyperacute phase of AIS and hemorrhagic stroke.

microRNAs

microRNAs are small non-coding RNAs with a length of ∼22 nucleotides that regulate gene expression by destabilizing and repression translation of complementary mRNAs. They are characterized by a high expression in mammalian brains and are involved in modulation of excitotoxicity, microglia polarization, oxidative stress, neuronal apoptosis, and oxidative stress, all together mechanisms orchestrating secondary brain damage and thereby regulating functional recovery following AIS (5, 6). There is growing evidence from animal models that miRNA-based treatments with enhancers and inhibitors are able to penetrate the blood-brain barrier (BBB) using specific carriers (e.g., exosomes, liposomes, and lentiviruses) (7) and beneficially modulate brain ischemia. They might therefore be incorporated in novel therapeutic strategies to improve functional outcome in stroke patients by targeting detrimental mechanisms in the hyperacute and subacute phase of stroke.

From a diagnostic point of view, an increasing number of studies have identified stroke-specific patterns of circulating miRNAs that were also associated with symptom severity as well as infarct volume and predicted functional outcome. Patients with AIS showed higher circulating levels of miR-125a-5p, miR-125b-5p, and miRNA-143-3p compared to patients with transient ischemic attack (TIA) or neurologically normal subjects with a return to control levels 2 days after symptom onset, supporting the utility of these miRNAs for early stroke diagnosis in the emergency setting (8). Furthermore, several miRNAs may help in providing prognostic information for consultations with patients and relatives; and guide treatment decisions, as they are associated with functional outcome and mortality following stroke (9, 10).

In a translational approach, Cepparulo et al. compared miRNA levels between animal models of ischemic stroke (middle cerebral artery occlusion—MCAO) and hemorrhagic stroke (collagenase-induced hemorrhagic stroke). They demonstrated upregulation of specific miRNAs as early as 3 h after the procedure in these distinct animal models, pointing toward a potential clinical usage in differentiating ischemic from hemorrhagic stroke in the clinical setting.

OMIC-APPROACH

Besides miRNA, "Omics" reflecting specific pathophysiologic aspects of ischemic as well as hemorrhagic stroke have been investigated as potential biomarkers for stroke diagnosis, differentiation of ischemic vs. hemorrhagic stroke, prediction of functional outcome, and risk of stroke recurrence.

Within this Topic Section collection of articles, Malicek et al. aimed to unravel potential new candidates for differentiation of ischemic and hemorrhagic stroke by using an exploratory proteomic-based pilot study. They identified nine potential candidates connected with the immune system, the coagulation cascade and apoptotic processes. These markers now have to be validated in larger cohorts during the hyperacute phase and with additional analysis such as ELISA, Western Blot, and Mass spectrometry. These efforts aim to shorten treatment delay in stroke patients eligible for recanalization therapies by enabling prehospital differentiation of stroke subtypes.

In order to improve individualized stroke treatment, biomarkers enabling assessment of patients' specific stroke outcome are needed. In this article collection on blood-based biomarkers of acute ischemic and hemorrhagic stroke (11), the usefulness of inflammatory markers (Kirzinger et al.; Li et al.; Sun et al.), markers for oxidative stress (Kuwashiro et al.), vasoactive peptides (Westphal et al.), and BBB function (Müller et al.) are investigated. In addition to these markers reflecting specific pathophysiologic aspects of ischemic/hemorrhagic stroke, individual patient outcome is additionally determined by stroke-associated complications such as infections (Gens et al.; Zhang et al.) and cardiac comorbidities (Lin et al.). Beside their usefulness in predicting stroke prognosis, markers of cardiac pathology might also be useful in uncovering stroke etiology, as it is reported for natriuretic peptides and their role in identifying patients with atrial fibrillation (12). In addition, by taking advantage of coagulation cascade assessment via D-Dimer, risk of in-hospital mortality could be estimated in stroke patients with concomitant COVID-19 infections as demonstrated by Kim et al.

Patients suffering from stroke experience a higher rate of stroke recurrence. Identifying patients with increased risk of stroke recurrence might help to improve secondary preventive strategies and to select patients for intensified stroke etiology work-up and prevention support programs. Pable Hervella et al. identified soluble tumor necrosis factor-like inducer of apoptosis (sTWEAK) as a marker for endothelial dysfunction, to be associated with stroke recurrence and progression of cerebral white matter lesions.

EXOSOMES

Exosomes (30–150 nm) belong to the family of extracellular vesicles, together with shedding microvesicles (or ectosomes 10–1,000 nm) and apopototic bodies (50–5,000 nm). Emerging evidence underlines a potential role of exosomes as diagnostic, therapeutic, and prognostic marker in stroke. Exosomes are endosome-derived vesicles; the following steps describe their formation: initiation, endocytosis, multivesicular body formation, and exosome secretion. Exosome secretion means the final process, in which the previously formed multivesicular bodies are fused with the plasma membrane and are finally secreted by their cell of origin. As the intracellular origin of

secreted exosomes is seldom demonstrated, the term "small extracellular vesicle" may be more accurate (13), but "exosome" is nonetheless frequently used (14). After release, exosomes can interact with recipient cells by different biochemical processes, such as endocytosis, fusion, and ligand–receptor interaction (15).

Due to the fact that exosomes are abundantly secreted by most human cells, they play an exceptional role in intercellular signaling via cell-to-cell communication. Moreover, they are characterized by a lipid bilayer with an aqueous core, thereby harboring the ability to cross the BBB and transport various molecules across the BBB (16). This feature represents a prerequisite for both, transport of potential treatment to the target site within the CNS, as well as transport of markers mimicking pathophysiological CNS processes from CNS compartments to extra-CNS compartment (e.g., blood stream), where they can be easily accessed by venous puncture. Altogether, exosomes are a promising way of enabling the interaction between components of the neurovascular unit (neurons, glial cells, brain vessels).

With this in mind, exosome-derived markers might be a powerful tool for distinction of stroke subtypes and obtaining information on the pathophysiologic state within the CNS. So far, multiple exosome-derived miRNAs were associated with ischemic stroke outcome [e.g., miRNA-223 (17), miRNA-134 (18)] and might be able to identify patients who could benefit from reperfusion therapies in the hyperacute phase of ischemic stroke (19), as well as differentiate hemorrhagic from ischemic stroke patients (20). Exosomes are therefore a promising strategy to exclude hemorrhagic stroke and enable timely application of reperfusion therapies especially intravenous thrombolysis in the prehospital setting of AIS. Nonetheless, exosomes are more extensively studied as potential therapeutics (21), compared to approaches to use them as diagnostic tools, at least in the area of stroke pathology.

Within the current article collection, Jödicke et al. analyzed dynamic changes of extracellular vesicles in the biomarker and perfusion-training-induced changes after stroke (BAPTISe) study and uncovered an association with functional outcome in patients with subacute stroke.

SUMMARY

The current Research Topic, "Blood-Based Biomarkers in Acute Ischemic Stroke and Hemorrhagic Stroke" (11) provides a collection of articles that identify and validate blood-based biomarkers that deepen our understanding of stroke pathophysiology and that might support clinical decision-making for patients with ischemic as well as hemorrhagic stroke in the future.

AUTHOR CONTRIBUTIONS

TU, HA, MG, ST, and RK edited the Research Topic and drafted and revised the editorial manuscript. All authors have read and approved the final version of the manuscript.

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ORIGINAL RESEARCH
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C-Terminal-Pro-Endothelin-1 Adds Incremental Prognostic Value for Risk Stratification After Ischemic Stroke

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Background and Aims: Endothelins have shown to play a role in the pathophysiology of ischemic stroke. We aimed at evaluating the incremental prognostic value of C-terminal-pro-endothelin-1 (CT-pro-ET-1) in a well-described cohort of acute stroke patients.

Methods: We performed serial measurements of CT-pro-ET-1 in 361 consecutively enrolled ischemic stroke patients and assessed functional outcome and mortality after 90 days. As we found peak levels of CT-pro-ET-1 and the most prominent association with mortality on day 1 after admission ($n = 312$), we focused on this time point for further outcome analyses. We calculated logistic regression and cox proportional hazards models to estimate the association of CT-pro-ET-1 with our outcome measures after adjusting for demographic and clinical risk factors. To evaluate the incremental value of CT-pro-ET-1, we calculated the area under the receiver operating characteristics (AUC) curve and the continuous net reclassification index (cNRI) comparing the model with and without the biomarker CT-pro-ET-1.

Results: In the univariate analysis CT-pro-ET-1 with a peak on day 1 after admission was associated with unfavorable outcome with an OR of 1.32 (95% CI, 1.16–1.51, $p < 0.001$) and with mortality with a HR of 1.45 (95% CI, 1.29–1.63, $p < 0.001$). After adjusting, CT-pro-ET-1 remained an independent predictor of mortality with an adjusted HR of 1.50 (95% CI, 1.29–1.74, $p < 0.001$), but not for functional outcome. Adding CT-pro-ET-1 to the cox-regression model for mortality, the discriminatory accuracy improved from 0.89 (95% CI, 0.84–0.94) to 0.92 (95% CI, 0.88–0.96) $p < 0.001$, and the cNRI was 0.72 (95% CI, 0.17–1.13).

Conclusion: CT-pro-ET-1 with a peak level on day 1 was an independent predictor of mortality adding incremental prognostic value beyond traditional risk factors.

Keywords: stroke, biomarker, C-terminal-pro-endothelin-1, outcome, mortality, risk stratification

INTRODUCTION

Blood based biomarkers in the setting of acute ischemic stroke are in demand to optimize risk stratification and treatment decisions of stroke patients. Endothelins are a group of vasoactive endogenous peptides existing in three isoforms CT-pro-ET-1, −2, and −3 (1). CT-pro-ET-1 is expressed in endothelial cells, vascular smooth muscles and the central nervous system mediating vaso- and bronchoconstriction with a long duration of action (1). CT-pro-ET-1-synthesis can be stimulated via humoral factors, such as cytokines and activated platelets, homeostatic factors, such as hypoxia and hypovolemia, and also mechanically via tangential sheering-stress of arteries (1–3). Normal plasma levels range between 0.4 and 8.1 pg/ml (4). Elevated CT-pro-ET-1 levels have been shown to be linked to unfavorable outcome in ischemic stroke in a recent pilot study of 60 patients (5).

We aimed to evaluate the incremental prognostic value of CT-pro-ET-1 in a well-described, larger and independent validation cohort of acute stroke patients compared to the above-mentioned studies. Due to the vasoconstrictive effect of CT-pro-ET-1, we hypothesized an association of CT-pro-ET-1 with unfavorable outcome and mortality in acute ischemic stroke.

MATERIALS AND METHODS

Study Design and Setting

This report adheres to the consolidated standards for the reporting of observational studies. In this prospective cohort-study we enrolled 361 ischemic stroke patients (6) and measured CT-pro-ET-1 on admission, day 1, 3, and 5 (see below). Patients were included with a clinically diagnosed acute ischemic stroke referring to the World Health Organization criteria (7) and a symptom onset within 72 h. The local Ethics Committee approved the study protocol. Informed consent was obtained in all patients. The study was conducted according to the World Medical Association Declaration of Helsinki. The primary outcome of the study was defined as favorable functional outcome after 90 days assessed by the modified Rankin Scale (mRS) score (8), (0–2 favorable, 3–6 unfavorable outcome), whereas secondary outcome was defined as death from any cause within a 90-day-follow-up period.

Clinical Variables and Imaging

On admission to hospital, demographic and clinical data, laboratory findings, comorbidities assessed by the Charlson comorbidity index (CCI) and known cardiovascular risk factors were registered (**Table 1**). The severity of stroke was assessed by the National Institutes of Health Stroke Scale (NIHSS) score (9) performed by a trained stroke physician (0–42 points with higher scores indicating an increased stroke severity). We assessed the clinical stroke syndrome by applying the Oxford Community Stroke Project (10). Stroke causative factors were included after a cardiovascular work-up according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification (11). The assessment of outcome parameters was performed by two trained medical students blinded to CT-pro-ET-1 levels with follow-up interviews with the patient or, if not possible, with the closest relative or family physician. Magnetic resonance diffusion-weighted imaging (MR-DWI) lesion volumes were measured by consensus of two experienced raters unaware of clinical and laboratory findings. A semi-quantitative method validated for ischemic stroke lesions was used to calculate the lesion size (12). Lesions were categorized into three sizes to represent typical stroke patterns: (1) small lesion with a volume of <10 mm^3, (2) medium lesion of 10–100 mm^3, (3) large lesion with a volume of more than 100 mm^3 (13).

Assays

Blood samples of the acute stroke patients were taken on admission (i.e., day 0) within 0–72 h from symptom onset with a vast majority of samples (74%, $n = 267$) taken until 12 h after symptom onset. Additionally to admission ($n = 335$), samples of CT-pro-ET-1 were taken on day 1 ($n = 312$), day 3 ($n = 264$) and day 5 ($n = 262$). The routine blood analyses were recorded and plasma stored at −80° Celsius. For the analyses a single batch with a commercial sandwich immunoluminometric assay (B.R.A.H.M.S LUMItest CT-proAVP, B.R.A.H.M.S AG, Henningsdorf/Berlin, Germany) was applied as described in detail elsewhere (12). The assay (mean reference range, 44.3 ± 10.6 pmol/l) has an analytical detection limit of 0.4 pmol/l (14).

Statistical Analysis

Discrete variables are summarized as counts (percentages), continuous variables as medians and interquartile ranges (IQR). To obtain normal distribution for skewed variables (i.e., CT-pro-ET-1 concentrations), we transformed the data by taking the square root. The Fisher's-exact test, respectively the Mann-Whitney U test were applied for two-group and the Kruskal Wallis test for multiple group comparisons. For the analysis of CT-pro-ET-1 and its association with stroke severity defined by the NIHSS on admission, we dichotomized patients into NIHSS$\leq$6 vs. NIHSS $\geq$7 in line with previous publications (6, 13). For the analysis of the association of CT-pro-ET-1 with lesion size, we conducted a bivariate regression analysis in the subgroup of patients with available information on lesion size.

We calculated logistic regression and cox proportional hazards models adjusting for significant outcome predictors. In the multivariate model analyzing mortality at 90 days after stroke onset, we adjusted for all risk factors, which where significant in the univariate analysis after bonferroni correction for multiple testing. These variables were chosen according to their magnitude of association with mortality in the univariate analysis and based on the Bonferroni corrected significance level of $p < 0.00217$.

To assess the discriminatory accuracy and incremental value of CT-pro-ET-1 beyond known risk factors, Receiver Operating Characteristic (ROC) curves and the area under the ROC curve (AUC) as an overall discriminatory measure were calculated for the model with and without CT-pro-ET-1 on day 1. The likelihood ratio test was used to compare the AUCs of nested vs. whole models. The whole model included all predictors that remained significant in the multivariate model. In addition, the cNRI was assessed considering only those changes in estimated prediction probabilities that imply a change from one risk

TABLE 1 | Baseline characteristics of stroke patients, stratified by mortality after 90 days.

		Mortality		
	All (*n* = 362)	**Alive** (*n* = 317)	**Dead** (*n* = 44)	*p*
Demographic factors				
Age, median (IQR)	75 (63–83)	74 (61–82)	83 (78–87)	**<0.001**
Female sex, *n* (%)	149 (41)	128 (40)	21 (48)	n.s.
Risk factors, *n* (%)				
Hypertension	276 (76)	238 (75)	37 (84)	n.s.
Atrial fibrillation	75 (21)	57 (18)	18 (41)	**0.001**
Current smoking	125 (35)	113 (36)	11 (25)	n.s.
Diabetes mellitus	71 (20)	62 (20)	9 (20)	n.s.
Coronary heart disease	91 (25)	74 (23)	17 (39)	**<0.05**
Dyslipidemia	93 (26)	82 (26)	35 (25)	n.s.
Prior cerebrovascular event	88 (24)	80 (25)	8 (18)	n.s.
Modified Charlson Index (IQR)	1 (0–2)	1 (0–2)	1 (0–2)	n.s.
Clinical data, median (IQR)				
NIHSS on admission	5 (2–10)	4 (2–8)	17 (8–25)	**<0.001**
Laboratory values, median (IQR)				
CT-pro-ET-1 day 0 in pg/ml	8.4 (7.3–9.6)	8.4 (7.3–9.4)	9.6 (8.3–10.9)	**<0.001**
CT-pro-ET-1 day 1 (*, *n* = 312)	8.5 (7.7–9.6)	8.4 (7.6–9.4)	10.2 (9.2–11.4)	**<0.001**
CT-pro-ET-1 day 3 (*)	8.3 (7.6–9.3)	8.3 (7.6–9.2)	9.8 (8.4–11.4)	**<0.001**
CT-pro-ET-1 day 5 (*)	8.2 (7.4–9.4)	8.1 (7.4–9.2)	10.1 (7.8–14.2)	**0.001**
Lesion size on MRI, DWI (*n* = 198), *n* (%)				
Small (1–10 mm^3) – size 1	136 (69)	131 (70)	4 (36)	**<0.05**
Medium (>10–100 mm^3) – size 2	50 (25)	46 (25)	4 (36)	n.s.
Large (>100 mm^3) – size 3	12 (6)	9 (5)	3 (27)	**<0.05**
Stroke etiology, *n* (%)				
Large-vessel disease	65 (18)	60 (19)	5 (11)	n.s.
Cardio-embolic (**)	131 (36)	111 (35)	20 (46)	n.s.
Small-artery disease	55 (15)	54 (17)	1 (2)	**<0.01**
Multiple causes	17 (5)	15 (5)	1 (2)	n.s.
Undetermined	94 (26)	77 (24)	17 (39)	**<0.05**

**Data normalization by square root model; **including atrial fibrillation, atrial flutter, congestive heart failure, patent foramen ovale; IQR, Interquartile range; NIHSS, National Institutes of Health Stroke Scale; MR, Magnetic Resonance; DWI, Diffusion Weighted Imaging; p, p-value; n.s., not significant. The bold values highlight significant values.*

category to another. A cut-off was identified by classifying sensitivity and specificity for CT-pro-ET-1 levels choosing the cut-off at the highest possible sensitivity for detection with still enough specificity to predict mortality. For Kaplan-Meier survival estimates, patients were stratified by the selected cut-off level for CT-pro-ET-1 of <8.8 pg/ml (57%) and ≥8.8 pg/ml (43%) measured on day 1 after admission. Groups were compared by means of the log-rank test. *P*-values ≤0.05 were considered to be statistically significant. All calculations were performed using STATA 14.1.

RESULTS

An acute ischemic stroke was diagnosed in 362 patients with 361 completing follow-up. These 361 patients were analyzed for baseline characteristics and stratified for mortality after 90 days as shown in **Table 1**.

CT-Pro-ET-1 Over Time

When assessing CT-pro-ET-1 sequentially on admission (i.e., day 0), day 1, 3, and 5, we observed a nominal increase of CT-pro-ET-1 over time with a peak on day 1 (8.5 pg/ml, IQR 7.7–9.6) followed by a gradual decrease over the next days with a minimum on day 5 (8.2 pg/ml, IQR 7.4–9.4), see **Table 1** and **Figure 1**. Thus, for further analyses, we concentrated on the predictive value of CT-pro-ET-1 on day 1 as at this point of time after stroke the association of CT-pro-ET-1 levels with mortality within 90 days was most prominent.

CT-pro-ET-1 levels on day 1 were available in 312 out of 362 patients. When conducting a sensitivity analysis between the original cohort of 361 patients, which was used for the analysis of baseline characteristics, and the cohort with available CT-pro-ET-1 measurements on day 1 ($n = 312$), we found no significant difference between both cohorts regarding the distribution of baseline risk factors (data not shown).

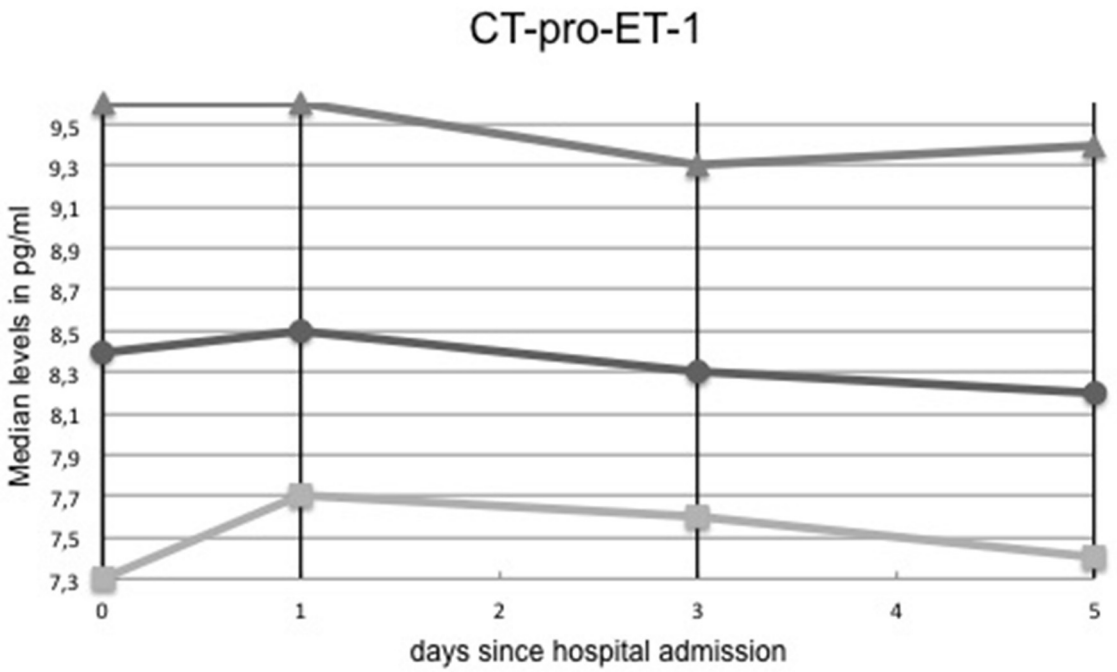

FIGURE 1 | CT-pro-ET-1 levels over time. Circles indicate median CT-pro-ET-1 levels, squares lower 95%-confidence interval limits and arrows upper confidence interval limits, respectively.

CT-Pro-ET-1 and Its Association With Baseline Risk Factors for Mortality

CT-pro-ET-1 levels on day 1 were higher in patients suffering from severe strokes compared to moderate strokes, but this difference was not statistically significant (NIHSS ≤6: CT-pro-ET-1 on day 1 8.4 pg/ml, IQR 7.7–9.4, vs. NIHSS ≥7: 8.8 pg/ml, IQR 7.6–9.9, $p = 0.16$).

A moderate to large ischemic lesion (>10 mm^3 on MR-DWI) was associated with higher CT-pro-ET-1 levels compared to small lesions (<10 mm^3: CT-pro-ET-1 on day 1 8.1 pg/ml, IQR 7.5–8.8, vs. >10 mm^3: 8.7 pg/ml, IQR 7.6–10.2, $p = 0.02$) in a subgroup of patients with available imaging ($n = 182$).

CT-Pro-ET-1 and Its Association With Stroke Etiology

We found only a borderline association of CT-pro-ET-1 levels on day 1 and cardio-embolic stroke etiology according to the TOAST criteria. However, after adjustment for multiple testing (comparison of all TOAST subgroups with each other), the association of CT-pro-ET-1 with cardio-embolic stroke etiology could not be confirmed ($p > 0.05$).

Prediction of Functional Outcome and Mortality After 90 Days

CT-pro-ET-1 on day 1 was associated with an unfavorable functional outcome with an odds ratio (OR) of 1.32 (95% CI, 1.16–1.51). Adjustment for significant outcome predictors (age, NHISS on admission and atrial fibrillation) in the multivariate analysis attenuated the association with an OR of 1.05 (95% CI, 0.88–1.25, $p = 0.59$).

TABLE 2 | Multivariate analysis: cox proportional hazards model for survival after 90 days.

Predictors	Mortality		
	HR	**95% CI**	***P***
CT-pro-ET-1 day 1 (*)	1.5	1.29–1.74	**<0.001**
Age (increase per unit)	1.04	1.01–1.08	**0.006**
NIHSS (increase per unit)	1.12	1.09–1.16	**<0.001**
Atrial fibrillation	0.54	0.24–1.21	0.13

**Data normalization by square root model; HR, hazard ratio; CI, confidence interval; p, p-value; NIHSS, National Institutes of Health Stroke Scale. The bold values highlight significant values.*

CT-pro-ET-1 on day 1 was associated with mortality with a hazards ratio (HR) of 1.45 (95% CI, 1.29–1.63). After adjusting, CT-pro-ET-1 remained an independent predictor of mortality with an adjusted HR of 1.50 (95% CI, 1.29–1.74, $p < 0.001$, **Table 2**). CT-pro-ET-1 on day 1 ≥8.8 pg/ml had a sensitivity of 89% and a specificity of 63% to predict mortality. Adding CT-pro-ET-1 to the regression model for mortality, the discriminatory accuracy improved from 0.89 (95% CI, 0.84–0.94) to 0.92 (95% CI, 0.88–0.96), $p < 0.001$, see **Figure 2**. The combination of CT-pro-ET-1 with the regression model led to a cNRI of 0.72 (95% CI, 0.17–1.13). Overall, Kaplan-Meier survival curves of patients stratified according to the above-mentioned CT-pro-ET-1 cut-off

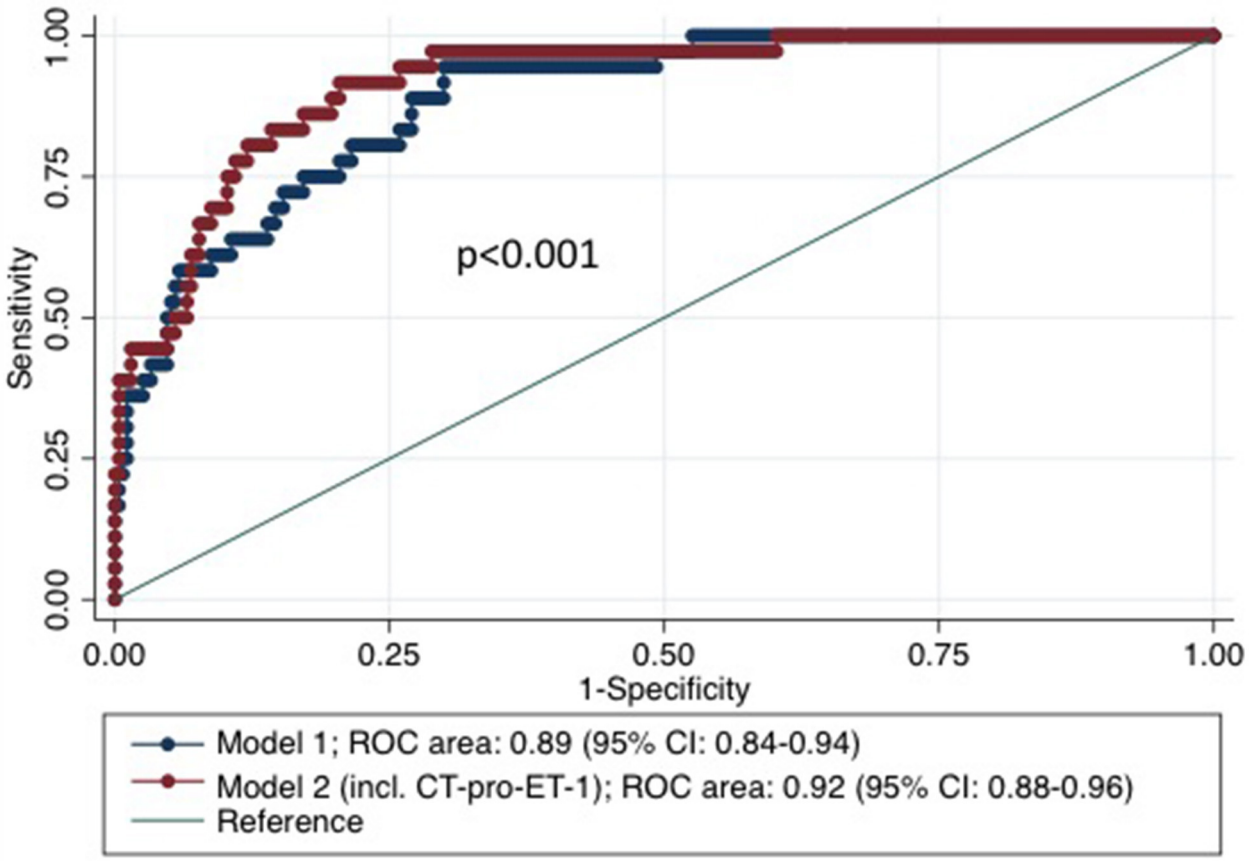

FIGURE 2 | Receiver Operator Characteristic (ROC)-curves based on regression models for mortality within 3 months after stroke with and without CT-pro-ET-1. Model 1 represents the cox proportional hazards model for mortality within 3 months after stroke including the risk factors age and NIHSS on admission. Model 2 represents the analysis adding CT-pro-ET-1 on day 1 after admission to the model.

level <8.8 and ≥8.8 pg/ml differed ($p < 0.001$, log-rank test) (**Figure 3**).

In a bivariate analysis within the subgroup of patients with available MRI data ($n = 182$) CT-pro-ET-1 on day 1 also remained associated with mortality with an HR of 1.49 (95% CI 1.21–1.84, $p < 0.001$) after adjusting for lesion size.

DISCUSSION

This prospective single-center cohort study revealed the following key findings: CT-pro-ET-1 with peak levels on day 1 after admission was positively associated with unfavorable outcome and most remarkably and independently with mortality after 3 months. CT-pro-ET-1 improved the discriminatory accuracy of the NIHSS alone as well as compared to the multivariate model without the biomarker as shown by an increase of the respective AUC and cNRI.

Circulating CT-pro-ET-1 levels have been described to be very low in normal conditions (15). During acute ischemic events several mechanisms have been discussed leading to an increase of CT-pro-ET-1. Among these, CT-pro-ET-1 is released as an acute phase reactant following severe physical stress caused by hypoxia (16). Furthermore, the hypercoagulability and platelet activation in ischemic lesions have been described to accelerate pre-proendothelin production (17). Via activation of inflammatory cells such as neutrophils, mast cells and macrophages CT-pro-ET-1 might exert pro-inflammatory effects (18) and thus besides the potent vasoconstrictive capability this pro-inflammatory aspect may contribute also to a worse outcome in stroke patients.

CT-pro-ET-1 has been linked particularly to cardiovascular disease previously. In the Leicester Acute Myocardial Infarction Peptide (LAMP) Study Khan et al. found increased CT-pro-ET-1 levels to be independently associated with a higher rate of heart failure and mortality in a large cohort of 983 acute myocardial infarction patients (19). In line with our results, peak plasma concentrations have been described to appear with a little delay on day 2 after myocardial infarction (19). Adverse outcome effects in cardiovascular disease have been linked to a reduction of coronary blood flow (20).

However, regarding the association of CT-pro-ET-1 and unfavorable outcome in acute ischemic stroke, the available

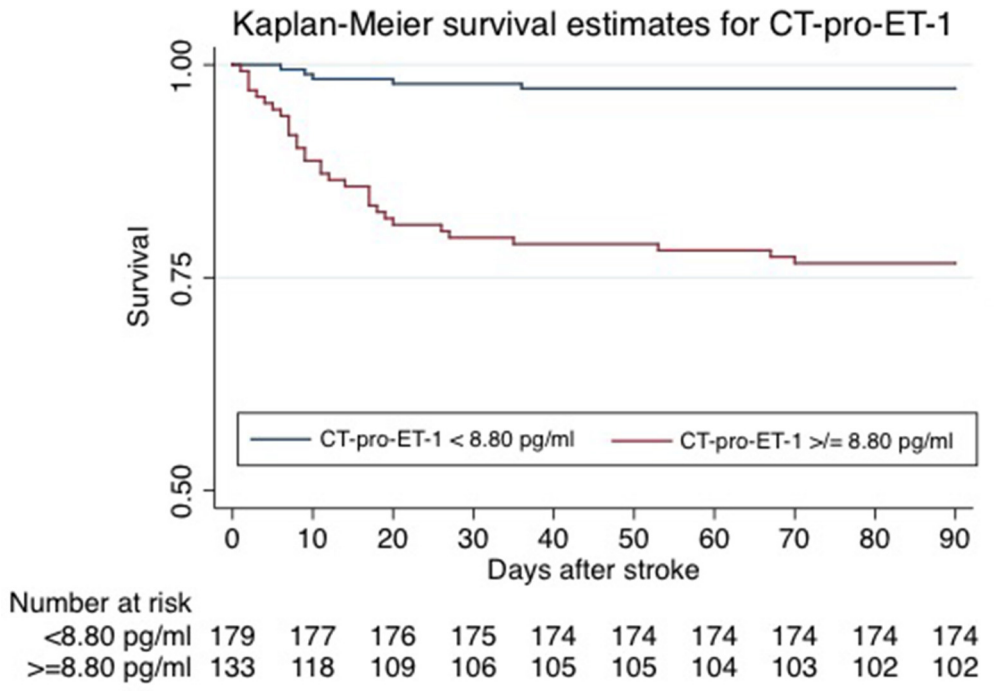

FIGURE 3 | Kaplan-Meier survival estimates of patients stratified by CT-pro-ET-1 on day 1 after admission. Kaplan-Meier survival curves of patients stratified per CT-pro-ET-1 cut-off levels <8.80 and ≥8.80 pg/ml differed significantly ($p = <0.001$, log-rank test).

studies remain to some extent controversial. A previous smaller pilot study consisting of 60 stroke patients has shown higher CT-pro-ET-1 levels associated with unfavorable outcome and mortality (5), whereas an older case-control study did not show a significant difference in CT-pro-ET-1 levels between healthy controls and ischemic stroke patients (21). Another case-control study with 30 sex- and age-matched patients found in line with our results higher CT-pro-ET-1 plasma levels on admission compared to day 7 after stroke onset and healthy controls, but could not find any correlation with infarct size, stroke severity or degree of clinical neurological deficit (22).

The strengths of this study are the prospective study design, the clinically well-characterized relatively large stroke patient cohort, a very low lost to follow-up-rate and blinded CT-pro-ET-1-measures. Additionally, we have shown serial measurements of CT-pro-ET-1 in the first hours up to 5 days after stroke onset in order to deduct the best time for measurement after stroke. By defining a cut-off value for CT-pro-ET-1 with levels > 8.8 pg/ml representing a 89%-sensitivity and 63%-specificity to predict mortality, the parameter could be applied for risk stratification in a clinical setting after external validation of this cut-off. This additional information beyond traditional risk factors could help physicians in clinical decision making, specifically to triage ischemic stroke patients, e.g., for intensified monitoring with regards to post-stroke complications. As we could also prove the association of CT-pro-ET-1 with mortality within 3 months after stroke onset in the subgroup of patients with available MRI data, the predictive value of CT-pro-ET-1 on mortality can be considered reliable supporting the independent additive prognostic information gained by measuring the biomarker. When compared to other thromboinflammatory biomarkers from the literature such as S100B, which has been associated with tissue damage, post-stroke infections and consecutively mortality (23), CT-pro-ET-1 predicted overall mortality in ischemic stroke patients independently of known risk factors.

As a limitation of the study, the role of CT-pro-ET-1 in hyper-acute treatment decisions is at least partly restricted since the peak of CT-pro-ET-1 was measured on day 1 after admission. Furthermore, time points of serial blood collections of CT-pro-ET-1 were classified in days after hospital admission and not in hours after stroke onset except for the day of hospital admission (day 0). However, as the majority of blood samples were taken until 12 h after stroke onset (74%), we can assume that for subsequent blood samples the days after hospital admission correspond largely with the days after stroke onset. Serial measurements of CT-pro-ET-1 levels in stroke survivors revealed only marginal changes over time (see **Table 1**), most likely as these patients had less severe strokes and therefore less complications over time. Thus, the stimulus of CT-pro-ET-1 production over time stayed stable in the group of stroke survivors. Additionally, since we are the first to propose a cut-off, there is need for an external validation of exactly the proposed cut off. Yet, as mentioned smaller studies in the past have shown overall association with mortality after stroke, therefore our study can also be interpreted as external validation of previous smaller studies.

In summary, CT-pro-ET-1 can be considered as an independent predictor of mortality in acute ischemic stroke

patients. Further studies should assess the suggested cut-off value in larger and multi-center validation cohorts to prove the benefit in clinical decision making.

DATA AVAILABILITY STATEMENT

Anonymized data will be shared by request from any qualified investigator.

ETHICS STATEMENT

The study involving human participants was reviewed and approved by the local Ethics Committee of Basel, Switzerland. The patients/participants provided their written informed consent to participate in this study.

DISCLOSURE

MK received the KITs for the measurement of CT-pro-ET-1 from BRAHMS GmbH, Henningsdorf, Germany.

AUTHOR CONTRIBUTIONS

LPW conducted data processing and interpretation, performed the statistical analysis, and wrote the first draft of the manuscript. JS was involved in the statistical analysis. FF, GMDM, and MC-C were involved in data collection and critically revised the manuscript. ARL critically revised the manuscript. MK designed the study, was involved in data analysis, and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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YKL-40 Is Associated With Ultrasound-Determined Carotid Atherosclerotic Plaque Instability

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Background and Aims: YKL-40, an inflammatory biomarker, has been reported to be involved in the process and progression of atherosclerosis. Several studies have investigated the association between YKL-40 and plaque and suggested YKL-40 might be a potential biomarker for plaque instability. This study aimed to investigate the association between YKL-40 and carotid plaque instability.

Methods: Based on a community-based study in Beijing from February 2014 to May 2016, 1,132 participants with carotid plaques were enrolled in this study. Data on demographics and medical history were collected through face-to-face interviews, and fasting blood samples were collected and stored. We used ultrasound to evaluate the presence of carotid plaque and its instability. The level of YKL-40 was measured by enzyme-linked immunosorbent assay (ELISA). Multivariate logistic regression analysis was performed to investigate the association between YKL-40 level and carotid atherosclerotic plaque instability.

Results: The mean age of the 1,132 participants was 58.0 (52.0–64.0) years, and 560 (49.5%) were male. Unstable plaques were detected in 855 (75.53%) participants. YKL-40 level was classified into four groups according to its quartile: quartile 1: <25.47 ng/mL, quartile 2: 25.47–39.53 ng/mL, quartile 3: 39.53–70.55 ng/mL, quartile 4: ≥70.55 ng/mL. After adjusting for age, sex, smoking, alcohol drinking, medical history, triglycerides, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, homocysteine, high-sensitivity C-reactive protein, and plaque thickness, the top quartiles of YKL-40 level were significantly associated with unstable plaque (quartile 3: OR 2.10, 95% CI 1.29–3.40; quartile 4: OR 1.70, 95% CI 1.04–2.80).

Conclusion: This study found that YKL-40 was associated with carotid plaque instability determined by ultrasound. Individuals with high YKL-40 may have a higher risk of unstable carotid plaque.

Keywords: YKL-40, plaque instability, association, carotid plaque, atheroscelorsis

INTRODUCTION

Stroke has already caused a heavy burden worldwide because of its high incidence, high disability rate, and high mortality, especially in China (1, 2). The rupture of a carotid atherosclerotic plaque could lead to ischemic stroke (3). YKL-40, a new inflammatory factor, is involved in the pathogenesis of atherosclerotic plaques (4). It plays a vital role in the process of matrix remodeling, cell proliferation and differentiation, new blood vessel formation, anti-apoptosis, and promotion of tissue fibrosis. Several studies have reported that high YKL-40 is associated with an increased risk of ischemic stroke, but not myocardial infarction (4–8), suggesting that YKL-40 might be a promising biomarker of plaque instability (5, 9, 10).

This study aimed to investigate the association between YKL-40 and carotid atherosclerotic plaque instability in a community-based population.

MATERIALS AND METHODS

Study Design and Population

We enrolled 1,132 participants with carotid plaques from a community-based, cross-sectional study in Beijing from February 2014 to May 2016. The inclusion criteria for the participants were: (1) age between 24 and 75 years; (2) carotid atherosclerotic plaques detected using carotid artery color-ultrasonography; and (3) signed written informed consent was obtained. Individuals with (1) severe inflammatory diseases such as acute and chronic infections, rheumatoid arthritis, osteoarthritis, and liver cirrhosis; (2) a history of stenting, percutaneous coronary stenting, and coronary artery bypass grafting; (3) severe clinical conditions including a recent trauma, surgery, severe heart failure, hepatic and renal insufficiency, autoimmune diseases, hematologic diseases, cerebrovascular diseases, and peripheral vascular diseases; and (4) any known malignant tumors were excluded from the study.

This study was approved by the Beijing Tiantan Hospital Research Ethics Committee. All participants provided signed written informed consent to participate in this study.

Data Collection

All participants were interviewed face-to-face with a structured questionnaire by trained interviewers. The questionnaire included questions on demographics (sex, age, body mass index, cigarette smoking, and alcohol consumption), medical history (diabetes mellitus, hypertension, dyslipidemia, coronary heart disease, and atrial fibrillation), and medications taken in the last 12 months. Height, weight, systolic blood pressure, and diastolic blood pressure were measured using standard operating procedures.

Fasting venous blood samples were drawn for routine blood examinations, measurement of lipid, fasting blood glucose, hypersensitive C-reactive protein (hsCRP), and homocysteine.

Assessment of Carotid Plaque

Ultrasound examinations were performed by trained and certified sonographers using standard equipment (iU22 xMatrix, Philips). Bilateral carotid arteries were scanned, focusing on the near and far walls. The scanning range was 15 mm before and 10 mm after the bifurcation of the common carotid artery. Carotid plaque was defined as a thickness $\geq$ 1.5 mm measured from the media-adventitia interface to the intima-lumen interface or a focal structure that encroaches into the arterial lumen for at least 0.5 mm or 50% of the surrounding intima-media thickness (IMT) value (11). Based on the morphology and echogenicity of the plaques detected by ultrasound, they were categorized as (1) hypoechoic lipid soft spots; (2) moderately echogenic fibrous flat plaques abundant in collagen tissue; (3) strong acoustic echo-like calcified hard plaques; or (4) ulcerative mixed plaques with different echoes. In this study, plaques with hypoechoic or heterogeneous echoes were defined as unstable (12).

Measurement of YKL-40

The serum YKL-40 levels were measured by an enzyme-linked immunosorbent assay (R&D Systems, China). We used the mean value of duplicate measurements. The detection limit was 20 ng/mL, while the intra-assay and inter-assay coefficients of variation were both <6%.

Statistical Analysis

Continuous variables with normal distribution were expressed as means ± standard deviations (SDs). Non-normal variables were presented as median (inter-quartile range). Categorical variables were expressed as frequency and percentage. The student's *t*-test and Wilcoxon test were used to evaluate the difference between groups of continuous variables. Categorical variables were compared using the χ^2 tests (the chi-squared tests). We performed univariate and multivariate logistic regression analysis to evaluate the association between YKL-40 and carotid artery plaque instability. While model 1 was unadjusted, model 2 was adjusted for age and sex, and model 3 was adjusted for the variables in model 2 plus BMI, medical history of hypertension, diabetes, coronary heart disease, dyslipidemia, smoking, and alcohol consumption. Model 4 was adjusted for the variables in model 3 plus fasting blood glucose, triglyceride, low-density lipoprotein, high-density lipoprotein, homocysteine, and high-sensitivity C-reactive protein. In Model 5, plaque thickness was added to Model 4. The significance level was defined as $P \leq 0.05$. All statistical analyses were performed using SAS 9.4 software (SAS Institute Inc, Cary, North Carolina).

RESULTS

A total of 1,132 participants were recruited in this study. The mean age of the participants was 58 (52–64) years, and 560 (49.5%) of them were men. Unstable plaques were detected in 855 (75.53%) participants. **Table 1** summarizes the characteristics of the participants. Compared to participants with stable plaques, those with unstable plaques were more likely to be men, and smokers. The two groups showed significant differences in plaque thickness, diabetes mellitus, dyslipidemia, white blood cell count, and levels of fasting glucose, high-density

TABLE 1 | Comparison of clinical data between stable and unstable plaque groups.

Variable	Total (%) (n = 1132)	Stable plaque group (n = 277)	Unstable plaque group (n = 855)	p-value
Age (years)	58.0 (52.0–64.0)	59.0 (52.0–67.0)	58.0 (52.0–64.0)	0.24
Male, *n* (%)	560 (49.5)	108 (39.0)	452 (52.9)	<0.001
BMI (kg/m^2)	26.1 (23.8–28.4)	25.1 (23.0–27.5)	26.4 (24.1–28.6)	<0.001
<25	417 (36.8)	134 (48.4)	283 (33.1)	<0.001
25–30	546 (48.3)	116 (41.9)	430 (50.4)	
≥30	168 (14.9)	27 (9.7)	141 (16.5)	
Smoking, *n* (%)				<0.001
Yes	804 (71.0)	51 (18.4)	277 (32.4)	
No	328 (29.0)	226 (81.6)	578 (67.6)	
Alcohol consumption, *n* (%)				0.99
Yes	413 (36.5)	101 (36.5)	312 (36.5)	
No	719 (63.5)	176 (63.5)	543 (63.5)	
Medical history, *n* (%)				
Diabetes mellitus	167 (14.8)	29 (10.5)	138 (16.1)	0.02
Hypertension	469 (41.4)	117 (42.2)	352 (41.2)	0.75
Dyslipidemia	209 (18.5)	84 (30.3)	125 (14.6)	<0.001
Coronary heart disease	67 (5.9)	15 (5.4)	52 (6.1)	0.68
Atrial fibrillation	12 (1.1)	5 (1.8)	7 (0.8)	0.16
Laboratory examination				
White blood cell count (×10^{12}/L)	6.04 (5.17–7.20)	5.60 (4.86–6.85)	6.20 (5.24–7.30)	<0.001
Fasting glucose (mmol/L)	5.80 (5.20–6.64)	5.25 (4.60–6.00)	5.96 (5.40–6.86)	<0.001
Triglycerides (mmol/L)	1.39 (1.00–1.99)	1.44 (1.03–2.01)	1.38 (0.98–1.96)	0.15
Total cholesterol (mmol/L)	5.23 (4.55–5.90)	5.21 (4.54–5.75)	5.24 (4.58–5.96)	0.06
High-density lipoprotein cholesterol (mmol/L)	1.44 (1.23–1.70)	1.49 (1.28–1.74)	1.43 (1.22–1.68)	0.02
Low-density lipoprotein cholesterol (mmol/L)	3.20 (2.62–3.72)	3.22 (2.67–3.60)	3.18 (2.62–3.75)	0.80
Homocysteine (umol/L)	14.7 (12.3–18.4)	14.9 (12.5–18.2)	14.7 (12.2–18.5)	0.68
High-sensitivity C-reactive protein (mg/ml)	1.7 (1.0–3.2)	1.4 (0.8–3.0)	1.8 (1.1–3.2)	<0.001
Plaque thickness (mm)	2.20 (1.80–2.70)	1.99 (1.60–2.40)	2.20 (1.80–2.70)	<0.001

BMI indicates body mass index, BMI (kg/m^2) = weight/height^2.

lipoprotein cholesterol and high-sensitivity C-reactive protein (**Table 1**). YKL-40 levels were stratified into four quartiles: quartile 1: < 25.47 ng/mL, quartile 2: 25.47–39.53 ng/mL, quartile 3: 39.53–70.55 ng/mL, quartile 4: ≥70.55 ng/mL. Participants with unstable plaques had a higher median concentration of YKL-40 (40.23 ng/mL) compared to those with stable plaques (31.44 ng/mL) (**Table 2**).

The univariate logistic analysis found male sex, history of diabetes mellitus, dyslipidemia, BMI, and plaque thickness associated with unstable plaques. Furthermore, YKL-40 levels (quartile 3: OR 2.51 95% CI 1.70–3.70 and quartile 4: OR 2.11 95% CI 1.41–3.14) were also associated with unstable carotid plaques (**Table 3**, Model 1).

The multivariate logistic analysis showed an age- and sex-adjusted association between YKL-40 and carotid plaque instability in Model 2 (quartile 3: OR 2.55; 95% CI 1.71–3.81; quartile 4: OR 2.18 95% CI 1.45–3.29). After adjustment for age, sex, BMI, medical history of hypertension, diabetes, coronary heart disease, dyslipidemia, smoking, and alcohol consumption, YKL-40 level remained significantly associated with carotid plaque instability (quartile 3: OR 2.71, 95% CI 1.78–4.11; quartile 4: OR 2.14, 95% CI 1.39–3.28) in Model 3. The association remained statistically significant after adjusting for all variables in Model 4 (quartile 3: OR 2.53, 95% CI 1.65–3.89; quartile 4: OR 1.97, 95% CI 1.27–3.11), and Model 5 (quartile 3: OR 2.10, 95% CI 1.29–3.40; quartile 4: OR 1.70, 95% CI 1.04–2.80).

DISCUSSION

This study investigated the association between YKL-40 level and carotid plaque instability in a community-based population. We found that high-levels of YKL-40 were associated with carotid plaque instability.

Recently, several studies have explored the relationship between YKL-40 and atherosclerotic plaque instability. Michelsen et al. found that serum YKL-40 was significantly elevated in patients with carotid atherosclerosis, especially in symptomatic patients, suggesting it may be a marker of plaque instability by causing macrophage activation and matrix degradation (9). However, this study had a small size and lack

TABLE 2 | Comparison of YKL-40 levels between stable and unstable plaque groups.

Variable	Total (%) (n = 1,132)	Stable plaque group (n = 277)	Unstable plaque group (n = 855)	p-value
YKL-40 (ng/mL)	37.61 (24.87–62.52)	31.44 (18.96–52.72)	40.23 (26.53–65.93)	<0.001
<25.47	297 (26.24)	100 (36.10)	197 (23.04)	<0.001
25.47–39.53	302 (26.68)	81 (29.24)	221 (25.85)	
39.53–70.55	291 (25.70)	49 (17.69)	242 (28.30)	
≥70.55	242 (21.38)	47 (16.97)	195 (22.81)	

TABLE 3 | Logistic regression analysis of the association between YKL-40 and carotid atherosclerotic plaque instability.

	Model 1		Model 2		Model 3		Model 4		Model 5	
	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
Q1	Reference	–	Reference	–	Reference	–	Reference	–	Reference	–
Q2	1.39 (0.98–1.97)	0.07	1.38 (0.97–1.96)	0.08	1.43 (0.99–2.07)	0.06	1.32 (0.90–1.93)	0.16	1.15 (0.74–1.79)	0.54
Q3	2.51 (1.70–3.70)	<0.001	2.55 (1.71–3.81)	<0.001	2.71 (1.78–4.11)	<0.001	2.53 (1.65–3.89)	<0.001	2.10 (1.29–3.40)	0.003
Q4	2.11 (1.41–3.14)	<0.001	2.18 (1.45–3.29)	<0.001	2.14 (1.39–3.28)	<0.001	1.98 (1.27–3.11)	<0.01	1.70 (1.04–2.80)	0.036

Model 1 is unadjusted.
Model 2 is adjusted for age and gender.
Model 3 is adjusted for model 2 and BMI, medical history of hypertension, diabetes, coronary heart disease, dyslipidemia, smoking, and alcohol consumption.
Model 4 is adjusted for model 3 and fasting blood glucose, triglyceride, low-density lipoprotein, high-density lipoprotein, homocysteine and high-sensitivity C-reactive protein.
Model 5 adjusted for model 4 and plaque thickness.

prospective data. Wu et al. evaluated YKL-40 in 168 patients with carotid atherosclerosis and found thicker carotid intima-media and more unstable plaques in patients with high serum levels of YKL-40. However, these findings were limited to patients with Helicobacter pylori-positive cytotoxic-associated gene A (CagA), and therefore, the association might have been related to Helicobacter pylori infection, which needs to be further verified (13).

It is well-known that the rupture of an unstable carotid atherosclerotic plaque is one of the leading causes of ischemic stroke (14, 15). Some studies have been accessed the association between YKL-40 and ischemic stroke. Kjaergaard et al. detected plasma YKL-40 in 8,899 general participants and followed them up for 18 years. They found that increase in YKL-40 led to an increased risk for ischemic stroke, but not for atherosclerosis myocardial infarction. The differences in the mechanisms of ischemic stroke and myocardial infarction indicated that YKL-40 might play a crucial role in thromboembolism rather than affecting the formation of local thrombus and atherosclerosis, suggestive of its association with plaque instability (4, 8). Rathcke et al. in a 15-years follow-up study of 2,656 Danes reported similar findings. They found that high levels of YKL-40 were associated with increased ischemic stroke-related mortality but were inversely related to the risk of ischemic heart disease (7). These studies elucidated that YKL-40 plays different roles in thromboembolism and local thrombosis by macrophage activation and matrix degradation within the atherosclerotic lesion. Recently, Hjalmarsson et al. found that patients with National Institutes of Health Stroke Scale (NIHSS) $\geq$ 5 had significantly higher levels of YKL-40 compared to those with a score $<$ 5, illustrating the significant association of YKL-40 with stroke severity. YKL-40, therefore, might be an important biomarker of carotid plaque instability and a warning sign for the pathogenesis and prognosis of acute ischemic stroke (16), which is consistent with our results.

YKL-40, a biomarker of inflammation, may be involved in the pathological progression of atherosclerosis (13, 17–19). Inflammation plays an important role in atherosclerosis and atherothrombotic events. An *in vivo* study by Rathcke and Vestergaard further confirmed the expression of YKL-40 protein in the smooth muscle cells in human atherosclerotic plaques (20). YKL-40 regulated angiogenesis and reorganization of the extracellular matrix by controlling vascularized smooth muscle cells and vascular endothelial cells. An increase in YKL-40 levels activate endothelial cells to express vascular adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), further injuring the vascular endothelial cells, and promoting the development of atherosclerosis (20–23). Boot et al. and Fach et al. showed an increase in YKL-40 expression in the macrophages in atherosclerotic plaques (24, 25). YKL-40 potentially is involved in macrophage activation and matrix degradation within the atherosclerotic lesion, supporting its role in thromboembolisms (7).

Our study has several limitations. First is its cross-sectional design, with no follow-up information on stroke events. Therefore, only an association and not a causal relationship between YKL-40 levels and carotid atherosclerotic plaque instability could be investigated. Second, as all the study participants were recruited from a community in Beijing, there may have been a selection bias. These findings, therefore, need to be verified in a more representative community-based population study. Third, the sample size was not large enough. Large-scale, more representative, and prospective studies are needed to further investigate the role of YKL-40 in carotid atherosclerotic

plaque formation and obtain higher-level evidence. Fourth, we did not collect data on medication, which could be a potential confounder because stain and antiplatelet therapy might affect the plaques' features. Fifth, in our study, the prevalence of unstable carotid plaque was 75.53%, which is a little higher than in a previous study (26). This discrepancy could be due to differences in sex distribution and the definition of plaque instability. Finally, in this study, plaque instability was defined by ultrasound, which can be largely influenced by the operator's skills, limiting the generalizability of our results. However, to minimize the functional heterogeneity, the ultrasound operators in this study were required to have more than five years of clinical experience. We also conducted uniform standardized training for all ultrasound operators before this study.

In conclusion, this study found that YKL-40 is associated with carotid plaque instability. High levels of YKL-40 are associated with a higher risk of carotid plaque instability, suggesting that YKL-40 is potentially a marker for plaque instability.

DATA AVAILABILITY STATEMENT

The datasets used and analyzed during the current study will be made available by the corresponding author upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Beijing Tiantan Hospital research ethics committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GL designed and conceived the study. YW and BL analyzed the data and drafted the article. YJ, RZ, and XZ contributed in the data collection and interpretation. XM, XZ, and YW critically revised the manuscript. All authors approved the final version of the article.

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Glycated Hemoglobin as a Marker for Predicting Outcomes of Patients With Stroke (Ischemic and Hemorrhagic): A Systematic Review and Meta-Analysis

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Background: Glycated hemoglobin (HbA1c) has emerged as a useful biochemical marker reflecting the average glycemic control over the last 3 months, and the values are not affected by short-term transient changes in blood glucose levels. However, its prognostic value in the acute neurological conditions such as stroke is still not well-established. The present meta-analysis was conducted to assess the relationship of HbA1c with outcomes such as mortality, early neurological complications, and functional dependence in stroke patients.

Methods: A systematic search was conducted for the PubMed, Scopus, and Google Scholar databases. Studies, either retrospective or prospective in design that examined the relationship between HbA1c with outcomes of interest and presented the strength of association in the form of adjusted odds ratio/hazard ratios were included in the review. Statistical analysis was done using STATA version 13.0.

Results: A total of 22 studies (15 studies on acute ischemic stroke and seven studies on hemorrhagic stroke) were included in the meta-analysis. For patients with acute ischemic stroke, each unit increase in HbA1c was found to be associated with an increased risk of mortality within 1 year, increased risk of poor functional outcome at 3 months, and an increased risk of symptomatic intracranial hemorrhage (sICH) within 24 h of admission. In those with HbA1c $\geq$ 6.5%, there was an increased risk of mortality within 1 year of admission, increased risk of poor functional outcomes at 3 and 12 months as well as an increased risk of symptomatic intracranial hemorrhage (sICH) within 24 h of admission. In patients with hemorrhagic stroke, each unit increase in HbA1c was found to be associated with increased risk of poor functional outcome within the first 3 months from the time of admission for stroke. In those with HbA1c $\geq$ 6.5%, there was an increased risk of poor functional outcome at 12 months.

Conclusions: The findings indicate that glycated hemoglobin (HbA1c) could serve as a useful marker to predict the outcomes in patients with stroke and aid in the implementation of adequate preventive management strategies at the earliest.

Keywords: glycated hemoglobin, stroke, glycemic control, ischemic stroke, meta-analysis

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INTRODUCTION

Diabetes mellitus is an increasingly growing medical condition that is estimated to affect nearly 400 million people globally as per the year 2015 estimates (1). Studies have predicted that by the year 2040, around 600 million people would have this chronic disease (1, 2). It is suggested that a substantial proportion of patients with stroke may have comorbid diabetes mellitus, and this is because diabetes is a well-established risk factor for neurovascular disease (3, 4). A recent meta-analysis found a significant association of acute hyperglycemia and diabetes with poor outcomes after stroke, both ischemic and hemorrhagic (5). Using around 27,000 subjects, a large multi-centric study found diabetes to be present in one-fifth of patients with acute stroke, whereas this proportion was less (22%) in those with no stroke (6). Further, the study also noted a higher magnitude of association of diabetes with ischemic stroke, as against hemorrhagic stroke (6).

Studies have shown that the presence of diabetes is associated with increased death, duration of stay at hospital, rates of readmission and poorer post-stroke functional and recovery outcomes (7–10). On the other hand, there are studies that have observed no substantial variations between subjects with or without diabetes in post-stroke outcomes (11, 12). Glycated hemoglobin (HbA1c) has emerged as a useful biochemical marker reflecting the average glycemic control over the last 3 months or 120 days (13, 14). The added advantage is that the possibility of misdiagnosis due to stress hyperglycemia is greatly reduced, and also, the values are not affected by short-term transient changes in blood glucose levels (13, 14). The measurement of HbA1c does not require overnight fasting, and the amount of blood required is also small (14). These characteristics probably make the testing for HbA1c feasible for routine screening of diabetes mellitus, particularly in hospital-based settings.

HbA1c has been shown to be a biochemical marker and a good predictor of vascular disruption is patients with diabetes (15, 16). It has also been shown to associate well with diabetic complications (15–17). However, its prognostic value in the acute neurological conditions such as stroke is still not well-substantiated. Studies have attempted to document the relationship of HbA1c levels and outcomes of patients with both ischemic and hemorrhagic stroke. However, the current understanding is not enough to inform the guidelines. There is a need for high-quality evidence through pooling of findings of studies in order to make an informed decision on the use of HbA1c for prediction of outcomes of stroke patients. With these considerations, the current meta-analysis was conducted to assess the relationship of HbA1c and outcomes (mortality, early neurological and functional) of stroke patients, both ischemic and hemorrhagic.

MATERIALS AND METHODS

Search Strategy

The study was designed and conducted based on PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyses) guidelines. A systematic search of English language papers published until November 30, 2020 was carried out through PubMed, Scopus, and Google academic databases. The search strategy included medical topic heading (MeSH) terminology and free text words. **Supplementary Table 1** includes the details of the search strategy used. The literature search was directed toward identifying studies that reported on the association of HbA1c levels with mortality and/or functional outcomes and/or neurological complications in patients with stroke, either acute ischemic or hemorrhagic.

Selection Criteria and Methods

Two subject experts from the team reviewed the studies identified on literature search. The titles and abstracts were screened as a first step, after elimination of the duplicates. The full text of possible studies was subsequently reviewed. Any disagreements in the inclusion of the studies were resolved through discussions between the study authors. Only the studies that complied with the inclusion criteria were chosen for the meta-analysis. For additional studies, the bibliographic list of included studies and related reviews on the subject were reviewed.

Inclusion Criteria

Studies that were either retrospective record-based study or prospective in design were considered for inclusion. For a study to be included in the meta-analysis, it should have examined the relationship between HbA1c with outcomes of interest (i.e., mortality, functional dependence, symptomatic intracranial hemorrhage, and neurological complications). Further, the study should also have reported on the strength of association in the form of adjusted odds ratio/hazard ratios.

Exclusion Criteria

Studies such as case-reports or review articles were excluded. Also, those studies that did not provide data on the outcomes of interest or did not present an adjusted estimate of association between HbA1c and the outcomes were excluded.

Data Extraction and Quality Assessment

Two authors separately extracted relevant data from the included studies using a data extraction sheet. Data extracted from qualifying studies mainly included the study identifier, i.e., the name of the first author along with the year the research was conducted; study setting, i.e., the country where the study was carried out; and other aspects of the study such as the design, subject characteristics, overall sample size, exposure variable of interest, and the main findings. The quality assessment of the included studies was done through the use of Newcastle–Ottawa Quality Assessment Scale, which has been adapted for use in observational studies (18).

Statistical Analysis

This meta-analysis, using STATA version 16.0, reported effect sizes as pooled odds ratio with 95% CI (confidence intervals). Analysis was done for acute ischemic stroke and hemorrhagic stroke separately. Subgroup analysis was done based on different reported cutoff of HbA1c. I^2 was used as a measure to denote heterogeneity, and in instances where the value of I^2 exceeded

40%, random effects model was used. For reporting statistical significance, a *p*-value of <0.05 was considered. Egger's test was employed to assess for presence or absence of publication bias, and this was further supported by visual inspection of funnel plots.

RESULTS

Selection of Articles, Study Characteristics, and Quality of Included Studies

Using the search strategy and after removal of the duplicates, overall, 784 citations were obtained (**Figure 1**). Screening of the titles led to the removal of 612 studies. Out of the remaining 172 citations, 142 were omitted after reading the abstract. The remaining 30 papers were reviewed in detail, and finally, 22 articles were included in the meta-analysis with 15 studies on acute ischemic stroke and seven studies on hemorrhagic stroke (19–40). **Tables 1**, **2** present the details of the included studies. Among the studies that included patients with acute ischemic stroke, majority were conducted in China (6/15). One study each was conducted in New Zealand, Taiwan, Italy, India, South Korea, Sweden, Japan, Germany, and USA. Among these studies, nearly half (8/15) had a prospective design, while the remaining had a retrospective design. For studies with acute hemorrhagic stroke patients, majority were done in China (5/7) and one study each in USA and Japan. All the studies on acute hemorrhagic stroke were prospective in design. Almost all the studies were done in elderly subjects aged above 60 years.

The primary outcomes for this meta-analysis were mortality and functional dependence. The secondary outcomes were risk of symptomatic intracranial hemorrhage (sICH), early neurological complications, and stroke recurrence. Mortality was reported by studies as within 1 year of stroke, whereas functional outcomes were reported at or within 3 and 12 months from the onset of stroke. Symptomatic intracranial hemorrhage and early neurological deterioration/complications were reported by majority of studies within 24 h of stroke onset. Out of the five studies that reported sICH, four studied intracranial hemorrhage after recanalization therapies (20, 28, 31, 32), and one reported hemorrhagic transformation

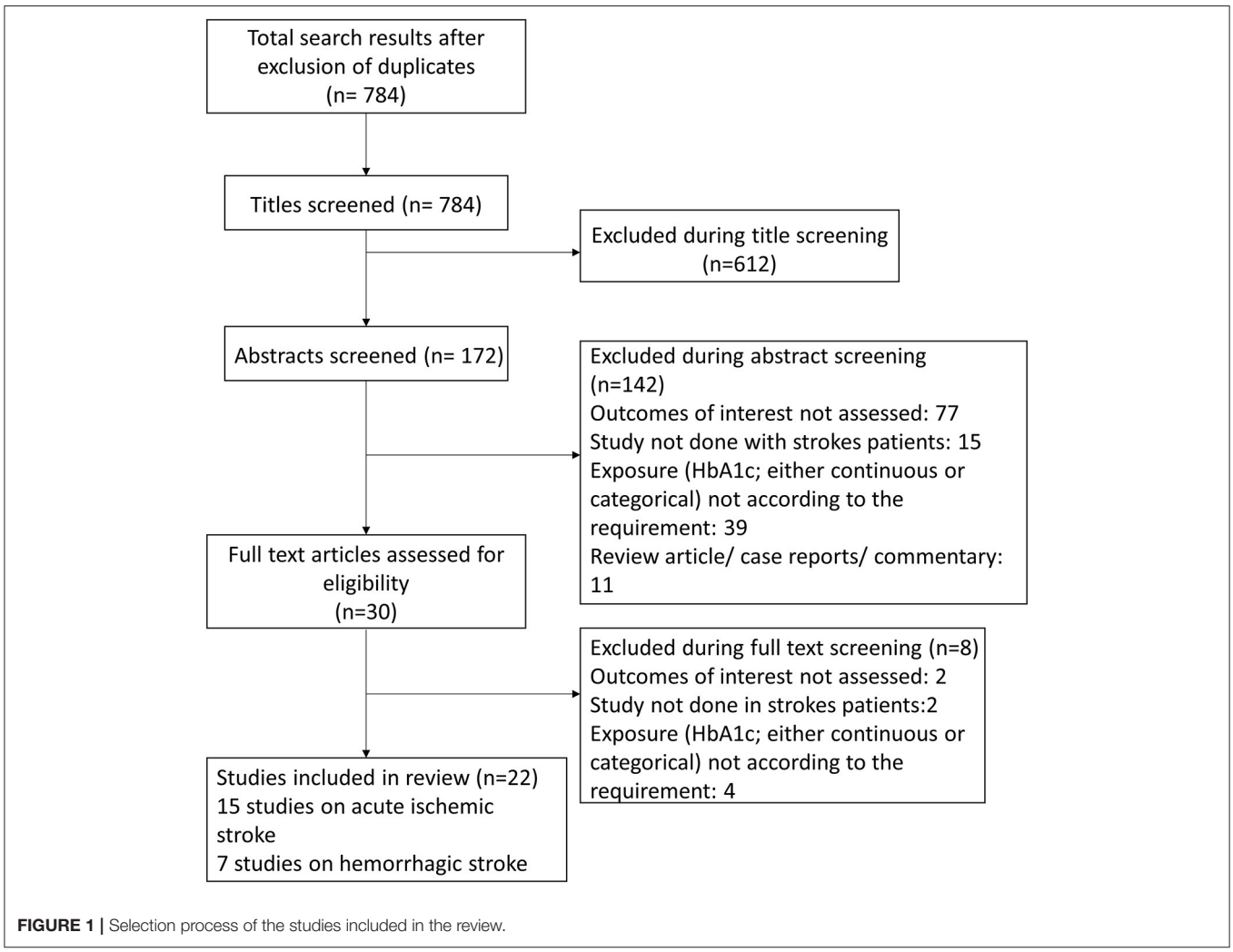

FIGURE 1 | Selection process of the studies included in the review.

TABLE 1 | Characteristics of the studies included in the meta-analysis (for acute ischemic stroke).

References	Study design	Country	Participant characteristics	Sample size	Exposure variable of interest	Key outcome
Diprose et al. (19)	Prospective follow-up	New Zealand	Patients undergoing endovascular thrombectomy for ischemic stroke Mean (SD) age: 64.5 (14.6) years; 61% males	223	HbA1c as continuous variable	3-month mortality: OR 1.26 (95% CI: 1.01, 1.57) 3-month functional dependence: OR 1.32 (95% CI: 1.04, 1.67) 24-h neurological deterioration: OR 1.16 (95% CI:0.95, 1.41) Successful reperfusion: OR 1.11 (95% CI: 0.83, 1.49) Symptomatic intracerebral hemorrhage: OR 1.33 (95% CI: 1.03, 1.71)
Jing et al. (20)	Prospective cohort study	China	Ischemic stroke patients enrolled in the "ACROSS-China" cohort Mean (SD) age of participants with no diabetes: 60.6 (12.8) years; 67% male participants Mean (SD) age of participants with HbA1c ≥ 6.5%: 64.2 (11.6) years; 68% male participants	853 (712 with no diabetes; 141 with diabetes diagnosed as HbA1c ≥ 6.5%)	HbA1c ≥ 6.5%	12-month mortality *(N = 853)*: OR 1.02 (95% CI: 0.85, 1.22) 12-month stroke recurrence *(N = 838)*: OR 1.03 (95% CI: 0.86, 1.24) 12-month poor functional outcome *(N = 837)*: OR 1.19 (95% CI:0.74, 1.91)
Wang et al. (21)	Prospective follow-up study	China	Patients with first-ever ischemic stroke were enrolled and followed-up for neurological outcome assessment at 3 months post-stroke. Total sample of 408 subjects with mean (SD) age of 63.8 (11.5) years and 64% males	Overall 408; Low HbA1c (153) Moderate HbA1c (126) High HbA1c (129)	Low (<5.7%); moderate (5.7–6.4%) and high (≥6.5%) HbA1c values HbA1c as continuous variable	Moderate HbA1c: 3-month poor functional outcome (*N* = 279): OR 1.79 (95% CI:1.01, 3.21) High HbA1c 3-month poor functional outcome (*N* = 282): OR 2.39 (95% CI:1.20, 4.74) Continuous HbA1c: 3-month poor functional outcome (*N* = 408): OR 1.38 (95% CI:1.08, 1.78)
Zhang et al. (22)	Prospective cohort	China	Patients with acute anterior circulation stroke; mean age was around 65 years and around 60% subjects were males	426 subjects	HbA1c as continuous variable	Symptomatic intracerebral hemorrhage: OR 1.29 (95% CI: 1.09, 1.53) Poor functional outcome; short term: OR 1.48 (95% CI:1.23, 1.79)
Yang et al. (23)	Retrospective observational study	Taiwan	Patients with acute ischemic stroke Mean age was around 64 years and around 62% subjects were males	309 subjects	HbA1c ≥ 7.0%	Mortality: OR 0.42 (95% CI: 0.16, 1.13) Poor functional outcome: OR 0.83 (95% CI: 0.51, 1.35) Neurological complications: OR 0.60 (95% CI: 0.35, 1.03)
Wu et al. (24)	Prospective cohort	China	Patients with acute ischemic stroke; mean age of subjects around 65 years and around 60% males	Total of 2,186 subjects	HbA1C categorized as: <5.5%; 5.5–<6.1%; 6.1–<7.2%, and ≥7.2%	Mortality within 1 year HbA1c 5.5–6.1% (*N* = 507*)*: OR 1.07 (95% CI: 0.57, 2.01) HbA1c 6.1–<7.2% (*N* = 579*)*: OR 1.01 (95% CI: 0.53, 1.86) HbA1c ≥ 7.2% (*N* = 560): OR 2.45 (95% CI: 1.30, 4.62)

(Continued)

TABLE 1 | Continued

References	Study design	Country	Participant characteristics	Sample size	Exposure variable of interest	Key outcome
						Poor functional outcome at 3 months HbA1c 5.5–6.1% (*N* = 507*)*: OR 1.57 (95% CI: 1.06, 2.33) HbA1c 6.1–<7.2% (*N* = 579*)*: OR 1.30 (95% CI: 0.87, 1.93) HbA1c ≥ 7.2% (*N* = 560): OR 1.36 (95% CI: 0.84, 2.19) Poor functional outcome at 12 months HbA1c 5.5–6.1% (*N* = 507): OR 1.05 (95% CI: 0.63, 1.75) HbA1c 6.1–<7.2% (*N* = 579): OR 0.88 (95% CI: 0.54, 1.46) HbA1c ≥ 7.2% (*N* = 560): OR 1.20 (95% CI: 0.66, 2.19)
Lattanzi et al. (25)	Retrospective cohort	Italy	Patients with acute ischemic stroke; mean age of subjects around 70 years and around 60% males	112 subjects	HbA1C categorized as: <7.0% and ≥7.0%	Poor functional outcome at 3 months HbA1c ≥7.0%: OR 6.22 (95% CI: 1.94, 19.98)
Sunanda et al. (26)	Prospective case control study	India	Patients with acute ischemic stroke; mean (SD) age of subjects 56.7 (12.9) years; 72% males	130 subjects	HbA1C categorized as: <7.0% and ≥7.0%	Poor functional outcome at 3 months HbA1c ≥ 7.0%: OR 19.4 (95% CI: 5.9, 63.2)
Choi et al. (27)	Prospective cohort	South Korea	Patients with acute ischemic stroke (large vessel occlusion) treated with mechanical thrombectomy; subjects around 69 years of age and nearly 50% male	534 subjects	HbA1C categorized as: <6.5% and ≥6.5%	Poor functional outcome at 3 months HbA1c ≥ 6.5%: OR 2.22 (95% CI: 1.43, 3.45) Mortality within 3 months HbA1c ≥ 6.5%: OR 4.32 (95% CI: 2.41, 7.75) Symptomatic intracerebral hemorrhage HbA1c ≥ 6.5%: OR 1.50 (95% CI: 0.68, 3.30) Early neurological deterioration HbA1c ≥ 6.5%: OR 2.11 (95% CI: 1.31, 3.38)
Hjalmarsson et al. (28)	Retrospective analysis of patient data	Sweden	Patients with acute ischemic stroke; subjects around 75 years of age	501 subjects	HbA1C categorized as: ≤6.0% and >6.0% Continuous HbA1C	Mortality within 12 months HbA1c > 6.0%: OR 3.40 (95% CI: 1.40, 8.22) Continuous HbA1c: OR 1.29 (95% CI: 1.03, 1.62) Poor functional outcome at 12 months HbA1c > 6.0%: OR 2.68 (95% CI: 1.14, 6.03)

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TABLE 1 | Continued

References	Study design	Country	Participant characteristics	Sample size	Exposure variable of interest	Key outcome
Kamouchi et al. (29)	Both prospective and retrospective cohort; data from multicenter hospital-based registry	Japan	Patients with acute ischemic stroke; mean (SD) age of participants 69 (12) years and 37.7% were women	3,627 subjects	HbA1C categorized as: Excellent (<6.2%) Good (6.2% to <6.9%) Fair (6.9% to <8.4%) Poor (≥8.4%)	Short term outcomes Neurological deterioration: HbA1c ≥ 6.9%: OR 1.65 (95% CI: 1.31, 2.06) Mortality: HbA1c ≥ 6.9%: OR 1.20 (95% CI: 0.79, 1.84) Poor functional outcome: HbA1c ≥ 6.9%: OR 1.35 (95% CI: 1.16, 1.58) Additional findings: Neurological deterioration: HbA1c Good: OR 1.02 (95% CI: 0.70, 1.46) HbA1c Fair: OR 1.66 (95% CI: 1.12, 2.43) HbA1c Poor: OR 2.32 (95% CI: 1.39, 3.83) Mortality: HbA1c Good: OR 1.20 (95% CI: 0.60, 2.64) HbA1c Fair: OR 1.02 (95% CI: 0.46, 2.45) HbA1c Poor: OR 0.46 (95% CI: 0.16, 1.38) Poor functional outcome: HbA1c Good: OR 1.16 (95% CI: 0.90, 1.51) HbA1c Fair: OR 1.26 (95% CI: 0.94, 1.71) HbA1c Poor: OR 2.30 (95% CI: 1.56, 3.40)
Rocco et al. (30)	Retrospective single-center study	Germany	Patients with acute ischemic stroke Mean age of participants around 68 years and around 60% subjects were males	112 subjects	Continuous HbA1c	Symptomatic intracerebral hemorrhage (within 24 h): OR 10.3 (95% CI: 3.89, 27.3) 3 month-mortality: OR 1.45 (95% CI: 1.25, 1.69) 3 month-poor functional outcome: OR 1.31 (95% CI: 1.15, 1.46)
Masrur et al. (31)	Retrospective analysis using GWTG-stroke database	USA	Patients with acute ischemic stroke; Median age of 72 years and 50% females	72,909 subjects	HbA1c categorized as ≤6.5% and >6.5%	Symptomatic intracerebral hemorrhage (within 24h): OR 1.25 (95% CI: 1.07, 1.46) In hospital-mortality: OR 1.36 (95% CI: 1.21, 1.53) Poor functional outcome at discharge: OR 1.29 (95% CI: 1.19, 1.39)

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TABLE 1 | Continued

References	Study design	Country	Participant characteristics	Sample size	Exposure variable of interest	Key outcome
Gao et al. (32)	Retrospective review of data from hospital-based registry	China	Patients with acute ischemic stroke; mean age of 62 years and 70% males	793 subjects	HbA1c categorized as <5.9%; 5.9–6.7% and ≥6.7%	3-month poor functional outcome HbA1c 5.9–6.7%: OR 1.63 (95% CI: 0.89, 2.98) HbA1c ≥ 6.7%: OR 2.10 (95% CI: 1.16, 3.79)
Lei et al. (33)	Chengdu stroke registry with prospective follow-up	China	Patients with acute ischemic stroke; mean age of 65 years and 60% males	1,877 subjects	HbA1c categorized as 4.7–6.7%; 6.8% to 8.2% and >8.2%	3-month poor functional outcome HbA1c 6.8–8.2%: OR 1.22 (95% CI: 0.86, 1.55) HbA1c >8.2%: OR 1.43 (95% CI: 1.15, 2.39) 3-month mortality HbA1c 6.8–8.2%: OR 1.32 (95% CI: 0.63, 3.01) HbA1c >8.2%: OR 1.43 (95% CI: 1.01, 1.98) 12-month poor functional outcome HbA1c 6.8–8.2%: OR 1.02 (95% CI: 0.52, 1.69) HbA1c >8.2%: OR 1.17 (95% CI: 1.01, 1.83) 12-month mortality HbA1c 6.8–8.2%: OR 1.22 (95% CI: 0.59, 1.65) HbA1c >8.2%: OR 1.48 (95% CI: 1.03, 2.30)

TABLE 2 | Characteristics of the studies included in the meta-analysis (for acute hemorrhagic stroke).

References	Study design	Country	Participant characteristics	Sample size	Exposure variable of interest	Key outcome
Kang et al. (34)	Multicenter prospective observational cohort study	China	Patients with spontaneous intracranial hemorrhage (sICH)	1,515 subjects	HbA1c with following cut-offs <6.0%; 6.0–7.9%; ≥8.0%	3-month poor functional outcome HbA1c ≥ 6.0%: OR 0.94 (95% CI: 0.67, 1.32) HbA1c 6.0–7.9%: OR 0.79 (95% CI: 0.32, 1.97) HbA1c ≥ 8.0%: OR 0.69 (95% CI: 0.14, 3.30)
Dandapat et al. (35)	GWTG-Stroke prospective registry of patients with ICH	USA	Patients with spontaneous intracranial hemorrhage (sICH); mean age around 67 years and 45% females	75,455 subjects	HbA1c with following cut-offs <5.7%; 5.7–6.5%; 6.5–8.0%; >8.0%	In-hospital mortality HbA1c ≥ 6.5%: OR 0.91 (95% CI: 0.87, 0.95) HbA1c > 8.0%: OR 0.78 (95% CI: 0.65, 0.93) HbA1c 6.5–8.0%: OR 0.73 (95% CI: 0.62, 0.87) HbA1c 5.7–6.5%: OR 0.72 (95% CI: 0.60, 0.87) Poor functional outcome at discharge HbA1c ≥ 6.5%: OR 0.96 (95% CI: 0.93, 0.99) HbA1c > 8.0%: OR 0.78 (95% CI: 0.57, 1.06) HbA1c 6.5–8.0%: OR 0.75 (95% CI: 0.55, 1.02) HbA1c 5.7–6.5%: OR 0.79 (95% CI: 0.56, 1.10)
Koga et al. (36)	Prospective multicenter observational study	Japan	Patients with hyperacute ICH; mean age of around 65 years with 60% males	176 subjects	Continuous HbA1c (per unit increase)	3-month mortality OR 1.04 (95% CI: 0.46, 1.97) 3-month functional outcome OR 1.54 (95% CI: 0.90, 2.94)
Zhang et al. (37)	Nationwide prospective cohort study	China	Patients with ICH; around 62% males and mean age of around 60 years	357	HbA1c categorized as <6.5% and ≥6.5%	12-month mortality HbA1c ≥ 6.5%: OR 1.08 (95% CI: 0.85, 1.37) 12-month stroke recurrence HbA1c ≥ 6.5%: OR 1.15 (95% CI: 0.90, 1.46) 12-month poor functional outcome HbA1c ≥ 6.5%: OR 1.93 (95% CI: 1.10, 3.38)
Zhang et al. (38)	Prospective registry study	China	Patients with spontaneous ICH; mean (SD) age of 59.8 (12.2) and 61% males	288	Continuous HbA1c (per unit increase)	Poor functional outcome at hospital discharge OR 1.28 (95% CI: 1.01, 1.33)

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TABLE 2 | Continued

References	Study design	Country	Participant characteristics	Sample size	Exposure variable of interest	Key outcome
Liu et al. (39)	Prospective multicenter cohort study	China	Patients with spontaneous ICH; mean (SD) age of 59 years and 65% males	416	HbA1c categorized as <5.7%; 5.7–6.4% and ≥6.5%	12-month poor functional outcome HbA1c ≥ 6.5%: OR 2.35 (95% CI: 1.28, 4.29) HbA1c 5.7–6.4%: OR 1.11 (95% CI: 0.62, 2.00) 12-month mortality HbA1c ≥ 6.5%: OR 2.63 (95% CI: 1.34, 5.15) HbA1c 5.7–6.4%: OR 1.35 (95% CI: 0.64, 2.84)
Wang et al. (40)	Prospective cohort study	China	Patients with spontaneous ICH; mean (SD) age of 60 years and 40% females	150	HbA1c categorized as <5.7%; 5.7–6.4% and ≥6.5%	1-month poor functional outcome HbA1c ≥ 6.5%: OR 8.6 (95% CI: 1.77, 41.7) HbA1c 5.7–6.4%: OR 6.17 (95% CI: 1.40, 27.1)

of the ischemic infarct independently of recanalization therapies (23).

The results of the quality evaluation of the included studies are provided in **Supplementary Table 2**. Overall, the quality of the included studies was judged to be good. Majority of studies reported on appropriate selection of participants, ascertainment of exposure and outcome, and had controlled for baseline differences in the cohorts.

Findings for Acute Ischemic Stroke

HbA1c as Continuous

Upon pooling of relevant studies, each unit increase in HbA1c was found to be associated with an increased risk of mortality within 1 year (OR 1.36; 95% CI: 1.22, 1.52; $I^2 = 0.0\%$; no. of studies, $N = 3$), increase risk of poor functional outcome or functional dependence at 3 months (OR 1.35; 95% CI: 1.24, 1.48; $I^2 = 0.0\%$; $N = 4$), and an increased risk of symptomatic intracranial hemorrhage (sICH) within 24 h of admission (OR 1.89; 95% CI: 1.11, 3.23; $I^2 = 88.2\%$; $N = 3$) (**Figure 2**). There was no evidence of publication bias using Egger's test, for any of the outcomes considered ($P = 0.72$ for mortality; $P = 0.31$ for poor functional outcome and $P = 0.18$ for sICH). Funnel plot is presented as **Supplementary Figures 1–3**.

HbA1c as Categorical

The pooled effect sizes for HbA1c ≥ 6.5% indicate increased mortality within 1 year of admission for stroke (OR 1.42; 95% CI: 1.12, 1.80; $I^2 = 74.0\%$; $N = 8$) and increased risk of poor functional outcomes at 3 months (OR 1.51; 95% CI: 1.27, 1.79; $I^2 = 72.0\%$; $N = 10$) and 12 months (OR 1.28; 95% CI: 1.06, 1.55; $I^2 = 0.0\%$; $N = 4$) after the event of stroke (**Figures 3**, **4**). On subgroup analysis based on the design of the studies, i.e., prospective or retrospective, a significant association was found between HbA1c ≥ 6.5%, and risk of mortality when studies that were prospective in design were pooled (OR 1.59; 95% CI: 1.04, 2.42; $I^2 = 81.9\%$; $N = 5$) but not when studies done retrospectively were pooled (OR 1.28; 95% CI: 0.89, 1.84; $I^2 = 60.3\%$; $N = 4$) (**Supplementary Figure 4**). Further, a significant association was found between HbA1c ≥ 6.5% and risk of poor functional outcome at both 3 and 12 months when studies that were prospective in design were pooled (at 3 months: OR 1.96; 95% CI: 1.29, 3.00; $I^2 = 80.0\%$; $N = 5$; at 12 months: OR 1.23; 95% CI: 1.01, 1.50; $I^2 = 0.0\%$; $N = 3$). Similar findings for risk of poor functional outcome were observed when studies that were retrospective in design were pooled (at 3 months: OR 1.33; 95% CI: 1.13, 1.56; $I^2 = 57.9\%$; $N = 5$; at 12 months: OR 2.68; 95% CI: 1.17, 6.16; $N = 1$) (**Supplementary Figures 5**, **6**).

A significant association was also found between HbA1c ≥ 6.5% and risk of symptomatic intracranial hemorrhage (sICH) within 24 h of admission (OR 1.26; 95% CI: 1.08, 1.47; $I^2 = 0.0\%$; $N = 2$) (**Figure 5**). There was no signification association between high HbA1c values (i.e., HbA1c ≥ 6.5%) and risk of early neurological complications (OR 1.31; 95% CI: 0.71, 2.43; $I^2 = 85.4\%$; $N = 3$) (**Figure 5**). There was no evidence of publication bias using Egger's test, for any of the outcomes considered ($P = 0.29$ for mortality; $P = 0.66$ for poor functional outcome; $P = 0.81$ for sICH, and $P = 0.54$ for

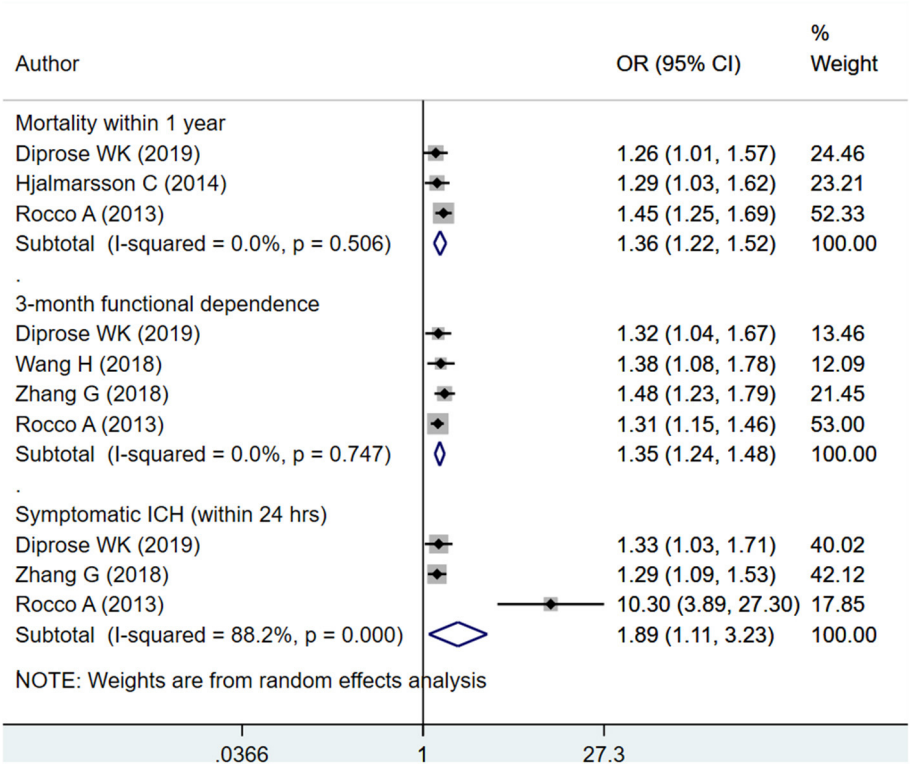

FIGURE 2 | Pooled association of glycated hemoglobin (HbA1c) (continuous) with outcomes (mortality, functional dependence, and symptomatic intracranial hemorrhage) in patients with acute ischemic stroke.

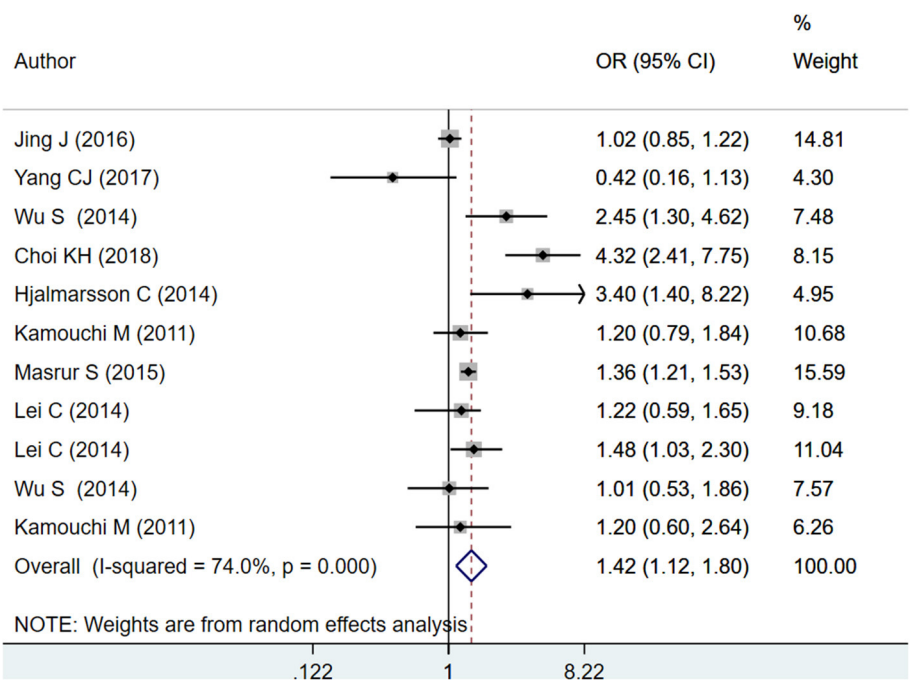

FIGURE 3 | Pooled association of HbA1c ≥ 6.5% with mortality within 1 year of admission for acute ischemic stroke.

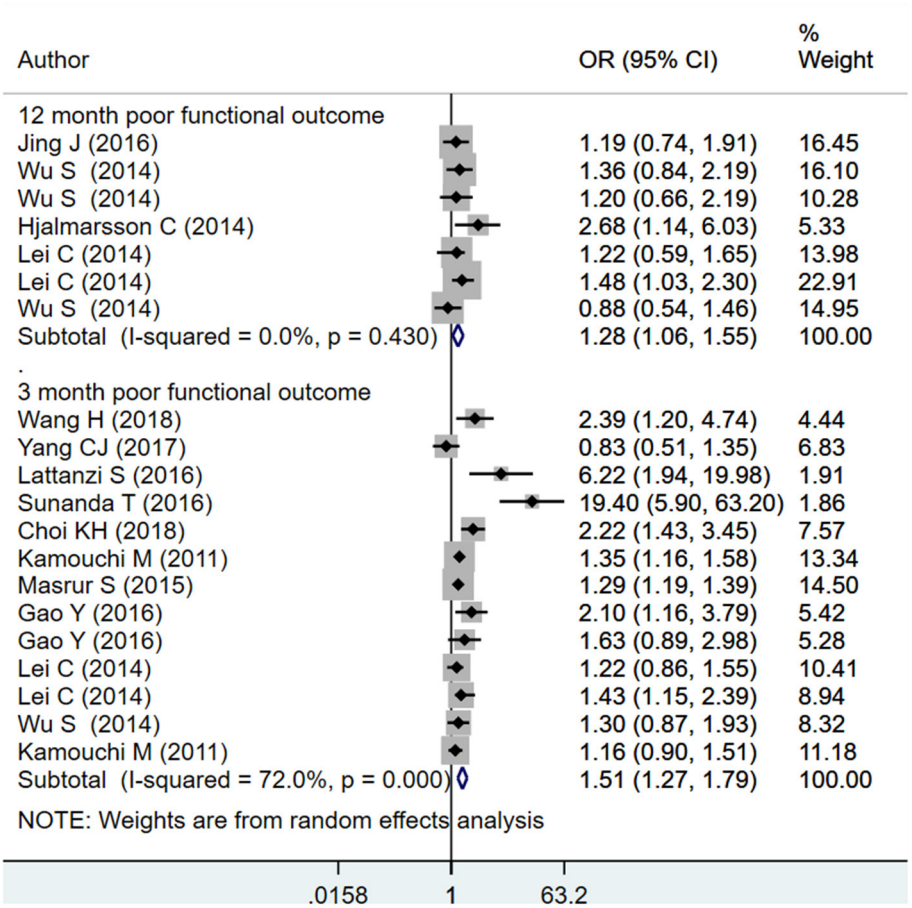

FIGURE 4 | Pooled association of HbA1c ≥ 6.5% with poor functional outcomes at 3 and 12 months after the event of acute ischemic stroke.

early neurological complications). Funnel plot is presented as **Supplementary Figures 7–10**.

On subgroup analysis, based on different values of HbA1c, there was no significant association with mortality within 1 year of admission for stroke (**Supplementary Figure 11**). This could be because of very few studies reporting association between subgroups based on different HbA1c values and mortality. The 3-month poor functional outcome was significantly associated with different subgroups based on HbA1c values (*HbA1c 5.9–6.7%:* OR 1.24; 95% CI: 1.01, 1.52, $N = 3$; *HbA1c 6.8–8.2%:* OR 1.24; 95% CI: 1.00, 1.53, $N = 2$; *HbA1c > 8.2%:* OR 1.80; 95% CI: 1.13, 2.87, $N = 2$) (**Supplementary Figures 12–14**). Further, a dose–response relationship was observed with the magnitude of association being maximum in the subgroup with HbA1c >8.2%. Possibly due to very few studies that reported on 12-month functional outcome within subgroups based on HbA1c, the pooled association was non-significant.

Findings for Hemorrhagic Stroke

HbA1c as Continuous

Each unit increase in HbA1c was found to be associated with increased risk of poor functional outcome within the first 3 months from the time of admission for stroke (OR 1.29; 95% CI: 1.13, 1.48; $I^2 = 0.0\%$; $N = 2$) (**Figure 6**). However, such significant association was not observed with 3-month mortality (OR 1.04; 95% CI: 0.50, 2.15; $N = 1$). The risk of "any" complication, i.e., either mortality or poor functional outcome, increased with each unit increase in HbA1c (OR 1.28; 95% CI: 1.12, 1.46; $I^2 = 0.0\%$; $N = 2$) (**Figure 6**). There was no evidence of publication bias using Egger's test, for any of the outcomes considered ($P = 0.29$ for mortality; $P = 0.33$ for poor functional outcome). Funnel plot is presented as **Supplementary Figure 15**.

HbA1c as Categorical

The pooled effect sizes indicate that among patients with hemorrhagic stroke, HbA1c ≥ 6.5% is associated with increased risk of poor functional outcome at 12 months (OR 2.11; 95% CI: 1.40, 3.19; $I^2 = 0.0\%$; $N = 2$) but not poor functional outcome within 3 months (OR 1.08; 95% CI: 0.72, 1.62; $I^2 = 73.0\%$; $N = 3$) or mortality within 12 months (OR 1.15; 95% CI: 0.82, 1.61; $I^2 = 82.3\%$; $N = 3$) (**Figure 7**). There was no evidence of publication bias for any of the outcomes considered ($P = 0.17$ for mortality; $P = 0.25$ for poor functional outcomes). Funnel plot is presented as **Supplementary Figure 16**.

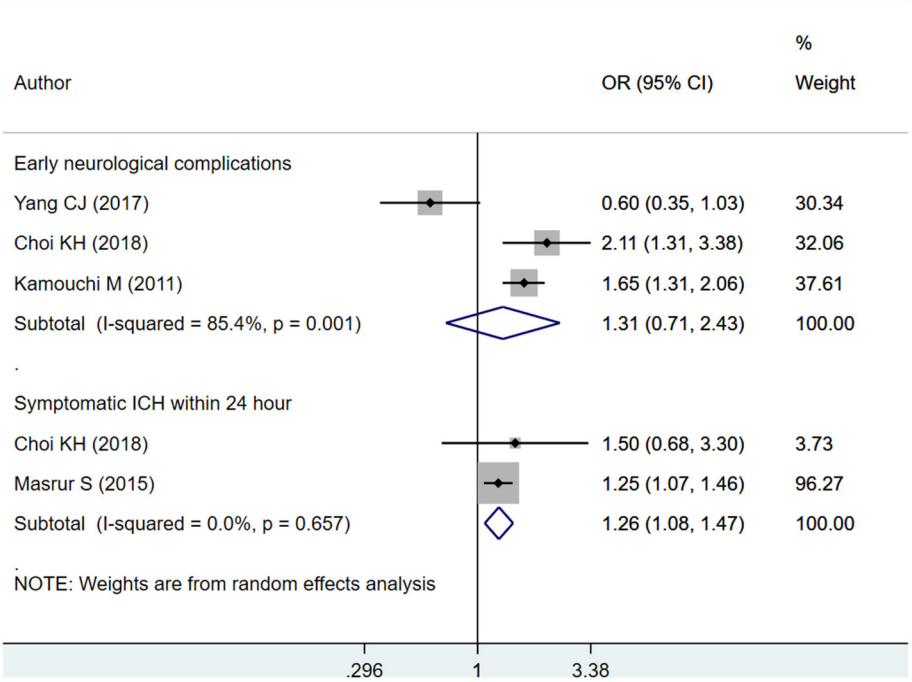

FIGURE 5 | Pooled association of HbA1c ≥ 6.5% with early neurological complications and symptomatic intracranial hemorrhage (sICH) within 24 h of admission, in patients with acute ischemic stroke.

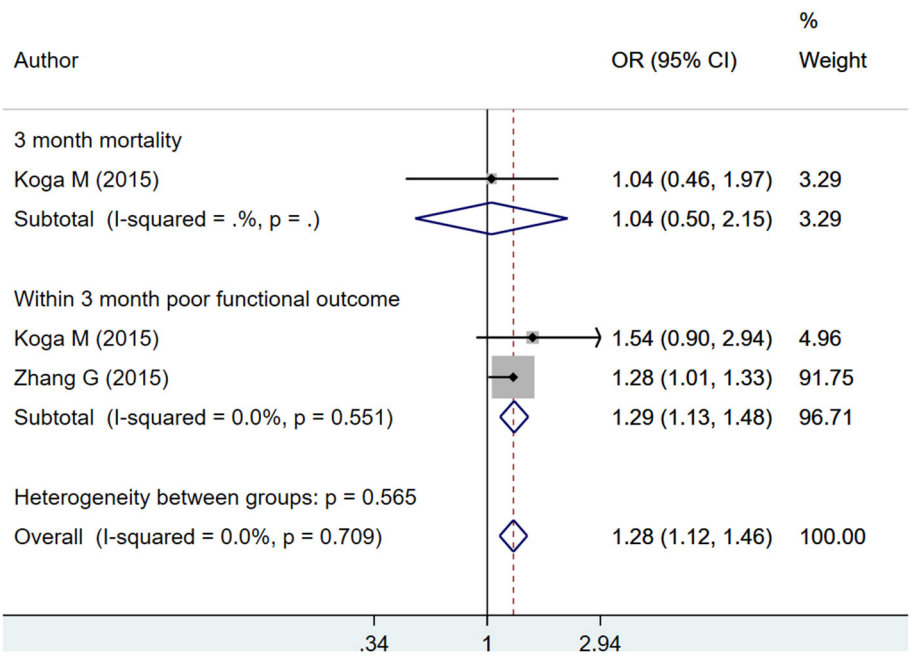

FIGURE 6 | Pooled association of continuous HbA1c with mortality and poor functional outcomes at or within 3 months from time of admission for stroke, in patients with hemorrhagic stroke.

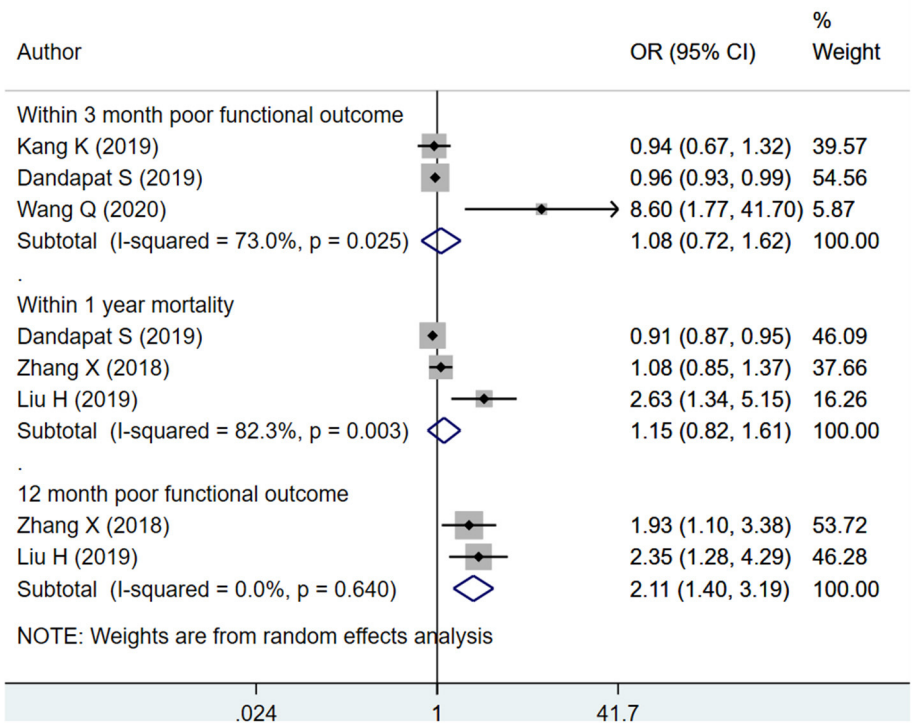

FIGURE 7 | Pooled association of HbA1c ≥ 6.5% with functional outcomes and mortality in patients with hemorrhagic stroke.

On subgroup analysis, there was no significant association with mortality within 1 year as well as functional outcomes at 3 and 12 months in subgroup with HbA1c between 5.7 and 6.4% (**Supplementary Figure 17**). In the subgroup of HbA1c between 6.0 and 7.9%, there was a significantly reduced risk of poor functional outcome within 3 months (OR 0.75; 95% CI: 0.56, 1.01; $I^2 = 0.0\%$; $N = 2$) and mortality within 12 months of stroke (OR 0.73; 95% CI: 0.62, 0.86; $N = 1$) (**Supplementary Figure 18**). Similarly, in the subgroup of HbA1c > 8.2%, although not statistically significant, there was a reduced risk of poor functional outcome within 3 months (OR 0.78; 95% CI: 0.57, 1.05; $I^2 = 0.0\%$; $N = 2$) (**Supplementary Figure 19**). The risk of mortality with 12 months of stroke (OR 0.78; 95% CI: 0.65, 0.93; $N = 1$) was significantly reduced (**Supplementary Figure 19**).

DISCUSSION

Glycated hemoglobin (HbA1c) has been shown to be associated with increased risk of first-onset stroke (41). A systematic review of 29 articles and around 500,000 participants showed that compared with HbA1c of <5.7%, HbA1c of ≥6.5% was associated with an increased risk of first-ever stroke (hazard ratio 2.15; 95% CI: 1.76, 2.63). The review also documented that for every 1% increment in HbA1c, there was a higher associated risk of first-ever ischemic stroke (hazard ratio 1.49; 95% CI: 1.32, 1.69) (41). So, while we understand well that increase in HbA1c is associated with increased risk of stroke, we do not understand the strength and nature of association of HbA1c with outcomes of stroke. The current meta-analysis was conducted with the intent to examine the association, if any, between HbA1c values and outcomes in patients with ischemic and hemorrhagic stroke. The findings indicate that each unit increase in HbA1c (continuous) and HbA1c ≥ 6.5% was found to be associated with an increased risk of mortality within 1 year, increased risk of poor functional outcome, and increased risk of symptomatic intracranial hemorrhage (sICH) in patients with ischemic stroke. In patients with hemorrhagic stroke, each unit increase in HbA1c was found to be associated with increased risk of poor functional outcome, but no significant association was observed with mortality. Similarly, HbA1c ≥ 6.5% was associated with increased risk of poor functional outcome but not with mortality. These findings indicate that glycated hemoglobin could serve as a useful marker to predict the outcomes in patients with stroke and consequently, the required management could be instituted.

Most of the included studies were consistent and showed high HbA1c to be associated with poor outcomes, yet there were few studies that reported findings in the opposite direction, particularly in hemorrhagic stroke patients. The findings of our subgroup analysis also show similar patterns. We did a subgroup analysis based on different cutoffs for HbA1c. Among those with ischemic stroke, no significant association was noted for any of the HbA1c categories with mortality within 1 year of admission. This might be because of very few studies reporting this association. Nonetheless, there was a clear dose–response

relationship between different subgroups based on HbA1c values (*HbA1c 5.9–6.7%*; *HbA1c 6.8–8.2%*; *HbA1c > 8.2%*) and short-term poor functional outcomes (within 3 months of admission for stroke). The magnitude of association was maximum in the subgroup with HbA1c > 8.2%. In those with hemorrhagic stroke, contrary to the current belief that higher HbA1c values will be associated with poor outcomes, in the subgroup of HbA1c between 6.0 and 7.9%, there was a significantly reduced risk of poor functional outcome within 3 months and mortality within 12 months of stroke. Similarly, in the subgroup of HbA1c > 8.2%, there was a reduced risk of poor functional outcome within 3 months, and the risk of mortality with 12 months of stroke was also significantly reduced. Studies have indicated low HbA1c to be associated with liver disease, low fibrinogen, and anemia, all of which could be expected to raise the risk of bleeding and increased hematoma volume (35, 42, 43). With high HbA1c levels, these might be averted, and this might explain the observed paradox.

Levels of blood glucose at the time of admission for stroke has been shown to have a positive association with the levels of HbA1c (44–46). The effect of uncontrolled blood glucose on infarct size and severity of the stroke is thought to be mediated through triggering of inflammatory pathways (44, 46). A poor glycemic status before and in the hyperacute stage of the stroke can therefore lead to worsening of the ischemic damage and consequent poor recovery. The high concentrations of HbA1c may also be an expression of unattention to a healthy lifestyle and poor adherence to treatment for coexisting vascular risk factors and related medical conditions. All these put together can have a detrimental impact on the outcomes of patients. The findings of the study serve as a useful evidence to support clinical programs aimed at better glycemic control in patients with diabetes as adequate pre-stroke glycemic control was found to decrease the risk of unfavorable outcomes. The findings also indicate that measurement of HbA1c could be a good addition to the decision support tools for endovascular thrombectomy; however, the efficacy of this approach needs further evaluation.

As discussed above, a major thrust of the current treatment practice is to focus on intensive and tight glucose control at the time of admission for a stroke event. While that is important, care must be instituted that an event of hypoglycemia does not ensue as this might lead to poor clinical outcomes. Apart from hyperglycemia and hypoglycemia, there is another important, yet overlooked, form of dysglycemia known as glycemic variability (GV) (47). It denotes the degree of fluctuation in the glucose levels over a period of time. Empirical studies have shown GV to be associated with poor functional outcomes, particularly in patients with acute ischemic stroke. However, it must be acknowledged that currently, there is no harmonized and universally accepted index to express GV, and until the time, more data are available on the relationship between GV and outcomes in stroke patients. It is preferred that continuous glucose monitoring is included in the management protocol for stroke (47).

There were some limitations of the study. Studies had used different cutoffs for categorizing HbA1c, and that posed difficulty in performing the analysis, particularly the subgroup analysis. Further, the timing of measurement of HbA1c varied between different studies and that could also have contributed to the heterogeneity observed in the meta-analysis. For some of the outcomes, such as 12-month functional outcome within subgroups based on HbA1c for patients with ischemic stroke, there were very limited number of studies (as low as one in number) that curtailed appropriate pooling of findings and also led to non-significant pooled estimates. Adjusted odd's ratios, as presented in the included studies, were pooled; the variables adjusted in the model may be different for different studies, and that may contribute to the heterogeneity in the pooled findings. Also, quite a few studies included in the analysis had a retrospective design, which may have led to selection bias.

HbA1c is clinically easy to measure, reflects long-term glycemic control, and is unaffected by transient changes in blood glucose levels. The findings of the meta-analysis provide evidence that HbA1c could be used as a marker to predict poor outcomes in patients with ischemic or hemorrhagic stroke. The findings call for regular monitoring and routine HbA1c testing at admission.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

YB conceived and designed the study and wrote the paper. YB and DG were involved in literature search, data collection, and analyzed the data. DG reviewed and edited the manuscript. Both authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.642899/full#supplementary-material

Supplementary Figure 1 | Funnel plot for publication bias with respect to HbA1c (continuous) and mortality within 1 year of admission for acute ischemic stroke.

Supplementary Figure 2 | Funnel plot for publication bias with respect to HbA1c (continuous) and functional outcomes in patients with acute ischemic stroke.

Supplementary Figure 3 | Funnel plot for publication bias with respect to HbA1c (continuous) and symptomatic intracranial hemorrhage in patients with acute ischemic stroke.

Supplementary Figure 4 | Subgroup analysis for mortality based on prospective/retrospective studies.

Supplementary Figure 5 | Subgroup analysis for functional outcome based on prospective/retrospective studies (at 3 months).

Supplementary Figure 6 | Subgroup analysis for functional outcome based on prospective/retrospective studies (at 12 months).

Supplementary Figure 7 | Funnel plot for publication bias with respect to HbA1c (categorical; ≥6.5%) and mortality within 1 year in patients with acute ischemic stroke.

Supplementary Figure 8 | Funnel plot for publication bias with respect to HbA1c (categorical; ≥6.5%) and functional outcomes in patients with acute ischemic stroke.

Supplementary Figure 9 | Funnel plot for publication bias with respect to HbA1c (categorical; ≥6.5%) and symptomatic intracerebral hemorrhage in patients with acute ischemic stroke.

Supplementary Figure 10 | Funnel plot for publication bias with respect to HbA1c (categorical; ≥6.5%) and early neurological deterioration in patients with acute ischemic stroke.

Supplementary Figure 11 | Forest plot with respect to subgroups based on different HbA1c categories and mortality within 1 year in patients with acute ischemic stroke.

Supplementary Figure 12 | Pooled association between HbA1c value ranging from 5.9 to 6.7% with functional outcomes at 3 and 12 months after acute ischemic stroke.

Supplementary Figure 13 | Pooled association between HbA1c value ranging from 6.8 to 8.2% with functional outcomes at 3 and 12 months after acute ischemic stroke.

Supplementary Figure 14 | Pooled association between HbA1c value > 8.2% with functional outcomes at 3 and 12 months after acute ischemic stroke.

Supplementary Figure 15 | Funnel plot for publication bias with respect to HbA1c (continuous) and mortality as well functional outcomes at or within 3 months from time of admission for stroke, in patients with hemorrhagic stroke.

Supplementary Figure 16 | Funnel plot for publication bias with respect to HbA1c ≥ 6.5% and mortality within 12 months as well functional outcomes within 3 months or at 12 months from time of admission for stroke, in patients with hemorrhagic stroke.

Supplementary Figure 17 | Pooled association between HbA1c value ranging from 5.7 to 6.4% with mortality and functional outcomes, in patients with hemorrhagic stroke.

Supplementary Figure 18 | Pooled association between HbA1c value ranging from 6.0 to 7.9% with mortality and functional outcomes, in patients with hemorrhagic stroke.

Supplementary Figure 19 | Pooled association between HbA1c value > 8.2% with mortality and functional outcomes, in patients with hemorrhagic stroke.

Supplementary Table 1 | Search strategy for identification of studies to be included in the review.

Supplementary Table 2 | Author's judgements about study quality using the adapted Ottawa-Newcastle Risk of Bias Assessment tool.

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Serum Phosphate and 1-Year Outcome in Patients With Acute Ischemic Stroke and Transient Ischemic Attack

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Objective: To determine the association between serum phosphate level and 1-year clinical outcomes in patients with acute ischemic stroke and transient ischemic attack.

Methods: We included 7,353 patients with acute ischemic stroke and transient ischemic attack from the China National Stroke Registry III for analysis. Participants were divided into 4 groups according to serum phosphate quartiles. Composite end point included recurrent stroke, myocardial infarction, other ischemic vascular events, and all-cause mortality. Poor functional outcome is defined as modified Rankin Scale score of 3 to 6. Multivariable Cox regression or logistic regression was used to evaluate the independent association of serum phosphate with 1-year all-cause mortality, recurrent stroke, composite end point and poor functional outcome.

Results: The mean age of the included 7,353 patients was 62.5 years, and 68.6% of them were men. Plotting hazard ratios over phosphate levels suggested a U-shaped association especially for recurrent stroke and composite end point, and therefore the third quartile group was set as reference group. Compared with the third quartile of phosphate (1.06–1.20 mmol/L), the adjusted hazard ratios/odds ratios (95% CI) of the lowest quartile (<0.94 mmol/L) were 0.98 (0.67–1.42) for all-cause mortality, 1.31 (1.05–1.64) for stroke recurrence, 1.26 (1.02–1.57) for composite end point, and 1.27 (1.01–1.61) for poor functional outcome, and the adjusted odds ratio of the highest quartile (≥1.2 mmol/L) was 1.40 (1.11–1.77) for poor functional outcome.

Conclusions: Serum phosphate may be an independent predictor of stroke recurrence, composite end point and poor functional outcome after ischemic stroke.

Keywords: serum phosphate, stroke, recurrence, mortality, outcome

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INTRODUCTION

Phosphorus plays an important role in multiple biological functions, including cellular signal transduction, mineral metabolism, and energy exchange. Serum phosphorus mainly occurs as inorganic phosphate in human body. The level of serum phosphate is tightly regulated by several pathways including dietary absorption, bone formation, renal excretion, and intracellular stores (1–3). The underlying pathological effects of elevated phosphate on cardiovascular organs include vascular calcification and endothelial dysfunction (4). Higher phosphate level is associated with higher rates of cardiovascular events or cardiovascular disease related mortality in general population (5–8) and individuals with underlying coronary artery disease (9), while low serum phosphate level is associated with hypertension and metabolic syndrome in general population (10–12). Although studies investigating dialysis patients (10, 13) focused on the relationships between hyperphosphatemia, hypophosphatemia and the cardiovascular outcomes in the beginning, more and more studies (5–9, 11, 12) found the relationship even within relatively normal ranges.

Current data about the effects of serum phosphate on clinical outcomes in patients after ischemic stroke is limited. Although one study showed a U-shaped association between phosphate and in-hospital mortality with significantly increased risk among ischemic stroke patients with lower phosphate level (14), another study found no association between phosphate and 3-month functional outcome in patients with acute ischemic stroke (15). To date, no comprehensive study of the association between serum phosphate and long-term clinical outcomes of ischemic stroke patients has been investigated.

In this study, we aimed to determine the association between serum phosphate level and 1-year clinical outcomes including all-cause mortality, recurrent stroke, composite end point, and functional outcome, in patients with acute ischemic stroke and transient ischemic attack (TIA).

METHODS

Study Population

This study was conducted on the basis of the CNSR III study (China National Stroke Registry III), which was a nationwide, hospital-based, prospective cohort study enrolling patients with acute ischemic cerebrovascular events between August 2015 and March 2018 in China. Patients were eligible if they met the following criteria: age 18 years or older; diagnosis within 7 days of the index event of ischemic stroke or TIA. The design, rationale and baseline patient characteristics of CNSR III study have been published previously (16). [15] A total of 201 study sites participated in this study with a median number of patients recruited as 55 (minimum, 3; maximum, 321). Among the 15,166 patients in the registry, 7,353 were analyzed after excluding patients with missing serum phosphate value (n = 7,288), missing serum creatinine or lipid value for key covariates (n = 312) and lost during the 1-year follow-up (n = 213; **Figure 1**).

Standard Protocol Approvals and Patient Consents

The CNSR III study was approved by ethics committee at Beijing Tiantan Hospital (IRB approval number: KY2015-001-01), and written informed consent was obtained from patients or their legally authorized representatives. The study complied with the principles of the Declaration of Helsinki.

Baseline Data Collection

Baseline information of included patients, including demographics, vascular risk factors, important laboratory data, treatment and complications were collected by trained research coordinators at each study center. Fasting blood samples were drawn within 24 h of admission and assessed as baseline laboratory data. Risk factors contained stroke history, hypertension, diabetes mellitus, hypercholesterolemia, coronary heart disease, previous or current smoking, and heavy alcohol consumption. Hypertension, diabetes mellitus, and hypercholesterolemia were defined according to (1) documented or self-reported history or (2) receiving medication for corresponding diseases or (3) clinical or laboratory examination (systolic blood pressure $\geq$140 mmHg or diastolic blood pressure $\geq$90 mmHg on repeated measurements for a diagnosis of hypertension (17), fasting glucose level $\geq$126 mg/dl or 2-h plasma glucose $\geq$200 mg/dl during oral glucose tolerance test (75 g) or random plasma glucose $\geq$200 mg/dl in persons with symptoms of hyperglycemia or hyperglycemic crisis for diabetes mellitus (18), total cholesterol>240 mg/dl or serum triglyceride $\geq$200 mg/dl or low-density lipoprotein cholesterol $\geq$160 mg/dl or high-density lipoprotein cholesterol $\leq$ 40 mg/dl for dyslipidemia (19)), or (4) new diagnosis at discharge. Heavy alcohol consumption was defined as consuming $\geq$2 standardized alcohol drinks per day. Estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration creatinine equation with adjusted coefficient of 1.1 for the Asian population (20, 21).

Serum Phosphate Testing

Fasting blood samples were drawn within 24 h of admission, and serum phosphate levels were assessed with an ammonium molybdate assay using unfrozen samples in each center. Briefly, the phosphate ions react with ammonium molybdate and then reduced to blue molybdenum which is finally colorimetric measured.

Clinical Outcome Assessment

Patients were followed up over telephone at 12-month after disease onset by trained research coordinators who were blinded to baseline clinical status. Data on clinical outcomes were collected. We defined adverse clinical outcomes as recurrent stroke, all-cause mortality, and poor functional outcome. Recurrent stroke includes ischemic stroke, intracranial hemorrhage, and subarachnoid hemorrhage. Composite end point was comprised of recurrent stroke, myocardial infarction, other ischemic vascular events, and all-cause mortality. Poor functional outcome is defined as modified Rankin Scale (mRS)

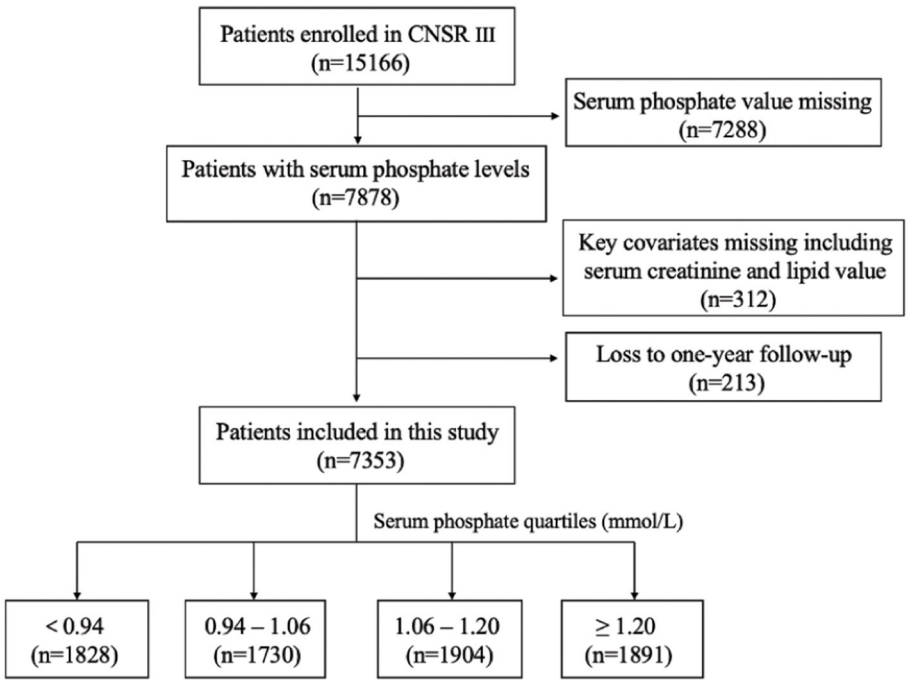

FIGURE 1 | Patient flow diagram. CNSR III, China National Stroke Registry III.

score of 3 to 6 [mRS score ranges from 0 (no symptoms) to 6 (death)].

Statistical Analysis

Proportions were used to describe the categorical variables; means with SD or median with the interquartile range (IQR) were used for continuous variables. We used $\chi 2$ test for categorical variables; 1-way analysis of variance or Kruskal-Wallis test were adopted for continuous variables. Univariate and multivariable Cox regression models were performed to estimate the association between serum phosphate and all-cause mortality, recurrent stroke and composite end point, with third quartile of serum phosphate as reference group (14), while univariate and multivariable logistic regression models were performed to estimate the association between serum phosphate and poor functional outcome. Odds ratios (ORs) or hazard ratios (HRs) with their 95% CIs were reported. Variables included in the multivariable model were selected based on baseline characteristics differences between different quartile groups or based on previous studies. These variables were age, sex, history of stroke, hypertension, diabetes mellitus, hypercholesterolemia, coronary heart disease, current or previous smoking, heavy drinker, body mass index (BMI), National Institutes of Health Stroke Scale (NIHSS) score at admission, hemoglobin, serum calcium, serum potassium, serum albumin, estimated glomerular filtration rate, serum creatinine, total cholesterol, triglycerides, mRS at discharge, antihypertensive drugs, lipid-lowering drugs, hypoglycemia drugs, and pneumonia during hospitalization. In addition, we further explored the pattern of association between serum phosphate levels and risk of stroke outcomes using a logistic regression model with restricted cubic splines for serum phosphate adjusting for covariates with 5 knots (at the 5th, 25th, 50th, 75th and 95th percentiles). Furthermore, C statistics, net reclassification index, and integrated discrimination improvement were used to evaluate the incremental prognostic value of serum phosphate levels beyond conventional risk factors (22). All analyses were conducted with SAS version 9.4 software (SAS institute), and 2-tailed *P*-values of <0.05 were considered to be statistically significant.

RESULTS

Baseline Characteristics

Compared to patients excluded ($n = 7{,}813$), the patients included in this analysis were slightly older and more likely to have diabetes history, to be a heavy drinker, and to have lower mRS score at discharge (**Supplementary Table 1**). Baseline characteristics of included patients are summarized in **Table 1**. Of the total 7,353 patients, the mean age was 62.5 years, and 68.6% were men. Patients with higher quartiles of serum phosphate were younger, and there were more females, more patients with history of diabetes mellitus, hypercholesterolemia while less smokers. They also had slightly higher BMI and lower NIHSS score. The levels of serum calcium, serum potassium, serum albumin, eGFR, total cholesterol, low-density lipoprotein cholesterol and triglycerides increased along with the levels of serum phosphate, while the levels of hemoglobin and serum creatinine decreased. History of stroke, hypertension, coronary

TABLE 1 | Baseline characteristics of the patients according to quartiles of serum phosphate level.

Characteristics	Overall (*n* = 7,353)	Serum phosphate level, mmol/L				*P*-value
		Q1 (<0.94) (*n* = 1,828)	Q2 (0.94-1.06) (*n* = 1,730)	Q3 (1.06-1.20) (*n* = 1,904)	Q4 (≥1.20) (*n* = 1891)	
Age (mean ± SD), y	62.5 ± 11.4	64.7 ± 11.2	63.5 ± 11.0	61.6 ± 11.3	60.4 ± 11.6	<0.0001
Male sex, *n* (%)	5046 (68.6)	1488 (81.4)	1278 (73.9)	1244 (65.3)	1036 (54.8)	<0.0001
Risk factors, *n* (%)						
Previous stroke	1577 (21.5)	422 (23.1)	378 (21.9)	404 (21.2)	373 (19.7)	0.09
Hypertension	4647 (63.2)	1147 (62.8)	1090 (63.0)	1197 (62.9)	1213 (64.2)	0.80
Diabetes mellitus	1764 (24.0)	380 (20.8)	397 (23.0)	467 (24.5)	520 (27.5)	<0.0001
Hypercholesterolemia	589 (8.0)	114 (6.2)	139 (8.0)	167 (8.8)	169 (8.9)	0.01
CHD	777 (10.6)	208 (11.4)	188 (10.9)	179 (9.4)	202 (10.7)	0.24
Current or previous smoking	3514 (47.8)	949 (51.9)	840 (48.6)	915 (48.1)	810 (42.8)	<0.0001
Heavy drinker	1084 (14.7)	280 (15.3)	248 (14.3)	294 (15.4)	262 (13.9)	0.45
BMI (mean ± SD)	24.7 ± 3.4	24.4 ± 3.2	24.6 ± 3.3	24.8 ± 3.5	24.9 ± 3.5	<0.0001
NIHSS score at admission, median (IQR)	3 (1-6)	4 (2-7)	3 (1-6)	3 (1-6)	3 (1-6)	<0.0001
Laboratory parameters, (mean ± SD)						
Hemoglobin, g/L	141.0 ± 17.1	143.1 ± 16.9	141.1 ± 16.5	140.8 ± 16.5	138.9 ± 18.1	<0.0001
Serum phosphate, mmol/L	1.07 ± 0.21	0.82 ± 0.09	1.00 ± 0.03	1.12 ± 0.04	1.33 ± 0.17	<0.0001
Serum calcium, mmol/L	2.2 ± 0.1	2.2 ± 0.2	2.2 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	<0.0001
Serum potassium, mmol/L	3.9 ± 0.4	3.9 ± 0.4	3.9 ± 0.4	3.9 ± 0.4	4.0 ± 0.4	<0.0001
Serum albumin, g/L	40.5 ± 4.1	40.1 ± 4.2	40.4 ± 4.0	40.7 ± 3.9	40.7 ± 4.4	<0.0001
Kidney function						
eGFR, mL/min/1.73 m2	90.7 ± 30.8	89.2 ± 29.7	89.6 ± 28.5	91.6 ± 27.5	92.4 ± 36.5	0.003
Serum creatinine, μmol/L	74.1 ± 28.4	75.7 ± 22.9	75.1 ± 26.0	72.4 ± 24.9	73.4 ± 37.3	0.001
Lipid status (mean ± SD), mmol/L						
Total cholesterol	4.3 ± 1.2	4.2 ± 1.2	4.3 ± 1.2	4.4 ± 1.3	4.4 ± 1.3	<0.0001
LDL cholesterol	2.6 ± 1.1	2.5 ± 1.0	2.6 ± 1.0	2.6 ± 1.1	2.7 ± 1.1	0.0001
HDL cholesterol	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.3	0.30
Triglycerides	1.6 ± 1.1	1.5 ± 0.9	1.6 ± 1.0	1.7 ± 1.3	1.8 ± 1.2	<0.0001
mRS score at discharge, *n* (%)						
0–2	5977 (81.6)	1415 (77.7)	1421 (82.4)	1578 (83.2)	1563 (82.9)	<0.0001
3–5	1351 (18.4)	406 (22.3)	304 (17.6)	319 (16.8)	322 (17.1)	
Medication during hospitalization, *n* (%)						
Antihypertensive drugs	3412 (46.4)	846 (46.3)	805 (46.5)	862 (45.3)	899 (47.5)	0.58
Hypoglycemia drugs	1897 (25.8)	399 (21.8)	429 (24.8)	515 (27.1)	554 (29.3)	<0.0001
Lipid-lowering drugs	6964 (94.7)	1734 (94.9)	1638 (94.7)	1797 (94.4)	1795 (94.9)	0.88
Pneumonia during hospitalization	432 (5.9)	143 (7.8)	111 (6.4)	96 (5.0)	82 (4.3)	<0.0001

BMI, body mass index; CHD, coronary heart disease; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; mRS, modified Rankin Scale score; NIHSS, National Institutes of Health Stroke Scale; Q, Quartiles.

heart disease, alcohol consumption, and level of high-density lipoprotein cholesterol were not significantly different among the quartiles.

One-year Outcomes Among Patients Grouped by Quartiles of Serum Phosphate

The 1-year incidences of clinical outcomes are shown in **Table 2**. The 1-year rates of recurrent stroke, composite endpoint and poor functional outcome were lowest in third quartile group ($p = 0.03$ for recurrent stroke; $p = 0.03$ for composite end point; $p < 0.0001$ for poor functional outcome). There was no significant difference in all-cause mortality among groups ($p = 0.06$). In the lowest serum phosphate quartile, the incidence rates of outcomes including all-cause mortality, recurrent stroke, composite endpoint, and poor functional outcomes were 4.4, 10.8, 11.6, 17.6%, respectively.

Association of Serum Phosphate Levels With Adverse Clinical Outcomes

Crude and adjusted ORs or HRs with 95% CIs of serum phosphate levels for adverse clinical outcomes are presented in **Table 3**. Compared with the third quartile of phosphate

TABLE 2 | Rates of 1-year outcomes according to quartiles of serum phosphate level.

Outcomes	Overall	Serum phosphate level, mmol/L				*P*-value
		Q1(<0.94)	Q2(0.94-1.06)	Q3(1.06-1.20)	Q4(≥1.20)	
All-cause mortality, *n* (%)	266 (3.6)	81 (4.4)	51 (3.0)	60 (3.1)	74 (3.9)	0.06
Recurrent stroke, *n* (%)	674 (9.2)	197 (10.8)	155 (9.0)	154 (8.1)	168 (8.9)	0.03
Composite end point, *n* (%)	723 (9.8)	212 (11.6)	164 (9.5)	168 (8.8)	179 (9.5)	0.03
Poor functional outcome, *n* (%)	1087 (14.8)	321 (17.6)	261 (15.1)	225 (11.8)	280 (14.8)	<0.0001

Q, quartiles.

TABLE 3 | Association between serum phosphate level and clinical outcomes.

	Unadjusted		Age- and sex-adjusted		Multivariable-adjusted[†]	
	OR/HR (95% CI)	*P*-value	OR/HR (95% CI)	*P*-Value	OR/HR (95% CI)	*P*-value
All-cause mortality						
Q1 (<0.94 mmol/L)	1.41 (1.01-1.97)	0.04	1.17 (0.83-1.64)	0.37	0.98 (0.67-1.42)	0.90
Q2 (0.94-1.06 mmol/L)	0.93 (0.64-1.36)	0.72	0.83 (0.57-1.20)	0.32	0.78 (0.52-1.18)	0.24
Q3 (1.06-1.20 mmol/L)	1.00 (reference)		1.00 (reference)		1.00 (reference)	
Q4 (≥1.20 mmol/L)	1.25 (0.89-1.75)	0.21	1.32 (0.94-1.87)	0.11	1.28 (0.87-1.86)	0.21
Recurrent stroke						
Q1 (<0.94 mmol/L)	1.36 (1.10-1.67)	0.005	1.34 (1.08-1.66)	0.008	1.31 (1.05-1.64)	0.02
Q2 (0.94-1.06 mmol/L)	1.11 (0.89-1.39)	0.35	1.10 (0.88-1.38)	0.40	1.13 (0.90-1.43)	0.31
Q3 (1.06-1.20 mmol/L)	1.00 (reference)		1.00 (reference)		1.00 (reference)	
Q4 (≥1.20 mmol/L)	1.11 (0.89-1.38)	0.37	1.11 (0.89-1.38)	0.37	1.11 (0.88-1.40)	0.37
Composite end point						
Q1 (<0.94 mmol/L)	1.34 (1.09-1.64)	0.005	1.30 (1.06-1.60)	0.01	1.26 (1.02-1.57)	0.03
Q2 (0.94-1.06 mmol/L)	1.08 (0.87-1.34)	0.49	1.06 (0.85-1.32)	0.60	1.08 (0.87-1.36)	0.48
Q3 (1.06-1.20 mmol/L)	1.00 (reference)		1.00 (reference)		1.00 (reference)	
Q4 (≥1.20 mmol/L)	1.08 (0.88 −1.33)	0.48	1.09 (0.88-1.34)	0.44	1.09 (0.87-1.35)	0.46
Poor functional outcome						
Q1 (<0.94 mmol/L)	1.59 (1.32-1.91)	<0.0001	1.42 (1.17-1.72)	0.0003	1.27 (1.01-1.61)	0.04
Q2 (0.94-1.06 mmol/L)	1.33 (1.09-1.61)	0.004	1.24 (1.02-1.51)	0.03	1.24 (0.98-1.57)	0.07
Q3 (1.06-1.20 mmol/L)	1.00 (reference)		1.00 (reference)		1.00 (reference)	
Q4 (≥1.20 mmol/L)	1.30 (1.07-1.57)	0.007	1.36 (1.12-1.65)	0.002	1.40 (1.11-1.77)	0.005

[†] *In multivariable analysis, adjusted variables included age, sex, history of stroke, hypertension, diabetes mellitus, hypercholesterolemia, coronary heart disease, current or previous smoking, heavy alcohol, National Institutes of Health Stroke Scale score at admission, modified Rankin Scale score at discharge, body mass index, hemoglobin, serum calcium, serum potassium, serum albumin, estimated glomerular filtration rate, serum creatinine, total cholesterol, triglycerides, antihypertensive drugs, lipid-lowering drugs, hypoglycemia drugs, and pneumonia during hospitalization.*

CI, confidence interval; HR, hazard ratios; OR, odds ratios; Q, quartiles.

(1.06–1.20 mmol/L), the adjusted ORs/ HRs (95% confidence interval) of the lowest quartile (<0.94 mmol/L) were 0.98 (0.67–1.42) for all-cause mortality, 1.31 (1.05–1.64) for stroke recurrence, 1.26 (1.02–1.57) for composite end point, and 1.27 (1.01–1.61) for poor functional outcome, and the adjusted ORs/HRs of the highest quartile (≥1.20 mmol/L) was 1.28 (0.87–1.86) for all-cause mortality, 1.11 (0.88–1.40) for stroke recurrence, 1.09 (0.87–1.35) for composite end point, and 1.40 (1.11–1.77) for poor functional outcome. Further analyses using restricted cubic spline regression showed a U-shaped relationship between serum phosphate levels and poor functional outcome and that low serum phosphate levels were significantly associated with increased risk of recurrent stroke and composite end point (**Figure 2**).

In patients with recurrent stroke ($n = 674$), 64 (9.5%) patients had hemorrhage stroke (**Supplementary Table 2**). Analyses for association between serum phosphate levels and 1-year hemorrhage stroke did not show statistical significance among groups (**Supplementary Table 3**).

Further analysis for association of serum phosphate levels with one-year all-cause mortality and poor functional outcome in patients without recurrent stroke (n = 6,679) did not show statistical difference between serum phosphate quartiles after adjusting for age, sex and other covariates. (**Supplementary Table 4**).

We further investigated whether adding serum phosphate to conventional risk factors improved the risk prediction of stroke recurrence, composite endpoint and poor functional

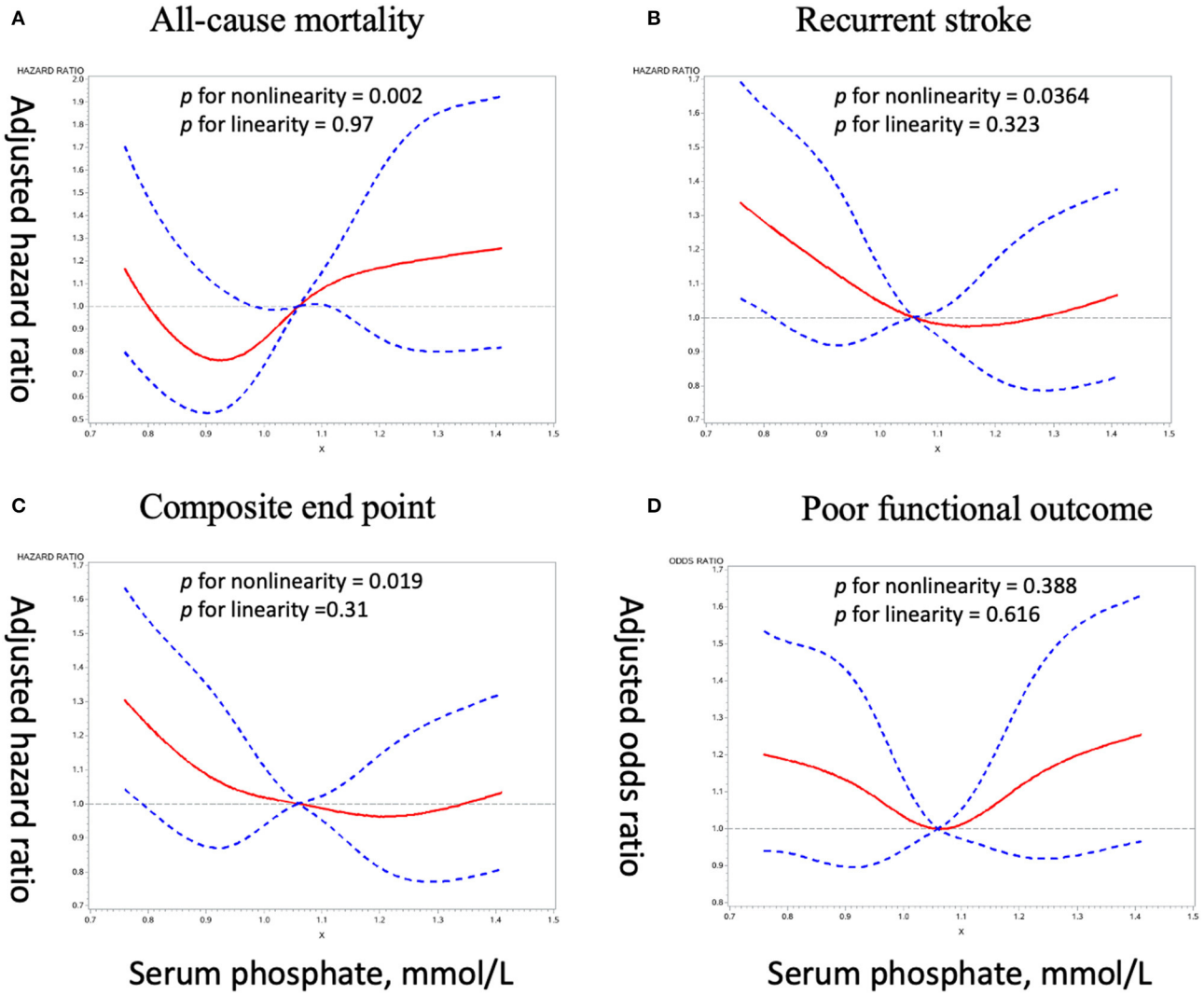

FIGURE 2 | Adjusted dose–response association between serum phosphate levels and adverse clinical outcomes. **(A)**, All-cause mortality. **(B)**, Recurrent stroke. **(C)**, Composite end point. **(D)**, Poor functional outcome.

outcome (**Supplementary Table 5**). Adding serum phosphate to a model containing conventional risk factors significantly improves risk reclassification for stroke recurrence (categorical net reclassification index was 8.9%, $p = 0.03$), composite end point (8.7%, $p = 0.03$) and poor functional outcome (14.3%, $p < 0.001$).

DISCUSSION

In this large observational study of patients with ischemic stroke and TIA, we found lower serum phosphate levels were associated with higher risk for stroke recurrence, composite end point and poor functional outcome after stroke, even after adjusting for potential covariates. Besides, higher serum phosphate levels were associated with higher risk for poor functional outcome. Our results provide new evidence for the relationship between serum phosphate levels and clinical outcomes after stroke.

Previous studies have demonstrated that both high and low serum phosphate levels could be associated with adverse cardiovascular events and related mortality (5–7, 9), and especially high serum phosphate received more attention (4, 23). However, the relationship between serum phosphate and adverse outcomes in patients with ischemic stroke has not been elucidated. Kim et al. (15) found that there is no association between serum phosphate levels and 3-month functional outcome in 1,034 patients with acute ischemic stroke. Zhong et al. (14) reported a U-shape relationship between serum phosphate and all-cause mortality in 2,944 acute ischemic stroke patients, and their results indicated that lower serum phosphate levels are associated with increased risk of in-hospital all-cause mortality. Our results also showed U-shaped relationships between serum phosphate and outcomes. We found that lower serum phosphate levels are associated with increased risk of 1-year stroke recurrence, composite end point and

poor functional outcome, while higher serum phosphate levels are associated with increased risk of poor functional outcome. However, the association between serum phosphate levels and poor functional outcome lost significance when we further tested the relationship in patients without recurrent stroke. We speculated that the association between serum phosphate levels and poor functional outcome could be influenced by stroke recurrence since patients with recurrent stroke usually have poorer functional outcome (24).

The potential pathophysiological mechanisms about the association between low serum phosphate and adverse outcomes after stroke are unclear. There are several possible explanations. Lower serum phosphate levels may affect brain vascular biology considering phosphate as a component of cell membranes and it is important in mediating intracellular signaling (25). In addition, low serum phosphate is a manifestation of malnutrition and low physical activity (10–12). Therefore, further studies are warranted to determine whether the effect of serum phosphate on ischemic stroke outcomes is directly or it is just a manifestation of malnutrition in acute ischemic stroke patients. Furthermore, low phosphate was suggested to be related with hypertension, reduced insulin sensitivity, and metabolic syndrome (10, 26), which might help, although not directly, explain the relationship. More studies are needed to investigate the mechanism underlying the relationship between low phosphate and adverse outcomes. Previous studies considered the underlying pathological effects of elevated phosphate on cardiovascular organs involved vascular calcification and endothelial dysfunction (4, 27). Although restricting dietary phosphate intake was recommended for the benefit of cardiovascular interests (28), our results suggest that maintaining the serum phosphate within an appropriate level, instead of achieving a low phosphate level target, is important to help prevent stroke recurrence, composite end point and poor functional outcome. Further studies are needed to identify the most appropriate range of serum phosphate level.

The study had some limitations. First, this is an observational study. Although several important potential covariates had been adjusted in multivariable regression models, we could not rule out the possibility of residual confounding. For example, we could not adjust for pre-morbid nutrition status since our dataset did not measure pre-morbid function, nutrition intake or "frailty." In addition, due to the fluctuation of serum phosphate with dietary intake, a single admission blood sample might not be representative of the serum phosphate profile over months and therefore interval samples depicting serum phosphate profiles are needed to clarify the relationship in the future study. Second, serum phosphate testing was performed at each study site. However, the results would be comparable because the same method based on the formation of ammonium phosphomolybdate using unfrozen samples was employed. Third, given that we excluded patients lacking serum phosphate value which partly depends on the clinical practice at individual participating hospitals, key covariate value and follow-up information, the selection bias might occur. Fourth, although the metabolism between serum phosphate and other serum electrolytes was closely related, due to that a large proportion of centers in our cohort did not test these variables such as serum magnesium routinely, we cannot further adjust for these variables. Finally, our study focused on Chinese population and the results might not be generalized to other populations with ischemic stroke.

CONCLUSIONS

In summary, in patients with ischemic stroke and TIA, low serum phosphate levels were associated with increased risk of stroke recurrence, composite end point and poor functional outcome while high serum phosphate levels were associated with increased risk of poor functional outcome. Serum phosphate might serve as a predictor for stroke outcomes after ischemic stroke.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee at Beijing Tiantan Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

J-FZ, JJ, XM, YP, Y-LW, X-QZ, J-XL, Y-CW, and Y-JW contributed to the conception and design of the study. J-FZ, JJ, XM, YP, Y-LW, X-QZ, J-XL, X-SH, B-BS, Z-CJ, S-DW, X-FC, W-JX, CA, Y-CW, and Y-JW contributed to the acquisition and analysis of data. J-FZ, JJ, XM, and YP contributed to drafting the text and preparing the figures. All authors edited and revised the manuscript and approved final submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.652941/full#supplementary-material

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Relationship Between Glycosylated Hemoglobin and Short-Term Mortality of Spontaneous Intracerebral Hemorrhage

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Background: The relationship between glycosylated hemoglobin (HbA1c) and prognosis of spontaneous intracerebral hemorrhage (SICH) patients has not been fully elucidated. This study aimed to reveal the relationship between HbA1c levels and short-term mortality after patient admission with SICH.

Methods: It was a large-scale, multicenter, cross-sectional study. From August 1, 2015, to July 31, 2019, a total of 41910 SICH patients were included in the study from the Chinese Stroke Center Alliance (CSCA) program. Finally, we comprehensively analyzed the data from 21,116 patients with SICH. HbA1c was categorized into four groups by quartile. Univariate and multivariate logistic regression analyses were used to assess the association between HbA1c levels and short-term mortality in SICH patients.

Results: The average age of the 21,116 patients was 62.8 ± 13.2 years; 13,052 (61.8%) of them were male, and 507 (2.4%) of them died. Compared to the higher three quartiles of HbA1c, the lowest quartile (≤5.10%) had higher short-term mortality. In subgroup analysis with or without diabetes mellitus (DM) patients, the mortality of the Q3 group at 5.60–6.10% was significantly lower than that of the Q1 group at ≤5.10%. After adjustment for potential influencing factors, the ROC curve of HbA1c can better predict the short-term mortality of patients with SICH (AUC = 0.6286 $P < 0.001$).

Conclusions: Therefore, we concluded that low or extremely low HbA1c levels (≤5.10%) after stroke were associated with higher short-term mortality in SICH patients, with or without DM.

Keywords: HbA1c, glucose, diabetes, intracerebral hemorrhage, mortality

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INTRODUCTION

Spontaneous intracerebral hemorrhage (SICH) accounts for 20–30% of all strokes. As a disabling type of stroke with poor prognosis, SICH contributes to an increase in the global burden of the disease (1, 2). The 30-day mortality rate of ICH was 35–52% (3). Half of the deaths occurred in the acute phase, especially in the first 2 days (4, 5).

Data from several studies suggested that hyperglycemia is associated with severe neurological impairment and poor prognosis in SICH patients (6–14). It is recommended that blood glucose levels should be measured and closely monitored to avoid hyperglycemia (15). However, in patients with SICH (16–18), subarachnoid hemorrhage (19), and ischemic stroke (20), early intensive insulin hypoglycemic therapy did not improve functional prognosis. Recent studies demonstrated that hyperglycemia is only the result of severe nervous system damage, which may be caused by a stress response, mainly adrenergic stress, and relative insulin deficiency (21, 22). Thus, it suggests that blood glucose level measured after the onset of SICH is not an ideal prognostic indicator for stroke patients. In contrast, glycosylated hemoglobin (HbA1c) is a measure of average blood glucose across 2–3 months before stroke. HbA1c possesses a high stability than random blood glucose after stroke, without the need to be measured or compared in a specific time (23, 24). Therefore, HbA1c could be considered as a potential biomarker for the prognosis of SICH. Some studies revealed that HbA1c is a better predictor of adverse outcomes in patients with SICH (25–27). However, a study showed that HbA1c is not associated with clinical outcome in patients with SICH (28). The relationship between HbA1c and the prognosis of SICH patients is not yet fully elucidated.

Therefore, our study aimed to investigate the relationship between HbA1c levels and short-term mortality in SICH patients.

METHODS

Study Population

From August 1, 2015, to July 31, 2019, a total of 1,006,798 patients diagnosed with cerebral hemorrhage, subarachnoid hemorrhage, acute ischemic stroke, or transient ischemic attack were included in the Chinese Stroke Center Alliance (CSCA) program. The patients were over 18 years old within 7 days of symptom onset. In the CSCA program, there was no follow-up after discharge, and only in-hospital information was recorded. We comprehensively analyzed the data from SICH patients enrolled in the CSCA. We included all the SICH patients. Spontaneous intracerebral hemorrhage refers to intracerebral hemorrhage caused by spontaneous rupture of the cerebral artery, vein, and capillary, excluding traumatic cerebral hemorrhage. We excluded patients with (1) history of previous stroke events; (2) history of abnormal liver function and renal function; (3) bleeding history or tendency; (4) lack data of death; and (5) lack data of HbA1c.

The study was conducted in accordance with guidelines from the Helsinki Declaration. All participating hospitals of CSCA had the right to collect data without informed consent of patients or a waiver of authorization and exemption from their institutional review board.

Clinical Information

During the hospital admission of the selected patients for this study, the following information was collected: demographic

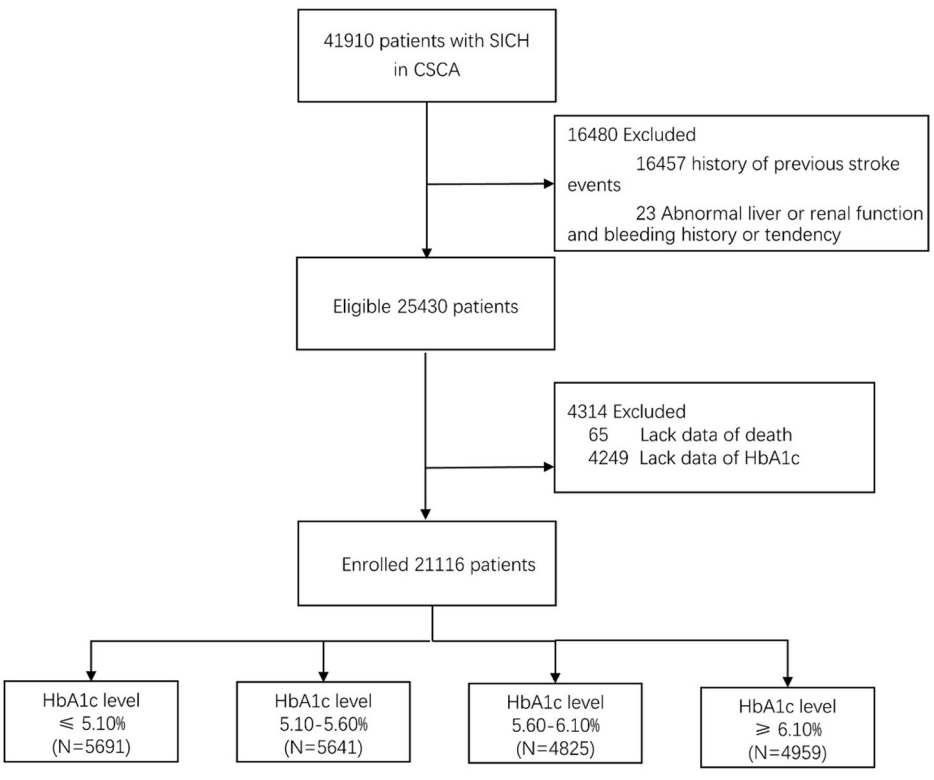

FIGURE 1 | Study population flowchart.

information, medical history [atrial fibrillation, coronary heart disease, hypertension, diabetes mellitus, dyslipidemia, peripheral vascular disease, chronic obstructive pulmonary disease (COPD), drinking, smoking, body mass index (BMI), medication history (hypotension, hypoglycemia, anticoagulation, antiplatelet agent), systolic blood pressure (SBP), and diastolic blood pressure (DBP)]. A history of diabetes is defined as a patient who was definitely diagnosed with diabetes before admission.

Baseline Neurological Assessment

The Glasgow Coma Scale (GCS) and National Institutes of Health Stroke Scale (NIHSS) were used to assess neurological deficit at admission. The GCS score was divided into mild coma (13–15) or moderate to severe coma (≤12). The NIHSS score was divided into mild to moderate disability (<16) or severe disability (≥16).

Laboratory Examinations

Venous blood was collected in a vacuum EDTA collection tube, and plasma was separated. HbA1c was assessed within 7 days after admission. Patients with SICH were divided into four groups according to the quartile of HbA1c: Q1, HbA1c ≤5.10%; Q2, HbA1c 5.10–5.60%; Q3, HbA1c 5.60–6.10%; and Q4, HbA1c ≥6.10%. The quartile was based on the data available in the study. There were significant differences in HbA1c levels among different quartile groups. Compared with the previously published data, the quartile level of HbA1c determined in this study is generally low.

Other laboratory tests, including FBG, bun, creatinine, low-density lipoprotein cholesterol (LDL-C), serum homocysteine (HCY), and international normalized ratio (INR) were also collected.

Clinical Outcomes

We analyzed the relationship between HbA1c levels and adverse outcome in patients with SICH. Adverse outcomes were defined as death during hospitalization.

Statistical Analysis

Continuous variables were expressed as mean ± standard deviation; categorical variables were presented as count (percentage). The median and quartile range expressed ordinal variables. Group differences were analyzed by the independent sample *t*-test or the Mann–Whitney *U*-test for continuous variables and by the chi-squared test for categorical variables. Logistic regression was used to analyze the relationship between different glycosylated hemoglobin levels and short-term mortality. The receiver operating characteristic (ROC) curve was used to evaluate the prognostic value of HbA1c. Sensitivity analysis was used to estimate the effects of potential unmeasured or uncontrolled confounding variables. First, we coded patients who lacked the NIHSS score or GCS score. Then, we analyzed patients with SICH who had NIHSS and GCS scores to determine if the results were similar. Odds ratios (ORs) and 95% confidence interval (95% CI) were expressed for the results and probability values. A two-sided value of $P < 0.05$ was considered statistically

TABLE 1 | Baseline characteristics of SICH patients according to HbA1c quartiles.

Variables	Q1: ≤5.10% (*N* = 5,691)	Q2: 5.10–5.60% (*N* = 5,641)	Q3: 5.60–6.10% (*N* = 4,825)	Q4: ≥6.10% (*N* = 4,959)	*P*-value
Age, years	61.1 ± 13.7	62.5 ± 13.4	63.8 ± 12.8	64.0 ± 12.4	<0.001
Male	3,621 (63.6)	3,566 (63.2)	2,951 (61.2)	2,914 (58.8)	<0.001
BMI	23.7 ± 5.0	23.7 ± 3.8	23.8 ± 3.7	24.3 ± 5.2	<0.001
Systolic BP (mmHg)	163.4 ± 29.4	164.0 ± 27.9	164.0 ± 28.2	164.5 ± 28.8	0.102
Diastolic BP (mmHg)	95.4 ± 17.3	95.0 ± 17.1	94.2 ± 16.8	94.0 ± 17.1	<0.001
Atrial fibrillation	70 (1.2)	87 (1.5)	102 (2.1)	118 (2.4)	<0.001
Myocardial infarction	41 (0.7)	40 (0.7)	49 (1.0)	70 (1.4)	<0.001
Hypertension	3,567 (62.7)	3,620 (64.2)	3,145 (65.2)	3,534 (71.3)	<0.001
Diabetes mellitus	150 (2.6)	114 (2.0)	201 (4.2)	1,498 (30.2)	<0.001
Dyslipidemia	151 (2.7)	125 (2.2)	129 (2.7)	260 (5.2)	<0.001
Peripheral vascular disorder	25 (0.4)	29 (0.5)	25 (0.5)	52 (1.0)	0.001
COPD	72 (1.3)	87 (1.5)	77 (1.6)	89 (1.8)	0.168
Antiplatelet	180 (3.2)	188 (3.3)	186 (3.9)	271 (5.5)	<0.001
Anticoagulation	76 (1.3)	69 (1.2)	53 (1.1)	81 (1.6)	0.009
Antihypertensive	2,098 (36.9)	2,208 (39.1)	1,982 (41.1)	2,447 (49.3)	<0.001
Diabetic medication	101 (1.8)	68 (1.2)	132 (2.7)	1,099 (22.2)	<0.001
Current smoking	1,263 (22.2)	1,304 (23.1)	975 (20.2)	884 (17.8)	<0.001
drinking	1,528 (26.8)	1,518 (26.9)	1,176 (24.4)	1,183 (23.9)	<0.001
NIHSS	6.0 (2.0-12.0)	6.0 (2.0-12.0)	5.0 (2.0-12.0)	6.0 (2.0-14.0)	<0.001
GCS	13.0 (7.0-15.0)	14.0 (8.0-15.0)	14.0 (8.0-15.0)	13.0 (7.0-15.0)	<0.001
Hypoglycemic treatment	114 (2.0)	87 (1.5)	158 (3.3)	1,585 (32.0)	<0.001

Continuous variables were expressed as mean ± SD; categories variables were expressed as n (%). BMI, body mass index; COPD, chronic obstructive pulmonary disease; NIHSS, National Institutes of Health Stroke Scale; GCS, Glasgow Coma Scale. Proportion of NIHSS missing (39.8, 38.0, 39.3, and 40.4%). Proportion of GCS missing (47, 46.4, 46, and 48.5%).

significant. All statistical analyses were performed using SAS software, version 9.4 (SAS Institute, Cary, NC, USA).

RESULTS

Baseline Characteristics

From August 1, 2015, to July 31, 2019, a total of 41,910 SICH patients were included in the study from the CSCA program. We excluded patients with history of previous stroke events, abnormal liver function, renal function, and bleeding history or tendency. A total of 25,430 patients with SICH were enrolled, and 21,116 (83.0%) patients of them had valid data of HbA1c and death while the data for others were missing (**Figure 1**). The average age of the patients having valid data was 62.8 ± 13.2 years; 13,087 (61.8%) of them were male, and 507 (2.4%) of them died. Age, atrial fibrillation, myocardial infarction, hypertension, diabetes, and hyperlipidemia in patients with HbA1c stratification of ≥6.10% were larger than in patients with HbA1c stratification of ≤5.10%. With regard to sex, the highest quartile group presented relatively few males. Patients with higher HbA1c levels were less likely to drink or smoke and more likely to be obese. **Table 1** shows the baseline characteristics of 21,116 patients with different HbA1c levels. Due to the missing HbA1c in more than 15%, a comparison of baseline characteristics between valid and missing HbA1cs was conducted. This comparison revealed significant differences in GCS and NIHSS scores between valid and missing HbA1c. We observed that GCS was relatively low and NIHSS was relatively high in the group lacking HbA1c data (**Table 2**).

TABLE 2 | Baseline characteristics between valid and missing HbA1c.

Variables	Missing (*N* = 4,244)	Valid (*N* = 21,116)	*P*-value
Age, years	61.7 ± 13.6	62.8 ± 13.2	<0.001
Male	2,619 (61.7)	13,052 (61.8)	0.902
BMI	23.8 ± 4.8	23.9 ± 4.5	0.638
Systolic BP (mmHg)	163.3 ± 30.7	163.9 ± 28.6	0.021
Diastolic BP (mmHg)	94.9 ± 17.5	94.7 ± 17.1	0.381
Atrial fibrillation	67 (1.6)	377 (1.8)	0.359
Myocardial infarction	47 (1.1)	200 (0.9)	0.608
Hypertension	2,607 (61.4)	13,866 (65.7)	<0.001
Diabetes mellitus	205 (4.8)	1,963 (9.3)	<0.001
Dyslipidemia	90 (2.1)	665 (3.1)	0.002
Peripheral vascular disorder	17 (0.4)	131 (0.6)	0.125
COPD	61 (1.4)	325 (1.5)	0.621
Antiplatelet	104 (2.5)	825 (3.9)	<0.001
Anticoagulation	50 (1.2)	279 (1.3)	0.281
Antihypertensive	1,536 (36.2)	8,735 (41.4)	<0.001
Diabetic medication	148 (3.5)	1,400 (6.6)	<0.001
Current smoking	952 (22.4)	4,426 (21.0)	<0.001
Drinking	1,044 (24.6)	5,405 (25.6)	0.394
NIHSS	7.0 (3.0-14.0)	6.0 (2.0-12.0)	<0.001
GCS	13.0 (7.0-15.0)	13.0 (8.0-15.0)	0.002
Hypoglycemic treatment	162 (3.8)	1,944 (9.2)	<0.001

Continuous variables were expressed as mean ± SD; categories variables were expressed as n (%). BMI, body mass index; COPD, chronic obstructive pulmonary disease; NIHSS, National Institutes of Health Stroke Scale; GCS, Glasgow Coma Scale; NIHSS and GCS in valid data of HbA1c were partially missing (39.3 and 47.0%).

Logistic Regression Analysis

Among the patients with different HbA1c levels, the univariate logistic regression analysis showed that in the HbA1c stratification of 5.10–5.60% and 5.60–6.10% of patients, the short-term mortality rate after admission was drastically lower than that of patients in the ≤5.10% group (OR respectively 0.74 95% CI $P = 0.014$ and 0.66 95% CI $P = 0.002$). However, no significant association was found in the ≥6.10% group compared with the ≤5.10% group (OR: 1.15 95% CI, $P = 0.244$). The results were consistent after the imbalance factors were adjusted (**Table 3**).

Subgroup Analysis

A stratified analysis of the association between HbA1c levels and short-term mortality after admission in patients of SICH with or without DM was performed. In univariate analysis of the patients with DM, a significant difference of the short-term mortality was found in the 5.60–6.10% group compared with the ≤5.10% group ($P > 0.05$). However, there was no significant correlation between HbA1c 5.10–5.60% and ≥6.10% groups compared with the ≤5.10% ($P > 0.05$) group. In the patients without DM, there was a significant correlation between the HbA1c 5.10–5.60% and 5.60–6.10% groups compared with ≤5.10% ($P < 0.05$). However, compared with the ≤5.10% group, no significant association was

TABLE 3 | Association between HbA1c quartiles with mortality after intracerebral hemorrhage.

HbA1c	*N*	Death, *n* (%)	Univariate analysis		Multivariate analysis*	
			P-value	OR (95%CI)	*P*-value	aOR (95%CI)
⩽5.10%	5,691	154 (2.71)	-	-		
5.10–5.60%	5,641	113 (2.00)	0.014	0.74 (0.58–0.94)	0.006	0.70 (0.55–0.90)
5.60–6.10%	4,825	87 (1.80)	0.002	0.66 (0.51–0.86)	0.001	0.63 (0.48–0.83)
⩾6.10%	4,959	153 (3.09)	0.244	1.15 (0.91–1.44)	0.925	0.99 (0.77–1.27)

The multivariate model was adjusted for age, sex, BMI, diastolic blood pressure, atrial fibrillation, myocardial infarction, hypertension history, diabetes history, lipid metabolism disorder, peripheral vascular disease, antiplatelet drugs, antihypertensive drugs, hypoglycemic drugs, smoking history, drinking history, and hypoglycemic therapy after the onset of the disease. GCS and NIHSS are not included.*

TABLE 4 | Stratified analysis of association between HbA1c levels and short-term mortality after admission in patients of SICH with or without DM.

HbA1c	*N*	Death, *n* (%)	DM				*N*	Death, *n* (%)	Non-DM			
			Univariate analysis		Multivariate analysis*				Univariate analysis		Multivariate analysis*	
			P-value	OR (95%CI)	*P*-value	aOR (95%CI)			*P*-value	OR (95%CI)	*P*-value	aOR (95%CI)
⩽5.10%	150	9 (6.00)	–	–	–	–	5,541	145 (2.62)	–	–	–	–
5.10–5.60%	114	2 (1.75)	0.108	0.28 (0.06–1.32)	0.112	0.28 (0.06–1.35)	5,527	111 (2.01)	0.034	0.76 (0.59–0.98)	0.017	0.73 (0.57–0.95)
5.60–6.10%	201	2 (1.00)	0.019	0.16 (0.03–0.74)	0.012	0.13 (0.03–0.64)	4,624	85 (1.84)	0.009	0.70 (0.53–0.91)	0.005	0.68 (0.51–0.89)
⩾6.10%	1,498	53 (3.54)	0.136	0.58 (0.28–1.19)	0.074	0.50 (0.24–1.07)	3,461	100 (2.89)	0.438	1.11 (0.86–1.43)	0.812	1.03 (0.79–1.35)

The multivariate model was adjusted for age, male, BMI, diastolic blood pressure, atrial fibrillation, myocardial infarction, hypertension history, diabetes history, lipid metabolism disorder, peripheral vascular disease, antiplatelet drugs, antihypertensive drugs, hypoglycemic drugs, smoking history, drinking history, and hypoglycemic therapy after the onset of the disease. GCS and NIHSS are not included.*

found in the ⩾6.10% group ($P > 0.05$). The conclusions were consistent after adjusting the imbalance factors (**Table 4**).

The ROC Curve

The AUC was 0.5596 ($P < 0.001$). In addition, the ROC curve of HbA1c combined with age, male, BMI, diastolic blood pressure, atrial fibrillation, myocardial infarction, hypertension history, diabetes history, lipid metabolism disorder, peripheral vascular disease, antiplatelet drugs, antihypertensive drugs, hypoglycemic drugs, smoking history, drinking history, NIHSS score, GCS, and hypoglycemic therapy after the onset of the disease can better predict the short-term mortality of patients with SICH (AUC = 0.6286 $P < 0.001$).

DISCUSSION

In this large, multicenter, cross-sectional study, we concluded that low or extremely low HbA1c after the stroke is associated with higher short-term mortality after SICH, regardless of whether patients have DM or not.

In accordance with the present results, previous studies have shown that very low HbA1c (<5.0 or <4.0%) is an independent risk factor for all-cause mortality (29, 30). The mechanism between low or very low HbA1c and higher short-term mortality after SICH has not been fully elucidated, which will be speculated as follows. Accounting for 20% of the total body energy consumption, brain energy consumption is mainly through sugar to provide energy. In light of the limited energy storage material, it is easy to be affected by the decrease of substrate supply (31). The increase of blood glucose is regulated by glucagon, adrenaline, and other hormones, of which glucagon and epinephrine mainly act on the liver, promoting the decomposition of liver glycogen into the blood and increasing the gluconeogenesis. Low HbA1c represents that the blood glucose level before stroke is at a low level for a long time, which transfers the blood glucose threshold of adrenaline to a low plasma glucose level, reducing the response level of elevated blood glucose and slowing the response of glucagon (32). It may lead to "brain energy crisis" (18, 33–35) when SICH occurs in this group of people by virtue of the low blood glucose threshold of adrenaline and the slow response of glucagon, hereby resulting in the increase of brain anaerobic metabolism and increasing the short-term mortality. It is found that the metabolic demand is the highest on the third day of intracranial injury (36, 37). If the patient has liver fibrosis or cirrhosis at the same time, the ability of the liver to regulate glucose will be affected. In this case, intrahepatic gluconeogenesis and glycogen decomposition will be further reduced, which will aggravate intracranial glucose deficiency. At the same time, the levels of procoagulant factor and anticoagulant factor were decreased and vitamin K was deficient in patients with abnormal liver function, which may generate larger hematoma volume and higher risk of rebleeding for patients with SICH.

Low or extremely low HbA1c after stroke may be related to the following aspects. To begin with, low or extremely low HbA1c may be associated with potential liver disease (38). Although we excluded patients with a history of liver dysfunction that had been

diagnosed before admission, potential liver dysfunction or liver vulnerability may exist in the included population. Additionally, a very low HbA1c level may indicate the long-term weakness and malnutrition before the onset of SICH. Finally, long-term intensive glycemic control may be about low or extremely low HbA1c after SICH. Regular health monitoring should be carried out for people with low or very low HbA1c levels, and the causes of abnormal HbA1c levels should be identified. HbA1c detection at least twice a year is recommended for patients with high risk of ICH, which may benefit them with early intervention. Early intervention refers to the following: (1) It is needed to improve the liver function examination early, being alert for potential liver dysfunction. On the basis of other studies, it has been shown that there is a lag in the increase of liver enzymes after liver function injury. Albumin dysfunction can be adopted as a new potential indicator of liver function injury (39). (2) We should look for malabsorption problems such as eating disorders or celiac disease to improve the nutritional status. (3) Being alert for severe hypoglycemia, we attempt to use appropriate antidiabetic drugs for individualized treatment. Previous randomized controlled trials revealed that intensive glycemic control does not reduce the cardiovascular risk in patients with diabetes (40–42). Meanwhile, strict glycemic control increased the risk of severe hypoglycemia, and the incidence of severe hypoglycemia events related to intensive treatment was two to three times higher than that of the non-intensive treatment group (43–45). From this point of view, long-term blood glucose should not be overcontrolled in diabetic patients with a high risk of SICH (46). Likewise, routine monitoring and routine HbA1c detection at admission are of great significance for patients with SICH to possibly prevent severe damage. At 3 months, the blood glucose threshold of symptoms and neuroendocrine response returned to normal with the recovery of glucagon response (47). For SICH patients with a low HbA1c level, it may be necessary to ensure enough brain energy and actively give non-surgical interventions including hypertonic drugs, antihypertensive drugs, and surgical interventions such as craniotomy or minimally invasive surgery (MIS).

HbA1c is a biomarker used to monitor blood glucose, which can evaluate the average blood glucose status within 2 to 3 months. Compared with other diabetes detection (such as fasting blood glucose or glucose tolerance test), HbA1c measurement does not need fasting, has high stability, is less affected by acute physiological disorders, and is more convenient for clinical application (23, 24). Some studies have shown that HbA1c is related to poor prognosis in patients with SICH (48, 49). These results are consistent with earlier studies indicating that patients with newly diagnosed diabetes based on the HbA1c criteria have a poor long-term prognosis after acute cerebral hemorrhage (50). Small cohort studies suggested that HbA1c is a better predictor of adverse outcomes in patients with SICH (25–27). Besides, HbA1c can better predict the symptomatic hemorrhagic transformation of acute ischemic stroke after thrombolysis (26, 51). These findings support the results of a large cohort study showing a J-type relationship between HbA1c and the risk of SICH. The lowest risk observed in HbA1c was 6.5% (52). Taken together, HbA1c levels might affect the incidence and prognosis of an acute cerebral hemorrhage, which is consistent with our findings. However, our outcome is contrary to a previous study showing that HbA1c was not associated with clinical outcome in patients with SICH (28).

At the same time, our study showed that when HbA1c was 5.60–6.10%, compared with patients without DM, DM patients with reasonable pre-stroke glycemic control had a lower mortality rate after SICH, but there was no statistical difference (1.00 vs. 1.84%, $p = 0.543$). This positive relationship could be linked to speculations that basement membrane thickening and endothelial cell proliferation caused by long-term diabetes before stroke reduce the risk of rebleeding after cerebral vascular rupture. In diabetic patients, increased levels of coagulation and plasminogen activator inhibitors are associated with the decreased fibrinolytic activity, as well as reduced bleeding volume and risk of rebleeding (53). However, this evidence contradicts previous studies that diabetes is a predictor of poor outcome after SICH (7, 54, 55).

There are several limitations in our current research. First of all, there is no follow-up data in this study, so long-term prognosis data are not available. At the same time, there were no imaging data and blood samples. The volume and location of the hematoma were not recorded. Secondly, in this study, patients without or with HbA1c measurement presented major baseline differences (**Table 1**). At the same time, the correlation between HbA1c and mortality was not statistically significant in the sensitivity analysis among patients with GCS and NIHSS scores (full details are given in **Appendices 1, 2**). The reason may be that patients with missing values have more severe neurological deficits and higher mortality, leading to possible selection bias.

CONCLUSION

In summary, this study mainly discusses the effect of low HbA1c on the prognosis of patients with SICH and expounds the possible mechanism. This study demonstrates that low or extremely low HbA1c is proportionally related to higher short-term mortality after SICH, regardless of whether patients have DM or not. Low or extremely low HbA1c may be associated with liver disease, long-term malnutrition, and excessive glycemic control in diabetes. It is very important for clinical decision-making to find the cause of low or extremely low HbA1c. These findings and their clinical significance need to be evaluated by future randomized clinical trials.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this

study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

PL and LC: study design, analysis and interpretation, and primary responsibility for writing the manuscript. YW, KK, HG, ZL, LL, YW, and XZ: study design, data interpretation, critical revision of the manuscript for important intellectual content, and supervision of the study. HG: data statistics. All authors: contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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An Analysis of the Potential Relationship of Triglyceride Glucose and Body Mass Index With Stroke Prognosis

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Background: The inverse association between obesity and outcome in stroke patients (known as the obesity paradox) has been widely reported, yet mechanistic details explaining the paradox are limited. The triglyceride glucose (TYG) index has been proposed as a marker of insulin resistance. We sought to explore possible associations of the TYG index, body mass index (BMI), and stroke outcome.

Methods: We identified 12,964 ischemic stroke patients without a history of diabetes mellitus from the China National Stroke Registry and classified patients as either low/normal weight, defined as a BMI < 25 kg/m^2, or overweight/obese, defined as a BMI ≥ 25 kg/m^2. We calculated TYG index and based on which the patients were divided into four groups. A Cox or logistic regression model was used to evaluate the association between BMI and TYG index and its influence on stroke outcomes, including stroke recurrence all-cause mortality and poor outcome (modified Rankin Scale score of 3–6) at 12 months.

Results: Among the patients, 63.3% were male, and 36.7% were female, and the mean age of the patient cohort was 64.8 years old. The median TYG index was 8.62 (interquartile range, 8.25–9.05). After adjusting for multiple potential covariates, the all-cause mortality of overweight/obese patients was significantly lower than that of the low/normal weight patients (6.17 vs. 9.32%; adjusted hazard ratio, 0.847; 95% CI 0.732–0.981). The difference in mortality in overweight/obese and low/normal weight patients with ischemic stroke was not associated with TYG index, and no association between BMI and TYG index was found.

Conclusion: Overweight/obese patients with ischemic stroke have better survival than patients with low/normal weight. The association of BMI and stroke outcome is not changed by TYG index.

Keywords: triglyceride glucose index, stroke, prognosis, body mass index, insulin resistance 3

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INTRODUCTION

Stroke is a leading cause of death and disability worldwide (1). Obesity is known to raise the risk of stroke (2). However, numerous reports have shown an inverse association between obesity and outcome in stroke patients, which is in contrast to the general population and is known as the stroke-obesity paradox (3–5). Although it is unknown if the stroke-obesity paradox is related to insulin sensitivity/resistance (IR), IR has been theorized to contribute to this phenomenon (6).

IR is a physiologic state in which a normal amount of insulin produces a subnormal physiologic response (7). IR is closely associated with obesity (8) and is an independent risk factor for mortality and major disability after stroke (9–11). Several mechanisms have been proposed to explain this association, including enhanced local inflammatory and prothrombotic responses and accelerated atherosclerotic development (12). The gold standard for measuring IR is the euglycemic hyperinsulinemia clamp (13). However, this method is difficult to apply in large population studies and clinical settings because of its costly, time-consuming and complex nature. In response to this limitation, large-scale epidemiological studies have resorted to the homeostasis model assessment of IR (HOMA-IR) index (9, 14), while the application of HOMR-IR is limited by the need for the fasting immunoreactive insulin.

A potential surrogate method of measuring IR has been developed, known as the triglyceride glucose (TYG) index (15). TYG index can be used in large-scale observational and/or interventional cohorts by comparing with the hyperinsulinemia-euglycemic clamp (16). Previous studies have shown that TYG index is positively correlated with ischemic stroke, and ischemic stroke patients with a high TYG index have a higher risk of dying or poorer outcome (17). Therefore, the aim of our study is to evaluate if the association between body mass index (BMI) and stroke outcomes is modified by TYG index.

METHODS

Study Population

Launched in 2012 by the Chinese Ministry of Health, the China National Stroke Registry II (CNSR II) is a prospective cohort study conducted nationwide aimed at establishing a reliable national stroke database for evaluating stroke care delivery in clinical practice (18). The criteria for site selection in China National Stroke Registry I (CNSR I) have been previously published (19) and were replicated in the CNSR II study. Patient's included in the study must have met the following criteria: age $\geq$ 18 years old, diagnosed within 7 days of the index event of ischemic stroke, transient ischemic attack (TIA), spontaneous intracerebral hemorrhage, or subarachnoid hemorrhage confirmed by brain imaging, or direct hospital admission from a physician's clinic or emergency department. Of the 25,018 patients in the CNSR II, 15,544 were diagnosed with ischemic stroke without a history of diabetes mellitus. Ultimately, 12,964 were analyzed after excluding patients without necessary clinical data including weight, height, fasting triglyceride or fasting glucose at admission ($n = 848$), as well as those who were lost to follow-up at 12 months ($n = 1{,}732$; **Figure 1**).

The study was approved by the central Institutional Review Board at Beijing Tiantan Hospital and prior to the enrollment, all patients were provided written informed consent.

Data Collection and Management

Patient baseline data were collected by trained research coordinators at each study center including basic patient characteristics, laboratory data, vascular risk factors, prior diagnoses and treatments. Vascular risk factors included stroke history, diabetes mellitus, hypertension, coronary heart disease, atrial fibrillation and prior/current smoking history. Past medication history included antihypertensive, antiplatelet drugs, anticoagulation, lipid-lowing, and hypoglycemic drugs.

BMI was measured by trained nurses at each clinical center at the time of patient enrollment. The delineation between low/normal and overweight/obese patients was set at 25 kg/m^2, based on World Health Organization standards (20). Fasting blood samples were collected for all participants within 24 h of admission. Using an automated enzymatic method, triglyceride and glucose levels were tested at each research center. TYG index was calculated as ln[fasting triglycerides (mg/dl) $\times$ fasting glucose (mg/dl)/2] (21).

Patient Follow-Up and Outcome Assessment

The primary outcomes of this study were stroke recurrence, all-cause mortality, and modified Rankin Scale (mRS) score, which were obtained by trained research coordinators that were blinded to the patients' baseline characteristics. Instances of recurrent stroke included ischemic stroke, intracranial hemorrhage, and subarachnoid hemorrhage. Poor functional outcomes were defined as a modified Rankin Scale score of 3–6. Modified Rankin Scale scores range from 0 (no symptoms) to 6 (death). At 3, 6, and 12 months after stroke, trained research personnel contacted patients by telephone following standard scripts, and collect information on death, stroke recurrence, and disability. During the follow-up, to ensure reliable diagnosis, stroke recurrences associated with rehospitalization were sourced to the corresponding hospitals. Adjudication was performed by the research coordinators together with the principal investigator in case of a suspected recurrent cerebrovascular event without hospitalization. Follow-up procedures for this study performed as previously described (22).

Statistical Analysis

Continuous variables are presented as mean $\pm$ standard deviation or median with interquartile range (IQR). Categorical variables are presented as frequencies and percentages. We used a student's *t*-test to compare groups with normally distributed parameters, a Wilcoxon test for non-normally distributed parameters, and a χ^2 test for categorical variables. A Cox proportional hazard analysis was conducted to estimate the hazard ratio (HR) and the 95% confidence interval (CI) of death

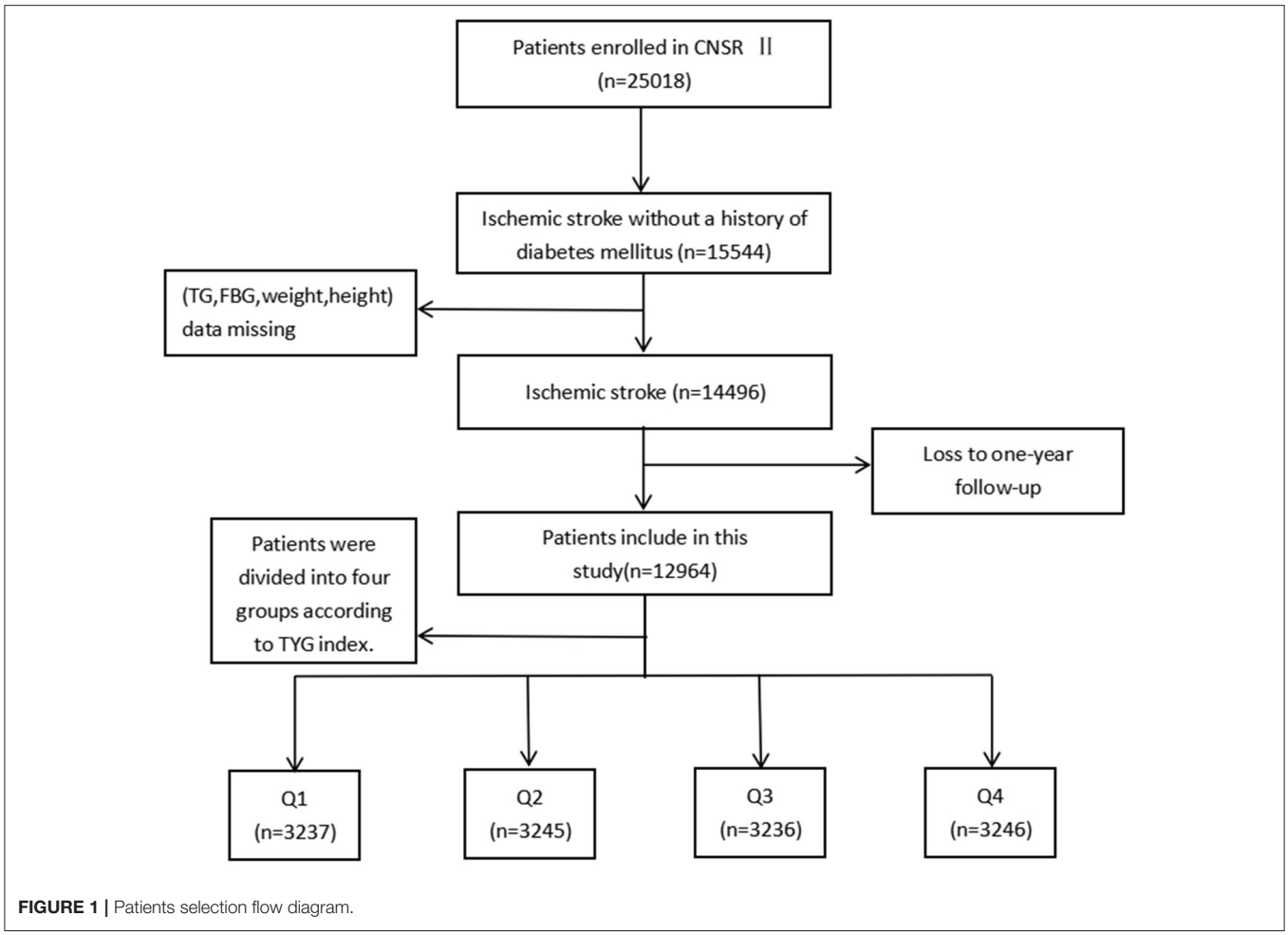

FIGURE 1 | Patients selection flow diagram.

and stroke recurrence. Odds ratios (OR) with 95% CIs were calculated for poor functional outcomes at 12 months and were estimated using a logistic regression model. Adjusted variables for HR with 95% CIs were sex, age, NIHSS score at admission, IV thrombolytic administration, prior/current smoking history, medical history (i.e., myocardial infarction, atrial fibrillation, hypertension, hyperlipidemia), medications (from the previously listed categories), and laboratory examination (i.e., total cholesterol, high density lipoprotein, low density lipoprotein). The impact of BMI and IR status on the risk of developing poor outcomes was also tested using Cox or logistic regressions. Kaplan-Meier analyses were used to assess survival from time of initial diagnosis until stroke recurrence during the 12-month follow-up period and a log-rank test was used to compare groups. We also examined the potential impact of age on the association between BMI and outcomes by separating patients into two groups using a cutoff of ≥65 years old. We assessed interactions between weight status in each age category using weight status × age category in multivariable models.

Statistical analyses were performed using SAS software (version 9.4, SAS Institute Inc, Cary, NC). Results were deemed statistically significant at a two-sided ($p < 0.05$).

RESULTS

Baseline Patient Characteristics

Twelve thousand nine hundred sixty-four patients were included in the study, with an average age of 64.83 ± 12.2 years old and the gender distribution was 65.17% male and 34.83% female. The median TYG index was 8.62 (IQR, 8.25–9.05). We separated patients into four groups based on TYG index. Other baseline patient characteristics are summarized in **Table 1**. In comparing to low/normal patients, obese/overweight patients were typically younger, more likely to have a history of hypertension and received the treatment for hypertension prior to admission. Within the overweight/obese cohort, patients with a high TYG index were more likely to have a history of hyperlipidemia. Significant differences in the sex, NIHSS score, and history of anticoagulation therapy were also observed within groups.

Association of BMI With Outcome

One thousand seventy-nine patients died in the 12 months following their initial stroke occurrence, including 832 (9.32%) overweight/obese and 247 (6.12%) low/normal weight patients. The prognosis of patients with ischemic stroke at 12 months in

TABLE 1 | Baseline characteristics of patients stratified by TYG index and BMI status.

Characteristic	TYG Q1(n = 3,237)			TYG Q2(n = 3,245)			TYG Q3(n = 3,236)			TYG Q4(n = 3,246)		
	BMI<25 kg/m^2	BMI≥25 kg/m^2	*P*-value	BMI<25 kg/m^2	BMI≥25 kg/m^2	*P*-value	BMI<25 kg/m^2	BMI≥25 kg/m^2	*P*-value	BMI<25 kg/m^2	BMI≥25 kg/m^2	*P*-value
Age (years) mean ± SD	68.28 ± 12.09	64.70 ± 13.08	< 0.0001	66.32 ± 11.8	64.21 ± 12.44	< 0.0001	65.19 ± 12.01	61.83 ± 11.83	< 0.0001	63.4 ± 11.1	61.0 ± 11.4	< 0.0001
Male, n (%)	1,836 (70.79)	425 (65.38)	0.0055	783 (33.66)	353 (38.41)	0.0106	1,338 (64.36)	772 (62.40)	0.2678	1204 (62.22)	814 (62.09)	0.9392
NHISS score median (IQR)	4 (2–7)	3.5 (2–6)	0.0043	4 (2–7)	4 (2–6)	0.4723	4 (2–7)	4 (2–6)	0.0557	4 (2–7)	4 (2–6)	0.0029
thrombolysis (%)	54 (2.09)	3 (0.46)	0.0048	36 (1.55)	15 (1.63)	0.8616	19 (0.91)	26 (2.25)	0.0019	35 (1.81)	14 (1.07)	0.0894
Prior/current smoker (%)	1,257 (48.59)	302 (46.46)	0.3318	1,070 (46.00)	387 (42.11)	0.0447	984 (47.33)	521 (45.03)	0.2086	887 (45.84)	595 (45.39)	0.7986
Medical history (%)												
Myocardial infarction	57 (2.20)	7 (1.08)	0.0652	48 (2.06)	24 (2.61)	0.3397	37 (1.78)	29 (2.51)	0.1610	38 (1.96)	26 (1.98)	0.9689
Atrial fibrillation	245 (9.47)	65 (10.00)	0.6817	148 (6.36)	75 (8.16)	0.0681	148 (7.12)	57 (4.93)	0.0141	115 (5.94)	68 (5.19)	0.3593
Hypertension	1,382 (53.42)	408 (62.77)	< 0.0001	1,390 (59.76)	626 (68.12)	< 0.0001	1,270 (61.09)	837 (72.34)	< 0.0001	1201 (62.07)	945 (72.08)	< 0.0001
Hyperlipidemia	203 (7.85)	61 (9.38)	0.2004	199 (8.56)	103 (11.21)	0.0191	189 (9.09)	136 (11.75)	0.0157	216 (11.16)	184 (14.04)	0.0146
Mediation History Prior to Stroke Hospitalization (%)												
Antihypertensive drugs	904 (34.84)	280 (43.08)	< 0.0001	886 (38.09)	433 (47.12)	< 0.0001	841 (40.45)	576 (49.78)	< 0.0001	800 (41.34)	620 (47.29)	0.0008
Antiplatelet drugs	456 (17.63)	128 (19.69)	0.2208	413 (17.76)	187 (20.35)	0.0865	330 (15.87)	215 (18.58)	0.0484	304 (15.71)	220 (16.78)	0.4160
Anticoagulation drugs	37 (1.43)	2 (0.31)	0.0190	25 (1.07)	14 (1.52)	0.2907	15 (0.72)	12 (1.04)	0.3441	12 (0.62)	11 (0.84)	0.4657
Lipid-lowing drugs	140 (5.41)	36 (5.54)	0.8986	134 (5.76)	57 (6.20)	0.6303	97 (4.67)	70 (6.05)	0.0880	101 (5.22)	77 (5.87)	0.4221
Laboratory examination												
TG (mg/dL)	66.38 (55.76–76.00)	69.03 (57.52–79.65)	0.0031	99.12 (87.62–111.51)	100.01 (89.39–113.28)	0.0246	136.29 (119.48–155.76)	138.06 (120.36–157.53)	0.1134	212.40 (176.12–271.70)	221.25 (179.66–285.86)	0.0027
TC(mg/dl)	158.12 (135.70–182.86)	158.51 (136.08–181.70)	0.9872	171.26 (148.84–196.00)	174.36 (149.23–196.39)	0.3158	180.54 (157.35–208.76)	183.25 (169.67–209.15)	0.2714	194.46 (168.94–225.77)	195.23 (170.10–223.45)	0.9685
HDL(mg/dl)	48.71 (40.98–58.38)	46.39 (39.82–54.51)	< 0.0001	46.01 (38.27–54.90)	44.07 (38.27–52.58)	0.0024	44.07 (36.73–51.80)	42.53 (36.34–51.03)	0.0382	41.37 (35.18–49.48)	40.98 (35.18–49.10)	0.8380
LDL(mg/dl)	91.62 (73.07–11.34)	95.49 (76.93–115.21)	0.0074	104.00 (85.44–124.49)	107.86 (88.53–127.58)	0.0006	110.57 (88.92–133.76)	113.66 (92.40–134.54)	0.0210	114.43 (91.62–139.56)	114.43 (93.17–139.18)	0.4526
FBG (mg/dL)	86.58 (79.20–95.40)	88.20 (80.10–95.40)	0.0912	92.88 (84.60–102.60)	91.98 (84.42–102.60)	0.1875	102.6 (91.3–121.9)	103.1 (90.9–121.1)	0.5167	108.18 (95.04–136.80)	108.00 (95.22–132.48)	0.4949

TYG index, triglyceride glucose index; NIHSS, National Institutes of Health Stroke Scale; BMI, body mass index; TG, Triglyceride; FBG, Fasting blood glucose; TC, total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; Q1–Q4, TYG index quartiles; SD, standard deviation; IQR, interquartile range.

TABLE 2 | Outcomes at 12 months follow up of ischemic stroke, stratified by BMI.

Outcome	BMI	n (%) of Events	Crude HR/OR (95% CI)	Adjusted HR/OR (95% CI)*	*P*-Value
Stroke recurrence	BMI < 25 kg/m^2	609 (6.82)	1	1	0.3078
	BMI ≥ 25 kg/m^2	286 (7.08)	0.6219 (0.900–1.912)	1.079 (0.932–1.248)	
[a]Poor outcome	BMI < 25 kg/m^2	2,064 (23.12)	1	1	0.2738
	BMI ≥ 25 kg/m^2	749 (18.55)	0.757 (0.690–0.832)	0.941 (0.843–1.049)	
Death	BMI < 25 kg/m^2	832 (9.32)	1	1	0.0269
	BMI ≥ 25 kg/m^2	247 (6.12)	0.647 (0.562–0.746)	0.847 (0.732–0.981)	

TYG index ln[fasting triglycerides (mg/dl) × fasting glucose(mg/ dl)/2]; BMI [weight in kilograms divided by height in meters squared].
**adjusted for sex, age, NIHSS score at admission, IV thrombolysis, smoking, medical history (Diabetes, Myocardial infarction, Atrial fibrillation, Hypertension, Hyperlipidemia), medication (Antihypertensive drugs, Antiplatelet drugs, Anticoagulation drugs, Lipid-lowing drugs, Hypoglycemic drugs), Laboratory examination (TG, TC, HDL, LDL, FBG).*
[a]Poor outcome, modified Rankin Scale score 3–6.

TABLE 3 | Association between BMI and stroke outcomes stratified by TYG index.

Outcome	TYG index	n (%) of Events		Crude HR/OR (95% CI)	Adjusted HR/OR (95% CI)*	*P*-Value	*P*-Value for interaction
		BMI < 25 kg/m^2	BMI ≥ 25 kgm^2				
Stroke recurrence	Q1	173 (6.69)	55 (8.46)	1.280 (0.945–1.733)	1.333 (0.974–1.824)	0.0726	0.2449
	Q2	153 (6.58)	60 (6.53)	0.987 (0.732–1.330)	1.049 (0.772–1.425)	0.7593	
	Q3	135 (6.49)	75 (6.48)	0.992 (0.748–1.315)	1.083 (0.810–1.450)	0.5895	
	Q4	148 (7.65)	96 (7.32)	0.952 (0.737–1.231)	0.962 (0.735–1.258)	0.7758	
[a]Poor outcome	Q1	675 (26.09)	140 (21.54)	0.778 (0.632–0.956)	0.997 (0.785–1.267)	0.9819	0.7887
	Q2	546 (23.47)	194 (21.11)	0.872 (0.725–1.050)	0.981 (0.790–1.219)	0.8651	
	Q3	472 (22.70)	211 (18.24)	0.759 (0.634–0.910)	0.905 (0.733–1.118)	0.3546	
	Q4	371 (19.17)	204 (15.56)	0.777 (0.644–0.937)	0.958 (0.768–1.196)	0.7042	
Death	Q1	277 (10.71)	52 (8.00)	0.740 (0.550–0.995)	0.892 (0.658–1.208)	0.4590	0.7542
	Q2	219 (9.42)	63 (6.86)	0.717 (0.542–0.948)	0.859 (0.643–1.147)	0.3021	
	Q3	190 (9.14)	64 (5.53)	0.594 (0.448–0.789)	0.827 (0.617–1.107)	0.2015	
	Q4	146 (7.55)	68 (5.19)	0.683 (0.512–0.910)	0.853 (0.631–1.154)	0.3039	

TYG index ln[fasting triglycerides (mg/dl) × fasting glucose(mg/ dl)/2]; BMI [weight in kilograms divided by height in meters squared].
**adjusted for sex, age, NIHSS score at admission, IV thrombolysis, smoking, medical history (Diabetes, Myocardial infarction, Atrial fibrillation, Hypertension, Hyperlipidemia), medication (Antihypertensive drugs, Antiplatelet drugs, Anticoagulation drugs, Lipid-lowing drugs, Hypoglycemic drugs), Laboratory examination (TG, TC, HDL, LDL, FBG).*
[a]Poor outcome, modified Rankin Scale score 3–6.

each of the BMI categories was shown in **Table 2**. Compared to patients classified as low/normal weight, overweight/obese patients had a lower risk of death at 12 months after adjusting for potential covariates (9.32 vs. 6.12%; adj. HR 0.847, CI 95% 0.732–0.981). No significant association was found between being overweight/obese and poor functional outcome or stroke recurrence ($p = 0.2738$ and 0.3078). There was an interaction effect of age group by BMI for the risk of stroke or death ($p = 0.0431$ and 0.0173, respectively; **Supplementary Table 1**). An inverse relationship between BMI and stroke mortality (obesity paradox) was observed in patients older than 65 years, whereas no association between them was observed in young patients.

Association Between BMI and Stroke Outcome in Patients Stratified by TYG Index

Patients were divided into quartiles according to TYG index: Q1 (TYG<8.33), Q2 (TYG 8.34–8.73), Q3 (TYG8.74–9.20), Q4 (TYG>9.21). Overweight and obese patients had a lower risk of death in all groups (Q1, crude HR 0.740, CI 95% 0.550–0.995; Q2, crude HR 0.717, CI 95% 0.542–0.948; Q3, crude HR 0.594 CI 95% 0.448–0.789; Q4, crude HR 0.683 CI 95% 0.512–0.910; **Table 3**, **Figure 2**), with no significant correlation after adjusting for all potential covariates (Q1, adj HR 0.892, CI 95% 0.658–1.208; Q2, adj HR 0.859, CI 95% 0.643–1.147; Q3, adj HR 0.827 CI 95% 0.617–1.107; Q4, adj HR 0.853 CI 95% 0.613–1.154). Similar results were found for the endpoints indicating a poor outcome. No significant association was found between BMI and stroke recurrence in the four groups. There was also no interaction between BMI and TYG index on the risk of stroke recurrence ($p = 0.2449$), mortality ($p = 0.7887$) and poor functional outcome ($p = 0.7452$; **Table 3**). In the analyses of continuous TyG index, BMI was associated with stroke mortality (adj HR 0.841, CI 95% 0.725–0.974, $p = 0.0210$), and there was no interaction between TYG index and BMI on stroke outcome (**Table 4**). Multivariate analysis of stroke outcomes by BMI and TYG index showed that overweight and obese patients had a lower risk of death at 12 months, and TYG index was associated with poor stroke outcomes ($p = 0.0237$, $p = 0.0260$; **Table 5**).

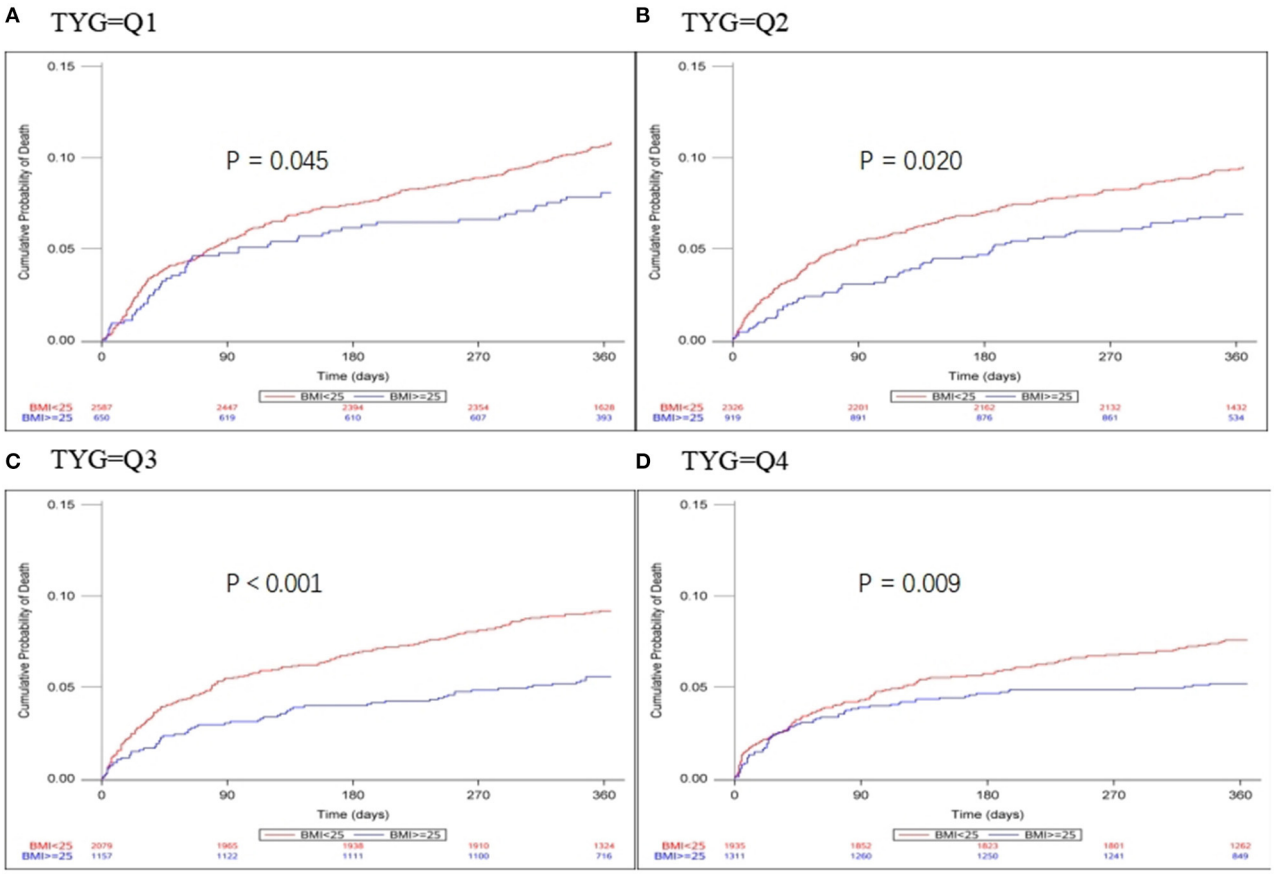

FIGURE 2 | Kaplan-Meier time-to-event curves and log-rank tests for 1-year mortality in each BMI group, stratified by TYG index. **(A–D)** represent TYG = Q1, TYG = Q2, and TYG = Q3, respectively.

TABLE 4 | Influence of TYG index as a continuous variable on the relationship between BMI and stroke.

Outcome	BMI	Crude HR/OR (95% CI)	Adjusted HR/OR (95% CI)*	*P*-Value	*P*-Value for interaction
Stroke recurrence	BMI< 25 kg/m^2	1	1	0.4270	0.2529
	BMI ≥ 25 kg/m^2	1.026 (0.890–1.184)	1.061 (0.916–1.229)		
[a]Poor outcome	BMI < 25 kg/m^2	1	1	0.3935	0.9176
	BMI ≥ 25 kg/m^2	0.798 (0.726–0.877)	0.953 (0.854–1.064)		
Death	BMI < 25 kg/m^2	1	1	0.0210	0.9359
	BMI ≥ 25 kg/m^2	0.680 (0.589–0.785)	0.841 (0.725–0.974)		

TYG index ln[fasting triglycerides (mg/dl) × fasting glucose(mg/ dl)/2]; BMI [weight in kilograms divided by height in meters squared].
**adjusted for sex, age, NIHSS score at admission, IV thrombolysis, smoking, medical history (Diabetes, Myocardial infarction, Atrial fibrillation, Hypertension, Hyperlipidemia), medication (Antihypertensive drugs, Antiplatelet drugs, Anticoagulation drugs, Lipid-lowing drugs, Hypoglycemic drugs), Laboratory examination (TG, TC, HDL, LDL, FBG).*
[a]*Poor outcome, modif.*

DISCUSSION

We found that overweight/obese stroke patients had a remarkably lower mortality compared to patients with normal/low BMI. There is an interaction between age and BMI. We found that association between BMI and stroke outcome was modified by age. However, we found no difference in outcomes in overweight/obese and normal/low-weight stroke patients that were stratified by TYG index and we did not observe an association between BMI and TYG index in ischemic stroke patients. A previous study examined IR using HOMA-IR suggested that the obesity paradox for mortality exists in insulin-resistant patients but not insulin-sensitive patients (6), suggesting IR may be part of the underlying mechanism of the obesity-stroke paradox. In this study, however, we did not observe a relationship between IR and the obesity-stroke

TABLE 5 | Multivariate analysis of BMI and TYG index on stroke outcome.

Variable	Stroke recurrence		[a]Poor outcome		Death	
	HR (95% CI)	*P*-Value	OR (95% CI)	*P*-Value	HR (95% CI)	*P*-Value
BMI≥25 kg/m^2	1.065 (0.920–1.233)	0.4001	0.954 (0.854–1.064)	0.3972	0.844 (0.728–0.978)	0.0237
TYG index Q2	0.974 (0.803–1.180)	0.7844	0.971 (0.849–1.111)	0.6682	1.059 (0.897–1.249)	0.4982
TYG index Q3	0.970 (0.794–1.185)	0.7648	0.930 (0.806–1.072)	0.3158	1.038 (0.870–1.239)	0.6770
TYG index Q4	1.176 (0.957–1.446)	0.1228	0.838 (0.717–0979)	0.0262	1.071 (0.879–1.304)	0.4954
Age	1.022 (1.015–1.028)	<0.0001	1.070 (1.065–1.076)	<0.0001	1.072 (1.065–1.079)	<0.0001
Male,	1.009 (0.853–1.193)	0.9167	1.117 (0.990–1.260)	0.0715	0.957 (0.826–1.109)	0.5578
NHISS score median (IQR)	1.043 (1.034–1.053)	<0.0001	1.172 (1.160–1.830)	<0.0001	1.091 (1.085–1.098)	<0.0001
thrombolysis	0.867 (0.509–1.475)	0.5980	1.066 (0.742–1.531)	0.7303	0.697 (0.423–1.149)	0.1571
Prior/current smoker (%)	1.067 (0.907–1.256)	0.4330	0.953 (0.847–1.071)	0.4180	1.014 (0.876–1.174)	0.8519
Myocardial infarction	1.559 (1.088–2.233)	0.0156	1.038 (0.755–1.427)	0.8171	1.413 (0.996–2.004)	0.0526
Atrial fibrillation	1.804 (1.475–2.206)	<0.0001	1.211 (1.025–1.454)	0.0250	1.804 (1.475–2.206)	<0.0001
Hypertension	1.365 (1.138–1.637)	0.0008	1.292 (1.132–−1.474)	0.0001	1.365 (1.138–1.637)	0.0008
Hyperlipidemia	1.047 (0.762–1.438)	0.7767	0.685 (0.526–0.892)	0.0050	1.047 (0.762–1.438)	0.7767
Antihypertensive drugs	0.897 (0.755–1.065)	0.2141	0.827 (0.727–0.941)	0.0039	0.897 (0.755–1.065)	0.2141
Antiplatelet drugs	1.147 (0.958–1.347)	0.1347	1.303 (1.140–1.490)	0.0001	1.147 (0.958–1.374)	0.1347
Anticoagulation drugs	0.928 (0.506–1.704)	0.8096	1.214 (0.753–1.957)	0.4258	0.928 (0.506–1.704)	0.8096
Lipid-lowing drugs	0.977 (0.647–1.475)	0.9115	1.348 (0.965–1.881)	0.0796	0.977 (0.647–1.475)	0.9115
TC(mg/dl)	1.001 (0.999–1.003)	0.4254	1.001 (0.999–1.002)	0.5651	1.001 (0.999–1.003)	0.4254
HDL(mg/dl)	1.003 (0.998–1.007)	0.2232	0.999 (0.996–1.003)	0.6850	1.003 (0.998–1.007)	0.2232
LDL(mg/dl)	1.000 (0.998–1.002)	0.9268	1.001 (1.000–1.003)	0.0891	1.000 (0.998–−1.002)	0.9268

TYG index ln[fasting triglycerides (mg/dl) × fasting glucose(mg/ dl)/2]; BMI [weight in kilograms divided by height in meters squared].
[a]Poor outcome, modified Rankin Scale score 3–6.

paradox. Possible explanations for this result include that obesity is a fundamental risk factor for the onset and development of insulin resistance, and the majority of the population with IR is obese or overweight. IR results in excessive release of free fatty acids (FFAs) into circulation by unrestrained lipolysis (23). The increased flux of FFAs increases triglyceride synthesis in the liver and the synthesized triglyceride is then released into plasma, which raises the risk of hypertriglyceridemia and subsequent metabolic syndrome (23). When the storage capacity of adipose tissue reaches full capacity, the energy surplus presenting in obese patients leads to an increased efflux of FFAs and an ectopic accumulation of fat in the liver (24). As such, numerous studies have demonstrated that the TYG index is highly associated with the incidence of non-alcoholic fatty liver disease (NAFLD) (25–27). An epidemiological survey of a Chinese population also found that an elevation of the TYG index might predict an increased incidence of NAFLD (28). A high TYG index is more likely to reflect metabolic abnormalities after the occurrence of the IR. Previous studies have also shown that visceral fat (VAT) rather than subcutaneous fat (SAT) is correlated with the occurrence of cardiovascular diseases (29). Kim et al. found that a low VAT proportion is associated with favorable and excellent outcomes in acute ischemic stroke patients treated with intravenous thrombolysis (30). Another study found that better clinical outcomes in obese patients are associated with a lower proportion of VAT (30). Conversely, higher visceral and liver fat amounts have been found in IR obesity (31), suggesting that IR may counteract the paradoxically protective effects of obesity in stroke patients. Second, the presence of metabolically healthy obese (MHO) individuals, a previously identified subset of obese subjects without metabolic abnormalities (including IR and a proatherogenic lipoprotein profile) may also be responsible for the controversial results. Although MHO criteria have not been clearly defined, normal glucose and lipid metabolism parameters, in addition to the absence of hypertension, is considered typical MHO diagnostic criteria (32). For example, Sánchez-Iñigo et al. found that metabolically unhealthy obese/non-obese (MUNO/MUO) individuals exhibit a greater risk of ischemic stroke than metabolically healthy non-obese, in which the TYG index is used to define a metabolically healthy state (MUNO, HR 1.55, CI 95% 1.36–1.77; MUO, HR 1.86, CI 95% 1.57–2.21) (33). Last, TYG index does not fully represent IR. Many studies have suggested that TYG index can be used as a surrogate of insulin resistance in otherwise healthy-appearing patients (15, 21, 34, 35). However, these findings lack consistency and a systematic review deemed the evidence for the usefulness of the TYG index as a surrogate biochemical marker of IR as "moderate-to-low quality."

We identified the obesity paradox in all four groups before the covariates were adjusted, but the results were no longer significant after adjustment. Indeed, whether or not the obesity paradox is a true phenomenon is still the subject of much debate. Some have proposed that the paradox is caused by physiological factors that reduce risk in obese participants

(36), whereas others suggested that it is an artifact resulting from selection bias in observational studies (37). From clinical and epidemiological observations, many contributing factors have been identified that lead to lower mortality, such as demographic characteristics (e.g., age, sex, educational status) and physical conditions (e.g., severity and duration of heart disease, initial neurological severity, and cachexia). In the present study, we found that overweight/obese patients are more likely to use antihypertensive, lipid-lowering, and antiplatelet drugs at baseline. We adjusted for patient characteristics that include sex, age, NIHSS score at admission, IV thrombolytic therapy, smoking, medical history (i.e., myocardial infarction, atrial fibrillation, hypertension, hyperlipidemia), and medication (i.e., antihypertensive, antiplatelet, anticoagulation, lipid-lowing, and hypoglycemic drugs). Despite the adjustments for these potentially confounding factors, the obesity paradox remained, with an adjusted HR for overall mortality in the overweight/obese group being 0.847 (0.732–0.981). A previous study including a cohort of 21,884 patients from the Danish National Indicator Project registry, found that post-stroke mortality in the 5-year follow-up period is lower in overweight (BMI 25.0–29.9 kg/m^2) and obese (BMI 30.0–34.9 kg/m^2) patients compared to those with normal weight (BMI 18.5–24.9 kg/m^2) and underweight (BMI<18.5 kg/m^2) (5). We also identified a BMI-age interaction since obese or overweight stroke patients older than 65 have a lower mortality rate, while the interaction is not presented in patients younger than 65. In contrast to our results, Vemmos et al. found that the protective effect of BMI on mortality decreases with increasing age (3). However, a *post-hoc* analysis of a randomized controlled trial in China (Clopidogrel in High-risk patients with Acute Non-disabling Cerebrovascular Events) did not identify the obesity paradox or observe an influence of age on the association of weight status with the prognosis of stroke (38). In a national wide representative survey of U.S. adults, an effect of age among stroke survivors was discovered, whereby a higher BMI was associated with a marginally increased mortality in younger individuals that reduced linearly with increasing age. This is similar to our findings (39).

We acknowledge several limitations with our findings. In particular, as a *post-hoc* analysis, several variables of interest were not accessible for analysis because they were not recorded. Additionally, a comparison of BMI with the waist-to-hip ratio cannot be evaluated. Our findings also use baseline BMI, fasting triglyceride and glucose, which may have confounding effects on the results as the collection of consecutive data about body weight, fasting glucose, and triglyceride at the 12 months follow-up is unavailable. Measurement errors across multiple centers may also slightly impact patients' fasting triglyceride and glucose levels, yet the laboratory values of these studies should still be comparable as the measurement techniques in all centers are based on the recommendation of the International Federation of Clinical Chemistry and Laboratory Medicine. A final limitation of our study is the relatively short follow-up period of 12 months. Several other studies have tracked patients much longer. For example, Korean Stroke Registry included 2,317 patients with ischemic stroke with 7.5-year follow-up (40) and the Prospective investigation of Greece included 2,785 first-ever acute stroke patients with a 10-year follow up (3).

In conclusion, we found that overweight and obese stroke patients have significantly lower mortality compared to patients with normal BMI, in line with previous reports of the stroke obesity paradox. Additionally, our findings suggest that age may influence the relationship between BMI and stroke prognosis. Stratification by TYG index eliminated the presence of the obesity paradox in this patient cohort, and thus TYG index, or its correlate IR, may not be beneficial in understating the metabolic mechanisms responsible for the stroke obesity paradox.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: The datasets generated for this study are available on request to the corresponding author. Requests to access these datasets should be directed to YW, yongjunwang@ncrcnd.org.cn.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Central Institutional Review Board at Beijing Tiantan Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZH, YP, YY, XY, and YoW contributed to the conception and design of the study. YiW and XZ assisted with data acquisition and interpretation. ZL and XM coordinated the study. YP and XX conducted the statistical analysis. ZH contributed to drafting. YoW is the guarantor for this paper. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.630140/full#supplementary-material

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ORIGINAL RESEARCH
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Association of Plasma Glucose to Potassium Ratio and Mortality After Aneurysmal Subarachnoid Hemorrhage

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Objectives: Hyperglycemia and hypokalemia are common problems in patients with aneurysmal subarachnoid hemorrhage (aSAH). The aim of this study was to determine whether the plasma glucose to potassium ratio (GPR) predicts mortality due to aSAH.

Methods: We prospectively recruited aSAH patients and healthy controls between March 2007 and May 2017. Clinical outcomes included mortality and poor outcome (modified Rankin scale score of 3-6) after 3 months. Multivariable analysis was used to determine the association between plasma GPR and 3-month mortality in aSAH patients.

Results: A total of 553 patients were recruited, and the mortality rate was 11%. The GPR was significantly elevated in aSAH patients compared with controls, in patients with a poor outcome than with a good outcome and in non-survivals than in survivals. Multivariable analysis showed that the plasma GPR was an independent factor associated with 3-month mortality. The area under the curve of the GPR was 0.747 in predicting 3-month mortality.

Conclusion: The plasma GPR on admission has potential as a predictor of 3-month mortality in patients with aSAH.

Keywords: aneurysmal subarachnoid hemorrhage, biomarkers, glucose, potassium, prognosis

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INTRODUCTION

Aneurysmal subarachnoid hemorrhage (aSAH) has a poor prognosis despite significant advances in early diagnostic modalities and treatment (1). aSAH counts for ∼5% of all acute stroke cases and has a high mortality rate of 32–44% (2). Assessment of the severity of aSAH and predicting patients' prognosis are required for the optimal treatment of aSAH. Multiple clinically based risk prediction scales are currently used to classify the severity of aSAH; the Hunt and Hess (HH) and the World Federation of Neurological Surgeons (WFNS) scale are commonly used scales for predicting the prognosis of aSAH.

Biomarkers may help clinicians to make therapeutic decisions when considering the severity of aSAH and in being able to predict whether a patient will have a poor prognosis, which may improve outcomes. Numerous biomarkers such as D-dimer, lactate, and C-reactive protein (CRP) have been reported to be the most promising biomarkers in aSAH patients (1, 3, 4). In addition, some clinical studies have reported that hyperglycemia is related with the risk of poor outcome

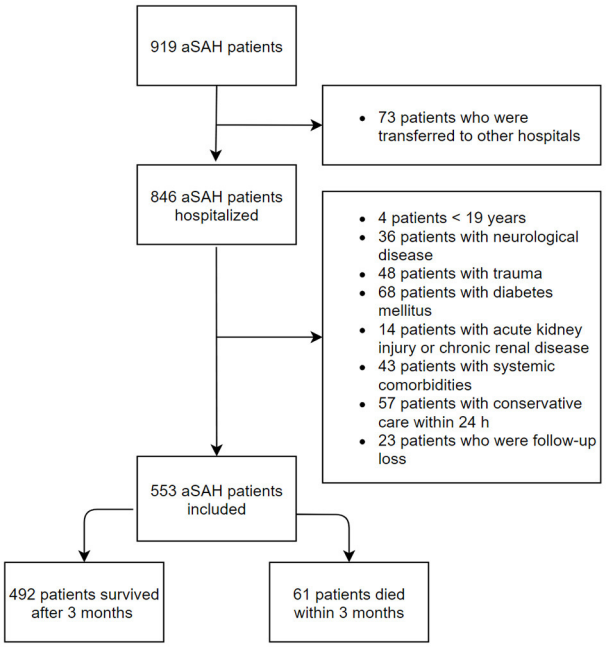

FIGURE 1 | Study population flow chart.

and delayed cerebral ischemia (DCI) after aSAH (5, 6). Recent reports have suggested that the glucose to potassium ratio (GPR) is significantly correlated with cerebral vasospasm and functional outcome (7, 8). The purpose of the present study was to calculate the plasma GPR in patients with aSAH and to determine whether this ratio is associated with the risk of 3-month mortality.

MATERIALS AND METHODS

Study Population

This was observational study undertaken from March 2007 to May 2017 in the emergency department (ED) at tertiary hospital, an 835-bed institution in Seoul with ∼58,000 ED visits annually.

The inclusion criteria were age ≥ 19 years and non-traumatic aSAH patients who were admitted to the ED within 24 h of symptom onset. Age- and sex-matched healthy individuals who visited our medical center for a medical examination were enrolled as the control group. The control group were checked for the absence of acute or chronic illness *via* a health questionnaire and a medical examination. The exclusion criteria were age of <19 years, a history of neurological disease including hemorrhagic or ischemic stroke and trauma, diabetes mellitus, acute kidney injury or chronic kidney disease, and concurrent systemic comorbidities including malignancy and liver cirrhosis (**Figure 1**).

Treatment for enrolled patients was performed according to protocols from the Korean Society of Cerebrovascular Surgeons, which included prevention of rebleeding and the management of vasospasm, delayed cerebral ischemia, seizure, and other medical complications (9). Treatment modalities included endovascular coiling or surgical clipping, as judged by both experienced endovascular specialists and neurosurgeons, which were determined based on the patients- and facility-related factors. Plasma glucose levels were checked four times per day. When the glucose level was persistently ≥ 200 mg/dl, the patients was managed using a sliding-scale insulin protocol.

The protocol of this study was approved by the Institutional Review Board of our hospital, and individual informed consent was waived owing to the routinely measured laboratory data during the process of the diagnosis and treatment in the ED.

Data Collection and Endpoints

Peripheral venous blood samples were collected within 30 min of admission. The plasma glucose and potassium levels were measured using a TBA-200FR Neo (Toshiba Medical Systems, Tokyo, Japan). There was no equipment replacement during the study period. The reference range of the plasma glucose level in our institutions was 70–99 mg/dl, and the reference range of the plasma potassium level was 3.5–5.5 mmol/l. The patients with hypokalemia were classified as mild hypokalemia (3.1–3.4 mmol/l), moderate hypokalemia (2.5–3.0 mmol/l), and sever hypokalemia (<2.5 mmol/l).

Patients demographic characteristics, clinical features, and laboratory data were collected. Clinical severity of aSAH on ED admission was evaluated according to the HH scale. We defined severe aSAH as HH scale score 4–5. The primary endpoint of interest was death within 3 months. The secondary endpoint was poor functional outcome at 3 months following ED admission; poor functional outcome was assessed using the modified Rankin scale (mRS score of 3–6). To assess the outcomes after 3 months, a review of the medical records or a telephone interview were conducted by a physician who was blinded to the clinical information.

Statistical Analysis

IBM SPSS Statistics 25 (IBM, Armonk, NY, USA), R version 3.6.3 (The R foundation, Vienna, Austria), and MedCalc version 16.4.3 (MedCalc Software, Ostend, Belgium) were used for all statistical analysis. Categorical variables were expressed as numbers and percentages, and proportions were compared with the χ^2 test. Non-normally distributed continuous variables were expressed as median and interquartile range (IQRs) and were analyzed using the Mann–Whitney *U*-test and Kruskal–Wallis test for intergroup differences. The Spearman's Coefficient test was performed to assess the relationships between GPR and white blood cell (WBC) count, HH score, Glasgow Coma Scale (GCS) score, and time from symptom onset to ED admission.

The aSAH patients were divided into four groups according to their plasma GPR quartile, and overall survival was evaluated until 3 months using the Kaplan–Meier survival curves. A receiver-operating characteristics (ROC) curve was applied to identify an optimal cutoff of plasma GPR for predicting 3-month mortality, and the area under the curve (AUC) and 95% confidence interval (CI) were reported.

A multivariable regression analysis was carried out to identify independent prognostic variables for 3-month mortality in

TABLE 1 | Characteristics and outcomes of the study population.

Characteristics	*N* = 553
Age (years)	54 (46–63)
Female (no.)	318 (57.5)
Time from onset to ED admission (min)	120 (49–280)
Hunt and Hess score	
Non-severe (1–3)	419 (75.8)
Severe (4–5)	134 (24.2)
Modified Fisher scoreGCS score	3 (3–4) 14 (12–15) 146 (125–171)
Laboratory results	
WBC ($\times 10^3/\mu l$)	10.6 (8.2–14.3)
Glucose (mg/dl)	146 (125–171)
Hypoglycemia (<70)	0 (0)
Normoglycemia (70–99)	9 (1.6)
Hyperglycemia (>99)	544 (98.4)
Potassium (mmol/l)	3.7 (3.4–4.0)
Hypokalemia (<3.5)	144 (26.0)
Normokalemia (3.5–5.5)	406 (73.4)
Hyperkalemia (>5.5)	3 (0.5)
Glucose/potassium ratio	38.7 (32.6–46.6)
Hemoglobin (g/dl)	13.5 (12.7–14.7)
PLT ($\times 10^3/\mu l$)	231.0 (194.5–271.0)
CRP (mg/dl)	0.09 (0.04–0.24)
Treatment modality (no.)	
Endovascular coiling	285 (51.5)
Neurosurgical clipping	237 (42.9)
RBC transfusion	170 (30.7)
Functional outcome	
Good (mRS 0–2)	399 (72.2)
Poor (mRS 3–6)	154 (27.8)
3-Month mortality rate	61 (11.0)

Data are presented as median with interquartile ranges or number (%).
GCS, Glasgow Coma Scale; WBC, white blood cells; PLT, platelet count; CRP, C-reactive protein; RBC, red blood cell; mRS, modified Rankin scale.

patients with aSAH. All statistical testing was two-sided, and $p < 0.05$ was considered statistically significant mortality.

RESULTS

Baseline Characteristics

During the study period, 553 aSAH patients were admitted to the study in accordance with the inclusion criteria. Simultaneously, 553 age- and sex-matched control were recruited. The median (IQR) plasma glucose levels were remarkably increased in patients with aSAH compared with controls: 146 (126–172) vs. 96 (88–105) mg/dl, respectively ($p < 0.001$). Plasma potassium concentrations were lower in aSAH patients than in healthy controls: 3.7 (3.4–4.0) vs. 4.2 (3.9–4.5) mmol/l, respectively ($p < 0.001$). The plasma GPR at ED admission was significantly higher in patients with aSAH than controls: 38.9 (32.8–47.2) vs. 22.8 (20.5–26.3) ($p < 0.001$).

The demographic characteristics, clinical features, and laboratory data of enrolled subjects are summarized in **Table 1**.

TABLE 2 | Clinical features according to GPR quartiles.

	Q1 (*n* = 138)	Q2 (*n* = 138)	Q3 (*n* = 136)	Q4 (*n* = 141)
Age (years)	51 (43–61)	56 (47–64)	56 (46–63)	55 (46–66)
Female (no.)	75 (54.3%)	84 (60.9%)	78 (57.4%)	81 (57.4%)
Hunt and Hess scale	2 (1,2)[b,c,d]	2 (2)[a,c,d]	2 (2,3)[a,b,d]	3 (2,4)[a,b,c]
Modified Fisher scale	2 (1,3)[b,c,d]	3 (2,3)[a,d]	3 (3)[a,d]	3 (3,4)[a,b,c]
GCS score	15 (15)[b,c,d]	15 (14,15)[a,d]	15 (13,15)[a]	13 (9,15)[a,b]
Poor outcome (no.)	20 (14.5%)[c,d]	32 (23.2%)[d]	43 (31.6%)[a]	58 (41.1%)[a,b]
3-Month mortality (no.)	2 (1.4%)[c,d]	8 (5.8%)[c,d]	22 (16.2%) [a,b]	29 (20.6%)[a,b]

Data are presented as median with interquartile ranges or number (%).
GPR, glucose to potassium ratio; Q, quartile; GCS, Glasgow Coma Scale.
[a]$p < 0.05$, *vs. Q1.*
[b]$p < 0.05$, *vs. Q2.*
[c]$p < 0.05$, *vs. Q3.*
[d]$p < 0.05$, *vs. Q4.*

The median age was 54 (46–63) years, there were 235 (42.5%) males and 318 (57.5%) females. The median time from symptom onset to ED admission was 120 (49–280) min for all patients. The median time from symptom onset to ED admission was significantly shorter in the non-survivors than in survivors [60 (30–197) vs. 120 (56–286) min, respectively; $p = 0.048$]. Among all aSAH patients, the severe group (HH score 4–5) accounted for 24.2%. A total of 285 (51.5%) patients underwent endovascular coiling for the aneurysm, 237 (42.9%) patients underwent neurosurgical clipping, and 170 (30.7%) patients were transfused with packed red blood cells.

A total of 544 (98.4%) patients had an elevated plasma glucose level (>99 mg/dl) on ED admission. Hypokalemia was present in 144 (26.0%) patients, but hyperkalemia was detected in only three (0.5%) patients. A lower percentage of patients suffered moderate hypokalemia (3.7%) than mild hypokalemia (22.8%), and none of patients fell into the severe hypokalemia. The plasma glucose and GPR were significantly higher in the severe group than they were in the non-severe group (180 vs. 138 mg/dl, $p < 0.001$, and 50.3 vs. 36.2, $p < 0.001$, respectively). The potassium concentration was significantly higher in the non-severe group than it was in the severe group (3.8 vs. 3.5 mmol/l, $p < 0.001$).

3-Month Functional Outcomes and Mortality

All enrolled subjects were categorized into four groups according to their plasma GPR quartile (Q): (1) Q1 (<32.7), (2) Q2 (32.7–38.8), (3) Q3 (38.9–47.2), and (4) Q4 (>47.2). The patients' characteristics and clinical features are presented according to GPR quartile in **Table 2**. Severity of aSAH, poor functional outcome, and 3-month mortality were significantly higher in the Q4 group than in the Q1 group.

The survival analysis showed that the higher GPR quartile group (Q4) at ED admission had the worst 3-month survival rate whereas the lower GPR quartile group (Q1) had the best 3-month survival rate. The 3-month mortality in Q4 (GPR > 47.2) was 20.6% whereas the 3-month mortality in Q1 (GPR < 32.7) was 1.4% (**Figure 2**).

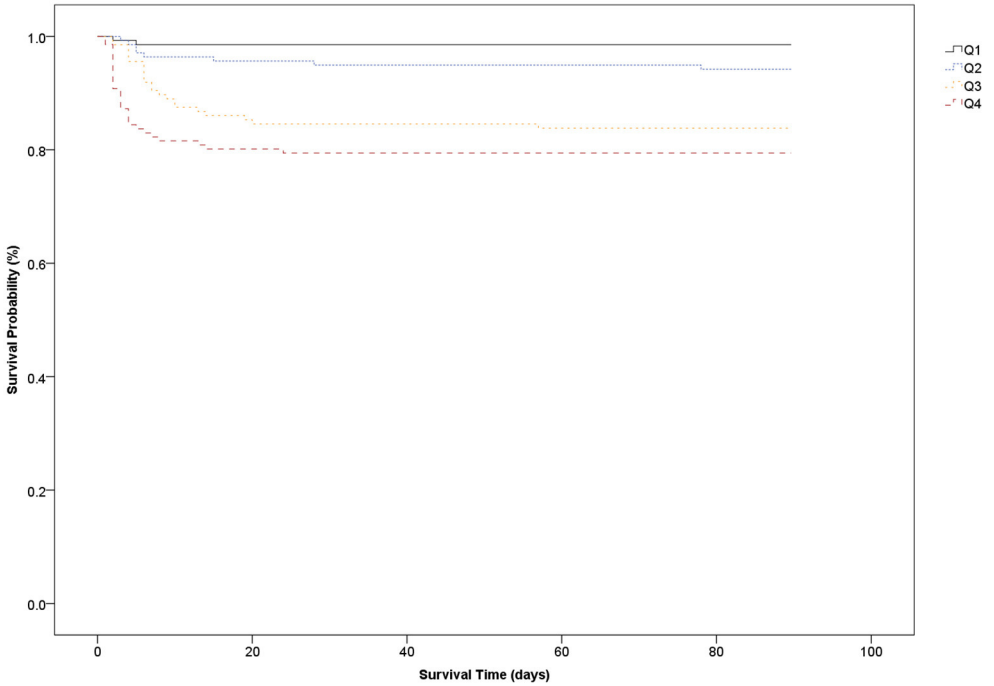

FIGURE 2 | The Kaplan–Meier survival analysis based on the glucose/potassium ratio.

TABLE 3 | Comparison of glucose and potassium levels and other biomarkers according to outcome.

	Functional outcome			Survival		
	Good	**Poor**	***P*-value**	**Survivors**	**Non-survivors**	***P*-value**
WBC ($\times 10^3/\mu l$)	10.0 (8.1–13.5)	11.6 (8.7–16.1)	0.058	10.2 (8.1–13.4)	14.0 (10.5–19.4)	<0.001
Hemoglobin (g/dl)	13.6 (12.8–14.6)	13.2 (11.9–14.1)	0.066	13.5 (12.8–14.5)	12.4 (11.3–16.2)	0.048
CRP (mg/dl)	0.07 (0.04–0.16)	0.15 (0.05–0.25)	<0.001	0.08 (0.04–0.21)	0.08 (0.05–0.23)	0.105
D-dimer (μg/ml)	0.99 (0.55–2.30)	1.16 (0.61–2.69)	0.366	1.13 (0.57–2.51)	0.65 (0.35–1.22)	0.034
Glucose (mg/dl)	142 (121–165)	158 (137–194)	<0.001	142 (122–167)	182 (151–219)	<0.001
Potassium (mmol/l)	3.7 (3.5–4.0)	3.7 (3.3–4.0)	0.028	3.7 (3.4–4.0)	3.6 (3.2–4.0)	0.087
Glucose/potassium ratio	37.5 (31.4–45.8)	42.8 (36.7–54.1)	<0.001	37.8 (31.8–46.2)	46.7 (41.3–61.1)	<0.001

Data are presented as median with interquartile ranges.

The Spearman rank correlation test of the relationships between plasma GPR, HH score, GCS score, and WBC count produced correlation coefficients of 0.383 (GPR and HH score), −0.339 (GPR and GCS score), 0.250 (GPR and WBC count), and −0.320 (GPR and time from symptom onset to ED admission) (all $p < 0.001$). The plasma potassium level and glucose level at ED admission produced a correlation coefficient of −0.303 ($p < 0.001$).

Sixty-one (11.0%) patients died, and 154 (27.8%) patients had poor functional outcome. The median time from ED admission to death was 4 (IQR 2–8, range 2–78) days. The initial median glucose concentration, potassium concentration, GPR, and other biomarkers are presented in **Table 3**.

The plasma glucose concentration and GPR were significantly increased in the poor outcome group compared with the good outcome group. The median glucose concentration and GPR were significantly increased in the non-survival patients than in the survival patients ($p < 0.001$). The potassium concentration differed significantly between the good outcome group and poor outcome group ($p = 0.028$); however, the potassium concentration did not significantly differ between the non-survivors and survivors ($p = 0.087$).

Regarding the ROC curve for predicting 3-month mortality, the AUC for GPR was 0.747 (95% CI 0.709–0.783). The suitable cutoff of GPR for predicting mortality was determined to be 37.8 (sensitivity, 90.2%; specificity, 51.0%; negative likelihood ratio, 0.19; positive likelihood ratio, 1.84) (**Figure 3**).

Age, sex, HH score, modified Fisher score, GCS score, WBC count, hemoglobin level, D-dimer level, glucose level, potassium level, and GPR were included in the logistic regression analysis.

In univariable analysis revealed that age, HH score, modified Fisher score, GCS score, hemoglobin level, glucose level, and GPR were significantly related to 3-month mortality. In multivariable analysis, independent predictors for 3-month mortality were as follows: GPR (OR 1.070, 95% CI 1.047–1.093; $p < 0.001$), and GCS score (OR 0.644, 95% CI 0.544–0.763; $p < 0.001$) (**Table 4**).

We evaluated the incremental benefit of adding the GCS score to the GPR by calculating the AUC; adding the GCS score to the GPR improved the predictive value for 3-month mortality (AUC = 0.925, 95% CI 0.888–0.963; $p < 0.001$).

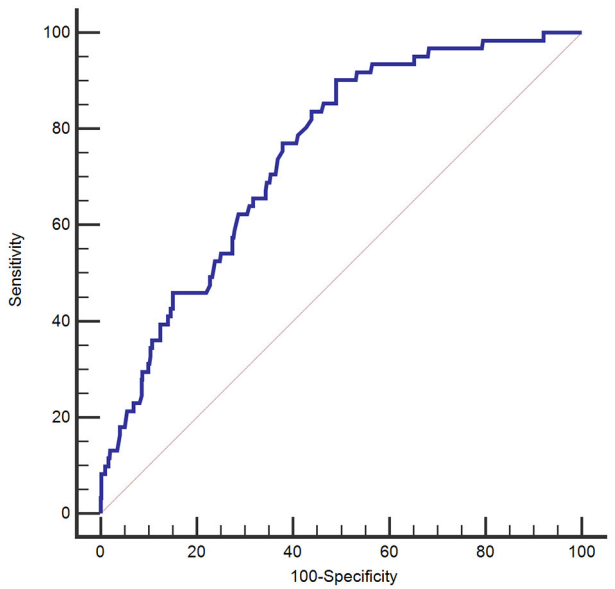

FIGURE 3 | Receiver operating characteristic curve of the plasma glucose to potassium ratio for 3-month mortality of patients with aneurysmal subarachnoid hemorrhage.

Discussion

This study investigated the association of plasma GPR with mortality among patients with aSAH. The plasma GPR on ED admission was significantly higher in non-survivors and was an independent predictor for 3-month mortality. Therefore, the plasma GPR was useful for predicting mortality in these aSAH patients.

Many factors stimulate the sympathetic nervous system in aSAH patients. Sympathetic activation leads to an increased release of catecholamine and cortisol into the systemic circulation, which begins a few minutes after an aSAH and can last for up to 10 days. These hormones stimulate gluconeogenesis, glycogenolysis, lipolysis, and proteolysis, which lead to glucose overproduction (10).

Previous studies have suggested that hyperglycemia is related with poor outcome in aSAH patients. Beseoglu and Steiger reported that hyperglycemia correlated with the initial neurological status and unfavorable outcome after 6 months (11). In the study by McGirt et al., patients with persistent high blood glucose levels were seven-fold more likely to have a poor prognosis than patients with good glucose control, although an isolated hyperglycemic event was not predictor for poor outcome (12). In our study, the plasma glucose level *at ED admission* was significantly higher in patients with a poor outcome and non-survivors; however, it was not an independent predictor for 3-month mortality. Unfortunately, our study did not evaluate the effect of glucose management on the prognosis of patients with aSAH during hospitalization.

A previous study reported that the difference in the risk of unfavorable outcomes between patients in the lowest and highest quartiles of glucose level was more than double (13). We found a 2.8-fold difference in the risk of poor outcomes and a 14.7-fold difference in mortality between the lowest GPR (<32.7) and the highest GPR (>47.2) quartiles.

Various factors such as systemic inflammatory response syndrome, sepsis, metabolic crisis, and insulin treatment may

TABLE 4 | Variables associated with 3-month mortality identified by logistic regression analysis.

	Univariable			Multiple		
	Odds ratio	**95% CI**	***P*-value**	**Odds ratio**	**95% CI**	***P*-value**
Age	1.043	1.021–1.065	<0.001	1.026	0.970–1.085	0.364
Sex	0.994	0.580–1.703	0.983			
Time from onset to ED admission	0.997	0.994–1.001	0.103			
Hunt and Hess score	6.153	3.966–9.546	<0.001	1.655	0.360–7.616	0.518
Modified Fisher score	5.782	3.122–10.711	<0.001	1.291	0.370–4.503	0.688
GCS score	0.632	0.573–0.699	<0.001	0.644	0.544–0.763	<0.001
Glucose	1.021	1.014–1.027	<0.001	1.033	0.995–1.072	0.094
Potassium	0.652	0.362–1.173	0.153			
Glucose/potassium ratio	1.052	1.035–1.069	<0.001	1.070	1.047–1.093	<0.001
WBC	1.000	1.000–1.011	0.652			
Hemoglobin	0.802	0.659–0.975	0.027	0.820	0.584–1.236	0.395
D-dimer	0.715	0.428–1.194	0.200			

GCS, Glasgow Coma Scale; WBC, white blood cell.

influence glucose control in patients with SAH (14). A previous study found that the infection rate was significantly lower in patients who were treated with intensive insulin therapy than it was in those without such treatment, but neurological outcome and mortality were not affected by intensive insulin therapy (15). Implementation of strict glucose control lowered the average glucose levels and incidence of hyperglycemia in patients compared with those without such control, but had no effect on hospital mortality (16). By contrast, good glucose management significantly reduced the incidence of poor outcome compared with that in patients with poor glucose management (17). Whether, control of hyperglycemia improves outcomes, including hypoglycemia, which can occur due to the control of hyperglycemia, or increases mortality remains unclear. Therefore, control of blood glucose levels above 180 mg/dl is currently recommended (18).

Hypokalemia is common electrolyte imbalance in aSAH patients. It is believed that hypokalemia associated with aSAH is induced by a catecholamine surge. A high catecholamine level causes overactivation of the Na^+/K^+-ATPase pump, which causes a shift in potassium ions from extracellular to intracellular spaces (19). Previous research found that about 50% of SAH patients had hypokalemia (20). In the study by Zhang et al., the prevalence of hypokalemia among aSAH patients was 35% (21). In our study, 26% of patients had hypokalemia, but this rate did not differ significantly between survivor and non-survivors. Only 20 patients in our study had moderate hypokalemia and no patients with severe hypokalemia. Tam et al. found that neither hypokalemia nor hyperkalemia were independent predictors for poor outcome at 3 months after SAH onset (22). By contrast, others found that only 2% of the patients included were hypokalemic on ED admission, and hypokalemia in the subacute phase (days 7–10) correlated with poor outcome at 3 months after discharge (23). The effect of a low potassium level on the outcome in aSAH patients remains controversial.

Recently, Fujiki et al. reported that serum GPR, glucose and potassium level at admission were significantly correlated with poor outcome at 3 months (7). Our results do not entirely coincide with their results. Interestingly, our study showed that plasma glucose was significantly higher in non-survivors than in survivors, but it was not an independent predictor of 3-month mortality in aSAH patients. These differences in results may be due to differences in study protocol and enrolled criteria, particularly the different severity of patients with aSAH. In the study by Fujiki et al., patients with various onset times from 1 h to 16 days were included, and 42.1% patients were classified as severe aSAH. On the other hand, our study included only aSAH patients whose onset time was within 24 h, and the proportion of severe patients was 24.2%.

The pathophysiological mechanism for the relationship between high plasma GPR and mortality in aSAH patients remains unclear. Hyperglycemia after SAH is associated with cardiac dysfunction, pulmonary edema, brain stem compression, bloodstream infection, and an increased risk of death (24). Furthermore, hyperglycemia induced intracellular acidosis, mitochondrial dysfunction, formation of free radicals, and brain edema (25). Severe hypokalemia increases the risk of cardiac arrhythmias, especially tachyarrhythmias. High GPR has been shown to be associated with the severity of SAH (7). In accordance with previous findings, our data also demonstrated that plasma GPR was significantly elevated in patients with severe aSAH compared with patients with non-severe aSAH. Whether, hyperglycemia and hypokalemia are factors that adversely affect the outcome of patients with aSAH or are simply surrogate markers of underlying etiological factors for poor outcome in clinical course remains controversial.

However, the plasma GPR was significantly elevated in non-survival patients and was an independent predictor for 3-month mortality. The relationship between 3-month mortality and glucose level on ED admission was weaker than that of the GPR and was no longer significant after multiple logistic regression analysis.

In our study, the AUC of the plasma GPR ranged from 0.709 to 0.783. For a cutoff value of 37.8 for the GPR, the sensitivity and specificity for predicting 3-month mortality were 90.2 and 51.0%, respectively. The Kaplan–Meier survival curves for 3-month mortality showed that patients in the group with the highest GPR (>47.2) had a significantly shorter overall survival time than those in the group with the lowest GPR (<32.7).

Our study has several limitations. First, glucose level can be influenced by catecholamine, cortisol, and glucagon levels, but we did not measure these. Second, glucose and potassium levels may be influenced by various factors such as a timing of last meal, use of beta-blockers, or potassium wasting diuretics, but we did not consider these factors. In addition, the effects of the management of glucose and potassium during hospitalization were not considered. Third, the plasma GPR was calculated only once based on the initial values obtained on admission to the ED, and it remains unclear whether serial measurement of GPR may provide additional prognostic information. In addition, the present data represents only a single tertiary hospital, and no *post-hoc* analysis was performed. Therefore, the outcomes cannot be generalized to all aSAH patients. Further prospective large-scale studies are required to confirm the relationship between GPR and mortality in aSAH patients.

In conclusion, plasma glucose and potassium level tests are inexpensive measures that are readily available from the routine blood analysis obtained at the time of admission to the ED. The plasma GPR was significantly higher in aSAH non-survivor than in survivor and has potential to be a prognostic predictor of 3-month mortality in aSAH patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Konkuk University Medical Center. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. Written informed consent was not obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

DH and HJ conceived and designed the study. DH, JP, and SK collected and complied data. DH, HJ, JP, and SK performed statistical analysis and interpreted the data. HJ and DH wrote the report. All authors contributed to the article and approved the submitted version.

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sTWEAK as Predictor of Stroke Recurrence in Ischemic Stroke Patients Treated With Reperfusion Therapies

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Aim: The purpose of this study was to investigate clinical and neuroimaging factors associated with stroke recurrence in reperfused ischemic stroke patients, as well as the influence of specific biomarkers of inflammation and endothelial dysfunction.

Methods: We conducted a retrospective analysis on a prospectively registered database. Of the 875 patients eligible for this study (53.9% males; mean age 69.6 ± 11.8 years vs. 46.1% females; mean age 74.9 ± 12.6 years), 710 underwent systemic thrombolysis, 87 thrombectomy and in 78, systemic or intra-arterial thrombolysis together with thrombectomy was applied. Plasma levels of interleukin 6 (IL-6) and tumor necrosis factor alpha (TNFα) were analyzed as markers of inflammation, and soluble tumor necrosis factor-like inducer of apoptosis (sTWEAK) as an endothelial dysfunction marker. The main outcome variables of the study were the presence and severity of leukoaraiosis (LA) and stroke recurrence.

Results: The average follow-up time of the study was 25 ± 13 months, during which 127 patients (14.5%) showed stroke recurrence. The presence and severity of LA was more severe in the second stroke episode (Grade III of the Fazekas 28.3 vs. 52.8%; $p < 0.0001$). IL-6 levels at the first admission and before reperfusion treatment in patients with and without subsequent recurrence were similar (9.9 ± 10.4 vs. 9.1 ± 7.0 pg/mL, $p = 0.439$), but different for TNFα (14.7 ± 5.6 vs. 15.9 ± 5.7 pg/mL, $p = 0.031$) and sTWEAK (5,970.8 ± 4,330.4 vs. 8,660.7 ± 5,119.0 pg/mL, $p < 0.0001$). sTWEAK values ≥7,000 pg/mL determined in the first stroke were independently associated to recurrence (OR 2.79; CI 95%: 1.87–4.16, $p < 0.0001$).

Conclusions: The severity and the progression of LA are the main neuroimaging factors associated with stroke recurrence. Likewise, sTWEAK levels were independently associated to stroke recurrence, so further studies are necessary to investigate sTWEAK as a therapeutic target.

Keywords: MRI, prognosis, stroke prevention–primary & secondary, leukoaraiosis, stroke recurrence

INTRODUCTION

Since the implementation of the new approach to stroke as a neurological emergency, which has led to the progressive creation of Stroke Units and the development of new reperfusion therapies, short-term outcome has improved in developed countries (1–4) both in terms of mortality and functional outcome. The new guidelines, however, have focused mainly on patient care in the acute phase, but we have not seen many developments in post-hospital care, or secondary prevention, and some data suggest an increase in late disability in stroke patients (2).

A large part of early, medium and late morbimortality is associated with stroke recurrence, which affects 40% at 5 years and 50% at 10 years after the first cerebrovascular episode, both ischemic and hemorrhagic (5–9). The control of vascular risk factors, antiplatelet agents and statins has not significantly modified therapeutic strategy, although direct oral anticoagulant drugs have fundamentally demonstrated fewer hemorrhagic complications (10, 11).

The influence of reperfusion therapies in acute phase on stroke recurrence has not been well-established. It seems, however, that in patients undergoing mechanical thrombectomy early recurrence is lower, but in patients that receive intravenous thrombolysis medium and long-term recurrence is similar. In both cases, reperfused patients seem to have a better long-term progress as compared to non-reperfused patients (1, 12–14).

On the other hand, there is clinical evidence that moderate to severe leukoaraiosis ((LA) or white matter lesions) presence may be related with endothelial dysfunction and blood brain barrier (BBB) damage (15–18). LA presence is known to contribute to long-term functional decline, morbidity, and death in independent outpatients and in stroke patients (19). We have recently identified an endothelial dysfunction marker, the soluble tumor necrosis factor-like inducer of apoptosis (sTWEAK), as a possible biomarker independently associated with hemorrhagic transformation and poor functional outcome in patients with IS undergoing reperfusion therapies through the presence of LA (20). sTWEAK is constitutively expressed by monocytes, tumor cell lines, and endothelial cells. Via binding to fibroblast growth factor-inducible 14 [Fn14]), sTWEAK can function as an inflammatory cytokine. In this line, previous studies have shown that patients with IS had high sTWEAK levels. However, no correlation was found between sTWEAK and an ischemic area volume during acute stroke (21, 22).

At present, the primary goal of secondary prevention strategies after IS is to reduce the risk of recurrent stroke, and information on stroke recurrence and survival is useful to assess the effect of secondary prevention and risk factors for recurrence and death. In this scenario, it would be useful to identify biomarkers that could become therapeutic targets for developing future treatments or diagnostic indicators for stroke recurrence prevention; which would allow more accurate post-hospital follow-up/care, as this would lead to lower disability and mortality in medium and long-term outcome.

We hypothesized that elevated serum levels of sTWEAK might be involved in a higher frequency of stroke recurrence through the presence of LA. In the present study, we intend to investigate the possible relationship among sTWEAK—LA—stroke recurrence in reperfused IS patients; compare results with other inflammation biomarkers and evaluate the functional outcome at 3 months.

MATERIALS AND METHODS

Patient Screening

For this study, we enrolled the stroke patients admitted to the Stroke Unit of the Hospital Clínico Universitario of Santiago de Compostela (Spain), who were prospectively registered in an approved data bank (BICHUS), and received reperfusion therapies (both intravenous and endovascular) during the acute phase. All patients were treated by expert neurologists according to national and international guidelines. Exclusion criteria for this analysis were: (1) latency time (from the onset of symptoms to hospital care) >4.5 h; (2) previous modified Rankin scale (mRS) >1; (3) history of chronic inflammatory diseases; (4) lack of at least two neuroimaging studies in the 1st week; (5) lost to follow-up patients (personal interview or telephone) at 3 months. The analysis of the data for this study was retrospective, using the period between September 2007 and September 2017.

For the estimation of stroke recurrence (ischemic stroke (IS) or intracerebral hemorrhage (ICH) patients) after the first ischemic stroke, the same database (BICHUS) was used in patients re-admitted to the same Stroke Unit. All the patients under care in Galicia (Spanish region on the northwest of the Iberian Peninsula) by the Servizo Galego de Saúde (SERGAS) are registered in a computer medical history (IANUS) that was used for patients who presented recurrence and who were seen by primary care doctors or other hospitals in the public network. Patients treated in private centers or outside Galicia were not registered and consequently excluded.

Clinical Variables and Neuroimaging Studies

The registry includes demographic variables, vascular risk factors, time from stroke onset to reperfusion therapies, comorbidities and associated treatments, axillary temperature and blood pressure, blood count and coagulation test, and biochemical variables. The clinical picture was evaluated by certified neurologists using the National Institute of Health Stroke Scale (NIHSS) at admission, every 6 h during the 1st day, and every 24 h during hospitalization; modified Rankin Scale (mRS) was used to evaluate functional outcome at discharge and at 3 months. Effective reperfusion was defined as ≤ 8 points in the NIHSS during the first 24 h. Poor outcome was defined as mRS > 2 at 3 months. Stroke diagnosis was made using the TOAST classification (23).

In the first episode, Computed Tomography (CT) was performed in all patients and Magnetic Resonance Imaging (MRI) in selected patients at admission. Follow-up CT scan after fibrinolysis or thrombectomy was performed in all patients at 24 h, and CT at 48 h or at any time if neurological deterioration (increase ≥ 4 points in the NIHSS) was detected; and between the 4th and 7th day. The presence and severity of LA was assessed

using Fazekas scale (24) with a total score of 0 to 6 (Fazecas I or Grade I, 1-2; Fazecas II or Grade II, 3-4; Fazecas III or Grade III, 5-6) by MRI/CT. Hemorrhagic transformation was defined according to ECASS II criteria (25). All neuroimaging tests were analyzed by a neuro-radiologist supervised by the same researcher (JMP). The neuroimaging study was completed in 786 (89.8%) patients. In the recurrence episode, in 94 (74.0%) patients only one CT was performed at admission and in 68 (53.5%) patients a further study was performed between the 4th−7th day.

Biomarkers

We used plasma levels of interleukin 6 (IL-6) and tumor necrosis factor alpha (TNFα) as markers of inflammation, and soluble tumor necrosis factor-like inducer of apoptosis (sTWEAK) as marker of endothelial dysfunction (26, 27). The blood sample to measure biomarkers was collected before the administration of the reperfusion treatment in the first stroke, and in the case of a recurrent stroke, in the 1st h following admission to the Stroke Unit of the Hospital Clínico Universitario of Santiago de Compostela. In the first episode, IL-6 measurements were performed in 843 patients (96.3%), TNFα in 828 (94.6%) and sTWEAK in 869 (99.3%). In the recurrences, the percentage of patients with a sample to measure biomarkers was lower (IL-6 71.6%; TNFα 56.7%; and sTWEAK 67.7%).

Biochemistry, hematology, and coagulation tests were assessed in the central laboratory of the Hospital Clínico Universitario of Santiago de Compostela blinded to clinical and neuroimaging data. IL-6, TNFα and sTWEAK measurements were performed in the Clinical Neurosciences Research Laboratory by researchers blinded to clinical and neuroimaging data. Serum levels of IL-6 and sTWEAK were measured by enzyme linked immunosorbent assay (ELISA) technique following manufacturer's instructions. IL-6 ELISA kit (BioLegend, San Diego, USA) minimum assay sensitivity was 1.6 pg/ml with an intra- and inter-assay coefficient of variation (CV) of 5.0 and 6.8%, respectively. sTWEAK Kit (Human TWEAK ELISA Kit (Elabscience, Texas, USA) minimum assay sensitivity was 4.69 pg/mL with an intra- and inter-assay CV of 5.06 and 5.21%, respectively. TNFα was measured using an immunodiagnostic IMMULITE 1000 System (Siemens Healthcare Global, Los Angeles, USA). Minimum assay sensitivity was 1.7 pg/mL, with an inter-assay CV of 6.5% and intra-assay CV of 3.5%. Biomarkers were evaluated within the first 3 months after blood sample collection.

Endpoints

The main outcome variables were stroke recurrence and the presence and severity of LA evaluated by neuroimaging within the first 48 h after an episode. Secondary endpoints were the association between stroke recurrence and plasma levels of IL-6; TNFα, and sTWEAK.

Statistical Analysis

For the descriptive study of the quantitative variables we used the mean ± one standard deviation or the median [range] according to the type of distribution determined by the Kolmogorov-Smirnov test for a sample with the significance correction of Lilliefors. The significance of the differences was estimated using the student's *t*-test or the Mann-Whitney U test. One-sided analysis of variance (ANOVA) was used to compare differences between more than two groups. The qualitative variables were expressed as percentages and for the differences the chi-square test and, if applicable, the uncertainty coefficient were used. The estimation of the independent variables associated with stroke recurrence was carried out using multiple regression models, identifying the continuous or categorical variables determined in the first stroke. First, we carried out logistic regression models including all variables with significant differences in univariate studies grouped according to demographic and background data, clinical and progression data and neuroimaging data. With the variables selected, a new logistic regression model was developed, which finally included the results of the biomarker analysis. To detect the ability of biomarkers to classify the values associated with stroke recurrence, ROC (Receiver Operating Characteristic) curves were developed, converting continuous variables into categorical ones for a value that offers maximum sensitivity and specificity. The results were expressed as odds ratio (OR) with 95% confidence intervals (95% CI). Significant values of $p < 0.05$ were considered. Analyzes were performed with IBM SPSS v.25 for Mac.

RESULTS

The first patient was enrolled in January 2008 and until the end of the enrollment period (December 2017) 986 reperfused IS patients were registered. **Figure 1** lists flowchart of patient groups. We excluded 27 patients who died during the first 24 h and 84 patients for whom no follow-up through either personal interview or IANUS was available. Of the 875 patients eligible for this study (53.9% males; mean age 69.6 ± 11.8 years vs. 46.1% females; mean age 74.9 ± 12.6 years), 710 patients underwent intravenous thrombolysis, 87 endovascular therapy (intraarterial thrombolysis or mechanical thrombectomy) and 78 underwent both intravenous and endovascular therapy. According to the TOAST classification, 206 patients were classified as atherothrombotic (23.5%), 381 as cardioembolic (43.5%), 11 as lacunar (1.3%) and 277 as undetermined (31.7%). Symptomatic hemorrhagic transformation (HT) was noted in 280 (32%) patients during the first admission; of which, 127 suffered stroke recurrence.

The average follow-up time of the study was 25 ± 13 months, during which 127 patients (14.5%) showed stroke recurrence. In the second stroke, 24 patients were classified as atherothrombotic (19.3%), 74 as cardioembolic (59.0%), 25 as undetermined (19.5%) and 3 intracerebral hemorrhages (2.2%). Recurrence was lower in patients with effective reperfusion (4.5 vs. 10.0%, $p < 0.0001$), and in patients undergoing endovascular treatment (6.9%), than in those who received intravenous thrombolysis (14.9%) or those who received both reperfusion therapies (19.2%, $p < 0.0001$). Functional outcome after the second stroke was worse than after the first stroke (mRS at 3 months after the first stroke 1[0, 3] vs. 4 [3, 6] after the second, $p < 0.0001$). Consequently, rates of poor functional outcome (26.1 vs. 85.8%,

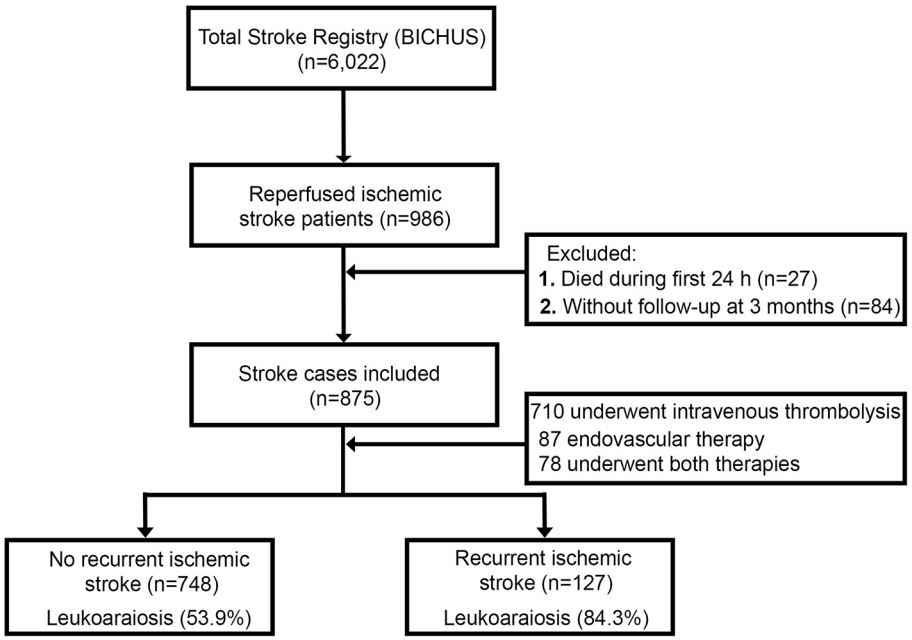

FIGURE 1 | Flowchart of patient groups and LA.

TABLE 1 | Univariate analysis of demographic variables obtained in the first admission among patients who presented or not a stroke recurrence ($n = 875$).

	No recurrence ($n = 748$)	Recurrence ($n = 127$)	*p*-value
Demographic variables			
Age, years	71.4 ± 12.7	75.7 ± 10.7	<0.0001
Female gender, %	46.3	44.1	0.700
Arterial hypertension, %	62.0	74.0	0.009
Diabetes, %	22.6	24.4	0.649
Smoking, %	24.5	11.0	0.001
Alcohol consumption, %	4.7	11.2	0.026
Hyperlipidemia, %	38.6	43.3	0.327
Peripheral arterial disease, %	6.3	9.4	0.183
Ischemic heart disease, %	13.1	10.2	0.470
Atrial fibrillation, %	21.1	33.1	0.004
Heart failure, %	4.0	6.3	0.240
Carotid disease, %	0.8	0.8	1.000
Latency time, min*	162.1 ± 61.2	160.5 ± 61.5	0.786
Previous antiaggregants, %	25.1	47.2	<0.0001
Previous Anticoagulants, %	6.3	19.7	<0.0001

**Time between the onset of symptoms and the onset of reperfusion treatment.*

$p < 0.0001$) and mortality (7.1 vs. 28.3%, $p < 0.0001$) at 3 months were higher after the second stroke.

Primary Endpoints: The Percentage of Stroke Recurrence and the Severity of LA

The univariate analysis of variables obtained at the first admission among patients who did not present with stroke recurrence are expressed in **Tables 1**, **2**. The relationship between the percentage of recurrence (84.2%) and the severity of LA (53.9%) is especially significant (**Figure 2A**). In the second stroke, the presence of LA was more severe. Grade III of the Fazekas scale went up from 28.3% in the first study to 52.8% in the second ($p < 0.0001$) (**Figure 2B**). The multivariate model shows that those patients treated with anticoagulant drugs (OR: 3.55; CI 95%: 1.01–6.40; $p < 0.0001$), those with a higher white blood cell count (OR: 1.08; CI 95%: 1.01–1.15; $p = 0.015$) and a greater severity of LA (OR: 23.31; CI 95%: 11.29–48.13; $p < 0.0001$) were independently associated with higher probability of stroke recurrence (**Table 3**).

Secondary Endpoints: IL-6, TNFα and sTWEAK

IL-6 levels analyzed in the blood sample collected at the first episode at admission and before reperfusion therapies in patients with and without stroke recurrence were similar (9.9 ± 10.4 pg/mL vs. 9.1 ± 7.0 pg/mL, $p = 0.439$), but different for TNFα (14.7 ± 5.6 pg/mL vs. 15.9 ± 5.7 pg/mL, $p = 0.031$) and sTWEAK (5,970.8 ± 4,330.4 pg/mL vs. 8,660.7 ± 5,119.0 pg/mL, $p < 0.0001$). Biomarker levels were similar in different types of stroke, both in patients with and without stroke recurrence. ANOVA tests were performed for IL-6 ($p = 0.532$ vs. $p = 0.943$), for TNFα ($p = 0.422$ vs. $p = 0.857$) and for sTWEAK ($p = 0.461$ vs. $p = 0.441$). sTWEAK levels were higher in all types of recurrent strokes, but similar for IL-6 and TNFα (**Figure 3**). In recurrent strokes, biomarker measurements were similar in the sample collected in the first and in the second episode (IL-6, 9.1 ± 6.9 pg/mL vs. 9.4 ± 7.5 pg/mL, $p = 0.330$; TNFα, 15.8 ± 5.8 pg/mL vs. 16.0 ± 7.0 pg/mL, $p = 0.168$; sTWEAK, 8,763.4 ± 5,167.3 pg/mL vs. 8,767.3 ± 4,126.0 pg/mL, $p = 0.992$).

TABLE 2 | Univariate analysis of clinical, neuroimaging variables, and molecular markers obtained in the first admission among patients who presented or not a stroke recurrence ($n = 875$).

	No recurrence ($n = 748$)	Recurrence ($n = 127$)	*p*-value
Clinical, Neuroimaging variables			
Previous mRS	0 [0, 0]	0 [0, 1]	<0.0001
NIHSS at admission	17 (12, 22)	18 (14, 22)	0.041
Early neurological improvement, %	46.3	30.7	0.001
Early neurological deterioration, %	8.5	18.5	0.002
mRS at 3 months	1 [0, 3]	2 [0, 3]	<0.0001
Axillary temperature at admission, °C	36.4 ± 0.7	36.8 ± 0.7	<0.0001
Stroke volume, mL	45.9 ± 70.1	81.0 ± 98.4	<0.0001
Leukoaraiosis, %	53.9	84.3	<0.0001
Leukoaraiosis degree			<0.0001
- No, %	46.1	15.7	
- Grade I, %	37.7	29.1	
- Grade II, %	13.0	26.8	
- Grade III, %	3.2	28.3	
Hemorrhagic transformation			<0.0001
- No, % (patients)	71.1 (532)	49.6 (63)	
- IH1, %	21.5 (161)	20.5 (26)	
- IH2, %	3.9 (29)	13.4 (17)	
- PH1, %	1.9 (14)	9.4 (12)	
- PH2, %	1.6 (12)	7.1 (9)	
TOAST			0.744
- Atherothrombotic, %	23.4	24.4	
- Cardioembolic, %	43.6	43.3	
- Lacunar, %	1.5	–	
- Undetermined, %	31.6	32.3	
Molecular markers			
Blood glucose, mg/dl	136.4 ± 53.3	149.3 ± 67.2	0.020
Leukocytes, $x10^3$/mL	8.2 ± 3.1	9.6 ± 67.2	0.020
Platelets, $x10^3$/mL	204.3 ± 66.8	197.0 ± 69.9	0.257
Fibrinogen, mg/dl	415.3 ± 102.1	438.5 ± 92.6	0.017
C-reactive protein, mg/l	3.9 ± 4.2	4.9 ± 4.6	0.048
Microalbuminuria, mg/24 h	5.3 ± 4.2	7.7 ± 9.4	0.003
LDL-cholesterol, mg/dl	109.6 ± 40.5	98.4 ± 41.0	0.123
HDL-cholesterol, mg/dl	41.7 ± 14.6	41.6 ± 16.7	0.971
Triglycerides, mg/dl	115.4 ± 53.3	102.3 ± 41.0	0.028
Erythrocyte sediment, mm	18.3 ± 20.4	22.3 ± 18.3	0.041

Modified Rankin scale (mRS); National Institute of Health Stroke Scale (NIHSS).

We demonstrated a correlation between sTWEAK levels and the severity of LA at the first admission (Spearman's coefficient $p < 0.0001$) (**Figure 4A**) that does not exist with the other biomarkers, and that the levels of sTWEAK measured in the second episode at admission increased in those patients in whom the severity of LA progressed between the two or more episodes as shown in **Figure 4B** (Spearman's coefficient $p < 0.0001$).

The ROC curve analysis of sTWEAK for stroke recurrence shows an area under the curve of 0.651; CI 95%: 0.596–0.705; $p < 0.0001$. For a cut-off point of 7,000 pg/mL, sensitivity is 63% and specificity 64%. In a logistic regression model adjusted for all biomarkers, only the sTWEAK values ≥7,000 pg/mL measured in the first stroke were independently associated with stroke recurrence (OR: 2.79; CI 95%: 1.87–4.16; $p < 0.0001$).

When the sTWEAK categorized variable was introduced into the logistic regression model, but not LA, sTWEAK multiplied the risk of recurrence by 2.48 (**Table 4**, Model A). It was demonstrated that if we include LA as a simple categorical variable, levels of sTWEAK, measured at the onset of the first stroke, ≥7,000 pg/mL multiplies by 1.62 the risk of presenting a recurrent stroke (**Table 4**, Model B). Importantly, however, if we include the different degrees of LA severity, the value of sTWEAK ≥7,000 pg/mL as a predictor of recurrence risk is no longer independent and is subrogated to LA severity (**Table 4**, Model C).

DISCUSSION

Stroke recurrence is the first cause of increased mortality and non-motor sequelae, and this complication persists in patients undergoing reperfusion treatments (1, 5–9, 12–14, 28). In our series of patients with acute IS, who received the best possible treatment according to management guidelines, recurrence was 14.5% for an average follow-up time of 2 years. The outcome of patients with recurrence was poor in 86% of cases, with a mortality of 28%. Recurrence in our study is similar to that obtained by several authors (29), but higher than that referred in other studies. This may be explained by the fact that our follow-up time was longer, and the age of our patients was higher. Mortality, however, was similar in all the studies reviewed (6, 12, 30, 31).

The type of stroke did not influence the frequency of recurrence, although the second episode led to the reclassification of almost 50% of the undetermined strokes into cardioembolic, and three patients with cardioembolic strokes recurred as intracerebral hemorrhage. Recurrence has been significantly lower in patients undergoing thrombectomy than in the case of systemic thrombolysis, and much lower than when the procedure was combined. Previously published data are uneven (1, 12–14, 32, 33). In our cases, these results were not influenced by the time between the onset of symptoms and treatment ($p = 0.108$), or follow-up time ($p = 0.424$, data not shown). However, patients in whom an effective reperfusion was achieved presented with lower recurrence rates. In a previous research work, we found that the treatment with tPA without reperfusion is associated with a worse patient progression, possibly due to the toxic effect of the drug in these cases (34).

In our study, oral anticoagulation and white blood cell count in the first stroke were independent factors associated with stroke recurrence. Although in the first episode the frequency of cardioembolic subtype was similar in both groups, in the second episode half of undetermined strokes were reclassified into cardioembolic, which implies an undervaluation of the initial diagnosis of cardioembolic. The platelet count was similar

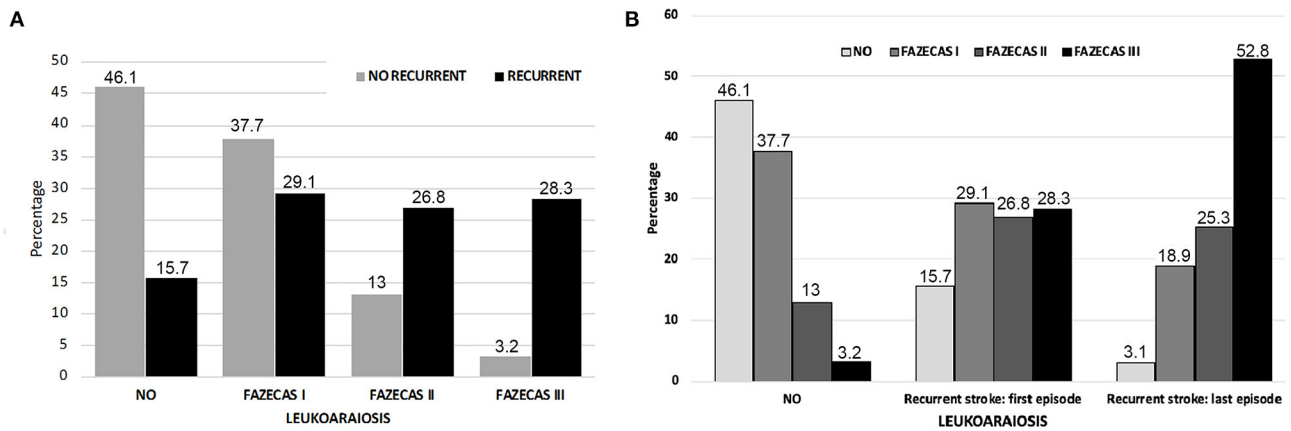

FIGURE 2 | (A) Percentage of patients with recurrence according to degree of LA in the initial MRI study. **(B)** Degree of LA in patients who did not present a new stroke; in those who presented a new stroke at the time of the first and in the study conducted following the second stroke.

TABLE 3 | Logistic regression analysis including anticoagulants, leukocytes, effective reperfusion, and leukoaraiosis degree.

	Not adjusted			Adjusted		
	OR	**CI 95%**	***P*-value**	**OR**	**CI 95%**	***P*-value**
Anticoagulants	3.65	2.16 - 6.20	<0.0001	3.55	1.01 - 6.40	<0.0001
Leukocytes	1.14	1.07 - 1.20	<0.0001	1.08	1.01 - 1.15	0.015
Effective reperfusion	0.51	0.34 - 0.77	0.001	0.97	0.60 - 1.58	0.921
Leukoaraiosis degree						
- Grade I	2.26	1.18–3.99	0.005	2.28	1.28–4.06	0.005
- Grade II	6.05	3.33–10.98	<0.0001	5.11	2.71–9.66	<0.0001
- Grade III	25.87	13.04–51.36	<0.0001	23.31	11.29–48.13	<0.0001

Dependent variable: Stroke recurrence.

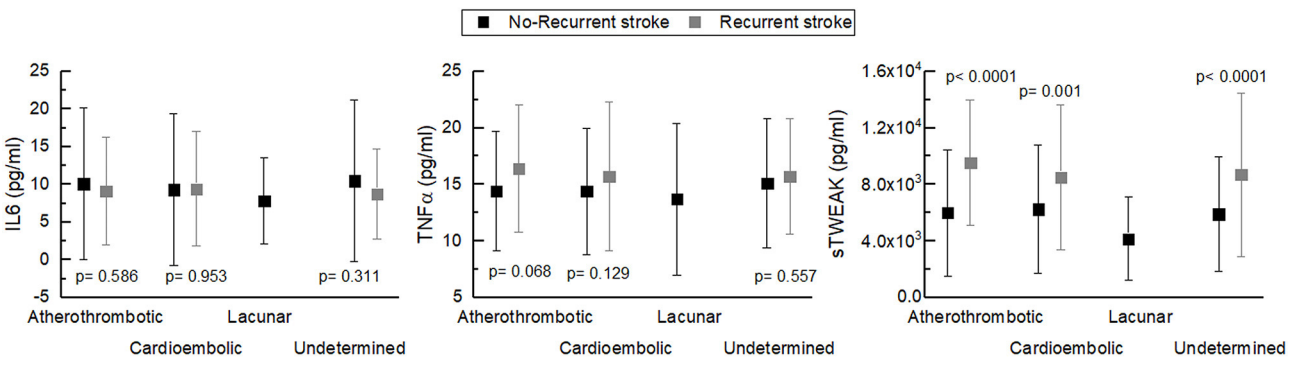

FIGURE 3 | Levels of biomarkers (IL-6, TNFα, and sTWEAK) analyzed in the sample collected during the first stroke, according to the different stroke types, both in those who did not recurred and in those who subsequently recurred.

in both groups and functional situation before stroke was worse in the patients who recurred, although this data did not reach independence in the multivariate model (13).

It is interesting that LA has been the strongest factor associated with stroke recurrence, and this association is directly related to the severity and extent of the white matter lesion. Despite the differences in the neuroimaging study and the method to quantify LA, this association is widely reported in the literature (35–41). There are, however, some differential data: (1) the association with lacunar infarctions (35) (in our series, only in reperfused patients, and none in the 11 lacunar infarctions recurred), and (2) the relationship with cardioembolic infarctions, which is

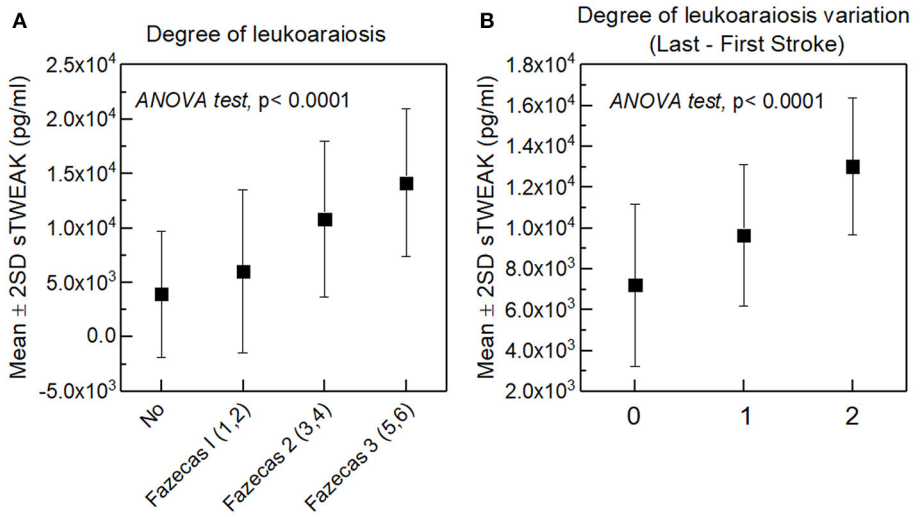

FIGURE 4 | (A) Relationship between sTWEAK levels and LA severity at first admission. **(B)** Relationship between sTWEAK levels measured in the last stroke and the variation between the degree of LA found in the last stroke in relation to the first.

TABLE 4 | Multivariate analysis including biomarkers, leukoaraiosis and leukoaraiosis degree. Dependent variable: Recurrence.

	Not adjusted			Adjusted		
	OR	**CI 95%**	**p value**	**OR**	**CI 95%**	**p value**
MODEL A						
Anticoagulants	3.65	2.16 - 6.20	<0.0001	3.64	2.09 - 6.31	<0.0001
Leukocytes	1.14	1.07 - 1.20	<0.0001	1.11	1.05 - 1.17	<0.0001
Effective reperfusion	0.51	0.34 - 0.77	0.001	0.64	0.42 - 0.98	0.041
sTWEAK ≥ 7,000 pg/mL	2.89	1.96 - 4.27	<0.0001	2.48	1.65 - 3.72	<0.0001
MODEL B						
Anticoagulants	3.65	2.16 - 6.20	<0.0001	3.64	2.08 - 6.41	<0.0001
Leukocytes	1.14	1.07 - 1.20	<0.0001	1.10	1.04 - 1.17	0.001
Effective reperfusion	0.51	0.34 - 0.77	0.001	0.68	0.44 - 1.05	0.083
Leukoaraiosis	4.58	2.78 - 7.54	<0.0001	3.34	1.94 - 5.76	<0.0001
sTWEAK ≥ 7,000 pg/mL	2.89	1.96 - 4.27	<0.0001	1.62	1.04 - 2.53	0.032
MODEL C						
Anticoagulants	3.65	2.16 - 6.20	<0.0001	3.52	1.96 - 6.34	<0.0001
Leukocytes	1.14	1.07 - 1.20	<0.0001	1.08	1.02 - 1.15	0.014
Effective reperfusion	0.51	0.34 - 0.77	0.001	0.96	0.59 - 1.56	0.872
Leukoaraiosis degree						
- Grade I	2.26	1.18 - 3.99	0.005	2.42	1.34 - 4.33	0.003
- Grade II	6.05	3.33 - 10.98	<0.0001	6.20	2.91 - 13.17	<0.0001
- Grade III	25.87	13.04 - 51.36	<0.0001	29.33	12.25 - 69.75	<0.0001
sTWEAK ≥ 7,000 pg/mL	2.89	1.96 - 4.27	<0.0001	0.76	0.43 - 1.35	0.350

not found in any study (38, 39). In our case, the association between LA and recurrence was similar in atherothrombotic, cardioembolic and undetermined strokes ($p = 0.383$). A possible explanation for this discrepancy might be that in our series the patients with cardioembolic strokes had a more advanced age (atherothrombotic 69.6 ± 12.6 years, cardioembolic 73.8 ± 11.8 years, lacunar 67.3 ± 11.8 years and undetermined 71.8 ± 12.9 years). Aside from theses discrepancies, LA is currently an important factor of poor outcome after a stroke.

Of the determined inflammatory markers (white blood cells, fibrinogen, C-reactive protein, sedimentation rate, IL-6 and TNFα), only white blood cells and TNFα maintained

statistical significance in the first regression models but they lost it when including all clinical factors and of neuroimaging. However, sTWEAK (levels ≥ 7,000 pg/mL) was associated independently with an increased risk of stroke recurrence. The strong relationship between the sTWEAK levels in the first stroke and the severity of LA suggests that sTWEAK is a surrogate marker for LA, and thus, when we included the severity of LA in the regression model, sTWEAK disappeared as an independent recurrence factor (**Table 4**, Model B).

sTWEAK is a type II transmembrane glycoprotein of the TNF (tumor necrosis factor) superfamily that acts by binding to Fn14 which is a small transmembrane type I protein. TWEAK-Fn14 is expressed in all cells that act in the Neurovascular Unit and overexpresses within a few hours of establishing a cerebral ischemia (42–45). TWEAK-Fn14 overexpression induces an inflammatory profile in brain endothelial cells with increased secretion of proinflammatory cytokines, production and activation of matrix metalloproteinases that will participate in the disruption of the blood-brain barrier and expression of intercellular adhesion molecules involved in the union of white blood cells to the endothelium (46, 47). This maintained expression could condition the development and progression of LA and could be the molecular marker associated with white matter disease associated with chronic cerebral ischemia. This hypothesis, however, remains to be demonstrated.

From a clinical point of view, the importance of sTWEAK as predictor of LA progression associated with the increase of stroke recurrence does not seem preferred, since neuroimaging is more sensitive and specific, at least with the method used (we have exclusively determined sTWEAK, and no sTWEAK-Fn14). However, the possibility of blocking the activation of the sTWEAK-Fn14 system (anti-sTWEAK or anti-Fn14 monoclonal antibodies, or through sTWEAK-Fn14 fusion blockade) makes this marker a hopeful therapeutic target that could decrease the progression of LA and stroke recurrence (48, 49).

This study has some limitations. First, our study presents the weaknesses of any retrospective study, even if its origin is prospective. Bias in the enrolment of patients was reduced as we enrolled all those registered in our hospital and followed-up in any hospital of the public system (in Galicia, the network of private hospitals is small). Second, sTWEAK measurements were not simultaneous and were made by different researchers, although measurements were always blind to the clinical and neuroradiological data and supervised by the same senior researchers, and the same is true of clinical and neuroradiological data. Three, it is important to note that LA is a gradual disease affected by different risk factors, and not associated with a unique pathological process (16, 50). There is the possibility that LA may be associated with factors in the study population other than stroke. It is known that in regions corresponding to LA on neuroimaging, the wall of penetrating arteries is thickened and hyalinized, and there is often narrowing, elongation, and tortuosity of small vessels, potentially leading to reduced cerebral blood flow, and permanent BBB damage. Furthermore, after the first stroke, Wallerian degeneration (WD) could develop and cause new white matter hyperintensities related with LA progression (51). Four, serum levels of sTWEAK do not represent a specific marker of a particular process; patients with multiple sclerosis, heart failure, or atherosclerosis show also variations in the sTWEAK levels (52). However, we investigate the possible relationship among sTWEAK—LA—stroke recurrence in reperfused IS patients. The strong points of this work are the unbiased screening of individuals, the high number of enrolled patients, and the large number of biomarkers assessed.

CONCLUSION

Stroke recurrence is associated with increased mortality, non-motor sequelae. Currently, preventive efficacy is limited. The presence of an advanced degree of LA, as well as its progression, is the main neuroimaging factor associated with stroke recurrence. sTWEAK (≥ 7,000 pg/mL) is a biomarker correlated with the progression of LA and stroke recurrence. sTWEAK could become a diagnostic boimarker and a potential therapeutic target in reducing stroke recurrence but further studies will be necessary.

DATA AVAILABILITY STATEMENT

The original contributions generated for this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by This research was carried out in accordance with the Declaration of Helsinki of the World Medical Association (2008) and approved by the Ethics Committee of Santiago de Compostela (2019/616). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PH, RI-R, and JC: conception and design of the study. FC, AdS-C, TS, and MP-M: data acquisition and analysis. JC, MR-Y, JP, and IL-D: clinical data acquisition and analysis. RI-R, TS, JC, PH, and AdS-C: handled funding and supervision. PH and JC: statistical analysis. RI-R, PH, and JC: manuscript drafting. MP-M, TS, JC, and FC: critical revision for important intellectual content. JC, MR-Y, JP, IL-D: supervision. All authors reviewed and approved the manuscript.

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Usefulness of the Neutrophil-to-Lymphocyte Ratio as a Predictor of Pneumonia and Urinary Tract Infection Within the First Week After Acute Ischemic Stroke

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Background: A high Neutrophil-to-Lymphocyte ratio (NLR) in patients with acute ischemic stroke (AIS) has been associated with post-stroke infections, but it's role as an early predictive biomarker for post-stroke pneumonia (PSP) and urinary tract infection (UTI) is not clear.

Aim: To investigate the usefulness of NLR obtained within 24 h after AIS for predicting PSP and UTI in the first week.

Methods: Clinical and laboratory data were retrieved from the University Hospital Brussels stroke database/electronic record system. Patients were divided into those who developed PSP or UTI within the first week after stroke onset and those who didn't. Receiver operating characteristics (ROC) curves and logistic regression analysis were used to identify independent predictors.

Results: Five hundred and fourteen patients were included, of which 15.4% ($n = 79$) developed PSP and 22% ($n = 115$) UTI. In univariate analysis, NLR was significantly higher in patients who developed PSP (4.1 vs. 2.8, $p < 0.001$) but not in those who developed UTI (3.3 vs. 2.9, $p = 0.074$). Multiple logistic regression analysis for PSP showed that NLR, male gender, dysphagia, and stroke severity measured by the National Institutes of Health Stroke Scale (NIHSS), were independent predictors of PSP. For NLR alone, the area under the curve (AUC) in the ROC curve was 0.66 (95% CI = 0.59–0.73). When combining NLR $\geq$ 4.7 with age >75 years, male gender, NIHSS > 7, and dysphagia, the AUC increased to 0.84 (95% CI = 0.79–0.89).

Conclusion: The NLR within 24 h after AIS appears to have no predictive value for post-stroke UTI, and is only a weak predictor for identifying patients at high risk for PSP. Its predictive value for PSP appears to be much stronger when incorporated in a prediction model including age, gender, NIHSS score, and dysphagia.

Keywords: acute ischemic stroke, post-stroke pneumonia, post-stroke urinary tract infection, post-stroke infections, neutrophil-to-lymphocyte ratio

INTRODUCTION

Pneumonia and urinary tract infections (UTI) are the most common infectious complications after acute ischemic stroke (AIS), with an incidence of 12 and 16%, respectively (1). Post-stroke infections have been associated with poor outcome and mortality (2, 3). Therefore, there is an interest in finding early predictors of these post-stroke infections, which may help to select high-risk patients to start interventions in time. Most prediction scoring models for post-stroke pneumonia (PSP) are based on clinical features including age, gender, stroke severity measured by the National Institutes of Health Stroke Scale (NIHSS) (4) and the presence of dysphagia (5–9). A recent meta-analysis showed that age, female gender and post-void residual volume >100 ml were predictors of post-stroke UTI (10).

Next to clinical factors, a number of inflammatory parameters including C-reactive protein (CRP), white blood cell count, procalcitonin and copeptin (11), interleukin-13 and interferon-γ (12), elevated monocyte count and interleukin-10 (13), and high circulating natural killer cell count within the first hours after stroke followed by a drop in all lymphocyte subsets (14) have been associated with post-stroke infections. However, it is unclear how these parameters should be applied in clinical practice.

A biomarker, which has gained interest over the last years, is the Neutrophil-to-Lymphocyte Ratio (NLR). It is a marker of inflammation that is simply calculated from blood cell counts obtained on admission in every AIS patient. Nam et al. (15) found that a higher NLR in patients with AIS who were admitted within 7 days of symptoms onset independently predicted PSP during that 7-day period. Wang et al. (16) found that the NRL at multiple time points with a peak at 36 h after stroke onset was independently associated with PSP but not with UTI. The NLR on admission was not used separately in their study. Three other studies in patients with AIS in whom blood was collected within 24 h of symptom onset did not discriminate between PSP, UTI, and other infections. Two of them found that a higher NLR was independently associated with post stroke infections (17, 18), whereas the third study could not confirm this association (19).

Since most of these infections already manifest within the first days after AIS, we wanted to investigate the predictive value of NLR obtained on admission within 24 h after stroke onset for PSP and UTI separately.

MATERIALS AND METHODS

Patients and Assessment Procedures

We extracted the data of 1,457 patients admitted to the Stroke Unit of the University Hospital Brussels (Belgium), which were prospectively collected in a database over a 6-year period. We included all patients with AIS, who had routine blood sampling within 24 h after stroke onset. AIS was defined as "a sudden onset of loss of global or focal cerebral function" (20) caused by brain ischemia of any origin, confirmed on cerebral computed tomography, or magnetic resonance imaging. Exclusion criteria were previous hematologic, inflammatory or autoimmune disorders, current cancer, infections preceding stroke, use of antibiotics <24 h before admission, use of immunosuppressants on admission, recent surgery, and stroke related death and/or palliative care started <48 h after stroke onset. A study population flowchart is shown in **Figure 1**. Demographic data (age, gender), medical history, use of beta-blockers prior to admission, pre-stroke modified Rankin Scale (mRS), NIHSS on admission, level of consciousness (LOC, determined by NIHSS subitem 1a) and information concerning intravenous thrombolysis (IVT) and endovascular therapy (EVT) were retrieved from the database. Dysphagia objectified by a professional speech therapist, nasogastric tube feeding, urinary catheter placement, and results of baseline blood measures (absolute neutrophil count, absolute lymphocyte count and CRP) were retrieved from the electronic record system.

Standard Protocol Approval

The study protocol was approved by the Ethics Committee of the University Hospital of Brussels (reference number B.U.N. 143201733949).

Neutrophil-to-Lymphocyte Ratio

The NLR was defined as the ratio of the absolute neutrophil count to the absolute lymphocyte count, which were counted in the peripheral blood sample on admission by use of fluorescent flowcytometric measurements (CELL-DYN Sapphire, Abbott Diagnostics, Abbott Park, IL) (14, 21).

Post-stroke Pneumonia

PSP during the first week after stroke onset was retrospectively diagnosed using Modified criteria of the US Center for Disease Control and Prevention: "at least one of the former and one of the latter criteria fulfilled: (A) abnormal respiratory examination, pulmonary infiltrates on chest x-rays; (B) productive cough with purulent sputum, microbiological cultures from lower respiratory tract or blood cultures, leukocytosis, elevated CRP" (14, 22).

Post-stroke UTI

UTI during the first week after stroke onset was retrospectively diagnosed and defined as having at least 2 of the 4 following criteria: urine sample positive for nitrite, urine culture with >100.0000 colonies/ml, urine culture with >25 white blood cells/μl or body temperature >38°C (22).

Statistics

Statistical analyses were performed using SPSS version 27.0 software package. Patients were divided into those who developed PSP/UTI and those who didn't. Normality was checked by using the Kolmogorov-Smirnov test and visual interpretation of histograms and Q-Q plots. Skewed variables were log-transformed to reach normality. Differences were detected using the Independent-Samples Student *T*-test (with back-transformation of the results, if applicable) and the Mann-Whitney U-test for continuous variables. The $\chi 2$- or Fisher Exact-test were used for categorical variables. Age and NIHSS on admission were dichotomized by using the values of the 50% percentile as cut-off. For NLR, the 75% percentile was used. Variables of clinical interest were enrolled in multiple logistic regression analysis (MLRA). The stepwise

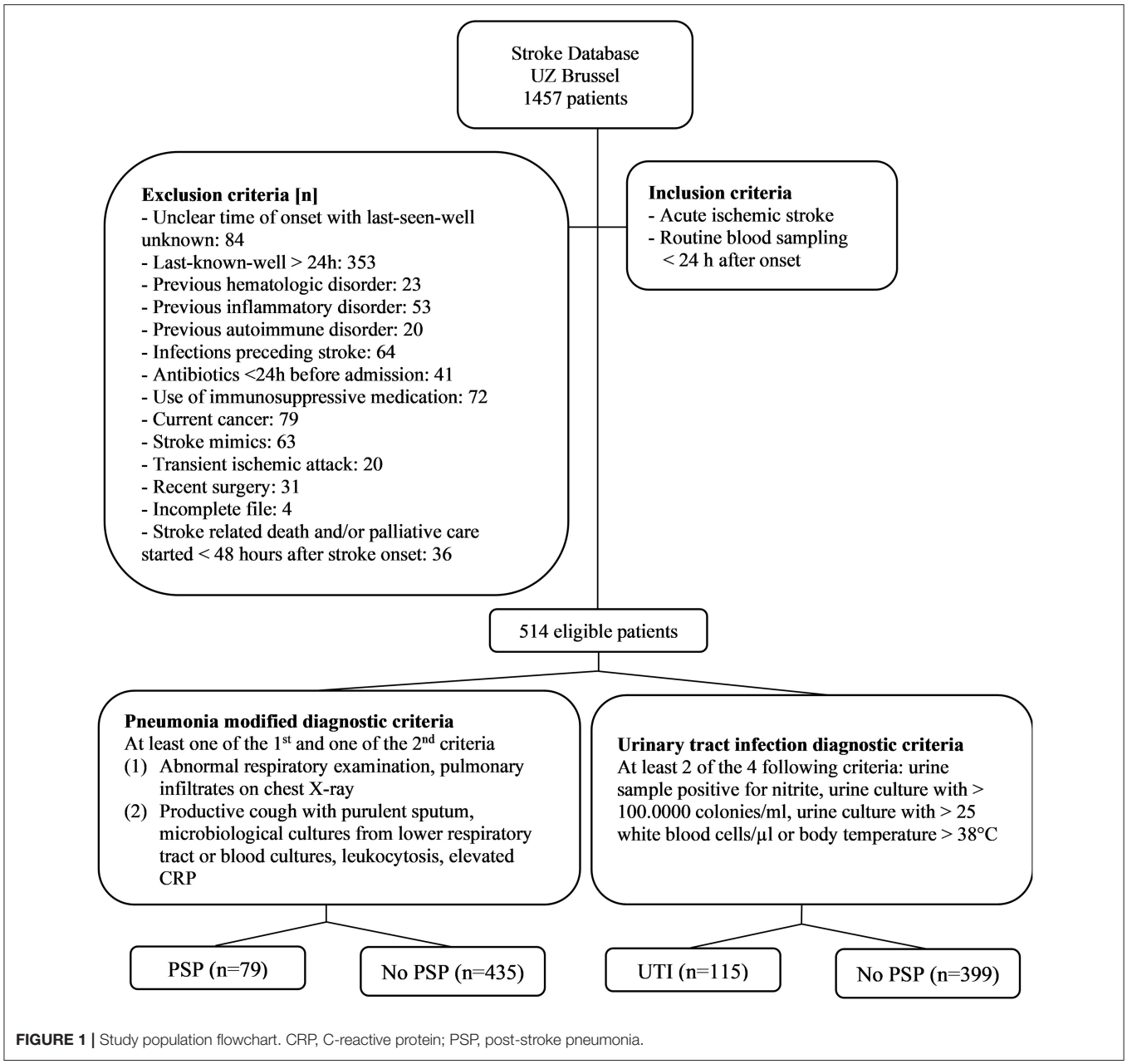

FIGURE 1 | Study population flowchart. CRP, C-reactive protein; PSP, post-stroke pneumonia.

Backward Wald method and ROC curves were used to identify independent predictors. Variables most accessible on admission were combined to create a prediction model.

RESULTS

Baseline Characteristics

Five hundred and fourteen patients met the selection criteria, of whom 15% (n = 79) developed PSP and 22% (n = 115) developed UTI (**Figure 1**). **Table 1** presents the baseline characteristics of patients with PSP vs. without PSP, and of patients with post-stroke UTI vs. without post-stroke UTI.

Post-stroke Pneumonia

In univariate analysis, age, male gender, NIHSS, altered LOC, treatment with IVT, dysphagia, tube feeding and urinary catheter placement were associated with PSP ($p < 0.05$). Patients who developed PSP had significantly lower lymphocyte counts on admission. CRP, neutrophil count, and NLR within 24 h after stroke onset were significantly higher in patients with vs. without PSP. The NLR was not significantly different between patients who developed PSP during the first 3 days (71% of PSP cases) of admission and those who developed PSP between day 4 and 7 (29% of cases) of admission (4.29 ± 2.07 vs. 3.59 ± 1.99 respectively, $p = 0.320$). Of all patients, 145 patients were discharged before day 7. The mean length of their hospital stay

TABLE 1 | Baseline characteristics of study population ($n = 514$).

Variables	Post-stroke pneumonia			Post-stroke UTI		
	PSP ($n = 79$)	No PSP ($n = 435$)	p-value	UTI ($n = 115$)	No UTI ($n = 399$)	p-value
Age, years[a]	79 (69–86)	74 (62–83)	0.005	79 (74–87)	72 (61–82)	< 0.001
Gender, male[b]	54 (68.4)	223 (51.3)	0.005	32 (27.8)	245 (61.4)	< 0.001
Known AHT[b]	62 (78.5)	313 (72.0)	0.230	89 (77.4)	286 (71.7)	0.224
Use of β-blockers[b]	36 (45.6)	166 (38.2)	0.215	50 (43.5)	152 (38.1)	0.298
Known DM[b]	19 (24.1)	86 (19.8)	0.385	25 (21.7)	80 (20.1)	0.692
NIHSS[a]	16 (8–21)	5 (2–12)	< 0.001	10 (5–18)	5 (2–14)	< 0.001
Altered LOC (NIHSS subitem 1a > 0)[b]	23 (29.1)	27 (6.6)	< 0.001	18 (16.2)	32 (8.4)	0.017
Dysphagia[b]	47 (59.5)	90 (20.7)	< 0.001	47 (41.6)	90 (23.1)	< 0.001
IVT[b]	39 (49.4)	120 (27.6)	< 0.001	34 (29.6)	125 (31.3)	0.719
EVT[b]	4 (6.3)	24 (5.5)	0.789	5 (5.1)	23 (5.8)	0.783
Tube feeding[b]	42 (53.2)	40 (9.2)	< 0.001	31 (27.2)	51 (12.8)	< 0.001
Urinary catheter	27 (34.2)	63 (14.5)	< 0.001	33 (28.9)	57 (14.3)	< 0.001
#Lymphocytes (/mm^3)[c]	1598 ± 1.7	1869 ± 1.6	0.344	1746 ± 1.6	1848 ± 1.6	0.245
#Neutrophils (/mm^3)[c]	6503 ± 1.55	5251 ± 1.53	< 0.001	5796 ± 1.6	5319± 1.5	0.064
NLR[c]	4.1 ± 2.1	2.8 ± 1.9	< 0.001	3.3 ± 2.2	2.9 ± 1.9	0.074
CRP (mg/l)[a]	3.2 (1.6–11.1)	2.6 (1.2–5.8)	0.035	2.9 (1.2–6.3)	2.9 (1.3–6.4)	0.703

Results are expressed as mean ± standard deviation (SD), median (interquartile range (IQR)) or n (%) when appropriate. PSP, post-stroke pneumonia; NIHSS, National Institutes of Health Stroke Scale; AHT, arterial hypertension; DM, diabetes mellitus; IVT, intravenous thrombolysis; EVT, endovascular therapy; NLR, Neutrophil-to-Lymphocyte Ratio; CRP, C-reactive protein.
[a] Mann-Whitney U-test.
[b] χ2-test.
[c] Independent-Samples Student t-test.

was 4.75 ± 1.2 days, which exceeded the mean time to onset of PSP of 2.9 ± 1.7 days for the entire study population. The mean time to event did not significantly differ between patients who had a hospital stay of 7 days or more vs. those who were discharged before day 7 (3.0 ± 1.8 vs. 2.4 ± 1.2 days, $p = 0.301$).

Post-stroke UTI

In univariate analysis, age, female gender, pre-stroke mRS, NIHSS, dysphagia, tube feeding, altered LOC, and urinary catheter placement were associated with post-stroke UTI ($p < 0.05$). NLR within 24 h after stroke onset was not significantly higher in patients with post-stroke UTI compared to patients without post-stroke UTI. The NLR was not predictive in both patients discharged before day 7 and those who stayed for 7 days or more.

Multiple Logistic Regression

Since NLR was not significant in univariate analysis for UTI, we opted to perform multivariate analysis for PSP only. The following variables were enrolled in MLRA: age, gender, smoking, chronic obstructive pulmonary disease (COPD), diabetes mellitus (DM), NIHSS, LOC, dysphagia, and NLR. The results indicated that NLR, next to age, male gender, NIHSS on admission, and dysphagia, was an independent predictor of PSP (**Table 2**). To create a more easy-to-use prediction model, we dichotomized "NIHSS on admission" and "age" by using the 50% percentile values as cut-offs, which were >7 and >75 years, respectively. The cut-off for NLR was determined by the 75% percentile value, which was ≥4.7. Based on the results of the first multivariate analysis and the clinical usefulness of the variables, we repeated MLRA using the following variables: age > 75 years, male gender, dysphagia, NIHSS > 7 and NLR ≥ 4.7, which shows a significant predictive value for each of these variables when using this model (**Table 2**).

ROC Curve Analyses

For NLR, age, NIHSS, and male gender, AUC was to 0.66 (95% CI = 0.59–0.73), 0.60 (95% CI = 0.53–0.66), 0.75 (95% CI = 0.68–0.81) and 0.59 (95% CI = 0.52–0.66), respectively (see **Figure 2**). For the dichotomized variables, NLR ≥ 4.7, age > 75 years, and NIHSS > 7, AUC was 0.64 (95% CI = 0.56–0.71), 0.58 (95% CI = 0.50–0.65), and 0.68 (95% CI = 0.62–0.75), respectively (**Figure 2**). For a 5-item prediction model, which combines age > 75, male gender, dysphagia, NIHSS > 7, and NLR ≥ 4.7, AUC was 0.84 (95% CI = 0.79–0.89) (**Figure 2**).

DISCUSSION

Previous studies have shown that NLR is a predictor of poor functional outcome and mortality after AIS, but the underlying mechanisms remain unclear (20, 23–26). Two studies found a link between the NLR and post-stroke infections but they lack information about the location of the infection (17, 18). A study by Nam et al. (15) found that a NLR cut-off value >2.43, which was based on the median of their cohort, was an independent predictor of PSP. However, NLR was determined within 7 days of stroke onset instead of 24 h. In another study, a higher NLR at different time points post-stroke, with a peak value at 36 h, has also been associated with post-stroke infection, and more specific

TABLE 2 | Stepwise MLRA for PSP (model 1a and 1b).

	Variables	OR	95% CI	*p*-value
Including continuous variables	Age	1.03	1.00–1.05	0.047
	Male gender	4.40	2.27–8.54	< 0.001
	Dysphagia	5.20	2.71–9.97	< 0.001
	NIHSS	1.08	1.04–1.13	< 0.001
	NLR	1.12	1.04–1.21	0.003
Prediction model	Age > 75 years	2.45	1.31–4.58	0.005
	Male gender	4.14	2.16–7.93	< 0.001
	Dysphagia	6.40	3.36–12.20	< 0.001
	NIHSS > 7	2.54	1.29–5.01	0.007
	NLR ≥ 4.7	2.89	1.60–5.22	< 0.001

MLRA, multiple logistic regression analysis; PSP, post-stroke pneumonia; OR, odds ratio; CI, confidence interval; NIHSS, National Institutes of Health Stroke Scale; NLR, Neutrophil-to-Lymphocyte ratio.

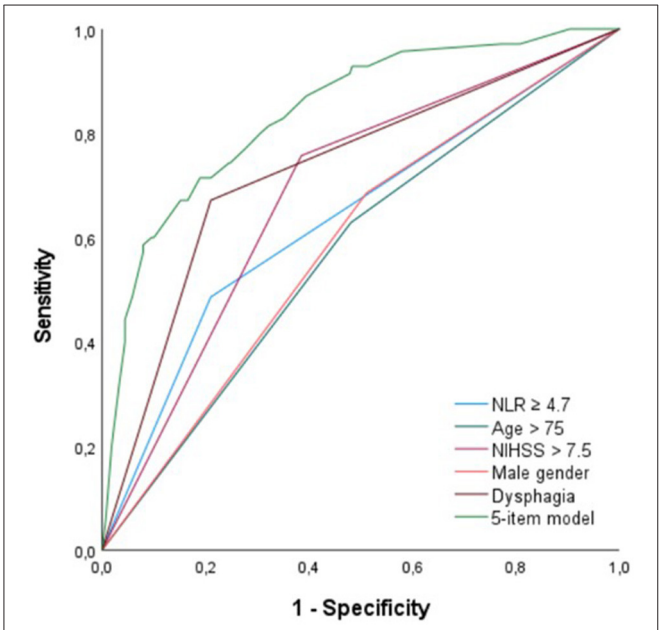

FIGURE 2 | ROC curve analysis for NLR ≥ 4.7, age > 75 years, NIHSS > 7, male gender, dysphagia, and a 5-item prediction model (NLR ≥ 4.7, age > 75 years, dysphagia, NIHSS > 7, male gender). ROC, receiver operating characteristics; NLR, neutrophil-to-lymphocyte ratio; NIHSS, National Institutes of Health Stroke Scale.

PSP (16). A study of van Gemmeren did not show an independent predictive value of the 24 h NLR for PSP, but because numbers were small the study was likely underpowered to detect such an effect (19).

Our results provide additional evidence for NLR as a significant and independent predictor for PSP, although, its predictive value appears to be quite weak. ROC curve analysis of NLR alone showed an AUC of 0.66 (95% CI = 0.59–0.73). This could be explained by the fact that immunological changes are only one of the mechanisms leading to PSP. Our results further showed that age, male gender, dysphagia, and stroke severity (NIHSS) were, albeit also weak, independent predictors of PSP, which is in line with previous studies (5–9, 27–29). Because of its rather low predictive value for PSP, we reperformed MLRA with only dichotomized variables, to make it more clinically useful. Based on the results of our first multivariate analysis and the immediate availability upon admission of the enrolled clinical variables, we created a 5-item prediction model using NLR ≥ 4.7, age > 75 years, male gender, dysphagia, and NIHSS > 7. In this model, the AUC increased to 0.84 (95% CI = 0.79–0.89), indicating that NLR is especially useful in predicting PSP when incorporated into a model with these four clinical predictive factors.

The NLR was not significantly different in patients who developed PSP within 3 days of admission and those who developed PSP during day 4–7 after admission, suggesting that a high admission NLR is not solely due to an inflammatory response caused by aspiration, or a pneumonia, that was already started on admission.

Our study found that NLR within 24 h after stroke onset was not a significant predictor of UTI. This confirms the findings of Wang et al. (16) who also did not find a significantly higher NLR in patients with post-stroke UTI, although, they did not use the NLR on admission. A plausible explanation why NLR is predictive for PSP but not for UTI, is that the underlying mechanisms of these infections are at least partially different. After AIS, neutrophil counts increase and lymphocyte counts decrease (28, 30, 31) as part of the post-stroke immunodepression phenomenon, activated by the sympathetic nervous system and hypothalamic-pituitary-adrenal axis (30, 32). This may be a mechanism to prevent further damage by reducing local brain inflammation. The role of neutrophils and lymphocytes seems to be dual, with both beneficial and harmful effects (3, 14, 31, 33). The NLR could be used to estimate the degree to which this post-stroke immunodepression occurs, with a higher NLR suggestive of a more pronounced immunodepression. Since both NLR and pneumonia have been associated with poor prognosis after ischemic stroke (20, 24, 34–36), we hypothesize that a higher degree of immunodepression makes patients more susceptible to systemic infections, such as pneumonia, leading to a worse outcome. Preclinical evidence shows that mice subjected to ischemic stroke were more susceptible to spontaneous bacteriemia and pneumonia compared to mice who underwent sham procedure (37). An explanation might be that the post-stroke immunodepression phenomenon favors bacterial translocation and dissemination of commensal bacteria from the host gut microbiota, leading to systemic infections (38). Whereas, these mechanisms might contribute to PSP, the occurrence of post-stroke UTI seems to rather depend on other factors. Urinary tract infections, which can be seen as rather local than systemic infections, seem to be mainly explained by mechanical factors such as bladder dysfunction causing urinary retention (39), use of urinary catheter (29, 40) and the presence of a short urethra (female predominance). In addition, they are less clearly associated with worse prognosis after ischemic stroke, since although preventive antibiotics reduced UTI frequency in the PASS-study, no effect was seen on outcome (36).

It has been hypothesized that sympathetic nervous system activation might be one of the underlying mechanisms of post-stroke immunodepression, and that therefore beta-blockers might theoretically prevent post-stroke infections (32). In mice, blockade of the sympathetic pathways by beta-blockers reduced post-stroke infections and improved stroke outcome (41). However, in human studies, results have been conflicting. Sykora et al. (42) reported that pre-stroke and on-stroke beta-blocker treatment reduced PSP frequency. On the other hand, Maier and coworkers reported that beta-blocker exposure had no effect on PSP frequency, but that it reduced UTI rates (43, 44). Dromerick et al. (45) found the use of beta-blockers to be a predictor of post-stroke UTI. In our study, we did not find an association between beta-blocker use prior to AIS and PSP or UTI.

There are some limitations to this study. First, although data were gathered prospectively, the diagnosis of PSP and UTI was checked retrospectively, which could have caused some diagnostic errors. By using the modified CDC criteria for retrospective diagnosis of pneumonia, a positive chest x-ray was not necessary to reach diagnostic criteria. Therefore, diagnosis could also be made based on clinical features only, which might have decreased diagnostic accuracy. Second, the NLR was only investigated for its predictive role regarding PSP/UTI. It is possible that patients developed other infectious or inflammatory complications that might have influenced NLR. Third, we did not intend to exclude patients discharged before day 7, as we wanted to explore the role of NLR and the subsequent combined model in a situation consistent with real-life in which we do not know in advance how long patients will stay. We may have missed a number of cases with PSP and UTI in patients who were discharged before day 7. However, because the majority of patients (72%) was hospitalized for 7 days or more, it is unlikely that this will affect our main conclusions. In addition, for UTI, the NLR was not predictive in both patients discharged before day 7 and those who stayed for 7 days or more. For PSP, we found that the mean length of hospital stay for those discharged before day 7 exceeded the mean time to onset of PSP, which is usually within the first 2 to 3 days after stroke onset.

Prospective studies are required to investigate whether our proposed prediction model, which incorporates NLR, too can, with a high degree of certainty, identify patients prone to develop PSP, who may therefore be candidates for prophylactic measures. Prophylactic antibiotic treatment significantly decreases overall post-stroke infection rate, but its effect on reducing the incidence of PSP has not been established (46). Identifying patients at risk will lead to a better selection of patients who could benefit from this kind of treatment. In addition, new therapeutic approaches other than prophylactic antibiotic administration, such as treatment of the underlying mechanisms of post-stroke immunodepression, should be considered (32).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Commissie Medische Ethiek UZ Brussel (reference number B.U.N. 143201733949). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

RG: design and conceptualized study, major role in the acquisition of data, analyzed the data, and drafted the manuscript for intellectual content. AO: design and conceptualized study, major role in the acquisition of data, analyzed the data, and drafted the manuscript for intellectual content. AD: major role in the acquisition of data and revised the manuscript for intellectual content. JD: revised the manuscript for intellectual content. SD: design and conceptualized study, major role in the acquisition of data, and drafted the manuscript for intellectual content. All authors contributed to the article and approved the submitted version.

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The Prognostic Value of the Acute Phase Systemic Immune–Inflammation Index in Patients With Intracerebral Hemorrhage

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Li Y, Wen D, Cui W, Chen Y, Zhang F, Yuan M, Xiao H, Li H, Ma L, Hu X and You C (2021) The Prognostic Value of the Acute Phase Systemic Immune–Inflammation Index in Patients With Intracerebral Hemorrhage. Front. Neurol. 12:628557. doi: 10.3389/fneur.2021.628557

Background and Purpose: The systemic immune–inflammation index (SII) is a novel prognostic index in various diseases. We evaluated the predictive value of SII in patients with intracerebral hemorrhage (ICH).

Methods: Patients with primary spontaneous ICH were enrolled. SII was constructed based on peripheral platelet (P), neutrophil (N), and lymphocyte (L) and defined as P*N/L. In addition to admission testing, acute phase SII was collected to analyze the potential dynamic change. Poor outcome was defined as modified Rankin Scale of more than 3 at 90 days.

Results: We included 291 patients; 98 (34%) achieved favorable functional outcomes. Day-1 SII was higher and was more related to poor outcome than was admission SII. Median time of day-1 SII was 29 h from onset. Day-1 SII had an OR in outcome (mRS >3) 1.74 (95% CI = 1.03–3.00, $p = 0.04$). The binary cutoff point of SII calculated using the area under the curve (AUC) method was $1{,}700 \times 10^9$/L, AUC 0.699 (95% CI = 0.627–0.774) (sensitivity 53.3%, specificity 77.3%) (OR = 2.36, 95% CI = 1.09–5.26, $p = 0.03$).

Conclusions: SII, especially day-1 SII, was highly associated with 90-day functional outcome in patients with ICH and could be used to predict outcomes.

Keywords: stroke, prognosis, blood platelets, intracerebral hemorhage, neutrophils lymphocyte ratio

INTRODUCTION

Spontaneous intracerebral hemorrhage (ICH) is associated with high mortality and poor outcome (1). Studies found various indicators for predicting outcome following ICH (2–4); however, few of these involve biochemical tests. The brain injury after ICH includes the primary injury, which is the mechanical damage of the adjacent tissues by hematoma within the first hours after ICH, and the secondary injury, which is initiated by the extravasation of blood products into the brain parenchyma (5). Mounting preclinical evidence has shown that inflammation after ICH plays an important role in the secondary brain injury (6). Furthermore, clinical

laboratory results that reflect inflammation have been reported to predict ICH outcome. Platelet (PLT) counts have been associated with growth edema (7), which predicts ICH outcome (8). The neutrophil–lymphocyte ratio (NLR) was reported (9, 10), as were PLT–lymphocyte ratio (PLR) (11), and lymphocyte–monocyte ratio (LMR) (12). These studies showed that levels of inflammation are highly related to the clinical outcome following ICH (13).

The systemic immune-inflammation index (SII), which was calculated as peripheral platelet*neutrophil/lymphocyte, was first reported as a prediction tool in cancers such as hepatocellular carcinoma (14–16). In 5,602 coronary artery disease patients after coronary intervention, SII was shown to have a better prediction for major cardiovascular events than traditional risk factor (17). In acute ischemic stroke, SII was reported as an independent risk factor for stroke severity (18). Moreover, dynamic changes of SII was suggested as a promising prognostic predictor for cancer patients such as colorectal cancer and hepatocellular carcinoma (19, 20). A recent study confirmed the value of SII for predicting short-term outcome following ICH (21). Nevertheless, the role of SII in predicting long-term outcome following ICH is unknown.

In the present study, we investigated acute phase SII and favorable outcome of ICH in recovery. We also studied dynamic changes of SII to identify a more precise way to predict outcome.

METHODS

The data and code that support the findings of this study are available from the corresponding author upon reasonable request. The study was approved by the local ethic committee.

We retrospectively collected data from patients admitted to West China Hospital, Sichuan University (Sichuan, China) from February 2018 to February 2019. The inclusion criteria were as follows: (1) over 18 years of age; (2) admission diagnosis of ICH based on brain CT scans; (3) <24 h from onset to admission; (4) available clinical data including at least one laboratory test of platelets, neutrophils, lymphocytes and monocytes; and (5) neuro-image to evaluate the characteristics of the hematoma. Exclusion criteria were as follows: (1) secondary ICH (aneurysm, vascular malformation, or tumor); (2) possible disease that may affect laboratory results (leukemia, lymphoma, or thrombocytopenia); and (3) unavailability of outcome data; (4) patients with coagulopathy or anticoagulant therapy; and (5) patients with active infection or autoimmune disease.

We recorded age, sex, clinical record, previous medical history, laboratory results (PLT), absolute neutrophil count (ANC), absolute lymphocyte count (ALC), absolute monocyte count (AMC), baseline CT imaging characteristics, and surgical information if available. ICH volume was measured based on the ABC/2 method (22). We collected all laboratory test results during hospitalization. SII was defined as platelet*neutrophil/lymphocyte.

The primary outcome was modified Rankin scale (mRS) at 90 days from onset. mRS was measured at outpatient visit or by telephone using a structured interview (23). We defined favorable outcome as mRS 0–3, and unfavorable outcome was mRS 4–6.

All statistical analyses were performed using R software (Version 4.0.2, R Core Team, Vienna, Austria). Continuous variables were tested using the Student's *t* test or Mann–Whitney test and were expressed as mean (standard deviation) or median (interquartile range) depending on their distribution. Categorical variables were defined as numbers and were analyzed using the χ^2 test or Fisher exact test. Receiver operating curves (ROCs) were generated and the area under curve (AUC) was calculated to estimate the ability of the SII and other factors to predict poor outcomes. The optimal cutoff point was calculated using the Youden's Index. Boxplots were performed to describe the distribution of admission and day-1 SII in ordinal mRS. Multivariate logistic regression analysis was used to analyze the association between factors and prognosis. Variables included in the model were selected based on the result of univariate analysis. Two-tailed $p < 0.05$ was considered significant.

RESULTS

We enrolled 291 patients (**Supplementary Figure 1**). Of these, 98 (34%) achieved favorable outcomes at 90 days. The poor outcome group included more females (38 vs. 23%, $p = 0.02$), older patients (59 ± 14 vs. 55 ± 13, $p = 0.0002$), lower Glasgow Coma Scales [8 (6–13) vs. 13 (13–15), $p < 0.0001$], larger ICH volumes [32 (14–59) vs. 12 (4–24), $p < 0.0001$], and more intraventricular hematomas (64 vs. 27%, $p < 0.001$) (**Table 1**). Admission ANC and day-1 ANC were significantly higher in patients with unfavorable outcome, and they tended to have a lower ALC on day-1. Admission SII and day-1 SII both showed significant differences between outcome groups (**Table 1** and **Supplementary Table 1**). The interval of the day-1 test in the full cohort was 29 (13–51) h from the onset; no difference was found between both groups.

The multivariate analysis was carried out considering factors including SII, sex, age, ICH volume (logarithm), IVH extension, hematoma location, and craniotomy. In multivariate analysis, day-1 SII independently predicted 90-day poor outcome (OR 1.74, 95% CI = 1.03–3.00, $p = 0.04$), while admission SII did not (OR 1.19, 95% 0.81–1.75, $p = 0.37$) (**Table 2**). Receiver operating characteristics yielded a cutoff of $1{,}315 \times 10^9$/L for admission SII (AUC 0.726, sensitivity 83.7%, specificity 56.9%) and $1{,}700 \times 10^9$/L for day-1 SII (AUC 0.699, sensitivity 53.3%, specificity 77.3%) with corresponding maximum Youden index for predicting 90-day outcome (**Supplementary Figure 2**). Multivariate analysis revealed that Day1-SII > $1{,}700 \times 10^9$/L (OR 2.36, 95% CI = 1.09–5.26, $p = 0.03$), but not admission SII > $1{,}315 \times 10^9$/L (OR 1.42, 95% CI = 0.72–2.82, $p = 0.31$), was significantly associated with poor 90-day functional outcome (**Supplementary Table 2**). Moreover, age, GCS, ICH volume, and location were also found as an independent predictor [detailed in(**Supplementary Table 2**)].

We performed a fitting curve based on the binary functional outcome and their individual ANC, ALC, PLT, and SII to display the trends (**Figure 1**). A peak of SII occurred at 24–48 h after

TABLE 1 | Baseline comparison between 90-day outcome groups.

	Full cohort ($n = 291$)	Favorable outcome ($n = 98$)	Poor outcome ($n = 193$)	*P*-value
Age, years	57 (14)	55 (13)	59 (14)	0.0002*
Male sex	194 (67%)	75 (77%)	119 (62%)	0.02†
Onset to admission time, h	5 (3–8)	6 (3–10)	5 (3–8)	0.20‡
Admission SBP, mmHg	166 (143–183)	163 (144–181)	166 (142–184)	0.71‡
Admission DBP, mmHg	94 (82–109)	95 (82–111)	93 (81–108)	0.41‡
Admission GCS	13 (7–14)	13 (13–15)	8 (6–13)	<0.0001‡
Admission SII, $\times 10^9$/L	1298 (658–2244)	989 (570–1867)	1440 (792–2422)	0.004‡
Day−1 SII, $\times 10^9$/L	1467 (884–2485)	969 (685–1564)	1833 (1170–2955)	<0.0001‡
Craniotomy	63 (22%)	12 (12%)	51 (26%)	0.008†
ICH volume, ml	24 (9–47)	12 (4–24)	32 (14–59)	<0.0001‡
Intraventricular hematoma	145 (50%)	22 (27%)	123 (64%)	<0.0001†
Lobar hematoma	52 (18%)	20 (12%)	32 (17%)	0.52†
Infratentorial hematoma	58 (20%)	16 (16%)	42 (22%)	0.35†

Data are mean (standard deviation) or median (interquartile range) for continuous variables, and n (%) for categorical variables. SBP, systolic blood pressure; DBP, diastolic blood pressure; GCS, glasgow coma scale; SII, systemic immune–inflammation index; ICH, intracerebral hemorrhage.

$\chi 2$ test or Fisher exact test.

†*Two-sample Student's t test.*

‡*ann–Whitney test.*

TABLE 2 | Relationship of SII and SII threshold with 90-day predicting poor outcome.

	Unadjusted			Adjusted*		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Admission SII†	1.47	1.12–1.96	0.007	1.19	0.81–1.75	0.37
Day-1 SII†	2.87	1.91–4.54	<0.0001	1.74	1.03–3.00	0.04
Admission SII >1315 × 10^9/L	2.14	1.31–3.55	0.003	1.42	0.72–2.82	0.31
Day-1 SII >1700 × 10^9/L	4.51	1.21–4.44	<0.0001	2.36	1.09–5.26	0.03

*SII, systemic immune–inflammation index; *Adjusted by sex, age, admission Glasgow Coma Scale, logarithm intracerebral hematoma volume, intraventricular hematoma occurrence, hematoma location and craniotomy;* †*Logarithm.*

stroke, and an obvious gap was identified after 24 h from ICH onset. ANC showed the same trend as SII, and ALC showed an inverse curve. PLT did not show a significant difference on the fitting curve. Furthermore, boxplots of admission and day-1 SII stratified by ordinal mRS revealed different distribution of SII, especially day-1 SII, in each mRS category (**Figure 2**).

DISCUSSION

We described the dynamic change of the SII in ICH patients and detected a peak at 24–48 h after ICH onset. The admission SII and day-1 SII was found to be associated with 90-day poor outcome. In the multivariate analysis, only day-1 SII independently predicted 90-day functional outcome with an optimal cutoff at 1,700 × 10^9/L. These findings suggest that the SII might serve as a new important indicator for prognosis prediction and risk stratification in ICH patients. To our knowledge, this was the first study that reported the dynamic change of SII following ICH and evaluated the predicting value of SII in long-term functional outcome in ICH patients.

There is accumulating evidence that inflammatory indices calculated based on routine blood count such as neutrophils and lymphocytes can provide valuable prognostic information in various diseases including ICH and ischemic stroke (9, 24–26). Since these inflammatory indices are easily obtainable and widely accessible, they could be added as simple predictive tools for risk stratification during clinical estimation. Meanwhile, the increase of these inflammatory indices might also reflect the acute inflammatory response to the primary and secondary brain injury.

After ICH occurs, plasma-derived factors (i.e., thrombin and vitronectin) and components released following erythrocyte lysis (i.e., hemoglobin, peroxiredoxin 2, and carbonic anhydrase 1) can activate macrophage/microglia and trigger the inflammatory cascade (27, 28). Activated macrophages/microglia further release pro-inflammatory cytokines and chemokines and promote infiltration of peripheral inflammatory cells (29). Neutrophils are the earliest white blood cells recruited from peripheral blood to the brain in response to acute inflammatory immune response (10). In animal models, neutrophil infiltration

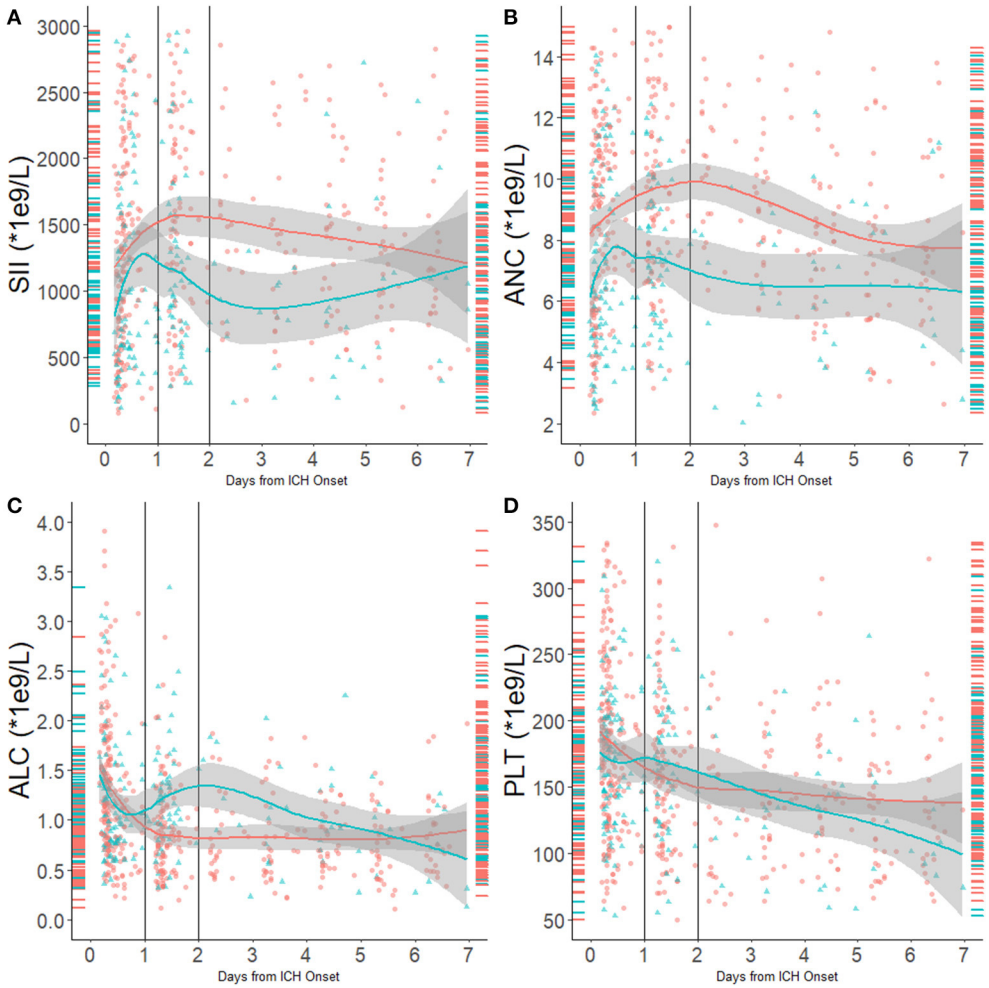

FIGURE 1 | (A) SII distribution and fitting curve according to 90-day outcome; **(B)** ANC distribution and fitting curve according to 90-day outcome; **(C)** ALC distribution and fitting curve according to 90-day outcome; **(D)** PLT distribution and fitting curve according to 90-day outcome. Red indicates patients with unfavorable outcomes, and blue indicates patients with favorable outcomes. Right-side rungs indicate SII distribution in the first 12 h from onset; the left side indicates SII distribution 24–48 h from onset.

was observed around the hematoma within 4 h and reached a peak in 1–3 days after ICH (6, 30). By analyzing the tissues surrounding the hematoma in patients with ICH, there was neutrophil infiltration within 8 h that further increased within 1 day (31). Neutrophils induce neurotoxicity by releasing pro-inflammatory cytokines (i.e., TNF-α and IL-1β), further contributing to increased capillary permeability, blood–brain barrier destruction, and aggravation of brain edema (32). In preclinical research, targeting neutrophil inhibition alleviated myelin fragmentation and axonal damage, further improving functional outcomes after ICH (33).

Platelets are an integral component of the hemostatic system (28). The balance of platelet aggregation is broken after ICH. The increase of platelet counts in the peripheral circulation induces a hypercoagulable state, which increases the risk of poor outcomes (34). Activated platelets release a series of potent chemical mediators (i.e., adenosine diphosphate, serotonin, thromboxane A2, and TGF β), all of which may potentially play important roles in brain damage and unfavorable prognosis (28).

In the acute phase after ICH, the sympathetic system and hypothalamic–pituitary–adrenal axis are overactivated and the levels of catecholamines and steroids increase, which contribute to systemic immunosuppression and further induce functional inactivation and apoptosis of peripheral lymphocytes (35). Lymphocytes play a crucial role in immune regulation and host defense against pathogens (10). Decreases in lymphocyte numbers reduces the immune capacity, increases the risk of infection after ICH, and may have an impact on functional outcomes (34, 36).

Based on previous studies, the inflammatory immune response may not be reflected in laboratory tests within the first few hours of onset (31). In our cohort, the median onset to admission time was 5 h, which is substantially shorter than that of a previous report (21); as a result, the immune response may not

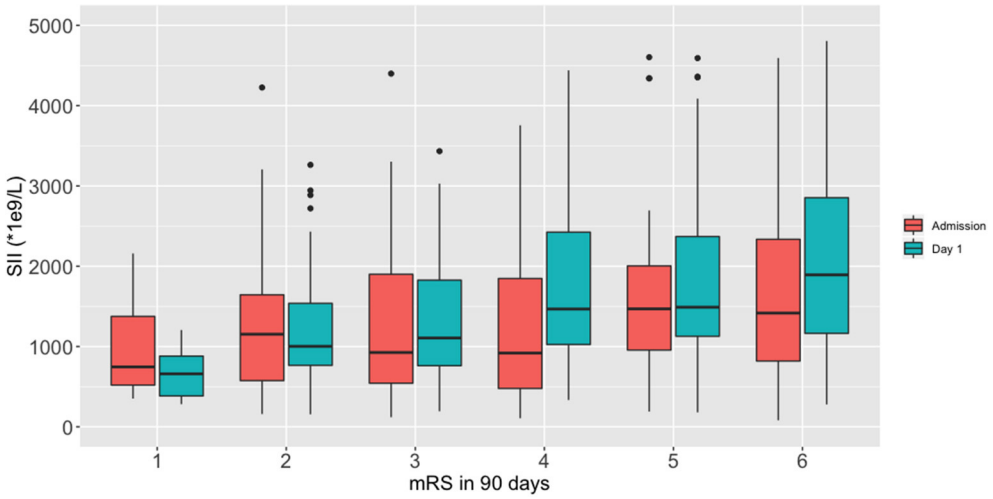

FIGURE 2 | Boxplots of admission and day-1 SII stratified by ordinal mRS.

fully reach the ultra-acute phase, giving rise to a different result. A more precise observation of inflammatory immune markers in stroke patients may be needed in the future.

There are some limitations in our study. A single-center retrospective cohort has potential biases. Other inflammatory markers such as edema volume and interleukins were not collected, and the interaction with other infective complications were not studied. The strengths of our report include the dynamic change of the immune–inflammation index. A previous study focused on the admission time point but did not consider the actual time from onset of stroke (21). We also had a relatively wide enrollment of patients with ICH, including all locations of hematoma and surgical patients.

CONCLUSION

SII is an easily calculated index that showed decent ability to predict outcome following ICH. Further investigations may increase the understanding of immune–inflammation processes in ICH and may guide clinical practice.

DATA AVAILABILITY STATEMENT

The data and code that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical ethics committee of Sichuan University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

YL and XH designed the study. YL, YC, FZ, MY, and HX collected the data. YL and DW analyzed the data. DW, WC, and XH did major revision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.628557/full#supplementary-material

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frontiers in Neurology

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Scalable Bio Marker Combinations for Early Stroke Diagnosis: A Systematic Review

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Background: Acute stroke treatment is a time-critical process in which every minute counts. Laboratory biomarkers are needed to aid clinical decisions in the diagnosis. Although imaging is critical for this process, these biomarkers may provide additional information to distinguish actual stroke from its mimics and monitor patient condition and the effect of potential neuroprotective strategies. For such biomarkers to be effectively scalable to public health in any economic setting, these must be cost-effective and non-invasive. We hypothesized that blood-based combinations (panels) of proteins might be the key to this approach and explored this possibility through a systematic review.

Methods: We followed the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) guidelines for systematic review. Initially, the broader search for biomarkers for early stroke diagnosis yielded 704 hits, and five were added manually. We then narrowed the search to combinations (panels) of the protein markers obtained from the blood.

Results: Twelve articles dealing with blood-based panels of protein biomarkers for stroke were included in the systematic review. We observed that NR2 peptide (antibody against the NR2 fragment) and glial fibrillary acidic protein (GFAP) are brain-specific markers related to stroke. Von Willebrand factor (vWF), matrix metalloproteinase 9 (MMP-9), and S100β have been widely used as biomarkers, whereas others such as the ischemia-modified albumin (IMA) index, antithrombin III (AT-III), and fibrinogen have not been evaluated in combination. We herein propose the following new combination of biomarkers for future validation: panel 1 (NR2 + GFAP + MMP-9 + vWF + S100β), panel 2 (NR2 + GFAP + MMP-9 + vWF + IMA index), and panel 3 (NR2 + GFAP + AT-III + fibrinogen).

Conclusions: More research is needed to validate, identify, and introduce these panels of biomarkers into medical practice for stroke recurrence and diagnosis in a scalable manner. The evidence indicates that the most promising approach is to combine different blood-based proteins to provide diagnostic precision for health interventions. Through our systematic review, we suggest three novel biomarker panels based on the results in the literature and an interpretation based on stroke pathophysiology.

Keywords: stroke, diagnosis, biomarker panels, serum biomarkers, neuroprotection

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BACKGROUND

Stroke remains to be the second leading cause of death worldwide, with a yearly death toll of 5.5 million (1, 2). Furthermore, approximately 116.4 million people are reportedly disabled because of stroke, resulting in stroke being one of the most important causes of disability in older people (3). Consequently, cerebrovascular diseases have substantial economic impact and significant social consequences. This impact is exacerbated in lower- and middle-income countries. Evidence suggests that this situation is due to insufficient and non-optimal strategies for the prevention of cerebrovascular diseases and due to reduced availability of equipment for the diagnosis and treatment in medical centers (4).

Many of the shortcomings in managing stroke and related diseases are due to the heterogeneity of these pathologies. The main subtypes of stroke are ischemic and hemorrhagic stroke. Ischemic stroke is characterized by a lack of blood supply to a part of the brain, whereas hemorrhagic stroke refers to a cerebral bleed due to a blood vessel's rupture (5). Ischemic stroke in turn comprises different subtypes such as transient ischemic attack (TIA), which is transitory and reversible in nature. We followed the classification system: Trial of Org 10172 in Acute Stroke Treatment (TOAST) developed by Adams et al. (6), and we further distinguished large-artery atherosclerosis, cardioembolic (CE), lacunar, undetermined etiology, and other determined etiology.

Several studies have shown that subjects with TIA have a much higher probability of future strokes than the general population (7–9). In fact, the recurrence estimated by the Oxfordshire Community Stroke Project varies between 8 and 12% at 7 days, 11 and 15% at 1 month, and 15 and 19% at 3 months (8). Notably, recurrent events tend to become more disabling or fatal than the first stroke or TIA (9). Therefore, the first occurrence of TIA constitutes a warning signal for future stroke, offering a unique opportunity for early interventions and stroke prevention, including neuroprotective strategies (**Figure 1**). As one of the reviewers have highlighted, "acute stroke treatment is a time-critical process where every minute counts."

Unfortunately, physicians may neglect these warning signals for recurrent future cerebrovascular events. In addition, misdiagnosis and untimely discharge are also relatively frequent (10). A TIA is a predictive factor for recurrence (11), and therefore, there is a strong need to determine the predictors of recurrence after the first TIA event. Early identification of patients at a higher risk for stroke recurrence may offer critical insights for urgent management and recurrence prevention.

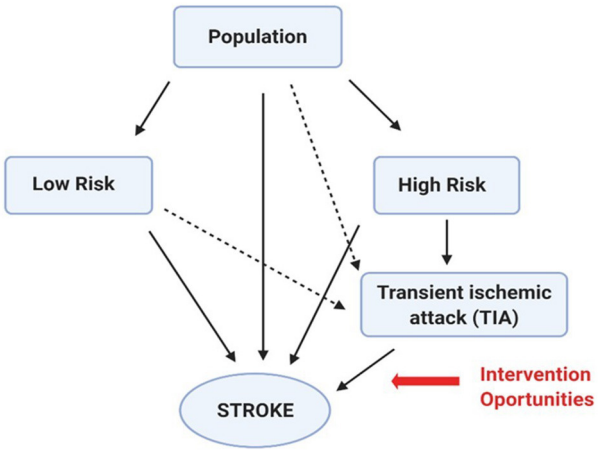

FIGURE 1 | Changing risks of stroke recurrence in a population. Schematic diagram of the risk of stroke recurrence in the general population. There are mainly two groups: those with low risk (without any disease apparently) and those with high risk (people with prior cardiovascular diseases, diabetes, and hypertension). The probability of experiencing a transient ischemic attack (TIA) was higher in the high-risk group than that in the low-risk group. In addition, a TIA event increases the probability of recurrent stroke. Recognition of this risk structure provides a unique opportunity for early health interventions and stroke prevention.

Stroke onset in patients requires additional factors that, ideally, are differentiation from stroke mimics, classification of stroke subtypes, and monitoring patient progression.

Early identification of such aspects is the goal of precision medicine for all diseases. This approach leverages disease progression models whose stages are identifiable using biomarkers (12, 13). In this framework, a biomarker is a parameter that may indicate the likelihood of disease progression or clinical events in subjects with a specific medical condition (14).

Regrettably, stroke remains to be a condition without well-established biomarkers, which, alone or in combination, are precise enough for a useful prediction. This situation seems contradictory, as an increasing number of biomarker candidates are continuously being proposed (15). However, selecting specific stroke biomarkers remains challenging for several reasons.

Stroke, as mentioned before, is a heterogeneous disease that involves diverse mechanisms that affect the specificity and sensitivity of potential biomarkers (16, 17). These mechanisms include disruption of the blood–brain barrier, thrombus formation, neuronal death, excitotoxicity, mitochondrial dysfunction, and immune system activation [(18); **Figure 2**]. Biomarkers may be sensitive to different facets of pathophysiology and may change over time.

Stroke diagnosis depends crucially on neuroimaging; computed tomography (CT) remains an essential component of stroke management, although it is not always available. Some areas of stroke management have been neglected, such as using

Abbreviations: AIS, acute ischemic stroke; IS, ischemic stroke; ICH, intracerebral hemorrhage; TIA, transient ischemic attack; SAH, subarachnoid hemorrhage; VCAM, vascular cell adhesion molecule; vWF, von Willebrand factor; BNGF, B-type neurotrophic growth factor; CRP, C-reactive protein; sRAGE, soluble receptor for advanced glycation end products; MMP-9, matrix metalloproteinase 9; BNP, brain natriuretic peptide; TIMP-4, metalloproteinase inhibitor-4; UCH-L1, ubiquitin C-terminal hydrolase 1; CE, cardioembolic stroke subtype; LVD, large-vessel disease stroke subtype; SVD, small-vessel disease; UDE, undetermined etiology; ELISAs, enzyme-linked immunosorbent assay.

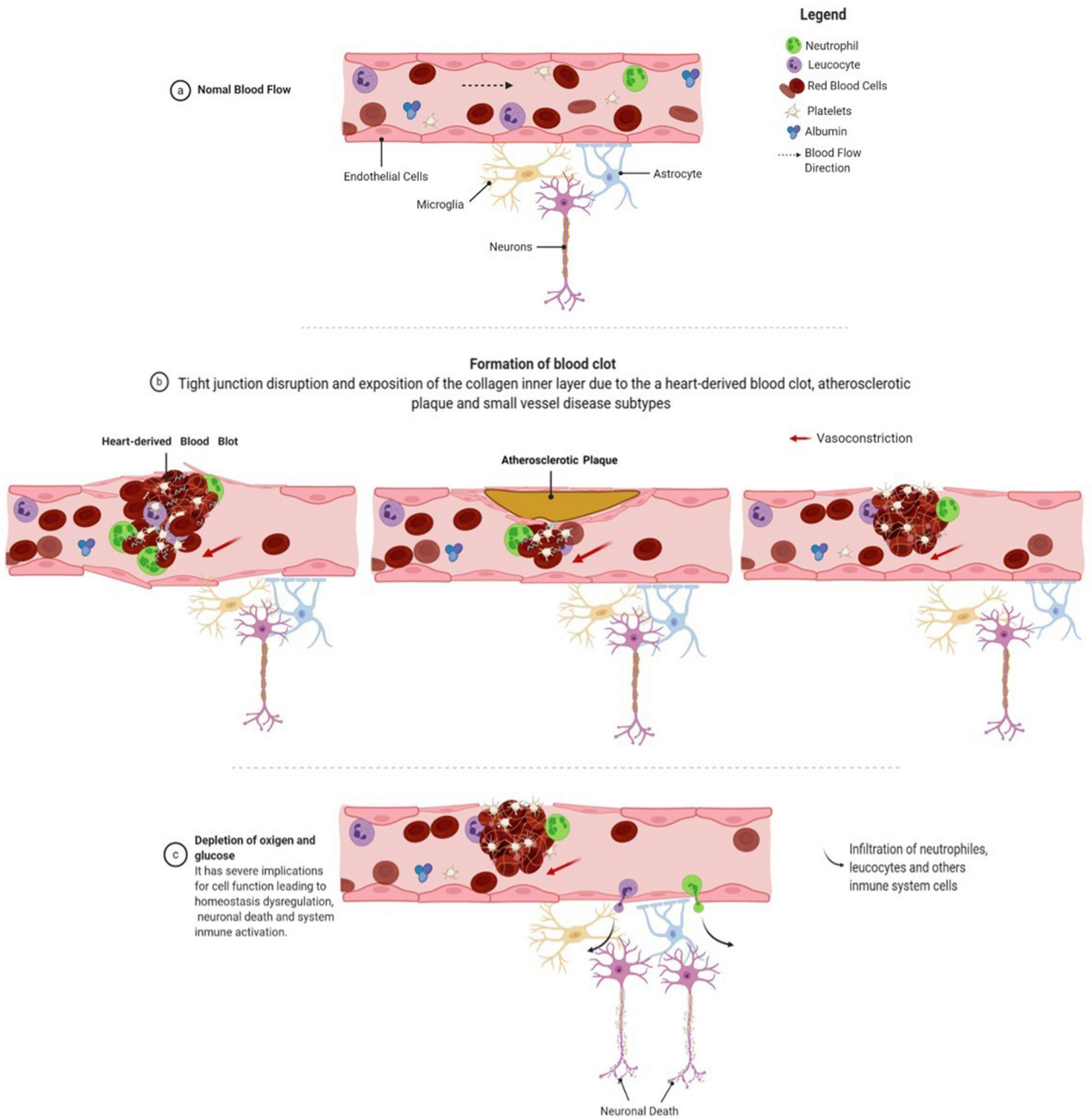

FIGURE 2 | Stroke pathophysiology. The clot formation process starts with disrupting the blood–brain barrier due to a rupture of the endothelial cell layer, exposing the blood vessel's inner collagen layer. Collagen exposure is recognized by circulating platelets in the bloodstream, which initiates the process of aggregation. Fibrinogen is released from the liver to the bloodstream and is cleaved by thrombin at the damaged site, resulting in fibrin formation. Fibrin is one of the main constituents of blood clots, providing remarkable biochemical and mechanical stability. The blood clot is also composed of neutrophils and leukocytes that arrive at the injured site and form a solid structure that obstructs or reduces blood flow. The reduction in blood flow leads to a decrease in oxygen and glucose levels. These conditions favor a shift in the neuron's metabolic conditions, mitochondrial dysfunction, excitotoxicity, ion imbalance, and neuronal death. Figure was drawn using Biorender.com.

biomarkers to predict stroke after TIA, and only a few studies have evaluated the risk of recurrent events in TIA subjects. Consequently, there is no clinical setting in which the use of a biomarker might help an individual patient. Acute stroke therapy is guided by the severity of the clinical symptoms and imaging. A preliminary study of the literature also indicated that single biomarkers achieved relatively low diagnostic accuracy.

To summarize, the use of biomarkers for stroke diagnosis is a challenging issue because, unlike for myocardial infarction, cerebral imaging remains the gold standard for stroke diagnosis.

Therefore, expectations regarding the use of biomarkers in stroke patients should be realistic. We suggest that the primary use of biomarkers in stroke patients is to provide additional laboratory information to effectively distinguish between actual stroke and its mimics and to monitor patient condition and the effect of potential neuroprotective strategies.

To highlight promising directions, we present a systematic review of the literature on stroke biomarkers for the purposes mentioned above. This review comprises the following:

1. A preliminary review of the literature indicated that combinations of stroke biomarkers ("panels") showed increased diagnostic accuracy. Thus, we focused on panels of biomarkers instead of isolated determinations.
2. We limited our attention to only those studies that reported the area under the receiver operating characteristic (ROC) curve (AUC). This choice allowed for quantitative comparisons of accuracy.
3. Because of the paucity of studies reported in the literature on subtype classification of stroke through biomarker combinations, we narrowed our search to the small-vessel-disease subtype of stroke.
4. We also focused our review on blood-based biomarkers as they seem to offer several advantages in terms of cost and ease of scalability (12).

As a consequence of our review, we propose new combinations that highlight the pathophysiological processes related to the selected biomarkers.

Overall, we adopted this scope for our review because of the geographical distribution of stroke. The highest incidence of stroke has been reported in high-income countries. Better reporting and shifting demographics place the onus on the developing world, with an increase of 91.4 million disability-adjusted life-years and 4.85 million deaths in proportion to all global causes (4, 19). Thus, technologies that are deployable without advanced analytical or imaging technologies need to be explored in more detail. Blood-based biomarker panels may therefore contribute in providing valuable information for the management of stroke.

METHODS

Article Search

We developed a search strategy with assistance from a research committee formed by neurologists, molecular biologists, mathematicians, and bioinformaticians. The search strategy was established using a combination of standardized MeSH (Medical Subject Headings) terms and keywords, including but not limited to (-cerebrovascular disorder or brain vascular disorders or vascular diseases, intracranial or intracranial vascular disease or cerebrovascular occlusion or cerebrovascular accident or intracranial embolism, and thrombosis or cerebrovascular insufficiencies) **AND** (- ischemia or Stroke or infarction or brain infarction or hypoxia-ischemia or brain ischemia or ischemic attack) **AND** (-intracerebral hemorrhage, cerebral hemorrhage, or intracranial hemorrhage) **AND** (- biological marker or biomarker or biologic marker or marker, biological, or biomarker panel) **AND** (- blood plasma sample, serum plasma sample, cerebrospinal fluid, blood proteins, plasma, blood, marker, serum, or serum marker or laboratory markers) **AND** (- diagnoses or diagnostic or examinations). The search encompassed studies conducted between 1966 and June 2020 for studies in patients with suspected stroke; the inclusion and exclusion criteria are provided below. The PubMed search was conducted on October 10, 2020, at 12:48:21 P.M.

Eligibility Criteria

The studies that were included met the following criteria: (1) case–control studies; (2) patients aged ≥18 years; (3) magnetic resonance imaging or CT performed to confirm the clinical diagnosis of ischemic stroke; (4) a blood or serum biomarker assessed within 0–24 h after symptom onset; (5) the study reported the relationship between biomarker level and diagnostic accuracy; (6) the study included two or more biomarkers because the use of a biomarker panel improved the sensitivity and specificity for identifying cases of stroke in comparison with a single biomarker (20); and (7) the study reported the sensitivity, specificity, and AUC for the model for stroke diagnosis. We selected articles written in English or Spanish. Reviews, conference abstracts, and editorial letters were excluded. Mendeley was the reference management software used for the identification, elimination of duplicates, and screening purposes. The studies were selected based on the title and abstract for one author in the first phase (SB). In the second phase, we read the full text of the preselected articles and included studies matching the eligibility criteria (**Figure 3**). Disagreements were resolved by consensus.

Data Extraction

One of the authors (SB) extracted all the data needed to meet the review goals, including publication year, first author, sample size (*n*), biomarkers used, assays used to measure the biomarker, biomarker cutoff value used (if available), blood draw time, and the values of AUC, sensitivity, and specificity. This review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines for a systematic review (21).

RESULTS

Search Results

A total of 704 articles emerged from the initial search process as potentially relevant records, and five were added manually based on the author's recommendations; 698 studies remained after the manual removal of duplicates. The screening process ruled out 645 articles based on abstract and title for the following reasons: articles related to cardiovascular diseases or other diseases (pulmonary embolism, Alzheimer disease, renal disorders, and others); articles evaluating the risk of stroke; and reviews and meta-analyses involving outcome and mortality and being related to genetic biomarkers. Finally, 53 full reads were selected and assessed for eligibility, and 41 were eliminated because of evaluating individual biomarkers, drawing blood after 24 h, and not reporting AUC, sensitivity, or specificity. Finally, 12

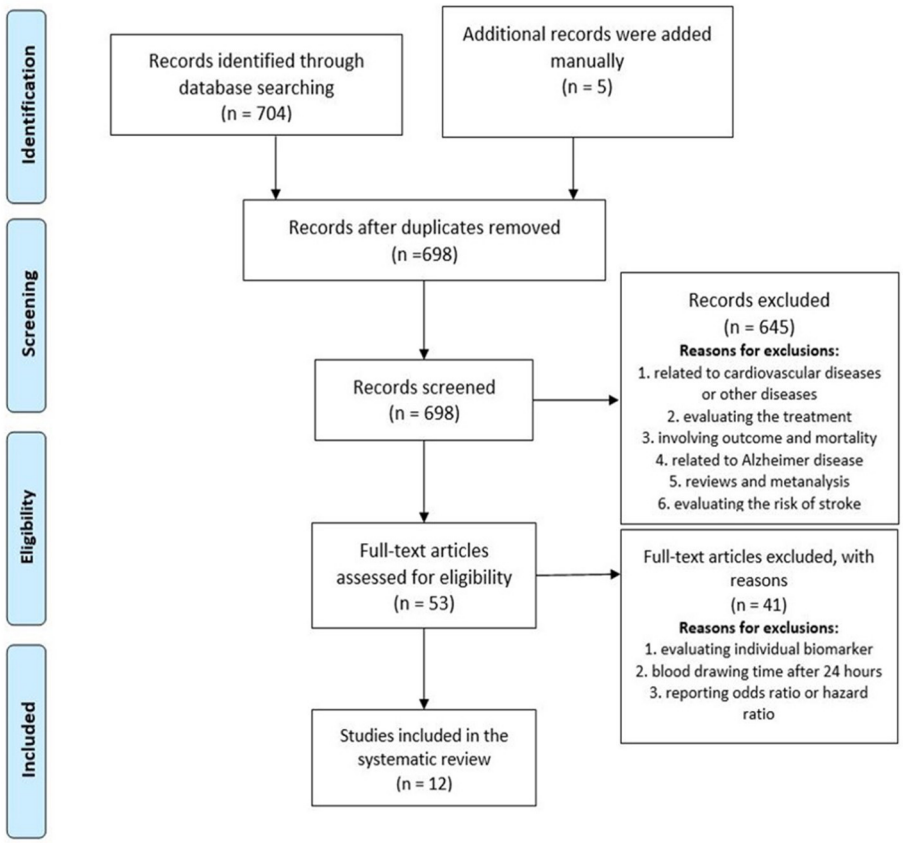

FIGURE 3 | PRISMA diagram. Flow diagram of the search and screening process. PubMed research yielded 704 articles at baseline, and five were added manually. No other sources of article identification were identified.

articles were included in the systematic review. A PRISMA flow diagram describing the search and screening process is shown in **Figure 3**.

An example of a systematic review that was not included in our evaluation because of a lack of statistical measures of diagnostic accuracy was a recent meta-analysis evaluating several biomarkers (25). Most of the biomarkers evaluated in this study are reported in the literature and are reviewed as potential candidates and are added in several panels below; however, von Willebrand factor (vWF) and NR2 were omitted. Note that glial fibrillary acidic protein (GFAP) was the most promising biomarker in the study of separate ischemic stroke (IS), intracerebral hemorrhage (ICH), and healthy controls. The same study mentions D-dimer, matrix metalloproteinase 9 (MMP-9), brain natriuretic peptide (BNP), and protein S100-β (S100β) derived from the meta-regression analysis as significant markers to be evaluated within 6 and 24 h of symptom onset (22).

Study Characteristics

The main features of the selected studies are listed in **Table 1**. Regarding sample characteristics, all the studies were case–control, which included control participants (without stroke), patients with acute IS (AIS), ICH, TIA, mimics, closed-head injuries (CLHs), or subarachnoid hemorrhage (SAH). All the studies involved subjects aged ≥18 years, and in the majority, immunoassays were used to evaluate the levels of biomarkers. Most of them reported their values of sensitivity, specificity, or AUC obtained within the first 6 h of symptom onset using multivariate or univariate regression logistic analyses.

Biomarker Analysis Based on Selected Studies

One of the biomarker panels frequently evaluated for the identification of AIS is composed of four proteins: BNP, D-dimer, S100β, and MMP-9 (23–25). The results of previous studies have been mixed as follows:

- Laskowitz et al. (23) showed that combining these four proteins outperformed other biomarkers in differentiating mimics from intracranial hemorrhage cases, with $c = 0.76$. This result was validated in a study of 293 subjects, 361 mimics, and 197 TIA with a validation cohort of 343 suspected stroke cases. The study's global results to classify stroke cases exhibited a high sensitivity of ∼90% but a low specificity of ∼45%, and 91% sensitivity and 45% specificity for differentiating specific IS.

TABLE 1 | Summary of the 12 articles included in the systematic review.

Biomarker panel	Sample size (*n*)/groups	Time	Essays	Cut off	Specificity	Sensitivity	Area under the curve	References
BNP, D-dimer, MMP-9, and S100β ICH vs. mimics	*n* = 946 subjects 293 (IS), 95 (ICH), 197 (TIA), and 361 (mimics) 343 subjects for the validation cohort: 87 (IS), 64 (ICH), 40 (TIA), and 152 (mimics) **ICH vs. mimics**	0–3 h 3–6 h	Triage Stroke Panel (fluorescence immunoassay)	MMP-9: 25–1,300 ng/mL D-dimer: 150–5,000 ng/mL S100β: 100–8,000 pg/mL BNP: 10–5,000 pg/mL	Stroke vs. mimics: 45% ICH vs. mimics: 38%	Stroke vs. mimics: 90% ICH vs. mimics: 88%	Stroke vs. mimics: 0.75 ICH vs. mimics: 0.81	(23)
BNP, D-dimer, MMP-9, S100β, and multimarker index (MMX)	*n* = 139 subjects 89 (AIS), 11 (ICH), and 39 (brain disorders) **AIS vs. others (control, brain diseases)**	<6 h	Triage® Meter (Biosite Inc.) fluorescence immunoassay	MMP-9: 25–1,300 ng/mL D-dimer: 150–5,000 ng/mL S100β: 100–8,000 pg/mL BNP: 10–5,000 pg/mL	21.5%	91.0%	0.714	(24)
BNP, D-dimer, MMP-9, S100β, and multimarker index (MMX)	*n* = 174 subjects 100 (IS), 49 (mimics), and 25 (TIA) **IS vs. mimics**	<6 h	Triage® Stroke Panel	—	33%	86%	0.59	(25)
Eotaxin, EGFR, S100A12, TIMP-4, and prolactin	*n* = 167 subjects 57 (IS), 32 (ICH), 41 (TIA), and 37 (mimics) **IS + ICH vs. mimics**	<24 h	—	—	84% IS vs. mimics (*c* = 0.92)	90%	0.97	(26)
D-dimer, caspase-3, sRAGE, MMP-9 chimerin, and secretagogin	*n* = 1,005 subjects 776 (IS), 139 (ICH), and 90 (mimics) **IS vs. mimics**	<6 h	ELISA immunoassays	Caspase-3: 1.962 ng/mL D-dimer: 0.275 μg/mL sRAGE: 0.91 ng/mL chimerin: 1.11 ng/mL secretagogin: 0.24 ng/mL MMP-9: 199 ng/mL	63%	84%	0.810 (0.757–0.863)	(27)
S100β, MMP-9, VCAM, and vWF	*n* = 80 subjects 44 (IS), 21 (controls), 13 (TIA), 1 (syncope), 1 other conditions **Stroke vs. controls**	<6 h 6–24 h	Biosite Inc.	—	90%	90%	—	(28)
S100β, BNGF, vWF, MMP-9, and MCP-1	*n* = 274 subjects 82 (ISCH), 65 (SAH), 38 (ICH), 38 (closed head injuries, CLH), and 51 (TIA) **AIS vs. other groups** **ICH vs.other groups**	<6 h	ELISA-immunoassays	—	93%	AIS: 91.7% ICH: 80%	—	(29)
IMA index IMA	*n* = 52 subjects 28 (IS) and 24 (No-Stroke) **IS vs. no stroke**	<6 h	Albumin–cobalt binding (ACB) test	91.4 U/mL	96.4%	95.8%	0.990 (0.970–1.000)	(30)

(Continued)

TABLE 1 | Continued

Biomarker panel	Sample size (*n*)/groups	Time	Essays	Cut off	Specificity	Sensitivity	Area under the curve	References
AT-III Fibrinogen	*n* = 198 subjects 152 (IS) and 46 (mimics) **IS vs. no stroke**	4.5 h	AT-III: chromogenic assay Fib: immunoturbidimetry assay	AT-III: 210% Fib: 4 g/L	AT-III:93.62% F: 82.61%	AT-III: 97.37% F: 96.05%	—	(31)
Ab NR2 and GFAP	*n* = 124 subjects 49 (IS), 23 (ICH), 52 controls **IS vs. ICH**	<12 h	GFAP: ELISA kit AB NR2: Gold Dot NR2 Antibody Assay kit (ELISA)	—	91%	94%	—	(32)
GFAP/UCH-L1 GFAP	*n* = 184 subjects 45 (ICH), 79 (IS), 5 (SAH), 3 (TIA), and 57 controls **IS vs. controls** **ICH vs. controls** **IS vs. ICH**	<4.5 h	ELISA immunoassays	GFAP: 0.34 ng/mL	— —	— —	0.875 0.71 **(IS vs. C)** 0.95 **(ICH vs. C)** 0.86 **(IS vs. ICH)**	(33)
ApoC-III, NT-proBNP, and FasL (21 biomarkers)	*n* = 1,308 subjects 941 (IS), 193 (mimics) and 174 (hemorrhagic) 767 validation cohort **IS vs. mimics** **IS vs. ICH**	<6 h	ELISA immunoassays	—	—	—	**IS vs. mimics** 0.742 (0.686–0.797) **IS vs. ICH** 0.757 (0.691–0.823)	(34)

ROC analysis includes clinical variables selected by the authors. Bold values indicate groups compared in the studies analyzed by Receiver Operating Curve analysis (ROC).

- Kim et al. (24) validated the previous biomarker panel using the composite multimarker index (MMX), which combines the individual marker values into a single index value. It exhibited good performance in the discrimination of patients with acute infarction; at 6 h, it could differentiate AIS ($p < 0.001$) but with insufficient precision. With 91.0% sensitivity, 21.3% specificity, and 71.4% AUC, the analysis exhibited a modest discriminatory power for acute stroke. According to the MMX values, there was no significant difference between the subjects with AIS and those with ICH ($p = 0.884$).
- These promising results should be considered with caution. Knauer et al. (25) evaluated MMX in a cohort of 174 cases in which 100 patients had stroke, 49 were mimics, and 25 had TIA. They advised against the use of the panel BNP, D-dimer, S100β, and MMP-9 in this assay because of (1) the low significance of MMX values to differentiate the IS group (MMX = 3.6 ± 2.0) from mimics (MMX = 4.2 ± 1.7) and (2) the low significance of MMX values when the analysis for the individual biomarkers was replicated. The 2.3 cutoff value of MMX was reported to have 86% sensitivity with a low specificity of ∼33% and an AUC of ∼59%.

A panel comprising eotaxin, EGFR, S100A12, metalloproteinase inhibitor-4 (TIMP-4), and prolactin was found to be elevated in a study of 167 cases with neurologic deficits, allowing the differentiation of IS cases from mimics ($c = 0.92$) (26). The study used a time window of 24 h and reported a high specificity of ∼84%, sensitivity of ∼90%, and AUC of ∼97%.

Another panel, caspase-3, D-dimer, chimerin-II, MMP-9, secretagogin, and sRAGE, was assessed in a large cohort of 1,005 cases where 915 had strokes and 90 had stroke-mimicking conditions, but only proteins could discriminate between the two groups (27). At 6 h after symptom onset, these protein levels had moderate sensitivity values of ∼84% but a low specificity of ∼63%.

The panel S100β, MMP-9, vascular cell adhesion molecule (VCAM), and vWF proved to have good discriminatory power as it differentiated 44 AIS cases from 21 controls within the first 6 h after symptom onset with high sensitivity (∼90%) and specificity (∼90%).

A panel slightly modified from the previous one comprising S100β, MMP-9, and vWF with two other markers including monocyte chemoattractant protein-1 (MCP-1) and B-type neurotrophic growth factor (BNGF) had similar discriminatory power (29). The levels for the panel in samples of 82 ISCH (IS with ICH), 65 SAH, 38 ICH, 38 CLH, and 51 TIA patients, at 6 h from symptom onset and using multivariate logistic regression model, had elevated sensitivity of ∼92% and specificity of ∼93% in the classification of ischemic events and a specificity of ∼93% and sensitivity of ∼80% for the prediction of hemorrhagic stroke.

The protein GFAP has been of interest in combination with other biomarkers. The combination of antibodies (Ab) against NR2 and GFAP exhibited the best predictive power for comparing 49 IS subjects from 23 ICH patients and 52 controls. A sensitivity of ∼91% and specificity of ∼94% were reported within 12 h of symptom onset (32). The use of GFAP and UCH-L1 for the identification of ICH vs. IS was tested in 129 stroke subjects, three TIA patients, and 57 controls (33). Notably, GFAP alone was capable of distinguishing between the condition with an AUC ∼0.86, sensitivity of 61%, and specificity of 96% (33).

Recently, a panel consisting of apolipoprotein CIII (Apo C-III), NT-proBNP, and FasL was selected as the best combination after an extensive study of 21 biomarkers in 1,308 cases to differentiate real stroke from mimics, within 6 h after stroke onset (34). This study was replicated with a smaller sample size for a different group of subjects, giving a modest accuracy of 0.742 (0.686–0.797). Despite being one of the studies that screened the largest number of biomarkers, this, in our opinion, has some limitations. GFAP was not assayed, and the levels of MMP-9 were not measured in the entire cohort because they were not deemed discriminative (34).

Finally, it is worth mentioning the two articles that screened several biomarkers, although they did not combine them. They can be integrated into an optimized panel in the future. The studies and biomarkers are as follows:

- Ischemia-modified albumin (IMA): in a small number of patients ($n = 28$) with stroke compared to the no-stroke group ($n = 24$) where the albumin-adjusted IMA index and IMA were measured within 6 h after symptom onset (30). Furthermore, the IMA index (98 U/mL) was even more sensitive (sensitivity, ∼95.8%; specificity, ∼96.4%; and AUC, ∼99%) than the conventional IMA value (sensitivity, ∼87.5%; specificity, 89.3%; and AUC, 92.8%) for the detection of patients with cerebral ischemia.
- The levels of antithrombin III (AT-III) in a study with 152 stroke patients and 46 mimics reported the highest sensitivity of ∼97.37% and specificity of ∼93.62% using a cutoff of 210%, whereas 4 g/L of fibrinogen reached a sensitivity of ∼96.05% with a specificity of ∼82.61% (31).

Observations From the Selected Studies

(1) Only two biomarkers, NR2 peptide (Ab against NR2 fragment) and GFAP, have been reported as brain-specific markers linked to the progression of stroke, reaching the highest predictive power when evaluated together (32).

(2) Two proteins that have been widely used as biomarkers are vWF and MMP-9; however, they are not specific to stroke. Although they were evaluated in a combined panel with higher accuracy (28, 29), Reynolds et al. reported that vWF and MMP-9 alone could not be used for diagnosis. However, it has good univariate discrimination of non-diseased vs. diseased, with an added discriminatory capacity to the logistic regression model [$p < 0.0001$; (29)].

(3) S100β is one of the most evaluated biomarkers; however, it is also not specific to stroke. Along with GFAP, it is one of the strong candidates for the differentiation of hemorrhagic and ischemic subtypes in the acute phase of stroke (35).

(4) An IMA index is the IMA value multiplied by individual serum albumin concentration/median albumin of the study population (36). The IMA index seems to be more specific than IMA in the differentiation of IS, but it has not been used in combination in previous studies.

(5) According to previous studies, the use of AT-III and fibrinogen might help in distinguishing between conditions with high accuracy in individual analyses (31).
(6) Usually, ROC curves are the statistical method to compare two groups of patients: ICH vs. mimics, AIS vs. other groups, IS vs. mimics, and IS + ICH vs. mimics.
(7) Note that none of these results have been approved for advanced clinical trials.

PROTEINS DERIVED FROM THE SYSTEMATIC SEARCH AND THEIR RELATION TO THE PATHOPHYSIOLOGY OF STROKE

We have selectively outlined concepts of stroke pathophysiology that justified our proposals for novel biomarker panels for clinical prediction explained with graphical details in **Figure 4**:

(1) When rupture of the endothelial layer of the vessel occurs, the inner collagen layer is exposed. The exposure of collagen with blood is recognized by platelets, forming a sticky plug that initiates clot formation (37). Consequently, endothelial cells release vWF, MMP-9, P-selectin, E-selectin, and inflammatory mediators (38). vWF promotes platelet adhesion to the damaged site by forming a molecular bridge between the subendothelial collagen matrix and the platelet-surface receptor complex GPIb-IX-V (39). Fibrinogen is released from the liver to the bloodstream and is cleaved by thrombin at the damaged site, resulting in fibrin formation. Fibrin is one of the main constituents of blood clots and provides remarkable biochemical and mechanical stability (40). The conversion of fibrinogen to fibrin by thrombin is inhibited by the enzyme AT-III, which is downregulated during an ischemic event. Neutrophils and leukocytes also adhere to the injury site and form a solid structure that obstructs or reduces blood (**Figure 2**).
(2) Flow reduction leads to the depletion of oxygen and glucose, which has severe implications for cell function, resulting in dysregulation of neuronal homeostasis. The process of intracellular medium acidification is reported when metabolism changes to anaerobic conditions (41). Mitochondrial dysfunction plays a significant role in inducing neuronal death caused by an increase in enzymes that favor the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as xanthine oxidase, NADPH oxidases, nitric oxide synthase, and a decrease in detoxifying systems (42, 43). Note that ROS and RNS pass into the bloodstream and are hypothesized to modify the N-terminal of albumin (44).
(3) Mitochondrial dysfunction affects ATP production, which induces a failure in the activity of the Na^+–K^+ pump. Na^+–K^+ pump activity depends on ATP hydrolysis (45), disappearing the electrical gradient in the cellular membrane and causing the influx of Na^{2+} into the neuron, resulting in membrane depolarization. Simultaneously, the activation of ASC1a, NCX, and TRP allows the influx of Ca^{2+} into the neuron, a process known as calcium overloading (46). Calcium overloading favors glutamate release into the extracellular medium and causes swelling due to the influx of water. NR2 is a subunit of the *N*-methyl-D-aspartate (NMDA) receptor and is a ligand-gated ion channel with high calcium permeability, which is cleaved by serine proteases under ischemic conditions (47).
(4) All these processes lead to neuronal death by necrosis or necroptosis, characterized by the loss of membrane integrity, damage to cellular structures, swelling, and release of cellular content, resulting in an acute inflammatory response (48). Consequently, there is an activation of astrocytes and microglia, which induces morphological changes and mediates the inflammatory response (49–51). These cells release proteins such as S100β and GFAP, reflecting structural and functional damage in the central nervous system (CNS) (35). MMP-9 is released by endothelial cells, astrocytes, and microglia and is activated by high nitric oxide concentrations. It degrades type IV collagen present in the endothelial blood–brain barrier, increasing parenchymal destruction, and is related to the inflammatory response after stroke (52).

PROPOSAL FOR NEW BIOMARKER PANELS

Based on our reviewed pathophysiology, we suggest further studies of three different panels (**Table 2**). We have included NR2 peptide and GFAP in all proposed panels because they seem to be the most promising brain-specific biomarkers related to stroke. We have included other biomarkers in the suggested panels under *Observations From the Selected Studies* described above because they seem promising in light of the pathophysiological process of stroke that have been previously evaluated (**Table 3**).

Panel 1: NR2 + GFAP + MMP-9 + vWF + S100β

- **NR2**: Precisely, the NR2 subunit is the only biomarker reported with the highest specificity (96%) and sensitivity (92%) at 12 h using a cutoff value of 1.0 μg/L in 101 IS and 91 no-stroke patients (60). When a lower cutoff value of 0.5 ng/mL was tested within 0.5–4.5 h, it revealed high sensitivity and specificity of 88 and 99%, respectively, for the differentiation between mild traumatic brain injury, AIS, ICH, healthy controls, and subjects at risk of TIA (vascular risk factors) (https://www.ahajournals.org/doi/10.1161/str.44.suppl_1.A30). Additionally, the concentrations of NR2 were found to be significantly elevated in IS subjects compared with patients without cerebral damage and were also related to the size of infarct and were used as a blood test for the validation of Cortexin treatment, a neurocytoprotector (61).
- **GFAP**: The predictive value of GFAP to determine the types of stroke was assessed by Foerch, who used a cutoff value of 2.9 ng/L, obtaining a sensitivity of 79% and a specificity of 98% (53). The same author, after several years, ratified GFAP as an efficient marker to differentiate ICH from IS, including stroke mimics [AUC = ∼0.915; 95% confidence interval (CI) = 0.847–0.982; $p < 0.001$; (62)]. Recently, the potential of using a value of 0.43 ng/mL was found, achieving

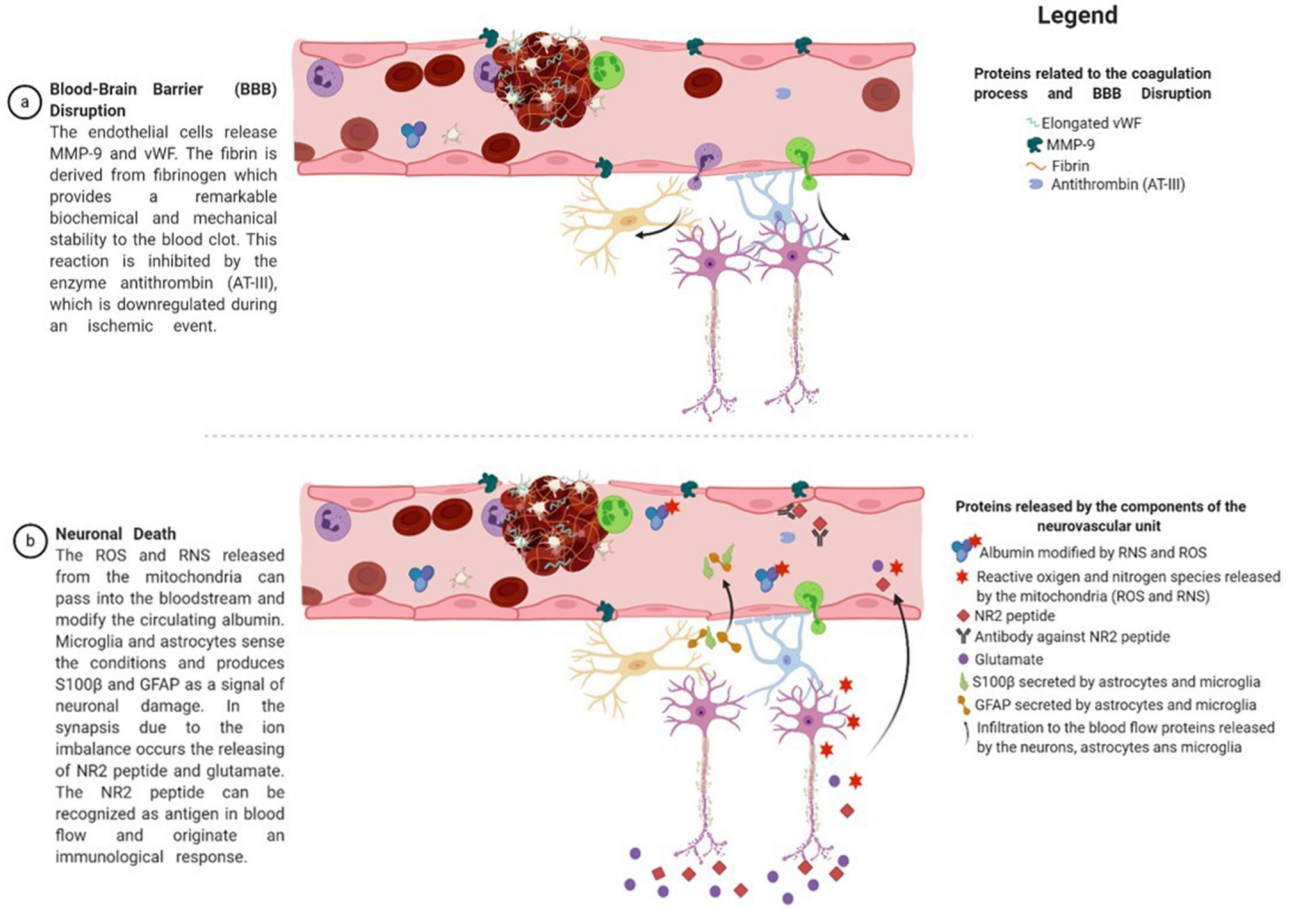

FIGURE 4 | Proteins derived from the proposed panel of biomarkers and their relation to the pathophysiology of stroke. After an injury, endothelial cells release MMP-9, which plays an essential role in the extracellular matrix and local proteolysis of leukocyte migration. In addition, endothelial cells release vWF in a globular form, which is then transformed into an elongated form by the activity of the ADAMTS13 enzyme. vWF promotes platelet adhesion to the damaged site by forming a molecular bridge between the subendothelial collagen matrix and the platelet-surface receptor complex GPIb-IX-V. Fibrinogen is released from the liver to the bloodstream and is cleaved by thrombin at the damaged site, resulting in fibrin formation. Fibrin is one of the main constituents of blood clots and provides remarkable biochemical and mechanical stability. The conversion of fibrinogen to fibrin by thrombin is inhibited by the enzyme antithrombin (AT-III), which is downregulated during an ischemic event. In addition, neutrophils and leukocytes arrive at the injured site and join with the previous proteins to form a solid structure that obstructs or reduces blood flow. These conditions lead to a shift in the neuron's metabolic conditions, mitochondrial dysfunction, excitotoxicity, and ion imbalance. The reactive oxygen and nitrogen species released from the mitochondria can pass into the bloodstream and modify the circulating albumin. On the other hand, microglia and astrocytes sense the conditions and produce S100β and GFAP to signal neuronal damage. In synapsis, because of ion imbalance, the release of the NR2 peptide and glutamate occurs. The NR2 peptide can be recognized as an antigen in the blood flow and induces an immunological response. Figure was drawn using Biorender.com.

the highest diagnostic accuracy for the differentiation between ICH and AIS (sensitivity ∼91%, specificity ∼97%) within 6 h after symptom onset (63). Ren et al. demonstrated that the median concentration of this protein in patients with IS with no history of stroke was lower than that in cases with a history of stroke (0.015 vs. 0.07 ng/mL, respectively, $p = 0.004$) (33).

- **vWF** was found to be higher in stroke patients, and it has been associated with the CE and large-vessel disease (LVD) subtypes (64). Additionally, its levels have been related to the severity of arterial thrombus formation and poor functional outcomes (65, 66). In a proteomics prospective clinical study, vWF could differentiate TIA and minor stroke from non-cerebrovascular (mimic) conditions [1.256 (1.034–1.527)] (67). Furthermore, vWF can be a sign of the response following thrombolytic therapy or endovascular treatment in IS patients (65, 68).
- **MMP-9**: Higher concentrations of this protein have been observed early, in the acute phase, and later in stroke (69). MMP-9 levels are correlated with infarct volume, neurological deficits, and infarct progression (70, 71). This could be a measure of the transformation to hemorrhagic stroke after thrombolytic treatment (69, 72, 73). MMP-9 levels may be indicative of an inflammatory response after stroke (55), endothelial dysfunction (52), and response to thrombolytic treatment after IS (69, 72, 74). Recently, Misra et al. concluded that its levels could differentiate between IS, ICH, stroke mimics, and control subjects ($p < 0.05$) in a systematic review

TABLE 2 | Proposal of biomarker panels for stroke recurrence.

Biomarker panel proposal
NR2+ GFAP+ MMP-9 + vWF + S100β
NR2 + GFAP + MMP-9 + vWF +IMA index
NR2+ GFAP+ AT-III + fibrinogen

and meta-analysis of studies realized at 24 h. However, within 6 h, IS could not be differentiated from other groups (ICH, stroke mimics, and controls) (22).

- **S100β** is released early into the peripheral blood and is correlated with the National Institutes of Health Stroke Scale scores, infarct volume, and severity (29, 75). Foerch et al. reported that it could be used for <6 h as an indirect time and for successful clot lysis (75). At days 2 to 4 after acute stroke, S100β can be predictive of the disease's course with higher accuracy, associating the higher levels with the worst functional status, making its evaluation an effective recurrent biomarker (76, 77). S100β was able to separate IS from mimics [standardized mean difference (SMD) = 0.41; 95% CI = 0.18–0.63] but failed to identify healthy controls, as well as ICH at 6 h (22). In addition, no significant differences were reported in an analysis that compared biomarker values between different conditions (34).

Panel 2: NR2 + GFAP + MMP-9 + vWF+ IMA index

We have decided to eliminate S100β in the second and third panels because GFAP is a much more brain-specific biomarker (35). We have added the IMA index in this panel because it has not been previously assayed.

- **IMA index:** Several studies have probed the use of IMA as a diagnostic marker of acute coronary syndrome and AIS (30, 56, 78). Their admission levels were also associated with people suffering from acute aortic dissection (79) and can differentiate between ICH and IS patients (80).

Panel 3: NR2 + GFAP+ AT-III + fibrinogen

We have decided to eliminate MMP-9 and vWF in the third panel because AT-III and fibrinogen are also biomarkers of the coagulation process and reflect the thrombotic status. Both proteins were individually evaluated in a meta-analysis, which did not show significant differences (22), but we recommend their assessment together in a multivariate regression logistic model.

- **Fibrinogen** is one of the markers with an essential role in the thrombosis process because it is related to platelet aggregation after injury and inflammation (40, 59). In the case of an ischemic event, an association between elevated levels of this protein and increased risk has been reported (81, 82). Fibrinogen has been used to evaluate the long-term outcome and the size of the clot burden in patients after stroke (83, 84).
- **AT-III** is involved in the blood coagulation cascade, and inactive AT-III-thrombin complexes are formed during the acute phase of stroke (57). Peripheral blood concentrations are correlated with infarct severity and predict clinical outcomes and recurrence (58).

DISCUSSION

Main Findings

To the best of our knowledge, this is the first systematic and comprehensive study to summarize current evidence regarding the use of combinations of biomarkers in the early stages of stroke. A sobering observation is that, despite numerous published studies, none of the protein biomarkers reported, alone or in combination, have been approved for the clinical management of stroke. It seems that, at best, these biomarkers can serve as support for clinical and imaging evaluation of patients. The objective of this study was to facilitate the identification of stroke mimics and allow dynamic follow-up of a patient's state to guide neuroprotective interventions. The diagnostic accuracy of stroke biomarkers must be accurate and time-sensitive to allow such dynamic follow-up. A significant result from our review is that combinations of biomarkers exhibit higher diagnostic accuracy than isolated biomarkers. Thus, there seems to be a substantial area for improvement by employing biomarker panels.

Based on our review, we suggest using three new panels of protein biomarkers to evaluate the pathophysiology of stroke. We have noted that the combination of GFAP and NR2 is included to determine neuronal damage with high accuracy in all three proposed panels. NR2 peptide is a brain-specific biomarker that has shown promising results for the distinguishing stroke, with one of the highest reported accuracies. Therefore, it is surprising that few studies have used it in combination with other proteins. The suggestion to include it in all three panels is based merely on this fact.

An intriguing possibility is that we might be able to monitor people with hemorrhagic and IS using a combination of S100β, GFAP, and IMA indices. S100β and GFAP have the same kinetics during cerebrovascular events. During ICH, both proteins peak early, before 24 h. The peak was reached later for the ischemic events. This difference in kinetics suggests that early peaking of blood levels of S100β and GFAP could be the hallmark of ICH during the acute phase of stroke (35). This crucial, yet still unresolved, distinction between IS and ICH is essential to make a decision about interventions. Likewise, blood proteins can contribute to treatment optimization for ISs by providing detailed information about hemostatic conditions involving pathways of coagulation activation, fibrinolysis, and endothelial function (85).

Of course, future studies might be based on panels other than those proposed in this study. Other promising biomarkers, such as glycogen phosphorylase isoenzyme BB (GPBB) (86), APOA1-UP (87), and platelet basic protein, have been described previously (88). These proteins have emerged as possible candidates showing high accuracy for distinguishing different conditions; however, more research is required to achieve the desired results of sensitivity and specificity during the process of validation.

Limitations of Our Review

There are certain limitations to our study:

1. We only focused on studies conducted on IS caused by small-vessel disease due to the small number of studies reported

TABLE 3 | Biomarkers derived from the biomarker panel proposal and their possible role identified in stroke.

Proteins	Gene name	Protein name	Description	Functions	References
NR2 peptide	GRIN1 GRIN2A GRIN2B GRIN2C	Glutamate receptor ionotropic, NMDA 2A, and NMDA 2B	It is a ligand-gated ion channel with high calcium permeability and voltage-dependent sensitivity to magnesium. It is essential in the process of neuronal synapses.	As a response to the brain's ischemic conditions, serine proteases are activated, which causes the cleavage of the NR2 subunit of NMDA receptors (NMDARs). Then its subunit is released to the blood, being a marker of neuronal damage.	(47)
GFAP	GFAP	Glial fibrillary acidic protein	It is an intermediate filament class-III. It is classified as a glial marker.	After an injury, trauma, disease, genetic disorders, or chemical insult GFAP is released from reactive astrocytes. The process is named astrogliosis and reflects structural and functional damage in the CNS.	(51, 53)
S100β	S100β	Protein S100-β	It is a protein-related to calcium metabolism. Also, it participates in the transmission of intracellular signals through second messengers. It is involved in the development and maintenance of the CNS. It is classified as a glial marker.	It is released from astrocytes and microglia after an injury or trauma, reflecting the CNS's structural and functional damage. It is directly related to the volume of lesions, clinical status, and functional outcome.	(29, 50)
vWF	VWF	von Willebrand factor	It plays an essential role in the maintenance of hemostasis, blood coagulation, and cell adhesion.	It is secreted by the endothelial cell activated in response to injury and can adhere to circulating platelets and contributing to thrombus formation.	(54)
MMP-9	MMP9	Matrix metallopeptidase 9	It is a proteolytic enzyme belonging to the group of gelatinases. It has an essential role in local proteolysis of the extracellular matrix and leukocyte migration.	It is activated by high concentrations of oxide nitric and degrades the type IV collagen present in the endothelial blood–brain barrier, increasing its parenchymal destruction. It is related to the inflammatory response after stroke.	(52, 55)
IMA index (albumin)	ALB	Albumin	It is the major transporter of Zn, Ca^{2+}, Mg in plasma and binds water Na^{+}, K^{+}, fatty acids, hormones, etc. It regulates the colloidal osmotic pressure of blood.	Under ischemic conditions, it is a measure of oxidative stress, where the NH_2 terminus of human albumin may be modified for the free radicals, but the precise mechanism is yet unknown.	(30, 44, 56)
AT-III	SERPINC1	Antithrombin III	It is a plasma serine protease inhibitor that regulates the blood coagulation cascade and inhibits the thrombin.	Inactive AT-III–thrombin complexes are formed during the acute phase of stroke.	(57, 58)
Fibrinogen	FGA, FGB, FGG	Fibrinogen	It is a blood glycoprotein essential in coagulation and determines the plasma viscosity.	It is related to the thrombosis process favoring the platelet aggregation after injury, being one of the primary components of blood clots.	(40, 59)

in the literature regarding the subtype classification of stroke through biomarker combinations.

2. For the same reason, we evaluated only case–control studies, although this selection was intentional.
3. We did not carry out meta-analyses because of the high heterogeneity of the reported data and the wide variety of proteins used. Future studies must include statistical methods that ensure sufficient power to detect valid effects.
4. We could not identify a sufficient number of studies on biomarkers or panel biomarkers in stroke subtypes. Possible covariables interfering with the specificity of the biomarker [e.g., age, medications, lifestyle factors, and diseases; (16)] must also be incorporated into the statistical model.

However, this review highlights additional general methodological issues when studying stroke biomarker panels. These, of course, generate open questions that we have briefly discussed. We also comment on the design issues for testing the proposed panels.

Considerations on the Statistical Methods in the Literature

Many studies do not provide complete information on the accuracy of diagnostic procedures to distinguish between two patient groups. Most articles report only sensitivity and specificity, which are threshold-dependent. We only

TABLE 4 | Summary of the biomarkers for the determination of the subtypes of stroke.

Biomarkers	Etiology	Sample/Methods	Cutoff/Time of blood drawing	Specificity	Sensitivity	References
BNP	Cardioembolic **CE vs. other strokes subtypes**	200 patients (LVD = 18, CE = 82, SVD = 31, and other stroke = 69) Chemiluminescence enzyme immunoassay	140.0 pg/mL 24 h	80.5%, AUC = 0.87	80.5%, AUC = 0.87	(91)
BNP	Cardioembolic **CE vs. all stroke subtypes**	707 IS (LVD = 151, CE = 259, SVD = 128, and UE = 169) ELISA	76 pg/mL <24 h	69%	72%	(92)
BNP and DD	Cardioembolic **CE vs. all stroke subtypes**	707 IS (LVD = 151, CE = 259, SVD = 128, and UE = 169) ELISA	BNP>76 pg/mL DD> 0.96 μg/mL <24 h	91.3% AUC = 0.89	66.5% AUC = 0.89	(92)
D-Dimer	Cardioembolic **CE vs. all stroke subtypes**	707 IS (LVD = 151,CE = 259, SVD = 128, and UE = 169) ELISA	0.96 μg/mL <24 h	64%	56%	(92)
D-Dimer	Cardioembolic **CE vs. LVD + SVD**	126 IS (LVD = 34,CE = 34, SVD = 31, and UE = 27), and controls = 63 STA Liatest d-Dimer immunoassay (immunoturbidimetric technology)	2.00μg/mL >24 h	93.2%	59.3%	(93)
NT-proBNP	Cardioembolic **CE vs. all stroke subtypes**	114 IS (LVD = 27,CE = 34, SVD = 19, and UE = 34) Human RIAKit Phoenix Pharmaceuticals	200 pg/mL <6 h	82%	65%	(94)
Albumin/globulin ratio (G/A ratio)	Cardioembolic **CE vs. all stroke subtypes**	114 IS (LVD = 27,CE = 34, SVD = 19, and UE = 34) Immunoassay or colorimetric assay	0.7 <6 h	31%	91%	(94)
NT-proBNP and G/A ratio.	Cardioembolic **CE vs. all stroke subtypes**	114 IS (LVD = 27,CE = 34, SVD = 19, and UE = 34) Immunoassay or colorimetric assay	NT-proBNP >200 pg/mL G/A = 0.7 <6 h	AUC = 0.91 with Atrial Fibrillation (AF) AUC = 0.84 without AF		(94)
Pro-BNP	Cardioembolic **CE vs. all stroke subtypes**	262 IS (LVD = 44, CE = 100, SVD = 36, and UE = 82) Electrochemiluminescence immunoassay "ECLIA"	360 pg/mL <12 h	83% AUC = 0.921	87% AUC = 0.921	(95)
Pro-ANP	Cardioembolic **CE vs. all stroke subtypes**	262 IS (LVD = 44, CE = 100, SVD = 36, UE = 86) ELISA	2,266.6 fmol/mL <12 h	70% AUC = 0.735	62% AUC = 0.735	(95)
CK-MB	Cardioembolic **CE vs. all stroke subtypes**	262 IS (LVD = 44, CE = 100, SVD = 36, and UE = 86) Electrochemiluminescence immunoassay "ECLIA"	2.6 ng/mL <12 h	80% AUC = 0.731	62% AUC = 0.731	(95)
NT-proBNP	Cardioembolic **CE vs. no-CE**	Meta-analysis: six studies NT-proBNP prospective cohort	200–360 pg/mL <72 h	93% AUC = 0.87	55% AUC = 0.87	(96)
BNP	Cardioembolic **CE vs. no-CE**	Meta-analysis: ten studies BNP prospective cohort	64–155 pg/mL <24 h	85% AUC = 0.87	65% AUC = 0.87	(96)
Troponin	Embolic stroke of unknown source (ESUS) **ESUS vs. CE, non-CE**	1,120 IS [CE = 371, non-CE = 310, and embolic stroke of unknown source (ESUS) = 439] Sandwich immunoassay	ng/mL <24 h	95 %	12%	(97)
Troponin	Cardioembolic **CE vs. ESUS, non-CE**	1,120 IS [CE = 371, non-CE = 310, and embolic stroke of unknown source (ESUS) = 439] Sandwich immunoassay	ng/mL <24 h	95 %	17%	(97)

(Continued)

TABLE 4 | Continued

Biomarkers	Etiology	Sample/Methods	Cutoff/Time of blood drawing	Specificity	Sensitivity	References
D-Dimer	SVD (lacunar) **SVD vs. CE + LVD**	126 patients (LVD = 34,CE = 34, SVD = 31, and UE = 27) STA Liatest d-Dimer immunoassay (immunoturbidimetric technology)	0.54 μg/mL >24 h	96.2%	61.3%	(93)
Homocysteine (Hcy)	Lacunar (SVD) **SVD vs. controls**	197 acute lacunar infarction patients and 192 controls –	15.5 μmol/L <24 h	100% AUC = 0.881	65% AUC = 0.881	(98)
Fibrinogen	Lacunar (SVD) **SVD vs. controls**	197 acute lacunar infarction patients and 192 controls –	228.55 μg/dL <24 h	58.3% AUC = 0.688	83.2% AUC = 0.688	(98)
Hcy/fibrinogen	Lacunar (SVD) **SVD vs. controls**	197 acute lacunar infarction patients and 192 controls –	15.5 μmol/L 228.55 μg/dL <24 h	58.3% AUC = 0.766	94.9% AUC = 0.766	(98)
GFAP/d-dimer preprint	LVD **LVD vs. other strokes**	128 patients (LVD = 23, non-LVD = 42, HS = 16, stroke mimic = 31, and TIA = 16) ELISA	d-dimer +GFAP = 0.33	92% AUC = 0.81	57% AUC = 0.81	(99)

Bold values indicate groups compared in the studies analyzed by Receiver Operating Curve analysis (ROC).

included those providing the AUC in our review (89). See **Supplementary Table 4** for the comparison. However, even this reporting level is insufficient because it is only useful to distinguish between the two groups. Critical clinical questions are therefore left unanswered when they require a distinction between several patient categories. To answer such questions involving three or more diagnostic groups, more sophisticated techniques are needed. Examples include the Youden index test proposed to generalize ROC curves by Obuchowski et al. (89) and Luo and Xiong (90).

Unanswered Clinical Questions

- **Are biomarker panels useful for stratifying stroke risk levels?** For example, this question was examined by Laskowitz et al., who classified patients who applied logistic regression to a combination of biomarkers. These results showed that the odds ratio might be a good predictor of stroke risk. A similar evaluation was included in the evaluation protocol of the biomarker panels.
- **Are biomarker panels able to discriminate between small and large vessel strokes?** This question is crucial because these conditions require entirely different therapeutic or vascular surgical treatment approaches. Significantly, the accuracy of a biomarker panel might depend on this etiological difference. Unfortunately, few studies have addressed this issue quantitatively. In **Table 4**, we report the results of protein biomarkers with differential sensitivity to small-vessel disease and LVD. Note that our proposed panels include several biomarkers (as detailed in **Supplementary Table 1**), thus having the potential for this distinction.
- **Are biomarker panels able to discriminate CE and LVD stroke etiology?** Note that the accuracy of a biomarker panel might depend on the etiology of the IS. However, only a few studies have considered this issue (**Table 4**). The selected proteins for our proposed panels could have great potential because of their differential expression of serum levels in these stroke subtypes (**Supplementary Table 3**).
- **Are biomarker panels useful for the follow-up of mixed stroke cases?** Here, we refer to both hemorrhagic transformations of IS and secondary ischemic injury after ICH. Indeed, specific proteins have been explored in this context, as shown in **Supplementary Table 2**. These proteins have been included in our proposed panels, and studies to evaluate them will consider this aspect.

We hope that this review will stimulate additional proposals of other biomarker panels that might contribute to the long-term objective of stroke precision medicine. We emphasize again that we have concentrated on biomarkers obtainable from plasma at a low cost with scalable technologies in any economic setting. If not these panels of biomarkers for stroke, then similar ones might be the key to tackling the global burden of disease due to stroke.

CONCLUSIONS

More research is needed to validate, identify, and introduce into medical practice useful biomarkers for stroke recurrence or diagnosis in a scalable manner. The most promising approach is to combine a panel of different blood-based proteins to provide acceptable diagnostic precision for health interventions. After a systematic review, we suggest three novel biomarker panels based on the results in the literature with an interpretation based on stroke pathophysiology.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**,

further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SB, JL, and PV-S: study concept and design and drafted the manuscript. AH-S, DG, and SB: acquisition, analysis, or interpretation of the data. PV-S and GG administered the project. PV-S and MB-V supervised the study. All authors critically revised the manuscript. All authors had full access to all the data in the study. They take responsibility for the integrity and accuracy of the analysis and results.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.638693/full#supplementary-material

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Prognostic Value of Elevated Cardiac Troponin I After Aneurysmal Subarachnoid Hemorrhage

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Object: Patients with aneurysmal subarachnoid hemorrhage (aSAH) have an increased incidence of cardiac events and short-term unfavorable neurological outcomes during the acute phase of bleeding. We studied whether troponin I elevation after ictus can predict future major adverse cardiac events (MACEs) and long-term neurological outcomes after 2 years.

Methods: Consecutive aSAH patients within 3 days of bleeding were eligible for review from a prospective observational cohort (ClinicalTrials.gov Identifier: NCT04785976). Potential predictors of future MACEs and unfavorable long-term neurological outcomes were calculated by Cox and logistic regression analyses. Additional Kaplan–Meier curves were performed.

Results: A total of 213 patients were enrolled with an average follow-up duration of 34.3 months. Individuals were divided into two groups: elevated cTnI group and unelevated cTnI group. By the last available follow-up, 20 patients had died, with an overall all-cause mortality rate of 9.4% and an annual all-cause mortality rate of 3.8%. Patients with elevated cTnI had a significantly higher risk of future MACEs (10.6 vs. 2.1%, $p = 0.024$, and 95% CI: 1.256–23.875) and unfavorable neurological outcomes at discharge, 3-month, 1-, 2-years, and last follow-up ($p = 0.001$, $p < 0.001$, $p = 0.001$, $p < 0.001$, and $p < 0.001$, respectively). In the Cox analysis for future MACE, elevated cTnI was the only independent predictor (HR = 5.980; 95% CI: 1.428–25.407, and $p = 0.014$). In the multivariable logistic analysis for unfavorable neurological outcomes, peak cTnI was significant (OR = 2.951; 95% CI: 1.376–6.323; $p = 0.005$). Kaplan–Meier analysis indicated that the elevated cTnI was correlated with future MACE (log-rank test, $p = 0.007$) and subsequent death (log-rank test, $p = 0.004$).

Conclusion: cTnI elevation after aSAH could predict future MACEs and unfavorable neurological outcomes.

Keywords: aneurysmal subarachnoid hemorrhage, troponin, prognostic, major adverse cardiac event, outcome

INTRODUCTION

The brain-heart connection loses balance after stroke (1). Cardiac complication has been shown to frequently occur in the emergency aneurysmal subarachnoid hemorrhage (aSAH) (2–4). A series of previous studies have shown that circulating cardiac biomarkers, including creatine phosphokinase isoenzyme-MB (CK-MB), troponin, brain natriuretic peptide (BNP), and N-terminal pro-B-type natriuretic peptide (NT-proBNP), are associated with delayed cerebral ischemia (DCI), short-term unfavorable neurological outcomes, and in-hospital mortality at the acute phase after aSAH (5–8). Among the cardiac biomarkers listed above, the incremental level of cardiac troponin I (cTnI) on admission was reported in 21–68% of emergency aSAH patients (6, 9), and the cardiac troponin has been shown to reach high sensitivity and specificity in the identification of cardiac abnormalities indicating subsequent major adverse cardiac events (MACEs) at the acute phase of aSAH, though conflicting results have been reported (2, 3, 10–12).

However, the long-term prognostic value of troponin elevation after emergency aSAH remains unclear. In this study, we aimed to explore whether the admission cTnI of emergency aSAH patient at the acute phase could predict future MACEs and long-term unfavorable neurological outcomes.

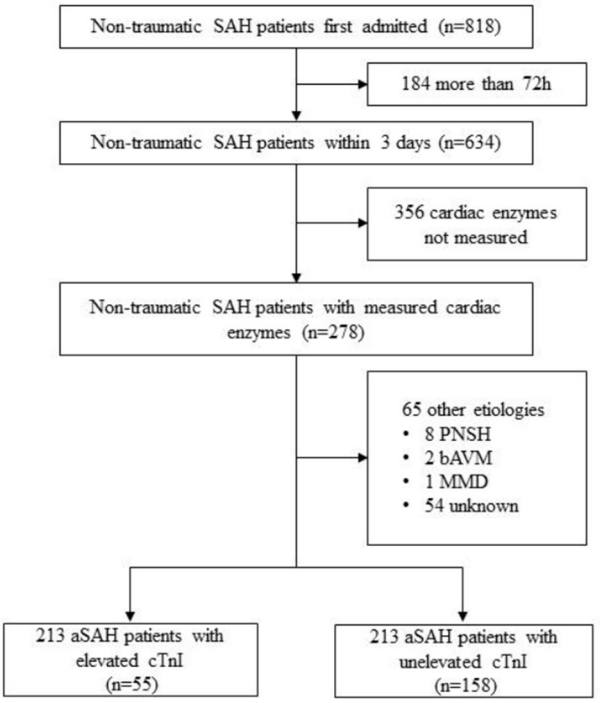

FIGURE 1 | Flow chart showing inclusion/ exclusion of patients.

MATERIALS AND METHODS

Study Design

Consecutive aSAH patients who conducted cardiac enzymes laboratory tests within 72 h of bleeding were eligible for review from a single-center prospective cohort study of intracranial aneurysms in Beijing Tiantan Hospital between January 2016 and December 2017 (**Figure 1**; ClinicalTrials.gov Identifier: NCT04785976). The diagnosis confirmed at the first interview was based on the 2012 guidelines for aSAH (13).

Data Collection and Definitions

Clinical data were retrospectively reviewed, including demographic, laboratory, radiological, and treatment-related information. Comorbid conditions were taken into consideration and conducted by the Charlson Comorbidity Index (CCI). The neurological status was evaluated by the modified Rankin Scale (mRS), and the clinical severity of SAH was assessed by the Hunt-Hess scale. Imaging characteristics, including Fisher score and intraventricular hemorrhage (IVH) involvement, were verified by at least two radiologists who had more than 5 years of clinical experience in the radiology center of our institute. The highest level of cTnI within 3 days after the rupture event was selected as the parameter of interest. Diverse cTnI assays were applied for the last decades. And according to the result of cardiac enzyme kit used in this study period (the lower limits of detectable cTnI level, 0.016 ng/mL), patients were dichotomized into two groups: the cTnI elevated group (cTnI value $>$ 0.016 ng/mL) and the cTnI unelevated group (cTnI value $\leq$ 0.016 ng/mL).

In-hospital complications were defined as major adverse cardiac events (MACEs, with the occurrence of an arrhythmia, myocardial injury, acute heart failure, repeat revascularization, and cardiac arrest, or as defined in our electronic medical records) (14), novel DCI (15)/cerebral infarction (CI), hydrocephalus, seizure, intracranial infection (ICI), pneumonia, and deep venous thrombosis (DVT). The primary goal of interest was future MACEs occurring after discharge. The secondary goal was dichotomized into favorable (mRS of 0–3) and unfavorable (mRS of 4–6). Follow-up was conducted in the first 3–6 months and annually after surgery by clinical visits and telephone interviews. Evaluation of the MACEs and mRS score was conducted by neurosurgeons who had at least 5 years' experience in clinical practice, and a training program was administered to ensure the measurement accuracy. Researchers who performed follow-up assessments were blinded to the different cTnI subgroups. Patients who were lost to follow-up were not included in the prognostic analyses of 3-month, 1-, and 2-year, but not excluded at their last available follow-up from the statistical analysis.

Statistical Analysis

The categorical variables are presented as counts (with percentages), and the continuous variables are presented as the means $\pm$ standard deviations (SD). Two-tailed *t*-tests were used for the continuous variable with Gaussian distribution. The Mann–Whitney *U* (Wilcoxon) test was used to compare non-normal distribution continuous variables. The Pearson chi-square test or Fisher exact test was used to compare categorical variables as appropriate. Multivariable Cox regression model was used to examine the risk factors associated with future MACEs. A multivariable logistic regression model was built to predict

the unfavorable neurological outcomes based on the covariables, including age, sex, Fisher score (ordinal), Hunt-Hess scale on admission (ordinal), and troponin elevation (dichotomized). Hazard ratios (HRs) or odds ratios (ORs) and 95% confidence intervals (CI) for potential risk factors of MACEs and unfavorable mRS were calculated. Variables' $p < 0.10$ in univariate analysis were selected for the multivariate model using a manual forward model building strategy. Kaplan–Meier curves with log-rank were performed to estimate the risk of MACE and death after aSAH for the dichotomized troponin elevation groups. A $p < 0.05$ (two-sided) was considered as statistically significant. All statistical analyses were performed with SPSS for Windows (version 25.0; IBM, New York, USA).

RESULTS

A total of 213 cases of aSAH that met the inclusion criteria were included between January 2016 and December 2017. After the rupture event occurred, the highest level of troponin was analyzed (9, 16) 159 patients (74.6%) received troponin tests on post-hemorrhagic day 1, 36 (16.9%) on day 2, and 18 (8.5%) on day 3. Individuals were divided into two groups based on whether cTnI value elevated or not, namely elevated cTnI group ($n = 55$, 25.8%) and unelevated cTnI group ($n = 158$, 74.2%).

Baseline Characteristics

The baseline characteristics of the 213 aSAH patients were summarized in **Table 1**. The mean (±SD) age was 57.4 ± 12.1 years, with an average follow-up duration of 34.3 months. 71.4% of them were evaluated as Hunt-Hess grade 1–2 on admission, and 57.7% were classified as Fisher score 1–2 based on the admission CT scan. Most patients (61.5%) had preoperative hypertension, and 36 (16.9%) had a confirmed cardiac disease history, including 33 (15.5%) coronary artery disease, two (0.9%) heart failure, and five (2.3%) arrhythmia. Only 14 cases (6.6%) took ACEI/ARB drugs regularly before the rupture event. The percentage of other elevated cardiac laboratory indicators was 53.1% (BNP), 25.4% (CK-MB), and 31.0% (Myoglobin, Myo), respectively. Finally, 18 cases (8.4%) received conservative management, 49.8% underwent craniotomy clipping, and 41.8% received endovascular embolization.

In the subgroup comparison, age, cardiac diseases history, and CCI did not have significant correlations with the elevated cTnI after aSAH (59.3 ± 11.2 vs. 56.8 ± 12.4, $p = 0.192$; 18.2 vs. 16.5, $p = 0.769$; 3.7 ± 2.0 vs. 3.3 ± 2.0, $p = 0.193$, respectively). However, patients with elevated cTnI were more likely to be female (80.0 vs. 55.1%, $p = 0.001$), have a higher Hunt-Hess grade (HH grade 3–5, 50.9 vs. 20.9%, and $p < 0.001$), and higher Fisher score (Fisher score 3–4, 56.4 vs. 37.7%, and $p = 0.014$). Other cardiac laboratory indicators were synchronized with the elevated cTnI (BNP, 83.6 vs. 42.4%, $p < 0.001$; CK-MB, 63.6 vs. 12.0%, $p < 0.001$; Myo, 58.2 vs. 21.5%, $p < 0.001$; respectively). There were no significantly statistical differences in other demographic characteristics and radiological matters in patients with and without detectable elevation of cTnI.

Outcomes

Clinical outcomes were presented in **Table 2**. The common in-hospital complications were pneumonia (72 patients, 33.8%) and DVT (65 patients, 30.5%). Of 61 (28.6%) patients who developed MACEs during hospitalization, 30 patients (14.1%) had acute heart failures, 27 (12.7%) had arrythmia, 19 (8.9%) had myocardial injuries, and two patients (0.9%) experienced cardiac arrests. In addition, we found a higher incidence of MACE (43.6 vs. 23.4%, $p = 0.004$), DCI (30.9 vs. 13.9%, $p = 0.005$), and DVT (49.1 vs. 24.1%, $p = 0.001$) in the elevated cTnI group. The mortality during hospitalization in the whole cohort was 3.8% ($n = 8$; elevated cTnI group vs. unelevated cTnI group: 10.9 vs. 1.3%, $p = 0.001$).

After discharge, eight future MACEs occurred in seven patients (3.3%), yielding an annual incidence of cardiac events of 1.5%. Patients with elevated cTnI had a significantly higher rate of future MACEs than the unelevated group (10.6 vs. 2.1%, $p = 0.024$, and 95% CI: 1.256–23.875). This finding was also statistically significant in the Kaplan–Meier analysis between these two groups (log-rank, $p = 0.007$; **Figure 2**). In addition, we found that MACEs mostly occurred within 1 year after aneurysmal rupture (seven in the first year, one in the second year).

During the clinical follow-up, we observed a significant correlation between elevated cTnI and unfavorable neurological outcomes (mRS > 3) at discharge, 3-month, 1-, 2-year, and last follow-up (45.5 vs. 22.2%, $p = 0.001$; 39.1 vs. 13.5%, $p < 0.001$; 37.0 vs. 14.9%, $p = 0.001$; 39.1 vs. 12.8%, $p < 0.001$; 41.8 vs. 14.6%, $p < 0.001$, respectively; **Figure 3**). By the last available follow-up, 20 patients had died, with an overall mortality of 9.4% and an annual death rate of 3.8% (8.9 vs. 2.3%, $p = 0.005$). In addition, the Kaplan–Meier analysis of mortality during the clinical follow-up was 21.7% with elevated cTnI and 7.1% without (log-rank, $p = 0.004$; **Figure 4**).

Predictors of Future MACEs and Unfavorable Long-Term Outcomes

A total of 27 patients were lost to follow-up, namely 26 after discharge and one at 1-year, with a missing rate of 12.7%. The remaining 186 patients were included in the survival analysis, amounting to 532 follow-up patient-years.

During hospitalization, 61 (28.6%) patients experienced MACEs. The univariable Cox analysis showed that age (HR = 0.927; 95% CI: 0.861–0.998, and $p = 0.044$), elevated cTnI (HR = 9.603; 95% CI: 1.487–55.209, and $p = 0.016$), and in-hospital pneumonia (HR = 0.104; 95% CI: 0.013–0.837, and $p = 0.033$) were significantly correlated with future MACEs (**Table 3**). When clinical confounding variables (age by decades, Hunt-Hess scale, Fisher score, and in-hospitalization complications) were incorporated into the multivariable Cox regression model, only elevated cTnI was statistically significant (HR = 5.974; 95% CI: 1.426–25.019, and $p = 0.014$).

The univariable logistic analysis showed that elevated cTnI was associated with unfavorable long-term outcomes at the last follow-up (OR = 4.218; 95% CI: 2.106–8.450; $p < 0.001$).

TABLE 1 | Baseline characteristics between patients with or without elevation of troponin I.

Variable	Total (%) *n* = 213	Elevated cTnI (%) *n* = 55	Unelevated cTnI (%) *n* = 158	*P*-value
Age (Years)	57.4 ± 12.1	59.3 ± 11.2	56.8 ± 12.4	0.192
Female	131/213 (61.5)	44/55 (80.0)	87/158 (55.1)	0.001
Medical history				
Diabetes mellitus	17 (8.0)	4 (7.3)	13 (8.2)	1.000
Hypertension	131 (61.5)	31 (56.4)	100 (63.3)	0.363
Dyslipidemia	27 (12.7)	10 (18.2)	17 (10.8)	0.154
Hyperhomocycteinemia	17 (8.0)	2 (3.6)	15 (9.5)	0.275
Stroke	36 (16.9)	11 (20.0)	25 (15.8)	0.476
*CCI-i	3.4 ± 2.0	3.7 ± 2.0	3.3 ± 2.0	0.193
Cardiac history				
Coronary artery disease	33 (15.5)	10 (18.2)	23 (14.6)	0.522
Heart failure	2 (0.9)	1 (1.8)	1 (0.6)	0.451
Arrhythmia	5 (2.3)	1 (1.8)	4 (2.5)	1.000
History of brain operation	1 (0.5)	1 (1.8)	0 (0)	0.258
History of cardiac surgery	9 (4.2)	1 (1.8)	8 (5.1)	0.521
ACEI/ARB	14 (6.6)	2 (3.6)	12 (7.6)	0.481
Anticoagulant drugs	2 (0.9)	1 (1.8)	1 (0.6)	0.451
Antiplatelet drugs	20 (9.4)	4 (7.3)	16 (10.1)	0.532
peak cTnI	0.007 (0.002–0.075)	0.366 (0.058–1.380)	0.002 (0.001–0.007)	0.000
Elevated BNP	113 (53.1)	46 (83.6)	67 (42.4)	0.000
Elevated CK-MB	54 (25.4)	35 (63.6)	19 (12.0)	0.000
Elevated Myo	66 (31.0)	32 (58.2)	34 (21.5)	0.000
Hunt-Hess grade				0.000
1–2	152 (71.4)	27 (49.1)	125 (79.1)	
3–5	61 (28.6)	28 (50.9)	33 (20.9)	
Fisher score				0.014
1–2	123 (57.7)	24 (43.6)	99 (62.7)	
3–4	90 (42.3)	31 (56.4)	59 (37.7)	
IVH involvement	121 (56.8)	35 (63.6)	86 (54.4)	0.235
Treatment				0.175
Microsurgery	106 (49.8)	24 (43.6)	82 (51.9)	
Endovascular treatment	89 (41.8)	23 (41.8)	66 (41.8)	
Medication	18 (8.4)	8 (14.5)	10 (6.3)	

**CCI-i, Charlson Comorbidity Index-i; BNP, brain natriuretic peptide; CK-MB, creatine phosphokinase isoenzyme—MB; Myo, myoglobin; ACEI, angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor blocker; IVH, intraventricular hemorrhage; peak.cTnI, peak value of cardiac troponin I.*

Age, history of stroke, Hunt-Hess grade 3–5, Fisher score 3–4, perioperative pneumonia, and DVT had a $p < 0.10$. Those variables were verified to have had a major impact on outcome in previous studies (13) and were selected in the reduced regression model. In the multivariable analysis, peak cTnI (OR = 2.951; 95% CI: 1.376–6.323; $p = 0.005$), age (OR = 1.046; 95% CI: 1.012–1.081; $p = 0.008$), and Hunt-Hess grade (OR = 4.017; 95% CI: 1.909–8.453; $p < 0.001$) were independent predictors for unfavorable long-term outcomes (**Table 4**).

DISCUSSION

In this study cohort involving aSAH patients with elevated cTnI on admission, brain-heart interactions were investigated. The major findings were that the predictor analysis showed that abnormal troponin level was associated with future MACEs and unfavorable long-term outcomes. Additionally, the survival analysis showed that aberrant cTnI was related to an increased risk of future MACEs and deaths after aSAH. The risk increased by 195% for death and by 498% for MACE, respectively.

The results of this study offer valuable information to emergency and intensive care clinicians to aid them in deciding whether or not future unexpected cardiac events could happen in patients with elevated cTnI during hospitalization or at follow-up (17, 18). To our knowledge, this is the most extensive series reporting survival and outcome in this particular subgroup. Meanwhile, we explained the continuous brain-heart interaction with detailed clinical data. Admittedly, subarachnoid hemorrhage-induced mortality in the acute phase was dreadful

TABLE 2 | Outcomes between patients with or without elevation of troponin I.

Variable	Total (%) *n* = 213	Elevated cTnI (%) *n* = 55	Unelevated cTnI (%) *n* = 158	*P*-value
In-hospital complications				
*MACE	61 (28.6)	24 (43.6)	37 (23.4)	0.004
Myocardial injury	19 (8.9)	10 (18.2)	9 (5.7)	0.012
Acute heart failure	30 (14.1)	15 (27.3)	15 (9.5)	0.001
Arrythmia	27 (12.7)	5 (9.1)	22 (13.9)	0.353
Cardiac arrest	2 (0.9)	1 (1.8)	1 (0.6)	1.000
DCI/CI	39 (18.3)	17 (30.9)	22 (13.9)	0.005
Hydrocephalus	15 (7.0)	6 (10.9)	9 (5.7)	0.320
Seizure	6 (2.8)	2 (3.6)	4 (2.5)	1.000
ICI	16 (7.5)	3 (5.5)	13 (8.2)	0.708
Pneumonia	72 (33.8)	20 (36.4)	52 (32.9)	0.631
DVT	65 (30.5)	27 (49.1)	38 (24.1)	0.001
Hospitalization duration	15.5 ± 10.2	16.7 ± 10.0	15.1 ± 10.3	0.338
In-hospital mortality	8 (3.8)	6 (10.9)	2 (1.3)	0.001
Discharge mRS > 3	60/213 (28.2)	25/55 (45.5)	35/158 (22.2)	0.001
3-month mRS > 3	37/187 (19.8)	18/46 (39.1)	19/141 (13.5)	0.000
1-year mRS > 3	38/187 (20.3)	17/46 (37.0)	21/141 (14.9)	0.001
2-year mRS > 3	35/186 (18.8)	18/46 (39.1)	17/140 (12.1)	0.000
Last follow-up mRS > 3	46/213 (21.6)	23/55 (41.8)	23/158 (14.6)	0.000

**MACE, major adverse cardiac event; DCI, delayed cerebral ischemia; CI, cerebral infarction; ICI, intracranial infection; DVT, deep venous thrombosis.*

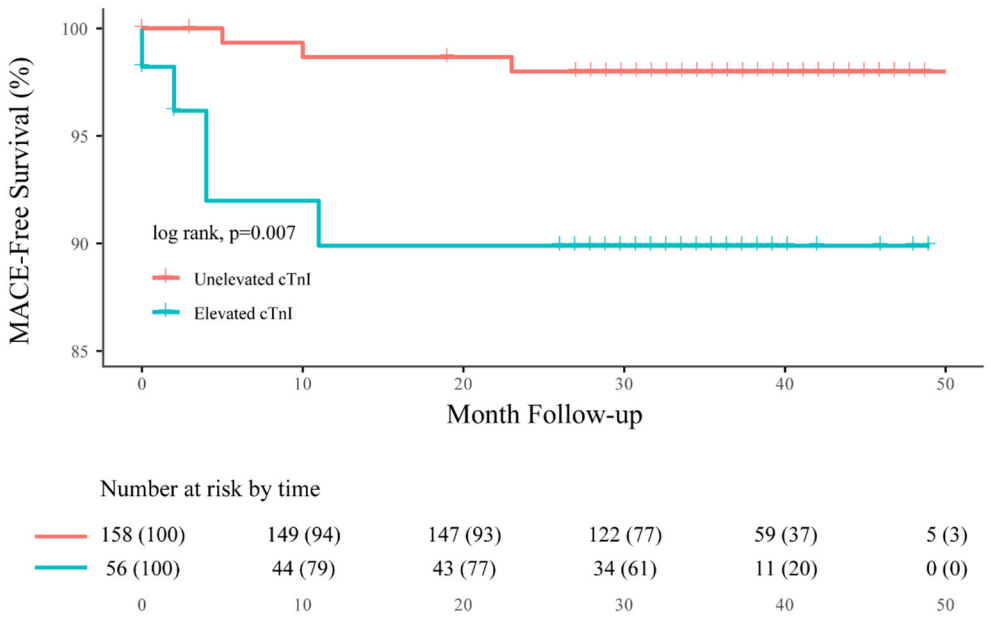

FIGURE 2 | Overall Kaplan–Meier estimates for freedom from MACE, comparing patients who had elevated cTnI (blue line) with unelevated cTnI (orange line; log-rank test, $p = 0.007$).

and following sublethal complications cannot be underestimated. In addition to the factors, such as DCI and DVT, which have been broadly verified by investigators, cardiac events secondary to the protopathy disturbed the diagnosis and treatment, in terms of both doctors and patients (19).

Recently, some clinicians have begun to pay attention to the impact of cardiovascular risk caused by aSAH. The cTnI elevation observed in this population is in keeping with the data produced by van der Bilt et al. (6) and Zhang et al. (7). Numerous studies have reported the reasonable predictive

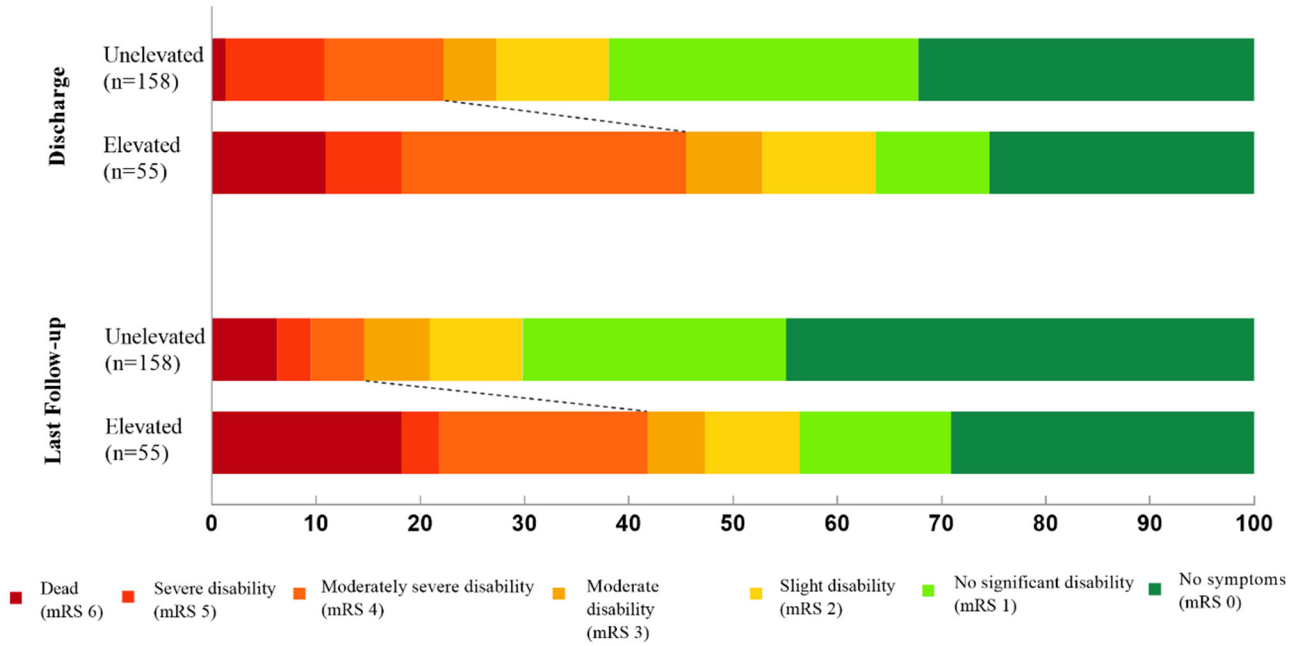

FIGURE 3 | Distribution of modified Rankin Scale (mRS) scores at discharge and last follow-up after aneurysmal subarachnoid hemorrhage.

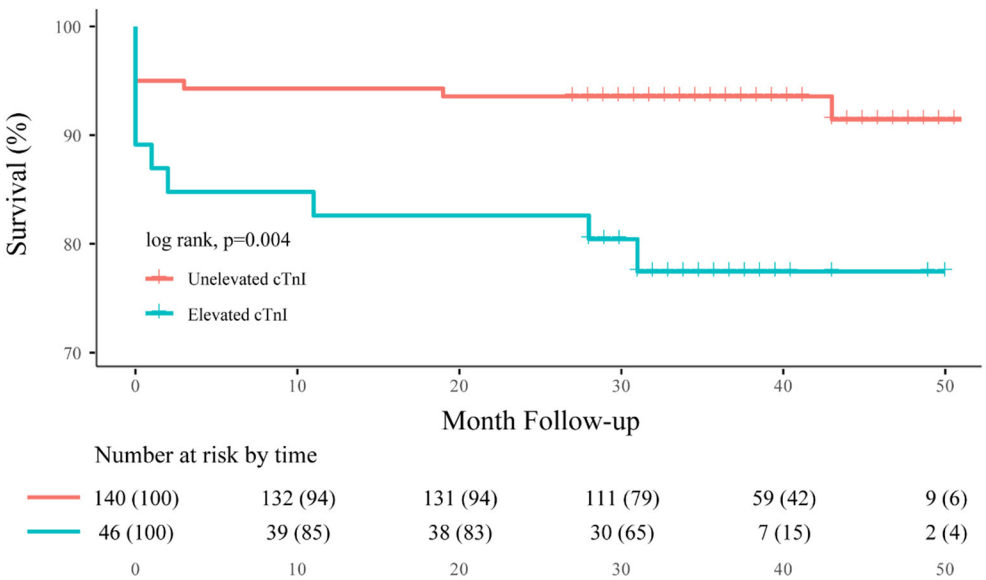

FIGURE 4 | Overall Kaplan–Meier estimates for freedom from death, comparing patients who had elevated cTnI (blue line) vs. unelevated cTnI (orange line; log-rank test, $p = 0.004$).

potential of admission elevated-troponin in aSAH patients, but lots of studies focused on the short-term neurological outcomes (7, 9, 20, 21), although several teams started to foresee the sensitivity and specificity of cTnI on long-term outcomes (7, 10, 16). However, none of the studies were performed for longer than 1 year. Studies that reported outcome separately for elevated cTnI and unelevated cTnI patients is in line with our findings at 3-month and 1-year. However, the disparity between studies is unescapable if outcome appraisal tools were applied differently (16). Furthermore, we conducted regular surveillance on surviving aSAH patients (mean follow-up of 34.3 ± 12.4 months) and found more unfavorable outcomes occurred in the elevated cTnI group (41.8 vs. 14.6%, $p < 0.001$).

TABLE 3 | Cox regression analysis of predictors for future MACEs.

	Primary outcome characteristics		Univariate		Multivariate	
Variable	**MACE**	**Non-MACE**	***p*-value**	**HR (95% CI)**	***p*-value**	**OR (95% CI)**
Age, years	53.5 ± 9.8	58.0 ± 12.5	0.044	0.927 (0.861–0.998)	0.238	
Gender	6 (75.0)	113 (62.8)	0.184	6.613 (0.405–107.921)		
HH grade 3–5	3 (37.5)	51 (28.3)	0.248	0.222 (0.017–2.862)		
*FS 3–4	4 (50.0)	73 (40.6)	0.728	1.436 (0.185–11.13)		
IVH involvement	5 (62.5)	101 (56.1)	0.518	1.818 (0.295–11.191)		
Smoke	2 (25.0)	56 (31.1)	0.43	0.388 (0.037–4.079)		
History of stroke	2 (25.0)	31 (17.2)	0.226	0.302 (0.043–2.098)		
Hypertension	4 (50.0)	112 (62.2)	0.239	3.31 (0.449–24.361)		
HR	82.9 ± 18.1	75.6 ± 13.5	0.405	1.027 (0.964–1.093)		
SBP	159.9 ± 26.0	161.3 ± 24.2	0.634	1.008 (0.975–1.042)		
Elevated cTnI	5 (62.5)	42 (23.3)	0.016	9.063 (1.487–55.209)	0.014	5.980 (1.428–25.407)
ih.MACE	5 (62.5)	49 (27.2)	0.064	4.528 (0.91–22.512)	0.064	
ih.DCI/CI	1 (12.5)	33 (18.3)	0.246	0.236 (0.02–2.711)		
ih.pneumonia	4 (50.0)	58 (32.2)	0.033	0.104 (0.013–0.837)	0.211	
ih.DVT	4 (50.0)	52 (28.9)	0.512	0.521 (0.074–3.656)	0.622	

**FS, Fisher score; HR, admission heart rate; SBP, admission systolic blood pressure; cTnI, cardiac troponin I; ih.MACE, in hospitalization major adverse cardiac event; ih.DCI/CI, in hospitalization delayed cerebral ischemia/ cerebral infarction; ih.pneumonia, in hospitalization pneumonia; ih.DVT, in hospitalization deep venous thrombosis.*

TABLE 4 | Logistic regression analysis of predictors for last follow-up outcomes.

	Last follow-up outcomes		Univariate		Multivariate	
Variable	**Unfavorable**	**Favorable**	***p*-value**	**OR (95% CI)**	***p*-value**	**OR (95% CI)**
Age, years	62.5 ± 12.1	56.0 ± 11.8	0.002	1.049 (1.018–1.081)	0.008	1.046 (1.012–1.081)
Gender	31 (67.4)	100 (59.9)	0.355	1.384 (0.694–2.759)		
HH grade 3–5	27 (58.7)	34 (20.4)	0.000	5.558 (2.767–11.160)	0.000	4.017 (1.909–8.453)
*FS 3–4	30 (65.2)	60 (35.9)	0.001	3.343 (1.686–6.627)		
IVH involvement	28 (60.9)	93 (55.7)	0.530	0.807 (0.414–1.572)		
Smoke	12 (26.1)	59 (35.3)	0.241	1.547 (0.745–3.213)		
History of stroke	12 (26.1)	24 (14.4)	0.064	0.475 (0.216–1.045)		
Hypertension	32 (69.6)	99 (59.3)	0.206	0.636 (0.316–1.282)		
HR	76.9 ± 14.5	74.8 ± 13.2	0.353	1.011 (0.987–1.036)		
SBP	162.1 ± 26.3	161.2 ± 24.3	0.813	1.001 (0.988–1.014)		
Elevated cTnI	23 (50.0)	32 (19.2)	0.000	4.218 (2.106–8.450)	0.005	2.951 (1.377–6.323)
ih.MACE	15 (32.6)	46 (27.5)	0.502	1.272 (0.629–2.572)		
ih.DCI/CI	10 (21.7)	29 (17.4)	0.498	1.321 (0.589–2.962)		
ih.pneumonia	25 (54.3)	47 (28.1)	0.001	0.329 (0.168–0.643)		
ih.DVT	21 (45.7)	44 (26.3)	0.013	0.425 (0.216–0.836)		

**FS, Fisher score; HR, admission heart rate; SBP, admission systolic blood pressure; cTnI, cardiac troponin I; ih.MACE, in hospitalization major adverse cardiac event; ih.DCI/CI, in hospitalization delayed cerebral ischemia/cerebral infarction; ih.pneumonia, in hospitalization pneumonia; ih.DVT, in hospitalization deep venous thrombosis.*

Few studies have offered an insight into the predictive value of admission troponin elevation in future MACEs, which has been studied in general noncardiac surgery and ischemic stroke population by degrees (5, 22). Akkermans et al. (11) concluded that patients with postinterventional cTnI elevation have a higher risk of MACE within the first year, but failed to build a multivariable regression model, which is consistent with our findings concerning admission cTnI elevation (HR = 5.980; 95% CI: 1.428–25.407; $p = 0.014$). Furthermore, none of the above-cited studies used survival analysis to evaluate the level of admission cTnI for predicting future MACEs and unfavorable 2-year outcomes. One could speculate that our notion of relations between elevated cTnI and the primary and secondary outcome is accidental and unintentional. However, the independent prognostic value of elevated cTnI was also successfully established when adjusted for known predictors. Unexpectedly, the history of stroke and heart diseases had nothing to do with future MACEs (25.0 vs. 17.2%, $p = 0.928$;

12.5 vs. 17.8%, $p = 1.000$, respectively). We proposed that the ischemic preconditioning, a powerful endogenous mechanism, could be a rational mechanism to monitor the ischemic events in both subsequent brain and heart events (18, 23, 24).

The severity of brain injury was widely regarded as the leading cause of a poor outcome in aSAH patients. Cardiac complications are not the most important factor for the eventual outcome (20), but they are the second most important (1). Proposed mechanisms of brain-heart interaction after aSAH have grown ever more important. A more generally accepted hypothesis is that an increased sympathetic tone determines catecholamines discharge and subsequently SIC ensues (25). As a matter of fact, there are some other possible explanations regarding neurocardiac injuries, including right insular cortex damage, decreased focal and global cerebral perfusion, instable autoregulation, existed cardiac diseases, and impaired blood-brain barrier (26–28). Interestingly, the above-mentioned mechanisms could interpret our data to some extent in the early phase after aSAH, but some may question the phenomenon that troponin elevation was strongly associated with future MACEs and long-term neurological outcomes. Exogenous administration of norepinephrine (29), perioperative cardiac injury after aneurysm occlusion (11), and neurogenic stunned myocardium (30) could be possible answers. Future studies should offer further proof of whether there is a causal relationship between the onset of troponin discharge and MACEs and outcomes within 2 years.

In the case of aSAH-related cardiac dysfunction, it is acceptable to treat the underlying neurological condition. Due to the low recognition rate, misdiagnosis (19), and lack of randomized trials, additional management of neurocardiogenic injury remains entirely empirical in each individual case. Recognized ominous clinical factors including higher age (31) and unfavorable admission neurological status concerned with poor outcome were also statistically significant in the present study (higher age, OR = 1.046, 95% CI: 1.012–1.081, and $p = 0.008$; higher Hunt-Hess grade, OR = 4.017, 95% CI: 1.909–8.453, $p < 0.001$). Nevertheless, no cohort study has ever been published relating the troponin elevation to future MACEs and 2-year outcomes. The findings of this study may not only improve clinical practice but also direct secondary prevention cost-effectively.

The present study had some limitations. First, the study population was too small to fulfill an accurate prediction model, though our retrospectively reviewed cohort showed the independent predictive value on future MACEs and 2-year outcomes. Second, although previous studies suggest that intervention methods have no significant effect on troponin release, with the significant progress of endovascular and microsurgical procedures, the impact of different treatments on MACEs after aSAH needs to be further explored (22). Finally, the laboratory updated cTnI settings once during the study period, and absolute value to stratification was impractical. Emergency and intensive care unit clinicians made decisions based on the thresholds laboratory offered, so different assays did not influence our results. Further quantitative analysis of sensitivity and specificity of troponin needs to be investigated.

CONCLUSION

CTnI elevation after the ictus of a ruptured intracranial aneurysm can predict the occurrence of MACEs and unfavorable outcomes within 2 years after aSAH. Although admission troponin elevation can be recognized as a biomarker to identify aSAH patients at high risk of neurocardiac injuries, further investigation into clinical management is needed to prevent cardiac complications and improve outcomes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Beijing Tiantan Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FL and YC: conception and design and drafted the article. FL, YC, QH, CZe, and CZh: acquisition of the data. CZe and CZh: analysis and interpretation of the data. XC and SW: critically revised the article. XC, YZ, SW, and JZ: reviewed the submitted version of the manuscript. All authors approved the final version of the manuscript.

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Monocyte to High-Density Lipoprotein Ratio Is Associated With Early Neurological Deterioration in Acute Isolated Pontine Infarction

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Objectives: The monocyte to high-density lipoprotein ratio (MHR) has been considered to be a novel inflammatory marker of atherosclerotic cardiovascular disease. However, its role in the acute phase of acute isolated pontine infarctions remains elusive. We explored whether an association existed between elevated MHR levels and early neurological deterioration (END) in patients with isolated pontine infarction.

Methods: Data from 212 patients with acute isolated pontine infarction were retrospectively analyzed. We examined the MHR in quartiles of increasing levels to evaluate for possible threshold effects. END was defined as an elevation in the total National Institutes of Health Stroke Scale (NIHSS) score ≥2 or an increase in NIHSS score ≥1 in motor power within the first week after symptom onset. Patients were divided into an END group and a non-END group. The association of MHR on END following pontine infarction was examined by logistic regression models after adjusting for age, NIHSS at admission, basilar artery stenosis, history of hypertension or hyperlipidemia or stroke, infarct size, fasting blood glucose, and paramedian pontine infarction.

Results: The mean MHR was 0.44 ± 0.22. A total of 58 (27.36%) patients were diagnosed with END. END occurred within the first 48 h after hospitalization in 38 patients (65.52%). After adjusting for confounding and risk factors, the multivariate logistic regression analysis showed NIHSS at admission [odds ratio (OR), 1.228; 95% confidence interval (CI), 1.036–1.456], basilar artery stenosis (OR, 2.843; 95% CI, 1.205–6.727), and fasting blood glucose (OR, 1.296; 95% CI, 1.004–1.672) were independently associated with END. The odds ratio of END increased as the quartile level of MHR increased, with the lowest quartile used as the reference value. Compared to the first quartile of MHR, the third and fourth quartiles were associated with 4.847-fold (95% CI, 1.532–15.336) and 5.824-fold (95% CI, 1.845–18.385) higher odds of END in multivariate analysis.

Conclusions: Elevated MHR levels may be valuable as a biomarker of END in patients with isolated pontine infarction. The elevated MHR was independently associated with END in isolated pontine infarction.

Keywords: acute isolated pontine infarction, early neurological deterioration, monocyte to high-density lipoprotein ratio, monocyte, high-density lipoprotein

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INTRODUCTION

Worsening neurological deficits, also known as early neurological deterioration (END), occur in up to one-third of patients with acute ischemic stroke and have been shown to be associated with increased mortality and subsequent functional disabilities (1, 2). Pontine infarctions account for ∼7% of all ischemic strokes, and isolated pontine infarctions are the most common type related to the posterior circulation, accounting for ∼15% of cases (3). Extensive studies regarding END prediction in isolated pontine infarction have been performed to enable physicians to better predict END occurrence (4–6). With the popularity of magnetic resonance imaging (MRI) in clinical practice, the correlation between neurological impairment and topographic location has been deeply studied (7, 8). There are also some studies concerning the treatment and prognosis of ischemic stroke (9, 10). However, there are few studies on hematological indexes in the study of the aggravation of nervous system function. Recently, the monocyte to high-density lipoprotein ratio (MHR) has been considered to be a novel inflammatory marker of atherosclerotic cardiovascular disease, especially coronary artery disease (11). It has been reported to be related to the prediction of ischemic stroke from the general population (12) and carotid artery intima-media thickness in patients with type 2 diabetes (13). In stroke-related studies, MHR has been reported to be a good predictive value of stroke-associated pneumonia (14) and mortality in patients with ischemic stroke (15). However, there are no studies exploring the value of MHR in predicting END in patients with acute isolated pontine infarction. Therefore, the aim of our study is to elucidate the association between MHR with END after acute isolated pontine infarction.

MATERIALS AND METHODS

Patients

A total of 2,789 consecutive patients with ischemic stroke registered at the Department of Neurology, Beijing Shijitan Hospital, Capital Medical University from January 2015 to December 2020 were retrieved. Patients were included in the analysis if they met the following criteria: (a) hospitalization within 48 h after the onset of symptoms, (b) acute ischemic lesions within the unilateral pons on diffusion-weighted imaging (DWI), and (c) modified Rankin scale (mRS) score <2 before admission. Patients were excluded if they (a) had no available DWI within 72 h of initial presentation, (b) had infraction of the anterior circulation infarction and/or other parts of the vertebrobasilar system on DWI, and (c) had deterioration due to extracerebral illnesses, such as infection, aspiration pneumonia, hypotension, metabolic disturbances, dehydration, and/or respiratory/heart failure. A flow chart of patient inclusion is shown in **Figure 1**.

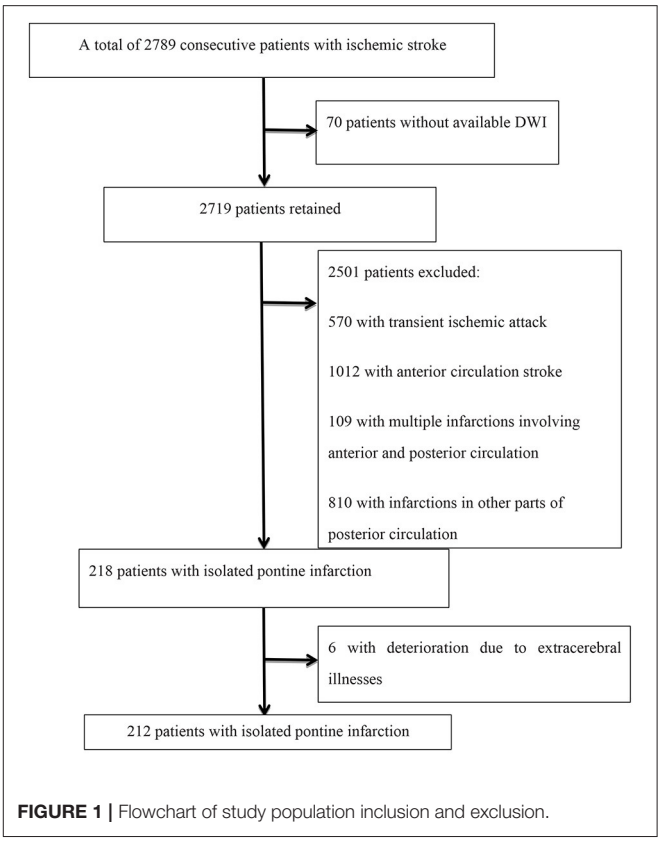

FIGURE 1 | Flowchart of study population inclusion and exclusion.

Clinical Information and Assessment

The following clinical data were retrospectively obtained: age, sex, and vascular risk factors including diabetes mellitus, hypertension, coronary heart disease, hyperlipidemia, previous stroke, and the presence of current smoking. On admission, all patients received brain MRI, magnetic resonance angiography (MRA), carotid artery color Doppler ultrasound, and transcranial Doppler. The following criteria were considered to be vascular risk factors: history of stroke was defined as prior ischemic stroke or transient ischemic attack. Smoking was defined as smoking ≥1 cigarette per day continuously for at least 1 year.

Blood samples were collected on the second day in the morning within 24 h of hospital admission after an 8-h fasting period. Biochemical variables, including serum high-density lipoprotein cholesterol (HDL-C), were measured using an AU5832 automatic biochemical analyzer (Beckman Coulter, Tokyo, Japan). White blood cell (WBC) and monocyte levels were analyzed using a Xe5000 automatic hematology analyzer (SYSMEX, Kobe, Japan). The MHR was calculated as the ratio of the monocyte ($\times 10^9$/L) count to HDL-C (mmol/L) level.

Severity of neurological impairment was assessed using the National Institutes of Health Stroke Scale (NIHSS) score immediately before MRI scans on admission, within the first 7 days after symptom onset. END was defined as an elevation in the total NIHSS score ≥2 or an increase in NIHSS score ≥1 in motor power within the first week after symptom onset (7). Patients were divided into an END group and a non-END group based on the incremental increase in NIHSS score. The neurological status of patients was evaluated by trained neurologists on a daily basis.

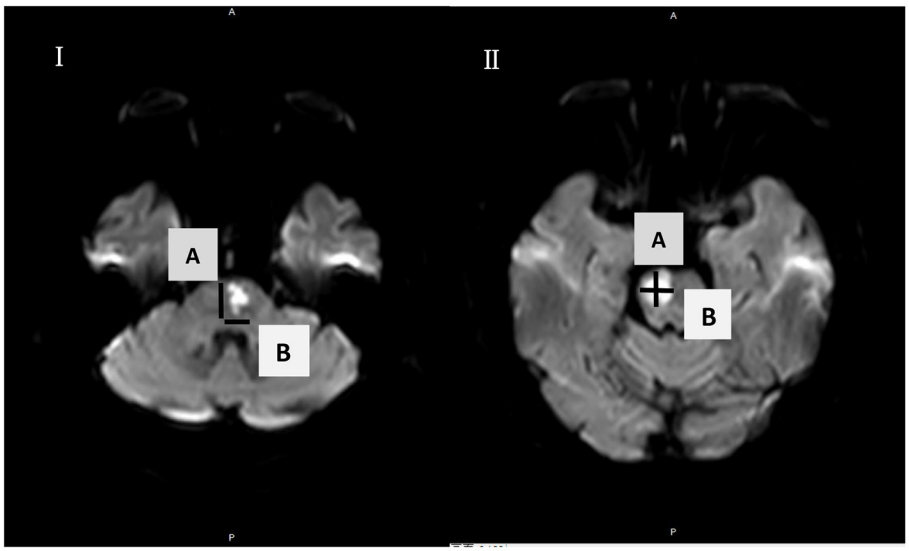

FIGURE 2 | Acute pontine infarctions on axial diffusion-weighted imaging are shown in I and II. The maximal ventrodorsal **(A)** length and **(B)** width of each infarct were measured on axial MRI. A × B was used to define the infarction size (IS).

Imaging Protocol and Morphometric Analysis

MRI was performed within 48 h after admission using a 3.0-T MRI unit (Ingenia, Philips, Best, the Netherlands). Morphometric measurement was performed on axial DWI with the following imaging parameters: repetition time = 2,800 ms, echo time = 90 ms, slice/gap = 5 mm/0.5 mm, voxel = 1.0 × 1.0 mm^2, field-of-view = 230 × 230 mm^2, and scan time = 35 min. The diffusion sensitivity coefficient B was set to 0 and 1,000 s/mm^2. DWI was positive for DWI with a high b value and had low a signal according to the apparent diffusion coefficient (ADC). Infarct size (IS) was measured at the axial position on DWI. The maximal ventrodorsal length (A) and width (maximum dimension in a direction perpendicular to the ventrodorsal length, B) of each infarct on axial DWI were measured. We used A × B to represent IS. All morphometric measurements were performed twice, and the mean value was used (**Figure 2**).

Basilar artery stenosis was defined as a reduction in the caliber of the basilar artery by at least 50% or occlusion of the basilar artery. Isolated pontine infarctions were divided into paramedian pontine infarction (PPI) and lacunar pontine infarction (LPI) (16). PPI was defined as a lesion that extends to the anterior surface of the pons, and LPI was defined as a lesion that does not extend to the basal surface of the pons. The morphometric analysis was performed by at least two neurologists and radiologists.

Statistical Analysis

We examined the total MHR in quartiles of increasing levels to evaluate for possible threshold effects. Patients were divided into quartiles based on the MHR (Q1, <0.24; Q2, 0.24–0.42; Q3, 0.43–0.55; and Q4, ≥0.56). For group comparisons, analysis of variance or the Kruskal–Wallis rank-sum test was used to compare continuous variables, and the chi-square test was applied for categorical variables. According to the END and non-END groups, baseline characteristics and risk factors were compared using Student's *t*-test (continuous variables) or the χ^2 test or Fisher's exact test (categorical variables), as appropriate. Continuous variables and categorical variables are expressed as mean (± SD) and frequency (percentage), respectively. Multivariate analyses were performed to determine independent factors associated with END, and the lowest quartile was used as the reference. Considering the close correlation between MHR and monocyte count and HDL level, only the MHR was included in the logistic regression. A receiver-operating characteristic curve was constructed to assess the sensitivity, specificity, and area under the curve of possible contributing factors to discriminate the END group from the non-END group. Spearman correlation was used to judge the relationship between the level of MHR and NIHSS. SPSS version 23.0 for Windows was used for statistical analysis. A two-sided $P < 0.05$ was considered statistically significant.

RESULTS

Among 2,789 consecutive patients with ischemic stroke, 218 (7.82%) were diagnosed with an isolated pontine infarction, 6 of whom experienced aggravation due to extracerebral illness. A total of 212 patients with acute isolated pontine infarctions were included in the final analysis. The mean age of patients was 68.27 ± 11.57 years, and 127 (59.9%) were male. The mean NIHSS score was 3.59 ± 2.25. A total of 41 (19.3%) patients had basilar artery stenosis. END was diagnosed in 58 (27.36%) patients. END

TABLE 1 | Characteristics of patients with isolated pontine infarction according to monocyte to high-density lipoprotein ratio quartile.

Characteristic	MHR				*P*
	<0.24 (*N* = 52)	0.24–0.42 (*N* = 55)	0.43–0.55 (*N* = 53)	≥0.56 (*N* = 52)	
Age, years	67.89 ± 11.54	67.33 ± 13.74	71.28 ± 10.96	66.50 ± 9.19	0.157
Sex, male	39 (31.2)	30 (32.9)	31 (31.8)	27 (31.2)	0.071
Risk factors					
Current smoking	13 (25.0)	16 (29.1)	16 (30.2)	13 (52.0)	0.899
History of hypertension	28 (53.8)	18 (32.7)	32 (60.4)	25 (25.3)	0.028
History of diabetes mellitus	14 (26.9)	19 (34.5)	24 (45.3)	15 (28.8)	0.188
History of stroke	5 (9.6)	9 (16.4)	9 (17.0)	11 (21.2)	0.461
History of atrial fibrillation	3 (5.8)	1 (1.8)	5 (9.4)	5 (9.6)	0.277
History of coronary heart disease	16 (30.8)	12 (21.8)	12 (22.6)	7 (13.5)	0.21
History of hyperlipidemia	37 (33.4)	33 (35.3)	33 (34.0)	33 (33.4)	0.656
Basilar artery stenosis	4 (7.7)	10 (18.2)	11 (20.8)	16 (30.8)	0.028
Initial SBP, mmHg	147.69 ± 16.58	144.58 ± 18.30	140.87 ± 16.70	141.56 ± 16.43	0.155
Initial DBP, mmHg	83.25 ± 8.78	82.85 ± 8.84	80.96 ± 8.22	82.23 ± 9.19	0.559
NIHSS at admission	3.44 ± 2.08	3.18 ± 2.03	3.71 ± 2.35	4.02 ± 2.51	0.254
HbA_{1C}	5.64 ± 0.95	5.77 ± 0.88	5.57 ± 0.89	5.95 ± 1.18	0.243
IS, mm^2	1.16 ± 0.56	0.88 ± 0.57	0.94 ± 0.58	1.32 ± 0.89	0.11
FBG, mmol/L	6.05 ± 1.21	5.95 ± 1.08	6.09 ± 1.43	6.40 ± 1.83	0.914
TOB, h	18.71 ± 11.84	20.29 ± 11.37	21.94 ± 11.19	22.40 ± 12.10	0.352
END	6 (11.5)	11 (20.0)	18 (34.0)	23 (44.2)	0.001

MHR, monocyte to high-density lipoprotein ratio; END, early neurological deterioration; SBP, systolic blood pressure; DBP, diastolic blood pressure; PPI, paramedian pontine infarction; LPI, lacunar pontine infarction; NIHSS, National Institutes of Health Stroke Scale; HbA1C, glycosylated hemoglobin; TOB, time from onset to blood sample collection.

TABLE 2 | Comparison of demographic and clinical characteristics between the END non-END groups.

Characteristics	All patients (*N* = 212)	END (*N* = 58)	Non-END (*N* = 154)	*P*
Age, years	68.27 ± 11.57	68.54 ± 11.80	67.57 ± 11.00	0.587
Sex, male	127 (59.9)	29 (50.0)	98 (63.6)	0.071
BMI, kg/m^2	23.98 ± 2.01	24.12 ± 2.00	23.61 ± 1.98	0.096
Risk factors				
Current smoking	58 (27.4)	20 (34.5)	38 (24.7)	0.153
History of hypertension	103 (48.6)	28 (48.3)	75 (48.7)	0.956
History of diabetes mellitus	72 (34.0)	18 (31.0)	54 (35.1)	0.581
History of stroke	34 (16.0)	14 (24.1)	20 (13.0)	0.049
History of atrial fibrillation	14 (6.6)	6 (10.3)	8 (5.2)	0.215
History of coronary heart disease	47 (22.2)	9 (15.5)	38 (24.7)	0.152
History of hyperlipidemia	136 (64.2)	37 (63.8)	99 (64.3)	0.947
Basilar artery stenosis	41 (19.3)	19 (32.8)	22 (14.3)	0.002
Initial SBP, mmHg	143.67 ± 17.13	144.53 ± 17.79	143.35 ± 16.92	0.655
Initial DBP, mmHg	82.33 ± 8.74	81.53 ± 9.69	82.62 ± 8.37	0.42
NIHSS at admission	3.59 ± 2.25	4.64 ± 2.31	3.19 ± 2.10	<0.001
PPI	91 (42.9)	36 (62.1)	55 (35.7)	0.001
LPI	121 (57.1)	22 (37.9)	99 (64.3)	0.001
TOB, h	20.83 ± 11.63	19.02 ± 10.19	21.52 ± 12.09	0.133

END, early neurological deterioration; SBP, systolic blood pressure; DBP, diastolic blood pressure; PPI, paramedian pontine infarction; LPI, lacunar pontine infarction; NIHSS, National Institutes of Health Stroke Scale; BMI, body mass index; TOB, time from onset to blood sample collection.

occurred within the first 48 h after admission in 38 patients (65.52%). The mean MHR level was 0.44 ± 0.22. Detailed demographic data are summarized in **Table 1**. As expected, the presence of basilar artery stenosis ($P = 0.028$), hypertension ($P = 0.028$), and END ($P = 0.001$) was significantly different between groups.

TABLE 3 | Laboratory and imaging data in the END and non-END groups.

Data	END ($N = 58$)	Non-END ($N = 154$)	P
FBG, mmol/L	6.62 ± 1.73	5.92 ± 1.22	0.006
Platelet, $\times 10^9$/L	209.15 ± 777.61	227.05 ± 69.90	0.108
D-dimer, ng/dl	168.91 ± 70.45	179.03 ± 74.49	0.372
Cr, μmol/L	71.71 ± 16.86	70.70 ± 15.36	0.680
WBC, $\times 10^9$/L	6.89 ± 1.85	6.58 ± 1.86	0.282
Neutrophil, $\times 10^9$/L	4.34 ± 1.55	4.02 ± 1.57	0.176
Monocyte, $\times 10^9$/L	0.55 ± 0.28	0.41 ± 0.16	0.001
Lymphocyte, $\times 10^9$/L	1.83 ± 0.79	1.72 ± 0.56	0.335
Hemoglobin, g/L	143.57 ± 36.73	134.61 ± 24.84	0.090
Total protein, g/L	74.93 ± 52.09	71.78 ± 45.45	0.665
HDL, mmol/L	1.01 ± 0.31	1.19 ± 0.57	0.024
LDL, mmol/L	2.29 ± 0.96	2.39 ± 1.02	0.515
MHR	0.57 ± 0.25	0.39 ± 0.19	<0.001
IS, m^2	1.29 ± 0.78	0.99 ± 0.62	0.004

FBG, fasting blood glucose; IS, infarct size; BMI, body mass index; WBC, white blood cell; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

TABLE 4 | Evaluation of the effect of MHR on END following pontine infarction using multivariate logistic regression models.

Variables	OR	95% CI		P
NIHSS at admission	1.228	1.036	1.456	0.018
Basilar artery stenosis	2.843	1.205	6.707	0.021
MHR Q1 (reference)				
Q2	2.459	0.765	7.9	0.131
Q3	4.847	1.532	15.336	0.007
Q4	5.824	1.845	18.385	0.003
Age	0.987	0.954	1.022	0.466
History of hypertension	0.611	0.274	1.364	0.229
History of hyperlipidemia	1.352	0.583	3.133	0.483
History of stroke	2.463	0.988	6.141	0.053
IS	1.787	0.993	3.215	0.053
FBG	1.296	1.004	1.672	0.046
PPI	1.692	0.758	3.776	0.2

FBG, fasting blood glucose; IS, infarct size; NIHSS, national institutes of health stroke scale; PPI, paramedian pontine infarction.

Patient baseline clinical characteristics in the END and non-END groups are shown in **Table 2**. Basilar artery stenosis ($P = 0.002$), NIHSS at admission ($P < 0.001$), previous stroke ($P = 0.049$), and PPI ($P = 0.001$) were significantly higher in the END group than in the non-END group. Prevalence of hypertension, diabetes, coronary heart disease, hyperlipidemia, atrial fibrillation, and current smoking showed no significant difference between the two groups.

Patient laboratory test and imaging results are shown in **Table 3**. Patients in the END group had larger infarct size ($P = 0.004$), higher HR ($P < 0.001$), higher blood glucose level on admission ($P < 0.001$), and higher monocyte count ($P < 0.001$) than those patients in the non-END group. At the same time, compared with that in the non-END group, the HDL level in the END group was lower ($P = 0.024$).

As presented in **Table 4**, multivariate logistic regression analysis showed that NIHSS at admission [$P = 0.018$, odds ratio (OR) = 1.228, 95% confidence interval (CI) = 1.036–1.456], basilar artery stenosis ($P = 0.021$, OR = 2.843, 95% CI = 1.205–6.727), and fasting blood glucose ($P = 0.046$, OR = 1.296, 95% CI = 1.004–1.672) were independently associated with END. The odds ratio for END increased with increasing quartile of MHR with the lowest quartile used as the reference value. A high (≥0.43) MHR was independently associated with END (third quartile OR, 4.847; 95% CI, 1.532–15.336; fourth quartile OR, 5.824; 95% CI, 1.845–18.385) in multivariate analysis. The third and fourth highest quartiles of MHR levels were identified to be independently associated with END. Receiver-operating characteristic curve analysis suggested the sensitivity, specificity, and area under the curve for MHR to discriminate END from non-END were 65.5, 76.6, and 71.5% (95% CI, 64–79%; $P < 0.001$), respectively. Youden's index was 0.421. The cutoff was 0.51 (**Figure 3**).

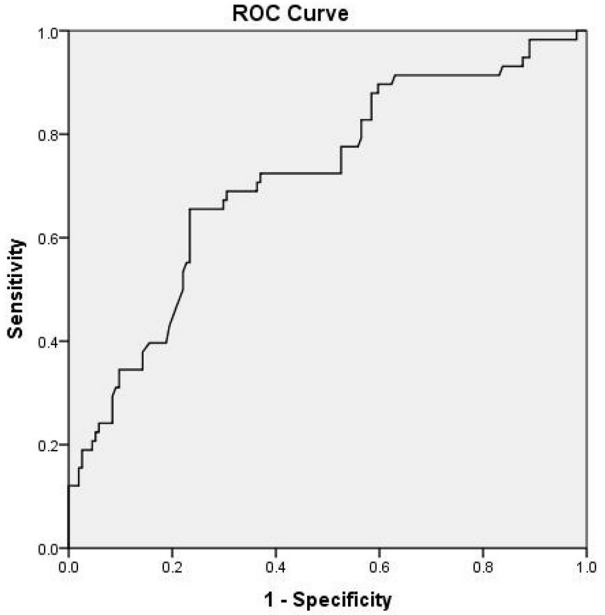

FIGURE 3 | Receiver operating characteristic (ROC) curve of the relationship between monocyte to high-density lipoprotein ratio (MHR) and early neurological deterioration (END).

Spearman's correlation was used to determine the relationship between MHR level and NIHSS at 7 days. The results showed that there was a correlation between MHR level and NIHSS at 7 days $r_s = 0.573$, $P < 0.001$.

DISCUSSION

There are limited reports describing the predictive value of MHR level for early deterioration during the acute phase of

an isolated pontine infarction. This study found that elevated MHR levels are associated with END and that the risk of END tended to increase with increasing MHR. It has been unequivocally shown that progressive neurological deficit after ischemic stroke may lead to increased mortality and morbidity (1). Progression of neurologic deficit, however, does not have an authoritative definition, as it could be considered to be either a neuropathological or a clinical event. Early neurological deterioration in patients with isolated pontine infarction is relatively common.

This study showed that 27.36% of the patients with isolated pontine infarction had END, which is consistent with previous studies that reported a prevalence of 20–58% in patients with acute stroke (17, 18). The incidence of END differs between studies according to its definition and the timing and duration of observation (3, 19–21). Although the majority of the relevant studies were based on an increase in NIHSS scores, the exact increase in NIHSS score used to define END has varied drastically. For example, some studies used an NIHSS score increase of 1–2 points combined with motor function impairment to define END, whereas others defined END as an NIHSS increase of 4 points. In terms of the timeframe, END has been defined to occur within 3 days, 5 days, or 1 week after symptom onset in different studies. In our study, END was defined as an elevation in the total NIHSS score $\geq$2 or an increase in NIHSS score $\geq$1 in motor power within the first week after symptom onset, which is congruent with most previous studies (7, 22).

Acute inflammation has been observed in brain injury caused by cerebral ischemic diseases, such as the production of inflammatory cells, release of proinflammatory mediators, and tissue infiltration (7, 22). In fact, there is growing evidence that inflammation exerts a prominent effect in the pathogenesis and progression of ischemic stroke (23, 24). A few hours after stroke onset, the number of circulating polymorphonuclear neutrophils increase in a stroke severity-dependent manner (25). Monocytes from the bloodstream reach the damaged site most abundantly 3–7 days after ischemia onset (26). In the early stages after brain injury, the number of total monocytes in the blood circulation shows an increasing trend (27). In addition, previous studies have reported an influx of different immune cells and cytokines produced in the brain, which play an immunomodulatory role in postischemic inflammation (27). Wang et al. suggested that high monocyte counts have the value in predicting the prognosis in various cardiovascular diseases (28). Monocytes play a pivotal role in the initiation and progression of the atherosclerotic process (29). Monocytes in the blood are involved in the start of the process of atherosclerosis by migrating to the intima and differentiating into macrophages under the action of cytokines (30). The increase in the number of macrophages and monocytes around vulnerable plaques can also lead to an increase in the monocyte count in the peripheral blood (31). This inflammatory response takes place during all subtypes of stroke. It could, at least in part, explain the more critical neurological symptomatology and worse outcomes (32). In contrast, HDL-C can control the activation of monocytes while inhibiting the migration of macrophages, protecting endothelial cells from inflammation and oxidative stress (33). Previous studies showed that impaired HDL-mediated cholesterol efflux and low HDL levels caused monocytosis proliferation, leading to a progression of the atherosclerotic plaque (34).

Intracranial atherosclerosis is the main feature of ischemic cerebrovascular disease (35, 36). Branch atherosclerosis, arterial embolism, and hypoperfusion after intracranial atherosclerosis are likely to lead to ischemic stroke (37, 38). Recent studies showed that the occurrence of END was also associated with the severity of basilar artery stenosis (7). By performing autopsies, Caplan identified the basis of pontine infarctions, such as plaque blocking the branch orifice within the parent artery, atherosclerotic plaques originating in the trunk and extending to the branches, and microatheroma originating in the orifice of branches (39). Atherosclerotic stenosis of the basilar trunk was observed in 50% of patients with isolated pontine infarction extending to the basal surface (40). Meanwhile, early neurological deterioration is one of the most concerning clinical problems in patients with branch atherosclerotic diseases. Progressive deficit has been associated with basilar artery branch disease and poor functional outcomes (41). Therefore, the progression of vascular stenosis or thrombosis caused by intracranial atherosclerosis is related to END.

The MHR has recently been used to predict a variety of cardiovascular abnormalities as a developed measure of inflammation and oxidative stress, which reflects the anti-inflammatory and antioxidative effects of HDL, as well as the balance of inflammation and oxidative stress caused by the proinflammatory effects of monocytes. At the same time, MHR has been used as a prognostic indicator in a series of studies. Compared to the control group, patients with acute ischemic stroke had higher MHR, and high values of MHR were found to be a significant independent variable predictive of 30-day mortality in patients with acute ischemic stroke (42). A higher MHR was found to be associated with an increased risk of disability or death at discharge and 3 months after intracerebral hemorrhage, whereas an increase in monocytes was only associated with an increased risk of disability or death after 3 months (43). There are few studies on the association between MHR and acute cerebrovascular disease, especially in the acute phase. We analyzed the correlation between MHR and END in the acute stage of pontine infarction. To our knowledge, this is the first time that inflammatory factors and infarct size have been considered together to study the factors related to END in ischemic cerebrovascular disease. Our study indicated that MHR was an effective and convenient measure in predicting neurological deficit aggravation following pontine infarction. The MHR is a simple and convenient measure that can be effectively applied in clinical practice and provides clinical utility in risk stratification in subjects presenting with isolated pontine stroke. These findings have implications for strategies aimed at lowering the MHR to prevent early neurological progression in patients with ischemic stroke.

Earlier studies have identified the presence of comorbidities (such as diabetes and hypertension), female sex, infarct size, and neurological severity at onset to be associated with progressive

deficit in patients with isolated pontine infarctions. However, some studies have reported inconsistent findings (41). In addition, the infarct area extending to the basal surface was 2.5 times greater than deep infarctions without extension to the basal surface (41). Compared to LPI, PPI was related to END in the univariate analysis in our study. In the present study, infarct size had a very high value in the crosstab analysis, which was consistent with many previous studies on infarct size and progression (18); however, in the multiple logistic regression analysis, there was no significant difference in infarct size between the two groups.

In the current study, hyperglycemia correlated with END in patients with isolated pontine infarction after adjusting for other confounding factors in the multivariate analysis. Poststroke hyperglycemia is a common finding among diabetic and nondiabetic patients as a stress response, which is also commonly known as stress hyperglycemia (44). Approximately one-third of stroke patients had hyperglycemia on admission, which was associated with a poor prognosis in patients treated with thrombolytic drugs after ischemic stroke. A recent study reported that stress hyperglycemia increases the risk of severe neurological dysfunction in patients with acute ischemic stroke and is associated with mortality within 1 year (45). Although the exact mechanism underlying the relationship between hyperglycemia and END remains unknown, studies have illuminated the involvement of endothelial injury, tissue acidosis, blood–brain barrier destruction, and production of excessive active oxygen species (46, 47). Therefore, additional studies are necessary to determine whether optimizing blood glucose control could improve the clinical outcomes of patients with pontine infarction.

END following ischemic stroke was a serious event associated with long-term functional outcomes, as reported previously. It was also related to composite event outcomes after discharge during the first year after stroke (48). As inflammatory and immune-mediated mechanisms of neuronal injury have received greater attention, anti-inflammatory treatments have been developed or tested in the preclinical studies and clinical trials, such as IL1-Ra (49, 50), statins (51), and edaravone (52). Despite clinical developments, no beneficial long-term interventions targeting inflammation are currently available.

Study Limitations

The present study has several limitations. First, this is a single-center retrospective study. Therefore, whether the findings of the present study could be extrapolated to other institutions remains unknown. Moreover, the rate of END in this study is relatively low, further limiting the robustness of the analysis. Our study is confined to isolated brainstem infarction. The next step is to analyze the anterior circulation and posterior circulation, including midbrain and medullary infarctions. Finally, according to previous reports, different inflammatory factors are activated at different times after stroke (24). Some patients may have had the MHR measured at the same time or even after the assessment of END. The change in MHR over time was not studied. Therefore, we only considered a simple association between the biomarker and END instead of any causal relationship. Although statistical associations may exist between some biomarkers and END, the prospective trials are still needed to determine their added value relative to radiological and clinical characteristics.

CONCLUSION

In conclusion, patients with acute isolated pontine infarction and elevated MHR levels are at increased risk for END. MHR could be a convenient and effective measure related to END following pontine infarction. In the future, prospective multicenter studies will be needed to conclusively determine the predictive value of MHR for END in acute isolated pontine infarctions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committees of Beijing Shijitan Hospital, Capital Medical University (No. sjtky11-1x-202091). The participants provided written informed consent to participate in this study. If the participant was unable to provide written informed consent due to illness, the informed consent was signed by the client instead.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SYSTEMATIC REVIEW
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A Systematic Review of the Predictive Value of Plasma D-Dimer Levels for Predicting Stroke Outcome

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Background: Stroke is a leading cause of morbidity and mortality. Over the past decade, plasma D-dimer levels have emerged as a biomarker for predicting stroke outcome. However, no consensus in the literature currently exists concerning its utility for predicting post-stroke functional outcome and mortality.

Objective: To systematically review the effectiveness of plasma D-dimer levels for predicting functional outcome and mortality following stroke.

Methods: Five academic databases were screened according to PRISMA guidelines for eligible studies. With these studies, we conducted a random-effect meta-analysis to evaluate the impact of plasma D-dimer levels for predicting functional outcome and mortality post-stroke. We also conducted subgroup analyses to evaluate differences in predictive capacity for different stroke subtypes.

Results: Nineteen studies were included, containing data on 5,781 stroke patients (mean age: 65.26 ± 6.4 years). Overall methodological quality for the included studies was high. Meta-analysis showed that increased D-dimer levels were predictive of worsened functional outcomes (Hazard ratio: 2.19, 95% CI: 1.63–2.93) and elevated overall mortality (2.29, 1.35–3.88). Subgroup analysis showed that plasma D-dimer levels were more predictive of poorer functional outcomes for ischemic (2.08, 1.36–3.18) stroke as compared to intracerebral hemorrhage (2.62, 1.65–4.17). We also noted that predictive capacity was similar when it came to mortality in patients with cryptogenic ischemic stroke (2.65, 0.87–8.08) and intracerebral hemorrhage (2.63, 1.50–4.59).

Conclusion: The study provides preliminary evidence concerning the capacity of plasma D-dimer levels for predicting functional outcomes and mortality following stroke and reports that higher D-dimer levels of are associated with poorer functional outcomes and higher mortality.

Keywords: D-dimer, cerebrovascular accident, prognosis, morbidity, mortality

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INTRODUCTION

Stroke is the second most common cause of death or disability worldwide (1, 2). Characterized as a cerebrovascular accident that hampers blood flow resulting in brain damage (3), stroke accounts for almost 5.5 million deaths and 116.4 million disability-adjusted life-years per year (4, 5).

Brain structural damage in stroke patients occurs due to either blood vessel occlusion or intracerebral hemorrhage (6, 7). The resultant ischemic damage then initiates a signaling cascade that triggers excitotoxic and/or inflammatory mechanisms eventually resulting in cellular apoptosis (8). Studies suggest that hemodynamic restoration is the primary mode for limiting neural injury (9, 10). However, this approach does not completely eliminate morbidity and mortality (7, 11). As such, preemptive diagnosis is imperative and is widely recommended (12–16).

D-dimers, such as circulating fibrin-degradation products, have recently been shown to be critical for predicting short- and long-term stroke-related outcomes (12, 17, 18). The presence of D-dimers can be representative of total fibrin concentrations, thereby serving as a biomarker for intravascular fibrinolysis and intravascular thrombus formation (19, 20). For stroke patients, this biomarker can detect disrupted vessels, dissolved clots, and the release of stroke-related tissue factors. D-dimers also serve as a good biomarker because of its prolonged stability, half-life, cost-effectiveness, and high sensitivity (> 97%) (21–24).

To date, only a few individual retrospective cohort studies have attempted to evaluate whether plasma D-dimer levels can predict future functional outcomes and mortality post-stroke (25–28). These studies have not established a consensus here. While some studies reported a positive correlation between mortality and plasma D-dimer levels (29–32), others have reported weaker or no correlation (27, 33, 34). Similarly, there is also no consensus concerning whether D-dimer levels are predictive for overall functional outcome. Some studies noted that plasma D-dimer levels were related to worse

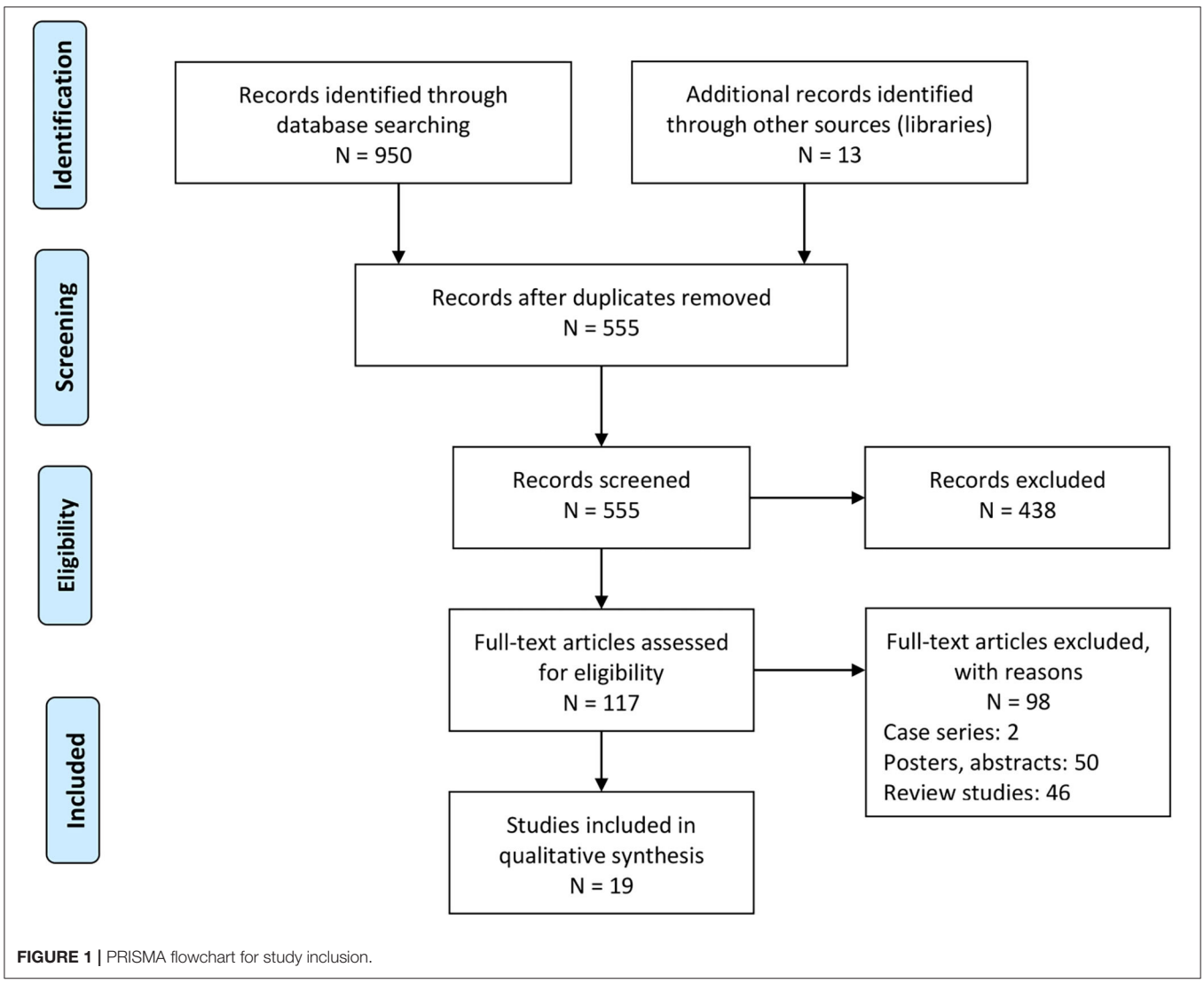

FIGURE 1 | PRISMA flowchart for study inclusion.

TABLE 1 | Study details.

References	Country	Type of study	Sample descriptive	Age (M ± S.D years)	Type of cerebrovascular stroke	D-dimer recorded	Assessment method of functional outcome	Follow-up functional outcome	D-dimer levels	Functional outcome (Hazard ratio, 95% CI, *p*-value)	Follow-up mortality	Mortality (Hazard ratio, 95% CI, *p*-value)
Hou et al. (46)	China	Prospective cohort study	10,518 (3,283F, 7,235M)	62.3 ± 11.4	Ischemic	At admission	Modified Rankin scale score ≥ 3	12 months	1.1 μg/mL	1.59 (1.32–1.91, <0.001)	–	–
Ye et al. (50)	China	Prospective cohort study	236 (91F, 145M)	70	Ischemic	At admission	Modified Rankin scale score > 2	1-month	0.45 mg/L	2.07 (1.49–2.88, <0.001)	–	–
Liu et al. (47)	China	Prospective cohort study	489	70.1 ± 11.9	Ischemic	–	–	6 months	1.83 ± 2.29 mg/L	–	–	3.06 (1.61–5.83, <0.001)
Sato et al. (48)	Japan	Prospective cohort study	130	–	Ischemic	At admission	Modified Rankin scale score ≥ 3	3 months	–	3.31 (1.14–9.61, <0.028)	–	–
Wang et al. (49)	China	Prospective cohort study	1,485 (997F, 488M)	63.9 ± 12.7	Ischemic	At admission	Modified Rankin scale score ≥ 3	3 months	0.93 ± 45.8 mg/L	2.93 (1.91–4.50, <0.0001)	–	–
Zhou et al. (28)	China	Retrospective cohort study	1,332 (694F, 638M)	65 ± 14	Intracerebral	1-h post admission	Modified Rankin scale score ≥ 3	3 months	–	1.48 (1.08–2.06, 0.1)	3 months	2.01 (1.18–3.42, 0.1)
Hutanu et al. (35)	Romania	Retrospective cohort study	89	71.9 ± 10	–	At admission	Modified Rankin scale score ≥ 3	3 months	185.1 (185.06–245.06) ng/mL	8.3 (1.4–47.6, 0.01)	–	–
Nezu et al. (27)	Japan	Retrospective cohort study	295 (143F, 152M)	72 ± 13	Cryptogenic ischemic stroke	–	–	–	–	–	36 months	1.35 (0.74–2.5, 0.33)
Fukuda et al. (25)	Canada	Retrospective cohort study	187 (37F, 150M)	62.45	Aneurysm, subarachnoid hemorrhage, intracerebral, intraventricular	At admission	Modified Rankin scale score ≥ 3	3 months	–	1.5 (1.1–2.0, 0.003)	–	–
Liu et al. (26)	China	Retrospective cohort study	146 (89F, 57M)	57	Subarachnoid hemorrhage	At admission	Glasgow coma scale, world Federation of Neurosurgical Societies stage IV to V	6 months	–	2.67 (1.66–4.45, <0.01)	–	–
Hsu et al. (44)	Taiwan	Retrospective cohort study	347 (140F, 207M)	67.6 ± 13.1	Intracerebral	24-h post stroke	Modified Rankin scale score ≥ 3	3 months	–	1.9 (1.27–2.86, 0.002)	–	–

(Continued)

TABLE 1 | Continued

References	Country	Type of study	Sample descriptive	Age (M ± S.D years)	Type of cerebrovascular stroke	D-dimer recorded	Assessment method of functional outcome	Follow-up functional outcome	D-dimer levels	Functional outcome (Hazard ratio, 95% CI, *p*-value)	Follow-up mortality	Mortality (Hazard ratio, 95% CI, *p*-value)
Chen et al. (29)	Taiwan	Prospective cohort study	43 (14F, 29M)	56.6 ± 15	Intraventricular	At admission	–	–	43.1 ± 45.8 μg/mL	–	–	30 (3–295, 0.0006)
Kim et al. (32)	South Korea	Retrospective cohort study	570 (214F, 356M)	60.8 ± 13.6	Cryptogenic ischemic stroke	At admission	–	–	–	–	34.0 ± 22.8 months	4.28 (1.79 – 10.27, 0.001)
Hu et al. (33)	China	Retrospective cohort study	259 (98F, 161M)	58 ± 14	Subarachnoid hemorrhage, intracerebral, intraventricular	At admission	Modified Rankin scale score ≥ 3	3 months	–	2.72 (1.13–6.59, 0.02)	7 days	1.23 (1.01–1.50, 0.033)
Yang et al. (51)	China	Prospective cohort study	220 (93F, 127M)	68	Ischemic	At admission	Modified Rankin scale score ≥ 3	3 months	1.36 (0.55–3.11) mg/L	4.25 (1.93–9.28, 0.001)	–	–
Chiu et al. (30)	Taiwan	Retrospective cohort study	170	65.9 ± 12.6	Intracerebral	At admission	Glasgow coma scale ≥ 2	72 h	1,231.9 ± 1,595.5 ng/mL	–	30 days	2.72 (1.08–6.9, 0.002)
Krarup et al. (45)	Norway	Retrospective cohort study	449 (218F, 231M)	80	Ischemic	–	Scandinavian stroke scale ≥ 3	48 h	–	0.99 (0.97–1.01, 0.34)	–	–
Üstündag et al. (34)	Turkey	Retrospective cohort study	91 (49F, 42M)	64.5 ± 12.7	–	–	–	–	–	–	–	0.51 (0.32–0.79, 0.003)
Delgado et al. (31)	Spain	Retrospective cohort study	98 (35F, 63M)	61–80	Intracerebral	At admission	NIH Stroke Scale ≥ 4	48 h	1,780 (354–2,655) ng/mL	6.8 (1.2–36.9, 0.02)	3 months	8.7 (1.4–54.1, 0.02)

functional outcomes (26, 31, 33, 35), other have reported limited correlations (25, 28). To date, we have located one systematic review that attempted to evaluate the predictive capacity for plasma D-dimers (12). However, this review failed to include a meta-analysis. Moreover, since it was published in 2009, an update centered around the current evidence is strongly warranted. While a recently published meta-analysis did attempt to evaluate the prognostic impact of plasma D-dimer levels on mortality, it only contained two studies (17). We therefore, in this present systematic review and meta-analysis, attempt to evaluate the capacity for plasma D-dimer levels to predict post-stroke functional outcome and mortality.

METHODS

Data Search Strategy

The database search for this meta-analysis was done according to PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (36). Five databases (Web of Science, MEDLINE, CENTRAL, EMBASE, and Scopus) were screened for studies published prior to February 2021. The search was performed across a combination of MeSH keywords, including "D-dimer," "stroke," "intracerebral stroke," "ischemic stroke," "cryptogenic stroke," "subarachnoid stroke," "hemorrhage," "cerebrovascular disease," "cerebrovascular accident," "functional outcome," and "mortality." A sample search strategy for EMBASE database has been provided in **Supplementary Table 1**. References cited in included studies were manually examined to identify further relevant hits. Study inclusion criteria were as follows:

a) Studies evaluating the impact of D-dimer levels in population groups following stroke.
b) Studies evaluating functional outcome and mortality outcome.
c) Studies investigating human participants.
d) Case-control studies, prospective trials, or retrospective cohort trials.
e) Studies published in peer-reviewed scientific journals.
f) Studies published in English.

Study screening and data collection was independently conducted by two reviewers. The extraction of data was done manually while using Microsoft excel. In cases of disagreements concerning eligibility of studies, discussions were held with a third independent reviewer. Moreover, in conditions where required data was not mentioned in the included studies, repeated attempts were made to contact respective corresponding authors for additional data. We extracted the following data from the included studies: author information, country of research, type of study, descriptive data of the sample, type of cerebrovascular incident, baseline D-dimer levels, functional outcomes, and mortality outcomes.

Quality Assessment

Risk of bias appraisal for included studies was performed using Cochrane's risk of bias assessment tool for non-randomized controlled trials (37). This tool evaluates study outcomes for possible selective reporting, confounding bias, measurement of outcomes, and incomplete data availability. Appraisal was carried out by two reviewers, with a third reviewer called in to arbitrate in case of disagreement. In addition, we also assessed the overall level of evidence presented in the literature by using Oxford Centre for Evidence Based Medicine tool (38).

Data Analysis

This study performed a within-group meta-analysis using Comprehensive Meta-analysis (CMA) software version 2.0 (39). This meta-analysis was conducted based on a random-effects model (40). Hazard ratios were calculated to determine the impact of D-dimer levels on functional outcomes and mortality following stroke. Heterogeneity among studies was assessed using I^2 statistics (0–25%: negligible heterogeneity, 25–75%: moderate heterogeneity, and ≥75%: substantial heterogeneity) (41). To ensure clinical heterogeneity we also carried out subgroup analyses on the basis of stroke subtypes i.e., intracerebral hemorrhage, subarachnoid hemorrhage, central nervous system infarction (including ischemic stroke and silent infarction). Besides, we also carried out subgroup analyses for two studies reporting the outcomes of cryptogenic ischemic stroke (i.e., a subtype of ischemic stroke). In the included studies cryptogenic ischemic stroke was defined as per the TOAST criteria which defines it as a brain infarction that is not attributable to a definite cardioembolism, large artery atherosclerosis, or small artery disease despite extensive vascular, cardiac, and serologic evaluation (42). Publication bias was evaluated using Duval and Tweedy's trim and fill procedure (43), which examines publication bias by adding studies on either side of the plotted graph. The significance level for this study was determined at 5%.

RESULTS

Database screening yielded 950 studies, while manual screening added another 13 to this total. After applying inclusion criteria, 19 studies remained (**Figure 1**). Thirteen of these were retrospective cohort studies (25–28, 30–35, 44, 45), while the other six were prospective cohort studies (29, 46–50). Relevant data from each study was extracted and tabulated (**Table 1**).

Participant Information

The 19 included studies featured data from 5,781 total patients (2,821 females and 2,701 males). Four studies did not report gender distributions (30, 35, 47, 48). Average patient age was 65.26 ± 6.4 years, with one study reporting age as only a range (31) and one omitting age altogether (48).

Quality Assessment for Included Non-randomized Controlled Trials

Risk of methodological bias for the included non-randomized controlled trials was assessed with the ROBINS-I tool (**Table 2**). Overall risk among the included studies was low, with missing data, selection of reported results, and selection bias the most prominent aspects (**Figure 2**). We also found that the overall level of evidence according to the Oxford Centre for Evidence Based Medicine to be 2b.

TABLE 2 | Risk of bias according to Cochrane's risk of bias assessment tool for included non-randomized controlled trials.

References	Confounding bias	Selection bias	Deviation from intended intervention	Missing data	Measurement in outcome	Selection of reported result	Classification of intervention	Level of evidence
Hou et al. (46)	+	+	+	?	+	−	+	2b
Ye et al. (50)	+	+	+	?	+	−	+	2b
Liu et al. (47)	+	+	+	?	+	−	+	2b
Sato et al. (48)	+	−	+	?	+	−	+	2b
Wang et al. (49)	+	+	?	+	+	?	+	2b
Zhou et al. (28)	+	+	+	+	+	+	+	2b
Hutanu et al. (35)	+	−	+	+	?	−	+	2b
Nezu et al. (27)	+	?	+	−	+	?	+	2b
Fukuda et al. (25)	+	?	+	−	+	?	+	2b
Liu et al. (26)	+	−	+	?	+	−	+	2b
Hsu et al. (44)	+	?	+	?	+	+	+	2b
Chen et al. (29)	+	?	+	−	+	?	+	2b
Kim et al. (32)	+	?	+	+	+	+	+	2b
Hu et al. (33)	+	+	+	+	+	+	−	2b
Yang et al. (51)	+	+	+	+	+	+	+	2b
Chiu et al. (30)	+	?	+	+	+	+	+	2b
Krarup et al. (45)	+	?	+	−	+	?	+	2b
Üstündag et al. (34)	+	−	+	+	+	−	−	2b
Delgado et al. (31)	+	−	+	+	+	−	+	2b

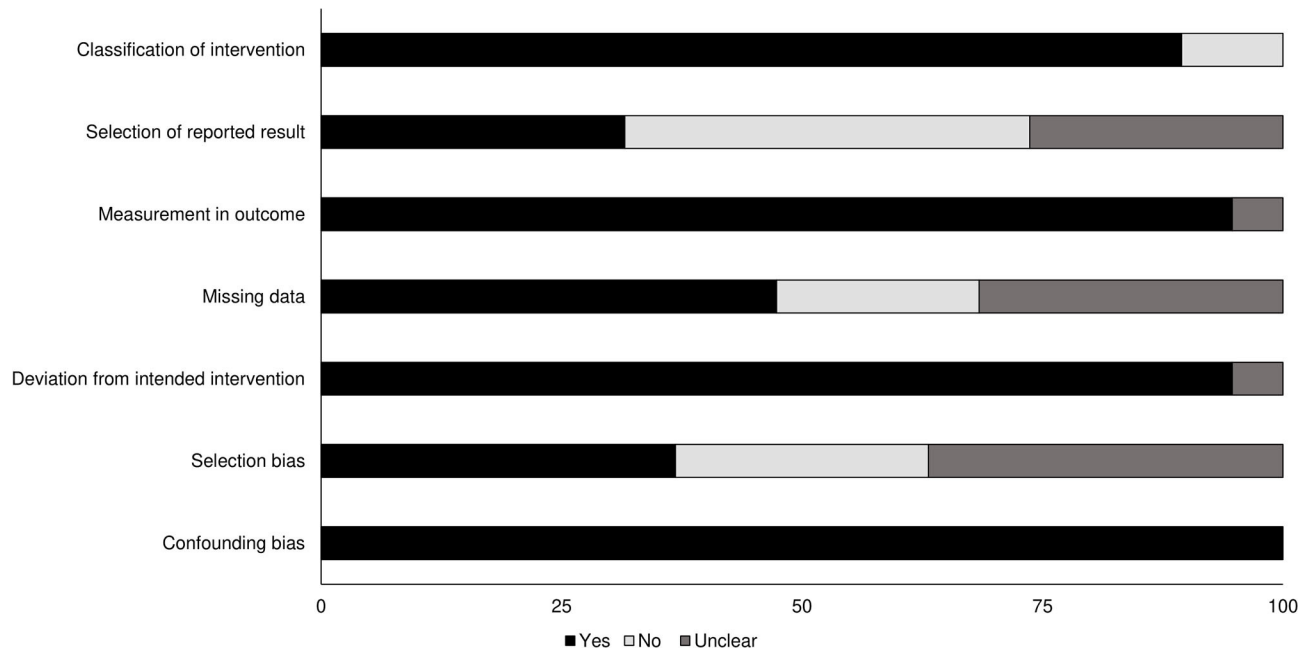

FIGURE 2 | Risk of bias for non-randomized controlled trials according to the Cochrane risk of bias assessment.

Publication Bias

Duval and Tweedy's trim and fill method was used to determine if studies were missing from either side of the mean effect. The method observed that six studies were missing on the left side of the mean effect. The overall random effects model determined point estimates and 95% confidence intervals for all studies combined as 2.13 (95% CI: 1.69–2.67). Imputed point estimate using

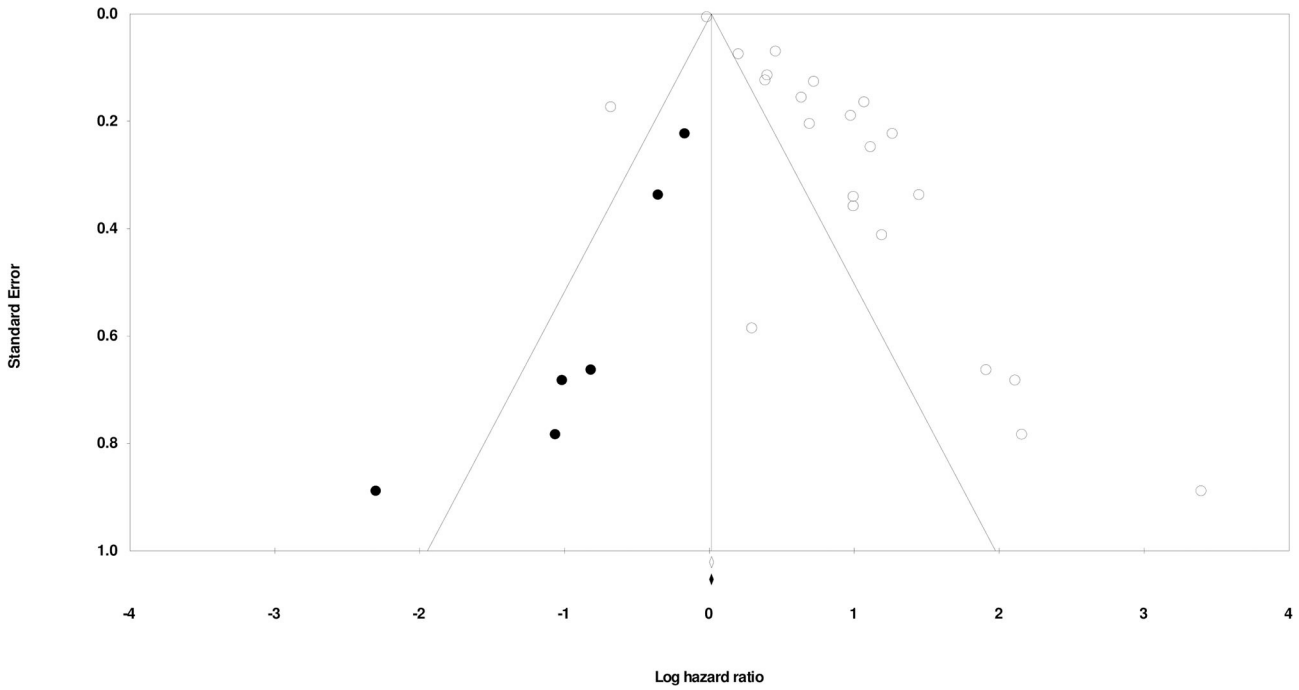

FIGURE 3 | Publication bias as determined using Duval & Tweedy's trim and fill method.

the trim and fill method was 1.74 (95% CI: 1.41–2.15) (**Figure 3**).

Meta-analysis Report

Functional Outcomes

Thirteen studies examined the impact of D-dimer levels on post-stroke functional outcome (25, 26, 28, 31, 33, 35, 44, 49, 51). Hazard ratio was 2.19 (95% CI: 1.63–2.93, $p < 0.001$) with no heterogeneity (I^2: 0%) (**Figure 4**).

Further subgroup analysis for functional outcome post-stroke was carried out to examine the effect of stroke type. Six studies reported functional outcomes for patients with ischemic stroke (Hazard ratio: 2.08, 95% CI: 1.36–3.18, $p = 0.001$; I^2: 0%; **Figure 5**) while three included studies evaluated outcomes for intracerebral hemorrhage patients with negligible heterogeneity (Hazard ratio: 2.62, 95% CI: 1.65–4.17, $p = 0.001$; I^2: 23.52%; **Figure 6**).

We also conducted two subgroup analyses based on different follow-up periods and assessment methods. Firstly, we identified only six studies that had reported a uniform follow-up of 3 months and they had used modified rankin scale for assessing functional outcome. We observed increased mortality outcomes for patients with moderate heterogeneity (Hazard ratio: 2.08, 95% CI: 1.53–2.84, $p < 0.001$; **Figure 7**; I^2:31.1%). Secondly, we identified two studies that had reported a uniform follow-up of 2 months and they had also used modified rankin scale for assessing functional outcome. We observed increased mortality outcomes for patients with no heterogeneity (Hazard ratio: 3.28, 95% CI: 2.27–4.74, $p < 0.001$; **Figure 8**; I^2:0%).

Mortality Outcomes

Nine studies evaluated the impact of D-dimer levels on post-stroke mortality (26–34). A hazard ratio of 2.29 (95% CI: 1.35–3.88, $p = 0.002$, **Figure 9**) was observed, with moderate heterogeneity (I^2: 39.03%).

Further subgroup analysis for overall mortality was carried out examining the impact of stroke type. Two studies reported mortality outcomes for patients with cryptogenic ischemic stroke (Hazard ratio: 2.65, 95% CI: 0.87–8.08, $p = 0.08$; **Figure 10**; I^2: 0%) while three included studies evaluated mortality outcomes for intracerebral hemorrhage patients with negligible heterogeneity (Hazard ratio: 2.63, 95% CI: 1.50–4.59, $p = 0.001$; **Figure 11**; I^2: 18.8%).

We also conducted subgroup analyses based on different follow-up periods. Here, we identified only two studies that had reported a uniform follow-up of 3 months. We observed increased mortality outcomes for patients (Hazard ratio: 3.43, 95% CI: 0.86–13.71, $p = 0.08$; **Figure 12**; I^2: 0%).

DISCUSSION

This systematic review and meta-analysis suggest that poorer functional outcome and increased mortality incidence following stroke is associated with increased plasma D-dimer levels. We also noted that the association between plasma D-dimer levels and functional outcomes was stronger for ischemic stroke than intracerebral hemorrhage. However, plasma D-dimer predictive capacity for mortality between patients with cryptogenic ischemic stroke and intracerebral hemorrhage was similar.

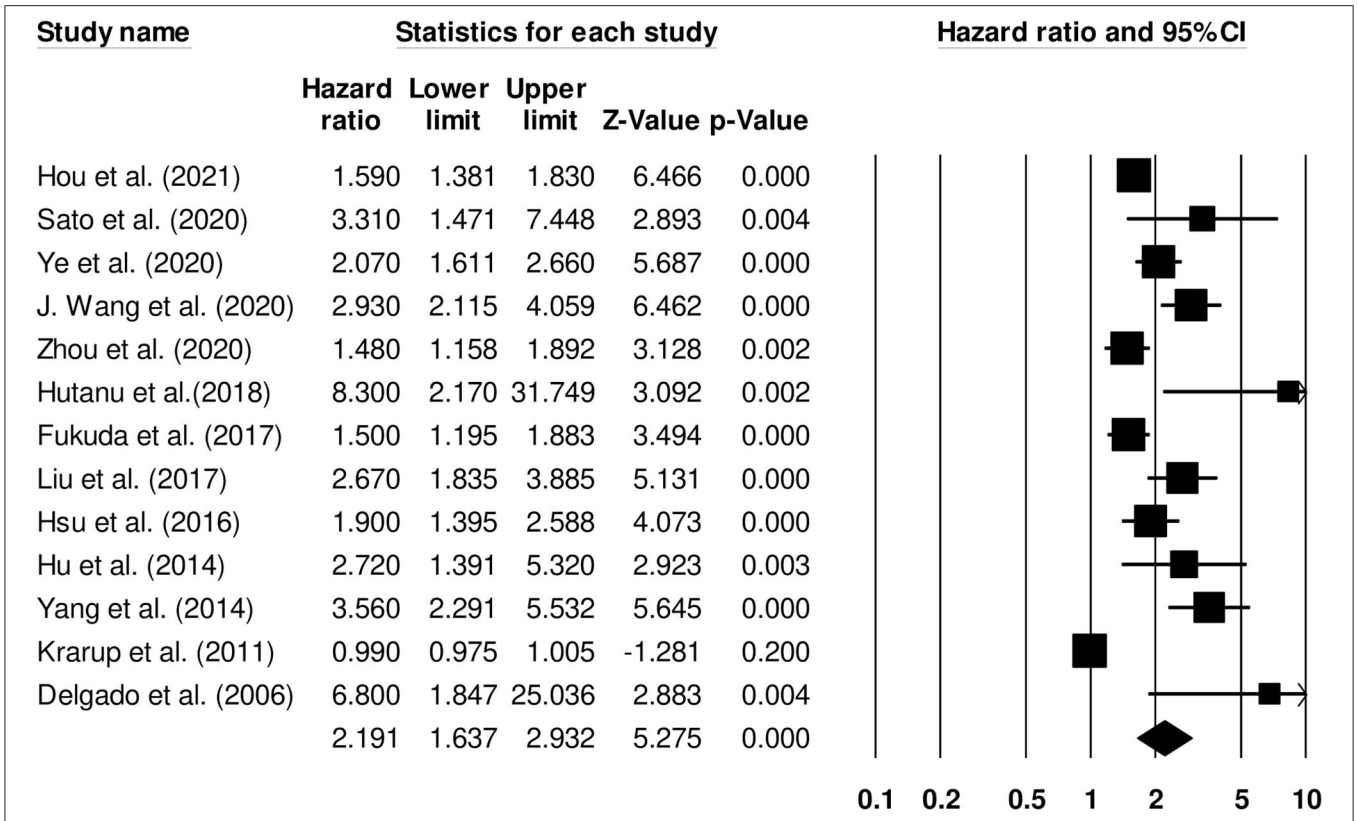

FIGURE 4 | Forest plot for studies evaluating the impact of D-dimer level on post-stroke functional outcomes. Hazard ratios are presented as black boxes while 95% confidence intervals are presented as whiskers. A small hazard ratio represents a lower influence of D-dimer levels on stroke patient functional outcome while a higher hazard ratio represents a higher influence.

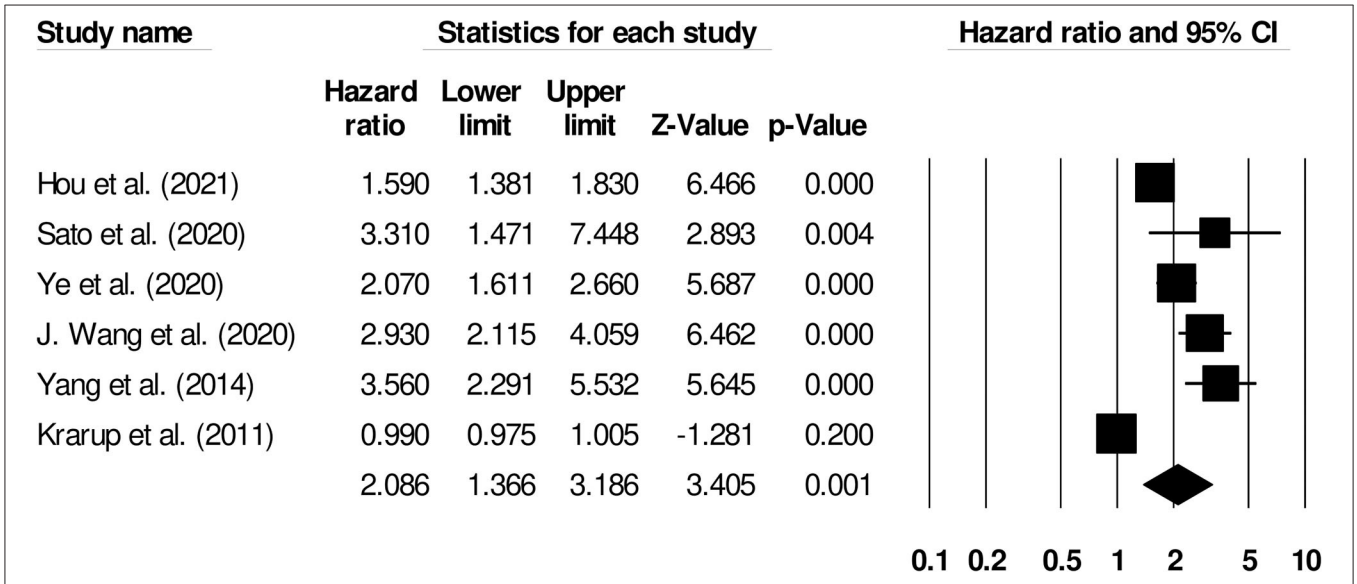

FIGURE 5 | Forest plot for studies evaluating the impact of D-dimer level on post-ischemic stroke functional outcomes. Hazard ratios are presented as black boxes while 95% confidence intervals are presented as whiskers. A small hazard ratio represents a lower influence of D-dimer levels on stroke patient functional outcome while a higher hazard ratio represents a higher influence.

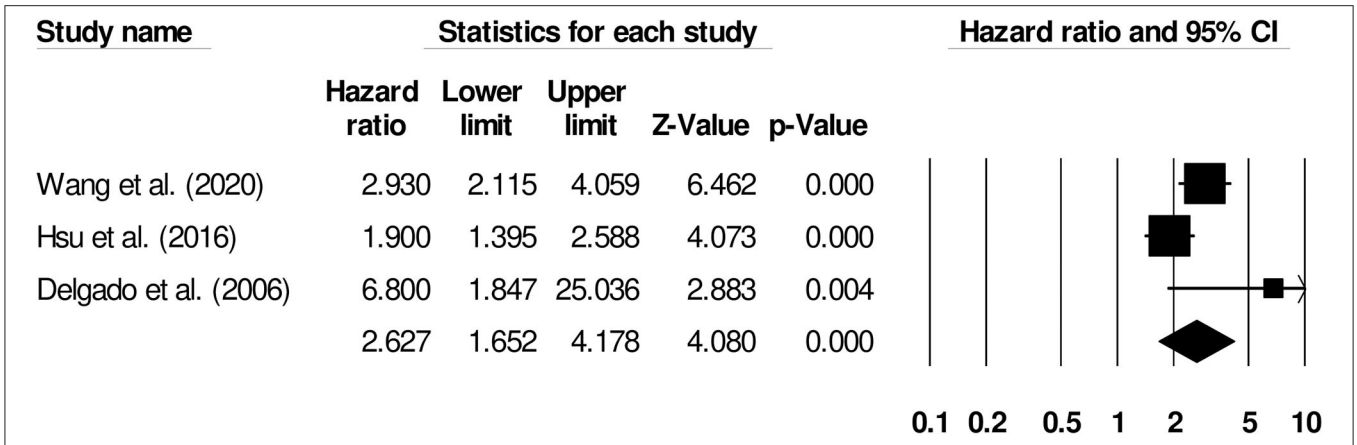

FIGURE 6 | Forest plot for studies evaluating the impact of D-dimer level on post-intracerebral hemorrhage functional outcomes. Hazard ratios are presented as black boxes while 95% confidence intervals are presented as whiskers. A small hazard ratio represents a lower influence of D-dimer levels on stroke patient functional outcome while a higher hazard ratio represents a higher influence.

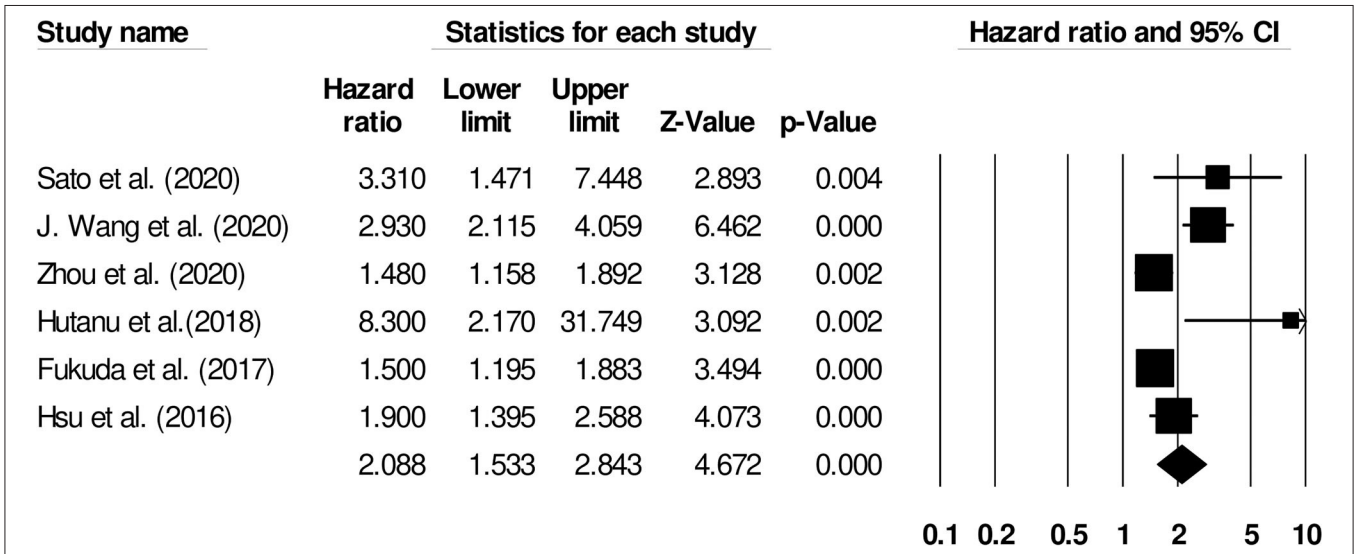

FIGURE 7 | Forest plot for studies evaluating the impact of D-dimer level on post-stroke functional outcomes for 3 months follow up. Hazard ratios are presented as black boxes while 95% confidence intervals are presented as whiskers. A small hazard ratio represents a lower influence of D-dimer levels on stroke patient functional outcome while a higher hazard ratio represents a higher influence.

Stroke management is challenging because of its atypical pathophysiology, poor prognosis, and heterogeneous manifestation (52, 53). In this light, preemptive prediction through biomarker detection has been widely recommended (54–56). Plasma D-dimer levels has been identified as a biomarker that was sensitive and specific for predicting short- and long-term functional outcomes, recurrence, and mortality post-stroke (12, 57). Johnson et al. (19) reported that D-dimer levels are indirectly indicative of hemostasis and thrombosis incidence. Furthermore, plasma D-dimers levels can be used to categorize increased risk for thromboembolic disorders (57, 58). Elevated plasma D-dimers could potentially boost interleukin-1 and 6 production (17, 59) precipitating worsened prognostic outcome following stroke (60). Nonetheless, despite pertaining several positive aspects, the routine use of plasma D-dimer in the current medical setting is complicated by its non-specificity. For instance, the plasma D-dimer levels are also susceptible to different inflammatory states, presence of infection, cancer, and venous thromboembolism (58, 61, 62). Therefore, the presence of a high plasma D-dimer at times could serve as a false positive with respect to stroke. Moreover, the clinical utility of plasma D-dimer is also limited perhaps because of limited clinical awareness this biomarker has in a stroke setting (i.e., plasma D-dimer evaluation not routinely demanded) (63).

This systematic review observed that plasma D-dimer levels could predict post-stroke functional outcome. These findings

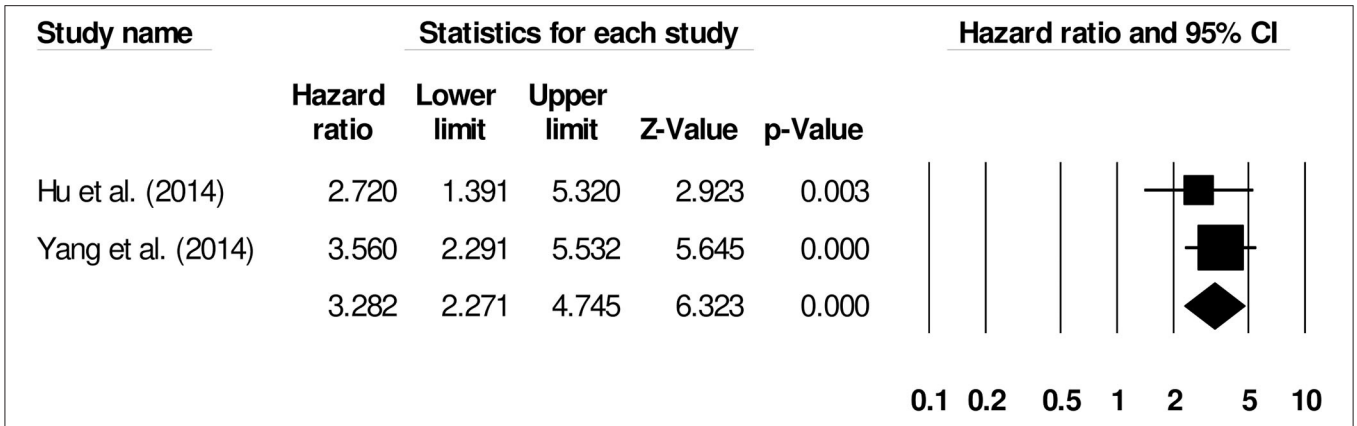

FIGURE 8 | Forest plot for studies evaluating the impact of D-dimer level on post-intracerebral hemorrhage functional outcomes for 2 months follow up. Hazard ratios are presented as black boxes while 95% confidence intervals are presented as whiskers. A small hazard ratio represents a lower influence of D-dimer levels on stroke patient functional outcome while a higher hazard ratio represents a higher influence.

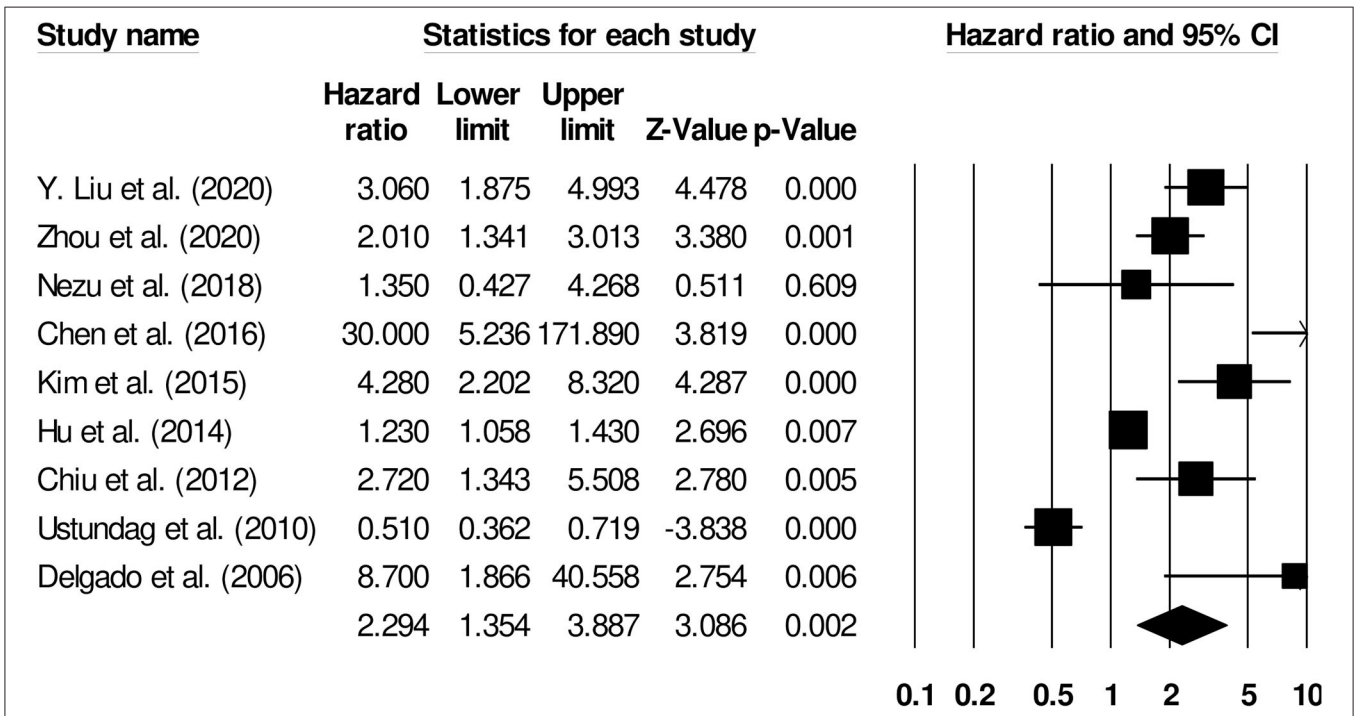

FIGURE 9 | Forest plot for studies evaluating the impact of D-dimer level on post-stroke mortality outcomes. Hazard ratios are presented as black boxes while 95% confidence intervals are presented as whiskers. A small hazard ratio represents a lower influence of D-dimer levels on stroke patient functional outcome while a higher hazard ratio represents a higher influence.

are aligned with other studies. Zhou et al. (28) showed that elevated plasma D-dimer levels measured 1-h post-hospital admission could predict poor 3-month functional outcomes for stroke patients with high precision and developed a scoring system for clinical practice. Furthermore, Hutanu et al. (35) found that plasma D-dimers could independently predict poor functional outcome in ischemic stroke patient outcomes whereas plasma c-reactive protein, neutrophil gelatinase associated lipocalin, the soluble receptor of tumor necrosis factor alfa, and neuron specific enolase could not.

We also examined the ability of plasma D-dimer levels to predict post-stroke mortality. The majority of included studies noted that plasma D-dimer levels were predictive for mortality. Hu et al. (33), for instance, noted that plasma D-dimer levels reliably predicted 7-day mortality with almost 88% sensitivity and 68% specificity—albeit the authors did note that plasma D-dimers were not as efficient as the standard Glasgow Coma

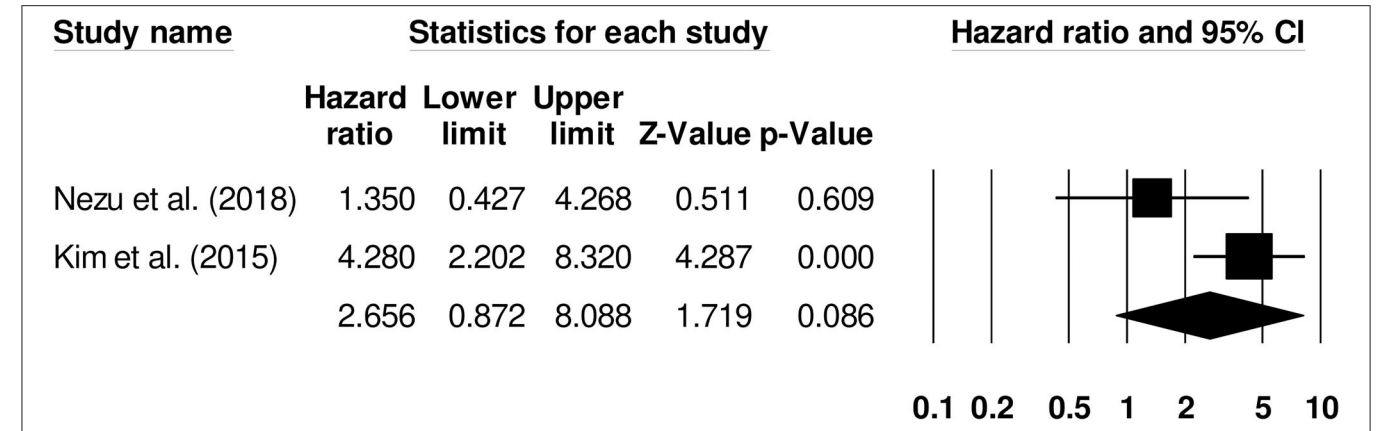

FIGURE 10 | Forest plot for studies evaluating the impact of D-dimer level on post-cryptogenic ischemic stroke mortality outcomes. Hazard ratios are presented as black boxes while 95% confidence intervals are presented as whiskers. A small hazard ratio represents a lower influence of D-dimer levels on stroke patient functional outcome while a higher hazard ratio represents a higher influence.

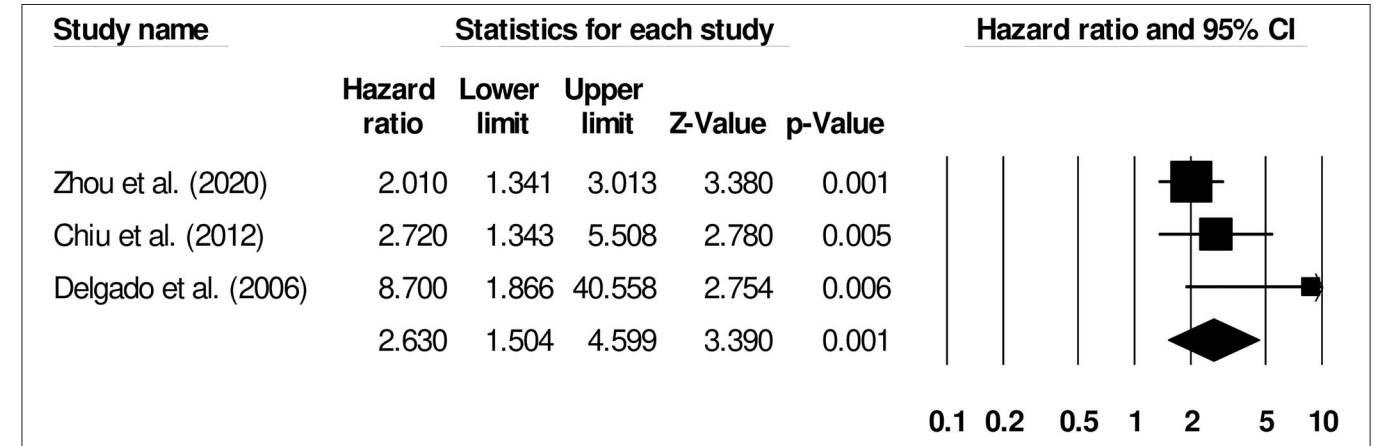

FIGURE 11 | Forest plot for studies evaluating the impact of D-dimer level on post-intracerebral hemorrhage mortality outcomes. Hazard ratios are presented as black boxes while 95% confidence intervals are presented as whiskers. A small hazard ratio represents a lower influence of D-dimer levels on stroke patient functional outcome while a higher hazard ratio represents a higher influence.

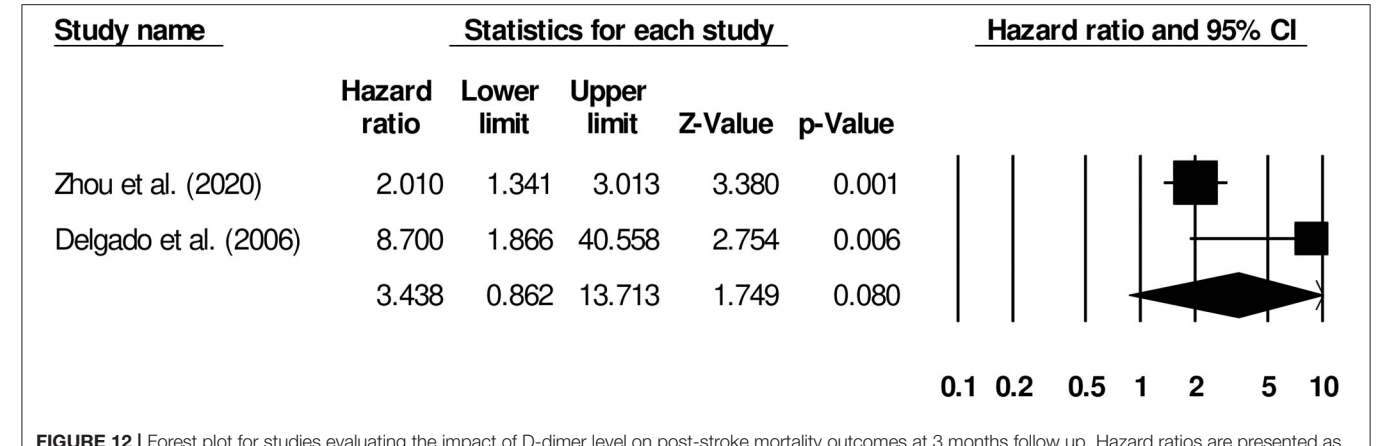

FIGURE 12 | Forest plot for studies evaluating the impact of D-dimer level on post-stroke mortality outcomes at 3 months follow up. Hazard ratios are presented as black boxes while 95% confidence intervals are presented as whiskers. A small hazard ratio represents a lower influence of D-dimer levels on stroke patient functional outcome while a higher hazard ratio represents a higher influence.

scale. Similarly, Nezu et al. (27) reported that plasma D-dimer levels recorded at admission not only correlated with the National Institute of Health Stroke Scale but also with mortality. It is possible that high plasma D-dimer levels may be predictive of post-stroke mortality because it can also capture conditions such as venous thrombus, malignancy, or atrial fibrillation (64). In a novel study, Chen et al. (29) found that cerebrospinal fluid D-dimer levels were highly sensitive (88%) and specific (81%) for predicting 30-day mortality in stroke patients. The authors suggest that cerebrospinal D-dimer levels could be used reliably in patients with intracerebral or intraventricular hemorrhage. Besides, in the subgroup analyses of mortality, we observed that the risks of mortality were higher for patients with cryptogenic ischemic stroke (i.e., 2.65) when compared with the overall analyses (i.e., 2.19). In our opinion, this difference could perhaps be attributed to the small number of studies included in the subgroup analysis of cryptogenic ischemic stroke (i.e., two studies).

This study is hampered by a few limitations. This study is not pre-registered in a systematic review repository such as PROSPERO York or the Joanna Briggs Institute (65). This was because the current COVID-19 pandemic crisis has extended registration queues to over 1 year. Besides, this review does not provide a list of studies that were excluded with reasoning. This was a major flaw on our behalf, and we request future studies to address this limitation. Additionally, because of data paucity, we were unable to carry out sub-group analyses for two important parameters: the relationship between functional outcome and stroke type and the relationship between plasma D-dimer levels and short- and long-term functional outcomes. Similarly, there was a huge discrepancy in the sample sizes between the studies we included (i.e., 10,518 participants in Hou et al., and 43 participants in Chen et al.). Additionally, although we conducted subgroup analyses based on the specific follow-up periods and assessment methodologies (i.e., for functional outcomes), we were only able to include studies that reported follow-up at 3 and 2 months. Other studies for instance had reported a varied range of follow-up (i.e., at 12 months, 1 month, 48 h, 72 h) and because these were only singular studies, we could not conduct subgroup analyses for them. We presume that this could be an important source of heterogeneity in the analyses we conducted and could possibly incur bias in our results. We therefore recommend future studies to focus on these areas where there is a knowledge gap.

CONCLUSION

In conclusion, we provide preliminary 2b level of evidence concerning the capacity of plasma D-dimer levels for predicting stroke patient functional outcome and mortality. We show that increased plasma D-dimer levels are predictive of poorer functional outcomes and increased mortality. The findings from the present study may have wider implications in developing best practice guidelines for predicting post-stroke prognostic outcomes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

PZ designed the project. CW and JW were involved in data collection and data analysis. SZ prepared the manuscript. JW edited the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

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Eosinophil-to-Neutrophil Ratio Predicts Poor Prognosis of Acute Ischemic Stroke Patients Treated With Intravenous Thrombolysis

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Background and Purpose: The eosinophil-to-neutrophil ratio (ENR) was recently reported as a novel inflammatory marker in acute ischemic stroke (AIS). However, few studies reported the predictive value of ENR in AIS patients, especially for those with intravenous thrombolysis.

Methods: Two hundred sixty-six AIS patients receiving intravenous thrombolysis were retrospectively recruited in this study and followed up for 3 months and 1 year. The Modified Rankin Scale (mRS) and the time of death were recorded. Poor outcome was defined as mRS 3–6. After excluding patients who were lost to follow-up, the remaining 250 patients were included in the 3-month prognosis analysis and the remaining 223 patients were included in the 1-year prognosis analysis.

Results: ENR levels in the patients were lower than those in the healthy controls. The optimal cutoff values for the ability of ENR $\times 10^2$ to predict 3-month poor outcome were 0.74 with 67.8% sensitivity and 77.3% specificity. Patients with ENR $\times 10^2 \geq 0.74$ have a lower baseline National Institutes of Health Stroke Scale (NIHSS) score (median: 7 vs. 11, $p < 0.001$). After multivariate adjustment, patients with ENR $\times 10^2 \geq 0.74$ were more likely to come to a better 3-month outcome (OR = 0.163; 95% CI, 0.076–0.348, $p < 0.001$). At the 1-year follow-up, the patients with ENR $\times 10^2 \geq 0.74$ showed a lower risk of mortality (HR = 0.314; 95% CI, 0.135–0.731; $p = 0.007$).

Conclusions: A lower ENR is independently associated with a 3-month poor outcome and a 3-month and 1-year mortality in AIS patients treated with intravenous thrombolysis.

Keywords: ischemic stroke, inflammation, prognosis, thrombolysis, eosinophil-to-neutrophil ratio

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INTRODUCTION

Stroke is one of the leading causes of mortality and morbidity worldwide (1). Intravenous thrombolysis with recombinant tissue plasminogen activator (r-tPA) was recommended for acute ischemic stroke (AIS) patients within 4.5 h of stroke onset, and an increasing trend of r-tPA treatment was discovered over the past 13 years (2). However, there were still nearly half of patients who went into major disability or died after 3 months of stroke onset. Hence, it was vital to find an accurate and concise prognostic marker to better distinguish patients who have a higher risk for poor outcome.

A strong neuro-inflammatory response is characteristic of ischemic stroke (3). Neutrophil plays an important role in the vascular innate immune system, and its distribution was highly influenced by the administration r-tPA (4). A higher neutrophil level after r-tPA infusion is a predictive factor for parenchymal hemorrhage and poor function outcome of AIS (5). Another notable aspect of the acute inflammatory response involves a sustained and rapid reduction of blood eosinophil count (6). A previous study reported that eosinopenia is associated with severe stroke and poor prognosis the day after admission (7). In addition, without concomitant eosinopenia, high neutrophil counts alone may not predict for a short-term risk of mortality of AIS patients (8), suggesting a potential interaction between eosinophils and neutrophils in ischemic stroke.

The eosinophil-to-neutrophil ratio (ENR) is a novel biomarker that was reported to be associated with in-hospital mortality of patients with chronic obstructive pulmonary disease (COPD) (9). A recent study reported that a neutrophil-to-eosinophil ratio represents systemic inflammation and a higher neutrophil-to-eosinophil ratio at admission is related to higher odds of in-hospital mortality in AIS patients (10). However, limited by the accuracy of the instrument, eosinophil count may show a number of 0 in some patients and excluding these patients could introduce some bias. Therefore, ENR may be a more stable biomarker than the neutrophil-to-eosinophil ratio. We performed this retrospective observational cohort study, aiming to analyze the predictive value of ENR for the 3-month and 1-year prognosis of AIS patients treated with r-tPA intravenous thrombolysis.

MATERIALS AND METHODS

Data Availability

The data that support the findings of this study are available from the corresponding author on reasonable request.

Study Population

The detailed selection criteria of the study patients are displayed in **Figure 1**. A total of 266 AIS patients who were treated with intravenous r-tPA (0.9 mg/kg body weight, maximum 90 mg, 10% of the dose as a bolus, followed by a 60-min infusion) from January 2016 to April 2019 at the Third Affiliated Hospital of Wenzhou Medical University and 2,196 healthy controls (HCs) were evaluated in this retrospective study. Patients were excluded if they have (1) a bridging therapy; (2) chronic inflammation; (3) immunology diseases; (4) tumor; (5) COPD or asthma; (6) parasitic infection, and (7) no full baseline data. We followed up each patient 3 months and 1 year after AIS onset. After excluding patients lost to follow-up, the remaining 250 patients were included in the 3-month prognosis analysis and the remaining 223 patients were included in the 1-year prognosis analysis. This study was approved by the Ethics Committee of the Third Affiliated Hospital of Wenzhou Medical University and was carried out in accordance with the Declaration of Helsinki.

Data Collection

Information of HCs was obtained from electronic examination reports. As for patients, the demographic data (age, sex) and medical history (smoking, hypertension, diabetes hyperlipidemia, atrial fibrillation, and prior stroke) were obtained from medical records. National Institutes of Health Stroke Scale (NIHSS) scores on admission and stroke subtypes were evaluated by experienced clinicians. Blood samples were collected on 24 h of admission. ENR was calculated using eosinophil counts divided by neutrophil counts. At 3 months and 1 year after onset of AIS, the prognoses of patients were assessed through telephone follow-up by two clinicians.

Diagnostic criteria

The etiology of AIS was classified on the basis of the Trial of Org 10,172 in Acute Stroke Treatment (TOAST) criteria: cardioembolic, atherosclerotic, small vessel or lacunar, and cryptogenic or others (11). Stroke severity was assessed using the NIHSS score. A good function outcome was defined as mRS scores of 0–2 while a poor function outcome was defined as mRS scores of 3–6. Outcomes included poor functional outcome and all-cause mortality. A 3-month poor function outcome was regarded as the primary outcome.

Statistical Analysis

Statistical analyses were performed *via* SPSS Statistics 24.0 software (SPSS Inc., Chicago, IL, USA), R version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria), and MedCalc Statistical Software version 15.2.2 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2015). Continuous variables were expressed as medians and interquartile range while categorical variables were expressed as frequencies and percentage. The intergroup difference of continuous variables was compared using the Mann–Whitney *U*-test. The chi-square test was performed for categorical variables. ENR levels between HCs and AIS patients were compared before and after age and sex matching. In AIS patients, the optimal cutoff value of ENR to predict the 3-month poor outcome was determined using receiver operating characteristic (ROC) curve analyses, and then patients were divided into a high-ENR group (ENR $\times 10^2 \geq 0.74$) and a low-ENR group (ENR $\times$ 10^2 $<$ 0.74). Univariate and multivariable logistic analyses were performed to estimate the association between ENR and AIS outcomes where variables with a $p < 0.10$ in univariate analysis were entered in the multivariable model. In addition, restricted cubic splines with four knots (at the 5th, 35th, 65th, and 95th percentiles) were performed to further

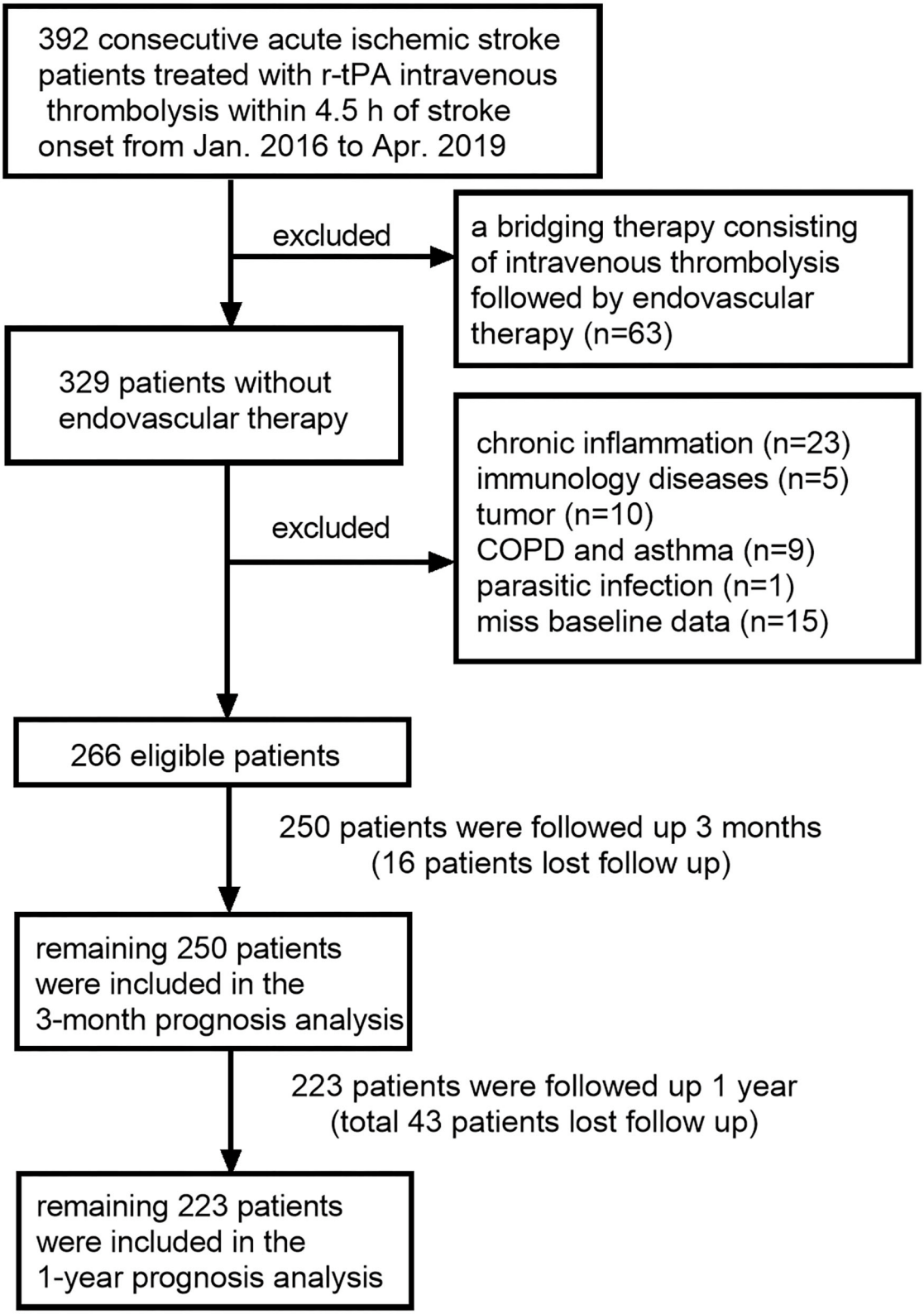

FIGURE 1 | Flowchart for patient selection.

investigate the relationship between ENR and AIS outcomes. C-statistics, net reclassification index (NRI), and integrated discrimination improvement (IDI) were employed to assess the incremental predictive ability of ENR. For clinical practice, 1-year mortality was presented graphically using Kaplan–Meier curves and we used log-rank tests to compare survival between high-ENR group and low-ENR group. Cox regression was used to determine whether ENR is a significant predictor for 1-year mortality. Statistical significance was set at $p < 0.05$.

TABLE 1 | Demographic and laboratory characteristics of AIS patients and healthy controls.

	AIS	HCs	*p*-value
Before matching	$n = 266$	$n = 2196$	
Age (years)	70 (60–79)	37 (30–46)	<0.001
Sex (male, *n*.%)	166 (62.4)	888 (40.4)	<0.001
Neutrophil ($\times 10^9$/l)	5.30 (3.88–7.03)	3.14 (2.56–3.90)	<0.001
Eosinophil ($\times 10^9$/l)	0.06 (0.02–0.12)	0.10 (0.06–0.17)	<0.001
ENR × 10^2	1.19 (0.28–2.90)	3.16 (1.91–5.38)	<0.001
After matching	$n = 153$	$n = 153$	
Age (years)	62 (56–68)	61 (55–68)	0.799
Sex (male, *n*.%)	91 (59.5)	91 (59.5)	1.000
Neutrophil ($\times 10^9$/l)	5.10 (3.80–6.80)	2.94 (2.46–3.61)	<0.001
Eosinophil ($\times 10^9$/l)	0.06 (0.02–0.12)	0.11 (0.06–0.18)	<0.001
ENR × 10^2	1.43 (0.32–2.94)	3.43 (2.27–5.81)	<0.001

ENR, eosinophil-to-neutrophil ratio.

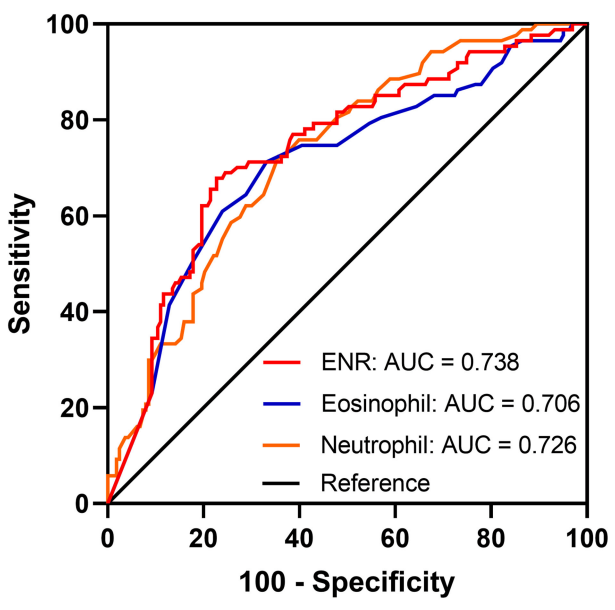

FIGURE 2 | Receiver operator characteristic curves for the prediction of 3-month poor outcome using neutrophil, eosinophil, and eosinophil-to-neutrophil ratio (ENR).

RESULTS

Baseline Characteristics of the Study Subjects

Among all enrolled subjects, 266 were AIS patients and 2,196 were HCs. The characteristics of the AIS patients and the HCs are displayed in **Table 1**. AIS patients were older, having a higher proportion of males than HCs. The higher level of neutrophil count and the lower level of eosinophil count led to lower ENR × 10^2 in AIS patients (1.19 [0.28–2.90] vs. 3.16 [1.91–5.38]; $p < 0.001$) compared to HCs. After matching of age and sex, ENR × 10^2 in AIS patients was still lower than that in HCs (1.43 [0.32–2.94] vs. 3.43 [2.27–5.81]; $p < 0.001$).

ENR Cutoff Points Distinguishing a 3-Month Poor Outcome

At the 3-month follow-up, 16 (6.0%) patients were lost to follow-up and the remaining 250 patients were included in the prognosis

TABLE 2 | Comparisons of baseline characteristics and 3-month outcomes between ENR groups.

Variable	ENR × 10^2 < 0.74 ($n = 96$)	ENR × 10^2 ≥ 0.74 ($n = 154$)	*p*-value
Demographic data			
Age, (years)	68 (59–80)	70 (60–77)	0.883
Sex, (male, *n*.%)	49 (51.0)	108 (70.1)	0.002
Stroke risk factors			
Current smoking, *n* (%)	17 (17.7)	39 (25.3)	0.160
Hypertension, *n* (%)	58 (60.4)	94 (61.0)	0.922
Diabetes, *n* (%)	14 (14.5)	32 (20.7)	0.219
Hyperlipidemia, *n* (%)	11 (11.4)	21 (13.6)	0.616
Atrial fibrillation, *n* (%)	35 (35.4)	38 (24.6)	0.046
Prior stroke, *n* (%)	11 (11.4)	15 (9.7)	0.665
Laboratory data			
Eosinophil, ($\times 10^9$/l)	0.01 (0–0.02)	0.10 (0.07–0.17)	<0.001
Neutrophil, ($\times 10^9$/l)	6.75 (5.25–8.98)	4.55 (3.40–5.62)	<0.001
ENR × 10^2	0.15 (0–0.47)	2.45 (1.46–3.75)	<0.001
Stroke subtype, *n* (%)			0.005
Cardioembolic	54 (56.2)	52 (33.7)	
Atherosclerotic	27 (28.1)	63 (40.9)	
Small vessel/lacunar	6 (6.2)	20 (12.9)	
Cryptogenic/others	9 (9.3)	19 (12.3)	
Onset to needle time (min)	163 (125–200)	150 (121–205)	0.270
Door to needle time (min)	60 (47–85)	58 (44–73)	0.206
Baseline NIHSS scores	11 (7–17)	7 (4–9)	<0.001
3-month mRS scores	3 (1–6)	1 (0–2)	<0.001

ENR, eosinophil-to-neutrophil ratio; NIHSS, National Institute of Health Stroke Scale; mRS, modified Rankin Scale. Among the eligible 266 patients, 16 patients were lost to follow-up and the remaining 250 patients were included in the 3-month prognosis analysis.

analysis. The optimal cutoff values of the ENR × 10^2 that best distinguished the 3-month poor outcome were 0.74 with 67.8% sensitivity and 77.3% specificity; the area under the curve (AUC) was 0.738 (95% CI = 0.679–0.792, $p < 0.001$). ENR had a better performance in discriminating patients at high risk and low risk of poor outcome than either eosinophil or neutrophil counts alone (AUC of eosinophil = 0.706; AUC of neutrophil = 0.726) (**Figure 2**). Patients were divided into a high-ENR group (n = 154) and a low-ENR group (n = 96) according to the ENR cutoff values. The median ENR × 10^2 was 2.45 in the high-ENR group and 0.15 in the low-ENR group. A significant higher proportion of male, eosinophil count, and percentage of atherosclerotic stroke and a significant lower percentage of atrial fibrillation, neutrophil count, percentage of cardioembolic stroke, baseline NIHSS score, and 3-month mRS scores were observed in the high-ENR group (**Table 2**).

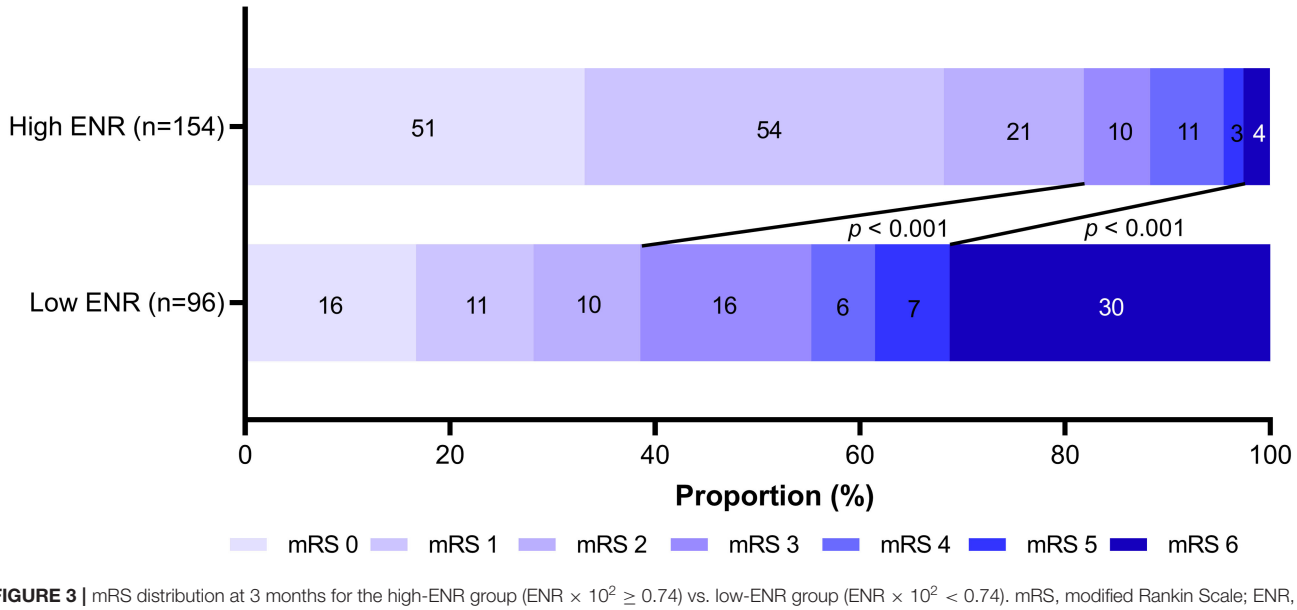

FIGURE 3 | mRS distribution at 3 months for the high-ENR group (ENR × $10^2 \geq 0.74$) vs. low-ENR group (ENR × $10^2 < 0.74$). mRS, modified Rankin Scale; ENR, eosinophil-to-neutrophil ratio.

TABLE 3 | Univariate and multivariate logistic regression analysis for 3-month poor outcome.

Variables	Univariate analysis		Model 1 + eosinophil		Model 1 + neutrophil		Model 1 + ENR	
	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
Age	1.059 (1.033–1.087)	<0.001	1.051 (1.016–1.087)	0.004	1.047 (1.013–1.084)	0.007	1.061 (1.024–1.101)	<0.001
Sex (male)	0.762 (0.446–1.300)	0.318						
Current smoking	0.380 (0.185–0.779)	0.008						
Hypertension	1.586 (0.918–2.739)	0.098						
Diabetes	0.785 (0.394–1.566)	0.492						
Hyperlipidemia	1.545 (0.728–3.280)	0.258						
Atrial fibrillation	1.879 (1.072–3.293)	0.028						
Prior stroke	2.017 (0.895–4.591)	0.090						
Baseline NIHSS score	1.260 (1.180–1.345)	<0.001	1.215 (1.131–1.305)	<0.001	1.197 (1.115–1.285)	<0.001	1.180 (1.096–1.271)	<0.001
Stroke subtype								
Cardioembolic	Reference							
Atherosclerotic	0.422 (0.233–0.764)	0.004						
Small vessel/lacunar	0.040 (0.005–0.318)	0.002						
Cryptogenic/others	0.415 (0.168–1.026)	0.057						
Eosinophil (per 0.01 increase)	0.922 (0.884–0.961)	<0.001	0.943 (0.900–0.987)	0.012				
Neutrophil	1.406 (1.237–1.598)	<0.001			1.391 (1.187–1.629)	<0.001		
ENR × 10^2 (≥0.74)	0.139 (0.078–0.249)	<0.001					0.163 (0.076–0.348)	<0.001

ENR, eosinophil-to-neutrophil ratio; NIHSS, National Institute of Health Stroke Scale.
Model 1 included age, current smoking, hypertension, atrial fibrillation, prior stroke, baseline NIHSS score, and stroke subtype.

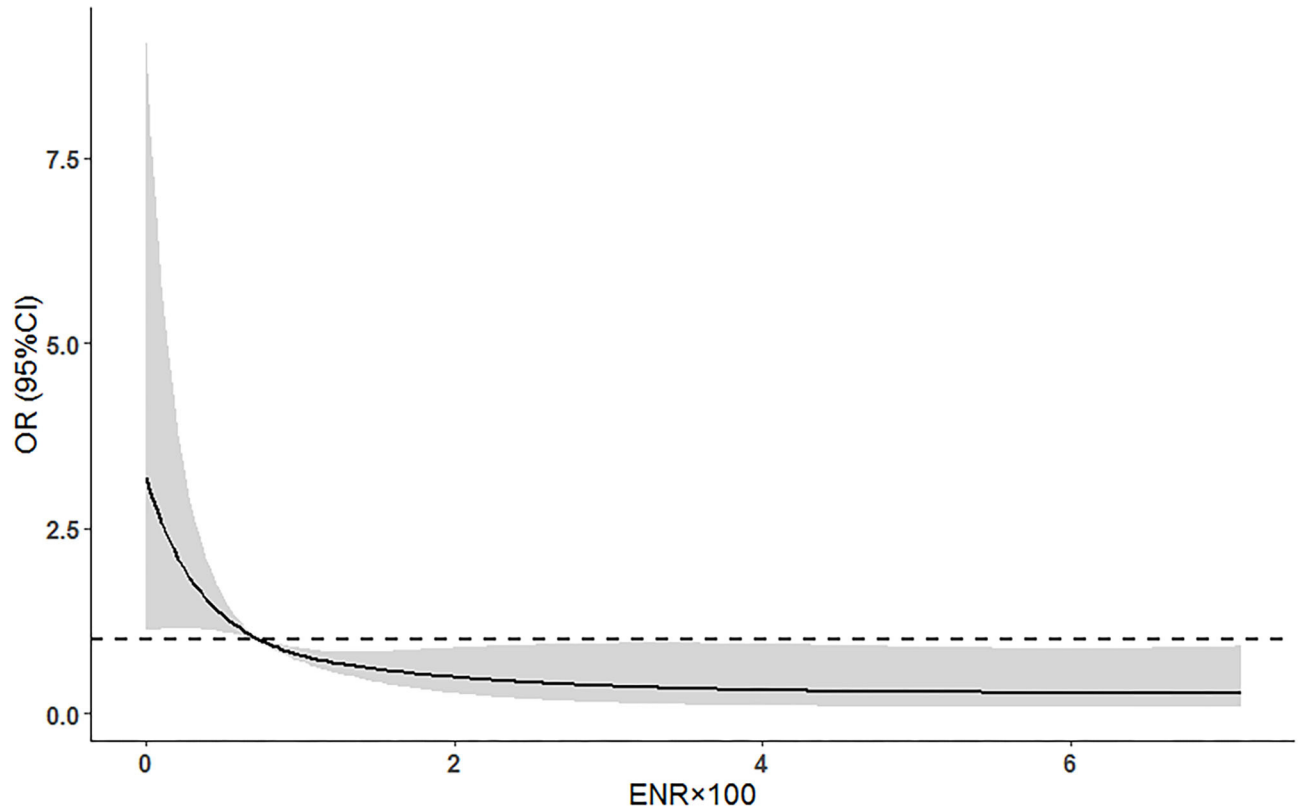

FIGURE 4 | Adjusted association of ENR with 3-month poor outcomes using multiple spline regression analyses with four knots (at the 5th, 35th, 65th, and 95th percentiles). The solid line indicates odds ratio while the shadow indicates 95% CIs. The dashed line is the reference line (odds ratio = 1). The reference of ENR was 0.74. Data were adjusted for age, current smoking, hypertension, atrial fibrillation, prior stroke, baseline NIHSS score, and stroke subtype. ENR, eosinophil-to-neutrophil ratio; NIHSS, National Institute of Health Stroke Scale.

Lower ENR Level Is Related to a 3-Month Poor Function Outcome

Among 250 AIS patients included in the 3-month prognosis analysis, 87 (34.8%) had poor function outcome. In this cohort, patients in the high-ENR group had a decreased 3-month poor outcome (28 [18.2%] vs. 59 [61.5%], $p < 0.001$) and mortality (4 [2.6%] vs. 30 [31.3%], $p < 0.001$; **Figure 3**) compared to those in the low-ENR group. After adjusting for potential confounders (age, current smoking, hypertension, atrial fibrillation, prior stroke, baseline NIHSS score, and stroke subtype), multivariate logistic regression showed that high neutrophil and low eosinophil are two independent risk factors for poor 3-month function outcome (**Table 3**). High ENR (ENR $\times$ $10^2 \geq 0.74$) was independently associated with 3-month function outcome (OR = 0.163, 95% CI 0.076–0.348, $p < 0.001$) and mortality (HR = 0.107, 95% CI 0.030–0.386, $p = 0.001$). Besides, the ENR as a continuous variable was also inversely associated with 3-month poor outcome (per one-point increase of ENR $\times$ 10^2, OR = 0.704, 95% CI 0.560–0.885, $p = 0.003$). In a multivariate logistic regression model with restricted cubic splines, the elevated ENR level was associated with lower odds of 3-month poor outcome (p overall association <0.001; **Figure 4**).

Secondary Analysis for the Primary Outcome

Sensitivity analyses were employed to test the robustness of our results. The association between ENR and poor 3-month outcome was significant in AIS patients admitted to the hospital during 2016–2017, AIS patients admitted to the hospital during 2018–2019, cardioembolic AIS patients, and non-cardioembolic AIS patients. In addition, these associations were highly robust across the range of decile ENR cutoffs. A higher ENR was associated with significantly better 3-month function outcomes for decile cutoffs from the 20th to 80th percentiles (**Table 4**). C-statistics, NRI, and IDI were used to verify whether adding ENR to a model containing conventional risk factors could improve the risk stratification of the poor 3-month outcome. Results show that the discriminatory ability of the model for primary outcome significantly improved after adding the ENR (AUC improved by 0.036, $p = 0.024$; NRI 86.71%, $p < 0.001$; IDI 7.92%, $p < 0.001$; **Table 5**).

Survival Analysis of ENR Levels and the 1-Year Prognosis

At the 1-year follow-up, 43 (16.2%) patients were lost to follow-up and the remaining 223 patients were included in the

TABLE 4 | OR (95% CI) of poor 3-month outcomes according to ENR: sensitivity analysis.

	OR (95% CI)	*p*-value
High ENR vs. low ENR (cutoff = 0.74)		
Patients from 2016 to 2017	0.277 (0.110–0.699)	0.007
Patients from 2018 to 2019	0.043 (0.008–0.217)	<0.001
Excluded cardioembolic AIS	0.085 (0.025–0.292)	<0.001
Only cardioembolic AIS	0.259 (0.090–0.745)	0.012
Using different ENR cutoff values		
ENR top 10% vs. bottom 90%	0.382 (0.098–1.496)	0.167
ENR top 20% vs. bottom 80%	0.326 (0.121–0.877)	0.026
ENR top 30% vs. bottom 40%	0.322 (0.143–0.727)	0.006
ENR top 40% vs. bottom 60%	0.287 (0.137–0.603)	0.001
ENR top 50% vs. bottom 50%	0.241 (0.119–0.488)	<0.001
ENR top 60% vs. bottom 40%	0.163 (0.076–0.348)	<0.001
ENR top 70% vs. bottom 30%	0.249 (0.116–0.531)	<0.001
ENR top 80% vs. bottom 20%	0.200 (0.083–0.479)	<0.001
ENR top 90% vs. bottom 10%	0.451 (0.172–1.183)	0.106

ENR, eosinophil-to-neutrophil ratio; NIHSS, National Institute of Health Stroke Scale. Adjusted for age, current smoking, hypertension, atrial fibrillation, prior stroke, baseline NIHSS score, and stroke subtypes (Model 1 in ***Table 3****, stroke subtypes were not adjusted in cardioembolic and non-cardioembolic AIS patient groups).*

prognosis analysis. Seventy-seven (34.5%) patients had a poor function outcome and 42 (18.8%) patients had died during the 1-year follow-up. After adjusting for age, current smoking, hypertension, atrial fibrillation, prior stroke, baseline NIHSS score, and stroke subtype, it is interesting that patients with ENR $\times 10^2 \geq 0.74$ were more likely to come to a good outcome then those with ENR $\times$ $10^2 < 0.74$ (OR = 0.282, 95% CI 0.124–0.639, $p = 0.002$), although no association was found between ENR and 1-year poor outcome when ENR was calculated as a continuous variable. Kaplan–Meier curves and the log-rank test indicated that patients in the high-ENR group had a lower incidence of mortality at the 1-year follow-up (**Figure 5**). Multivariate Cox regression proportional hazard model analyses were used after adjusting the potential confounders. Patients with a higher ENR were associated with a lower mortality risk (high ENR vs. low ENR: HR = 0.314; 95% CI, 0.135–0.731; $p = 0.007$ and per one-point increase of ENR $\times$ 10^2: HR = 0.586; 95% CI, 0.384–0.872; $p = 0.008$).

DISCUSSION

A significantly decreased ENR level was observed in the AIS patients compared with the healthy controls. The ROC curve showed that ENR was a fair prognostic biomarker for 3-month poor outcome and had a higher predictive power than either eosinophil or neutrophil count alone. Patients with lower ENR levels were more likely to develop cardioembolic stroke and severe symptoms. In addition, the multivariate adjusted model and restricted cubic splines showed that elevated ENR levels were associated with a lower risk of poor 3-month function outcome. Furthermore, addition of ENR to the conventional model led to the improvement in the model's ability to predict a 3-month poor outcome. Our study also demonstrated that ENR is an independent predictor of 3-month and 1-year mortality in patients with AIS.

ENR is a composite marker of absolute blood eosinophil and neutrophil counts. Neutrophils are most abundant circulating white blood cells and play a vital role during acute inflammatory responses (12). In AIS patients, neutrophils are rapidly recruited into the injury site after stroke onset and release reactive oxygen species (ROS), various proteases, and numerous inflammatory mediators which contribute to tissue damage within the ischemic area (13, 14). A recent study showed that the extracellular traps released by neutrophils are harmful to vascular remodeling after AIS, and an increased extravasation of immune cells and toxic proteins will be observed due to blood–brain barrier (BBB) disruption (15). In addition, the activation kinetics of neutrophils in response to r-tPA should be concerned. Administration of r-tPA can promote *in vitro* and potentially *in vivo* neutrophil degranulation (16). Degranulation products like matrix metalloproteinase-9 (MMP-9) and myeloperoxidase (MPO) are generally considered to be associated with the presence of hemorrhage and poor function outcomes after stroke (17). Maestrini et al. found that higher neutrophil counts and MPO levels were associated with 3-month worse outcomes and higher mortality rates, suggesting that MPO could be a potential therapeutic target (18). In our study, higher neutrophil counts were found in AIS patients compared with healthy controls.

Eosinophils are involved in local immune and inflammatory responses, and treatment targeting eosinophils may help to control a variety of diseases, including atopic diseases such as asthma and allergies, as well as diseases not primarily related to eosinophils, such as autoimmunity and malignancies (19). However, few studies have reported the role of eosinophil in stroke. Eosinophilia has been reported as a prothrombotic condition (20). It is interesting that lower eosinophil counts are associated with severe symptom and poor prognosis of AIS patients (21, 22). The underlying mechanism of eosinophils in stroke is complex, and whether eosinophils are beneficial or harmful depends on the patient's specific background. Enhanced procoagulant activity and impaired anticoagulant properties of the endothelial membrane may contribute to the thrombosis. Eosinophils can release fibroblast growth factor (FGF2), nerve growth factor (NGF), and vascular endothelial growth factor (VEGF), which are involved in vascular remodeling (23). It is worth noting that eosinophil-derived cytotoxic proteins also played an important role in AIS (24). Eosinophil infiltration may be an essential mechanism to explain why eosinophils decreased after stroke. Eosinopenia-producing substances by neutrophils might lead to local margination of eosinophils and thereby cause continued eosinopenia (6). Hence, we may miss the interaction between eosinophil and neutrophil and underestimate the role these cells played in the pathogenesis of AIS if we analyze them separately.

To the best of our knowledge, our study is the first to suggest the association between the ENR level and prognosis of AIS patients treated with intravenous thrombolysis. In regions with different levels of medical resources, a complete blood cell test is

TABLE 5 | C-statistics and reclassification analyses for ENR to improve the risk stratification of poor 3-month outcome.

	C-statistics		Continuous NRI, %		IDI, %	
	Estimate (95% CI)	***p*-value**	**Estimate (95% CI)**	***p*-value**	**Estimate (95% CI)**	***p*-value**
Model 1	0.845 (0.794–0.887)		Reference		Reference	
Model 1 + ENR	0.881 (0.834–0.918)	0.024	86.71 (63.02–110.39)	<0.001	7.92 (4.22–11.61)	<0.001

The model 1 was included age, current smoking, hypertension, atrial fibrillation, prior stroke, baseline NIHSS score, and stroke subtype.
ENR, eosinophil-to-neutrophil ratio; NRI, net reclassification improvement; IDI, integrated discrimination improvement; NIHSS, national institute of health stroke scale.

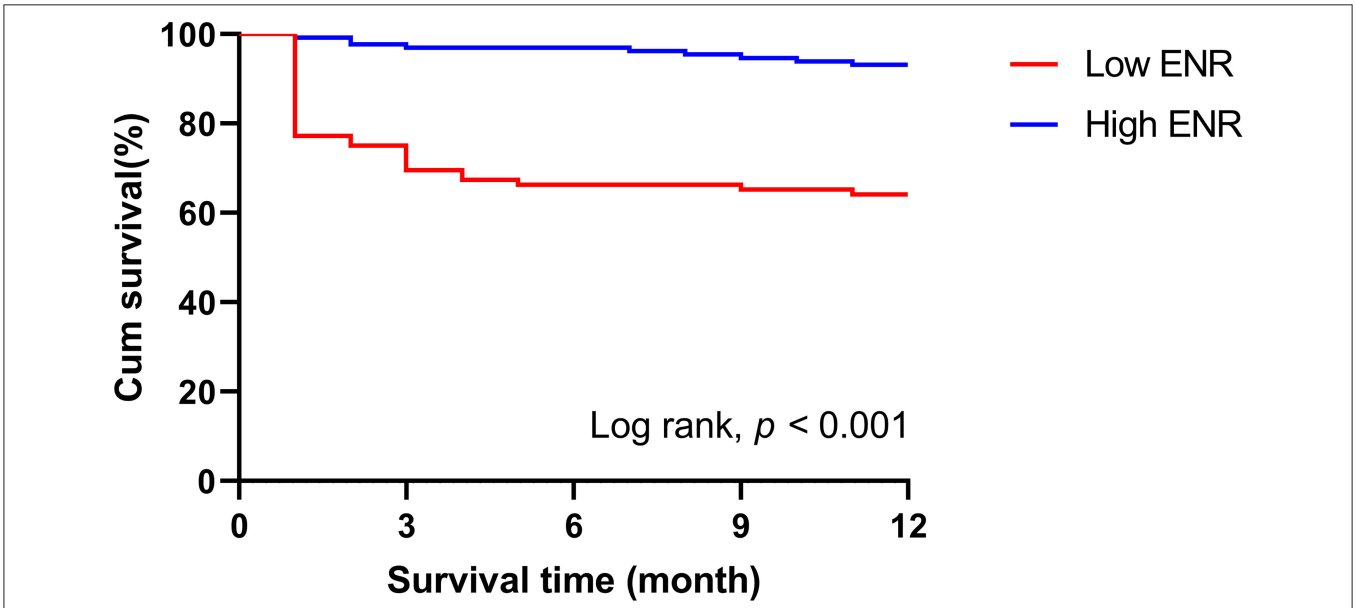

FIGURE 5 | Kaplan–Meier curves comparing the death rate of the two groups over the 1-year follow-up. ENR, eosinophil-to-neutrophil ratio.

widely used. Eosinophils and neutrophils could be obtained and calculated rapidly from a blood sample, which assists clinicians to judge the prognosis of patients at an early stage.

However, several limitations of our study should be acknowledged. First, this study is an observational study and residual confounding still remained. Therefore, the causal relationship between ENR and poor prognosis is unable to establish. Second, the sample size of our study was relatively small; among the 266 patients who met the inclusion criteria, only 250 (94.0%) patients finished the 3-month follow-up and 223 (83.8%) patients finished the 1-year follow-up. Furthermore, subjects of our study were selected from a single hospital so that selection bias may exist in our study.

CONCLUSION

Our study shows that a lower ENR is independently associated with 3-month poor outcome and 3-month and 1-year mortality in AIS patients treated by r-tPA intravenous thrombolysis. Monitoring ENR at an early stage might be helpful for risk stratification and making therapeutic decisions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Third Affiliated Hospital of Wenzhou Medical University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SZ and GC conceptualized this work and supervised the study. HC, HH, CY, JR, JW, BG, WP, FS, XZ, TZ, JH, and YC acquired the data. HC, HH, CY, and JR performed the statistical analysis and interpreted the data. HC, HH, and CY prepared

the manuscript. SZ, GC, HC, HH, CY, JR, JW, BG, WP, FS, XZ, TZ, JH, and YC revised the manuscript. All authors approved the protocol.

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BRIEF RESEARCH REPORT
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Predicting In-hospital Mortality Using D-Dimer in COVID-19 Patients With Acute Ischemic Stroke

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Background: Coronavirus disease 2019 (COVID-19) has been associated with coagulopathy, and D-dimer levels have been used to predict disease severity. However, the role of D-dimer in predicting mortality in COVID-19 patients with acute ischemic stroke (AIS) remains incompletely characterized.

Methods: We conducted a retrospective cohort study using the Optum® de-identified COVID-19 Electronic Health Record dataset. Patients were included if they were 18 or older, had been hospitalized within 7 days of confirmed COVID-19 positivity from March 1, 2020 to November 30, 2020. We determined the optimal threshold of D-dimer to predict in-hospital mortality and compared risks of in-hospital mortality between patients with D-dimer levels below and above the cutoff. Risk ratios (RRs) were estimated adjusting for baseline characteristics and clinical variables.

Results: Among 15,250 patients hospitalized with COVID-19 positivity, 285 presented with AIS at admission (2%). Patients with AIS were older [70 (60–79) vs. 64 (52–75), $p < 0.001$] and had greater D-dimer levels at admission [1.42 (0.76–3.96) vs. 0.94 (0.55–1.81) μg/ml FEU, $p < 0.001$]. Peak D-dimer level was a good predictor of in-hospital mortality among all patients [c-statistic 0.774 (95% CI 0.764–0.784)] and among patients with AIS [c-statistic 0.751 (95% CI 0.691–0.810)]. Among AIS patients, the optimum cutoff was identified at 5.15 μg/ml FEU with 73% sensitivity and 69% specificity. Elevated peak D-dimer level above this cut-off was associated with almost 3 times increased mortality [adjusted RR 2.89 (95% CI 1.87–4.47), $p < 0.001$].

Conclusions: COVID-19 patients with AIS present with greater D-dimer levels. Thresholds for outcomes prognostication should be higher in this population.

Keywords: D-dimer, COVID-19, stroke, mortality, coagulopathy, electronic medical records, coronavirus

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome Coronavirus-2 (SARS-CoV-2) is primarily a respiratory tract infection, but coagulopathy associated with its profound inflammatory response has been well-described (1–3). D-dimer, a degradation product of cross-linked fibrin that reflects ongoing activation of the coagulation cascade, has been linked with coagulopathy in COVID-19 infection. Elevated D-dimer level has been identified as a useful

predictor for mortality in patients with COVID-19 and several studies demonstrated its prognostic potential and optimal cutoff value (4–6). However, the prognostic value of D-dimer in predicting COVID-19 mortality has been tested mostly from single provider or pooled meta-analyses (4–7), and the performance and optimal cutoff value in patients with acute ischemic stroke (AIS), a condition that may independently elevate D-dimer (8, 9), remains uncharacterized. Here, we examine whether D-dimer remains useful to predict mortality in COVID-19 patients identified from a large multicenter sample and determine the optimal cutoff value to predict mortality in COVID-19 patients presenting with AIS. We study a broad time period including more recent COVID-19 cases and cover national level geographic regions to include multiple COVID-19 pandemic surges and viral strains.

METHODS

Data Source

We conducted a retrospective cohort study using the Optum® de-identified COVID-19 Electronic Health Record (EHR) dataset. Given the urgent need to clinically understand the novel virus of COVID 19, Optum developed a data pipeline that enables minimal data lag, while preserving as much clinical data as possible. The data is sourced from Optum's longitudinal EHR repository, which is derived from dozens of

TABLE 1 | Characteristics of patients in COVID with or without acute ischemic stroke.

	Total (N = 15,250)	No AIS at admission (n = 14,965)	AIS at admission (n = 285)	p-value
Age, median (IQR)	64 (52–75)	64 (52–75)	70 (60–79)	<0.001
Age ≥ 65, *n* (%)	7,525 (49.3)	7,340 (49.0)	185 (64.9)	<0.001
Male, *n* (%)	8,371 (54.9)	8,199 (54.8)	172 (60.4)	0.062
Race, *n* (%)				
African American	3,525 (23.1)	3,453 (23.1)	72 (25.3)	0.80
Asian	529 (3.5)	519 (3.5)	10 (3.5)	
Caucasian	8,106 (53.2)	7,956 (53.2)	150 (52.6)	
Other/unknown	3,090 (20.3)	3,037 (20.3)	53 (18.6)	
Ethnicity, *n* (%)				
Hispanic	1,872 (12.3)	1,852 (12.4)	20 (7.0)	<0.001
Non-Hispanic	11,531 (75.6)	11,319 (75.6)	212 (74.4)	
Unknown	1,847 (12.1)	1,794 (12.0)	53 (18.6)	
Region, *n* (%)				
Midwest	5,531 (36.3)	5,457 (36.5)	74 (26.0)	<0.001
Northeast	5,521 (36.2)	5,372 (35.9)	149 (52.3)	
South	3,339 (21.9)	3,296 (22.0)	43 (15.1)	
West	520 (3.4)	507 (3.4)	13 (4.6)	
Other/unknown	339 (2.2)	333 (2.2)	6 (2.1)	
Risk Factors, *n* (%)				
Congestive heart failure	3,295 (21.6)	3,190 (21.3)	105 (36.8)	<0.001
Hypertension	10,962 (71.9)	10,718 (71.6)	244 (85.6)	<0.001
Diabetes	6,812 (44.7)	6,655 (44.5)	157 (55.1)	<0.001
Vascular disease	3,683 (24.2)	3,549 (23.7)	134 (47.0)	<0.001
Atrial fibrillation	2,731 (17.9)	2,634 (17.6)	97 (34.0)	<0.001
Smoke	4,180 (27.4)	4,091 (27.3)	89 (31.2)	0.14
Labs at admission, median (IQR)				
D-Dimer (μg/ml feu)	0.95 (0.56–1.83)	0.94 (0.55–1.81)	1.42 (0.76–3.96)	<0.001
C-reactive protein (mg/L)	93 (43–159)	93 (44–159)	85 (24–165)	0.24
Ferritin (ng/ml)	551 (250–1,153)	551 (250–1,152)	551 (235–1,343)	0.90
Lactate dehydrogenase (u/L)	343 (257–468)	343 (257–467)	361 (254–503)	0.13
Lymphocyte ($\times 10^9$/L)	1.00 (0.70–1.40)	1.00 (0.70–1.40)	0.90 (0.60–1.50)	0.71
Neutrophil ($\times 10^9$/L)	5.4 (3.7–8.2)	5.4 (3.7–8.2)	6.7 (4.2–9.6)	<0.001
Platelet count ($\times 10^9$/L)	212 (165–275)	212 (165–274)	213 (169–293)	0.33
White blood cell count ($\times 10^9$/L)	7.3 (5.4–10.3)	7.3 (5.4–10.2)	9.0 (6.4–12.2)	<0.001
Medication administered, *n* (%)				
Antiplatelet	5,231 (34.3)	5,012 (33.5)	219 (76.8)	<0.001
Anticoagulant	2,058 (13.5)	1,957 (13.1)	101 (35.4)	<0.001

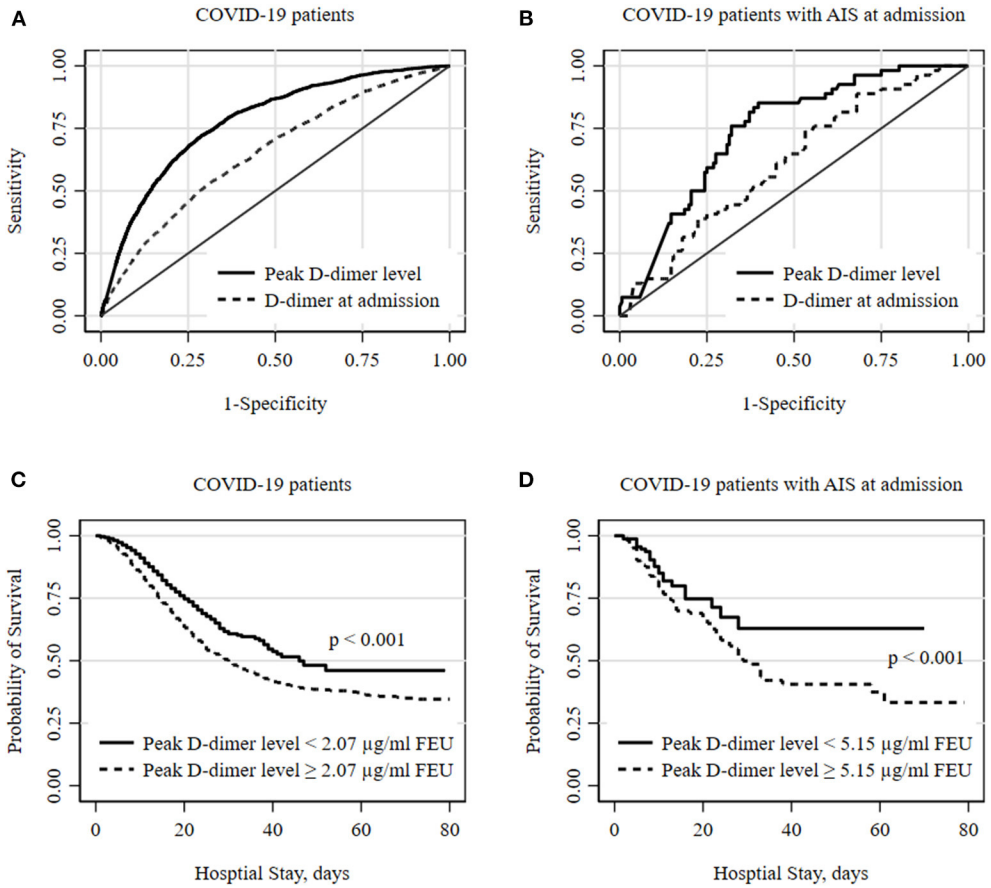

FIGURE 1 | Optimal D-dimer levels to predict in-hospital mortality among COVID patients. **(A,B)** show receiver operator characteristic (ROC) curves for optimal D-dimer levels to predict deaths in all COVID-19 hospitalized patients and subgroup of patients with acute ischemic stroke (AIS) at admission. The peak D-dimer level performs better to predict deaths compared to D-dimer level at admission. The optimum cutoff thresholds of peak D-dimer levels were defined as the point on the ROC curve nearest to the upper left corner (0, 1) and were 2.07 µg/ml FEU with 72.3% sensitivity and 69.8% specificity for all and 5.15 µg/ml FEU with 72.6% sensitivity and 68.7% of specificity for AIS subgroup. **(C,D)** show Kaplan-Meier survival curves for all-cause death during hospital stay. Cutoff values of 2.07 and 5.15 estimated from ROC analyses were used for all and a subgroup of patients with AIS at admission, respectively. Statistical differences in survival curves between peak D-dimer levels below and above the cutoff values were assessed using a log-rank test.

healthcare provider organizations in the United States, which include more than 700 hospitals and 7,000 clinics across the continuum of care. The COVID-19 dataset incorporates a wide swath of raw clinical data, including new, unmapped COVID-specific clinical data points from both Inpatient and Ambulatory electronic medical records, practice management systems, and numerous other internal systems. The Optum COVID-19 data elements include demographics, mortality, diagnoses, procedures, medications prescribed and administered, lab results, and other observable measurements.

Study Population

Patients were included if they had laboratory-confirmed COVID-19 between March 1, 2020, and November 30, 2020 ($n = 281,665$) and were hospitalized within 7 days of the positivity date ($n = 35,919$). Positive COVID-19 status was determined by the detection of SARS-CoV-2 in polymerase chain reaction (PCR) test, and the positivity date was based on the date of sample collected. We limited the study to individuals who were tested and had valid results for D-dimer at admission or during their hospitalization ($n = 15,313$). Patients who were younger than 18 years ($n = 52$) or had missing sex information ($n = 11$) were excluded.

Measurements of D-dimer and Other Variables

D-dimer values within 24 h of admission and the peak values recorded during hospital stay were tested to predict all-cause mortality during the index COVID-19 hospital stay. Because the data was sourced from multiple laboratories, D-dimer results varied in reporting units. D-dimer results can be reported using a fibrinogen equivalent unit (FEU) or using a D-dimer unit (DDU) depending on the molecular weight used. FEU reports D-dimer levels based on the molecular weight of fibrinogen, whereas DDU reports D-dimer levels based on its own molecular weight, which is about half that of fibrinogen. We approximated

TABLE 2 | Factors associated with mortality among hospitalized COVID-19 patients with acute ischemic stroke.

	COVID 19 patients with AIS at admission ($n = 285$)			
	Crude RR (95% CI)	***p*-value**	**Adjusted RR (95% CI)**	***p*-value**
Peak D-dimer ≥ 5.15 μg/ml feu)	3.44 (2.26–5.24)	<0.001	2.89 (1.87–4.47)	<0.001
Age group				
18–44	1.00 (reference)		1.00 (reference)	
45–64	1.95 (0.50–7.60)	0.34	2.70 (0.88–8.32)	0.08
65–74	3.27 (0.86–12.41)	0.08	4.13 (1.38–12.40)	0.01
≥75	2.62 (0.69–9.96)	0.16	4.38 (1.45–13.22)	0.009
Male sex	1.12 (0.77–1.63)	0.54	0.87 (0.60–1.26)	0.47
Race/ethnicity				
White	1.00 (reference)		1.00 (reference)	
Black	0.76 (0.44–1.31)	0.32	0.72 (0.45–1.16)	0.18
Hispanic	1.41 (0.77–2.59)	0.27	1.69 (0.95–3.00)	0.07
Other/unknown	1.24 (0.82–1.87)	0.32	1.09 (0.73–1.61)	0.68
Risk factors				
Congestive heart failure	1.00 (0.69–1.46)	0.99	1.11 (0.76–1.60)	0.59
Hypertension	0.77 (0.49–1.21)	0.26	0.62 (0.38–1.01)	0.06
Diabetes	1.47 (1.00–2.15)	0.049	1.47 (1.01–2.16)	0.045
Vascular disease	1.18 (0.82–1.69)	0.36	1.05 (0.76–1.45)	0.77
Atrial fibrillation	1.32 (0.92–1.89)	0.13	1.06 (0.76–1.49)	0.73
Smoke	1.42 (0.99–2.04)	0.054	1.60 (1.14–2.24)	0.007
Medication administered				
Antiplatelet	1.11 (0.71–1.72)	0.66	1.13 (0.76–1.69)	0.54
Anticoagulant	2.31 (1.62–3.31)	<0.001	1.60 (1.11–2.30)	0.01

D-dimer levels reported in DDU to those in FEU by multiplying by 2. AIS was identified using the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) codes (433.x1, 434.x1 and 436) and ICD-10-CM code (I63.x). Although ICD-9-CM codes have been replaced with ICD-10-CM as of October 2015, ICD-9 codes were included to identify AIS and comorbidities as some patient medical records were still reported using ICD-9 codes. We also examined medication usages, particularly antiplatelets (aspirin, clopidogrel, prasugrel, ticagrelor, dipyridamole, and eptifibatide) and anticoagulants given during hospitalization.

Statistical Analysis

The optimal D-dimer cutoff point and C-statistic of D-dimer levels were evaluated by receiver operator characteristic (ROC) curve for all COVID-positive patients and a subgroup of patients with AIS at admission. The probability of survival during the hospital stay was plotted for patients above and below the cutoff level of D-dimer using Kaplan-Meier survival functions, and the difference in survival curves was assessed using log-rank test. The prognostic value of D-dimer was assessed using a modified Poisson regression model, and risk ratio (RR) was estimated adjusting for age, sex, race/ethnicity, known risk factors, and medication use (10). Significance levels were set at $P < 0.05$ for 2-tailed tests and all analyses were performed using STATA 16.0 (StataCorp, College Station, TX).

RESULTS

Among 15,250 patients hospitalized with COVID-19 positivity, 285 presented with AIS at admission (2%). Patients with AIS were older [median age 70 (60–79) vs. 64 (52–75)] and had higher prevalence of congestive heart failure, hypertension, diabetes, vascular disease, and atrial fibrillation. D-dimer levels at admission were greater for patients presenting with AIS [median (IQR), 1.42 (0.76–3.96) μg/ml FEU] compared to those without AIS [0.94 (IQR 0.55–1.81) μg/ml FEU] and peak levels were also greater for patients with AIS [3.86 (IQR 1.23–15.58) vs. 1.42 (IQR 0.76–3.96) μg/ml FEU]. All other lab values on admission were similar between patients with and without AIS except neutrophil and white blood cell counts in AIS patients (**Table 1**).

The area under the ROC curve (C-statistic) using D-dimer levels at admission was 0.651 (95% CI, 0.637–0.664) for all COVID-positive patients and was 0.613 (95% CI, 0.529–0.697) for the AIS subgroup. Peak D-dimer levels performed better than admission level and were a good predictor for in-hospital mortality among all COVID-positive patients [c-statistic 0.774 (95% CI 0.764–0.784)] and among the AIS subgroup [c-statistic 0.751 (95% CI 0.691–0.810)] as shown in **Figures 1A,B**. The optimum cutoff values of peak D-dimer were identified as 2.07 μg/ml FEU with 72% sensitivity and 70% specificity for all COVID-positive patients and 5.15 μg/ml FEU with 73% sensitivity and 69% of specificity for the AIS subgroup.

Kaplan-Meier survival curves constructed using these cutoff values show that patients with elevated peak D-dimer level above the cutoff value are less likely to survive both in all and the AIS subgroup (**Figures 1C,D**). Among all COVID-positive patients, elevated peak D-dimer level above the cutoff value was associated with increased mortality with crude RR 4.48 (95% CI, 4.12–4.87, $p < 0.001$) and adjusted RR 3.00 (95% CI, 2.75–3.28, $p < 0.001$) accounting for age, sex, race/ethnicity, and comorbidities. Among the AIS subgroup (**Table 2**), in-hospital mortality for those with elevated peak D-dimer level $\geq$ 5.15 μg/ml FEU was more than 3 times higher compared to those with below the cutoff D-dimer level [crude RR 3.44 (95% CI, 2.26–5.24, $p < 0.001$). After adjusting for covariates, we still found the elevated D-dimer level is associated with a significantly higher risk for death with adjusted RR 2.89 (95% CI, 1.87–4.47, $p < 0.001$)] in the AIS subgroup (**Table 2**). Increasing age and anti-coagulant use during the hospitalization were also associated with an increased of mortality among patients with AIS (**Table 2**).

DISCUSSION

In this study of a large multicenter database of patients with COVID positivity, patients presenting with AIS had greater D-dimer levels compared to those without AIS, and thresholds to predict mortality were higher in the AIS population. In patients with AIS, peak values above 5.15 μg/ml FEU were associated with a nearly three-fold risk of in-hospital mortality.

A pro-inflammatory hypercoagulable state has been well-associated with the COVID-19 infection (11, 12). Elevated D-dimer levels have been found in COVID-19 patients with coagulopathy and several observational studies reported elevated D-dimer level was a good predictor of ICU admission or in-hospital death (4, 6, 13). Independently, D-dimer has been previously identified as a biomarker for AIS and associated with stroke severity (9, 14). Therefore, the prognostic value of D-dimer in COVID-19 could differ for COVID-19 patients presenting with AIS, in whom D-dimer levels may be independently elevated. Our study confirmed that D-dimer levels at admission were elevated among COVID-19 patients [0.95 (0.56–1.83) μg/ml FEU] beyond normal range (<0.5 μg/ml FEU) and greater elevations were observed among COVID-19 patients presenting with AIS [1.42 (0.76–3.96) μg/ml FEU] (15, 16).

We found the optimal cutoff values to predict mortality in COVID-19 patients were 2.07 μg/ml FEU with 72.3% sensitivity and 69.8% specificity for all. The cutoff value of 2.07 μg/ml FEU for all hospitalized COVID-19 patients is similar to previous findings. Zhang et al. reported an optimum cutoff value of D-dimer as 2.0 mg/ml within 24 h of hospital admission and Yao et al. reported D-dimer levels > 2.14 mg/ml on admission as a predictor of mortality (4, 5). However, most of these studies used the level of D-dimer on admission only and few studies discussed changes in D-dimer levels over time and showed an association between dynamic changes of D-dimer level with the prognosis of COVID-19 (11, 17). In our study, peak D-dimer levels performed better than admission level in predicting in-hospital mortality among all COVID-19 patients as well as the AIS subgroup. Since the time from COVID-19 onset to hospitalization varies across different patient characteristics and health care systems, the peak level reflects better dynamic changes of patient's progress and be more uniformed to be used than the D-dimer level on admission. Soni et al. also tested with both D-dimer levels on admission and with peak value during the hospital stay and found the peak level performs better and reported the cutoff value of 2.01 mg/ml with a sensitivity of 73.3% and a specificity of 70.0%, with a C-index of 0.789 (6). Importantly, the cutoff value for COVID-19 patients presenting with AIS was more than twice as high as the cutoff value for non-AIS patients, reflecting a greater elevation of D-dimer levels among AIS patients.

We assessed other lab values including inflammatory markers but found they were not significantly different between stroke and non-stroke COVID-19 patients except neutrophil and white blood cell counts. We also tested their optimal cutoff values and found they had similar or lower performance in predicting hospital mortality among all COVID-19 patients and the AIS subgroup. It is also worth noting that we observed an increased likelihood of mortality in AIS COVID-19 positive patients with increasing age and with anticoagulant use. The increased mortality associated with anticoagulant use may be secondary to increased usage in patients with more severe strokes or extensive thrombosis.

Our study has several limitations. Unlike single provider-based datasets, this multicenter database contained variations in D-dimer units across different hospitals, and as a result, we converted the reporting units to μg/ml FEU. In addition, our dataset contained limited descriptions of stroke subtypes and severity, precluding additional subgroup analyses. However, despite the potential heterogeneity and limited information, we found similar cutoff values compared to previous studies. Since we used a large EHR dataset covering patients across the country, we believe our study provides the external validity of the established cutoff value and presents the feasibility of conducting reliable observational studies using EHR data.

CONCLUSION

COVID-19 patients with AIS present with higher D-dimer levels compared to those without AIS. D-dimer functions well as a predictor of mortality in this subgroup, however the threshold for predicting this outcome is substantially greater.

DATA AVAILABILITY STATEMENT

The data analyzed in this study was obtained from Optum, the following licenses/restrictions apply: the data that support the findings of this study are available from Optum upon reasonable

request. Requests to access these datasets should be directed to Sunil A. Sheth, ssheth@post.harvard.edu.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Committee for the Protection of Human Subjects (CPHS) at The University of Texas Health Science Center at Houston. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

YK, SK, and SS drafted the manuscript. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Prognostic Value of Abnormal Liver Function Tests After Mechanical Thrombectomy for Acute Ischemic Stroke

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Objective: To determine the clinical significance of post-procedural abnormal liver function test (ALFT) on the functional outcomes at 90 days in acute ischemic stroke (AIS) treated with mechanical thrombectomy (MT).

Methods: In this retrospective observational study, patients with AIS undergoing MT were enrolled from the Nanjing Stroke Registry Program and the multicenter Captor trial. A favorable outcome was defined as a modified Rankin Scale score 0–2 at 90 days. Predictive models were established by multivariable logistic regression. Improved predictive value of models was assessed by continuous net reclassification improvement (NRI) and integrated discrimination improvement (IDI). In addition, multivariable logistic regression and restricted cubic spline were used to analyze dose–response correlations between the severity of ALFT and prognosis.

Results: Among 420 patients enrolled, 234 (55.7%) patients were diagnosed as post-procedural ALFT after MT. Patients with post-procedural ALFT had higher National Institute of Health Stroke Scale score on admission (median, 18 vs. 15, $p < 0.001$) and more pneumonia (65.4 vs. 38.2%, $p < 0.001$) than those without post-procedural ALFT. Post-procedural ALFT, rather than preprocedural ALFT, was independently associated with favorable outcome (adjusted odds ratio, 0.48; 95% CI 0.28–0.81; $p = 0.006$). The improvement of predictive model after adding post-procedural ALFT was significant [continuous NRI (value, 0.401; $p < 0.001$), IDI (value, 0.013; $p < 0.001$)]. However, the restricted cubic spline indicated no evidence of a dose–response relationship between the severity of post-procedural ALFT and prognosis.

Conclusions: In AIS patients treated by MT, post-procedural ALFT was associated with more severe stroke and served as an independent predictor of worse prognosis at 90 days.

Keywords: liver function tests, ischemic stroke, thrombectomy, prognosis, observational study

INTRODUCTION

Mechanical thrombectomy (MT) is a well-established treatment for acute ischemic stroke (AIS) caused by large vessel occlusions (LVO) (1). However, regardless of the proven validity of MT, only approximately 40% of patients undergoing MT could achieve functional independence at 90 days (1, 2). Despite the growing attention, factors affecting clinical outcomes have not been fully recognized yet. Conventional factors including age, baseline disability, history of hypertension, and hyperglycemia were known to be associated with worse prognosis in AIS patients treated with MT (3). Besides, radiological markers of chronic brain damage, such as cortical microinfarcts (4), leukoaraiosis (5), brain atrophy (6), and cerebral microbleeds (7), have recently been identified as risk factors of poor clinical outcomes. Comorbidities and complications such as malignant brain edema (8), pneumonia (9), and renal dysfunction (10) were reported as potential risk factors for worse prognosis, too.

Liver is a vital organ with essential biosynthetic and metabolic functions. It was reported that abnormal liver function test (ALFT) was associated with higher mortality risk in critically ill patients (11–14). ALFTs are extremely common in AIS patients, with an incidence of about 40% (15–18). A prospective study showed more severe stroke and worse outcomes in AIS patients with non-alcoholic fatty liver disease (15). However, there are scarce data regarding the association between ALFT and clinical outcomes in AIS-LVO patients undergoing MT.

The aims of this study were to (1) determine the association between periprocedural ALFT and the clinical outcomes, and (2) assess the potential dose–response relationship between liver function test levels and prognosis of AIS-LVO patients undergoing MT.

METHODS

Data Resources

Patients were screened from the Nanjing Stroke Registry Program (NSRP, between January 2014 and December 2019) and the Captor trial (a multi-center clinical trial between March 2018 and July 2019, register code: ChiCTR1900025256). Detailed descriptions of NSRP had been published elsewhere (19). The Captor trial was aimed to compare the Captor stent retriever with the Solitaire FR device for rapid flow restoration in AIS-LVO. The non-inferiority of the Captor retrievable stent was proved after enrolling 245 patients from 16 comprehensive stroke centers in China.

Inclusion and Exclusion Criteria

The key inclusion criteria were as follows: (1) age ≥18 years; (2) National Institute of Health Stroke Scale (NIHSS) score on admission ≥6; (3) occlusion of the intracranial large vessel (defined as diameter ≥2 mm) proved by CT angiography, magnetic resonance angiography, or digital subtraction angiography; (4) completion of groin-puncture within 8 h since stroke onset.

The key exclusion criteria were as follows: (1) active hemorrhage or hemorrhagic tendency; (2) severe organic diseases such as heart, lung, liver, and kidney failure; (3) occlusion attributed to arterial dissection or arteritis. The Ethics Committee of Jinling Hospital and each participating center approved this study according to the Declaration of Helsinki. The requirement for written informed consent was waived because of its retrospective nature.

Data Collections

Baseline demographic parameters, medical histories, laboratory tests, the NIHSS scores, and radiographic evaluation were obtained. The treatment profiles were also gathered, including intravenous thrombolysis (IVT), number of retrieval attempts, final reperfusion status, and procedural time. IVT was administrated within 4.5 h of stroke onset after excluding contraindications. Retrieval attempts were recommended no more than three times, but it was at the discretion of the interventionalists. Reperfusion was evaluated with the modified Thrombolysis In Cerebral Ischemia scale (mTICI) (20). Successful reperfusion was defined as the mTICI scale score of 2b or 3.

Post-procedural NIHSS scores of all patients were assessed by attending neurologists. Follow-up imaging examinations were performed within 24 ± 6 h after the procedure. Symptomatic intracranial hemorrhage (SICH) was defined as imaging evidence of intracranial hemorrhage (ICH) with an increase of ≥4 points on the NIHSS within 24 h. On day 7 ± 2 or hospital discharge if earlier, assessments of neurological severity were performed on the subjects again.

Post-procedural laboratory tests were carried out routinely in the acute phase (7 ± 2 days after MT) and/or in case of clinical deterioration. Liver function was assessed according to tests of serum aspartate aminotransferase [upper limit of normal (ULN): 40 U/L], alanine aminotransferase (ULN: 40 U/L), total bilirubin (ULN: 21 mmol/L), and direct bilirubin (ULN: 10 mmol/L). ALFT was defined as any elevated value above the ULN (21). $\text{Multiple}_{\text{max}}$ was defined as the maximum multiple of ULN in the four variables (aspartate aminotransferase, alanine aminotransferase, total bilirubin, and direct bilirubin) during hospitalization after MT.

Follow-up was performed by outpatient clinical visits or telephone contacts to record the modified Rankin Scale (mRS) score at 90 ± 14 days. A favorable outcome was defined as mRS 0–2 at 90 days, and an excellent outcome was referred to as mRS 0–1 at 90 days.

Statistical Analysis

Median imputation was performed to account for missing values [white blood cell: 6 (1.4%); creatinine: 9 (2.1%); glucose: 11 (2.6%); activated partial thromboplastin time: 17 (4.0%); partial thromboplastin time: 13 (3.1%); international normalized ratio: 13 (3.1%)].

Frequency (percentage) was used for categorical variables. Mean (SD) was adopted for continuous variables with normal distribution and median (interquartile range, IQR) for non-normal variables.

TABLE 1 | Clinical characteristics in the ALFT group and the non-ALFT group.

	All patients (*n* = 420)	ALFT (*n* = 234)	Non-ALFT (*n* = 186)	*P*-value
Demographics				
Age, median (IQR)	68 (58–76)	69 (58–76)	68 (58–75)	0.745
Male, *n* (%)	255 (60.7)	150 (64.1)	105 (56.5)	0.111
SBP, mean (SD)	140 (22)	140 (23)	140 (20)	0.660
Medical history, *n* (%)				
Hypertension	258 (61.4)	152 (65.0)	106 (57.0)	0.096
Diabetes	77 (18.3)	45 (19.2)	32 (17.2)	0.594
Hyperlipidemia	20 (4.8)	14 (6.0)	6 (3.2)	0.188
Coronary heart disease	78 (18.6)	49 (20.9)	29 (15.6)	0.161
Stroke	71 (16.9)	37 (15.8)	34 (18.3)	0.503
Smoke	128 (30.5)	74 (31.6)	54 (29.0)	0.567
Atrial fibrillation	184 (43.6)	112 (47.9)	71 (38.2)	0.047
Preprocedural ALFT*	124 (29.5)	94 (40.2)	30 (16.1)	<0.001
Baseline NIHSS score, median (IQR)	16 (13–21)	18 (13–23)	15 (12–19)	<0.001
Occlusion site, *n* (%)				
Intracarotid artery	137 (32.6)	84 (35.9)	53 (28.5)	0.108
Middle cerebral artery	236 (56.2)	115 (49.1)	121 (65.1)	0.001
Anterior cerebral artery	10 (2.4)	6 (2.6)	4 (2.2)	0.782
Vertebrobasilar artery	51 (12.1)	35 (15.0)	16 (8.6)	0.048
TOAST, *n* (%)				0.003
Large artery atherosclerosis	130 (31.0)	77 (32.9)	53 (28.5)	
Cardio-embolism	186 (44.3)	114 (48.7)	72 (38.7)	
Others	104 (24.8)	43 (18.4)	61 (32.8)	
Laboratory findings, median (IQR)				
WBC, 10^9/L	8.39 (6.69–10.4)	8.67 (6.91–10.60)	7.95 (6.45–9.90)	0.019
Glu, mmol/L	7.38 (6.20–8.49)	7.50 (6.40–8.85)	7.04 (6.00–8.20)	0.035
eGFR, ml/min/1.73 m^2	92 (76–103)	91 (74–103)	92 (79–103)	0.911
APTT, s	27.0 (23.7–32.9)	27.0 (23.2–32.3)	27.0 (24.4–33.7)	0.104
PT, s	12.2 (11.3–13.4)	12.3 (11.2–13.2)	12.2 (11.2–13.5)	0.699
INR	1.03 (0.97–1.10)	1.03 (0.97–1.11)	1.03 (0.97–1.18)	0.168
Operation procedures				
IVT, n (%)	133 (31.7)	80 (34.2)	53 (28.5)	0.213
ASITN/SIR, median (IQR)	1 (1–2)	1 (1–2)	1 (1–2)	0.784
mTICI 2b−3, *n* (%)	345 (82.1)	186 (79.5)	159 (85.5)	0.111
Operation time, min, median (IQR)	97 (69–136)	97 (71–144)	95 (67–130)	0.329
SICH, *n* (%)	35 (8.3)	28 (12.0)	7 (3.8)	0.003
Pneumonia, *n* (%)	224 (53.3)	153 (65.4)	71 (38.2)	<0.001
NIHSS score at 24 h, median (IQR)	15 (8–22)	19 (11–25)	11 (6–16)	<0.001
Clinical outcomes				
Mortality at 7 days, *n* (%)	43 (10.2)	28 (12.0)	15 (8.1)	0.190
7-day NIHSS score, median (IQR)	11 (4–20)	14 (8–24)	6 (2–14)	<0.001
Excellent prognosis, *n* (%)	113 (26.9)	36 (15.4)	77 (41.4)	<0.001
Favorable prognosis, *n* (%)	168 (40.0)	62 (26.5)	106 (57.0)	<0.001
Mortality at 90 days, *n* (%)	97 (29.1)	68 (29.1)	29 (15.6)	0.001
90-day mRS score, median (IQR)	3 (1–5)	4 (2–6)	2 (1–4)	<0.001

**For 26 patients without any results of preprocedural liver function tests, they were considered to be free of preprocedural ALFT.*

ALFT, abnormal liver function test; IQR, interquartile range; SBP, systolic blood pressure; NIHSS, National Institute Health Stroke Scale; TOAST, trial of org 10,172 in acute stroke treatment; WBC, white blood cell; eGFR, estimated glomerular filtration rate; APTT, activated partial thromboplastin time; PT, partial thromboplastin time; INR, international normalized ratio; IVT, intravenous thrombolysis; ASITN/SIR, American Society of Interventional and Therapeutic Neuroradiology/Society of Interventional Radiology System; mTICI, modified Thrombolysis In Cerebral Ischemia; SICH, symptomatic intracranial hemorrhage; mRS, modified Rankin scale.

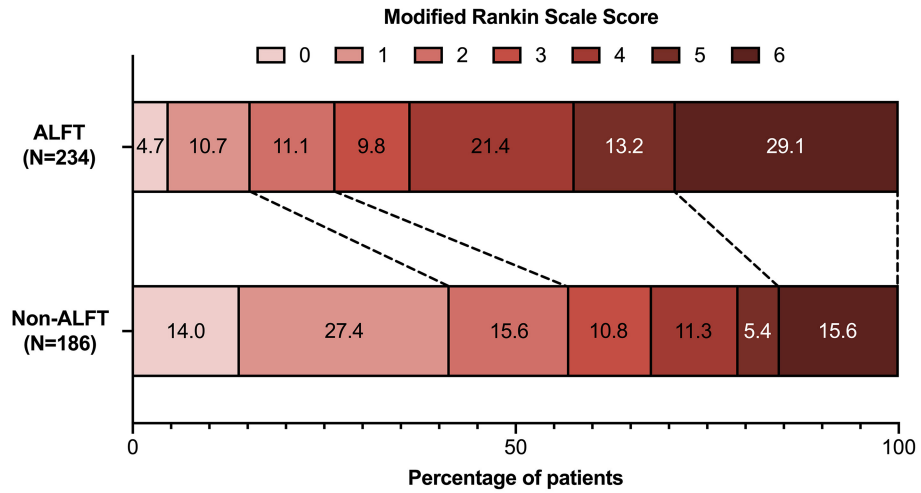

FIGURE 1 | Comparisons of modified Rankin scale scores at 90 days between the ALFT group and the non-ALFT group. ALFT, abnormal liver function test.

Univariate analysis was conducted with Student *t*-test, Mann–Whitney *U*, χ^2 test, or Fisher exact test as appropriate. Confounding factors and other variables with a statistical trend ($p \leq 0.1$) in the univariate analysis were included in multivariate logistic regression analysis (forward selection) to develop a basic model for the favorable prognosis. Composite model was established by factors of basic model and IVT, ASITN/SIR, recanalization, and SICH. Besides, the predictive value of adding preprocedural or post-procedural ALFT into the basic model was estimated by the area under curve (AUC). The improvements on the basic model were assessed by the continuous net reclassification index (NRI) and integrated discrimination improvement (IDI) of which values above 0 were regarded as significant (22). $\text{Multiple}_{\text{max}}$ was categorized based on quartiles to evaluate the association between $\text{Multiple}_{\text{max}}$ and prognosis. The pattern of the potential dose–response relationship between $\text{Multiple}_{\text{max}}$ and prognosis was revealed using a restricted cubic spline with 3 knots after adjusting for confounders. Odds ratio (OR) was reported with a 95% CI.

Subgroup analyses were performed to detect the heterogeneity in the effect of post-procedural ALFT on prognosis. Sensitivity analyses were conducted to explore the effect of ALFTs at different periods on prognosis. A two-tailed $p \leq 0.05$ was considered statistically significant. Statistical analyses were performed with the SPSS software package, version 26 (IBM, Armonk, NY) and R statistical software 3.6.3 [R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/].

TABLE 2 | Multivariable logistic regression for predicting favorable outcomes.

Variable	OR (95% CI)	*P*-value
Basic model*		
Age	0.96 (0.94–0.98)	<0.001
24-h NIHSS score	0.85 (0.82–0.88)	<0.001
Pneumonia	0.43 (0.25–0.72)	0.002
Glucose	0.81 (0.71–0.92)	0.001
Basic model + preprocedural ALFT		
Preprocedural ALFT	1.615 (0.902–2.891)	0.107
Basic model + post-procedural ALFT		
Post-procedural ALFT	0.48 (0.28–0.81)	0.006
Composite model# + post-procedural ALFT		
Post-procedural ALFT	0.45 (0.26–0.78)	0.004

**Basic model was established by confounders and variables with a statistical trend ($p \leq 0.1$) in the univariate analysis using the stepwise forward method.*

#Composite model was established by factors of basic model and IVT, ASITN/SIR, recanalization, and SICH.

OR, odds ratio; NIHSS, National Institute of Health Stroke Scale; IVT, intravenous thrombolysis; ALFT, abnormal liver function test; IVT, intravenous thrombolysis; ASITN/SIR, American Society of Interventional and Therapeutic Neuroradiology/Society of Interventional Radiology System; SICH, symptomatic intracranial hemorrhage.

RESULTS

Clinical Characteristics

The flowchart of this study is illustrated in **Supplementary Figure 1**. The demographic characteristics of all participants are displayed in **Table 1**. Of the 420 patients enrolled in this study, men accounted for 60.7% and the median age was 68 years. In total, 234 (55.7%) subjects presented as post-procedural ALFT, and 186 (44.3%) had normal liver function after MT.

Patients were divided into two groups (the ALFT group and the non-ALFT group) according to the post-procedural ALFT. Medical histories were balanced between the two groups. The ALFT group had a significantly larger proportion of preprocedural ALFT (40.2 vs. 16.1%, $p < 0.001$). Higher NIHSS score on admission (median, 18 vs. 15, $p < 0.001$), higher white blood cell count ($p = 0.019$), higher glucose level ($p = 0.035$), more pneumonia (65.4 vs. 38.2%, $p < 0.001$), and more SICH

TABLE 3 | Comparison of basic models and models adding ALFT for predicting favorable prognosis and excellent prognosis.

	AUC	*P*-value	Continuous NRI (95% CI)	*P*-value	IDI (95% CI)	*P*-value
Basic model	0.875	–	Reference	–	Reference	–
Preprocedural ALFT	0.878	0.106	0.068 (−0.112, −0.247)	0.460	0.005 (−0.016, −0.012)	0.133
Post-procedural ALFT	0.881	0.007	0.401 (0.211, 0.591)	<0.001	0.013 (0.013, 0.045)	<0.001

ALFT, abnormal liver function test; AUC, area under curve; NRI, net reclassification improvement; IDI, integrated discrimination improvement.

TABLE 4 | $Multiple_{max}$ for predicting favorable prognosis (modified Rankin Scale 0–2).

	mRS 0–2 *n* (%)	Univariable analysis OR (95% CI)	*P*-value	Multivariable analysis* OR (95% CI)	*P*-value
$Multiple_{max}$, quartiles			<0.001		0.019
(0–0.73) UNL	68 (63.6)	Reference		Reference	
(0.73–1.10) UNL	48 (44.9)	0.47 (0.27–0.81)	0.006	0.55 (0.28–1.07)	0.080
(1.10–1.80) UNL	32 (31.7)	0.27 (0.15–0.47)	<0.001	0.37 (0.18–0.76)	0.007
>1.80 UNL	20 (19.0)	0.14 (0.07–0.25)	<0.001	0.35 (0.16–0.75)	0.007

**Adjusted for age, dichotomous NIHSS score at 24 h (divided by median), pneumonia, and glucose level.*
mRS, modified Rankin Scale; OR, odds ratio; UNL, upper limit of normal.

(12.0 vs. 3.8%, $p < 0.001$) were detected in the ALFT group than the non-ALFT group. The ratio of IVT pretreatment (34.2 vs. 28.5%, $p = 0.213$) was similar between patients with normal and abnormal liver function. The comparisons between the two groups on median NIHSS scores at 24 h (19 in the ALFT group vs. 11 in the non-ALFT group, $p < 0.001$) and 7 days (14 in the ALFT group vs. 6 in the non-ALFT group, $p < 0.001$) showed similar trends with that of the admission NIHSS score (**Table 1**).

Clinical Outcomes

The mortality rate at 7 days was comparable between the two groups ($p = 0.190$). More deaths occurred in the ALFT group at 90 days (29.1 vs. 15.6%, $p = 0.001$, **Table 1**).

In total, 168 (40.0%) patients ranked 0–2 on mRS score at 90 days after MT, and the proportion of excellent prognosis was 26.9%. Compared with the non-ALFT subjects, the ALFT group had a lower percentage of favorable prognosis (26.5 vs. 57.0%, $p < 0.001$) and excellent prognosis (15.4 vs. 41.4%, $p < 0.001$) at 90 days (**Figure 1**). The density plot of *log-transformed* $Multiple_{max}$ distribution for dichotomous prognosis is shown in **Supplementary Figure 2**. Smaller $Multiple_{max}$ was detected in patients achieving better prognosis.

Multivariable Analyses

The basic model for predicting favorable prognosis was developed after adjusting for confounding factors and variables with a statistical trend ($p \leq 0.1$) in the univariate analysis. Finally, age [OR (95% CI): 0.96 (0.94–0.98), $p < 0.001$], the NIHSS score at 24 h [OR (95% CI): 0.85 (0.82–0.88), $p < 0.001$], pneumonia [OR (95% CI): 0.43 (0.25–0.72), $p = 0.002$], and glucose level [OR (95% CI): 0.81 (0.71–0.92), $p = 0.001$] on admission were included in the basic model (**Table 2**).

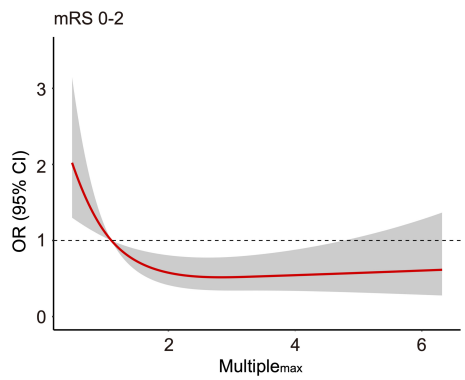

FIGURE 2 | The association between $Multiple_{max}$ and the odds ratio for favorable prognosis (90-day modified Rankin scale scores 0–2). Adjusted for age, median NIHSS score at 24 h, pneumonia, and glucose level. mRS, modified Rankin scale; OR, odds ratio; NIHSS, National Institute of Health Stroke Scale.

An improved model was established after adding ALFT into the basic model. As shown in the improved model, post-procedural ALFT had a significant correlation [OR (95% CI): 0.48 (0.28–0.81), $p = 0.006$] with mRS 0–2 at 90 days after adjusting for the factors involved in the basic model (**Table 2**). Compared with the basic model, the predictive value of the improved model on favorable prognosis showed a significant improvement when assessed by AUC (basic model: 0.875; improved model: 0.881, $p = 0.007$), continuous NRI (value: 0.401, $p < 0.001$), and IDI (value: 0.013, $p < 0.001$), as shown in **Table 3**. In addition, preprocedural ALFT was not a statistically significant predictor of clinical prognosis (all p-values > 0.05).

The role of $Multiple_{max}$ in predicting favorable prognosis was also assessed in multivariable analysis. After adjusting for

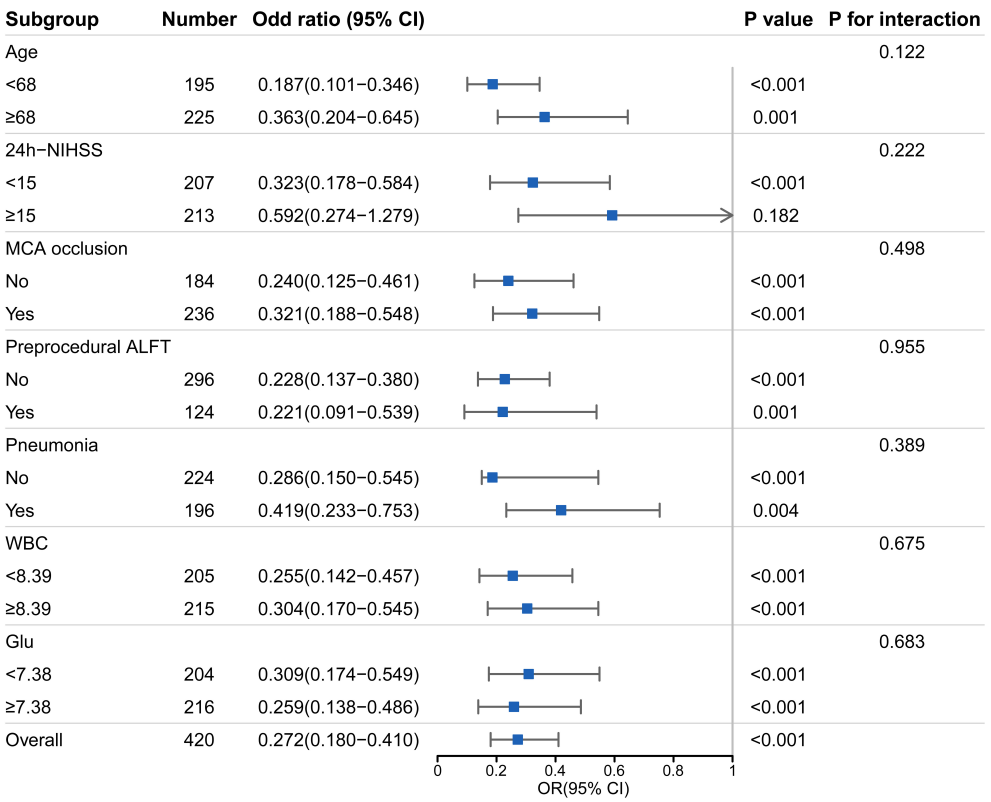

FIGURE 3 | Subgroup analyses of the association between post-procedural ALFT and the favorable prognosis. This forest plot shows the odds ratio for favorable prognosis (90-day modified Rankin scale scores 0–2) of post-procedural ALFT in subgroups. NIHSS, National Institute Health Stroke Scale; MCA, middle cerebral artery; ALFT, abnormal liver function; WBC, white blood cell; mRS, modified Rankin scale; OR, odds ratio.

age, pneumonia, glucose level, and median NIHSS score at 24 h, $\text{Multiple}_{\text{max}}$ remained an independent predictive factor of favorable prognosis ($p = 0.019$, **Table 4**). To explore potential dose–response association, the restricted cubic spline was drawn with three knots after adjusting for covariates mentioned previously. Interestingly, increasing $\text{Multiple}_{\text{max}}$ was no longer lower odds of favorable prognosis (**Figure 2**) further when $\text{Multiple}_{\text{max}}$ was more than 2.

Subgroup Analyses and Sensitivity Analyses

A dominant tendency toward a lower proportion of favorable prognosis in the ALFT group was elucidated in the subgroup analysis (all p-values < 0.01, except for subjects who had NIHSS score ≥15) (**Figure 3**). Of note, no significant interaction was detected between post-procedural ALFT and these clinical features on the favorable prognosis.

In sensitivity analyses, patients with post-procedural ALFTs alone had a lower proportion of favorable prognosis [OR (95% CI): 0.23 (0.14–0.38), $p < 0.001$] than patients with preprocedural ALFTs alone [OR (95% CI): 1.94 (0.84–4.52), $p = 0.120$] (**Figure 4**). They even had less favorable prognoses than patients with both pre- and post-procedural ALFTs [OR (95% CI): 0.23 (0.14–0.38) vs. 0.43 (0.25–0.73)].

DISCUSSION

In this multi-center retrospective study, 55.7% of all AIS patients undergoing MT were identified as post-procedural ALFT. Patients with post-procedural ALFT had more severe stroke, and higher WBC count and blood glucose values on admission. The presence of post-procedural ALFT, rather than the preprocedural ALFT, was independently associated with a decreased proportion of favorable outcomes at 90 days. However, there was no evidence of a dose–response relationship between the severity of post-procedural ALFT and functional prognosis.

The prevalence of post-procedural ALFT in patients with AIS in our study was 55.7%, higher than the previously reported frequency (about 40%) in AIS patients (15–18). Post-procedural ALFT may be a consequence of neuroendocrine dysregulation after stroke onset (23). Focal brain ischemia could induce systemic pathophysiologic reactions and contribute to hepatic inflammatory and apoptotic activation (24). Stroke-induced catecholamine surge promotes the endoplasmic reticulum stress and impairs hepatic insulin signaling (25). Moreover, elevated bilirubin and liver enzyme levels may also be attributed to the treatment measures of AIS, such as antibiotic drugs, statin, and endovascular therapy (21, 26). Therefore, metabolic imbalances and the reflection of treatment during

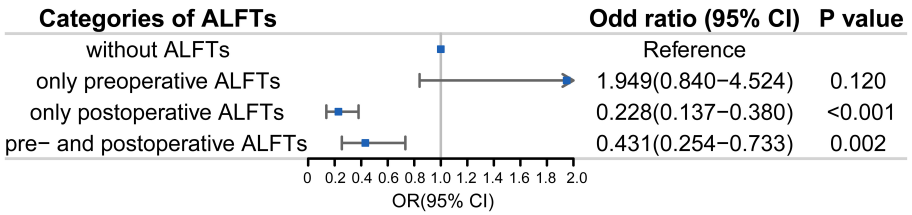

FIGURE 4 | Association between ALFTs at different periods and the favorable prognosis in patients with AIS. ALFT, abnormal liver function; OR, odds ratio.

hospitalization are conjectured to be major mechanisms of post-procedural ALFT.

This study demonstrated a significant correlation between post-procedural ALFT and the severity of stroke. Similar to the findings of previous researches, subjects with ALFT were associated with higher NIHSS scores during hospitalization, indicating more severe stroke (15, 18). Analogously, Muscari et al. elucidated that impaired metabolic homeostasis in the liver was associated with cerebral infarct volume in experimental stroke models (27).

Inspiringly, this study revealed the reliable prognostic value of post-procedural ALFT on predicting functional outcomes of AIS patients treated with MT. This finding was in line with previous reports on the prognostic role of ALFT in AIS (15, 18). Substantial evidence expounded that hepatic dysfunction served as a predictor of clinical outcomes in critically ill patients (11–14). In addition, a population-based research indicated abnormal liver tests were associated with increased all-cause mortality in elderly people (28). However, in our study, data regarding liver function tests before the procedure were limited, and no significant association between preprocedural ALFT and the prognosis was observed. There might exist bias regarding preprocedural ALFT, as patients with severe ALFTs might not receive MT. This might partly explain why the prognostic value of post-procedural ALFT was important than the preprocedural ALFT. Furthermore, results of post-procedural liver function tests may be more representative of the physiological state and restoration of metabolic homeostasis.

Despite unclear specific mechanisms, it is conceivable that ALFT affects functional outcomes since the liver is an important organ for metabolism and immunity (29). It is known that AIS patients suffer from potential immunodepression due to the post-stroke autonomic system activation (30, 31). Subsequent infections and impaired metabolic homeostasis contribute to organ abnormalities, ultimately resulting in the poor prognosis of AIS (23).

Several strengths of our study were noteworthy. Relatively intact data from multiple centers revealed the contemporary status of MT for AIS-LVO in China. Moreover, comprehensive analyses were performed to explore the relationship between ALFT and functional outcomes after MT. Most importantly, liver function tests serve as routine examinations during hospitalization. The availability of test results makes its predictive value more significant for clinical application.

Despite the advantages mentioned previously, our study also had some inevitable limitations. First, this study is subject to the inherent limitations of a retrospective study design. Second, the liver function tests containing only four liver chemistries. Incomprehensive assessments made limited contributions to revealing the detailed mechanism. Third, missing records of the adjuvant medicine, especially for liver protection drugs, was another limitation. Finally, only the Chinese population was enrolled in this study, which may limit the generalizability of the conclusions. Further researches based on different ethnic populations and larger sample sizes are warranted.

CONCLUSIONS

In this study, post-procedural ALFT occurred commonly in AIS patients treated with MT. It was associated with the severity of stroke and was an independent predictor for worse functional outcomes. More attention is needed for AIS patients who were diagnosed as ALFT after MT concerning the increased risk for poor prognosis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of Jinling Hospital and each participating center. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

RL, KH, and XL: concept and design. KH, MZ, MW, and QY: acquisition of data. KH, MZ, RL, and LX: analysis and interpretation of the data. KH, MZ, LX, JG, and JD: drafting of

the article. RL, KH, MZ, and XL: critical revision of the article for important intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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The Incremental Prognostic Value of Hepatocyte Growth Factor in First-Ever Acute Ischemic Stroke: An Early Link Between Growth Factor and Interleukins

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Hepatocyte growth factor (HGF) is a potential prognostic factor for acute ischemic stroke (AIS). In this study, we sought to validate its earlier predictive accuracy within 24 h for first-ever AIS. Moreover, as HGF interacts with interleukins, their associations may lead to novel immunomodulatory therapeutic strategies. Patients with first-ever AIS ($n = 202$) within 24 h were recruited. Plasma HGF and related interleukin concentrations were measured by multiplex immunoassays. The primary and secondary outcomes were major disability (modified Rankin scale score ≥3) at 3 months after AIS and death, respectively. Elastic net regression was applied to screen variables associated with stroke outcome; binary multivariable logistic analysis was then used to explore the relationship between HGF level and stroke outcome. After multivariate adjustment, upregulated HGF levels were associated with an increased risk of the primary outcome (odds ratio, 7.606; 95% confidence interval, 3.090–18.726; $p < 0.001$). Adding HGF to conventional risk factors significantly improved the predictive power for unfavorable outcomes (continuous net reclassification improvement 37.13%, $p < 0.001$; integrated discrimination improvement 8.71%, $p < 0.001$). The area under the receiver operating characteristic curve value of the traditional model was 0.8896 and reached 0.9210 when HGF was introduced into the model. An elevated HGF level may also be a risk factor for mortality within 3 months poststroke. The HGF level was also positively correlated with IL-10 and IL-16 levels, and HGF before interaction with all interleukins was markedly negatively correlated with the lymphocyte/neutrophil ratio. HGF within 24 h may have prognostic potential for AIS. Our findings reinforce the link between HGF and interleukins.

Keywords: acute ischemic stroke, HGF, IL-16, neutrophil, prognosis

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INTRODUCTION

Ischemic stroke is a prevalent disease with high disability and mortality. The current treatments for acute ischemic stroke (AIS) are intravenous administration of tissue plasminogen activator (t-PA) and endovascular treatment to recanalize the blood flow. The prediction of clinical outcomes after AIS is increasingly accepted by physicians in several steps of stroke evaluation for acute therapies, palliative care, or rehabilitation (1, 2). However, traditional risk factors are not comprehensive enough to predict the prognosis of AIS patients. Therefore, the accurate identification of novel biomarkers to improve risk stratification in patients with ischemic stroke is desirable to aid in making decisions regarding stroke care and management.

As hepatocyte growth factor (HGF) is present in the circulation after endothelial injury, its higher levels correlate with multiple cardio-cerebrovascular diseases, including atherosclerosis (3), diabetes mellitus (3), and acute myocardial infarction (4). HGF has received attention as a potential biomarker of AIS. First, circulating HGF levels can be referred to predict the risk of ischemic stroke (3). Second, HGF levels may be useful in diagnosing ischemic stroke as an earlier study shows that serum levels of HGF in patients with cerebral infarction are significantly increased during the early stage and remain elevated until 7 d poststroke and that higher HGF concentrations are correlated with lower gains in the Stroke Impairment Assessment Set in stroke rehabilitation. Recently, serum HGF levels were shown to be associated with poor prognosis at 3 months independent of stroke severity, especially in patients with AIS without heparin pretreatment (5). HGF was also independently associated with death and major disability in patients with AIS with dyslipidemia (6). However, these studies excluded patients undergoing anticoagulation therapy because heparin has antifibrotic activity, mediated by the cellular secretion of HGF (7), and HGF is activated by t-PA (8), which abates its prognostic value. In addition, the patients in the above studies were recruited within 48 h of symptom onset; however, HGF level is already increased within 24 h (9).

The causal role of HGF in cerebral vascular disease has not been fully elucidated. Recent research points to a link between HGF level and phenotypic transformation of immune cells. A link between HGF levels and basal metabolic rate was mediated by IL-16 in patients with obesity-related nonalcoholic fatty liver disease (10). In addition, HGF inhibited microglia activation and the expression of pro-inflammatory IL-1β in a rat model of cerebral ischemia (11); it also promoted M2 macrophage transition and the expression of anti-inflammatory IL-10 and facilitated muscle regeneration (12). Mesenchymal stem cell–secreted HGF contributed to the conversion of fully differentiated Th17 cells into functional T regulatory (Treg) cells (13); however, HGF decreased the levels of Th2 cytokines (IL-4, IL-5, and IL-13) in bronchoalveolar lavage fluid and attenuated airway hyperresponsiveness (14). Although interleukin levels (IL-16, IL-1β, IL-5, and IL-10) are detected in patients with AIS, their prognostic value or relationship with HGF is unknown. Therefore, the prognostic value of HGF within 24 h after stroke with functional outcome (modified Rankin scale and death at 90 d) in patients with first-ever AIS with or without heparin/t-PA treatment requires validation. Understanding the associations of the plasma HGF level and its related interleukins in clinical populations in the acute phase may offer novel immunomodulatory therapeutic options.

MATERIALS AND METHODS

Study Participants and Outcome Assessment

In the present study, we analyzed consecutive patients with first-ever AIS who presented to Xuanwu Hospital of Capital Medical University within 24 h after symptom onset between November 2018 and May 2019. Our study was approved by the Ethics Committee of Xuanwu Hospital, Capital Medical University. Written informed consent was provided by all patients or their immediate family members. According to the criteria of the Trial of Org 10,172 in Acute Stroke Treatment (TOAST), ischemic stroke is classified as large artery atherosclerosis (thrombotic), cardiac embolism (embolic), or small artery occlusion lacunae (lacunar). The inclusion criteria were (1) patients with focal or global neurological deficits, (2) brain computed tomography (CT) or magnetic resonance imaging (MRI) findings indicating a diagnosis of AIS, (3) clinical evaluation performed and recorded 90 d after stroke, and (4) no previous history of stroke. Patients with heart failure, renal failure, cancer, immune diseases, active infection, rheumatic heart disease, liver cirrhosis, epilepsy, and other neurological diseases as well as serious pancreas, intestine, thyroid, or lung disease were excluded from the present study. Finally, 202 patients with first-ever AIS were included for analysis. The primary outcome was defined as an unfavorable outcome [modified Rankin scale (mRS) score, 3–6] at 90 d after stroke, and the secondary outcome was death within 3 months after stroke. The follow-up was conducted by experienced neurologists blinded to the experimental design.

Clinical Data and Blood Collection

Baseline data on demographic characteristics; clinical features, including onset time and systolic and diastolic pressure; medical history; and routine laboratory examination (leukocyte number, glucose levels, blood lipids, etc.) at admission were recorded from the electronic medical record system. Stroke severity was evaluated using the NIH Stroke Scale (NIHSS) score by experienced neurologists at admission (15). Blood samples were collected from patients with AIS before they received any treatments. The blood samples were centrifuged at 3,000 rpm for 10 min to obtain plasma. All plasma samples were frozen at −80°C before the examination.

Measurement of Circulating Biomarker Levels

Plasma levels of HGF, IL-1β, IL-10, IL-5, and IL-16 were examined using a ProcartaPlex multiplex magnetic bead panel kit (Invitrogen, PPX-10) according to the manufacturer's protocol. The data were analyzed using ProcartaPlex Analyst 1.0 software. The experienced laboratory technicians who conducted the tests were blinded to the experimental design.

Statistical Analysis

Data were analyzed using IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, NY, USA) and R software (version 3.5.1), and $p < 0.05$ was defined as statistically significant. Continuous variables are expressed as the means ± standard deviations (SDs) and analyzed using Student's *t*-test (normal distribution) or medians with interquartile ranges (IQRs) and analyzed using Mann–Whitney U tests (nonnormal distribution). Frequencies and percentages in categorical variables were analyzed by chi-squared tests. Receiver operating characteristic (ROC) curves were generated to identify the predictive values of the five cytokines in predicting AIS and calculating the optimal cutoff values of HGF and the four inflammatory biomarkers. An elastic net regression model was used to select variables that were potentially related to stroke outcome with cross-validation applied to determine the optimal regularization parameters (16, 17). In logistic regression models that introduced penalty terms, variables with estimated coefficients close to zero were removed from the model for variable selection. Finally, seven variables were selected, and their corresponding odds ratios (ORs) and 95% confidence intervals (CIs) were reported. We considered variables with $p < 0.05$ to be statistically significant. The five significant variables selected from the elastic net regression model were then imported into the binary multivariable stepwise logistic regression analysis to explore the relationship between HGF level and stroke outcome.

Spearman rank correlation analysis was used to explore the relationship between HGF and other factors. Finally, net reclassification improvement (NRI) and integrated discrimination improvement (IDI) (18) were used to evaluate whether adding one or more inflammatory biomarkers to conventional risk factors improved the predictive power for the primary and secondary outcomes in patients with AIS.

RESULTS

Baseline Characteristics

The baseline clinical characteristics of the 202 patients with AIS are listed in **Table 1**. The mean patient age was 64.24 years, and 149 (73.76%) of the patients were male. The median NIHSS

TABLE 1 | Clinical characteristics and inflammatory cytokines of AIS patients with favorable and unfavorable outcomes.

Baseline characteristics	All (202)	Favorable outcome (mRS 0–2, *n* = 130)	Unfavorable outcome (mRS 3–6, *n* = 72)	*p*-value
Age, y	64.24 ± 13.31	61.92 ± 12.30	68.42 ± 14.11	0.001
Male, *n* (%)	149 (73.76)	98 (75.38)	51 (70.83)	0.481
Time from onset, h	2.95 (1.48–5.03)	2.85 (1.30–4.43)	3.15 (1.80–6.38)	0.108
Baseline systolic BP, mmHg	150 (140–170)	152.5 (140–170)	149 (140–169.5)	0.322
Baseline diastolic BP, mmHg	87.5 (78.0–93.0)	85.0 (77.8–94.0)	90.0 (79.0–92.0)	0.803
Baseline NIHSS	5.0 (3.0–11.0)	4.0 (2.0–6.0)	13.0 (8.3–17.0)	<0.001
Rt-PA administration, *n* (%)	92 (45.54)	68 (52.31)	24 (33.33)	0.009
Prior risk factors, *n* (%)				
Hypertension	133 (65.84)	83 (63.85)	50 (69.44)	0.422
Diabetes mellitus	83 (41.09)	47 (36.15)	36 (50.00)	0.055
Coronary heart disease	46 (22.77)	24 (18.46)	22 (30.56)	0.050
Atrial fibrillation	32 (15.84)	11 (8.46)	21 (29.17)	<0.001
Stroke etiology, n (%)				
Thrombotic	148 (73.27)	94 (72.31)	54 (75.00)	0.679
Embolic	15 (7.35)	10 (7.63)	5 (6.85)	0.846
Lacunar	39 (19.18)	26 (19.85)	13 (17.81)	0.737
Clinical parameters, median (IQR)				
NLR	2.88 (2.01–5.14)	2.55 (1.77–3.99)	4.48 (2.34–8.67)	<0.001
PLT	208.0 (170.8–246.5)	215.5 (175.3–257.0)	196.0 (164.0–229.8)	0.032
Baseline glucose, mmol/L	6.72 (5.71–8.91)	6.32 (5.56–8.04)	8.18 (6.35–11.18)	<0.001
TG, mmol/L	1.50 (0.96–2.48)	1.70 (1.07–2.69)	1.26 (0.79–1.81)	0.007
Total cholesterol, mmol/L	4.55 (3.82–5.44)	4.75 (3.93–5.59)	4.28 (3.60–5.11)	0.018
HDL, mmol/L	1.18 (1.00–1.39)	1.17 (0.97–1.39)	1.23 (1.05–1.39)	0.358
LDL, mmol/L	2.72 (2.05–3.44)	2.79 (2.05–3.62)	2.50 (2.01–3.15)	0.137
Biomarkers (pg/ml), median (IQR)				
HGF	101.11 (77.01–132.64)	89.47 (67.61–108.41)	138.96 (99.12–201.03)	<0.001
IL-1β	5.54 (3.46–9.54)	6.30 (3.70–10.69)	4.83 (3.03–8.63)	0.020
IL-5	63.18 (36.62–101.96)	64.49 (44.04–109.02)	57.92 (33.35–92.22)	0.102
IL-10	3.07 (1.89–4.79)	3.07 (1.89–4.68)	3.14 (1.89–4.58)	0.938
IL-16	68.44 (47.29–109.97)	55.86 (43.81–85.38)	89.65 (62.35–147.30)	<0.001

BP, blood pressure; NIHSS, NIH stroke scale; Rt-PA, recombinant tissue-plasminogen activator; IQR, interquartile range. TG, triglyceride; HDL, high-density lipoprotein; LDL, Low-density lipoprotein.

score at presentation was 5 (IQR 3–11), and the median time from stroke onset to blood collection was 2.95 h (IQR 1.48–5.03). According to their mRS scores, the patients were divided into two groups with good ($N = 130$, mRS score = 0–2) and poor ($N = 72$, mRS score = 3–6) prognoses. As shown in **Table 1**, compared with patients with a good prognosis, patients with a poor prognosis were older and had a higher NIHSS score, a higher neutrophil-to-lymphocyte ratio, higher blood glucose, lower triglycerides, lower total cholesterol, and higher HGF levels.

Expression Levels of Plasma HGF and Interleukins in Patients AIS With Unfavorable and Favorable Outcomes at 3 Months

At the 3-month follow-up, 72 (35.64%) patients had experienced the primary unfavorable outcome (**Table 2**). The clinical variables related to an unfavorable outcome were older age, higher admission NIHSS scores, history of atrial fibrillation, and higher baseline neutrophil-to-lymphocyte ratio. We observed significantly increased baseline HGF (138.96 vs. 89.47, $p < 0.001$) and IL-16 (89.65 vs. 55.86, $p < 0.001$) levels in patients with unfavorable outcome, and baseline IL-1β (4.83 vs. 6.30, $p = 0.020$) levels were decreased in patients with unfavorable outcomes compared with those in patients with favorable outcomes (**Table 1**).

Prognostic Function of Plasma HGF and Interleukin Levels for Poststroke Outcome at 3 Months

We next conducted a multivariable logistic regression analysis to determine whether HGF and its related interleukins could predict the outcome of patients with AIS. After adjusting for clinical variables possibly related to the primary outcome, only elevated HGF level remained significant for the prediction of unfavorable outcomes in binominal multivariate analysis ($p < 0.001$). The multivariable-adjusted OR (95% CI) for HGF (each 10 pg/mL increase) was 1.178 (1.087–1.277) (**Table 2**). HGF, IL-1β, IL-5, IL-10, and IL-16 levels were then dichotomized using ROC curves.

TABLE 2 | Univariable and multivariable logistic regression analyses depicting the associations of the four parameters and other baseline characteristics with unfavorable outcomes.

Facors	Model 1		Model 2	
	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
Age	1.034 (1.002–1.067)	0.040	1.036 (1.003–1.069)	0.030
NIHSS	1.300 (1.186–1.426)	<0.001	1.309 (1.196–1.432)	<0.001
Diabetes mellitus	2.680 (1.069–6.719)	0.036	2.945 (1.194–7.265)	0.019
Rt-PA administration	0.379 (0.152–0.944)	0.037	0.397 (0.162–0.972)	0.043
Biomarkers (as continuous variables)				
HGF, 10 pg/ml per increase	1.160 (1.070–1.257)	<0.001	1.178 (1.087–1.277)	<0.001
IL-1β, 1 pg/ml per increase				
IL-5, 10 pg/ml per increase				
IL-10, 1 pg/ml per increase			–	–
IL-16, 10 pg/ml per increase				
Biomarkers (as categorical variables)				
HGF, ≥117.745 pg/ml			7.606 (3.090–18.726)	<0.001
IL-1β, ≥4.895 pg/ml		–	–	–
IL-5, ≥125.960 pg/ml	–	–	–	–
IL-10, ≥3.660 pg/ml	–	–	–	–
IL-16, ≥60.190 pg/ml	–	–	–	–

Model 1 was elastic net regression model. Model 2 was adjusted for age, admission NIHSS score, history of diabetes mellitus, and rt-PA treatment.

TABLE 3 | Reclassification of the primary outcome by plasma HGF in AIS patients.

Models	NRI		IDI	
	Estimate (95% CI), %	*p*-value	Estimate (95% CI), %	*p*-value
Conventional model	Reference	–	Reference	–
Conventional model + HGF	37.13 (19.17–44.29)	<0.001	8.71 (3.75–13.67)	<0.001

CI, confidence interval; IDI, integrated discrimination index; NRI, net reclassification improvement. The conventional model included factors that are significant in multivariate logistic regression.

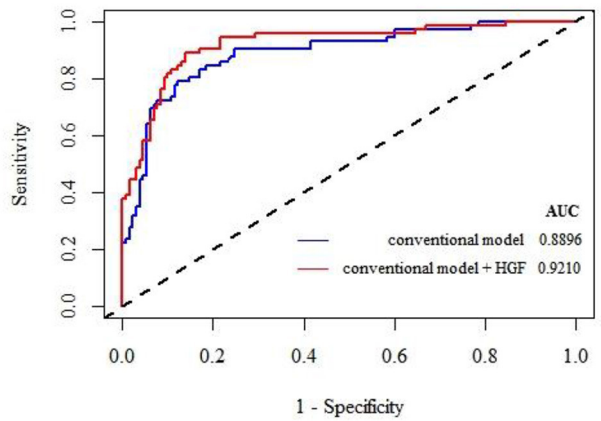

FIGURE 1 | The ROC curves of the two models are drawn to calculate the corresponding AUC value. It can be seen that the AUC value of the traditional model is 0.8896. When HGF is introduced into the model, the AUC value can reach 0.9210, with an increase of 3.1 percentage points.

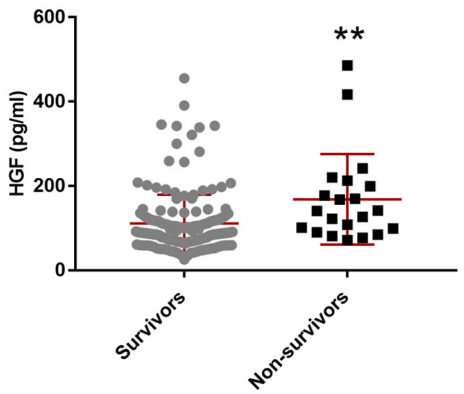

FIGURE 2 | Comparisons of plasma HGF between survivors and non-survivors within 3 months poststroke. The horizontal lines represent median levels and interquartile ranges (IQR). $N = 181$ in survivors, and $n = 21$ in non-survivors. $^{**}p < 0.01$.

The optimal cutoff values are shown in **Table 2**. Multivariable logistic regression analyses were then conducted as before using the dichotomized data. Following multivariable analysis, only plasma HGF remained an independent predictor of unfavorable outcome ($p < 0.001$) with a higher adjusted OR of 7.606 (3.090–18.726). None of the other four parameters were independently associated with the primary outcome (**Table 2**).

Incremental Predictive Value of Plasma HGF Level for Poor Prognosis of AIS

We then tested whether adding plasma HGF to the conventional model (including age, NIHSS score, diabetes, and t-PA treatment) could improve the predictive ability for unfavorable outcomes in patients with AIS. We calculated the NRI and IDI of the new model compared with the traditional model (**Table 3**). The results demonstrate that HGF may improve the reclassification of unfavorable outcome (NRI: 31.73%, $p < 0.001$; IDI: 8.71%, $p < 0.001$). We also generated ROC curves of the two models and calculated the corresponding area under the curve (AUC) values (**Figure 1**). The AUC value of the traditional model was 0.8896, which increased by 3.1 percentage points to 0.9210 when HGF was introduced into the model.

Plasma HGF Level Was Significantly Higher in Non-survivors of AIS Within 3 Months

Within 3 months after stroke, 21 patients (10.40%) died. The plasma HGF levels were significantly higher in non-survivors than those in survivors ($p = 0.0011$) (**Figure 2**). The five biomarkers were dichotomized using ROC curves, and their optimal cutoff values were determined (**Table 4**). After multiple logistic regression analysis, only plasma HGF was a risk factor for mortality (OR: 3.120, 95% CI: 1.042–9.343, $p = 0.042$) (**Table 4**).

HGF Was Closely Associated With Interleukin Levels and Inflammation in Patients With AIS

Correlation analysis to further explore the association of HGF with interleukins and inflammation in patients with AIS revealed that plasma HGF levels were positively related to both IL-10 and IL-16 (**Figure 3A**, $p < 0.05$). Then, HGF and IL-16 were negatively correlated with lymphocyte number, and IL-1β was positively correlated with lymphocyte number (**Figure 3B**, $p < 0.05$). Moreover, HGF levels before all interleukins were significantly negatively correlated with the lymphocyte-to-neutrophil ratio (**Figure 3C**, $p < 0.0001$).

DISCUSSION

This study investigated the prognostic value of baseline HGF and HGF-associated interleukins in 202 patients with acute-phase AIS and 76 healthy controls. The core findings of this study include (i) elevated plasma HGF levels within 24 h after stroke attack were associated with an increased risk of the primary outcomes at 3 months after stroke in a broader AIS population; moreover, the addition of HGF to a model containing conventional risk factors improved the risk stratification for primary outcomes. (ii) IL-10, IL-1β, IL-5, and IL-16 were not independently associated with unfavorable outcomes in patients with AIS. (iii) HGF was positively correlated with IL-10 and IL-16, and HGF levels before all interleukins and was negatively correlated with lymphocyte-to-neutrophil ratio.

Previously, Zhu et al. reported that serum HGF levels were associated with mortality but not disability at 3 months after ischemic stroke onset (5). However, they included patients within 48 h of symptom onset and excluded patients treated with rt-PA (5). By comparison, we collected blood samples within 24 h and before the patients had received any treatment. Thus, the HGF level could better reflect the pathological changes of AIS without the influence of clinical treatment. We also included patients who received rt-PA therapy for AIS within 4.5 h after symptom onset (19). As rt-PA treatment is a well-accepted strategy, incorporating those patients into this study made our findings more easily

TABLE 4 | Biomarkers and risk of the secondary outcome after AIS.

Factors	Model 1		Model 2	
	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
NIHSS	1.135 (1.071–1.202)	<0.001	1.115 (1.046–1.188)	0.001
Diabetes mellitus	2.577 (1.017–6.532)	0.046	3.148 (1.091–9.085)	0.034
Coronary heart disease	2.316 (0.895–5.992)	0.083	1.639 (0.528–5.093)	0.561
Atrial fibrillation	2.385 (0.848–6.705)	0.099	0.958 (0.238–3.855)	0.870
Leukocytes number	1.233 (1.087–1.399)	0.001	1.114 (0.942–1.317)	0.204
Biomarkers (as categorical variables)				
HGF, ≥140.005 pg/ml	6.011 (2.333–15.486)	<0.001	3.120 (1.042–9.343)	0.042
IL-1β, ≥7.035 pg/ml	1.703 (0.686–4.227)	0.251	–	–
IL-5, ≥83.87 pg/ml	1.928 (0.775–4.797)	0.158	–	–
IL-10, ≥2.465 pg/ml	2.163 (0.759–6.164)	0.149	–	–
IL-16, ≥60.190 pg/ml	4.111 (1.331–12.696)	0.014	1.179 (0.475–6.738)	0.413

Model 1 was an unadjusted logistic regression model. Model 2 was adjusted for admission NIHSS score, history of diabetes mellitus, coronary heart disease, and atrial fibrillation, leukocytes number.

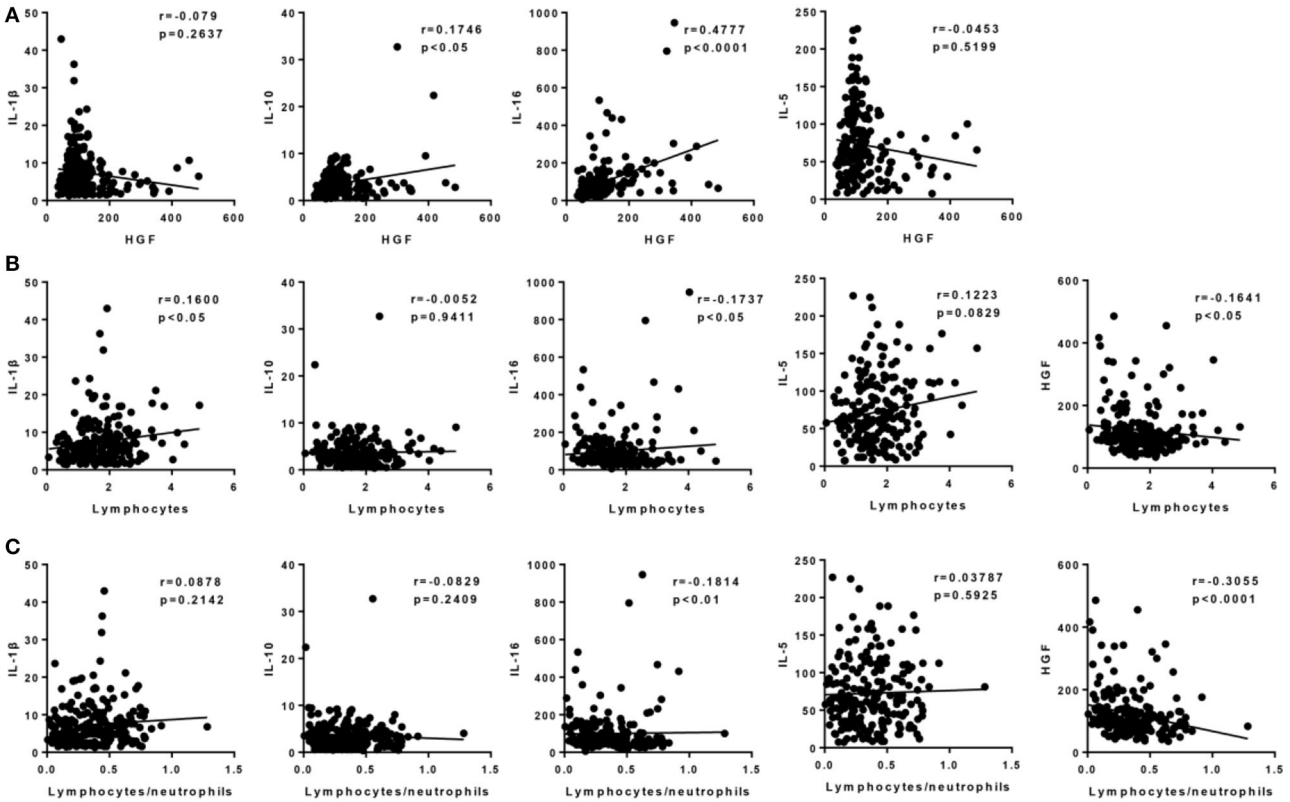

FIGURE 3 | Correlation analyses between circulating HGF and the related interleukins (IL-1β, IL-5, IL-10, and IL-16) in AIS patients. **(A)** Correlation between circulating HGF and other four parameters within 24 h after stroke attack. **(B)** Correlation between the number of circulating lymphocytes and the five parameters within 24 h after stroke attack. **(C)** Correlation between lymphocyte-to-neutrophil ratio and the five parameters within 24 h after stroke attack. $N = 202$.

generalized. In general, although the inclusion criteria of AIS patients differed somewhat, our results are consistent with those of previous clinical studies reporting that HGF is an independent risk factor for an unfavorable prognosis in patients with AIS.

HGF is a pleiotropic cytokine that can regulate different cellular functions. Delayed recanalization after middle cerebral artery occlusion ameliorated ischemic stroke by inhibiting apoptosis via the HGF/c-Met/STAT3/Bcl-2 pathway in rats (20).

However, clinical studies report that circulating HGF level is associated with stroke risk factors involved in endothelial dysfunction, including hypertension, diabetes mellitus, smoking, and age (21). HGF also accelerated the progression of atherosclerosis, and melatonin inhibited macrophage infiltration and promoted plaque stabilization by upregulating the anti-inflammatory HGF/c-Met system in atherosclerotic rabbit ultrasmall superparamagnetic iron oxides (USPIO)-enhanced MRI assessment (22, 23). Similarly, we also found that plasma HGF levels were associated with increased risks of unfavorable outcomes within 3 months in patients with AIS. Collectively, although HGF interventions have shown both good and bad effects in basic research, increased circulating HGF levels were related to the risk factors of stroke and predicted an adverse prognosis of stroke.

HGF was also associated with central and peripheral inflammation in ischemic stroke. HGF suppressed microglial activation and IL-1β expression in rats with ischemic stroke (11). An *in vitro* study also showed that HGF affected the phenotypic shift of macrophages by decreasing the levels of pro-inflammatory IL-1β and promoting the expression of anti-inflammatory IL-10 (12). Our results show the elevation of IL-1β and IL-10 and HGF levels are positively related to IL-10 expression, indicating that HGF may participate in the phenotypic transformation of microglia/macrophages in patients with AIS. Moreover, both HGF and IL-16 were associated with the regulation of Th17, which are important pro-inflammatory cells in AIS. In our study, HGF was strongly positively associated with IL-16 expression, both of which were negatively correlated with lymphocyte number. We speculate that HGF and IL-16 may synergistically regulate the Th17-associated inflammatory process in AIS. HGF levels before all interleukins were substantially negatively correlated with the lymphocyte-to-neutrophil ratio as well as unfavorable outcomes in patients with AIS. Therefore, we inferred that HGF was involved in multiple inflammatory responses in AIS and that interventions involving HGF may be a therapeutic strategy in AIS.

However, our study has some limitations. First, it lacks data on infarct volume in CT or MRI scans. Patients who met the criterion of intravenous therapy undergo CT scans to exclude cerebral hemorrhage and should be infused with thrombolysis drugs as early as possible (24). Thus, nearly half of the patients in our study did not undergo a premorbid MRI scan. However, infarcts are not obvious on early CT scans, and the severity of neurological prognosis is not always proportional to the size of infarct volume; hence, it is reasonable that we did not include infarct volume in our study. Second, our study was performed in a single center, and the sample size is somewhat small, which limits the generalizability of our results. Further testing in separate cohorts with larger sample sizes in multiple centers is needed to verify our findings. Third, the biomarkers included in this study were only examined once at admission. As the inflammatory process is dynamic after AIS, it is essential to monitor the changes of these biomarkers in further studies. Nevertheless, the present study extends the knowledge and clinical application of acute-phase circulating HGF levels in a broader AIS population.

In conclusion, our study results demonstrate that increased plasma HGF levels within 24 h were associated with unfavorable prognosis and mortality at 3 months after AIS. Adding plasma HGF to established risk factors substantially improves the risk prediction for an unfavorable prognosis in patients with AIS patients; thus, the results of the present study extend the prognostic significance of HGF in patients with AIS administered t-PA treatment within 24 h poststroke. In addition, we find that plasma HGF may be a node for phenotype transformation of inflammatory cells in AIS. However, our findings also have limitations, including the fact that, due to the characteristics of the prediction (its bidirectionality), we cannot determine which is the cause and which is the effect. However, this link provides a thought-provoking example of how apparently different biological properties could interact to determine a unique abnormal condition of disease. If this association is verified, interventions including HGF may offer a new, promising immunomodulatory therapeutic target for AIS.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Xuanwu Hospital, Capital Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FL and PL prepared the study protocol, collected, analyzed, interpreted the data, and prepared the manuscript. YH, LL, SZ, ZY, RW, ZT, ZH, JF, and YZ collected the data. FL and PL performed the cytometric assay and analyzed the data. HZ and YL prepared the study protocol, analyzed and interpreted the data, and supervised the study. All authors contributed to the article and approved the submitted version.

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The Relationship Between Elevated Serum Uric Acid and Risk of Stroke in Adult: An Updated and Dose–Response Meta-Analysis

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Background: Uric acid (UA) is proposed as a potential risk factor for stroke in adult, yet the results from published studies are not generally accordant.

Method: We included prospective studies that explored the relationship between serum UA (SUA) and strokes. In this study, strokes include ischemic stroke and hemorrhagic stroke, which consists of intracerebral hemorrhage and subarachnoid hemorrhage. The effect-size estimates were expressed as hazard ratio (HR) and 95% confidence interval (CI). Sensitivity and subgroup analyses were performed to assess the robustness of the pooled estimation and potential sources of heterogeneity between studies.

Results: We meta-analyzed 19 prospective cohort articles, which involve 37,386 males and 31,163 females. Overall analyses results showed a significant association between a 1 mg/dl increase in high levels of SUA and the risk of total stroke (HR = 1.13; 95% CI: 1.09–1.18; $P < 0.001$), ischemic stroke (HR = 1.15; 95% CI: 1.10–1.21; $P < 0.001$), and hemorrhagic stroke (HR = 1.07; 95% CI: 1.00 to 1.15; $P = 0.046$). No significant difference was found between ischemic stroke and hemorrhagic stroke. In the subgroup analyses, the association of high SUA levels and the risk of total stroke was statistically significant in females (HR = 1.19; 95% CI: 1.12–1.26; $P < 0.001$) and males (HR = 1.11; 95% CI: 1.05–1.17; $P < 0.001$). Coincidentally, the association was also statistically significant for ischemic stroke, both in females (HR = 1.26; 95% CI: 1.17–1.36; $P < 0.001$) and in males (HR = 1.12; 95% CI: 1.06–1.19; $P < 0.001$). However, for hemorrhagic stroke, it was only statistically significant in females (HR = 1.19; 95% CI: 1.04–1.35; $P = 0.01$). Our dose–response research indicated the J-shaped trend between the ascending SUA levels and the higher risk of suffering from a stroke.

Conclusions: Our findings indicate that elevated SUA is a significant risk factor for adult stroke, both for ischemic stroke and hemorrhagic stroke, and especially in females.

Keywords: risk factor, meta-analysis, serum uric acid, ischemic stroke, hemorrhagic stroke

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INTRODUCTION

Stroke is believed to be the second leading cause of death and a major contributor to disability-adjusted life-years (DALYs) lost worldwide (1). According to global statistics, together with ischemic heart disease, strokes account for nearly 15.2 million deaths in 2015 (1). In 2017, intracerebral hemorrhage and ischemic stroke caused 57.9 and 47.8 million DALYs lost, separately (2). Stroke is preventable. Multiple modifiable risk factors, such as hypertension, diabetes mellitus, atrial fibrillation, dyslipidemia, smoking, obesity, lack of physical activity, etc., have been widely observed in the prevention and treatment of stroke (1). However, the number of incidents of stroke, survivors, and stroke-related death, as well as DALYs, are still increasing globally (3). Therefore, a better understanding of more potential risk factors are needed to develop additional preventive strategies for stoke.

Uric acid (UA), one metabolic end product of purine, exists in the form of UA salt with high solubility in organisms. Regularly, serum UA (SUA) levels range from 1.5 to 6.0 mg/dl for women and 2.5 to 7.0 mg/dl for men under a healthy status, which is hard upon the upper limit of UA dissolution in serum (4). Up to date, controversial results regarding the correlation between SUA levels and the incidence of stroke have been reported. It was shown that UA is one of the most essential antioxidants in the blood whose concentration is 10 times greater than that of other antioxidants. UA provides an antioxidant defense against oxidant- and radical-caused damage in humans (5). Researches demonstrated that UA is an antioxidant factor to protect nerves from oxidative damage (6, 7), thereby possibly preventing stroke outcomes. Whereas, many studies found that high SUA levels might be a major risk factor for the onset of stroke (8–11). Zhong et al. explored the association between SUA levels and risk of stroke base on a meta-analysis (12). The study revealed that the elevated SUA levels were significantly related to the modestly increased risk of stroke, and there existed no significant gender differences. Meanwhile, the association between SUA and the risk of each subtype of stroke had been developed by different meta-analyses (13, 14). No studies were conducted to compare the effect of SUA levels on ischemic stroke and hemorrhagic stroke. It is widely accepted that hemorrhagic stroke is ascribed to the rupture of a blood vessel, and ischemic stroke is caused by blockage of an artery; both conditions cause local hypoxia that damages brain tissue. Ischemic stroke accounts for the majority of strokes, yet hemorrhagic stroke is responsible for more deaths and DALYs lost (15). Identifying the role of SUA levels in each type of stroke is vital for subsequent targeted treatment and prevention. In our study, we performed a meta-analysis of prospective studies to detect the association between elevated SUA levels and the risk of stroke and explored the differences between ischemic stroke and hemorrhagic stroke.

METHODS

This meta-analysis was carried out in line with the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) statement (16), which is presented in **Supplementary Table 1**.

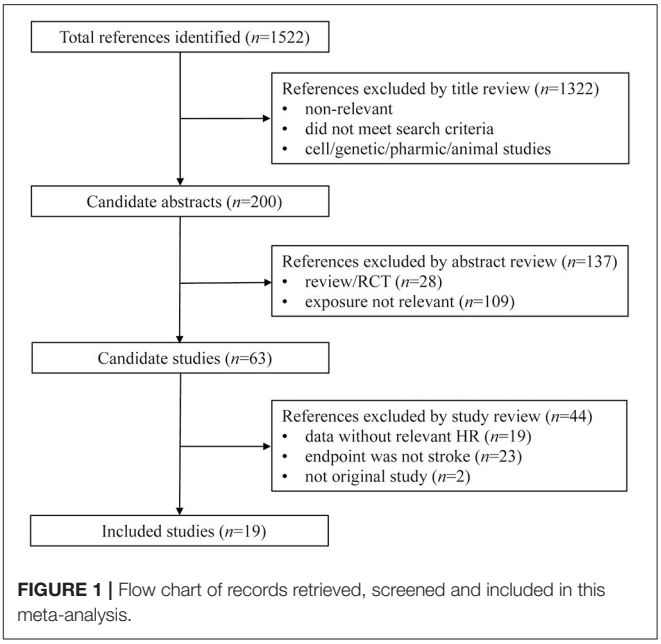

FIGURE 1 | Flow chart of records retrieved, screened and included in this meta-analysis.

Search Strategy

We finished literature search by looking through PubMed, EMBASE, and Web of Science databases as of December 26, 2020. The following medical nomenclature are considered: (uric acid OR ua OR urate OR hyperuricemia OR hyperuric OR ammonium acid urate [Title/Abstract]) AND (stroke OR cerebrovascular OR apoplexy OR brain vascular accident OR cerebral stroke OR ischemic stroke OR ischaemic stroke OR cryptogenic ischemic stroke OR cryptogenic stroke OR embolism stroke OR intracranial embolism OR intracranial infarction OR cerebral embolism OR cerebral infarction OR brain infarction OR intracranial hemorrhage OR brain hemorrhage OR hemorrhagic stroke OR cerebral hemorrhage [Title/Abstract]). In order to avoid underlying missing points, reference lists of retrieved articles and systematic reviews were scanned.

Two researchers (Tianci Qiao and Hongyun Wu) examined all retrieved articles independently, and they seriously assessed preliminary qualification based on the titles, abstracts, and full texts when necessary.

Inclusion/Exclusion Criteria

We included the articles when they met the following criteria: (1) the study has a prospective design (prospective cohort or prospective nested case-control study); (2) the study outcomes were stroke, including ischemic stroke and any kinds of hemorrhagic stroke (intracerebral hemorrhage and subarachnoid hemorrhage); (3) enrolled participants were free of stroke at baseline; (4) studies that reported the definition of outcomes in participants with stroke; and (5) hazard ratio (HR) and corresponding 95% confidence interval (CI) of the association between UA and stroke were reported. Articles were excluded

TABLE 1 | Baseline characters of the associations between UA levels and the risk of having stroke.

Author	Year	Location	Baseline age	Follow-up (year)	Sample size (*n*)	Stroke type	Sex	Case (*n*)	Uric acid levels (μmol/L)	HR (95% CI)
Sakata	2001	Japan	≥30	14	8,172	Total stroke	M	94	297–338	0.84 (0.45–1.59)
Sakata	2001	Japan	≥30	14	8,172	Total stroke	M	94	339–385	0.66 (0.33–1.33)
Sakata	2001	Japan	≥30	14	8,172	Total stroke	M	94	≥386	1.71 (0.92–3.17)
Sakata	2001	Japan	≥30	14	8,172	Total stroke	F	80	214–248	1.40 (0.54–3.63)
Sakata	2001	Japan	≥30	14	8,172	Total stroke	F	80	249–290	0.95 (0.37–2.45)
Sakata	2001	Japan	≥30	14	8,172	Total stroke	F	80	≥291	1.12 (0.46–2.74)
Chien	2005	China	>35	11	3,602	Total stroke	M	155	Per unit	1.13 (0.88–1.46)
Chien	2005	China	>35	11	3,602	Total stroke	F	155	Per unit	1.32 (1.01–1.73)
Bos	2006	Netherlands	≥55	8.4	4,385	Total stroke	M	132	310–375	1.78 (1.16–2.74)
Bos	2006	Netherlands	≥55	8.4	4,385	Total stroke	M	132	≥375	1.41 (0.90–2.23)
Bos	2006	Netherlands	≥55	8.4	4,385	Total stroke	M	132	Per unit	1.15 (0.95–1.38)
Bos	2006	Netherlands	≥55	8.4	4,385	Total stroke	F	249	263–321	1.45 (1.05–2.02)
Bos	2006	Netherlands	≥55	8.4	4,385	Total stroke	F	249	≥321	1.45 (1.05–2.01)
Bos	2006	Netherlands	≥55	8.4	4,385	Total stroke	F	249	Per unit	1.18 (1.05–1.34)
Bos	2006	Netherlands	≥55	8.4	4,385	IS	M	73	310–375	1.57 (0.88–2.79)
Bos	2006	Netherlands	≥55	8.4	4,385	IS	M	73	≥375	1.36 (0.74–2.48)
Bos	2006	Netherlands	≥55	8.4	4,385	IS	M	73	Per unit	1.18 (0.92–1.51)
Bos	2006	Netherlands	≥55	8.4	4,385	IS	F	132	263–321	1.44 (0.91–2.27)
Bos	2006	Netherlands	≥55	8.4	4,385	IS	F	132	≥321	1.68 (1.08–2.62)
Bos	2006	Netherlands	≥55	8.4	4,385	IS	F	132	Per unit	1.26 (1.07–1.49)
Bos	2006	Netherlands	≥55	8.4	4,385	HS	M	16	310–375	1.23 (0.38–4.04)
Bos	2006	Netherlands	≥55	8.4	4,385	HS	M	16	≥375	1.11 (0.32–3.83)
Bos	2006	Netherlands	≥55	8.4	4,385	HS	M	16	Per unit	0.97 (0.55–1.70)
Bos	2006	Netherlands	≥55	8.4	4,385	HS	F	30	263–321	1.22 (0.48–3.10)
Bos	2006	Netherlands	≥55	8.4	4,385	HS	F	30	≥321	1.32 (0.53–3.26)
Bos	2006	Netherlands	≥55	8.4	4,385	HS	F	30	Per unit	1.23 (0.87–1.74)
Gerber	2006	Israel	≥40	23	9,125	Total stroke	M	292	≤238	1.52 (1.04–2.23)
Gerber	2006	Israel	≥40	23	9,125	Total stroke	M	292	238–267	1.46 (1.00–2.12)
Gerber	2006	Israel	≥40	23	9,125	Total stroke	M	292	298–333	1.25 (0.85–1.84)
Gerber	2006	Israel	≥40	23	9,125	Total stroke	M	292	≥333	1.20 (0.81–1.78)
Gerber	2006	Israel	≥40	23	9,125	IS	M	292	≤238	1.34 (0.87–2.05)
Gerber	2006	Israel	≥40	23	9,125	IS	M	292	238–267	1.33 (0.89–2.01)
Gerber	2006	Israel	≥40	23	9,125	IS	M	292	298–333	1.21 (0.81–1.82)
Gerber	2006	Israel	≥40	23	9,125	IS	M	292	≥333	1.15 (0.75–1.74)
Gerber	2006	Israel	≥40	23	9,125	HS	M	292	≤238	3.27 (1.14–9.33)
Gerber	2006	Israel	≥40	23	9,125	HS	M	292	238–267	2.52 (0.87–7.29)
Gerber	2006	Israel	≥40	23	9,125	HS	M	292	298–333	1.55 (0.49–4.89)
Gerber	2006	Israel	≥40	23	9,125	HS	M	292	≥333	1.62 (0.51–5.18)
Hozawa	2006	USA	45–64	12.6	11,263	IS	All	381	286–351	0.86 (0.60–1.23)
Hozawa	2006	USA	45–64	12.6	11,263	IS	All	381	351–411	1.09 (0.79–1.49)
Hozawa	2006	USA	45–64	12.6	11,263	IS	All	381	≥411	1.25 (0.91–1.73)
Hozawa	2006	USA	45–64	12.6	11,263	IS	M	149	286–351	1.01 (0.48–2.13)
Hozawa	2006	USA	45–64	12.6	11,263	IS	M	149	351–411	1.30 (0.67–2.53)
Hozawa	2006	USA	45–64	12.6	11,263	IS	M	149	≥411	1.63 (0.83–3.19)
Hozawa	2006	USA	45–64	12.6	11,263	IS	F	118	286–351	0.85 (0.51–1.41)
Hozawa	2006	USA	45–64	12.6	11,263	IS	F	118	351–411	1.22 (0.75–1.99)
Hozawa	2006	USA	45–64	12.6	11,263	IS	F	118	≥411	1.27 (0.70–2.30)
Strasak1	2008	Austria	62.3	15.2	28,613	Total stroke	F	1,552	220–268	1.25 (0.99–1.57)
Strasak1	2008	Austria	62.3	15.2	28,613	Total stroke	F	1,552	268–322	1.48 (1.18–1.86)
Strasak1	2008	Austria	62.3	15.2	28,613	Total stroke	F	1,552	≥322	1.37 (1.09–1.74)
Strasak1	2008	Austria	62.3	15.2	28,613	Total stroke	F	1,552	Per unit	1.07 (1.01–1.13)

(Continued)

TABLE 1 | Continued

Author	Year	Location	Baseline age	Follow-up (year)	Sample size (*n*)	Stroke type	Sex	Case (*n*)	Uric acid levels (μmol/L)	HR (95% CI)
Strasak1	2008	Austria	62.3	15.2	28,613	HS	F	228	220–268	1.14 (0.65–2.01)
Strasak1	2008	Austria	62.3	15.2	28,613	HS	F	228	268–322	1.47 (0.83–2.52)
Strasak1	2008	Austria	62.3	15.2	28,613	HS	F	228	≥322	1.29 (0.71–2.4)
Strasak1	2008	Austria	62.3	15.2	28,613	HS	F	228	Per unit	1.06 (0.91–1.23)
Strasak1	2008	Austria	62.3	15.2	28,613	IS	F	422	220–268	1.33 (0.97–1.83)
Strasak1	2008	Austria	62.3	15.2	28,613	IS	F	422	268–322	1.66 (1.22–2.26)
Strasak1	2008	Austria	62.3	15.2	28,613	IS	F	422	≥322	1.53 (1.11–2.09)
Strasak1	2008	Austria	62.3	15.2	28,613	IS	F	422	Per unit	1.02 (0.91–1.14)
Strasak2	2008	Austria	41.6	13.6	83,683	Total stroke	M	645	273.82–315.48	1.00 (0.76–1.30)
Strasak2	2008	Austria	41.6	13.6	83,683	Total stroke	M	645	315.49–351.19	1.05 (0.80–1.38)
Strasak2	2008	Austria	41.6	13.6	83,683	Total stroke	M	645	351.2–398.81	1.02 (0.78–1.34)
Strasak2	2008	Austria	41.6	13.6	83,683	Total stroke	M	645	>398.81	1.59 (1.23–2.04)
Strasak2	2008	Austria	41.6	13.6	83,683	Total stroke	M	645	Per unit	1.11 (1.05–1.18)
Strasak2	2008	Austria	41.6	13.6	83,683	HS	M	147	273.82–315.48	1.02 (0.60–1.72)
Strasak2	2008	Austria	41.6	13.6	83,683	HS	M	147	315.49–351.19	0.89 (0.51–1.57)
Strasak2	2008	Austria	41.6	13.6	83,683	HS	M	147	351.2–398.81	0.92 (0.53–1.60)
Strasak2	2008	Austria	41.6	13.6	83,683	HS	M	147	>398.81	1.18 (0.70–2.01)
Strasak2	2008	Austria	41.6	13.6	83,683	HS	M	147	Per unit	1.06 (0.93–1.20)
Strasak2	2008	Austria	41.6	13.6	83,683	IS	M	147	273.82–315.48	0.92 (0.52–1.63)
Strasak2	2008	Austria	41.6	13.6	83,683	IS	M	147	315.49–351.19	1.19 (0.68–2.07)
Strasak2	2008	Austria	41.6	13.6	83,683	IS	M	147	351.2–398.81	1.01 (0.57–1.80)
Strasak2	2008	Austria	41.6	13.6	83,683	IS	M	147	>398.81	1.81 (1.07–3.04)
Strasak2	2008	Austria	41.6	13.6	83,683	IS	M	147	Per unit	1.13 (1.01–1.27)
Holme	2009	Sweden	30–85	11.8	417,734	Total stroke	M	9,324	281–319	1.03 (0.97–1.09)
Holme	2009	Sweden	30–85	11.8	417,734	Total stroke	M	9,324	319–362	1.09 (1.02–1.15)
Holme	2009	Sweden	30–85	11.8	417,734	Total stroke	M	9,324	>362	1.26 (1.19–1.34)
Holme	2009	Sweden	30–85	11.8	417,734	Total stroke	F	6,952	208–242	1.05 (0.97–1.15)
Holme	2009	Sweden	30–85	11.8	417,734	Total stroke	F	6,952	242–327	1.16 (1.07–1.26)
Holme	2009	Sweden	30–85	11.8	417,734	Total stroke	F	6,952	>327	1.41 (1.31–1.53)
Holme	2009	Sweden	30–85	11.8	417,734	HS	M	9,324	281–319	0.83 (0.71–0.96)
Holme	2009	Sweden	30–85	11.8	417,734	HS	M	9,324	319–362	0.92 (0.80–1.07)
Holme	2009	Sweden	30–85	11.8	417,734	HS	M	9,324	>362	1.10 (0.96–1.27)
Holme	2009	Sweden	30–85	11.8	417,734	HS	F	6,952	208–242	0.81 (0.64–1.01)
Holme	2009	Sweden	30–85	11.8	417,734	HS	F	6,952	242–327	1.01 (0.82–1.24)
Holme	2009	Sweden	30–85	11.8	417,734	HS	F	6,952	>327	1.13 (0.92–1.37)
Holme	2009	Sweden	30–85	11.8	417,734	IS	M	9,324	281–319	1.08 (1.01–1.16)
Holme	2009	Sweden	30–85	11.8	417,734	IS	M	9,324	319–362	1.10 (1.02–1.18)
Holme	2009	Sweden	30–85	11.8	417,734	IS	M	9,324	>362	1.30 (1.22–1.40)
Holme	2009	Sweden	30–85	11.8	417,734	IS	F	6,952	208–242	1.12 (1.01–1.24)
Holme	2009	Sweden	30–85	11.8	417,734	IS	F	6,952	242–327	1.27 (1.15–1.40)
Holme	2009	Sweden	30–85	11.8	417,734	IS	F	6,952	>327	1.56 (1.42–1.72)
Storhaug	2013	Norway	≥25	12.5	5,700	IS	M	430	Per unit	1.31 (1.14–1.50)
Storhaug	2013	Norway	≥25	12.5	5,700	IS	F	430	Per unit	1.13 (0.94–1.36)
Zhang	2016	Japan	35–89	10	36,313	Total stroke	M	301	279.7–315.4	0.83 (0.58–1.18)
Zhang	2016	Japan	35–89	10	36,313	Total stroke	M	301	315.4–351.1	0.77 (0.52–1.13)
Zhang	2016	Japan	35–89	10	36,313	Total stroke	M	301	351.1–398.7	0.77 (0.52–1.13)
Zhang	2016	Japan	35–89	10	36,313	Total stroke	M	301	398.7–952.2	1.19 (0.84–1.68)
Zhang	2016	Japan	35–89	10	36,313	IS	M	301	279.7–315.4	0.87 (0.54–1.40)
Zhang	2016	Japan	35–89	10	36,313	IS	M	301	315.4–351.1	0.75 (0.45–1.26)
Zhang	2016	Japan	35–89	10	36,313	IS	M	301	351.1–398.7	0.91 (0.55–1.50)
Zhang	2016	Japan	35–89	10	36,313	IS	M	301	398.7–952.2	1.19 (0.75–1.90)

(Continued)

TABLE 1 | Continued

Author	Year	Location	Baseline age	Follow-up (year)	Sample size (*n*)	Stroke type	Sex	Case (*n*)	Uric acid levels (μmol/L)	HR (95% CI)
Zhang	2016	Japan	35–89	10	36,313	HS	M	301	279.7–315.4	0.90 (0.46–1.77)
Zhang	2016	Japan	35–89	10	36,313	HS	M	301	315.4–351.1	1.07 (0.54–2.14)
Zhang	2016	Japan	35–89	10	36,313	HS	M	301	351.1–398.7	0.83 (0.41–1.68)
Zhang	2016	Japan	35–89	10	36,313	HS	M	301	398.7–952.2	1.41 (0.75–2.65)
Zhang	2016	Japan	35–89	10	36,313	Total stroke	F	293	202.3–232.1	1.27 (0.90–2.01)
Zhang	2016	Japan	35–89	10	36,313	Total stroke	F	293	232.1–261.8	0.98 (0.62–1.54)
Zhang	2016	Japan	35–89	10	36,313	Total stroke	F	293	261.8–303.5	1.05 (0.67–1.64)
Zhang	2016	Japan	35–89	10	36,313	Total stroke	F	293	303.5–642.7	1.46 (0.98–2.19)
Zhang	2016	Japan	35–89	10	36,313	IS	F	293	202.3–232.1	1.42 (0.74–2.74)
Zhang	2016	Japan	35–89	10	36,313	IS	F	293	232.1–261.8	0.80 (0.40–1.61)
Zhang	2016	Japan	35–89	10	36,313	IS	F	293	261.8–303.5	1.22 (0.65–2.30)
Zhang	2016	Japan	35–89	10	36,313	IS	F	293	303.5–642.7	1.35 (0.75–2.44)
Zhang	2016	Japan	35–89	10	36,313	HS	F	293	202.3–232.1	1.41 (0.64–3.13)
Zhang	2016	Japan	35–89	10	36,313	HS	F	293	232.1–261.8	1.33 (0.63–2.80)
Zhang	2016	Japan	35–89	10	36,313	HS	F	293	261.8–303.5	1.09 (0.48–2.43)
Zhang	2016	Japan	35–89	10	36,313	HS	F	293	303.5–642.7	1.54 (0.76–3.10)
Shi	2017	China	45–75	4.5	20,577	Total stroke	All	632	M: 279.7–327.3 F: 226.1–261.8	0.90 (0.72–1.13)
Shi	2017	China	45–75	4.5	20,577	Total stroke	All	632	M: 327.3–380.8 F: 261.8–309.5	0.90 (0.71–1.13)
Shi	2017	China	45–75	4.5	20,577	Total stroke	All	632	M: ≥380.8 F: ≥309.5	0.87 (0.69–1.11)
Shi	2017	China	45–75	4.5	20,577	IS	All	632	M: 279.7–327.3 F: 226.1–261.8	1.01 (0.78–1.30)
Shi	2017	China	45–75	4.5	20,577	IS	All	632	M: 327.3–380.8 F: 261.8–309.5	0.93 (0.71–1.20)
Shi	2017	China	45–75	4.5	20,577	IS	All	632	M: ≥380.8 F: ≥309.5	0.95 (0.73–1.25)
Shi	2017	China	45–75	4.5	20,577	HS	All	632	M: 279.7–327.3 F: 226.1–261.8	0.56 (0.32–0.97)
Shi	2017	China	45–75	4.5	20,577	HS	All	632	M: 327.3–380.8 F: 261.8–309.5	0.86 (0.52–1.41)
Shi	2017	China	45–75	4.5	20,577	HS	All	632	M: ≥380.8 F: ≥309.5	0.67 (0.38–1.16)
Shi	2017	China	45–75	4.5	20,577	Total stroke	M	300	279.7–327.3	0.86 (0.62–1.19)
Shi	2017	China	45–75	4.5	20,577	Total stroke	M	300	327.3–380.8	0.91 (0.66–1.27)
Shi	2017	China	45–75	4.5	20,577	Total stroke	M	300	≥380.8	0.80 (0.56–1.15)
Shi	2017	China	45–75	4.5	20,577	Total stroke	F	332	226.1–261.8	0.95 (0.69–1.31)
Shi	2017	China	45–75	4.5	20,577	Total stroke	F	332	261.8–309.5	0.90 (0.65–1.24)
Shi	2017	China	45–75	4.5	20,577	Total stroke	F	332	≥309.5	0.95 (0.68–1.32)
Tu	2019	China	≥65	3	3,243	Total stroke	M	1,309	273.7–309.5	1.10 (1.06–2.55)
Tu	2019	China	≥65	3	3,243	Total stroke	M	1,309	309.5–374.9	1.18 (1.07–2.17)
Tu	2019	China	≥65	3	3,243	Total stroke	M	1,309	≥374.9	2.09 (1.40–4.28)
Tu	2019	China	≥65	3	3,243	IS	M	1,309	273.7–309.5	1.09 (1.05–3.35)
Tu	2019	China	≥65	3	3,243	IS	M	1,309	309.5–374.9	1.13 (1.07–3.37)
Tu	2019	China	≥65	3	3,243	IS	M	1,309	≥374.9	1.69 (1.24–4.80)
Tu	2019	China	≥65	3	3,243	HS	M	1,309	273.7–309.5	1.09 (1.05–3.35)
Tu	2019	China	≥65	3	3,243	HS	M	1,309	309.5–374.9	1.13 (1.07–3.37)
Tu	2019	China	≥65	3	3,243	HS	M	1,309	≥374.9	1.69 (1.24–4.80)
Tu	2019	China	≥65	3	3,243	Total stroke	F	1,309	273.7–309.5	1.15 (1.06–2.39)
Tu	2019	China	≥65	3	3,243	Total stroke	F	1,309	309.5–374.9	1.18 (1.12–2.53)
Tu	2019	China	≥65	3	3,243	Total stroke	F	1,309	≥374.9	2.55 (1.28–5.44)
Tu	2019	China	≥65	3	3,243	IS	F	1,309	273.7–309.5	1.15 (1.06–2.39)

(Continued)

TABLE 1 | Continued

Author	Year	Location	Baseline age	Follow-up (year)	Sample size (*n*)	Stroke type	Sex	Case (*n*)	Uric acid levels (μmol/L)	HR (95% CI)
Tu	2019	China	≥65	3	3,243	IS	F	1,309	309.5–374.9	1.18 (1.12–2.53)
Tu	2019	China	≥65	3	3,243	IS	F	1,309	≥374.9	1.49 (1.18–4.24)
Tu	2019	China	≥65	3	3,243	HS	F	1,309	273.7–309.5	2.84 (1.33–6.93)
Tu	2019	China	≥65	3	3,243	HS	F	1,309	309.5–374.9	3.37 (1.55–8.82)
Tu	2019	China	≥65	3	3,243	HS	F	1,309	≥374.9	5.85 (1.99–9.81)
Chaudhary	2020	USA	≥45	4	30,239	Total stroke	M	430	357–404.7	2.11 (1.29–3.45)
Chaudhary	2020	USA	≥45	4	30,239	Total stroke	M	430	≥404.7	1.14 (0.75–1.73)
Chaudhary	2020	USA	≥45	4	30,239	Total stroke	F	389	357–404.7	0.78 (0.46–1.34)
Chaudhary	2020	USA	≥45	4	30,239	Total stroke	F	389	≥404.7	1.04 (0.62–1.73)
Li	2020	Japan	40–79	23.1	13,420	Total stroke	M	488	279.7–321.4	1.03 (0.78–1.36)
Li	2020	Japan	40–79	23.1	13,420	Total stroke	M	488	321.4–357.1	0.95 (0.71–1.27)
Li	2020	Japan	40–79	23.1	13,420	Total stroke	M	488	357.1–398.7	1.10 (0.82–1.48)
Li	2020	Japan	40–79	23.1	13,420	Total stroke	M	488	398.7–666.5	1.02 (0.74–1.35)
Li	2020	Japan	40–79	23.1	13,420	Total stroke	M	488	Per unit	1.02 (0.92–1.13)
Li	2020	Japan	40–79	23.1	13,420	HS	M	488	279.7–321,4	1.06 (0.57–1.98)
Li	2020	Japan	40–79	23.1	13,420	HS	M	488	321.4–357.1	1.23 (0.66–2.29)
Li	2020	Japan	40–79	23.1	13,420	HS	M	488	357.1–398.7	1.26 (0.67–2.41)
Li	2020	Japan	40–79	23.1	13,420	HS	M	488	398.7–666.5	0.83 (0.40–1.72)
Li	2020	Japan	40–79	23.1	13,420	HS	M	488	Per unit	0.95 (0.75–1.19)
Li	2020	Japan	40–79	23.1	13,420	IS	M	488	279.7–321,4	1.04 (0.74–1.45)
Li	2020	Japan	40–79	23.1	13,420	IS	M	488	321.4–357.1	0.89 (0.63–1.26)
Li	2020	Japan	40–79	23.1	13,420	IS	M	488	357.1–398.7	1.01 (0.71–1.44)
Li	2020	Japan	40–79	23.1	13,420	IS	M	488	398.7–666.5	1.01 (0.70–1.41)
Li	2020	Japan	40–79	23.1	13,420	IS	M	488	Per unit	1.02 (0.91–1.15)
Li	2020	Japan	40–79	23.1	13,420	Total stroke	F	530	214.2–244	1.02 (0.74–1.40)
Li	2020	Japan	40–79	23.1	13,420	Total stroke	F	530	244–273.7	1.20 (0.89–1.63)
Li	2020	Japan	40–79	23.1	13,420	Total stroke	F	530	273.7–309.5	1.15 (0.84–1.56)
Li	2020	Japan	40–79	23.1	13,420	Total stroke	F	530	309.5–613	1.45 (1.07–1.96)
Li	2020	Japan	40–79	23.1	13,420	Total stroke	F	530	Per unit	1.12 (1.03–1.22)
Li	2020	Japan	40–79	23.1	13,420	HS	F	530	214.2–244	0.64 (0.32–1.25)
Li	2020	Japan	40–79	23.1	13,420	HS	F	530	244–273.7	0.86 (0.47–1.59)
Li	2020	Japan	40–79	23.1	13,420	HS	F	530	273.7–309.5	1.22 (0.68–2.18)
Li	2020	Japan	40–79	23.1	13,420	HS	F	530	309.5–613	1.20 (0.65–2.20)
Li	2020	Japan	40–79	23.1	13,420	HS	F	530	Per unit	1.19 (0.99–1.42)
Li	2020	Japan	40–79	23.1	13,420	IS	F	530	214.2–244	1.33 (0.88–2.02)
Li	2020	Japan	40–79	23.1	13,420	IS	F	530	244–273.7	1.52 (1.02–2.26)
Li	2020	Japan	40–79	23.1	13,420	IS	F	530	273.7–309.5	1.12 (0.73–1.72)
Li	2020	Japan	40–79	23.1	13,420	IS	F	530	309.5–613	1.61 (1.07–2.41)
Li	2020	Japan	40–79	23.1	13,420	IS	F	530	Per unit	1.06 (0.95–1.18)
Norvik	2017	Norway	55–74	19	2,940	IS	All	271	Per unit	1.13 (1.02–1.25)
Chen	2011	China	19–85	1.5	226	IS	All	43	per unit	1.01 (0.99–1.01)
Chen	2009	China	≥35	8.2	5,427	IS	All	344	Per unit	1.35 (1.04–1.76)
Chen	2009	China	≥35	8.2	5,427	IS	M	344	>416.6	1.14 (0.83–1.57)
Chen	2009	China	≥35	8.2	5,427	IS	F	344	>416.6	1.83 (1.17–2.87)
Chen	2009	China	≥35	8.2	5,427	HS	All	200	Per unit	1.18 (0.83–1.67)
Chen	2009	China	≥35	8.2	5,427	HS	M	200	>416.6	1.18 (0.76–1.83)
Chen	2009	China	≥35	8.2	5,427	HS	F	200	>416.6	1.01 (0.55–1.88)
Koton	2008	UK	45–85	3.8	2,131	IS	All	259	Per unit	0.94 (0.83–1.06)
Koton	2008	UK	45–85	3.8	2,131	IS	M	259	Per unit	0.90 (0.78–1.04)
Koton	2008	UK	45–85	3.8	2,131	IS	F	259	Per unit	1.07 (0.83–1.38)
Lehto	1998	Finland	45–64	7	1,017	Total stroke	All	114	>295	1.91 (1.24–2.94)

HR, hazard ratio; 95% CI, 95% confidence interval; IS, Ischemic Stroke; HS, Hemorrhagic Stroke; UA: uric acid; UK, the united kingdom; USA, the United States; M, male; F, female; All, both male and female.

if they were reviews, proceedings, letters, case reports, or meta-analyses, or they were not reported in English languages, or the subjects of the studies were not stroke patients, or they were of duplicated publications or studies using overlapping data.

Data Extraction

Two investigators (Tianci Qiao and Hongyun Wu) excerpted data from each qualified article and imported them into a standardized Excel spreadsheet independently, including name of the first author, year of publication, location where study was conducted, sample size, sex, baseline age, follow-up period, ascertainment of UA and stroke, type of stroke, levels of UA, effect estimation, adjusted confounders, and other traditional risk factors, if available. The disagreements were resolved by reevaluating original articles jointly and, if necessary, by a third author (Wei Peng).

Statistical Analysis

Stata software version 14.1 for Windows (Stata Corp, College Station, TX, USA) was used to regulate and analyze the data. The random-effects model was employed without considering the magnitude of between-study heterogeneity. Effect size estimates were indicated by HR and its 95% CI. The difference between the two estimates was tested by using *Z*-test as reported by Altman and Bland (17). Generalized least squares regression proposed by Greenland and Longnecker (18) was used to examine the dose–response association for trend estimation of summarized dose–response data. In addition, non-linearity test between SUA levels and risk of stroke was conduct by restricted cubic splines of exposure distribution with three knots (25, 50, and 75th percentiles).

Heterogeneity between studies was assessed by inconsistency index (I^2), which represents the percentage of multiplicity observed between studies whose result is from chance rather than a casual result. A higher I^2 value indicates a higher degree of heterogeneity. If the I^2 value is higher than 50%, significant heterogeneity would be recorded. As for multiple sources of heterogeneity possibly from clinical and methodological fields, plenty of prespecified subgroups were analyzed according to the baseline age, gender, region, follow-up, factor correction, including whether body mass index (BMI) was adjusted, smoking status, hypertension or blood pressure, diabetes mellitus or blood glucose, hyperlipidemia or lipid, or renal factors.

Begg's funnel plots and Egger regression asymmetry tests were used to evaluate the potential publication bias at a significance level of 10%. In addition, the number of theoretically missing studies was estimated by trim and fill methods, respectively. Sensitivity analysis was conducted to test the stability of results.

RESULTS

Eligible Studies

A total of 1,522 articles were initially included. After searching the public databases with medical subject terms that were previously defined, there were 19 articles with data on association between SUA and risk of stroke that were eligible for inclusion (11, 19–36), including 37,386 males and 31,163 females in the final analysis. The detailed selection process including specific reasons for exclusion was tabulated in **Figure 1**.

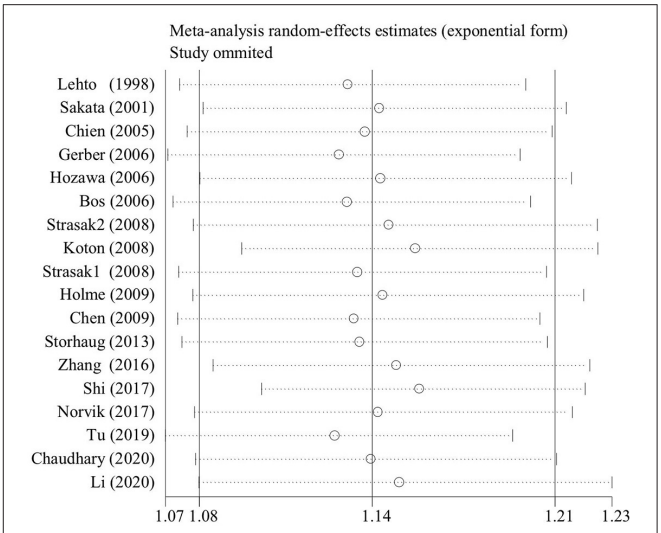

FIGURE 2 | The sensitive plot on the association of uric acid levels and risk of stroke with the exclusion of lower-quality studies.

Study Characteristics

The baseline characteristics of all cohort studies included in this meta-analysis are displayed in **Table 1** and **Supplementary Table 1**. Only four of 19 qualified articles analyzed the effect of per unit UA increase on stroke (11, 25, 30, 32). Seven articles described the association between different SUA levels and risk of stroke without out separate gender groups (19, 24, 25, 28, 30, 32, 33), and 10 articles specifically reported the effect of different levels of UA on different type of strokes (22, 23, 26–29, 31, 33, 34, 36). Based on geographic regions, all the eligible articles were classified into three categories, namely, America (24, 35), Europe (11, 19, 22, 25–27, 29, 32), and Asia (20, 21, 23, 28, 30, 31, 33, 34, 36). According to sensitive analysis with the exclusion of lower-quality study (30), the outcome was stable (**Figure 2**).

Quality Assessment

The Newcastle–Ottawa Scale (NOS) tool was used to assess the quality of the cohort studies, shown in **Table 2**, with the total scores ranging from 5 to 9 in this meta-analysis.

Overall Analyses

After pooling the results of all eligible prospective cohorts together (**Table 3**), there was a statistically significant association between SUA levels and the risk of total stroke (HR = 1.13; 95% CI: 1.09–1.18; $P < 0.001$), ischemic stroke (HR = 1.15; 95% CI: 1.10–1.21; $P < 0.001$), and hemorrhagic stroke (HR = 1.07; 95% CI: 1.00–1.15; $P = 0.046$) (**Table 3**). This association was obscured by significant between-study heterogeneity, with the corresponding I^2 of 59.0, 77.0, and 33.7%. No obvious distinction had been found between

TABLE 2 | The Newcastle-Ottawa Scale (NOS) for assessing the quality of cohort studies.

First author	Sakata	Jee	Chien	Bos	Gerber	Hozawa	Strasak1	Strasak2	Holme	Storhaug	Zhang	Shi	Tu	Chaudhary	Li	Norvik	Chen	Koton	Lehto
Year	2001	2004	2005	2006	2006	2006	2008	2008	2009	2013	2016	2017	2019	2020	2020	2017	2011	2008	1998
1. Representativeness of the exposed cohort	1	1	0	0	0	0	1	1	1	0	1	1	0	1	1	1	0	1	1
2. Selection of the non-exposed cohort	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
3. Ascertainment of exposure	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4. Demonstration that outcome of interest was not present at start study	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
5. Control for important cohort	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
6. Additional factors	1	0	0	0	0	1	1	1	0	1	0	0	1	1	0	0	0	1	0
7. Assessment of outcome	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
8. Follow up	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	0	1	1
9. Adequacy of follow up of cohorts	1	1	1	1	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0
Score	9	7	7	6	6	8	9	9	7	7	7	6	6	8	7	7	5	7	7

ischemic stroke and hemorrhagic stroke (two-sample Z-test $P = 0.095$).

Publication Bias

Begg's funnel plot was used to assess publication bias for the association between SUA levels and risk of stroke, and all of them seemed symmetrical, shown in **Figure 3**. As exposed by the Egger's test, there were strong evidence of publication bias for total stroke ($P = 0.00$), ischemic stroke ($P = 0.00$), and hemorrhagic stroke ($P = 0.05$). Further filled funnel plots showed that there was one potentially missing study in total stroke, 28 missing studies in ischemic stroke, and 13 missing studies in hemorrhagic stroke due to the publication bias to have a symmetrical plot.

Subgroup Analyses

A sequence of subgroup analyses was conducted to investigate the possible causes of between-study heterogeneity for SUA levels and risk of stroke (**Table 3**). By gender, the association of SUA levels and risk of total stroke was statistically significant in both women (HR = 1.19; 95% CI: 1.12–1.26; $P < 0.001$) and men (HR = 1.11; 95% CI: 1.05–1.17; $P < 0.001$) (two-sample Z-test $P = 0.088$). It was also statistically significant for ischemic stroke in women (HR = 1.26; 95% CI: 1.17–1.36; $P < 0.001$) and men (HR = 1.12; 95% CI: 1.06–1.19; $P < 0.001$) (two-sample Z-test $P = 0.015$). The association of SUA levels and risk of hemorrhagic stroke was statistically significant in women (HR = 1.19; 95% CI: 1.04–1.35; $P = 0.01$), but not in men (HR = 1.01; 95% CI: 0.95–1.07; $P = 0.81$) (two-sample Z-test $P = 0.025$).

By geographic locations, in Asia, there was a statistically significant in association between SUA levels and risk of total stroke (HR = 1.06; 95% CI: 1.01–1.13; $P = 0.03$), as well as ischemic stroke (HR = 1.08; 95% CI: 1.02–1.14; $P = 0.01$) and hemorrhagic stroke (HR = 1.17; 95% CI: 1.03–1.34; $P = 0.02$). In Europe, however, there was only statistically significant association for SUA levels and risk of total stroke (HR = 1.20; 95% CI: 1.13–1.27; $P < 0.001$) and ischemic stroke (HR = 1.19; 95% CI: 1.12–1.27; $P < 0.001$).

By follow-up years, in sector of (0, 10) years, significance was observed for association of the SUA levels and risk of total stroke (HR = 1.13; 95% CI: 1.02–1.25; $P = 0.02$) and ischemic stroke (HR = 1.10; 95% CI: 1.02–1.19; $P = 0.01$). For (10, 20) years, total stroke (HR = 1.15; 95% CI: 1.09–1.21; $P < 0.001$) and ischemic stroke (HR = 1.19; 95% CI: 1.12–1.26; $P < 0.001$) were observed to be statistically related to a high level of SUA. While for (20, 30) years as well, the association of the SUA levels and risk of total stroke (HR = 1.13; 95% CI: 1.09–1.18; $P < 0.001$) and ischemic stroke (HR = 1.15; 95% CI: 1.10–1.21; $P = 0.02$) was statistically significant.

By age, total stroke was significantly associated with SUA levels in all subgroups [(20, 40) years: HR = 1.12; 95% CI: 1.04–1.21; $P < 0.001$, (40, 50) years: HR = 1.08; 95% CI: 1.02–1.14; $P = 0.01$, and (50, 90) years: HR = 1.28; 95% CI: 1.17–1.40; $P < 0.001$]. Similarly, for ischemic stroke, statistically significance was observed [(20, 40) years: HR = 1.18; 95% CI: 1.08–1.30; $P < 0.001$, (40, 50) years: HR = 1.05; 95% CI: 1.00–1.10; $P < 0.001$, and (50, 90) years: HR = 1.23; 95% CI: 1.14–1.34; $P = 0.04$].

TABLE 3 | Overall and subgroup analyses of the association between UA levels and the risk of stroke.

Groups	Studies (*n*)	Total		IS		HS	
		HR (95% CI); *P*	I^2	HR (95% CI); *P*	I^2	HR (95% CI); *P*	I^2
Overall analysis							
	13/14/11	1.13 (1.09–1.18); <0.001	59.0%	1.15 (1.10–1.21); <0.001	77.0%	1.07 (1.00–1.15); 0.046	33.7%
Subgroup analysis							
By gender							
Female	10/10/7	1.19 (1.12–1.26); <0.001	55.1%	1.26 (1.17–1.36); <0.001	58.6%	1.19 (1.04–1.35); 0.01	49.5%
Male	11/12/8	1.11 (1.05–1.17); <0.001	56.9%	1.12 (1.06–1.19); <0.001	38.1%	1.01 (0.95–1.07); 0.81	0.0%
All	2/5/2	1.02 (0.79–1.31); 0.89	72.8%	1.02 (0.97–1.10); 0.38	40.3%	0.82 (0.58–1.16); 0.27	51.5%
By location							
Asia	7/6/6	1.06 (1.01–1.13); 0.03	25.5%	1.08 (1.02–1.14); 0.01	19.6%	1.17 (1.03–1.34); 0.02	41.2%
Europe	5/8/4	1.20 (1.13–1.27); <0.001	77.8%	1.19 (1.12–1.27); <0.001	75.5%	1.01 (0.95–1.07); 0.76	1.7%
America	1/1/NA	1.13 (1.10–1.18); 0.39	NA	1.10 (0.95–1.28); 0.19	NA	NA	NA
By follow up years							
(0, 10)	5/5/4	1.13 (1.02–1.25); 0.02	56.4%	1.10 (1.02–1.19); 0.01	50.3%	1.24 (0.99–1.54); 0.06	57.4%
(10, 20)	6/7/4	1.15 (1.09–1.21); <0.001	69.7%	1.19 (1.12–1.26); <0.001	60.9%	1.10 (0.96–1.07); 0.75	2.7%
(20, 30)	2/2/2	1.13 (1.09–1.18); <0.001	0.2%	1.15 (1.10–1.21); 0.02	0.0%	1.11 (0.98–1.26); 0.11	4.6%
By age							
(20, 40)	4/6/3	1.12 (1.04–1.21); <0.001	72.6%	1.18 (1.08–1.30); <0.001	91.1%	1.00 (0.92–1.08); 0.91	16.6%
(40, 50)	6/6/4	1.08 (1.02–1.14); 0.01	42.2%	1.05 (1.00–1.10); <0.001	3.7%	1.04 (0.94–1.14); 0.45	9.9%
(50, 90)	3/4/3	1.28 (1.17–1.40); <0.001	52.6%	1.23 (1.14–1.34); 0.04	25.9%	1.40 (1.14–1.72); <0.001	49.5%
By stroke severity							
Fatal	7/8/6	1.17 (1.10– 1.25); <0.001	37.7%	1.20 (1.13– 1.27); <0.001	12.0%	1.24 (1.10– 1.39); <0.001	31.7%
Non-fatal	5/6/3	1.16 (1.10– 1.23); <0.001	70.6%	1.14 (1.07– 1.22); <0.001	84.8%	1.00 (0.94– 1.07); 0.98	10.1%
Adjusted body mass index (BMI)							
Yes	11/12/8	1.11 (1.06–1.16); <0.001	40.3%	1.11 (1.07–1.17); <0.001	24.6%	1.31 (1.03–1.24); 0.01	29.5%
No	2/3/2	1.20 (1.11–1.18); <0.001	83.2%	1.23 (1.11–1.37); <0.001	94.0%	1.07 (1.00–1.15); 0.68	27.5%
Adjusted smoking status							
Yes	9/11/7	1.10 (1.04–1.17); <0.001	43.6%	1.12 (1.07–1.19); <0.001	27.5%	1.18 (1.05–1.33); 0.01	39.7%
No	4/4/3	1.17 (1.10–1.24); <0.001	73.3%	1.18 (1.09–1.28); <0.001	89.7%	1.00 (0.94–1.07); 0.98	10.1%
Adjusted hypertension or blood pressure							
Yes	10/14/9	1.12 (1.07–1.16); <0.001	61.8%	1.14 (1.09–1.20); <0.001	77.5%	1.07 (1.00–1.16); 0.07	40.0%
No	3/1/1	1.29 (1.14–1.46); <0.001	24.8%	1.30 (1.15–1.46); <0.001	0.0%	1.17 (0.91–1.52); 0.23	0.0%
Adjusted diabetes mellitus or blood glucose							
Yes	10/10/7	1.12 (1.07–1.17); <0.001	63.7%	1.15 (1.09–1.21); <0.001	81.7%	1.02 (0.96–1.09); 0.58	20.2%
No	3/4/3	1.20 (1.08–1.32); <0.001	38.5%	1.18 (1.10–1.26); <0.001	0.0%	1.37 (1.13–1.65); <0.001	30.9%
Adjusted hyperlipidemia or lipid							
Yes	12/13/8	1.12 (1.07–1.17); <0.001	60.0%	1.14 (1.09–1.19); <0.001	56.5%	1.07 (0.99–1.16); 0.09	43.1%
No	1/2/2	1.27 (1.13–1.41); <0.001	17.5%	1.28 (1.10–1.50); <0.001	73.1	1.16 (0.97–1.39); 0.10	0.0%
Adjusted renal factors							
Yes	3/3/8	0.99 (0.90–1.09); 0.87	62.7%	1.14 (1.04–1.25); <0.001	8.0%	1.03 (0.99–1.08); 0.15	79.5%
No	10/12/2	1.17 (1.12–1.22); <0.001	23.3%	1.16 (1.10–1.22); <0.001	79.1%	1.41 (0.88–2.26); 0.16	0.0%

HR, hazard ratio; 95% CI, 95% confidence interval; IS, Ischemic Stroke; HS, Hemorrhagic Stroke; UA: uric acid; BMI: body mass index; NA, not available.

While for hemorrhagic stroke, only marginal significance was observed among age group of 50–90 years (HR = 1.23; 95% CI: 1.14–1.34; $P = 0.04$).

By the stratification for stroke severity, we classified the severity of a stroke as fatal and non-fatal, and we found high SUA levels were significantly associated with both fatal and non-fatal stroke (fatal stroke: HR = 1.17; 95% CI: 1.10–1.25; $P < 0.001$, non-fatal stroke: HR = 1.16; 95% CI: 1.16–1.23; $P < 0.001$). The same trend was absorbed in ischemic stroke (fatal stroke: HR = 1.20; 95% CI: 1.13–1.27; $P < 0.001$, non-fatal stroke: HR = 1.14; 95% CI: 1.07–1.22; $P < 0.001$) and hemorrhagic stroke (fatal stroke: HR = 1.24; 95% CI: 1.10–1.39; $P < 0.001$, non-fatal stroke: HR = 1.00; 95% CI: 0.94–1.07; $P = 0.98$).

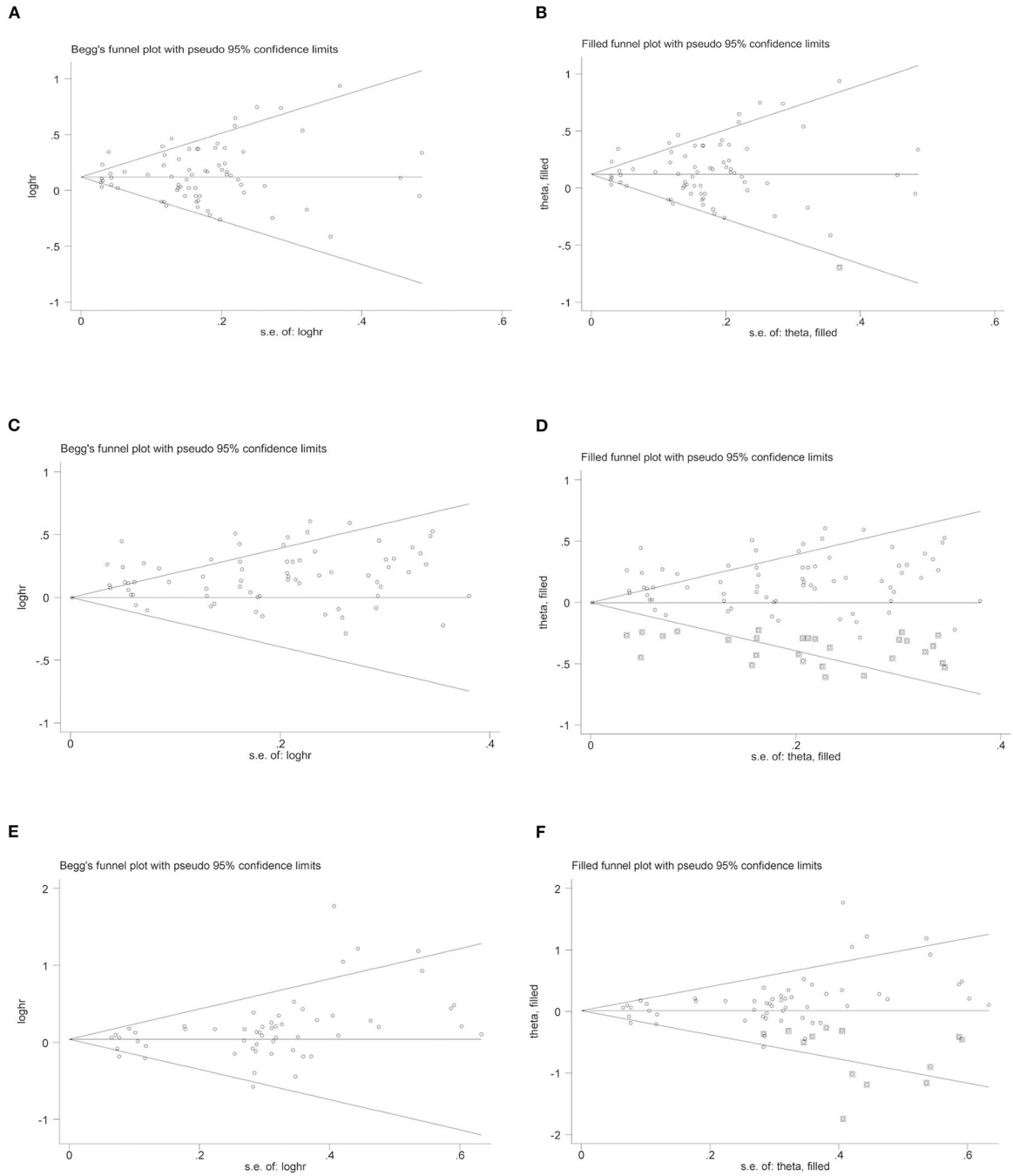

FIGURE 3 | Begg's and filled funnel plots on the association of uric acid levels and risk of stroke. **(A)** Begg's funnel plot and **(B)** Filled funnel plots: UA levels and total stroke. **(C)** Begg's funnel plot and **(D)** Filled funnel plots: UA levels and ischemic stroke. **(E)** Begg's funnel plot and **(F)** Filled funnel plots: UA levels and hemorrhagic stroke.

It should also be noticed that the significantly positive associations between SUA levels and risk of stroke that remained in subgroups had been found, which adjusted for potential confounders, including BMI, smoking status, hypertension, diabetes mellitus, hyperlipidemia, and renal factors.

Dose–Response Analyses

Our dose–response research indicated the *J*-shaped trend between the ascending SUA levels and the higher risk of suffering from stroke. In the dose–response analysis on total stroke, the risk of stroke obviously increased with the higher UA concentration. When the SUA reached 5.35 mg/dl, it started to

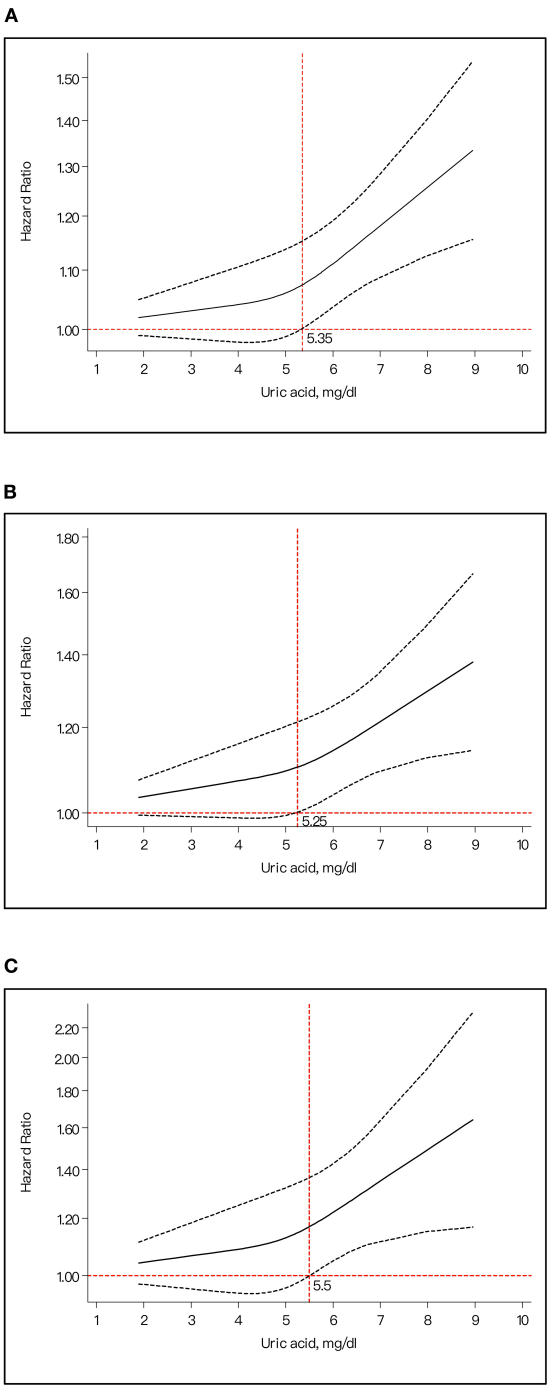

FIGURE 4 | The dose-response plot for the association of uric acid levels and risk of stroke. **(A)** Uric acid levels and risk of total stroke. **(B)** Uric acid levels and risk of ischemic stroke. **(C)** Uric acid levels and risk of hemorrhagic stroke.

become statistically significant (**Figure 4A**). The same pattern was also found in ischemic stroke (the dividing value was 5.25 mg/dl) (**Figure 4B**) and hemorrhagic stroke (5.5 mg/dl) (**Figure 4C**).

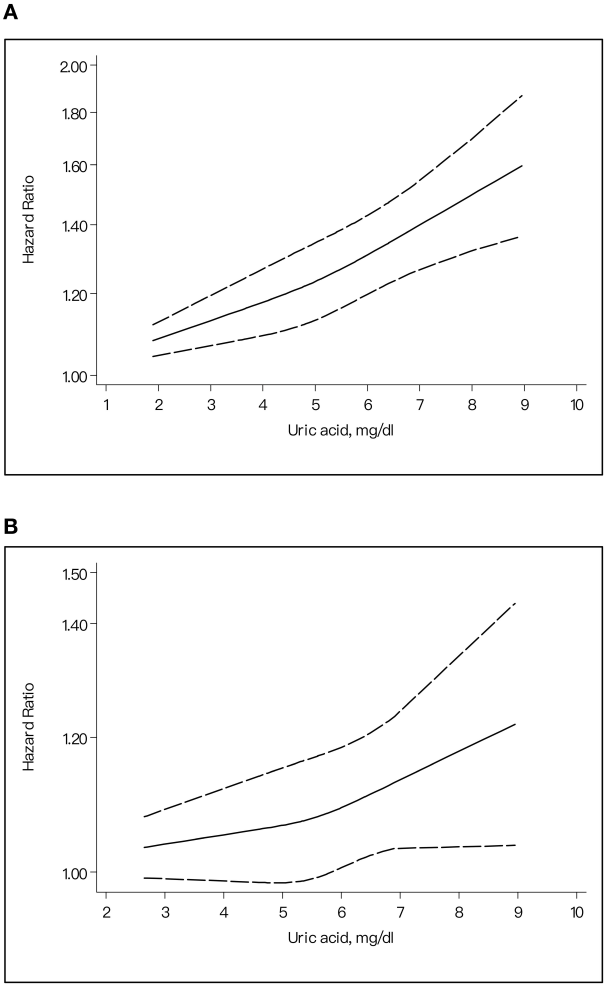

FIGURE 5 | The dose-response plot on the association of uric acid levels and risk of stroke for different gender. **(A)** Female. **(B)** Male.

In our dose–response dichotomized by gender, it indicated a *J*-shaped trend between the ascending SUA levels and the higher risk of stroke for males (p for non-linear trend = 0.39) (**Figure 5A**) and a liner trend (p for non-linear trend = 0.32) for females (**Figure 5B**).

DISCUSSION

To the best of our knowledge, this is to date the most panoptic meta-analysis that has investigated the association between SUA levels and risk for stroke. The key findings of this study are that elevated SUA is a significant risk factor for adult stroke, both for ischemic stroke and hemorrhagic stroke, and the risk is more evident in females than that in males. Our sensitivity analyses and subgroup analyses also revealed that the relationship between SUA and stroke was robust and not affected by multifactor correction. Moreover, dose–response analysis presented the *J*-shaped trend between the ascending SUA levels and the higher risk of stroke. However, no obvious distinction

was found between ischemic stroke and hemorrhagic stroke. More importantly, we found high SUA levels were significantly associated with an increased risk of fatal stroke. Our findings highlight the prominence and the necessity of closely regulating SUA, especially for elderly females, who have a high risk of suffering from cerebrovascular disease.

Several systematic reviews and meta-analyses have evaluated the impact of high SUA on the onset of stroke. Pooling the results of 13 prospective studies by Zhong et al. (12) showed that elevated serum SUA levels were significantly associated with modestly increased risk of stroke and have similar adverse effects on both sexes, whereas further subsidiary analyses by different types of stroke were lacking. Meanwhile, limited seven studies that involved SUA and the risk of stroke in males and seven studies in females had been included in Zhong et al.s' study. Researchers raised that if 10 or fewer studies are pooled in a meta-analysis, the possibility/capacity to detect statistical significance is low (37). At the same time, the study mixed risk ratio (RR) and HR as effect-size estimates for analysis, which is inaccurate and may affect the conclusions. Our work that was based on high-quality cohort studies have avoided these problems effectively and found the same significant relationship.

The concentration of UA is the key point of the mechanisms underlying the association of UA with development of stroke. As one of the most abundant antioxidant molecules in humans, UA has the valid ability to clear out peroxynitrite, nitric oxide, and hydroxyl radicals; hence, it can prevent protein nitration and lipid peroxidation (38, 39). Studies in animal models have shown that administration of UA or soluble UA analogs that retain the antioxidant properties of UA protects the brain against ischemic injury (40–42). However, once it exceeds the normal range, SUA would impact multiple systems, which in turn lead directly or indirectly to stroke. Possible mechanisms have been reported that elevated UA level was associated with carotid intima media thickness, as reported by the latest meta-analysis; high UA was related to carotid intima thickening (43); and the same trend was found in proximal extracranial artery stenosis (44). Meanwhile, it was demonstrated that elevated UA promoted atherosclerotic progression by increasing production of free radicals and facilitating low-density lipoprotein cholesterol (LDL-C) oxidation and lipid peroxidation (45). In addition, high levels of UA increased vascular endothelial dysfunction (46) and vascular smooth muscle cell proliferation, which could lead to preglomerular vascular disease and high blood pressure (47, 48). Potential mechanisms have also been reported that elevated UA level was involved in microvascular injury (47), increasing platelet aggregation and thrombus formation (49). Studies had revealed that UA could increase inflammatory cytokines such as C-reactive protein, interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α) (50). Simultaneously, clinical studies also suggested that high SUA levels increased the risk of total mortality and cardiovascular and cerebrovascular diseases. In Italy, a national multicenter retrospective cohort study (51) assessed that all-cause mortality was substantially increased when the UA levels were above 4.7 mg/dl (95% CI: 4.3–5.1 mg/dl), and the risk of cardiovascular mortality (CVM) ascended while the value of SUA is over 5.6 mg/dl (95% CI: 4.99–6.21 mg/dl). These findings from experimental, epidemiological, and clinical studies of UA suggested that elevated SUA could be associated with vascular diseases and clarified the important role played by SUA levels in illustrating the possible pathophysiological association with hypertension, atherosclerosis, and stoke.

In our study, we took ischemic stroke and hemorrhagic stroke as the main subtypes, and we found elevated SUA levels have similar adverse effects on the development of stroke in these two subtypes. Evidence showed that ischemic stroke and hemorrhagic stroke both cause local hypoxia that damage brain tissue, and they could be converted to each other. There was a high risk of hemorrhagic transformation during the treatment of ischemic stroke (52). However, the study presented that significant differences existed in body composition between hemorrhagic and ischemic stroke in humans, and individuals with ischemic stroke had significantly worse body composition (53). Further exploration of the molecular mechanisms of SUA and different types of stroke is noteworthy.

Sex differences in the association of elevated SUA with stroke-related risk factors were found in our study. Statistically, females have a higher risk of experiencing a stroke-related fatality than males. Meanwhile, a J-shaped trend between the ascending SUA levels and higher risk of stroke for men and a liner trend for women had been explored. It is universally acknowledged that stroke is a sexually dimorphic disease. For one reason, females have a longer average lifespan, which increases the odds that they will have a stroke. Besides, females suffer greater susceptibility to depression and anxiety and often report higher levels of stress than males do (54–56). Other unique risk factors that females are facing, such as gestational hypertension and climacteric syndrome, may also cause the difference. To conclude, differences in vascular biology, immunity, coagulation, hormonal profiles, lifestyle factors, and societal roles seem to contribute (57).

Some limitations for the present meta-analysis should be acknowledged. Firstly, we were unable to carry out further subgroup comparison of hemorrhagic stroke because the corresponding data were not available in the original articles. The mechanisms and risk factors for subarachnoid hemorrhage and intracerebral hemorrhage are different in important ways, as are treatment and outcomes (58). More clinical and mechanistic studies deserve further research. Secondly, even though the errors of dose–response analysis are unavoidable in secondary analysis, the overall *J*-shaped trend is worthy of our attention in the relationship of SUA and risk of stroke in this meta-analysis. Thirdly, although a large panel of subgroup analyses were undertaken to account for possible sources of heterogeneity, significance still persisted in some subgroups, limiting the interpretation of pooled effect-size estimates. Finally, similar to any observational studies, a causal relationship could not be fully established.

CONCLUSIONS

Our study found that elevated SUA is a significant risk factor for adult stroke, both for ischemic stroke and hemorrhagic stroke, especially in females. Our dose–response research revealed a *J*-shaped trend between the ascending SUA levels and the higher risk of suffering from stroke. Moreover, high SUA levels

are associated with an increased risk of fatal stroke. Further investigations on the molecular mechanisms linking SUA to adult stroke are also warranted.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

TQ reviewed the articles and wrote the manuscript. HW helped with the article review. WP was the editor of the manuscript and helped with the preliminary qualification. All authors contributed to the article and approved the submitted version.

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Serum Cystatin C Predicts Stroke Clinical Outcomes at 1 Year Independent of Renal Function

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Objective: Serum cystatin C (CysC) is a sensitive marker of renal function to predict cardiovascular diseases. We aimed to investigate the predictive value of CysC for clinical outcomes independent of renal function in patients with acute ischemic stroke (AIS).

Methods: We measured serum CysC levels in 10,256 AIS patients from Third China National Stroke Registry (CNSR-III). The primary outcome was a combination of all-cause mortality and major disability (modified Rankin scale score, 3–6). Secondary outcomes included stroke recurrence and combined vascular events at 1 year. Outcomes were analyzed using logistic regression and Cox proportional hazards models, respectively.

Results: The median CysC of included patients was 0.95 mg/l (interquartile range, 0.83–1.10 mg/l). A U-shaped association was observed between CysC and primary outcome (all-cause mortality or major disability) [quartile (Q)1 vs. Q2: adjusted odds ratio (aOR) 1.29, 95% CI 1.06–1.58, $p = 0.012$; Q3 vs. Q2: aOR 1.12, 95% CI 0.93–1.35, $p = 0.242$; Q4 vs. Q2: aOR 1.35, 95% CI 1.10–1.65, $p = 0.004$]. A similar trend also existed in "preserved renal function" patients. Adding CysC to a model containing conventional risk factors improved the model performance with integrated discrimination improvement (IDI) of 0.13% ($p = 0.016$) and net reclassification index (NRI) of 13.10% ($p < 0.001$) for primary outcome. No significant association was observed for stroke recurrence or combined vascular event rate in different CysC quartiles.

Conclusions: CysC showed a U-shaped correlation with 1-year stroke clinical outcome, suggesting that serum CysC may not only be a simple candidate marker of renal function.

Keywords: cystatin C, renal function, biomarker, ischemic stroke, clinical outcomes

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INTRODUCTION

Cystatin C (CysC), a protein inhibitor of cysteine protease, was generally considered an alternative to creatinine for kidney function measurement (1). It was also reported as a predictive marker of cardiovascular diseases (CVDs) (2, 3). Besides, CysC was independently associated with cerebral artery stenosis and mortality in stroke or CVD patients with estimated glomerular filtration rate (eGFR) ≥60 ml/min/1.73 m^2 (4, 5). Thus, it is suggested that CysC may act in versatile roles rather than a single index for glomerular filtration.

Since serum CysC was considered a marker of endothelial dysfunction in the glomerulus and throughout the vascular tree, elevated CysC levels may indicate a higher degree of cerebral vessel damage (6). On the other hand, since CysC is a potent competitive inhibitor of cysteine proteases, low levels of CysC are inevitably accompanied by an increase in cysteines protease (7), which has direct cytotoxic effects on brain tissue and leads to neuronal death (8). Thus, it is plausible to speculate that CysC's involvement in the clinical prognosis of stroke patients does not simply depend on renal function. However, evidence with a large sample size on this issue is limited (4).

In this analysis of The Third China National Stroke Registry (CNSR-III), we aimed to assess whether CysC was a potential biomarker in the prediction of clinical outcomes among acute ischemic stroke (AIS) patients independent of renal function and to explore the effect of CysC on stroke clinical prognosis in patients with "preserved renal function" [eGFRcreatinine (eGFRcr) $\geq$60 ml/min/1.73 m^2].

METHODS

Study Design and Subjects

This study was conducted based on CNSR-III, a nationwide, multicenter, prospective registry study launched in China between August 2015 and March 2018, aiming to evaluate the etiology, imaging, and biological markers of AIS. Detailed descriptions of the CNSR-III study have been reported previously (9). Blood samples were collected from 171 study sites for this prespecified biomarker subgroup analysis. Finally, 10,256 subjects were included in our main analytic sample (**Figure 1**). The protocol of the CNSR-III study was approved by the ethics committee of Beijing Tiantan Hospital.

Kidney function was estimated by GFR, which was calculated using the latest Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equations by creatinine (1). "Renal dysfunction" was defined as eGFR <60 ml/min/1.73 m^2 based on the National Kidney Foundation Kidney Disease Outcomes Quality Initiative working group definition of kidney disease (10). "Preserved renal function" was defined as eGFRcr $\geq$60 ml/min/1.73 m^2. The degree of stenosis was assessed according to computed tomographic angiography (CTA), magnetic resonance angiography (MRA) imaging, or conventional ultrasonography. More than 50% caliber reduction of the intracranial and extracranial artery was defined as intracranial artery stenosis (ICAS) and extracranial artery stenosis (ECAS), respectively. Stroke subtypes are classified according to the modified Trial of Org 10,172 in acute stroke treatment (TOAST). To ensure that the diagnosis standard was consistent, all images were independently evaluated by two trained neuroradiologists blinded to clinical information. A third neuroradiologist was involved for additional assessment if there was disagreement in certain cases.

Data Collection and Serum Biomarker Measurement

The blood samples were collected on the 1st day of enrollment and transported through the cold chain to the central laboratory in Beijing Tiantan Hospital, where all serum specimens were stored at −80°C until testing was performed. Blood samples were tested uniformly in the central laboratory of Beijing Tiantan Hospital according to the standardized methods. All measurements were performed by laboratory personnel blinded to the study status.

The value of CysC was measured by the immunoturbidimetric method (Roche Cobas c501 analyzer with cystatin C assay); coefficient of variation (CV) of CysC was 2%. Concentrations of serum creatinine were measured by the enzymatic method (sarcosine oxidase-PAP) using a commercial kit (Beckman Coulter, Brea, CA, USA) according to the manufacturer's protocol. The Beckman assay was calibrated to the Roche/Hitachi P module Creatinine Plus enzymatic assay (Roche Diagnostics, Basel, Switzerland), which has an approximate CV of 2%. Low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglyceride (TG) were measured by the enzymatic method. Total cholesterol (TC) testing was the cholesterol oxidase method. Levels of high-sensitivity C-reactive protein (hs-CRP) were measured on Cobas c501 analyzer using the cardiac CRP (latex) high-sensitive assay (Roche, Basel, Switzerland).

Outcome Assessment

The outcomes were obtained through clinic or telephone in 1-year follow-up. Assessment of endpoints was completed by trained research coordinators who were blinded to patients' baseline clinical information. Patients were contacted over the telephone by trained research coordinators after 1 year. The primary outcome was a combination of all-cause mortality and major disability [modified Rankin scale (mRS) score, 3–6]. Secondary outcomes included stroke recurrence and combined vascular events (including recurrent stroke, myocardial infarction, and vascular death). Stroke recurrence was defined as new onset of focal neurological deficit induced by cerebral ischemic or hemorrhagic events and confirmed by computed tomography/magnetic resonance imaging.

Statistical Analysis

Baseline characteristics were compared among quartiles of CysC (<0.83 mg/L, 0.83–0.95 mg/L, 0.95–1.10 mg/L, >1.10 mg/L) using the chi-square test for categorical variables and ANOVA or the Kruskal–Wallis test for continuous variables. Logistic regression models and Cox proportional hazards models were performed for stroke outcomes. Variables were adjusted in the multivariable analyses if established as traditional predictors for stroke or associated with CysC in univariate analysis with a value of $p < 0.05$. The backward selection method was adopted in multiple adjustments (**Supplementary Table 3**). Model 1 adjusted for age, gender, National Institutes of Health Stroke Scale (NIHSS) at admission, antihypertensive agents, hypoglycemic drugs, anticoagulants, ischemic stroke, coronary artery disease, smoking, atrial fibrillation, hs-CRP, TG, TC, and non-HDL-C. Model 2 further adjusted for TOAST subtypes. Model 3 further adjusted for eGFRcr. The association between CysC and stroke patients' clinical outcomes was evaluated using a regression model with restricted cubic splines. The Sankey

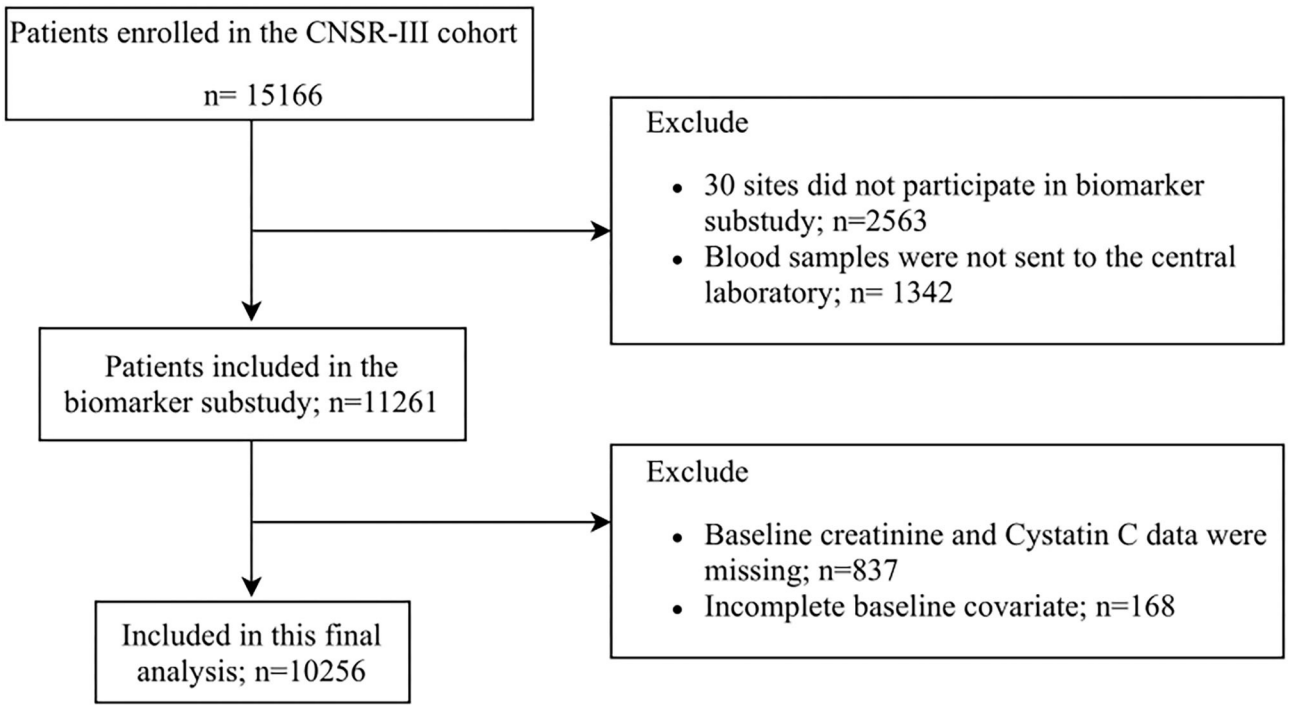

FIGURE 1 | Flowchart of the study.

diagram was used to visualize the mRS score distributions in different CysC quartiles. We performed a sensitivity analysis to explore the differences in the primary outcome between the patients' proportion in different subgroups. Besides, C statistics, integrated discrimination improvement, and net reclassification index were used to assess improvement in model performance by adding CysC to a conventional model (risk factors in model 3) to assess the incremental value of CysC in risk prediction for the prognosis.

All data were analyzed with the SAS version 9.4 software (SAS Institute Inc., Cary, NC). The level of significance was defined as $p < 0.05$ (two-sided).

RESULTS

Baseline Characteristics

Of 15,166 stroke patients enrolled in the CNSR-III, 10,256 were included in this analysis. The baseline characteristics of the biomarker cohort vs. those excluded from the overall study population were shown in **Supplementary Table 1**. Patients included in the analysis were more likely to have lower NIHSS score, a lower rate of statin and hypoglycemic drug uses as compared with excluded patients. Other factors did not differ significantly between the two groups. Among the included participants, 9,508 were "preserved renal function" patients at baseline.

The mean age of the study subjects was 63.0 years; 3,312 (31.7%) patients were female. The median CysC was 0.95 mg/l (interquartile range, 0.83–1.10 mg/l). Subject characteristics grouped by quartiles of serum CysC are listed in **Table 1**. The participants with higher serum CysC tended to be older, male; had higher prevalence of ischemic stroke, CVD, and atrial fibrillation; and had higher hs-CRP levels than those with lower serum CysC (**Table 1**).

Clinical Outcomes

A total of 1,321 participants (13.2%) experienced primary outcome (all-cause mortality or major disability) in 1-year follow-up (**Table 2**). The distribution of 1-year mRS score by CysC quartiles among all the included patients is shown in **Figure 2**. The cumulative rates of the primary outcome within 1 year among patients with ischemic stroke in the four quartiles of serum CysC (from low to high) were 10.6, 10.0, 12.9, and 19.3%, respectively (**Table 2**). After adjustment for conventional covariables (model 1) and further adjustment for TOAST subtypes in model 2 and eGFRcr in the full adjusted model (model 3), patients in the first and last CysC quartiles (Q1 and Q4) had worse clinical prognosis (mRS score, 3–6) compared with the second quartile [Q1 vs. Q2: adjusted odds ratio (aOR) 1.29, 95% CI 1.06–1.58, $p = 0.012$; Q3 vs. Q2: aOR 1.12, 95% CI 0.93–1.35, $p = 0.242$; Q4 vs. Q2: aOR 1.35, 95% CI 1.10–1.65, $p = 0.004$]. A U-shaped association was observed between CysC and primary outcome in all the included patients and the "preserved renal function" group (eGFRcr ≥60 ml/min/1.73 m^2) (**Figure 3**). Characteristics between the "renal dysfunction" group (eGFRcr <60 ml/min/1.73 m^2) and the "preserved renal

TABLE 1 | Characteristics of all enrolled patients according to CysC quartiles.

Characteristics*	Baseline CysC, mg/l				*p*-value
	Q1 (<0.83) *N* = 2,552 (24.9)	Q2 (0.83–0.95) *N* = 2,559 (25.0)	Q3 (0.95–1.10) *N* = 2,575 (25.1)	Q4 (>1.10) *N* = 2,570 (25.1)	
No. of patients	2,377 (25.00)	2,377 (25.00)	2,356 (24.78)	2,398 (25.22)	
Age, years, mean ± SD	56.7 ± 10.4	60.9 ± 10.3	63.6 ± 10.5	68.1 ± 10.9	<0.001
Male sex	1,528 (59.9)	1,742 (68.1)	1,856 (72.1)	1,875 (73.0)	<0.001
NIHSS at admission	3.0 (1.0–6.0)	3.0 (1.0–6.0)	3.0 (1.0–6.0)	3.0 (1.0–6.0)	<0.001
0–3	1,331 (52.2)	1,418 (55.4)	1,385 (53.8)	1,337 (52.0)	0.048
≥4	1,221 (47.8)	1,141 (44.6)	1,189 (46.2)	1,233 (48.0)	
BMI	24.6 (22.9–26.6)	24.5 (22.6–26.6)	24.4 (22.5–26.6)	24.5 (22.5–26.6)	0.195
Medical history					
Ischemic stroke	419 (16.4)	468 (18.3)	617 (24.0)	683 (26.6)	<0.001
Coronary artery disease	189 (7.4)	241 (9.4)	292 (11.3)	393 (15.3)	<0.001
Atrial fibrillation	70 (2.7)	135 (5.3)	180 (7.0)	329 (12.8)	<0.001
Smoking	719 (28.2)	862 (33.7)	886 (34.4)	774 (30.1)	<0.001
Alcohol drinking	329 (12.9)	410 (16.0)	386 (15.0)	326 (12.7)	<0.001
Laboratory data					
hs-CRP, mg/l	1.4 (0.7–3.7)	1.5 (0.8–3.8)	1.8 (0.8–4.4)	2.7 (1.1–7.7)	<0.001
TG, mmol/l	1.5 (1.1–2.1)	1.3 (1.0–1.8)	1.3 (1.0–1.8)	1.3 (1.0–1.8)	<0.001
TC, mmol/l	4.1 (3.4–4.9)	4.0 (3.3–4.6)	3.9 (3.3–4.7)	3.9 (3.3–4.7)	<0.001
LDL-C, mmol/l	2.3 (1.7–3.0)	2.3 (1.7–3.0)	2.4 (1.8–3.0)	2.3 (1.7–3.0)	0.011
HDL-C, mmol/l	0.9 (0.8–1.1)	0.9 (0.7–1.1)	0.9 (0.8–1.1)	0.9 (0.8–1.1)	0.257
Non-HDL-C, mmol/l	3.2 (2.5–3.9)	3.0 (2.3–3.7)	3.0 (2.4–3.7)	2.9 (2.3–3.7)	<0.001
eGFRcr, ml/min/1.73 m^2	103.4 (96.8–110.6)	96.5 (90.2–102.5)	90.2 (82.5–97.1)	74.6 (60.7–86.9)	<0.001
Concomitant medication					
Antihypertensive agents	1,063 (41.7)	1,133 (44.3)	1,180 (45.8)	1,382 (53.8)	<0.001
Statins	2,447 (95.9)	2,460 (96.1)	2,476 (96.2)	2,463 (95.8)	0.909
Hypoglycemic drugs	810 (31.7)	620 (24.2)	580 (22.5)	622 (24.2)	<0.001
Antiplatelets	2,454 (96.2)	2,476 (96.8)	2,480 (96.3)	2,455 (95.5)	0.142
Anticoagulants	251 (9.8)	231 (9.0)	250 (9.7)	298 (11.6)	0.017
TOAST subtypes, no. (%)					<0.001
LAA	678 (26.6)	597 (23.3)	678 (26.3)	644 (25.1)	
CE	0.86 (3.5)	129 (5.0)	161 (6.3)	274 (10.7)	
SVD	515 (20.2)	597 (23.3)	555 (21.6)	469 (18.3)	
Others	1,271 (49.8)	1,236 (48.3)	1,181 (45.9)	1,183 (46.0)	
ICAS or ECAS, no. (%)					<0.001
With	1,122 (51.1)	1,138 (51.8)	1,108 (49.4)	1,016 (45.4)	
Without	1,076 (49.0)	1,058 (48.2)	1,134 (50.6)	1,221 (54.6)	

SD, standard deviation; Q, quartile; CysC, cystatin C; NIHSS, National Institutes of Health Stroke Scale; BMI, body mass index; hs-CRP, high sensitivity C-reactive protein; TG, triglycerides; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; eGFRcr, creatinine-calculated glomerular filtration rate; LAA, large artery atherosclerosis; CE, cardioembolism; SVD, small vessel disease; ICAS, intracranial arteries stenosis; ECAS, extracranial arteries stenosis; TOAST, Trial of Org 10,172 in acute stroke treatment.
**Variables were presented as median (interquartile range) or counts (percentages) unless otherwise indicated.*

function" group (GFRcr ≥60 ml/min/1.73 m^2) were shown in **Supplementary Table 2**.

The cumulative rates of stroke recurrence at 1 year across the four quartiles of serum CysC (from low to high quartile) were 8.8% (n = 224), 9.3% (n = 239), 10.3% (n = 265), and 10.4% (n = 267), respectively, while the combined vascular event rates were 9.21% (n = 235), 9.61% (n = 246), 10.91% (n = 281), 11.28% (n = 290) respectively, but there was no statistical difference in the four groups for stroke recurrence and combined vascular events.

Sensitivity Analysis

Results of sensitivity analysis of the primary outcome are shown in the forest plot in **Supplementary Figure 1**. There was no heterogeneity in the effects of CysC levels on the primary outcome between subgroups classified by age, gender, TOAST subtypes, and previous stroke history. Of note, the U trend was more pronounced in male, the small artery occlusion group, and subjects without stroke history. Statistical interaction between CysC and gender did not show a significant difference (**Supplementary Table 4**).

TABLE 2 | Clinical outcomes according to quartiles of serum CysC at 1 year.

1-year outcomes	Event rate, no. (%)†	Unadjusted model		Adjusted model 1‡		Adjusted model 2§		Adjusted model 3‖	
		HR/OR (95% CI)*	*p*-value	HR/OR (95% CI)	*p*-value	HR/OR (95% CI)	*p*-value	HR/OR (95% CI)	*p*-value
Primary outcome: All-cause mortality or major disability (modified Rankin scale score, 3–6)									
Q1 (<0.83)	265 (10.6)	1.07 (0.89–1.29)	0.462	1.29 (1.06–1.57)	0.010	1.27 (1.04–1.55)	0.017	1.29 (1.06–1.58)	0.012
Q2 (0.83–0.95)	250 (10.0)	Reference		Reference		Reference		Reference	
Q3 (0.95–1.10)	323 (12.9)	1.33 (1.12–1.59)	0.001	1.14 (0.95–1.38)	0.156	1.13 (0.94–1.37)	0.190	1.12 (0.93–1.35)	0.242
Q4 (>1.10)	483 (19.3)	2.15 (1.83–2.54)	<0.001	1.42 (1.18–1.70)	<0.001	1.44 (1.18–1.70)	<0.001	1.35 (1.10–1.65)	0.004
Stroke recurrence									
Q1 (<0.83)	224 (8.8)	0.94 (0.78–1.13)	0.504	0.96 (0.80–1.16)	0.699	0.95 (0.79–1.14)	0.555	0.96 (0.80–1.16)	0.676
Q2 (0.83–0.95)	239 (9.3)	Reference		Reference		Reference		Reference	
Q3 (0.95–1.10)	265 (10.3)	1.11 (0.93–1.32)	0.239	1.05 (0.88–1.25)	0.628	1.03 (0.87–1.23)	0.719	1.02 (0.85–1.22)	0.827
Q4 (>1.10)	267 (10.4)	1.13 (0.95–1.34)	0.179	0.97 (0.81–1.16)	0.713	0.95 (0.79–1.14)	0.595	0.91 (0.74–1.11)	0.349
Combined vascular events									
Q1 (<0.83)	235 (9.21)	0.96 (0.80–1.15)	0.637	0.99 (0.83–1.19)	0.913	0.97 (0.81–1.17)	0.753	0.99 (0.82–1.19)	0.879
Q2 (0.83–0.95)	246 (9.61)	Reference		Reference		Reference		Reference	
Q3 (0.95–1.10)	281 (10.91)	1.15 (0.97–1.36)	0.120	1.07 (0.90–1.27)	0.457	1.06 (0.89–1.26)	0.537	1.04 (0.88–1.24)	0.630
Q4 (>1.10)	290 (11.28)	1.19 (1.00–1.41)	0.045	1.00 (0.84–1.19)	0.980	0.98 (0.82–1.17)	0.851	0.94 (0.77–1.15)	0.540

CI, confidence interval; Q, quartile; HR, hazard ratio; OR, odds ratio; CysC, cystatin C.

**OR for dependence; while HR for stroke recurrence and combined vascular events.*

†Event rate: no. of patients with event/total no.

‡Model 1 Adjusted for age, gender, NIHSS at admission, antihypertensive agents, hypoglycemic drugs, anticoagulants, ischemic stroke, coronary artery disease, smoking, atrial fibrillation, hs-CRP, TG, TC, Non-HDL-C.

§Model 2 Adjusted for Model 1 + TOAST subtypes.

‖Model 3 Adjusted for Model 2 + eGFRcr.

Besides, primary outcomes according to normal ranges of serum CysC (mg/l) (11) were shown in **Supplementary Figure 2**. Compared with the normal range of CysC, lower and higher range group patients indicated more risk of poor prognosis (Low vs. Normal: aOR 1.60, 95% CI 1.01–2.54, $p = 0.044$; High vs. Normal: aOR 1.10, 95% CI 1.10–1.44, $p < 0.001$). But there was no statistically significant difference among the age subgroups (age <50 or ≥50).

Incremental Predictive Value of Cystatin C for Prognosis

We evaluated whether CysC would further increase the predictive performance of the models with conventional risk factors on the prognosis of ischemic stroke (**Table 3**). For all-cause mortality or major disability (mRS score, 3–6) as the outcome of interest, the C statistic by the conventional model improved by the addition of CysC quartile (from 0.765 to 0.791, $p = 0.014$). The risk reclassification appeared to be substantially significant (integrated discrimination improvement 0.13%, $p = 0.016$; quartiles net reclassification index was 13.10%, $p < 0.001$).

DISCUSSION

There are several key findings in this study. First, we investigated the association between CysC levels and the prognosis of AIS at 1 year. We demonstrated a U-shaped correlation between CysC and clinical outcome (mortality or major disability) independent of eGFRcr. Second, adding CysC to conventional risk factors (including eGFRcr) could improve risk prediction for clinical outcomes. Another important observation from our study is the fact that half of the subjects in the subset with eGFRcr ≥60 ml/min/1.73 m^2 still demonstrate the same impact of CysC levels on stroke prognosis. Fourth, the U trend between CysC and the clinical prognosis was more pronounced in the small artery occlusion group and subjects without stroke history.

It was widely acknowledged that CysC is a prominent predictor of CVDs independent of eGFRcr (12–15). To date, several studies have shown the associations of CysC with prognosis and the recurrent vascular event in stroke patients (4, 5, 16, 17). In the previous case-control study, Ni et al. (5) showed that higher plasma CysC levels were independently associated with both ischemic and hemorrhagic stroke and death in 5 years' follow-up. Besides, CysC level was also a useful predictor for early neurological deterioration in AIS patients (17) and short-term outcomes for AIS patients after intravenous tissue plasminogen activator (IV-tPA) therapy (16). On the other hand, previous studies have shown that CysC may provide neuroprotective activities in stroke and neurodegenerative disorders (18, 19). Increasing pieces of evidence revealed that CysC was not only a simple candidate marker of impaired kidney function (20) but also closely associated with congestive heart failure (21, 22), inflammation (23), oxidative stress (19), carotid atherosclerosis (24), and peripheral vascular disease (25) superior to serum creatinine (26). In the current study, we further added evidence of bilateral effects for CysC levels on 1-year prognosis compared to previous studies. Seliger et al. (27) have suggested a quadratic U-shaped association between renal function and subclinical brain infarcts (SBIs) due to small-vessel arteries rather than large-vessel atherosclerosis. We also have discovered that the U-trend was more pronounced in the small artery occlusion group, suggesting the possible effect of small vessel injury on prognosis.

The U-shaped correlation between CysC levels and the clinical outcome means low concentrations of CysC is also detrimental to stroke patients. Additional underlying mechanisms for the seemingly paradoxical outcomes are suggested. CysC is a potent competitive inhibitor of cysteine proteases (28). The balance between cysteine protease and protease inhibitor (CysC) plays an important role in the pathogenesis of cerebral injury and functional rehabilitation (20, 29). Cysteine proteases released after traumatic injury would lead to neuronal death (8). Cathepsin B is a major lysosomal cysteine protease that plays an important role in aging, oxidative stress, inflammation, and apoptosis processes (18, 30). Imbalances between cathepsin B and CysC were involved in atherosclerosis, glomerulosclerosis, and cardiomyopathy with senescence-associated phenotypes (31). It is possible to hypothesize that low levels of CysC are accompanied by an increase in cathepsin content (32), which appears to reflect cell necrosis and brain tissue damage, leading to adverse clinical outcomes, as confirmed in this current real-world clinical cohort analysis.

Furthermore, we proved that serum CysC could significantly improve the predictive power for the primary outcome beyond established traditional risk factors (including eGFRcr), indicating that incremental improvement in risk prediction with CysC is due in part to its non-GFR determinants among ischemic stroke patients. However, there is no significant correlation between CysC levels and 1-year stroke recurrence and combined vascular events in our research.

Besides, patients with elevated CysC seemed more likely to be male, as Q4 has both more numbers and proportion of males than Q2, but there were no significant interactions. Of note, elevated CysC levels (Q4) were more likely to experience poor clinical outcome than Q2 in age ≥65 subgroup in the sensitivity analysis. A cohort from the China Health and Retirement Longitudinal Study also showed that the association between CysC levels and the incidence of ischemic stroke was more pronounced in males or the aged than in females or the young (33). The underlying mechanism needs to be confirmed by further research.

Several studies have investigated the relationship between CysC and the risk of stroke outcomes previously (5, 34, 35). However, evidence from large-scale studies on the relationship between CysC and stroke clinical prognosis is still insufficient. Compared with previous studies, we further added the evidence of a bilateral effect of CysC levels on clinical outcomes after AIS independent of eGFRcr. Our findings corroborated prior studies that suggested that CysC may improve overall risk prediction due in part to its non-GFR determinants. Nonetheless, there are some limitations that need to be interpreted. First, only baseline CysC was analyzed in our study, so we were unable to examine the association of CysC changes with prognosis; further studies with repeated measurement intervals are needed. Second, 4,910 patients of the CNSR-III trial were excluded,

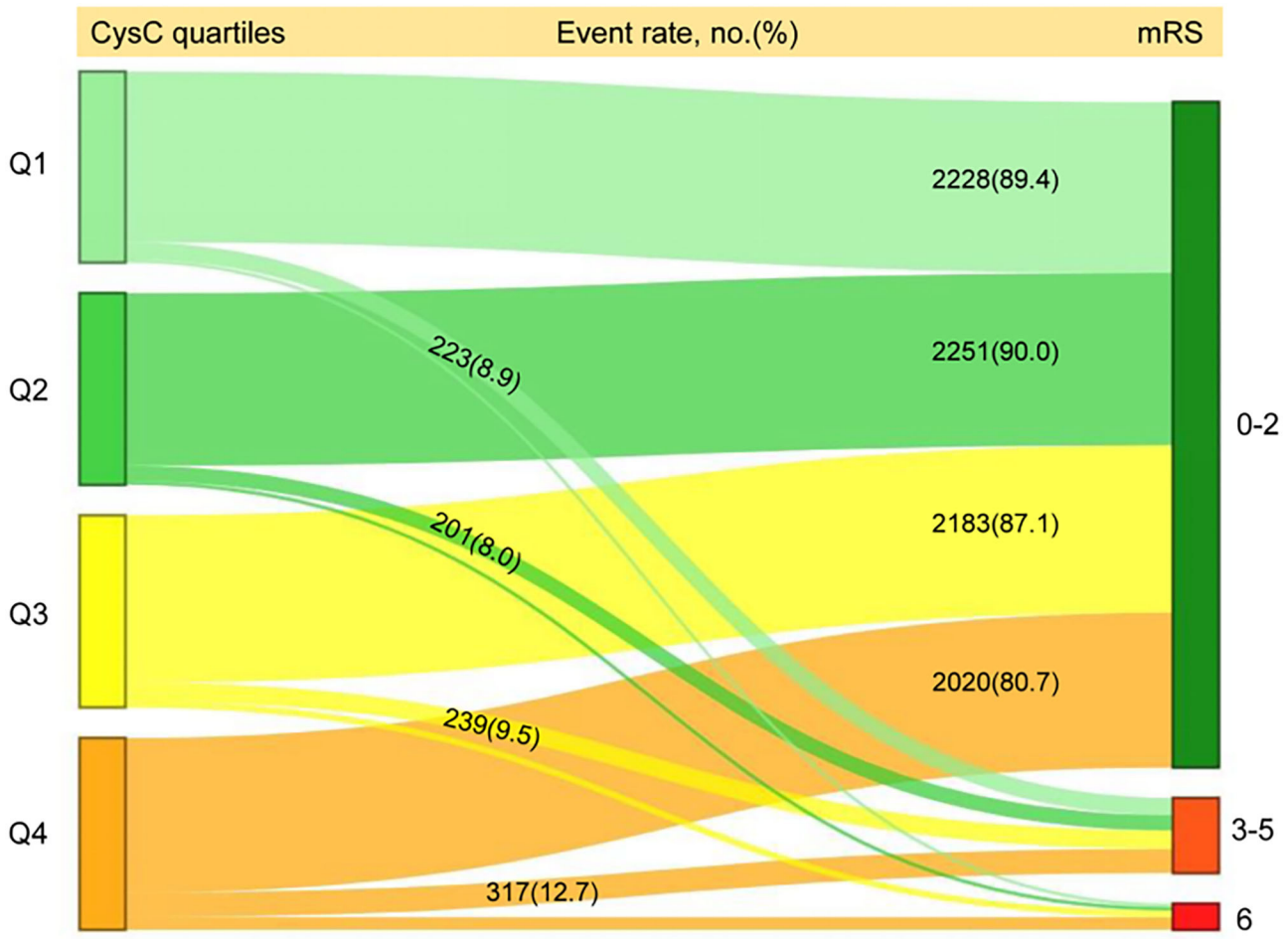

FIGURE 2 | Distribution of 1-year mRS score by CysC quartiles among all the included patients. mRS, modified Rankin Scale; Q, quartile; CysC, cystatin C.

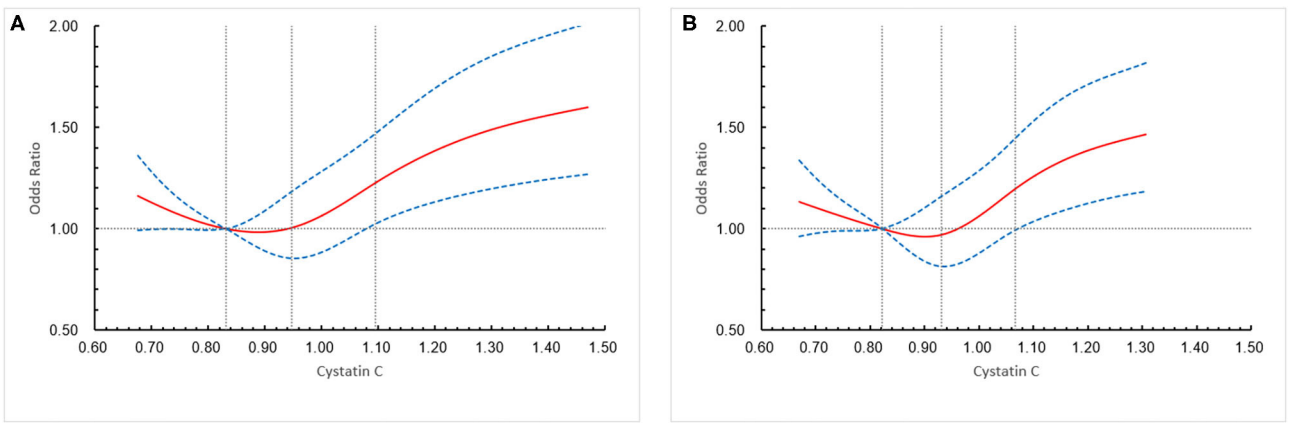

FIGURE 3 | Association of cystatin C (CysC) level with a combination of all-cause mortality and major disability (modified Rankin scale score, 3–6) in all the included patients and "preserved renal function" patients at 1 year. **(A)** All the included patients, **(B) "**preserved renal function" patients. The solid line indicates estimated hazard ratio/odds ratio and the dashed lines the 95% confidence interval bands. Reference is first quartile of CysC. The vertical dashed lines indicate the first, second, and third quartiles of CysC. Data were fitted using a logistic regression model of the restricted cubic spline with 5 knots (the 5th, 25th, 50th, 75th, 95th percentiles) for CysC, adjusting for potential covariates (Model 3). The lowest 5% and highest 5% of participants were not shown in the figures.

TABLE 3 | Reclassification and discrimination statistics for outcomes by CysC within 1 year.

1-year outcomes, no. (%)		C statistic		IDI, %		NRI,† %	
		Estimate (95% CI)	*p*-value	Estimate (95% CI)	*p*-value	Estimate (95% CI)	*p*-value
All-cause mortality or major disability (modified Rankin scale score, 3–6)	Conventional model*	0.776 (0.763–0.790)		–		–	
	Conventional model + CysC quartile	0.778 (0.765–0.791)	0.014	0.13 (0.02–0.23)	0.016	13.10 (7.33–18.86)	<0.001
Stroke recurrence	Conventional model*	0.622 (0.603–0.640)		–		–	
	Conventional model + CysC quartile	0.623 (0.604–0.641)	0.344	0.02 (−0.01 to 0.05)	0.179	1.45 (−5.09 to 7.99)	0.663
Combined vascular events	Conventional model*	0.628 (0.610–0.646)		–		–	
	Conventional model + CysC quartile	0.629 (0.611–0.646)	0.553	0.02 (−0.01 to 0.05)	0.233	1.09 (−5.28 to 7.46)	0.738

IDI, integrated discrimination improvement; NRI, net reclassification index; BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; NIHSS, National Institutes of Health Stroke Scale; CysC, cystatin C; hs-CRP, high sensitivity C-reactive protein; TG, triglycerides; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; eGFRcr, creatinine-calculated glomerular filtration rate; TOAST, Trial of Org 10 172 in acute stroke treatment.

**Conventional model: age, sex, body mass index, medical history of ischemic stroke, coronary artery disease, atrial fibrillation, smoking and alcohol drinking, NIHSS at admission, laboratory data of hs-CRP, LDL, HDL, TG, TC level, eGFRcr, ml/min/1.73 m^2, and TOAST subtype.*

†Patients were divided into four risk categories by CysC quartiles.

and a selection bias may unavoidably be present. However, the baseline characteristics of participants in this study were balanced, suggesting that the selection bias may be minimal. Third, our study has not relied on direct GFR measurement to exclude the compounded effect of GFR on the predictive role of CysC. Fourth, data at 3 months' follow-up were not available; we were unable to determine the relationship between CysC and short-term outcomes. Even when we have tried to adjust for possible confounders such as medication, there are still many factors influencing the long-term prognosis. Finally, only Chinese patients were enrolled in the trial. This limits the generalizability of the findings to a Western population with a different disease pattern or stroke subtypes. Further work is needed to validate our research and seek out possible mechanisms.

CONCLUSIONS

This sub-study of the CNSR-III trial suggests that CysC levels have a bilateral effect on 1-year clinical outcome independent of eGFRcr after ischemic stroke onset. Further prospective studies are needed to validate our findings and to elucidate the potential biological mechanisms.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee at Beijing Tiantan Hospital (IRB approval number: KY2015-001-01) and all participating centers. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YW had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis, designed, and conceptualized this study. YD performed the experiments and drafted the manuscript. LL interpreted the data. ZC revised the manuscript for intellectual content. HL interpreted the data and revised the manuscript. YP conducted the statistical analysis and interpreted the data. JW revised the manuscript for intellectual content. XM, JL, JJ, and XXie performed the experiments and interpreted the data. XXia conducted the statistical analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.676872/full#supplementary-material

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Higher Serum Levels of Lactate Dehydrogenase Before Microsurgery Predict Poor Outcome of Aneurysmal Subarachnoid Hemorrhage

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Introduction: We explored whether higher preoperative serum levels of lactate dehydrogenase (LDH) predicted outcome 3 months after surgery in patients with aneurysmal subarachnoid hemorrhage (aSAH) treated using microsurgical clipping in our institution.

Methods: Patients with aSAH treated at our institution between 2010 and 2018 were enrolled. The following parameters were recorded: age, sex, smoking and drinking history, medical history, Hunt–Hess and Fisher grades, aneurysm location, aneurysm size, surgical treatment, delayed cerebral ischemia (DCI), intracranial infection, hydrocephalus, pneumonia, and preoperative serum LDH levels within 24 h of aSAH. We investigated whether preoperative serum LDH levels were associated with Hunt–Hess grade, Fisher grade, and functional neurological outcome.

Results: In total, 2,054 patients with aSAH were enrolled, 874 of whom were treated using microsurgical clipping. The average serum LDH level (U/L) was significantly lower in the good outcome group (180.096 ± 50.237) than in the poor outcome group (227.554 ± 83.002; $p < 0.001$). After propensity score matching, the average serum LDH level (U/L) was still lower in the good outcome group (205.356 ± 76.785) than in the poor outcome group (227.119 ± 86.469; $p = 0.029$). The area under the receiver operating characteristic (ROC) curve was 0.702 (95% confidence interval [CI]: 0.650–0.754; $p < 0.001$). Based on the ROC curve, the optimal cutoff value for serum LDH levels as a predictor of poor 3-month outcome (modified Rankin Scale score > 2) was 201.5 U/L. The results revealed that Hunt–Hess grade, Fisher grade, DCI, pneumonia, and serum LDH (>201.5 U/L) were significantly associated with poor outcome. After propensity score matching, serum LDH levels > 201.5 U/L were still considered an independent risk factor for poor outcome (odds ratio: 2.426, 95% CI = 1.378–4.271, $p = 0.002$). Serum LDH levels were associated with Hunt–Hess and Fisher grades and were correlated with functional neurological outcomes ($p < 0.001$).

Conclusions: Our findings showed that higher preoperative serum levels of LDH correlated with Hunt–Hess grade, Fisher grade, and neurological functional outcome, and predicted the outcome of aSAH treated by microsurgical clipping at 3 months, which was involved in the related mechanisms of early brain injury and showed its potential clinical significance in patients with aSAH.

Keywords: serum lactate dehydrogenase level, aneurysmal subarachnoid hemorrhage, risk factor, biomaker, outcome

INTRODUCTION

Lactate dehydrogenase (LDH) is a glycolytic enzyme that occurs in all important human organs, including the liver, heart, skeletal muscle, kidney, lung, and brain (1, 2). It catalyzes the dehydrogenation of lactic acid to pyruvic acid, promotes anaerobic glycolysis, and prevents lactic acid accumulation; the latter are associated with unfavorable clinical outcomes of traumatic brain injury (3). When cytolysis occurs or the cell membrane is destroyed, LDH is released into the blood, resulting in an increase in serum LDH (4). LDH activity can be detected in malignant tumor tissues and leukemic cells (5), and serum LDH levels are correlated with the prognosis of adult T-cell leukemia-lymphoma (6), prostate cancer (7), acute myeloid leukemia (8), melanoma (9), neuroblastoma (10), glioblastoma multiforme (11), acute encephalopathy (12), and *Mycoplasma pneumoniae* pneumonia (13). Serum LDH levels reflect the degree of brain tissue injury, and Yu et al. demonstrated that serum LDH activity is associated with middle cerebral artery occlusion in a dose-dependent manner (14). Several reports have shown that LDH quantification predicts neuronal injury (15, 16) and may predict poor prognosis of traumatic brain injury (17) and neonatal intracranial hemorrhage (18).

Several risk factors contribute to the poor prognosis in aneurysmal subarachnoid hemorrhage (aSAH), such as hypertension, poor Hunt–Hess grade, higher Fisher grade, hydrocephalus, pneumonia, and treatment modalities (19–21). However, few reports have explored the clinical significance of serum LDH levels in patients with aSAH, and the role of LDH in aSAH has not been fully established. At least two sources may contribute to higher serum LDH levels in patients with aSAH: (1) apoptotic/necrotic/damaged neurons or glial cells, (2) lytic red blood cells (RBCs) after release into the cerebrospinal fluid (CSF). Lu et al. reported that the number of apoptotic/necrotic/damaged cells was positively correlated with clinical condition in patients with aSAH, as well as with their Hunt–Hess grade (22). Similarly, the number of RBCs in the cerebra cisterna, sulcus, and/or ventricle was correlated with Fisher grade. Frontera found that early brain ischemia injury was associated with worse Hunt–Hess grade, which indicates poor acute neurological status and is correlated with worse functional outcomes after SAH (23). Claassen et al. showed that SAH completely filling the cistern or fissure, as well as intraventricular hemorrhage (IVH) on computed tomography (CT), were risk factors for delayed cerebral ischemia (DCI) (24), which is correlated with poor outcomes after SAH. However, few reports have explored the relationship between serum LDH levels and the extent of cerebral tissue injury in patients with aSAH. In the present study, we explored the clinical significance of serum LDH in patients with aSAH treated using microsurgical clipping in our institution. We hypothesized that higher preoperative serum levels of LDH, which may be correlated with Hunt–Hess grade and Fisher grade, predict 3-month outcome in patients with aSAH treated using microsurgical clipping.

MATERIALS AND METHODS

Participants

Patients were enrolled in the study based on the following criteria: (1) diagnosis of SAH confirmed by CT; (2) presence of intracranial aneurysms confirmed using CT angiography (CTA) or digital subtraction angiography (DSA); (3) all aneurysms treated using microsurgical clipping; (4) CTA and/or DSA performed postoperatively. (5) patients were admitted 24 h after the onset of SAH. The exclusion criteria were as follows: (1) aSAH detected $>$ 24 h after occurrence; (2) other cerebrovascular diseases (such as cerebral arteriovenous malformations, intracranial arteriovenous fistula, or moyamoya syndrome/disease) or intracranial tumors; (3) history of myocardial infarction, hepatitis, malignant tumor, pulmonary infarction, leukemia, hemolytic anemia, kidney disease, or progressive muscular atrophy. The data of patients with aSAH at our institution between 2010 and 2018 were collected. The following parameters were recorded: age, sex, smoking and drinking history, medical history (hypertension, diabetes, coronary heart disease, cerebral stroke), Hunt–Hess and Fisher grades, aneurysm location, aneurysm size, surgical treatment (conventional or decompressive craniotomy), delayed cerebral ischemia (DCI), intracranial infection, hydrocephalus, pneumonia, and preoperative serum LDH levels within 24 h of aSAH.

Treatment

After confirmation, ruptured intracranial aneurysms were treated using microsurgical clipping within 72 h of aSAH onset. After surgery, patients were treated according to current aSAH guidelines (25), including prevention of cerebral arterial narrowing, improvement of cerebral blood flow, neurotrophic treatment, stress ulcer prevention, and nutritional support.

Follow-Up Visit and Definition of Outcome

Postoperative complications were evaluated using CT scanning within 24 h of surgery. The neurological outcome was assessed at the 3-month follow-up and classified according to the modified Rankin Scale (mRS) score. Good clinical outcome was defined as an mRS score of 0–2, while poor outcome was assigned to patients with an mRS score of 3–6. Functional outcome was divided into four levels according to mRS: no symptoms (mRS = 0), no significant to slight disability (mRS = 1–2), moderate to serious disability (mRS = 3–4), and severe disability to death (mRS = 5–6). To define the relationship between serum LDH levels and clinical outcome in patients with aSAH, we investigated whether preoperative serum LDH levels were associated with Hunt–Hess grade, Fisher grade, or the upper four functional outcomes.

Statistical Analysis

All statistical analyses were performed using SPSS for Windows (version 25.0; IBM Corp., Armonk, NY, USA). The Kolmogorov–Smirnov test was used to check whether the data had a normal distribution. One-way analysis of variance or the Student's *t*-test were used to determine the significance of differences in continuous data. The χ^2 test or Fisher's exact test was used to determine the significance of differences in qualitative data. Multivariable analysis was carried out using a backward stepwise logistic regression model that included all variables with a *p*-value of <0.10 in the univariate analysis. In the multivariable analysis model, age was divided into "≤ 60 years" and "> 60 years" (26), Hunt–Hess grade into "low grade" (I–III) and "high grade" (IV–V), Fisher grade into "low grade" (1–3) and "high grade" (4), and serum LDH levels into "≤ optimal cutoff value" and "> optimal cutoff value." Statistical significance was set at a *p*-value of <0.05. The receiver operating characteristics (ROC) curve (MedCalc for Windows version 15.2.2; Mariakerke, Belgium) was generated to analyze the specificity and sensitivity of serum LDH levels for mRS. Propensity-score matching (PSM) was performed to remove imbalances in basic clinical characteristics between the good outcome and poor outcome groups, as well as between the pneumonia and non-pneumonia groups. Conditional probability was estimated using a logistic regression model. The good outcome and poor outcome groups were matched at a ratio of 1:1 using the nearest neighboring matching algorithm.

RESULTS

A total of 2,054 patients with aSAH were treated in our institution between 2010 and 2018, and 874 patients treated using microsurgical clipping were enrolled based on the above criteria (**Figure 1**). The incidence of poor outcomes following aSAH was

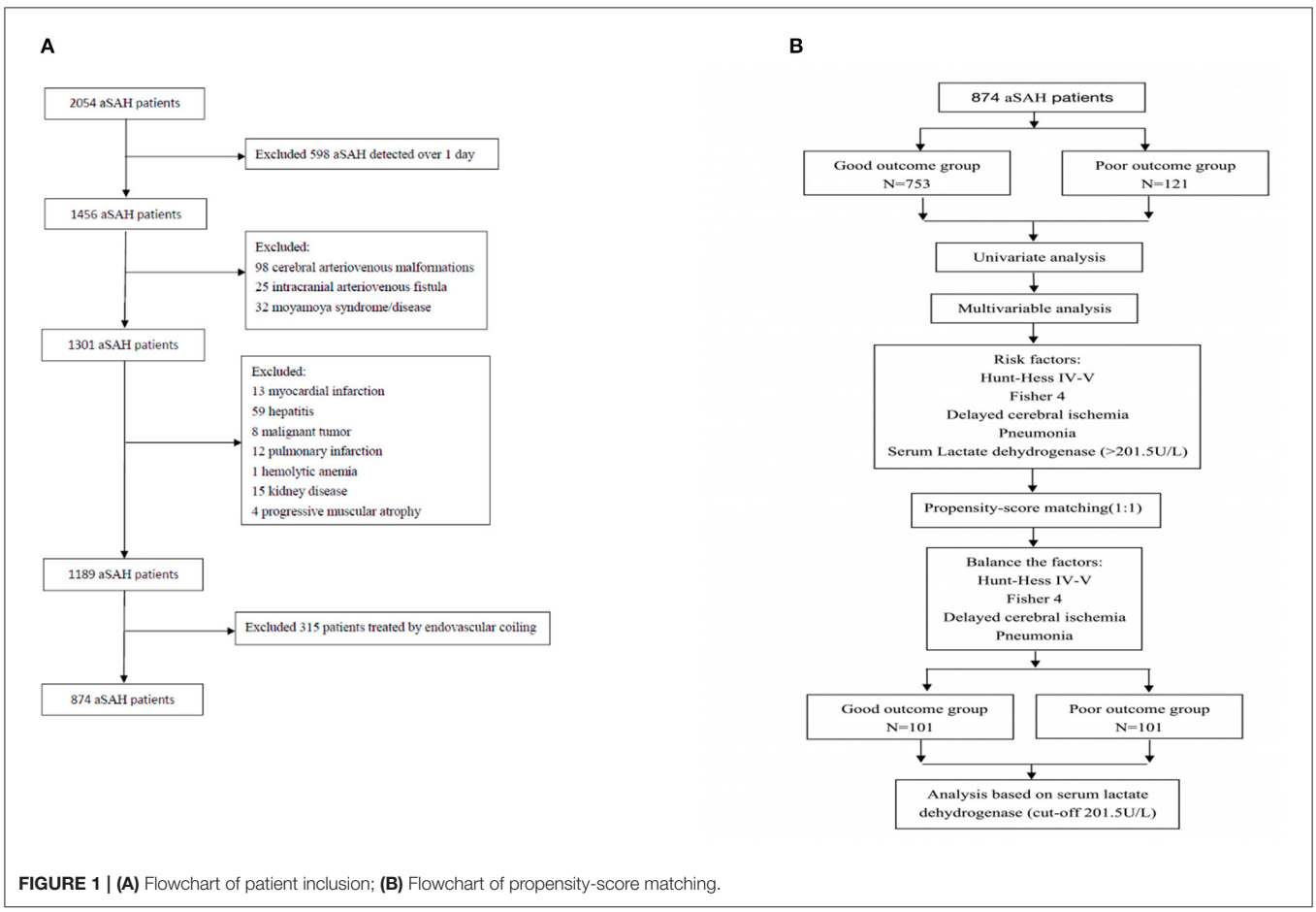

FIGURE 1 | (A) Flowchart of patient inclusion; **(B)** Flowchart of propensity-score matching.

13.8% (121/874). The basic clinical characteristics of patients with aSAH are shown in **Tables 1**, **2**. The average serum LDH level (U/L) was significantly lower in the good outcome group (180.096 ± 50.237) than in the poor outcome group (227.554 ± 83.002; $p < 0.001$). After PSM, the average serum LDH level (U/L) was still lower in the good outcome group (205.356 ± 76.785) than in the poor outcome group (227.119 ± 86.469; $p = 0.029$). The ROC curve of serum LDH levels for poor outcome

TABLE 1 | Basic clinical characteristics of patients with aneurysmsal subarachnoid hemorrhage before and after propensity-score matching.

General information	Before propensity-score matching			After propensity-score matching		
	Good outcome (*n* = 753)	Poor outcome (*n* = 121)	*P*-value	Good outcome (*n* = 101)	Poor outcome (*n* = 101)	*P*-value
Age			0.043			0.128
≤65 yrs	645	95		74	83	
>65 yrs	108	26		27	18	
Age range (y)	10–86	22–85		10–85	22–85	
Sex			0.193			0.240
Male	302	41		40	32	
Female	451	80		61	69	
Smoking	115	7	0.593	11	6	0.205
Drink	75	7	0.144	9	5	0.268
Medical history						
Hypertension	327	73	0.001	61	59	0.774
Diabetes	40	11	0.100	8	7	0.788
Coronary heart disease	9	2	0.675	2	1	0.561
Cerebral stroke	13	2	0.954	2	1	0.561
Hunt-Hess grade			0.000			0.196
0-III	677	58		65	56	
IV-V	76	63		36	45	
Fisher			0.000			0.396
1–3	621	57		59	53	
4	132	64		42	48	
Location of Aneurysm						
Internal carotid artery	141	25	0.614	12	20	0.123
Anterior choroidal artery	25	4	0.994	2	3	0.651
Ophthalmic artery	18	2	0.615	0	2	0.155
Posterior communicating artery	160	24	0.723	25	19	0.306
Middle cerebral artery	171	33	0.271	23	29	0.334
Anterior communicating artery	230	47	0.069	37	41	0.563
Basilar artery	4	3	0.026	0	1	0.316
Anterior cerebral artery	49	6	0.515	8	3	0.121
Posterior cerebral artery	6	2	0.359	0	2	0.155
Aneurysm size			0.510			0.731
<5 mm	451	83		69	76	
5–15 mm	328	55		32	37	
15–25 mm	19	6		5	7	
>25 mm	6	2		1	0	
Surgical treatment			0.246			0.346
Conventional craniotomy	711	111		89	93	
Decompressive craniotomy	42	10		12	8	
Delay ischemic neurological deficit	74	43	0.000	29	30	0.877
Hydrocephalus	117	54	0.000	38	40	0.773
Intracranial infection	53	13	0.152	12	11	0.825
Pneumonia	148	79	0.000	65	61	0.561
Serum Lactate dehydrogenase (>201.5U/L)	178	72	0.000	38	60	0.002

TABLE 2 | Predictors for poor outcome of aSAH in multivariable model.

	Univariate analysis OR (95%CI)				Multivariable analysis AOR (95%CI)				After propensity-score matching OR (95%CI)			
Independent Variable	**OR**	**lower**	**upper**	***P*-value**	**AOR**	**lower**	**upper**	***P*-value**	**OR**	**lower**	**upper**	***P*-value**
Age	1.025	1.008	1.043	0.004	1.019	0.998	1.040	0.075				
Hypertension	1.981	1.339	2.931	0.001	0.876	0.528	1.451	0.606				
Diabetes	1.782	0.888	3.578	0.104	1.056	0.436	2.557	0.904				
Hunt-Hess IV-V	2.746	2.244	3.360	0.000	1.637	1.266	2.118	0.000				
Fisher 4	2.445	1.994	2.998	0.000	1.517	1.182	1.946	0.001				
Anterior communicating artery aneurysm	1.444	0.971	2.148	0.070	1.048	0.636	1.727	0.855				
Basilar artery aneurysm	3.803	0.897	16.125	0.070	3.296	0.479	22.693	0.226				
Delayed cerebral ischemia	5.058	3.248	7.877	0.000	4.234	2.412	7.432	0.000				
Hydrocephalus	4.381	2.910	6.596	0.000	1.043	0.612	1.778	0.877				
Pneumonia	7.689	5.076	11.646	0.000	3.848	2.386	6.206	0.000				
Serum Lactate dehydrogenase (>201.5U/L)	4.747	3.182	7.081	0.000	2.702	1.645	4.440	0.000	2.426	1.378	4.271	0.002

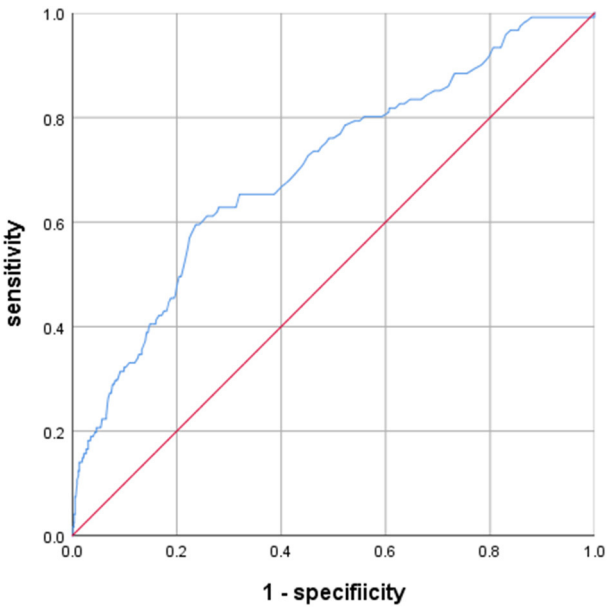

FIGURE 2 | Predictive values of LDH for 3-month modified Rankin Scale (mRS) >2 area under curve 0.702 (95% confidence interval [CI], 0.650–0.754; $p < 0.001$).

in patients with aSAH at the 3-month follow-up is shown in **Figure 2**. The area under the ROC curve (AUC) was 0.702 (95% confidence interval [CI]: 0.650–0.754; $p < 0.001$). The optimal cutoff value for serum LDH levels as a predictor of poor 3-month outcome (mRS > 2) was determined to be 201.5 U/L based on the ROC curve. At this level, the sensitivity was 59.5% and the specificity 76.4%.

To analyze the predictors of poor outcome in patients with aSAH, the following variables with a significance level of $p < 0.10$ were included in a univariate analysis: age, hypertension, diabetes, Hunt–Hess grade, Fisher grade, anterior communicating artery aneurysm, basilar artery aneurysm, DCI, hydrocephalus, pneumonia, and serum LDH levels of > 201.5 U/L. The results revealed that age, hypertension, Hunt–Hess grade, Fisher grade, DCI, hydrocephalus, pneumonia, and serum LDH levels > 201.5 U/L were associated with poor 3-month outcomes (**Table 2**; $p < 0.05$). In a multivariable analysis, Hunt–Hess grade, Fisher grade, DCI, pneumonia, and serum LDH levels >201.5 U/L were still significantly associated with outcome, whereas age, hypertension, diabetes, anterior communicating artery aneurysm, basilar artery aneurysm, and hydrocephalus were not. Patients with a Hunt–Hess grade of IV–V had a 1.6-fold increased risk of poor outcomes (odds ratio [OR]: 1.637; 95% CI: 1.266–2.118, $p < 0.001$). Those with a Fisher grade of 4 had a 1.5-fold increased risk of poor outcomes (OR: 1.517, 95% CI: 1.182–1.946, $p = 0.001$). DCI conferred a 4.2-fold increased risk of poor outcomes (OR: 4.234, 95% CI: 2.412–7.432, $p < 0.001$). Pneumonia was associated with a 3.8-fold increased risk of poor outcomes (OR: 3.848, 95% CI: 2.386–6.206, $p < 0.001$). Serum LDH levels >201.5 U/L showed a 2.7-fold increased risk of poor outcomes (OR: 2.702, 95% CI: 1.645–4.440, $p < 0.001$; **Table 2**). After PSM, there were no significant differences in Hunt–Hess grade, Fisher grade, DCI, or pneumonia between the good outcome and poor outcome groups (**Tables 1**, **2**). In the logistic regression model (**Table 2**), serum LDH levels >201.5 U/L were still considered an independent risk factor for poor outcome (OR: 2.426, 95% CI: 1.378–4.271, $p = 0.002$).

Interestingly, serum LDH levels were associated with Hunt–Hess and Fisher grade, with levels of 163.880 ± 35.571 U/L in the Hunt–Hess grade I group, lower than those in the grade II (174.981 ± 49.616), III (188.306 ± 50.702), IV (225.609 ± 69.509), and V groups (252.851 ± 93.302). There were significant differences among the groups in this regard ($p < 0.001$), and there was a marked trend whereby serum LDH levels increased

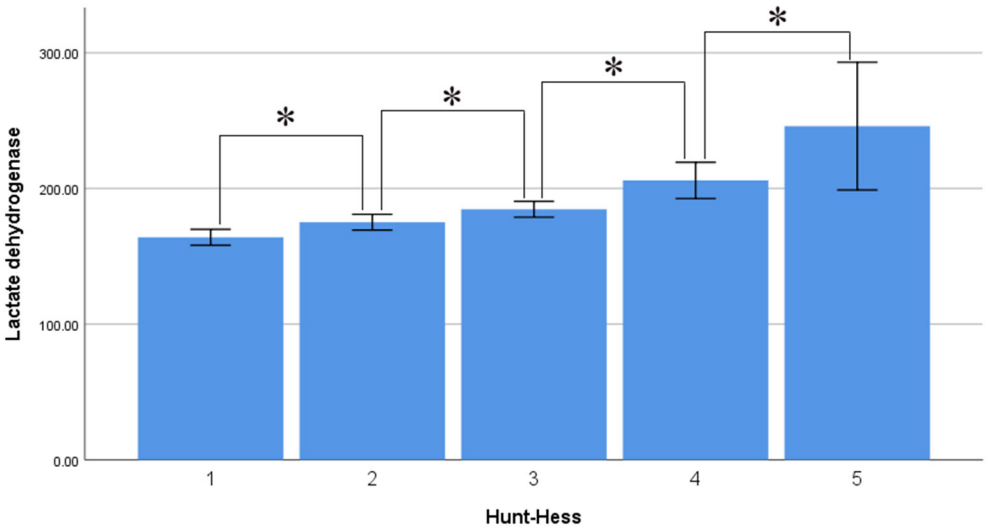

FIGURE 3 | Average level of serum LDH in different Hunt-Hess grade (asterisk represent statistically significant differences).

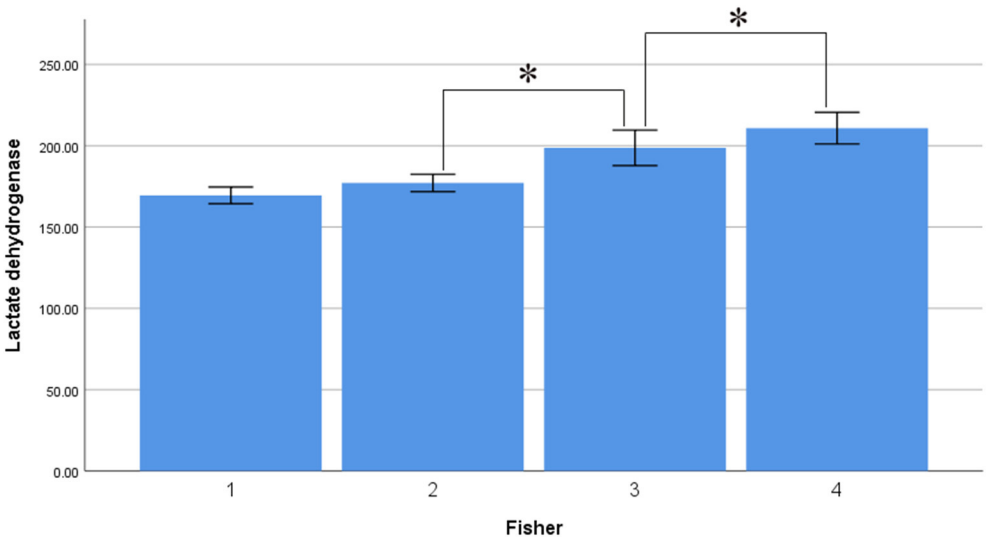

FIGURE 4 | Average level of serum LDH in different fisher grade (asterisk represent statistically significant differences).

alongside Hunt–Hess grade (**Figure 3**). Serum LDH levels were 169.492 ± 41.621 in the Fisher grade 1 group, lower than in the grade 2 (177.097 ± 42.621), grade 3 (198.709 ± 72.553), and grade 4 groups (210.811 ± 68.962). There were statistically significant differences between grades 4 and 3, grades 4 and 2, grades 4 and 1, grades 3 and 2, grades 3 and 1 ($p < 0.001$ in all cases). There was a marked trend whereby serum LDH levels increased alongside Fisher grade (**Figure 4**).

Serum LDH levels were also correlated with functional neurological outcome at the 3-month follow-up. The serum LDH levels were 179.247 ± 46.761 in the mRS 0 group, lower than in the no significant to slight disability (mRS 1–2; 193.977 ± 69.399), moderate to serious disability (mRS 3–4; 205.918 ± 59.203), and severe disability to death groups (mRS 5–6; 234.188 ± 108.336). There were significant differences among the groups in this regard ($p < 0.001$). There was a marked trend whereby serum LDH levels increased as neurological function deteriorated (**Figure 5**).

DISCUSSION

Our findings showed a marked trend whereby serum LDH levels increased alongside Hunt–Hess and Fisher grades. In addition, Hunt–Hess grade, Fisher grade, DCI, pneumonia, and higher serum LDH levels predicted and contributed to poor outcome

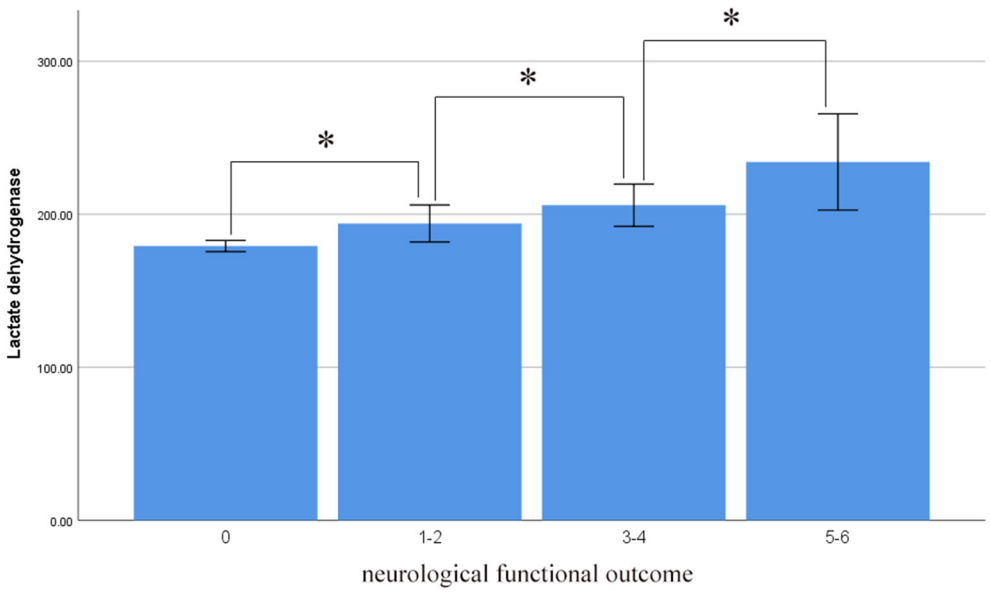

FIGURE 5 | Average level of serum LDH in different neurological functional outcome (asterisk represent statistically significant differences).

in patients with aSAH at 3 months. The optimal cutoff value for serum LDH levels as a predictor of 3-month poor outcome (mRS > 2) was 201.5 U/L. Moreover, serum LDH levels were correlated with functional neurological outcomes. There was a marked trend whereby serum LDH levels increased as neurological function deteriorated. After PSM, serum LDH (>201.5 U/L) was still considered an independent risk factor of poor outcome.

Serum LDH levels are correlated with the prognosis of adult T-cell leukemia-lymphoma (6), prostate cancer (7), acute myeloid leukemia (8), melanoma (9), neuroblastoma (10), glioblastoma multiforme (11), acute encephalopathy (12), and *Mycoplasma pneumoniae* pneumonia (13). However, few studies have investigated the relationship between LDH and aSAH. Regional cerebral blood flow and arteriovenous difference of oxygen are reduced due to primary aSAH injury (27), and cerebral ischemia causes an anaerobic shift of metabolism, leading to lactic acidosis and upregulation of serum LDH levels (28). There is a significant correlation between serum LDH and lactic acid levels, and both reflect the degree of tissue damage (27, 29).

Serum LDH levels can also reflect the severity of brain tissue injury. Neuronal apoptosis and necrosis have been observed 24 h after SAH (30, 31) and can result in cytolysis and cell membrane destruction. Subsequently, LDH is released into the blood from the damaged or dead cells, resulting in increased serum LDH (4). In the study by Yu et al. (14), serum LDH activity was associated with infarct volume and degree of middle cerebral artery occlusion in a dose-dependent manner. Several studies have shown that LDH can be quantified to predict neuronal damage (15, 16), and inhibition of LDH release may reduce neuronal apoptosis (14). Rao et al. reported that a significant increase in serum LDH levels was a predictor of severe brain damage and poor prognosis of traumatic brain injury (17), while Engelke et al. indicated that LDH was significantly correlated with subsequent seizures, hydrocephalus, and adverse long-term outcomes of neonatal intracranial hemorrhage (18).

In the present study, serum LDH levels increased alongside Hunt–Hess grade. We deduced that serum LDH levels were correlated with Hunt–Hess grade, and that they reflected the degree of early brain injury and the clinical condition of patients with aSAH. Furthermore, there was a marked trend whereby serum LDH levels increased as neurological function at the 3-month follow-up deteriorated. Subarachnoid clots in sulci/fissures induce spreading depolarizations and acute cerebral infarction of the adjacent cortex after cerebral aneurysm rupture (30). This is a mechanism of early brain injury after SAH (32) and contributes to the clinical condition of patients with aSAH. In a study by Frontera et al. (23), early ischemic brain injury was related to worse Hunt–Hess grade, higher rates of death, and severe disability/death (mRS 4–6) at the 3-month follow-up. Increased ischemic lesion volume has been associated higher Hunt–Hess grade and 3-month mRS (33), corroborating our own findings.

Our findings also showed a marked trend whereby serum LDH levels increased alongside Fisher grade. Fisher grade is higher when there is more blood in the subarachnoid space, and higher Fisher grade correlates with poor aSAH outcome (34, 35). After cerebral aneurysm rupture, the blood brain barrier is destroyed and RBCs are released into the subarachnoid space from the artery. These RBCs break down in the cerebrospinal fluid (CSF) (35) and LDH from the lysed RBCs is absorbed into the blood after being released into the CSF (17), increasing serum LDH levels. Therefore, higher serum LDH levels are associated with higher Fisher grade, which is closely related to poor outcome in patients with aSAH (34, 36, 37).

However, our study had some limitations. Firstly, LDH occurs in all important human organs and lacks specificity to the central nervous system. LDH was not collected and measured in the CSF in our patients, and serum LDH levels do not directly reflect the true levels in brain tissue. Secondly, imaging data were not available to confirm the relationship between serum LDH levels and the degree of brain tissue damage. Thirdly, serum LDH levels were within the normal range in some patients with poor outcomes, so they do not fully explain these patients' prognosis; the detailed mechanism needs further exploration.Fourthly, LDH, like C-reactive protein, neutrophil to lymphocyte ratio and white blood cell count, are related to the prognosis of SAH. They are biomarkers of the prognosis of SAH. However, the specific mechanism of LDH is still unclear. And there is still no effective treatment to reduce the mortality and disability rate of aSAH patients.

CONCLUSIONS

Our findings showed that higher preoperative serum levels of LDH correlated with Hunt–Hess grade, Fisher grade, and functional neurological outcome, and that they predicted the 3-month outcome in patients with aSAH, which is associated with mechanisms of early brain injury and may have clinical significance in patients with aSAH.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the First Affiliated Hospital of Fujian Medical University. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SZ, HW, GC, HS, and LY: acquisition of data and critical revision of manuscript for intellectual content. YL: study supervision. ZL, PY, and DK: study concept and design. PY and DK: analysis and interpretation of data and study supervision. All authors reviewed the manuscript.

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frontiers in Neurology

ORIGINAL RESEARCH
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Serum Bilirubin Levels and Extent of Symptomatic Intracranial Atherosclerotic Stenosis in Acute Ischemic Stroke: A Cross-Sectional Study

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Background: Bilirubin plays a paradoxical role in the pathological mechanism of stroke. To date, few clinical studies have investigated the effect of serum bilirubin on symptomatic intracranial atherosclerotic stenosis (sICAS). This study aims to evaluate the connection between serum bilirubin and sICAS.

Methods: From September 2015 to May 2020, 1,156 sICAS patients without hepatobiliary diseases admitted to our hospital were included. Patients were distributed into none-mild (0–49%), moderate (50–69%) and severe-occlusion sICAS groups (70–100%) by the degree of artery stenosis. Moderate and severe-occlusion sICAS patients were classified into three groups by the number of stenotic arteries (single-, two- and multiple-vessel stenosis). The relationship between serum bilirubin levels and sICAS was analyzed by logistic regression analysis.

Results: In univariable analyses, sICAS patients with severe and multiple atherosclerotic stenoses had lower levels of total bilirubin (Tbil), direct bilirubin (Dbil), and indirect bilirubin (Ibil). In multinomial logistic regression analyses, when compared with the highest tertile of bilirubin, lower levels of Tbil, Dbil, and Ibil showed higher risks of severe-occlusion sICAS (95% CI: 2.018–6.075 in tertile 1 for Tbil; 2.380–7.410 in tertile 1 for Dbil; 1.758–5.641 in tertile 1 for Ibil). Moreover, the logistic regression analyses showed that lower levels of Tbil, Dbil, and Ibil were related to multiple (≥3) atherosclerotic stenoses (95% CI: 2.365–5.298 in tertile 1 and 2.312–5.208 in tertile 2 for Tbil; 1.743–3.835 in tertile 1 and 1.416–3.144 in tertile 2 for Dbil; 2.361–5.345 in tertile 1 and 1.604–3.545 in tertile 2 for Ibil) when compared with tertile 3.

Conclusions: Our findings suggest that lower bilirubin levels may indicate severe and multiple intracranial atherosclerotic stenoses.

Keywords: ischemic stroke, symptomatic intracranial atherosclerotic stenosis, bilirubin, biomarkers, oxidative stress

INTRODUCTION

Symptomatic intracranial atherosclerosis (sICAS), a critical cause of ischemic stroke in China, refers to the stenosis ≥50% of one or more intracranial arteries (1). In Asia, ∼50% of patients with transient ischemic attack (TIA) and 40% of patients with ischemic stroke (IS) have ICAS (2, 3). sICAS is a developing and dynamically changing disease with a high recurrence risk, causing a huge social burden. Traditional risk factors such as age, ethnicity, obesity, hypertension, diabetes, hyperlipidemia, smoking, and metabolic syndrome have been reported to be closely related to sICAS (4). However, the relationship between circulating biomarkers and sICAS is less explored.

Bilirubin is produced by heme catabolism, including total bilirubin (Tbli), indirect bilirubin (Ibil), and direct bilirubin (Dbil). In the past few decades, bilirubin has been considered as a potentially toxic metabolite, which could damage the central nervous system once passing through the blood-brain barrier (5, 6). However, subsequent evidence has shown that bilirubin plays a dual role in oxidative stress and it may be a protective factor for atherosclerosis (7, 8). Accumulating research suggests that bilirubin can inhibit the production of oxidized low-density lipoprotein (ox-LDL), increase the solubility of serum cholesterol, inhibit protein kinase C activity in human fibroblasts, and capture oxygen free radicals, thus inhibiting the progression of atherosclerosis (9–11).

Several clinical observations have indicated that high bilirubin concentrations could reduce the risk of stroke (12–14). Besides, a small number of studies have revealed the negative associations between high bilirubin levels and the occurrence of asymptomatic intracranial atherosclerosis (aICAS) (15) and extracranial atherosclerosis (16). However, research to date has not yet determined the relationship between bilirubin and sICAS.

Therefore, this research aimed to explore the relationship between serum bilirubin concentrations (including Tbil, Dbil, and Ibil) and sICAS in the Chinese Han Population.

METHODS

Study Population

This study was a descriptive, retrospective, cross-sectional study. From September 2015 to May 2020, patients with TIA or acute ischemic stroke (AIS) caused by large artery atherosclerosis (LAA) within 14 days from symptom onset were enrolled from the Department of Neurology of Xiangya Hospital. The information of all patients was collected from the medical records. The diagnosis of AIS and TIA matched with the 2018 Chinese AIS guidelines (17). We assessed the stroke severity of AIS patients at admission using the National Institutes of Health Stroke Scale (NIHSS) score. All patients were subtyped by the Chinese ischemic stroke subclassification (CISS) system (18). We recruited 1,015 patients caused by intracranial atherosclerotic stenosis (stenosis ≥50%) and 141 patients attributed to atherosclerotic causes with none-mild intracranial stenosis (stenosis: 0–49%). Exclusion criteria were as follows: (1) under 18 years old; (2) other causes for TIA or IS such as small vessel occlusion or cardioembolism; (3) incomplete clinical information or laboratory tests; (4) patients with extracranial artery stenosis diagnosed by carotid contrast-enhanced magnetic resonance angiography (CE-MRA) or carotid computed tomography angiography (CTA) according to the methods used in the North American Symptomatic Carotid Endarterectomy Trial (19); (5) brain tumor, intracranial or systemic infection, congenital hypoplastic cerebrovascular disease, etc; (6) other diseases causing intracranial artery stenosis such as vascular malformation, moyamoya disease, artery dissection, vasculitis and syphilis (20); (7) suffering from hepatobiliary diseases (Tbil >34.2 μmol/L, alanine aminotransferase [ALT] ≥80 IU/L, aspartate aminotransferase ≥80 IU/L, serum albumin <3.5 g/dL) or other diseases that may affect bilirubin level such as Gilbert syndrome (Tbil >34.2 μmol/L, ALT <80 IU/L, aspartate aminotransferase <80 IU/L, γ-glutamyl transpeptidase <80 IU/L) (21). This study was approved by the Ethics Committees of Xiangya Hospital of Central South University, Changsha, Hunan Province, China (ethical approval number: 201503330). All patients or their family members signed the informed consent.

Demographics and Risk Factors

The following clinical information was collected via questionnaires and physical examinations: age, sex, hypertension, diabetes mellitus, hyperlipemia, alcohol use, smoking duration, and the history of coronary artery disease. Hypertension was defined as systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg or currently taking antihypertensive drugs (22). Diagnostic criteria for diabetes: random blood glucose ≥11.1 mmol/L, fasting blood glucose ≥7.0 mmol/L or using hypoglycemic drugs (23). Dyslipidemia was diagnosed as serum triglyceride ≥1.7 mmol/L, serum total cholesterol ≥5.2 mmol/L, serum low-density lipoprotein cholesterol ≥3.4 mmol/L, or serum high-density lipoprotein <1.0 mmol/L or using anti-hyperlipidemic drugs (24). Smoking was determined based on the self-report questionnaire at the time of admission, and the smoking amount was defined as pack-years in our study (25). The pack-years was measured based on the average smoking volume and the past and current smoking durations (25). All patients were divided into four groups according to smoking mount: group 1 (0, non-smoker), group 2 (0–15 pack-years), group 3 (15–30 pack-years), and group 4 (>30 pack-years) (25). The state of alcoholism was thought to be an average of more than 20 g of alcohol per day (26). Fasting overnight, the blood samples of all patients were collected the next morning after admission (within 14 days of stroke onset) and sent to the same laboratory department in our hospital. The data of white blood cell, Tbil, Dbil, Ibil, total cholesterol, triglyceride, high-density lipoprotein, low-density lipoprotein, fasting blood glucose, glycosylated hemoglobin A1c, uric acid, and homocysteine levels were derived from the medical records.

Radiological Assessment

On admission, magnetic resonance imaging (MRI) and time of flight magnetic resonance angiography (TOF-MRA) were performed for most patients. Computed tomography (CT) and computed tomography angiography (CTA) were performed for

patients with contraindications to MRI. In addition, CTA was performed when there were doubts about the results of MRA, and digital subtraction angiography (DSA) was performed when the results of MRA and CTA were inconsistent. Meanwhile, carotid CTA and carotid CE-MRA were used to exclude extracranial artery stenosis. All imaging data were evaluated by at least two neurologists with more than 5 years of experience. They knew nothing about the clinical information and reached a consensus. According to the results of MRA, CTA, DSA, or CE-MRA, ICAS was diagnosed as large intracranial artery stenosis (50–100%), including bilateral internal carotid artery (ICA), bilateral anterior cerebral artery (ACA), bilateral middle cerebral artery (MCA), bilateral posterior cerebral artery (PCA), bilateral basilar artery (BA) or bilateral vertebral artery (VA) (27). The degree of intracranial stenosis was assessed by MRA/CTA/DSA using Warfarin-Aspirin Symptomatic Intracranial Disease (WASID) method with reference to normal distal vessels (28). Patients with none-mild intracranial stenosis (stenosis: 0–49%) served as controls. In this study, participants were divided into none-mild group (0–49%), moderate group (50–69%), and severe-occlusion group (70–100%) based on the degree of artery stenosis. We then counted the number of intracranial stenotic arteries (stenosis $\geq$50%) of ICAS patients and classified patients into three groups (single-, two- and multiple-vessel stenosis) accordingly.

Statistical Analysis

The statistical analysis was conducted using IBM SPSS Statistics 22.0 (Chicago, USA), and all data were expressed as frequency (%) and the median (interquartile range, IQR). Characteristics of the objects were compared with a chi-square (χ^2) test for categorical variables and the Mann-Whitney *U* test or Kruskal-Wallis test for continuous variables. We also analyzed the association between bilirubin and sICAS in different groups according to the degree and number of vascular stenosis. Bilirubin levels were categorized into tertiles and the χ^2 test for trends was used to analyze the dose-effect of Tbil, Dbil, and Ibil. Factors with $P < 0.05$ in univariate analysis and reported confounding risk factors were included in multivariate logistic regression analysis to evaluate the independent influence of bilirubin. Tbli, Dbil, and Ibil were tested separately to avoid interaction. We used multinomial logistic regression instead of ordinal polytomous logistic regression because the test of the parallel lines hypothesis was rejected. The results were shown by odds ratio (OR) and 95% confidence interval (CI). In addition, receiver operating characteristic curve (ROC) analysis was conducted by MedCalc software (MedCalc Inc., Mariakerke, Belgium) to determine the predictability of bilirubin for discriminating the extent of ICAS. $P < 0.05$ was considered significant.

RESULTS

Clinical Characteristics of Patients With Ischemic Stroke

Clinical characteristics of all patients were presented in **Table 1**. A total of 1,156 subjects were finally included in the study. The average age of the participants was 61 (IQR, 53–68) years old and 65.5% were male. The median (IQR) of Tbil, Dbil, and Ibli levels was 10.34 (7.70–13.40), 4.50 (3.30–5.90), and 5.60 (4.91–7.18) μmol/L, separately.

TABLE 1 | Baseline characteristics of patients with ischemic stroke.

Characteristics	Value
Age years [IQR]	61 [53–68]
Sex (male, *N*, %)	757 (65.5)
Hypertension (*N*, %)	872 (75.4)
Diabetes mellitus (*N*, %)	389 (33.7)
Hyperlipidemia (*N*, %)	521 (45.1)
CAD (*N*, %)	178 (15.4)
Smoking duration (pack-years) (*N*, %)	
Group 1 (0)	638 (55.2)
Group 2 (>0, ≤15)	107 (9.3)
Group 3 (>15, ≤30)	184 (15.9)
Group 4 (>30)	227 (19.6)
Drinking (*N*, %)	376 (32.5)
NIHSS [IQR]	4 [2–8]
SBP mmHg [IQR]	144 [132–158]
DBP mmHg [IQR]	84 [76–93]
WBC, $\times 10^9$/L [IQR]	6.80 [5.60–8.40]
Tbil, μmol/L [IQR]	10.34 [7.70–13.40]
Dbil, μmol/L [IQR]	4.50 [3.30–5.90]
Ibil, μmol/L [IQR]	5.60 [4.00–7.90]
FBG, mmol/L [IQR]	5.60 [4.91–7.18]
HbA1c, % [IQR]	5.80 [5.70–6.60]
TC, mmol/L [IQR]	4.37 [3.55–5.18]
TG, mmol/L [IQR]	1.55 [1.16–2.12]
HDL, mmol/L [IQR]	1.02 [0.87–1.22]
LDL, mmol/L [IQR]	2.66 [2.05–3.30]
UA, μmol/L [IQR]	316.20 [256.20–382.15]
HCY, μmol/L [IQR]	13.25 [11.11–16.35]

IQR, Inter quartile range; CAD, coronary artery disease; NIHSS, National Institute of Health stroke scale; SBP, systolic blood pressure; DBP, diastolic blood pressure; WBC, white blood cell; Tbil, total bilirubin; Dbil, direct bilirubin; Ibil, indirect bilirubin; FBG, fasting blood glucose; HbA1c, glycosylated hemoglobin A1c; TC, total cholesterol; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein; UA, Uric acid; HCY, Homocysteine. Smoking duration: group 1 (0, non-smoker), group 2 (0–15 pack-years), group 3 (15–30 pack-years) and group 4 (>30 pack-years).

Baseline Characteristics of the Study Population According to the Severity of ICAS

According to the degree of intracranial artery stenosis, participants were divided into none-mild group (0–49%, $n = 141$), moderate group (50–69%, $n = 357$), and severe-occlusion group (70–100%, $n = 658$). Baseline clinical characteristics and laboratory tests of these 1,156 objects were shown in **Table 2**. There were significant differences among these three groups in sex, hypertension, hyperlipidemia, systolic blood pressure, diastolic blood pressure, and the levels of Tbil, Dbil, Ibil, uric acid, and homocysteine. Moreover, **Figures 1A–C** illustrates the median concentrations of Tbil, Dbil and Ibil in different

TABLE 2 | Baseline characteristics of the study population according to the severity of ICAS.

Characteristics	None-mild ($n = 141$)	Moderate ($n = 357$)	Severe-occlusion ($n = 658$)	*P*-value
Age, years [IQR]	62 [53–70]	62 [54–69]	61 [52–68]	0.054
Sex (male, *N*, %)	103 (73.0)	241 (67.5)	413 (62.8)	0.041
Hypertension (*N*, %)	99 (70.2%)	287 (80.4)	486 (73.9)	0.021
Diabetes mellitus (*N*, %)	43 (30.5)	131 (36.7)	215 (32.7)	0.303
HbA1c (yes), % [IQR]	7.20 [6.30–9.20]	7.10 [6.10–8.50]	7.30 [6.30–8.60]	0.557
HbA1c (no), % [IQR]	5.80 [5.60–6.03]	5.80 [5.50–5.80]	5.80 [5.50–5.80]	0.167
Hyperlipidemia (*N*, %)	37 (26.2%)	163 (45.7)	321 (48.8)	<0.001
LDL (yes), mmol/L [IQR]	3.01 [2.02–4.14]	3.13 [2.32–3.69]	2.95 [2.24–3.69]	0.794
LDL (no), mmol/L [IQR]	2.40 [2.00–3.13]	2.58 [2.02–3.04]	2.44 [1.99–2.90]	0.217
CAD (*N*, %)	14 (9.9)	58 (16.2)	105 (16.1)	0.158
Smoking duration (pack-years) (*N*, %)				0.054
Group 1 (0)	70 (49.6)	187 (52.4)	381 (57.9)	
Group 2 (>0, ≤15)	10 (7.1)	42 (11.8)	55 (8.4)	
Group 3 (>15, ≤30)	33 (23.4)	52 (14.6)	99 (15.0)	
Group 4 (>30)	28 (19.9)	76 (21.3)	123 (18.7)	
Drinking (*N*, %)	48 (34.0)	116 (32.5)	212 (32.2)	0.916
NIHSS [IQR]	4 [2–7]	4 [2–7]	4 [2–8]	0.143
SBP mmHg [IQR]	143 [134–158]	148 [134–162]	143 [131–155]	0.002
DBP mmHg [IQR]	87 [79–92]	85 [77–94]	82 [75–92]	0.008
WBC, $\times 10^9$/L [IQR]	6.55 [5.60–8.00]	6.80 [5.60–8.50]	6.85 [5.60–8.40]	0.599
Tbil, μmol/L [IQR]	11.10 [8.85–15.25]	11.20 [9.10–14.85]	9.50 [6.90–12.30]	<0.001
Dbil, μmol/L [IQR]	5.10 [4.05–6.95]	4.96 [3.80–6.30]	4.00 [3.00–5.40]	<0.001
Ibil, μmol/L [IQR]	5.90 [4.70–8.80]	6.30 [4.70–8.55]	5.20 [3.60–7.20]	<0.001
FBG, mmol/L [IQR]	5.50 [4.54–7.56]	5.72 [5.07–7.24]	5.56 [4.90–7.08]	0.053
HbA1c, % [IQR]	5.90 [5.80–6.80]	5.80 [5.70–6.55]	5.80 [5.60–6.50]	0.210
TC, mmol/L [IQR]	4.44 [3.51–5.42]	4.44 [3.62–5.15]	4.30 [3.50–5.17]	0.515
TG, mmol/L [IQR]	1.59 [1.16–2.18]	1.50 [1.15–2.05]	1.57 [1.18–2.22]	0.446
HDL, mmol/L [IQR]	1.04 [0.87–2.20]	1.02 [0.88–1.21]	1.01 [0.87–1.23]	0.851
LDL, mmol/L [IQR]	2.51 [2.00–3.33]	2.78 [2.10–3.32]	2.61 [2.08–3.29]	0.176
UA, μmol/L [IQR]	333.10 [269.30–403.03]	333.95 [273.03–390.08]	306.90 [250.40–371.80]	<0.001
HCY, μmol/L [IQR]	13.50 [11.72–16.06]	13.57 [11.28–16.46]	12.76 [10.82–16.20]	0.031

None-mild: stenosis 0–49%, moderate: stenosis 50–69%, severe-occlusion group: stenosis 70–100%. ICAS, intracranial atherosclerotic stenosis; IQR, Inter quartile range; HbA1c, glycosylated hemoglobin A1c; LDL, low density lipoprotein; CAD, coronary artery disease; NIHSS, National Institute of Health stroke scale; SBP, systolic blood pressure; DBP, diastolic blood pressure; WBC, white blood cell; Tbil, total bilirubin; Dbil, direct bilirubin; Ibil, indirect bilirubin; FBG, fasting blood glucose; TC, total cholesterol; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein; UA, uric acid; HCY, homocysteine. Smoking duration: group 1 (0, non-smoker), group 2 (0–15 pack-years), group 3 (15–30 pack-years) and group 4 (>30 pack-years).

groups according to the severity of ICAS. Lower bilirubin levels were found in the severe-occlusion group compared with the none-mild or moderate group. Bilirubin levels were categorized into tertiles, and the linear trends across the three categories were tested by chi-square linear trend test. The chi-square linear trend test yielded a significant result (*P*-trends <0.001) (**Figure 1D**), mostly due to the significant difference in bilirubin levels between the moderate and severe-occlusion ICAS groups. The serum bilirubin levels in patients with none-mild ICAS were similar compared with patients presenting with moderate ICAS. We tried to find clear cutoff values by ROC analyses to discriminate the severity of ICAS, however, the performance of these models was rather poor with AUCs of 0.627–0.640. The results were shown in **Supplementary Figure 1A** and **Supplementary Table 1**.

Baseline Characteristics of the Study Population Based on the Number of Stenotic Arteries

Based on the number of stenotic arteries, patients were stratified into three groups: single-vessel stenosis ($n = 294$), two-vessel stenosis ($n = 193$) and multiple-vessel stenosis ($n = 528$). **Table 3** provides the specific clinical information and laboratory results. There were significant differences among the three groups in age, hypertension, diabetes, hyperlipidemia, Tbil, Dbil, Ibil, fasting blood glucose, and glycosylated hemoglobin A1c. In addition, lower bilirubin levels were found in the multiple-vessel stenosis group compared with the single- and two-vessel stenosis groups (as shown in **Figures 1E–G**). The chi-square linear trend test yielded a significant result (*P*-trends <0.001) (**Figure 1H**), mostly due to the significant difference in bilirubin levels between the

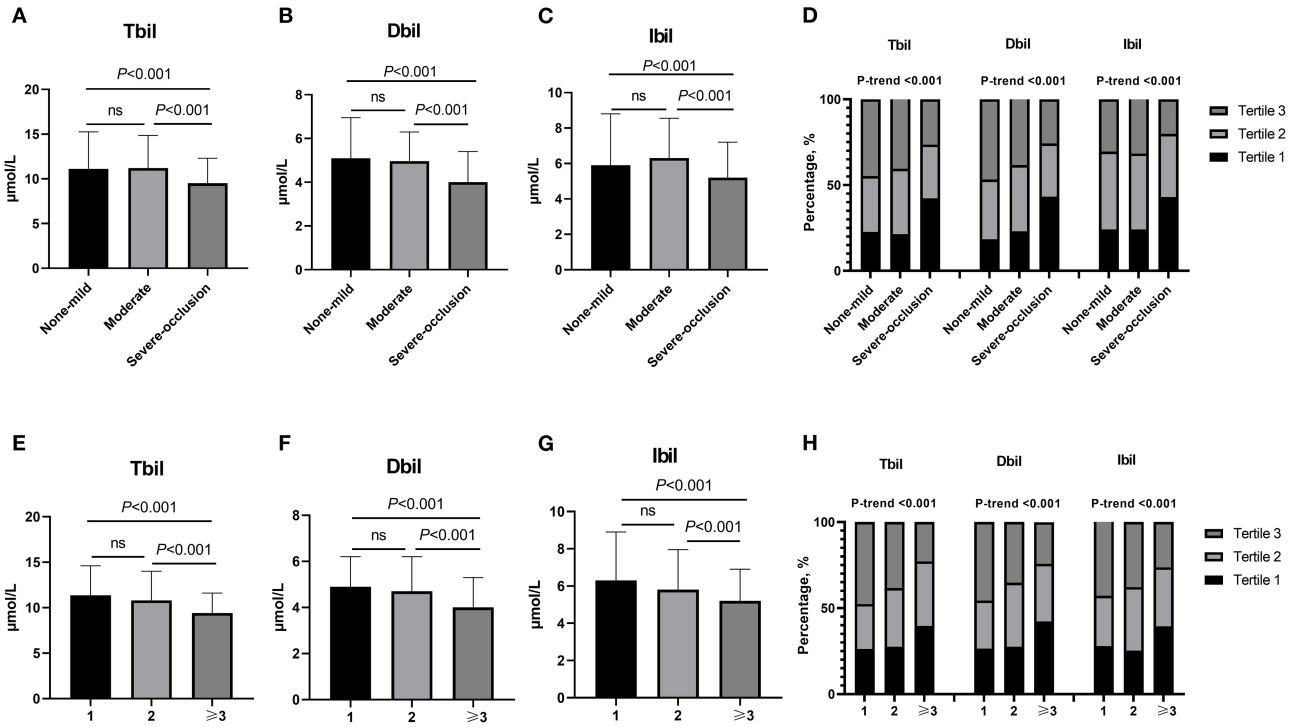

FIGURE 1 | (A–D) Bilirubin levels in different groups stratified by the severity of ICAS (none-mild group: stenosis 0–49%, moderate group: stenosis 50–69% and severe-occlusion group: stenosis 70–100%). **(A)** Different Tbli levels among non-mild, moderate and severe-occlusion groups.Tertile 1: <8.6 μmol/L, Tertile 2: 8.6–12.1 μmol/L, Tertile 3: >12.1 μmol/L. **(B)** Different Dbli levels among non-mild, moderate and severe-occlusion groups. Tertile 1: <3.7 μmol/L, Tertile 2: 3.7–5.3 μmol/L, Tertile 3: >5.3 μmol/L. **(C)** Different Ibli levels among non-mild, moderate and severe-occlusion groups. Tertile 1: <4.6 μmol/L, Tertile 2: 4.6–7.9 μmol/L, Tertile 3: >7.9 μmol/L. **(D)** The different percentages of Tbli, Dbli and Ibli tertiles among non-mild, moderate and severe-occlusion groups. **(E–H)** Different bilirubin levels in groups according to the number of stenoses (≥50%) (n = 1, 2, and ≥3). **(E)** Different Tbli levels among groups. Tertile 1: <8.4 μmol/L, Tertile 2: 8.4–11.9 μmol/L, Tertile 3: >11.9 μmol/L. **(F)** Different Dbli levels among groups. Tertile 1: <3.6 μmol/L, Tertile 2: 3.6–5.1 μmol/L, Tertile 3: >5.1 μmol/L. **(G)** Different Ibli levels among groups. Tertile 1: <4.5 μmol/L, Tertile 2: 4.5–6.9 μmol/L, Tertile 3: >6.9 μmol/L. **(H)** The different percentages of Tbli, Dbli and Ibli tertiles in groups according to the number of stenoses (≥50%) (n = 1, 2, and ≥3). n = 1, single-vessel stenosis; n = 2, two-vessel stenosis; n ≥ 3, multiple-vessel stenosis. ICAS, intracranial atherosclerotic stenosis; Tbil, total bilirubin; Dbil, direct bilirubin; Ibil, indirect bilirubin.

two- and multiple-vessel stenosis groups. ROC analyses were used to differentiate the number of stenotic arteries in patients with ICAS, unfortunately, the ROC methods demonstrated poor discriminatory capability for constructing cutoff values (AUCs: 0.600, 0.607, and 0.615). The results were presented in the **Supplementary Figure 1B** and **Supplementary Table 1**.

Multivariate Logistic Regression Analyses for Predictors of Severe ICAS

The findings obtained from the multivariate logistic regression analysis are presented in **Table 4**. The variables with $P < 0.05$ in univariate analysis in **Table 2** and other reported confounding factors were input into the multivariate model. Tbil, Dbil, and Ibil did not have significant influence on the degree of ICAS when comparing the group with moderate ICAS with the none-mild ICAS group. When comparing the severe-occlusion and none-mild groups, lower Tbil, Dbil, and Ibil levels were found to be independent factors (OR: 3.502, 95% CI: 2.018–6.075, $P < 0.001$ in tertile 1 for Tbil; OR: 4.199, 95% CI: 2.380–7.410, $P < 0.001$ in tertile 1 for Dbil; OR: 3.149, 95% CI: 1.758–5.641, $P < 0.001$ in tertile 1 for Ibil) compared to the highest levels of bilirubin.

Multivariate Logistic Regression Analyses for Predictors of Multi-Stenosis of ICAS

Table 5 shows the results of multinomial logistic regression analysis. We compared two- and multiple-vessel stenosis groups separately, with single-vessel stenosis group as the control. Using tertile 3 as a reference, we found that lower levels of Tbil, Dbil, and Ibil were related to multiple (two-vessel stenosis or multiple-vessel stenosis) atherosclerotic stenosis (when two- vs. single-vessel stenosis, OR: 2.052, 95% CI: 1.270–3.314, $P = 0.003$ in tertile 2 for Tbil; OR: 1.866, 95% CI: 1.167–2.986, $P = 0.009$ in tertile 2 for Ibil. When multiple- vs. single-vessel stenosis, OR: 3.540, 95% CI: 2.365–5.298, $P < 0.001$ in tertile 1 and OR: 3.470, 95% CI: 2.312–5.208, $P < 0.001$ in tertile 2 for Tbil; OR: 2.585, 95% CI: 1.734–3.835, $P < 0.001$ in tertile 1 and OR: 2.110, 95% CI: 1.416–3.144, $P < 0.001$ in tertile 2 for Dbil; OR: 3.552, 95% CI: 2.361–5.345, $P < 0.001$ in tertile 1 and OR: 2.384, 95% CI: 1.604–3.545, $P < 0.001$ in tertile 2 for Ibil).

TABLE 3 | Baseline characteristics of the participants according to the number of stenotic arteries.

Characteristics	1 ($n = 294$)	2 ($n = 193$)	≥3 ($n = 528$)	*P*-value
Age years [IQR]	57 [48–66]	62 [53–69]	63 [54–69]	<0.001
Sex (male, *N*, %)	204 (69.4)	127 (65.8)	323 (661.2)	0.056
Hypertension (*N*, %)	191 (65.0)	154 (79.8)	428 (81.1)	<0.001
Diabetes mellitus (*N*, %)	78 (26.5)	69 (35.8)	199 (37.7)	0.005
HbA1c (yes), % [IQR]	6.65 [5.80–7.80]	7.20 [6.20–8.70]	7.40 [6.40–8.60]	0.002
HbA1c (no), % [IQR]	5.80 [5.50–5.80]	5.80 [5.60–5.90]	5.80 [5.50–5.90]	0.183
Hyperlipidemia (*N*, %)	160 (54.4)	77 (39.9)	247 (46.8)	0.006
LDL (yes), mmol/L [IQR]	3.06 [2.25–3.70]	3.06 [2.20–3.55]	2.95 [2.28–3.72]	0.896
LDL (no), mmol/L [IQR]	2.45 [1.94–2.94]	2.56 [2.11–2.94]	2.47 [2.02–2.97]	0.400
CAD (*N*, %)	42 (14.3)	34 (17.6)	88 (16.7)	0.559
Smoking duration (pack-years) (*N*, %)				0.092
Group 1 (0)	152 (51.7)	105 (54.4)	311 (58.9)	
Group 2 (>0, ≤15)	31 (10.5)	17 (8.8)	49 (9.3)	
Group 3 (>15, ≤30)	40 (13.6)	27 (14.0)	84 (15.9)	
Group 4 (>30)	71 (24.1)	44 (22.8)	84 (15.9)	
Drinking (*N*, %)	94 (32.0)	71 (36.8)	163 (30.9)	0.319
NIHSS [IQR]	5 [2–8]	5 [2–8]	4 [2–7]	0.151
SBP mmHg [IQR]	144 [128–155]	143 [134–158]	145 [133–159]	0.078
DBP mmHg [IQR]	84 [76–95]	85 [77–93]	82 [75–91]	0.128
WBC, $\times 10^9$ /L [IQR]	6.90 [5.60–8.50]	7.20 [5.60–8.78]	6.70 [5.60–8.20]	0.220
Tbil, μmol/L [IQR]	11.35 [8.10–14.60]	10.80 [8.30–14.00]	9.40 [7.03–11.60]	<0.001
Dbil, μmol/L [IQR]	4.90 [3.58–6.20]	4.70 [3.60–6.20]	4.00 [3.00–5.30]	<0.001
Ibil, μmol/L [IQR]	6.30 [4.40–8.90]	5.80 [4.45–7.95]	5.20 [3.70–6.90]	<0.001
FBG, mmol/L [IQR]	5.50 [4.91–6.69]	5.92 [5.09–7.53]	5.67 [4.93–7.22]	0.032
HbA1c, % [IQR]	5.80 [5.60–6.10]	5.80 [5.70–6.95]	5.80 [5.70–6.78]	<0.001
TC, mmol/L [IQR]	4.38 [3.50–5.30]	4.41 [3.65–5.14]	4.34 [3.57–5.11]	0.498
TG, mmol/L [IQR]	1.63 [1.21–2.20]	1.50 [1.12–2.06]	1.54 [1.16–2.11]	0.218
HDL, mmol/L [IQR]	1.02 [0.88–1.24]	1.02 [0.87–1.25]	1.01 [0.87–1.19]	0.481
LDL, mmol/L [IQR]	2.71 [2.07–3.41]	2.75 [2.17–3.27]	2.60 [2.08–3.29]	0.760
UA, μmol/L [IQR]	316.55 [252.60–375.80]	315.45 [259.48–387.60]	310.30 [256.05–375.55]	0.858
HCY, μmol/L [IQR]	13.56 [10.96–16.54]	12.52 [11.13–14.90]	13.01 [10.93–16.69]	0.350

1, single-vessel stenosis; 2, two-vessel stenosis; ≥3, multiple-vessel stenosis. IQR, Inter quartile range; HbA1c, glycosylated hemoglobin A1c; CAD, coronary artery disease; NIHSS, National Institute of Health stroke scale; SBP, systolic blood pressure; DBP, diastolic blood pressure; WBC, white blood cell; Tbil, total bilirubin; Dbil, direct bilirubin; Ibil, indirect bilirubin; FBG, fasting blood glucose; TC, total cholesterol; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein; UA, uric acid; HCY, homocysteine. Smoking duration: group 1 (0, non-smoker), group 2 (0–15 pack-years), group 3 (15–30 pack-years) and group 4 (>30 pack-years).

DISCUSSION

The present study indicates that patients with severe and multiple sICAS had significantly lower serum bilirubin levels, even after adjusting for confounding factors such as age, sex, smoking status, hypertension, diabetes and hyperlipidemia.

ICAS is a leading cause of stroke occurrence and recurrence worldwide and is associated with higher risk for ischemic stroke and death. Since atherosclerosis is a chronic disease mediated by endothelial dysfunction, lipid deposition and inflammation, oxidative stress might play a crucial role in the pathological processes of sICAS (29–32). Prior studies have shown that endothelial dysfunction acts in the preclinical development of atherosclerosis, and inflammation could increase the vulnerability of plaques. Under oxidative stress, LDL could be transformed into oxidized low-density lipoprotein (ox-LDL), and vascular endothelial dysfunction and increased permeability could promote the deposition of ox-LDL in the intima (33). Furthermore, the accumulated ox-LDL contributes to the initiation of inflammatory reactions, infiltration of monocytes and T cells, and accumulation of extracellular matrix (34). T cells could recognize antigens and initiate the Type-1 immunity, causing local inflammation and plaque growth, leading to gradual narrowing of blood vessels and ICAS development (34).

Bilirubin has antioxidant and anti-inflammatory activities, reported to be inversely correlated with asymptomatic intracranial atherosclerosis (15, 35, 36). The mechanisms by which bilirubin functions in ICAS remains unclear, but prior studies indicate that bilirubin could inhibit atherosclerosis in several ways. First, reactive oxygen species promote lipid peroxidation, endothelial cell injury, smooth muscle cell proliferation and migration, inflammatory factor expression, and

TABLE 4 | Multivariate logistic regression analyses for predictors of severe-occlusion of ICAS.

	Moderate vs. None-mild		Severe-occlusion vs. None-mild		Moderate vs. None-mild		Severe-occlusion vs. None-mild		Moderate vs. None-mild		Severe-occlusion vs. None-mild	
	***P*-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**
Age, years	0.768	0.997 (0.975–1.019)	0.116	0.984 (0.964–1.004)	0.803	0.997 (0.976–1.019)	0.118	0.984 (0.964–1.004)	0.775	0.997 (0.975–1.019)	0.129	0.984 (0.964–1.005)
Sex, (Female vs. male)	0.416	0.771 (0.412–1.444)	0.926	1.029 (0.569–1.860)	0.538	0.820 (0.437–1.541)	0.908	1.036 (0.571–1.878)	0.433	0.778 (0.415–1.457)	0.967	1.013 (0.561–1.829)
Hypertension	0.045	1.773 (1.013–3.104)	0.236	1.364 (0.816–2.280)	0.062	1.700 (0.973–2.972)	0.238	1.362 (0.815–2.275)	0.042	1.788 (1.022–3.127)	0.206	1.392 (0.834–2.322)
Diabetes mellitus	0.376	1.245 (0.767–2.020)	0.999	1.000 (0.629–1.590)	0.450	1.206 (0.743–1.957)	0.875	0.963 (0.605–1.534)	0.356	1.255 (0.774–2.034)	0.884	1.035 (0.652–1.642)
Hyperlipidemia	0.001	2.270 (1.411–3.653)	<0.001	2.792 (1.772–4.399)	0.001	2.257 (1.403–3.631)	<0.001	2.548 (1.616–4.015)	0.001	2.272 (1.410–3.659)	<0.001	2.806 (1.781–4.422)
Smoking duration												
Group 1 (0)	Reference		Reference		Reference		Reference			Reference		
Group 2 (>0, ≤15)	0.087	2.158 (0.895–5.203)	0.991	1.005 (0.422–2.393)	0.098	2.106 (0.872–5.084)	0.860	1.081 (0.454–2.576)	0.092	2.131 (0.884–5.140)	0.979	1.012 (0.426–2.404)
Group 3 (>15, ≤30)	0.324	0.715 (0.367–1.393)	0.101	0.596 (0.321–1.106)	0.326	0.716 (0.367–1.396)	0.112	0.604 (0.325–1.125)	0.340	0.723 (0.371–1.408)	0.128	0.619 (0.334–1.147)
Group 4 (>30)	0.405	1.333 (0.678–2.617)	0.816	0.926 (0.486–1.764)	0.408	1.331 (0.676–2.623)	0.994	0.997 (0.523–1.903)	0.421	1.319 (0.672–2.591)	0.754	0.902 (0.475–1.716)
SBP, mmHg	0.228	1.009 (0.995–1.023)	0.767	1.002 (0.989–1.016)	0.224	1.009 (0.995–1.023)	0.925	1.001 (0.987–1.014)	0.204	1.009 (0.995–1.024)	0.733	1.002 (0.989–1.016)
DBP, mmHg	0.039	0.976 (0.954–0.999)	0.014	0.973 (0.952–0.994)	0.044	0.977 (0.955–0.999)	0.017	0.974 (0.953–0.995)	0.031	0.975 (0.953–0.998)	0.013	0.973 (0.952–0.994)
UA, μmol/L	0.931	1.000 (0.998–1.002)	0.015	0.997 (0.995–0.999)	0.785	1.000 (0.997–1.002)	0.010	0.997 (0.995–0.999)	0.931	1.000 (0.998–1.002)	0.020	0.997 (0.995–1.000)
HCY, μmol/L	0.696	0.996 (0.979–1.014)	0.961	1.000 (0.985–1.016)	0.775	0.997 (0.980–1.015)	0.905	1.001 (0.986–1.017)	0.675	0.996 (0.980–1.013)	0.974	1.000 (0.985–1.015)
Tbil, μmol/L	P-trend: 0.678		P-trend: <0.001									
Tertile1 (<8.6)	0.737	1.108 (0.611–2.009)	<0.001	3.502 (2.018–6.075)								
Tertile2 (8.6–12.1)	0.518	1.182 (0.712–1.961)	0.226	1.354 (0.829–2.213)								
Tertile3 (>12.1)	Reference		Reference									
Dbil, μmol/L					P-trend: 0.145		P-trend: <0.001					
Tertile 1 (<3.7)					0.161	1.544 (0.841–2.835)	<0.001	4.199 (2.380–7.410)				
Tertile 2 (3.7–5.3)					0.198	1.393 (0.841–2.307)	0.061	1.592 (0.979–2.589)				
Tertile 3 (>5.3)					Reference		Reference					
Ibil, μmol/L									P-trend: 0.751		P-trend: <0.001	
Tertile 1 (<4.6)									0.736	1.113 (0.598–2.069)	<0.001	3.149 (1.758–5.641)
Tertile 2 (4.6–7.9)									0.937	1.021 (0.605–1.724)	0.354	1.271 (0.765–2.113)
Tertile 3 (>7.9)									Reference		Reference	

None-mild: stenosis 0–49%, moderate: stenosis 50–69%, severe-occlusion group: stenosis 70–100%. ICAS, intracranial atherosclerotic stenosis; SBP, systolic blood pressure; DBP, diastolic blood pressure; UA, uric acid; HCY, homocysteine; Tbil, total bilirubin; Dbil, direct bilirubin; Ibil, indirect bilirubin. Smoking duration: group 1 (0, non-smoker), group 2 (0–15 pack-years), group 3 (15–30 pack-years) and group 4 (>30 pack-years).

TABLE 5 | Multivariate logistic regression analyses for predictors of multi-stenosis of ICAS.

	2 vs. 1		≥3 vs. 1		2 vs. 1		≥3 vs. 1		2 vs. 1		≥3 vs. 1	
	***P*-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**
Age, years	0.012	1.025 (1.006–1.045)	<0.001	1.046 (1.029–1.062)	0.017	1.024 (1.004–1.043)	<0.001	1.043 (1.027–1.059)	0.013	1.024 (1.005–1.044)	<0.001	1.044 (1.028–1.061)
Sex, (Female vs. male)	0.943	0.980 (0.565–1.700)	0.663	0.905 (0.577–1.419)	0.875	0.957 (0.553–1.656)	0.564	0.878 (0.564–1.366)	0.905	0.967 (0.558–1.676)	0.624	0.894 (0.573–1.397)
Hypertension	0.010	1.863 (1.159–2.997)	0.001	1.931 (1.319–2.827)	0.009	1.884 (1.171–3.029)	<0.001	1.962 (1.348–2.855)	0.013	1.826 (1.136–2.934)	0.001	1.930 (1.321–2.818)
Diabetes mellitus	0.729	1.101 (0.640–1.895)	0.561	1.143 (0.729–1.793)	0.847	1.055 (0.614–1.812)	0.693	1.094 (0.701–1.706)	0.795	1.075 (0.624–1.850)	0.626	1.118 (0.714–1.750)
Hyperlipidemia	0.003	0.525 (0.344–0.801)	0.135	0.769 (0.545–1.085)	0.003	0.531 (0.349–0.807)	0.118	0.764 (0.544–1.071)	0.003	0.526 (0.345–0.802)	0.160	0.782 (0.556–1.101)
Smoking duration												
Group 1 (0)	Reference		Reference		Reference		Reference		Reference		Reference	
Group 2 (>0, ≤15)	0.913	0.956 (0.427–2.142)	0.833	1.071 (0.564–2.036)	0.851	0.925 (0.414–2.070)	0.830	1.072 (0.569–2.019)	0.962	0.981 (0.438–2.194)	0.819	1.077 (0.569–2.038)
Group 3 (>15, ≤30)	0.739	1.124 (0.566–2.231)	0.421	1.259 (0.719–2.204)	0.693	1.147 (0.579–2.272)	0.357	1.296 (0.746–2.253)	0.669	1.161 (0.586–2.299)	0.324	1.322 (0.759–2.302)
Group 4 (>30)	0.925	1.029 (0.569–1861)	0.104	0.660 (0.399–1.089)	0.977	1.009 (0.560–1.818)	0.099	0.661 (0.404–1.081)	0.886	1.044 (0.578–1.888)	0.087	0.648 (0.394–1.066)
FBG, mmol/L	0.580	1.027 (0.934–1.131)	0.440	0.967 (0.887–1.054)	0.557	1.029 (0.936–1.131)	0.454	0.968 (0.890–1.054)	0.516	1.033 (0.937–1.137)	0.470	0.969 (0.890–1.055)
HbA1c, %	0.121	1.174 (0.959–1.436)	0.014	1.244 (1.045–1.481)	0.105	1.181 (0.966–1.444)	0.012	1.247 (1.050–1.482)	0.124	1.173 (0.957–1.437)	0.015	1.240 (1.042–1.475)
LDL, mmol/L	0.795	0.969 (0.765–1.228)	0.668	0.959 (0.792–1.161)	0.592	0.938 (0.741–1.186)	0.182	0.880 (0.729–1.062)	0.822	0.973 (0.766–1.236)	0.998	1.000 (0.825–1.213)
Tbil, μmol/L	P-trend: 0.101		P-trend: <0.001									
Tertile 1 (<8.4)	0.120	1.484 (0.902–2.441)	<0.001	3.540 (2.365–5.298)								
Tertile 2 (8.4-11.9)	0.003	2.052 (1.270–3.314)	<0.001	3.470 (2.312–5.208)								
Tertile 3 (>11.9)	Reference		Reference									
Dbil, μmol/L					P-trend: 0.610		P-trend: <0.001					
Tertile 1 (<3.6)					0.617	1.136 (0.689–1.874)	<0.001	2.585 (1.743–3.835)				
Tertile 2 (3.6–5.1)					0.068	1.552 (0.967–2.491)	<0.001	2.110 (1.416–3.144)				
Tertile 3 (>5.1)					Reference		Reference					
Ibil, μmol/L									P-trend: 0.136		P-trend: <0.001	
Tertile 1 (<4.5)									0.145	1.461 (0.877–2.434)	<0.001	3.552 (2.361–5.345)
Tertile 2 (4.5–6.9)									0.009	1.866 (1.167–2.986)	<0.001	2.384 (1.604–3.545)
Tertile 3 (>6.9)									Reference		Reference	

1, single-vessel stenosis; 2, two-vessel stenosis; ≥3, multiple-vessel stenosis. ICAS, intracranial atherosclerotic stenosis; FBG, fasting blood glucose; HbA1c, glycosylated hemoglobin A1c; LDL, low density lipoprotein; Tbil, total bilirubin; Dbil, direct bilirubin; Ibil, indirect bilirubin. Smoking duration: group 1 (0, non-smoker), group 2 (0–15 pack-years), group 3 (15–30 pack-years) and group 4 (>30 pack-years).

foam cell formation, leading to atherosclerosis and ultimately cerebral ischemia (37–39). Bilirubin, as the main end-product of heme metabolism, can scavenge free radicals and reduce the production of reactive oxygen species, thereby reducing the progression of atherosclerosis (40, 41). Second, a study by Vachharajani et al. showed that bilirubin could down-regulate the expression of P- and E- selectin induced by endotoxin, thus accounting for its anti-platelet aggregation effect (42). It has also been demonstrated that high concentrations of Ibil, as seen in Gilbert syndrome, could inhibit platelet aggregation induced by collagen and adenosine diphosphate (43). Third, bilirubin is negatively correlated with inflammatory markers, such as C-reactive protein, neutrophil-leukocyte ratio, and red cell distribution width, indicating that bilirubin could reduce pro-inflammatory cytokines and might also inhibit the inherent inflammatory process of atherosclerosis (44). Fourth, bilirubin could dissolve and transport cholesterol. Patients with hereditary diseases associated with elevated bilirubin levels have increased high-density lipoprotein/LDL ratio and decreased apolipoprotein B/apolipoprotein A-1 and total cholesterol levels (43). Fifth, previous research has established that bilirubin could delay the progression of atherosclerosis and improve vessel wall elasticity by down-regulating matrix metalloproteinase (45–47).

Accumulating clinical evidence proves that bilirubin has a protective impact on the carotid artery (48), cardiovascular system (9), and peripheral blood vessels (49). Nevertheless, there has been little discussion about the association between bilirubin and ICAS. In 2020, a population-based cross-sectional study pointed that serum Tbil, Dbil, and Ibil levels were negatively interrelated with aICAS, which is in accordance with our results (15). However, this paper focused on the connection between bilirubin (including Tbil, Dbil, and Ibil) and aICAS rather than sICAS. In addition, some scholars have examined the influence of serum Tbil on cerebral atherosclerosis and cerebral small vessel disease in the same subject and found that serum Tbil levels were negatively correlated with cerebral atherosclerosis (16). A study by Chen et al. revealed that Ibil concentrations increase with the exacerbation of intracranial or extracranial atherosclerotic stenosis, but decrease in patients with cranial vascular occlusion. In addition, there was no correlation between serum Tbil and Dbil levels and ICAS in their study (50). This is inconsistent with our results. A possible explanation for this might be that only 189 patients were recruited and they were divided into normal, mild (<50%), moderate (50–69%), severe (70–99%) and occlusion groups in their research. Meanwhile, the patients' gender, hypertension, smoking and alcohol consumption histories in each group were not completely matched in their study.

Hyperlipidemia is a well-established risk factor of macroangiopathy. However, our results showed that the presence of hyperlipidemia is a negative predictor for multi-stenosis of ICAS in the regression model of **Table 5**. One possible explanation for this paradox could be that the variable "hyperlipidemia" (yes/no) is rather imprecise when accounting a patient's risk for atherosclerosis. In our study, hyperlipidemia cases also included patients who had a history of hyperlipidemia or were currently receiving anti-hyperlipidemia therapy, while their present blood lipid levels may be within normal ranges. This was also supported by the unremarkable differences in LDL concentrations among the three groups (single-, two- and multiple-vessel stenosis) in patients with diagnosis of hyperlipidemia.

In this study, the bilirubin levels were significantly lower in patients with severe-occlusion or multiple-vessel stenosis, however, no clear linear dose-effect relationship between bilirubin levels and the extent of ICAS could be extrapolated from our data. The potential reasons are that the sample size of the none-mild group is relatively small and MRA was used to assess the degree of ICAS in the majority of patients, which is not the gold standard. MRA may amplify the extent of ICAS due to vascular tortuosity and various artifacts, thus causing diagnostic bias. Further research on the dose-effect-relationship between bilirubin levels and ICAS is needed.

There were some limitations in this study. First, it is a hospital-based, descriptive, retrospective cross-sectional study, and the results are unable to demonstrate a causal relationship between sICAS and serum bilirubin levels. Second, the participants were recruited from a single-center, so one should be cautious in inferring the results to other populations. Third, we only recorded the baseline levels of serum bilirubin, which might have a dynamic change during the development of ischemic stroke. Fourth, the evaluation of sICAS was based on different imaging methods, which might lead to diagnostic bias. Further studies, preferably with multicenter design, are needed to be conducted to confirm our findings.

CONCLUSION

In conclusion, we found that lower bilirubin levels might indicate severe and multiple atherosclerotic stenoses of patients with sICAS in a Chinese Han population.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Xiangya Hospital, Central South University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FY and LZ: methodology and writing—original draft preparation. ZL, YL, DL, and XF: investigation and data curation. JX: conceptualization and writing—reviewing and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.714098/full#supplementary-material

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Elevated Serum Inflammatory Markers in Subacute Stroke Are Associated With Clinical Outcome but Not Modified by Aerobic Fitness Training: Results of the Randomized Controlled *PHYS-STROKE* Trial

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Background: Inflammatory markers, such as C-reactive Protein (CRP), Interleukin-6 (IL-6), tumor necrosis factor (TNF)-alpha and fibrinogen, are upregulated following acute stroke. Studies have shown associations of these biomarkers with increased mortality, recurrent vascular risk, and poor functional outcome. It is suggested that physical fitness training may play a role in decreasing long-term inflammatory activity and supports tissue recovery.

Aim: We investigated the dynamics of selected inflammatory markers in the subacute phase following stroke and determined if fluctuations are associated with functional recovery up to 6 months. Further, we examined whether exposure to aerobic physical fitness training in the subacute phase influenced serum inflammatory markers over time.

Methods: This is an exploratory analysis of patients enrolled in the multicenter randomized-controlled *PHYS-STROKE* trial. Patients within 45 days of stroke onset were randomized to receive either four weeks of aerobic physical fitness training or relaxation sessions. Generalized estimating equation models were used to investigate the dynamics of inflammatory markers and the associations of exposure to fitness training with serum inflammatory markers over time. Multiple logistic regression models were used to explore associations between inflammatory marker levels at baseline and three months after stroke and outcome at 3- or 6-months.

Results: Irrespective of the intervention group, high sensitive CRP (hs-CRP), IL-6, and fibrinogen (but not TNF-alpha) were significantly lower at follow-up visits when compared to baseline (p all ≤ 0.01). In our cohort, exposure to aerobic physical fitness training did

not influence levels of inflammatory markers over time. In multivariate logistic regression analyses, increased baseline IL-6 and fibrinogen levels were inversely associated with worse outcome at 3 and 6 months. Increased levels of hs-CRP at 3 months after stroke were associated with impaired outcome at 6 months. We found no independent associations of TNF-alpha levels with investigated outcome parameters.

Conclusion: Serum markers of inflammation were elevated after stroke and decreased within 6 months. In our cohort, exposure to aerobic physical fitness training did not modify the dynamics of inflammatory markers over time. Elevated IL-6 and fibrinogen levels in early subacute stroke were associated with worse outcome up to 6-months after stroke.

Clinical Trial Registration: ClinicalTrials.gov, NCT01953549.

Keywords: stroke, inflammation, outcome, IL-6, crp, TNF-alpha, fibrinogen, biomarkers

INTRODUCTION

Over the past decades, progress in stroke treatment has led to lower mortality rates but at the same time, increased numbers of stroke survivors implicate an increasing need for post-stroke rehabilitation. Stroke is the third leading cause of Disability Adjusted Life Years; 40% of stroke survivors are disabled between 1 month and 5 years after the event (1). It is therefore crucial to classify ways that improve outcome after stroke.

Biomarkers have become a relevant topic in stroke research and multiple interesting markers have been identified (1). Amongst them, inflammatory biomarkers have been found to affect stroke etiology and outcome (2). In heart disease, an association of upregulated inflammation with a higher risk of recurrent events and impaired outcome is widely acknowledged (3, 4). Similar associations have been found for stroke patients (4). In the post-stroke brain, dying neuronal cells release damage signals and danger-associated molecular patterns are exposed, subsequently activating microglia and peripheral leucocytes, which both release inflammatory cytokines as a response (5). However, little is known about the long-term time course of inflammatory parameters after stroke (6–10). Most studies investigate inflammatory activity in the acute phase after stroke, only a few provide long-term measurements (9). Some studies suggest that elevated inflammatory markers are associated with the risk of recurrent events and poorer outcome after stroke and lead to poorer outcome (11, 12). Approaches of measuring inflammatory biomarkers to predict stroke outcome in the clinical setting have been discussed (1, 13–15). Their final role in stroke pathogenesis and functional recovery, however, remains uncertain (12, 16). While it has deleterious effects on the post-stroke brain, inflammation is crucial for post-stroke tissue recovery and neovascularization (17). Up to date, no effective ways to downregulate inflammatory activity have been established in the clinical setting (18, 19). Physical fitness training may downregulate inflammatory processes in the long term (20).

We investigated the dynamics of high-sensitive C-reactive protein (hs-CRP), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-alpha) and fibrinogen in the subacute phase following stroke. With our intervention group receiving a 4-week fitness training program, we explored whether aerobic physical fitness training in the subacute phase is associated with the course of inflammatory biomarkers. Furthermore, we determined if fluctuations in inflammatory markers are associated with functional outcome after stroke.

METHODS

Study Participants and Trial Design

This exploratory analysis is part of an observational biomarker sub-study nested within the multicenter, randomized controlled, endpoint blinded *PHYS-STROKE* trial. The details of the *PHYS-STROKE* study design and the main results have been published previously (21, 22). In total, 200 patients in the subacute phase of ischemic or hemorrhagic stroke (5–45 days after the index event) were recruited at 7 rehabilitation centers in and around Berlin, Germany, between 2013 and 2017. Patients were randomized 1:1 to receive either 4 weeks of treadmill-based, aerobic physical fitness training or a 4-week relaxation program in addition to standard rehabilitation therapy. For inclusion into the trial, patients had to be at least 18 years of age, present with a baseline Barthel Index (BI) of 65 or less and be capable of participating in an aerobic fitness program. In the clinical setting, the BI is a widely used tool to measure impairments in activities of daily living (23). Patients were excluded if they needed assistance in walking before stroke, were unable to sit unsupported for at least 30 s or had severe psychiatric or cardiac comorbidities. A full list of inclusion and exclusion criteria to the *PHYS-STROKE* study can be found in **Supplementary Table 1**. If patients met the eligibility criteria, written informed consent was obtained. During the intervention, all additional therapy sessions were recorded and therapists and patients were instructed to note the time of additional therapy they received outside of their participation in the *PHYS-STROKE* study (22). No standard treatment policy was set for the post-intervention period (22). Aerobic fitness training was performed using treadmill-based training to achieve a target heart rate using the formula "180 min years of age". A period of 4 weeks was chosen to ensure that intervention session took place during the in-patient stay at the rehabilitation centers (21). A subsample of patients were additionally enrolled in the accompanying observational

biomarker study *BAPTISe* ("Biomarkers And Perfusion – Training-Induced Changes After Stroke") and received additional blood biomarker measurements and MRI scans before and after the intervention (24). *PHYS-STROKE* participants with at least baseline inflammatory marker measurements were included in this analysis. Ethical approval was obtained from the institutional review board of Charité–Universitätsmedizin Berlin (*PHYS-STROKE*: EA1/138/13; *BAPTISe*: NCT01954797).

Follow-Up Visits and Outcomes

Clinical follow-up visits took place at study enrollment (baseline), after the 4-week trial intervention period (v1), as well as 3- (v2) and 6-months (v3) after stroke. Screening of patients was performed by the trial physician of the respective site. Study outcomes were assessed and documented by trained study assessors at each visit. Study assessors and the trial statistician were blinded to the intervention. Detailed descriptions of outcome parameters were reported previously (21, 22). For this project, functional outcome was analyzed using the modified Rankin Scale (mRS), BI and maximal walking speed (MWS) at 3 and 6 months after stroke. The mRS assesses the degree of handicap in the clinical setting (25).

Measurement of Inflammatory Markers

Fasting blood samples were retrieved at the recruiting rehabilitation center at each follow-up visit. Laboratory analyses of serum inflammatory markers were performed within 6 h at the "Labor Berlin" in Berlin, Germany. Blood levels of hs-CRP, IL-6 and TNF-alpha were determined using solid-phase, chemiluminescent immunometric assays (IMMULITE® 1,000, Siemens Healthcare Diagnostics). Measurement of fibrinogen levels was performed based on the Clauss method in citrated blood plasma by using the HemosIL® Q.F.A. Thrombin (Bovine) kit, Instrumentation Laboratories. Standard laboratory reference values were used to define elevated serum levels for each inflammatory marker: <3.0 mg/l for hs-CRP, <3.8 ng/l for IL-6, <8.1 pg/ml TNF-alpha, and 2–4 g/l for fibrinogen.

Statistical Analysis

Primary research questions of this project were the dynamics of inflammatory markers over a time course of 6 months and differences in inflammatory biomarkers between the two treatment groups. Secondary aims were the associations of inflammatory markers at baseline with functional outcome parameters at 3- and 6-months after stroke.

Using Generalized estimating equation (GEE)-models, we investigated the dynamics of inflammatory markers, as well as associations of levels of inflammatory markers over time with respective patient characteristics and cerebrovascular risk factors. GEE-models allow estimating associations for repeated measurements (26). We used an exchangeable correlation matrix and calculated models for each biomarker individually. For GEE-models, we used levels of inflammatory markers over time (from baseline to v3) as dependent variables. As independent variables we chose a list of clinically relevant baseline parameters and cerebrovascular risk factors: center, visits/time, age, sex, intervention group, TOAST-classification, baseline NIHSS, smoking, atrial fibrillation, diabetes mellitus, arterial hypertension, cerebro- and cardiovascular disease. Cerebrovascular disease was defined as previous stroke and/or transient ischemic attack (TIA). Cardiovascular disease was defined as history of chronic heart disease (CHD), peripheral artery disease (PAD) and/or myocardial infarction (MI).

We used multivariate logistic regression models to analyze associations between levels of inflammatory biomarkers at baseline and 3 months after stroke and functional outcome parameters up to 6 months. Functional outcome parameters (mRS, BS and MWS) were dichotomized by the median and used as dependent variables in the multivariate models. As independent variables, we used absolute levels of single inflammatory markers (as continuous variables). Furthermore, we added sex, National Institutes of Health Stroke Scale (NIHSS) scores at baseline and age, as well as arterial hypertension ($p \leq 0.1$ in univariate analyses) to our models. Multivariate models were calculated using backward selection.

For all statistical analyses we used SPSS Version 25 and 27. Non-normally distributed data were log-transformed. *P*-values $\leq$ 0.05 are considered significant. Due to the exploratory character of the study, no Bonferroni correction has been performed.

RESULTS

Baseline Characteristics of the PHYS-STROKE Cohort

Between September 2013 and April 2017, 200 participants were enrolled in the *PHYS-STROKE* study of which 105 (53%) were randomized in the fitness and 95 (48%) in the relaxation group. The mean age of the study cohort was 69 years (SD $\pm$ 12) and 119 (60%) of our participants were male. One hundred eighty-one (90.5%) participants suffered from ischemic stroke, 19 (9.5%) from hemorrhagic stroke. At study enrollment, our cohort presented with a median NIHSS of 8 (IQR: 5–12), a median mRS of 4 (IQR: 4.00–4.00), a median BI of 50 (IQR: 35–60) and a median MWS of 0.30 m/s (IQR: 0.13–0.66 m/s). No significant differences between the two groups regarding the baseline characteristics were determined. **Table 1** shows a detailed list of baseline characteristics of the study cohort.

Baseline Inflammatory Markers and Dynamics of Inflammatory Markers

The median time from stroke onset to the start of intervention was 28 days (IQR: 17–40). At baseline, the mean levels of all investigated inflammatory markers were above the clinical cut-offs. The mean serum concentrations of inflammatory markers showed a falling tendency over the 6-month observation period. The mean values of hs-CRP, IL-6, TNF-alpha, and fibrinogen over a time course of 6 months are depicted in **Figure 1**. In GEE-models, hs-CRP, IL-6, and fibrinogen were significantly lower at all follow-up visits when compared to baseline visits ($p < 0.01$ for all three inflammatory markers and all time points from v1 to v3 as compared to baseline). TNF-alpha was elevated post-stroke and declined over the study period; however, no statistically

TABLE 1 | Baseline characteristics of the *PHYS-STROKE* cohort.

	All ($n = 200$)	Fitness ($n = 105$)	Relaxation ($n = 95$)	p-value
Age (years, mean ± SD)	69 ± 12	69 ± 12	70 ±11	0.53
Male (*n*, %)	119 (59.5)	60 (57.1)	59 (62.1)	0.56
Ischemic stroke (*n*, %)	181 (90.5)	91 (86.7)	90 (94.7)	0.06
TOAST (*n*, %)				0.86
Large-artery atherosclerosis	36 (18.0)	17 (18.7)	19 (21.3)	
Cardioembolic	36 (18.0)	18 (19.8)	18 (20.2)	
Small-vessel occlusion	30 (15.0)	16 (17.6)	14 (15.7)	
Other determined etiology	7 (3.5)	3 (3.3)	4 (4.5)	
Undetermined etiology	62 (31.0)	34 (37.4)	28 (31.5)	
Two or more causes identified	9 (4.5)	3 (3.3)	6 (6.7)	
NIHSS [median (IQR)]; missing 1*	8 (7)	9 (7)	7 (6)	0.29
mRS [median (IQR)]	4 (4)	4 (4)	4 (3,4)	0.15
BI [median (IQR)]	50 (35–60)	50 (35–60)	55 (35–65)	0.19
MWS [m/s, median (IQR)]; missing 4	0.30 (0.13–0.66)	0.22 (0.13–0.56)	0.38 (0.14–0.71)	0.12
Stroke to intervention (days, mean ± SD); missing 4+	32.36 ± 40.44	28.06 ± 12.96	37.10 ± 56.88	0.77
Smoking until stroke (*n*, %); missing 1	63 (31.5)	32 (30.5)	31 (32.6)	0.94
Diabetes Mellitus (*n*, %)	63 (31.5)	32 (30.5)	31 (32.6)	0.76
Arterial hypertension (*n*, %)	166 (83.0)	86 (81.5)	80 (84.2)	0.71
Atrial fibrillation (*n*, %)	46 (23.0)	23 (21.9)	23 (24.2)	0.74
Hypercholesterolemia	80 (40.0)	43 (41.0)	37 (38.9)	0.89
Cardiovascular disease (CHD, MI, PAD; *n*, %)	34 (17.0)	13 (12.4)	21 (22.1)	0.09
CHD	29 (14.5)	11 (10.5)	18 (18.9)	
MI (> 120 days)	2 (1.0)	1 (1.0)	1 (1.1)	
PAD	10 (5.0)	4 (3.8)	6 (6.3)	
Cerebrovascular disease (stroke, TIA; *n*, %)	54 (27.0)	27 (25.7)	27 (28.4)	0.75
Stroke	38 (19.0)	20 (19.0)	18 (18.9)	
TIA	22 (11.0)	11 (10.5)	11 (11.6)	
hs-CRP at baseline (mg/l, mean ± SD); missing 5	12.04 ± 18.72	12.05 ± 18.69	12.03 ± 18.85	0.57
IL-6 at baseline (pg/ml, mean ± SD); missing 3	6.34 ± 11.72	5.70 ± 6.80	7.04 ± 15.43	0.59
TNF-alpha at baseline (pg/ml, mean ± SD); missing 7	9.34 ± 3.85	8.96 ± 3.68	9.77 ± 4.00	0.10
Fibrinogen (g/l, mean ± SD); missing 7	4.09 ± 1.15	4.12 ± 1.29	4.06 ± 0.98	0.85

TOAST, Trial of Org 10172 in Acute Stroke Treatment; NIHSS, National Institutes of Health Stroke Scale; mRS, modified Rankin Scale; BI, Barthel Index; MWS, Mean Walking Speed; CHD, Coronary heart disease; MI, Myocardial infarction; PAD, Peripheral artery disease; TIA, Transient ischemic attack; hs-CRP, high-sensitive C-reactive protein; IL-6, Interleukin 6; TNF-alpha, Tumor Necrosis Factor alpha.

**Hospital chart missing.*

+Excluded at screening.

significant decline was observed (v1: Coef. 0.01; v2: Coef. −0.001; v3: Coef. −0.02; p-values between 0.09 and 0.9).

Associations of Risk Factors With Level of Inflammatory Biomarkers Over Time

GEE models demonstrated that elevating hs-CRP (Coef. 0.01, $p = 0.03$), IL-6 (Coef. 0.01, $p < 0.01$) and TNF-alpha (Coef. 0.004, $p < 0.01$) levels over time correlated with increasing age. Additionally, levels of inflammatory markers over time were associated with prevalent cardiovascular risk factors at baseline: participants with pre-existing arterial hypertension had significantly increased levels of hs-CRP (Coef. 0.23, $p = 0.04$) and smokers had significantly elevated hs-CRP (Coef. 0.18, $p = 0.04$) and IL-6 (Coef. 0.09, $p = 0.04$) levels over the course of 6 months after stroke. Participants presenting with a history of cardiovascular, but not cerebrovascular, disease had significantly increased TNF-alpha levels over time (Coef. 0.07, $p = 0.02$), compared to participants without cardiovascular disease. Fibrinogen levels over time did not show any associations with investigated risk factors in GEE-models. **Table 2** shows an overview of the results of the GEE-models.

Effect of Physical Fitness Training on Inflammatory Markers

The dynamics of inflammatory biomarkers over the 6-month observation period separated by treatment groups are presented in **Figure 2**. In GEE-models, absolute levels of investigated inflammatory markers over a time course of 6 months did not significantly differ between the two treatment groups of *PHYS-STROKE* (hs-CRP: Coef. −0.04; IL-6: Coef. 0.004; TNF-alpha:

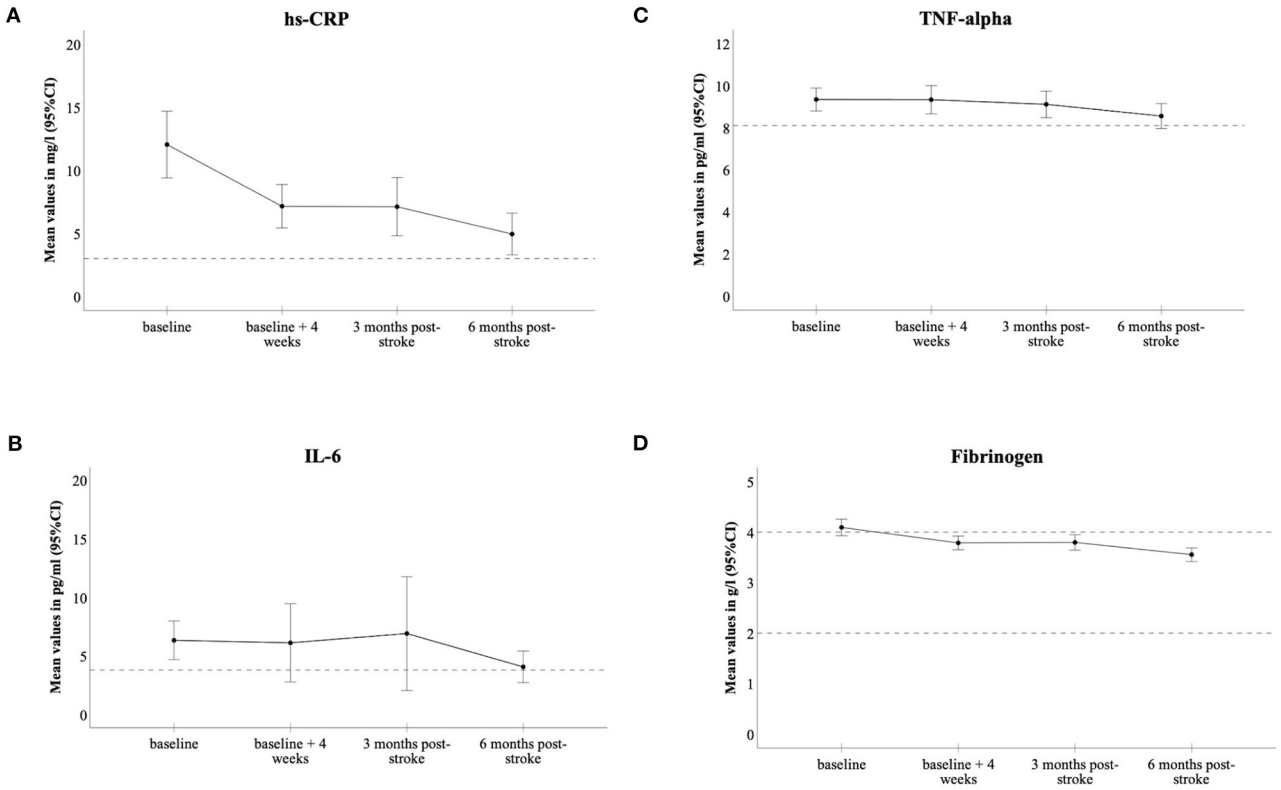

FIGURE 1 | Dynamics of inflammatory biomarkers in the PHYS-Stroke study cohort; hs-CRP **(A)**, IL-6 **(B)**, TNF-alpha **(C)**, fibrinogen **(D)**. Levels of markers are expressed as means, 95% CIs as error bars; horizontal dashed lines depict cut-off for normal ranges.

Coef. −0.02; fibrinogen: Coef. −0.03; *p*-values between 0.29 and 0.91).

Association of Inflammatory Markers With Outcome

In univariate models, increased baseline levels of all investigated markers were associated with worse functional outcome measurements at 3 or 6 months after stroke. Median splits of functional outcome parameters and the results of univariate analyses can be found in the Supplementary Material (**Supplementary Tables 3**, **4**). **Table 3** shows an overview of all results from multivariate logistic regression models. In multivariate logistic regression analyses, increased levels of IL-6 and fibrinogen at baseline were associated with a lower MWS (MWS $\leq$ 0.57 m/s) 6-months after stroke, independently of age, sex, baseline NIHSS and arterial hypertension [OR 0.34, 95% CI (0.13, 0.88); OR 0.70, 95% CI (0.51, 0.96), respectively]. Elevated baseline IL-6 and fibrinogen were also independently associated with a worse mRS (mRS $\geq$ 3) at 6 months after stroke [OR 3.02, 95% CI (1.01, 9.08); OR 1.59, 95% CI (1.08, 2.35), respectively]. In addition, elevated baseline levels of fibrinogen were associated with a lower BI (BI $\leq$ 90) 6 months post-stroke [OR 0.66, 95% CI (0.47, 0.94)]. Elevated fibrinogen at 3 months was independently associated with a worse outcome on all clinical scales at 3 and 6 months [mRS: OR 1.63, 95% CI (1.12, 2.38) and OR 1.75, 95% CI (1.14, 2.70); BI: OR 0.62, 95% CI (0.42, 0.92) and OR 0.59, 95% CI (0.34, 0.88); MWS: OR 0.65, 95% CI (0.45, 0.93) and OR 0.61, 95% CI (0.40, 0.91)]. Elevated hs-CRP at 3 months was associated with a higher mRS (mRS $\geq$ 3) at 6 months [OR 1.89, 95% CI (1.02, 3.51)] but no independent associations of baseline hs-CRP with outcome were identified. TNF-alpha did not show any independent associations with investigated outcome parameters at follow-up.

DISCUSSION

In the presented study we analyzed consecutive serum levels of inflammatory markers of patients with subacute stroke. We observed upregulated inflammatory activity after stroke and a decline over a period of 6-months. Levels of investigated inflammatory markers were not modified in magnitude or dynamic by early aerobic physical fitness training. Elevated levels of IL-6 and fibrinogen in the early subacute phase showed associations with functional impairment up to 6-months.

Inflammatory Markers

Hs-CRP is the marker that has received the most attention over the past decades when it comes to inflammatory biomarkers in stroke research. Many studies have underlined an elevation of hs-CRP after stroke and an association of higher levels with

TABLE 2 | Factors associated with levels of inflammatory biomarkers over time; GEE-models.

	hs-CRP		IL-6		TNF-alpha		Fibrinogen	
	Coef.	95% CI	Coef.	95% CI	Coef.	95% CI	Coef.	95% CI
Centre (reference: centres with early rehabilitation)	−0.14	(−0.31, 0.03)	−0.13*	(−0.21, −0.04)*	0.04	(−0.01, 0.09)	−0.2	(−0.40, 0.17)
Time (reference: baseline)								
baseline + 4 weeks	−0.20**	(−0.29, −0.10)**	−0.10**	(−0.16, −0.05)	0.01	(−0.01, 0.03)	−0.33**	(−0.50, −0.17)**
3 months post–stroke	−0.26**	(−0.36, −0.16)**	−0.12**	(−0.17, −0.06)	−0.001	(−0.023, 0.02)	−0.31**	(−0.50, −0.13)**
6 months post-stroke	−0.47**	(−0.57, −0.38)**	−0.19**	(−0.25, −0.13)	−0.02	(−0.04, 0.003)	−0.59**	(−0.76, −0.42)**
Age	0.01*	(0.00, 0.02)*	0.01**	(0.01, 0.02)	0.004*	(0.002, 0.01)**	0.01	(0.00, 0.03)
Gender (reference: male)	0.041	(−0.11, 0.19)	−0.03	(−0.11, 0.05)	0.01	(−003, 0.05)	−0.04	(−0.28, 0.20)
Intervention group (reference: relaxation)	−0.04	(−0.19, 0.10)	0.004	(−0.07, 0.08)	−0.02	(−0.06, 0.02)	−0.03	(−0.26, 0.20)
TOAST–classification (reference: undetermined etiology)								
Large–artery atherosclerosis	0.12	(−0.12, 0.35)	0.11	(−0.001, 0.22)	0.03	(−0.04, 0.09)	0.09	(−0.23, 0.41)
Cardioembolic	−0.12	(−0.50, 0.26)	0.12	(−0.20, 0.44)	0.02	(−0.07, 0.10)	−0.20	(−0.81, 0.40)
Small–vessel occlusion	−0.001	(−0.22, 0.22)	0.10	(−0.03, 0.22)	−0.004	(−0.06, 0.05)	0.35	(−0.43, 0.74)
Other determined etiology	−0.07	(−0.56, 0.42)	0.07	(−0.11, 0.25)	0.05	(−0.04, 0.13)	0.07	(−0.60, 0.74)
Two or more causes identified	−0.19	(−0.65, 0.28)	−0.03	(−0.34, 0.28)	−0.04	(−0.14, 0.07)	0.08	(−0.57, 0.71)
NIHSS at baseline	0.01	(−0.01, 0.03)	−0.002	(−0.01, 0.01)	−0.002	(−0.01, 0.003)	0.03	(−0.002, 0.05)
Smoking until stroke (reference: no smoking)	0.18*	(0.01, 0.35)*	0.09*	(0.002, 0.17)*	0.01	(−0.03, 0.06)	0.22	(−0.03, 0.48)
Atrial fibrillation (reference: no Atrial fibrillation)	0.23	(−0.14, 0.60)	−0.03	(−0.33, 0.27)	0.02	(−0.05, 0.10)	−0.04	(−0.62, 0.55)
Diabetes Mellitus (reference: no Diabetes Mellitus)	−0.10	(−0.27, 0.08)	0.04	(−0.05, 0.12)	0.04	(−0.02, 0.08)	0.09	(−0.18, 0.35)
Arterial hypertension (reference: no Arterial hypertension)	0.23*	(0.01, 0.45)*	−0.001	(−0.09, 0.09)	0.02	(−0.05, 0.08)	0.24	(−0.03, 0.51)
Cardiovascular disease (CHD, MI, PAD; reference: no Cardiovascular disease)	0.13	(−0.06, 0.32)	0.08	(−0.05, 0.20)	0.07*	(0.01, 0.12)*	0.12	(−0.21, 0.44)
Cerebrovascular disease (Stroke, TIA; reference: no Cerebrovascular disease)	0.02	(−0.17, 0.21)	0.06	(−0.05, 0.18)	−0.02	(−0.07, 0.03)	0.03	(−0.24, 0.30)

*$p < 0.05$; **$p < 0.001$.

hs-CRP, high-sensitive C-reactive protein; IL-6, Interleukin 6; TNF-alpha, Tumor Necrosis Factor alpha; TOAST, Trial of Org 10172 in Acute Stroke Treatment; NIHSS, National Institutes of Health Stroke Scale; CHD, Coronary heart disease; MI, Myocardial infarction; PAD, Peripheral artery disease; TIA, Transient ischemic attack.

an unfavorable outcome (12). We found elevated hs-CRP after stroke followed by a significant decrease up to 6-months after the event. These findings are in line with previous studies describing an elevation of a panel of inflammatory markers in stroke patients (6, 7). However, in contrast to the literature, we could not show independent associations of hs-CRP levels with functional outcome parameters. One possible explanation is that the time point of blood draws differs from previous studies. Most studies focused on CRP blood levels in the acute phase after stroke or on measuring peak levels of CRP (12, 27, 28). Moreover, post-stroke infections are associated with higher levels of CRP, especially in the early phase after stroke (29). In our study, baseline visits varied from five to 45 (median 28 days) days post-stroke and acute phase reactions (including levels of CRP) most likely have already decreased.

There is growing evidence that elevated blood levels of IL-6 in acute stroke are associated with stroke lesion volume, stroke severity, post-stroke infection as well as worse short- and long-term outcome and death (1, 13–15, 30–32). Whiteley et al. underlined that adding blood values of IL-6 and NTproBNP to a validated score including age and NIHSS could improve outcome prediction (1, 14). Nevertheless, the impact was not substantial enough for this model to be useful in clinical practice. In contrast, the Linz Stroke Study claimed that the combination of several inflammatory markers can be a useful approach to predict post-stroke outcome in a clinical setting (15). Mouse models, however, showed a substantial role of IL-6 for angiogenesis after middle cerebral artery occlusion (17). No favorable effects of increasing IL-6 levels were stated in this analysis. Instead, we provide further evidence that upregulated IL-6 after stroke is correlated with worse functional outcome. We can specify the potential advantages of measuring IL-6 for prediction of impaired outcome, yet its clinical significance needs to be further validated.

There is good evidence that fibrinogen is involved in cardiovascular disease and negatively associated with clinical outcome (33–36). Evidence about the role of fibrinogen is still inconsistent and various studies were not able to show an independent impact on outcome (37). Moreover, adding fibrinogen to a model of age and NIHSS did not improve outcome prediction in stroke patients (38). Del Zoppo et al. showed that stroke patients with initial hyperfibrinogenemia had a worse outcome up to 90 days (39). Our study contributes further evidence of understanding the dynamics of fibrinogen after stroke and the advantages of measuring fibrinogen levels

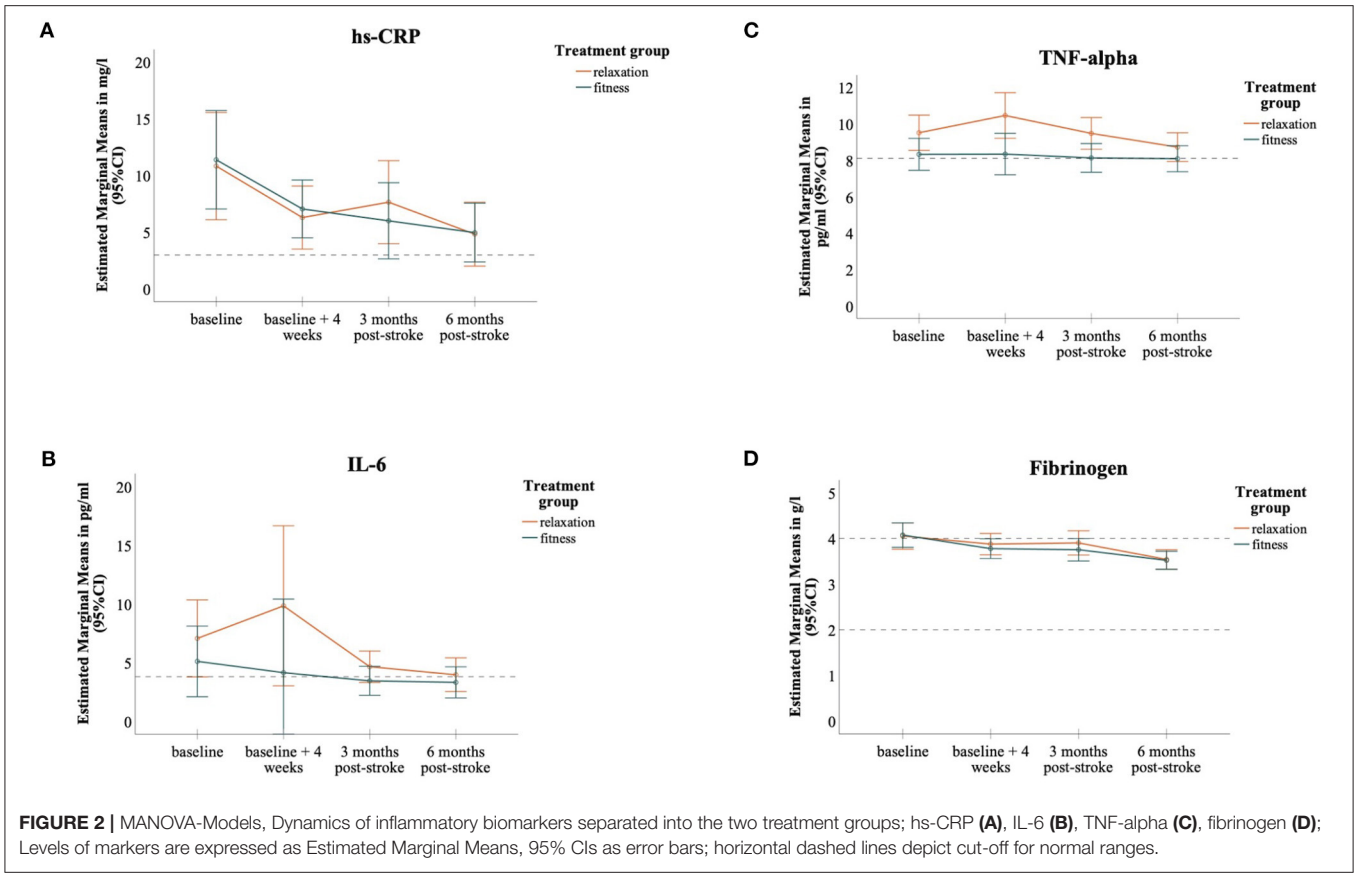

FIGURE 2 | MANOVA-Models, Dynamics of inflammatory biomarkers separated into the two treatment groups; hs-CRP **(A)**, IL-6 **(B)**, TNF-alpha **(C)**, fibrinogen **(D)**; Levels of markers are expressed as Estimated Marginal Means, 95% CIs as error bars; horizontal dashed lines depict cut-off for normal ranges.

TABLE 3 | Associations of inflammatory biomarkers with outcome parameters in multivariate logistic regression models.

	mRS		Barthel index		Mean walking speed	
	3 months after stroke	6 months after stroke	3 months after stroke	6 months after stroke	3 months after stroke	6 months after stroke
hs-CRP						
Baseline [OR, (95% CI)]	1.16 (0.68, 1.98)	1.63 (0.88, 3.02)	0.90 (0.52, 1.56)	0.62 (0.34, 1.13)	0.60 (0.35, 1.03)	0.80;(0.45, 1.43)
3 months post-stroke [OR, (95% C)]	1.27 (0.73, 2.20)	1.89 (1.02, 3.51)*	0.64 (0.36, 1.14)	0.61 (0.34, 1.12)	0.75 (0.43, 1.29)	0.65 (0.36, 1.16)
IL-6						
Baseline [OR, (95% CI)]	2.04 (0.80, 5.22)	3.02 (1.01, 9.08)*	0.64 (0.25, 1.64)	0.36 (0.13. 1.02)	0.34 (0.13, 0.88)*	0.42 (0.15, 1.17)
3 months post-stroke [OR, (95% CI)]	1.54 (0.58, 4.07)	3.02 (0.88, 10.42)	0.47 (0.16, 1.38)	0.45 (0.15, 1.38)	0.70 (0.27, 1.80)	0.39 (0.12, 1.26)
TNF-alpha						
Baseline [OR, (95% CI)]	1.25 (0.13, 11.71)	2.23 (0.17, 29.65)	0.87 (0.09, 8.69)	1.51 (0.12, 18.71)	0.40 (0.04, 3.64)	0.17 (0.02. 2.02)
3 months post-stroke [OR, (95% CI)]	2.34 (0.31, 17.59)	2.46 (0.27, 22.69)	0.49 (0.06, 3.72)	0.22 (0.02, 2.21)	0.35 (0.50, 2.55)	0.24 (0.03, 2.16)
Fibrinogen						
Baseline [OR, (95% CI)]	1.36 (1.00, 1.86)	1.59 (1.08, 2.35)*	0.90 (0.66, 1.22)	0.66 (0.47, 0.94) *	0.70 (0.51, 0.96)*	0.85 (0.61, 1.20)
3 months post-stroke [OR, (95% CI)]	1.63 (1.12, 2.38)*	1.75 (1.14, 2.70)*	0.62 (0.42, 0.92)*	0.59 (0.34, 0.88)*	0.65 (0.45, 0.93)*	0.61 (0.40, 0.91)*

*$p < 0.05$.
Adjusted for: age, sex, NIHSS baseline, arterial hypertension.
hs-CRP, high-sensitive C-reactive protein; IL-6, Interleukin 6; TNF-alpha, Tumor Necrosis Factor alpha.

in clinical stroke management. In our cohort, fibrinogen levels remained elevated in the subacute phase of stroke and associations between higher inflammatory marker levels and impaired outcome were most profound for fibrinogen (**Table 3**). In addition, in contrast to other inflammatory markers, levels of fibrinogen over time were not associated with (common) pre-existing comorbidities, which makes it a more reliable marker to measure in the clinical setting.

Studies on TNF-alpha and stroke have yielded inconsistent results. Zaremba et al. indicated that elevated levels of TNF-alpha within 24 h of stroke-onset (in cerebrospinal fluid and serum) are correlated with lower BI scores up to 2 weeks (40). In contrast to these findings, Vila et al. were unable to show a significant correlation between raised serum values of TNF-alpha on admission and early neurological worsening up until 48 h after stroke (41). Our study expanded the knowledge to the subacute phase after stroke and could demonstrate that levels of TNF-alpha showed no dynamic change over the course of 6 months after stroke. We were not able to detect a specific expression pattern of TNF-alpha in the blood of patients after stroke. Moreover, we could not find any independent associations of serum TNF-alpha levels and functional outcome.

Aerobic Fitness Training and Inflammatory Markers

Exercise potentially downregulates inflammatory activity in the long-term but only few interventional studies focus on its chronic effects on inflammatory markers. So far, study results either remain inconsistent or cannot show any favorable effects (42–44). In our cohort, fitness training did not have a substantial effect neither on levels of inflammatory biomarkers nor on clinical outcome. Furthermore, no significant effects of fitness training on primary outcome (MWS or BI) were observed in the main *PHYS-STROKE* study analyses (21). A possible reason for our lack of evidence might be that our aerobic fitness training sessions were not long and/or intense enough to initiate anti-inflammatory processes. Training-induced anti-inflammatory effects might be present only after longer, more intense training sessions. Given our study population with functional impairment post-stroke, longer training interventions could not have been performed.

Strengths and Limitations

We investigated levels of inflammatory biomarkers within the *PHYS-STROKE* study, representing a multicenter, high quality trial. To our knowledge, most studies investigating post-stroke inflammatory markers focused on blood levels in the acute phase of stroke and only a few provide serial long-term measurements. In this study, however, we collected blood samples at four defined time-points until 6 months after stroke. We were able to depict the progression of inflammatory markers more precisely, even beyond the acute phase, covering the early and late subacute as well as early chronic phase. Additionally, we were able to examine a cohort with relatively severe impairments after stroke.

Certainly, this study has some limitations. Firstly, inclusion and baseline visit vary from a period of five to 45 days after the index stroke. This results in variances in the timing of the first intervention, as well as the post-intervention visit (v1). Blood levels of inflammatory markers might therefor vary between participants, with participants included at a later time-point post-stroke showing decreased inflammatory activity. Secondly, the effect that aerobic fitness training has on inflammatory markers might differ with different time points of training-initiation, resulting in us finding no significant effects in our cohort. Thirdly, we did not adapt for possible differences in activities outside the study intervention, as well as activities post-intervention. That could have influenced our study results. Moreover, we did not exclude patients with a diagnosis of infection or other conditions that lead to an upregulation of blood inflammatory markers.

Impact and Future Research

Our study contributes to the literature on understanding the dynamics of inflammatory processes in stroke. In association with clinical outcome, the observed fluctuations can help to comprehend the role of inflammatory processes post-stroke. Including the measurement of inflammatory markers in routine stroke management can help identify patients that are at risk for long-term functional impairment. Models of outcome prediction including blood levels of inflammatory markers (single markers as well as a panel of markers) need to be validated in large, independent cohort studies. To understand the impact of fitness training on inflammation in stroke patients, researchers must first identify the ideal training program for counteracting inflammation. This research will support the important goal of identifying treatment options to blunt the neurotoxic effects of post-stroke inflammation in clinical practice. It remains unclear whether targeted anti-inflammatory treatment for stroke patients could prevent long-term stroke deterioration and this work is certainly deserving further research.

CONCLUSION

Serum levels of hs-CRP, IL-6 and fibrinogen decrease up to 6 months following stroke. Aerobic fitness training did not modify levels of inflammatory markers compared to relaxation over time. Increased IL-6 and fibrinogen levels in early and late subacute stroke are associated with worse outcome up to 6-months after stroke.

DATA AVAILABILITY STATEMENT

The raw data will be made available upon reasonable request to the authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional review board of Charité–Universitätsmedizin Berlin. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AF, MEb, AN, and TR conceived or designed the study and supervised the study. BK drafted the manuscript and contributed in statistical analysis. TR, AF, MEb, and MEn critically revised the manuscript for important intellectual content and gave final approval of the version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.713018/full#supplementary-material

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Association Between Alkaline Phosphatase and Clinical Outcomes in Patients With Spontaneous Intracerebral Hemorrhage

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Background: Spontaneous intracerebral hemorrhage (ICH) is associated with high rates of mortality and morbidity. Alkaline phosphatase (ALP) is related to increased risk of cardiovascular events and is also closely associated with adverse outcomes after ischemic or hemorrhagic stroke. However, there are limited data about the effect of ALP on clinical outcomes after ICH. Therefore, we aimed to investigate the relationship between serum ALP level and prognosis in ICH patients.

Methods: From January 2014 to September 2016, 939 patients with spontaneous ICH were enrolled in our study from 13 hospitals in Beijing. Patients were categorized into four groups based on the ALP quartiles (Q1, Q2, Q3, Q4). The main outcomes were 30-day, 90-day, and 1-year poor functional outcomes (modified Rankin Scale score of 3–6). Multivariable logistic regression and interaction analyses were performed to evaluate the relationships between ALP and clinical outcomes after ICH.

Results: In the logistic regression analysis, compared with the third quartile of ALP, the adjusted odds ratios of the Q1, Q2, and Q4 for 30-day poor functional outcome were 1.31 (0.80–2.15), 1.16 (0.71–1.89), and 2.16 (1.32–3.55). In terms of 90-day and 1-year poor functional outcomes, the risks were significantly higher in the highest quartile of ALP compared with the third quartile after adjusting the confounding factors [90-day: highest quartile OR = 1.86 (1.12–3.10); 1-year: highest quartile OR = 2.26 (1.34–3.80)]. Moreover, there was no significant interaction between ALP and variables like age or sex.

Conclusions: High ALP level (>94.8 U/L) was independently associated with 30-day, 90-day, and 1-year poor functional outcomes in ICH patients. Serum ALP might serve as a predictor for poor functional outcomes after ICH onset.

Keywords: alkaline phosphatase, spontaneous intracerebral hemorrhage, clinical outcomes, hemorrhagic, stroke

INTRODUCTION

Spontaneous intracerebral hemorrhage (ICH) is one of the most common stoke subtypes (1, 2), resulting in striking morbidity and mortality (1, 2). The age-adjusted incidence of ICH for individuals ≥55 years of age in China was generally higher than that in Western countries (3, 4). China Stroke Statistics 2019 reported that for ICH patients, in-hospital mortality was 19.5% (5). Given that there are limited therapeutic strategies in ICH patients (6), early identification and management of the risk factors for the poor prognosis are urgently needed.

Alkaline phosphatase (ALP), first discovered in 1923 (7), is an enzyme that catalyzes the hydrolysis of pyrophosphate from nucleotides and proteins (8, 9). Serum ALP has been implicated to regulate the balance between promoters and inhibitors of mineralization and enhanced vascular calcification (10, 11). Previous studies demonstrated that elevated ALP was associated with all-cause mortality and subsequent cardiovascular disease in myocardial infarction survivors, clinic populations, and general populations (9). Moreover, it has been reported that ALP could predict mortality, functional outcome, and stroke recurrence, especially in those with ischemic stroke (11–17). However, the role of ALP on clinical outcomes of ICH patients has not been fully interpreted in previous studies conducted in a single center with relatively small cohort, and the underlying mechanism remains unclear. Some suggested that vascular dysfunction and atherosclerotic process may play a crucial role in ICH clinical outcomes (16, 18).

Therefore, in this study, we aimed to investigate the association between serum ALP level and clinical outcomes in patients with spontaneous ICH.

MATERIALS AND METHODS

Study Design and Population

The study was a multicenter, prospective, observational cohort study, conducted in 13 hospitals in Beijing from January 2014 to September 2016. The study was carried out in compliance with the guidelines from the World Medical Association Declaration of Helsinki and was approved by the Institutional Review Board (IRB) of Beijing Tiantan Hospital. The ethics committee(s) approved consent by proxy in the ethics statement. Written informed consent was obtained from patients or their legally authorized representatives. Participating centers collected data and submitted it online to the coordinating center of Beijing Tiantan Hospital.

The inclusion criteria were as follows: (1) ICH patients diagnosed by the WHO standard and confirmed by CT scan, (2) first-ever acute-onset ICH, (3) age ≥ 18 years old, and (4) arriving at the hospital within 72 h after symptom onset.

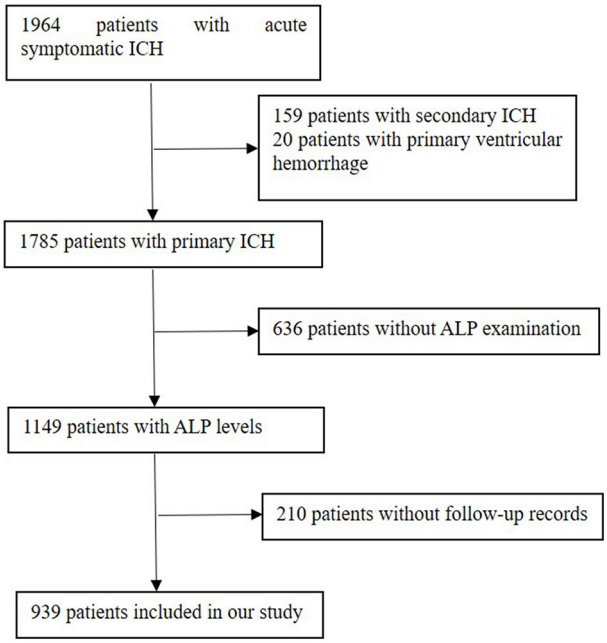

FIGURE 1 | Flow diagram of study patients. ICH, intracerebral hemorrhage; ALP, alkaline phosphatase.

The exclusion criteria were patients complicated with major comorbidities or late-stage diseases, which referred to liver failure (Child–Pugh score C), end-stage kidney disease [estimated glomerular filtration rate (eGFR) is <15 ml/min per 1.73 m^2], heart failure with reduced (≤40%) left ventricular ejection fraction, and malignant tumor with a life expectancy of <3 months. There were 1,964 ICH patients enrolled in our database. The additional exclusion criteria of this analysis were as follows: (1) patients with secondary ICH, which attribute to aneurysms, cerebrovascular malformations, cerebral venous thrombosis, trauma, tumor, or hemorrhagic transformation of ischemic stroke; (2) patients with primary ventricular hemorrhage; and (3) lack of serum ALP concentration and patients without follow-up records. As a result, 939 patients were finally enrolled in this study (**Figure 1**).

Baseline Information

Baseline information including age, sex, medical history (hypertension, diabetes mellitus, dyslipidemia, and cerebral infarction), health habits (smoking and alcohol consumption), and concomitant medications were collected using standard questionnaires. Hypertension was defined as a self-reported history, a systolic blood pressure ≥ 140 mmHg, or diastolic blood pressure ≥ 90 mmHg at baseline or taking any antihypertensive medicine. Diabetes mellitus was noted as a self-reported history, fasting blood glucose level ≥ 7.0 mmol/L at baseline, or treated with hypoglycemic drug or insulin. Dyslipidemia was defined as a self-reported history, current use of lipid-lowering agents, or

Abbreviations: ICH, intracerebral hemorrhage; ALP, alkaline phosphatase; IRB, Institutional Review Board; BMI, body mass index; GCS, Glasgow Coma Scale; NIHSS, National Institutes of Health Stroke Scale; mRS, modified Rankin Scale score; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FBG, fasting blood glucose; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; SD, standard deviation; IQR, interquartile range; CI, confidence interval; OR, odds ratio.

a total cholesterol level ≥ 6.22 mmol/L or triglyceride ≥ 2.26 mmol/L or low-density lipoprotein ≥ 4.14 mmol/L at baseline. Smoking was documented when a patient smoked at least one cigarette per day for over a year. Alcohol consumption was defined as an intake of at least 80 g of liquor a day for more than a year.

Neurological deficit was assessed using the Glasgow Coma Scale (GCS) and the National Institutes of Health Stroke Scale (NIHSS) at admission. We also recorded the hematoma location (lobar, basal ganglia, thalamus, brainstem, cerebellum) and volume (ABC/2 method) (19) based on the initial CT scan, which was completed within 24 h after admission.

ALP Testing and Other Laboratory Examinations

Blood samples were collected from an antecubital vein the next morning after overnight fasting for at least 8 h, and serum ALP levels were measured using unfrozen samples by an automated enzymatic method at each qualified study center.

Other laboratory examinations, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), fasting blood glucose (FBG), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C), were also measured during admission. eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration creatinine equation with an adjusted coefficient of 1.1 for the Asian population (20).

Follow-Up Information and Clinical Outcome

Telephone interviews were carried out for all the patients at 30 days, 90 days, and 1 year separately after ICH onset. Functional outcomes of the included patients were assessed using the modified Rankin Scale score (mRS) by trained research interviewers. A structured interview protocol was used in all telephone follow-ups, and the interviewers were blinded to the baseline characteristics and prognostic factors at each follow-up. For patients who were not reached at the first telephone interview, we made telephone follow-up interviews weekly until three missed calls were recorded; at this point, the follow-up was considered as lost. The clinical outcome was defined as 30-day, 90-day, and 1-year poor functional outcomes. Poor functional outcome, namely, death or disability, was defined as mRS of 3–6.

Statistics Analysis

The statistical analysis was conducted using the SAS software (version 9.4; SAS Institute, Cary, NC, USA). All the participants were categorized into four groups according to quartiles of serum ALP levels (Q1, Q2, Q3, Q4). Continuous variables were expressed as means ± standard deviation (SD) or medians (interquartile range, IQR) and were compared by variance analysis. Categorical variables were presented as numbers (proportions) and were compared using chi-squared tests. A multivariate logistic regression model analysis was performed to estimate the association between ALP levels and clinical outcomes. Variables associated with adverse clinical outcomes of ICH for theoretical considerations or variables based on differences in baseline characteristics between different ALP levels were finally entered in the multivariable models. These included age, sex, alcohol, hypertension, diabetes mellitus, dyslipidemia, history of cerebral infarction, prior antiplatelet use, prior anticoagulant use, body mass index (BMI), systolic blood pressure, diastolic blood pressure, GCS score, NIHSS score, location of hematoma, hematoma volume, ALT, AST, eGFR, fasting blood glucose, surgical treatment, and whether breaking into ventricle or subarachnoid. Odd ratios (ORs) and 95% confidence intervals (CIs) were calculated for each group with the third quartile as reference for ALP. All tests of significance were two-tailed, and a p-value < 0.05 was considered to be statistically significant.

RESULTS

Baseline Characteristics

Baseline characteristics by quartiles of ALP are provided in **Table 1**. The study included 939 patients, the mean age was 58.7 years old, 69.8% (655/939) were men, and 96.4% of patients were Han. The patients from the higher quartiles of ALP were more likely to have higher ALT and AST. However, no significant differences were observed in age, sex, BMI, smoking and drinking status, hypertension, diabetes mellitus, dyslipidemia, and history of cerebral infarction between different levels of ALP. In addition, there was no difference in blood pressure, GCS score, NIHSS score, location and volume of hematoma, other laboratory results, and whether underwent surgical treatment between groups. The basic characteristics between included and excluded patients in our study are shown in **Supplementary Table 1**. Patients excluded in our study tend to be younger and have lower proportions of hypertension, diabetes mellitus, dyslipidemia, and surgical treatment, as well as have relatively higher ALT, AST, and eGFR. We also discovered that in the excluded group, the GCS score was significantly lower, while the NIHSS score and hematoma volume were significantly higher.

Correlations Between ALP Levels and Clinical Outcomes

The incidences of the 30-day, 90-day, and 1-year poor functional outcomes were 61.4, 53.4, and 45.3%, respectively, among the highest ALP quartile. Compared with patients in the third quartile of ALP, the adjusted odds ratio of the highest quartile (>94.8 U/L) was 2.16 (1.32–3.55) for the 30-day poor functional outcome, 1.86 (1.12–3.10) for the 90-day poor functional outcome, and 2.26 (1.34–3.80) for 1-year poor functional outcome. However, a serum ALP in the lowest quartile (≤58.0 U/L) was not significantly correlated with 30-day, 90-day, and 1-year poor functional outcomes (**Table 2**). Subgroup analysis showed that age and sex had no interaction effect on the association between ALP levels and poor functional outcomes, although some ORs were significant in subgroups (**Table 3**).

DISCUSSION

In this prospective cohort study of patients with ICH, higher ALP levels were correlated with increased risk of 30-day, 90-day,

TABLE 1 | Baseline characteristics of the participants according to the quartiles of ALP levels.

	Overall (n = 939)	Q1 (≤58.0)	Q2 (58.0–75.0)	Q3 (75.0–94.8)	Q4 (>94.8)	p-value
Male, *n* (%)	655 (69.8)	170 (72.3)	159 (68.0)	169 (72.2)	157 (66.5)	0.40
Age (years)	58.7 ± 13.2	60.5 ± 13.6	58.4 ± 13.5	58.0 ± 13.0	57.9 ± 12.8	0.18
Ethnic Han, *n* (%)	905 (96.4)	215 (94.7)	227 (97.0)	220 (97.4)	243 (96.4)	0.44
Current smoking, *n* (%)	307 (32.7)	84 (35.7)	66 (28.2)	76 (32.5)	81 (34.3)	0.33
Alcohol, *n* (%)	352 (37.5)	82 (34.90)	81 (34.6)	96 (41.0)	93 (39.4)	0.37
Hypertension, *n* (%)	896 (95.4)	221 (94.0)	223 (95.3)	227 (97.0)	225 (95.3)	0.50
Diabetes mellitus, *n* (%)	323 (34.4)	84 (35.7)	78 (33.3)	84 (35.9)	77 (32.6)	0.83
Dyslipidemia, *n* (%)	308 (32.8)	73 (31.1)	72 (30.8)	82 (35.0)	81 (34.3)	0.67
History of cerebral infarction, *n* (%)	135 (14.4)	32 (13.6)	39 (16.7)	34 (14.5)	30 (12.7)	0.65
Prior antiplatelet use, *n* (%)	152 (16.2)	34 (14.5)	37 (15.8)	44 (18.8)	37 (15.7)	0.62
Prior anticoagulant use, *n* (%)	11 (1.2)	3 (1.3)	4 (1.7)	1 (0.4)	3 (1.3)	0.63
BMI	25.6 ± 3.6	25.5 ± 3.8	25.6 ± 3.8	26.0 ± 3.4	25.3 ± 3.3	0.16
SBP (mmHg)	163.5 (149.0–185.0)	163.5 (147.0–188.0)	163.5 (148.0–180.0)	163.3 (150.0–185.0)	163.5 (150.0–185.0)	0.80
DBP (mmHg)	97.0 (84.0–109.0)	98.0 (86.0–109.0)	96.0 (80.0–106.0)	96.0 (83.0–108.0)	97.0 (84.5–110.0)	0.33
GCS score	14 (11–15)	14 (9–15)	14 (10–15)	14 (11–15)	14 (10–15)	0.36
NIHSS score	10 (3–16)	10 (3–19)	10 (3–16)	10 (3–15)	9 (3–16)	0.55
Location of hematoma, *n* (%)						0.30
Lobar	161 (17.2)	40 (17.0)	39 (16.7)	41 (17.5)	41 (17.4)	
Deep	585 (62.3)	154 (65.5)	143 (61.1)	152 (65.0)	136 (57.6)	
Infratentorial	93 (9.9)	26 (11.1)	23 (9.8)	18 (7.7)	26 (11.0)	
Hematoma volume (ml)	14.6 (6.0–30.0)	14.6 (5.5–27.0)	15.1 (8.0–33.3)	14.6 (6.0–31.4)	13.5 (6.0–28.8)	0.31
Break into ventricle, *n* (%)	311 (33.1)	77 (32.8)	68 (29.1)	82 (35.0)	84 (35.6)	0.42
Break into subarachnoid, *n* (%)	96 (10.2)	27 (11.5)	24 (10.3)	20 (8.6)	25 (10.6)	0.76
ALT (U/L)	21.3 (14.0–31.0)	18.0 (11.0–26.0)	21.0 (14.0–29.2)	23.0 (15.0–33.0)	24.5 (17.0–35.6)	<0.0001
AST (U/L)	21.0 (17.0–27.4)	20.0 (16.0–25.0)	20.7 (16.4–26.9)	21.0 (17.0–26.0)	24.0 (19.0–31.0)	<0.0001
eGFR (ml/min)	54.4 (50.7–58.2)	53.7 (49.8–58.0)	54.6 (51.0–58.0)	54.7 (51.1–58.5)	54.7 (51.1–58.4)	0.18
FBG (mmol/L)	5.9 (5.0–7.1)	5.9 (5.0–7.2)	5.9 (5.1–7.0)	5.9 (5.1–7.2)	5.9 (4.8–6.8)	0.54
Surgical treatment, *n* (%)	200 (21.3)	55 (23.4)	45 (19.2)	55 (23.5)	45 (19.1)	0.46

Continuous variables are expressed as means ± (SD) or medians (IQR) as appropriate.
ALP, alkaline phosphatase; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; GCS, Glasgow Coma Scale; NIHSS, National Institutes of Health Stroke Scale; ALT, alanine aminotransferase; AST, aspartate aminotransferase; eGFR, estimated glomerular filtration rate; FBG, fasting blood glucose.

and 1-year poor functional outcomes, whereas no significant association was found between lower level of ALP and poor functional outcome. The association remained in the age and sex subgroups. Our findings suggest that the probability of high ALP concentration has an impact on the development and prognosis in patients with ICH.

Recently, the role of ALP has been highlighted in terms of its potential effects on various stroke outcomes. A large cohort study of patients with preserved kidney function suggested that higher serum ALP levels (>98 U/L) were connected with a 1.4-fold higher risk for 1-year all-cause mortality, stroke recurrence, composite endpoint, and poor functional outcome after stroke (12). Previous studies also suggested that a higher level of ALP was an independent prognostic factor for 3-month poor functional outcome and in-hospital mortality after acute cerebral infarction (17). Very limited research on the relationship between serum ALP and prognosis in patients with ICH has been carried out. A prospective study, including 221 ICH patients with a median follow-up period of 2.5 years, noted that a higher ALP (>97 U/L) was related to mortality rate after ICH (11). Findings from another prospective study in 639 patients indicated that elevated ALP (>96 U/L) was correlated with 30-day death and 90-day poor functional outcome after ICH (16). However, a prospective community-based study conducted in 10,754 participants with a median follow-up time of 16 years revealed that lower ALP levels were also related to increased risk of ischemic and hemorrhagic strokes (9). Different results in the literature may be explained by different study populations, sample size, and follow-up periods, and it is unclear whether both higher and lower ALP levels are correlated with poor prognosis after stroke onset. In our study, we found that higher ALP levels were associated with an increased risk of poor functional outcome in ICH patients, whereas no significant connection was observed between lower ALP levels and poor functional outcome.

Serum ALP levels might vary depending on sex (7). Shimizu et al. (9) noted that the associations of ALP levels and increased risk of stroke differed between males and females. The bone remodeling process, which could be regulated by ALP levels, increased in postmenopausal women due to estrogen deficiency (21, 22). Moreover, the study also suggested bone-type ALP

TABLE 2 | Crude and adjusted OR of ALP levels for 30-day, 90-day, and 1-year poor outcomes.

	Q1 (≤58.0)	Q2 (58.0–75.0)	Q3 (75.0–94.8)	Q4 (>94.8)
30-day poor outcome				
Events, *n* (%)	138 (58.7)	129 (55.1)	120 (51.3)	145 (61.4)
Crude OR (95% CI)	1.35 (0.94–1.95)	1.17 (0.81–1.68)	1.00 (reference)	1.51 (1.05–2.18)
Adjusted[a] OR (95% CI)	1.31 (0.80–2.15)	1.16 (0.71–1.89)	1.00 (reference)	2.16 (1.32–3.55)
90-day poor outcome				
Events, *n* (%)	121 (51.5)	115 (49.2)	107 (45.7)	126 (53.4)
Crude OR (95% CI)	1.26 (0.88–1.81)	1.15 (0.80–1.65)	1.00 (reference)	1.36 (0.95–1.95)
Adjusted[a] OR (95% CI)	1.15 (0.69–1.92)	1.14 (0.69–1.89)	1.00 (reference)	1.86 (1.12–3.10)
1-year poor outcome				
Events, *n* (%)	110 (46.8)	102 (43.6)	84 (35.9)	107 (45.3)
Crude OR (95% CI)	1.57 (1.09–2.28)	1.38 (0.95–2.00)	1.00 (reference)	1.48 (1.02–2.15)
Adjusted[a] OR (95% CI)	1.54 (0.92–2.59)	1.61 (0.96–2.70)	1.00 (reference)	2.26 (1.34–3.80)

ALP, alkaline phosphatase; OR, odd ratios; CI, confidence interval.

[a]Adjusted for age, sex, alcohol, hypertension, diabetes mellitus, dyslipidemia, history of cerebral infarction, prior antiplatelet use, prior anticoagulant use, BMI, systolic blood pressure, diastolic blood pressure, GCS score, NIHSS score, location of hematoma, hematoma volume, ALT levels, AST levels, eGFR, fasting blood glucose, surgical treatment, and whether breaking into ventricle or subarachnoid.

TABLE 3 | Multivariate-adjusted OR and 95% CI for poor outcome according to quartiles of ALP levels, stratified by age and sex.

Outcome	Subgroup	Q1 (≤58.0)	Q2 (58.0–75.0)	Q3 (75.0–94.8)	Q4 (>94.8)	*p* for interaction
30-day poor outcome	Age					0.46
	<70	1.41 (0.80–2.46)	1.31 (0.76–2.26)	1.00 (reference)	2.14 (1.24–3.69)	
	≥70	1.35 (0.38–4.81)	0.74 (0.22–2.51)	1.00 (reference)	4.20 (1.0–17.54)	
	Sex					0.23
	Male	1.29 (0.71–2.35)	0.99 (0.55–1.81)	1.00 (reference)	2.63 (1.43–4.82)	
	Female	1.59 (0.60–4.22)	1.70 (0.66–4.36)	1.00 (reference)	1.69 (0.65–4.36)	
90-day poor outcome	Age					0.71
	<70	1.10 (0.61–1.98)	1.22 (0.69–2.17)	1.00 (reference)	1.93 (1.10–3.40)	
	≥70	1.66 (0.44–6.18)	1.08 (0.30–3.94)	1.00 (reference)	2.77 (0.69–11.09)	
	Sex					0.57
	Male	1.11 (0.59–2.07)	1.13 (0.61–2.11)	1.00 (reference)	2.15 (1.15–4.00)	
	Female	1.55 (0.57–4.21)	1.34 (0.51–3.52)	1.00 (reference)	1.62 (0.61–4.25)	
1-year poor outcome	Age					0.57
	<70	1.46 (0.80–2.67)	1.37 (0.75–2.49)	1.00 (reference)	2.46 (1.37–4.41)	
	≥70	2.87 (0.75–10.96)	2.39 (0.64–8.92)	1.00 (reference)	1.60 (0.39–6.48)	
	Sex					0.08
	Male	1.26 (0.68–2.34)	1.36 (0.74–2.51)	1.00 (reference)	2.49 (1.34–4.60)	
	Female	2.61 (0.85–7.98)	2.37 (0.78–7.22)	1.00 (reference)	1.53 (0.49–4.83)	

Adjusted for age, sex, alcohol, hypertension, diabetes mellitus, dyslipidemia, history of cerebral infarction, prior antiplatelet use, prior anticoagulant use, BMI, systolic blood pressure, diastolic blood pressure, GCS score, NIHSS score, location of hematoma, hematoma volume, ALT levels, AST levels, eGFR, fasting blood glucose, surgery, and whether breaking into ventricle or subarachnoid.

ALP, alkaline phosphatase; OR, odds ratio; CI, confidence interval.

expressed in vascular smooth muscle cells (10), so we assumed that the accelerated bone remodeling process might induce vascular dysfunction and further increase the risk of stroke. However, in our subgroup analysis, we did not discover the effects of ALP on poor outcome in ICH patients stratified by sex. This discrepancy might be due to the unbalanced proportion of females: 61.8% in the former study compared with 30.2% in our study. So further studies are needed to explore the sex difference in the relationships between ALP and ICH outcomes. In addition, the relative lower percentages of females in this cohort might partially reveal that women may be less likely to suffer from ICH in China, which was consistent with research findings conducted in Asian populations (23–25). However, studies from most of the Western countries have demonstrated that the incidence of ICH was comparable for males and females (26, 27). This phenomenon could be possibly explained by the higher prevalence of uncontrolled hypertension, smoking, and alcohol consumption observed in Asian men (3, 24, 26, 28, 29).

Several different mechanisms underlying the correlation of high serum ALP with poor functional outcome after ICH

onset may be considered. First of all, ALP is often used as an early indicator of vascular calcification (15, 30, 31). Vascular calcification can further contribute to the process of atherosclerosis, which in turn results in vascular aging and increases the risk and extent of vessel rupture after stroke (7, 11, 16, 18). Moreover, studies indicated that vascular calcification especially occurring in intracranial internal carotid artery was an independent risk factor for hematoma enlargement (32) and closely related to deep cerebral microbleeds (33), which may result in poor outcomes after ICH. Secondly, serum ALP is identified as a surrogate marker of systematic inflammation (17, 34, 35). Higher levels of ALP may deteriorate the secondary cascade of injury associated with inflammation after ICH, which contribute to poor prognosis. Thirdly, ALP may be a reflection of malnutrition (12, 17). Increased ALP levels were associated with lower serum albumin levels and increased risk of infection-related mortality (11), contributing to adverse clinical outcomes in ICH patients. In addition, ALP may play an important role in principal functions of neural stem cells such as proliferation and differentiation (36–39). Neural progenitors have been found to express ALP and experiments *in vitro* have revealed that ALP knockdown reduces progenitor cell proliferation and differentiation (37, 38). Moreover, ALP has an impact on the process of axonal development (38, 40). Studies have shown that extracellular adenosine triphosphate (ATP) could repress axon growth and branching *via* the activation of P2X7 receptor (38, 40). ALP is able to hydrolyze the extracellular ATP and thus inhibit the activation of P2X7 receptor (38, 40). In this way, ALP could promote axonal growth. However, when ICH occurs, disruption of the neurons induced by hematomas and the damage of the blood–brain barrier (2) could further lead to the release of ALP expressed in neuronal membranes (41) into the plasma. Therefore, we speculate that higher serum ALP levels at the acute phase may reflect severe deficiency of ALP expressed in the brain and, thus, have an adverse effect on functions of neural stem cells and axonal development during the process of recovery after ICH, which leads to poor prognosis. The possible mechanisms underlying the relationship between lower ALP levels and poor ICH prognosis may be associated with impaired vascular homeostasis (9). The hematopoietic stem cells, which play a significant role in the maintenance of vasculature (42, 43), can be regulated by the activity of osteoblasts (44), and bone-type ALP expression, regulating the activity of osteoblasts, might further have an impact on the hematopoietic stem cells (9). Thus, lower ALP levels may be associated with impaired vascular homeostasis, which leads to unstable vasculature and a higher susceptibility to poor prognosis. Further analysis of the association between lower ALP levels and poor outcomes should be explored. These findings indicate that it is crucial to maintain optimal serum ALP levels for preventing poor prognosis after hemorrhagic stroke. Although in our study the increased risk did not reach statistical significance in the lowest quartile, we provide a new insight to better understand the association between serum ALP and poor functional outcomes in ICH patients.

There are some limitations in the present study. First, we recruited patients within 72 h after ICH onset and excluded more than 10% total number of patients lacking ALP levels and follow-up information. Given that deaths occurring within the first 48 h have been reported to be 11.3–27.5% (45–48) and patients with severe neurological deficits and larger hematoma may have not undergone the subsequent ALP testing and telephone follow-ups, our study exclude the most severe patients to some extent. Moreover, the majority of patients enrolled in our analysis were Han. Thus, the selection bias might exist and could have further limited the generalizability of our results to a population with more severe conditions and broader ethnic diversity. Secondly, around one-fifth of the patients in our research underwent surgery, which may have an impact on our analysis of the clinical outcomes. However, the Surgical Trial in Intracerebral Hemorrhage (STICH) trial and the Minimally Invasive Surgery Plus Alteplase in Intracerebral Hemorrhage Evacuation (MISTIE III) trials demonstrated that surgical treatment or minimally invasive surgery did not significantly improve the favorable functional outcome in patients with ICH (49–52). These findings need to be verified in further studies. Thirdly, the ALP isozymes were not tested in our study, so we could not evaluate which types of ALP were correlated with poor functional outcomes of ICH. Fourth, we only examined ALP levels at the acute period of ICH, but did not measure it before the onset of ICH or during hospitalization and the follow-up period. Thus, it is unknown whether the acute phase reaction accompanying ICH may influence ALP levels and whether changes of ALP levels may, in turn, have an impact on ICH outcomes. In addition, patients with elevated ALP levels had increased liver enzymes, indicating serum ALP levels could reflect hepatocellular injury (53, 54). Therefore, the presence of liver disease may influence our results. Although we excluded patients with liver failure and serum ALP levels still remained significantly associated with worse clinical outcomes after further adjustment for liver enzymes, limited information on systematic ultrasound examination for detecting subclinical liver disease could affect our results. Other potential factors such as dietary intake of vitamin D or related obstructive biliary diseases not collected in our study may have some residual confounding effect. Finally, a validation cohort might be needed to test and evaluate the efficiency of the ALP levels to predict the clinical outcomes in patients with ICH in our future analysis.

In conclusion, our results demonstrated that a high ALP level (>94.8 U/L) was independently associated with 30-day, 90-day, and 1-year poor functional outcomes in patients with ICH. Serum ALP might serve as a predictor for poor functional outcomes after ICH onset.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board (IRB) of Beijing Tiantan Hospital. The patients/participants provided their written informed consent to participate in this study. Written

informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

SL analyzed and interpreted the data and drafted the original manuscript. WW designed the research. QZ and YW analyzed and interpreted the data. AW conducted the statistical analyses. XZ designed the research and handled funding and supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.677696/full#supplementary-material

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Fibrinogen Level Combined With Platelet Count for Predicting Hemorrhagic Transformation in Acute Ischemic Stroke Patients Treated With Mechanical Thrombectomy

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A serious complication of acute ischemic stroke (AIS) after mechanical thrombectomy (MT) is hemorrhagic transformation (HT), which is potentially associated with clinical deterioration. This study examined predictors of HT following MT in AIS patients. Patients with AIS due to large artery occlusion in the anterior circulation, treated with MT and successfully recanalized (modified Thrombolysis in Cerebral Infarction score 2b/3), were studied retrospectively. HT was evaluated by computed tomography (CT) 24 h after MT and was diagnosed and classified into parenchymal hematoma (PH) and hemorrhagic infarction (HI). Multivariate logistic regression models were used to determine the risk factors for HT. Receiver operating characteristic (ROC) curve analysis was performed to determine the predictive utility of risk factors for HT. We enrolled 135 patients: 49 in the HT group and 86 in the non-HT group. The two groups differed significantly in baseline fibrinogen levels ($p = 0.003$) and platelet counts ($p = 0.006$). Multivariate logistic regression analyses showed that lower fibrinogen levels [odds ratio (OR), 0.41; 95% CI, 0.23–0.72; $p = 0.002$] and platelet counts (OR, 0.58; 95% CI, 0.33–0.99; $p = 0.048$) were independently associated with a higher risk of HT. Together, the binary variates fibrinogen and platelets well-predicted HT (area under the curve, 0.703; specificity, 77.9%; sensitivity, 55.1%). The combination of fibrinogen <2.165 g/L and platelets $<171.5 \times 10^9$/L was the strongest predictor of HT (OR, 23.17; 95% CI, 5.75–126.80; $p < 0.0001$). Our study suggests that lower baseline fibrinogen levels and platelet counts may be risk factors for HT in AIS patients following MT and reperfusion. Specifically, the combination of fibrinogen level and platelet count may predict the risk of HT after MT in these patients.

Keywords: hemorrhagic transformation, acute ischemic stroke, mechanical thrombectomy, fibrinogen, platelets

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INTRODUCTION

Acute ischemic stroke (AIS) is the leading cause of long-term disability in developed countries and the leading cause of mortality worldwide (1). Mechanical thrombectomy (MT) has become the standard of care for patients with acute intracranial large-vessel occlusion. With the DIFFUSE 3 and DAWN trials extending the time window to up to 24 h, more AIS patients are now eligible for MT (2, 3). Hemorrhagic transformation (HT), a common and severe complication, is usually associated with a poor functional outcome, or even death, after MT and has a reported incidence of up to 46.1% in clinical MT trials (4). Therefore, identifying risk factors for HT could help guide patient selection for MT, which will improve procedural safety and clinical outcomes.

Studies have examined possible risk factors for HT in the setting of MT in AIS patients. Li et al. (5) found that a higher National Institutes of Health Stroke Scale (NIHSS) score, increased systolic blood pressure, history of coronary heart disease, and use of intravenous thrombolysis or oral anti-platelet or anticoagulation drugs were associated with HT in patients undergoing MT. Moreover, ischemic volume, cerebral collateral circulation, baseline Alberta Stroke Program Early CT Score (ASPECTS), and delayed endovascular treatment are associated with an increased risk of HT after MT (6–8). However, most of these risk factors are assessed using clinical and imaging data (9) that are complex and subjective. Hence, it is necessary to identify blood biomarkers that can accurately predict HT after MT.

Studies of blood biomarkers have shown that blood glucose, lipid profiles, bilirubin, aminotransferase, alkaline phosphatase, globulin, biomarkers of disruption of the blood–brain barrier (BBB) (10), inflammation and oxidative stress (11), vasoreactivity (12), and coagulation/fibrinolysis disorder (13–15) are associated with HT in AIS patients (16). These biomarkers may reflect the pathophysiology of HT. However, most of these studies are on thrombolysis treatments, and there are limited data on blood biomarkers and the clinical relevance of HT in the setting of MT.

Platelet and fibrinogen are well-known biomarkers of the coagulation system. Fibrinogen level and platelet counts are proven to be associated with HT in AIS patients after thrombolysis (14, 17, 18). However, research about biomarkers and HT after AIS in the setting of MT is relatively less. Therefore, this study examined blood biomarkers that predict HT in AIS patients after reperfusion to provide reference data facilitating patient selection for MT.

MATERIALS AND METHODS

Study Population

This study recruited 135 AIS patients who had undergone MT and recanalization at the Department of Neurology, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine between October 2012 and May 2018. The inclusion criteria were a diagnosis of AIS confirmed by computed tomography (CT) or diffusion-weighted imaging (DWI), acute anterior circulation occlusion determined by CT angiography (CTA) or digital subtraction angiography (DSA), MT performed within 24 h of symptom onset following reperfusion graded using the modified Thrombolysis in Cerebral Infarction (mTICI) scale (2b/3) with or without intravenous thrombolysis, and routine blood tests before MT and follow-up CT 24 h after MT. Patients were excluded if they had no clinical or laboratory information or follow-up CT imaging.

Data Collection

Data on sex, age, history of stroke or transient ischemic attack (TIA), and vascular disease risk factors (e.g., smoking, drinking, hypertension, diabetes mellitus, coronary heart disease, and atrial fibrillation) were recorded. Stroke subtypes were based on the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) classification and included large-artery atherosclerosis (LAA), cardioembolism (CE), stroke of other determined cause (ODC), and stroke of undetermined etiology (SUE) (19). Blood pressure was recorded on admission. Neurological deficits were evaluated at baseline, i.e., preoperatively, using the NIHSS. Disability was assessed by the modified Rankin Scale (mRS) score at 90 days after MT. mRS $\leq$2 was considered a good clinical outcome, while mRS $\geq$3 was considered a bad clinical outcome.

Imaging parameters included the baseline ASPECTS (20). All patients underwent non-contrast CT and CTA before MT. HT was determined based on 24-h post-interventional non-contrast CT. The CT reading process was performed by two neurologists with more than 2-year working experience in our department. HT was diagnosed and classified into parenchymal hematoma (PH) and hemorrhagic infarction (HI) according to the recommendations of the European Cooperative Acute Stroke Study (ECASS)II classification (21). We had a uniform standard distinction between contrast extravasation after MT and HT. Hyperdensity meeting the following standards was considered to be HT: 1. Hyperdensity with Hounsfield units (HU) <90; 2. Hyperdensity persisting longer than 24 h and/or create mass effect with a hypoattenuation rim; 3. After 24 h, there was still visible hyperdensity on CT (22). Blood samples were obtained before treatments including MT and intravenous thrombolysis and included glucose level, routine blood counts, coagulation function, renal function, electrolytes, and myocardial enzymes. Treatments included intravenous thrombolysis and anticoagulant agents. The procedure time was recorded.

Endovascular Therapy

Patients were eligible for MT if acute occlusion of the anterior circulation was diagnosed by CTA. Some of the patients within the time window for intravenous thrombolysis, and without contraindications, were given intravenous alteplase as bridging therapy. Patients were treated directly with MT if there was any contraindication to thrombolysis or a heavy thrombus burden. After successful local anesthesia, the patient's femoral artery was punctured to determine the occlusion site. Using a coaxial catheter, the tip of a microcatheter (Rebar™ 21/27; EV3, USA) was placed at the distal end of the occluded artery under micro-guidewire guidance. Solitaire™ AB embolization stents (EV3) 4–6 mm in diameter and 15–30 mm long were selected, according to the diameter of the occluded blood vessels. The stent was introduced into the distal end of the occlusion and then released.

Then, contrast agent was injected for visualization, and negative pressure was applied to the catheter to withdraw the stent slowly and remove the thrombus. Recanalization of the main arteries was confirmed by reexamination showing thrombus removal. Evaluation of the grade of recanalization was based on mTICI grade, which is defined as: 0, No perfusion; 1, Minimal flow past the occlusion with little to no perfusion; 2a, Antegrade partial perfusion of less than half of the downstream ischemic territory; 2b, Antegrade partial perfusion of half or greater of the downstream ischemic territory; 3, Antegrade complete perfusion of the downstream ischemic territory. The mTICI scores of 2b/3 were considered recanalization after MT (23).

Statistical Analyses

Quantitative data were provided as medians and interquartile range (IQR) and categorical variables as frequencies and percentages. Differences in baseline characteristics between the non-HT and HT groups were compared using the Mann–Whitney *U*-test for quantitative data and Pearson chi-square test for categorical variables. Univariate and subsequent multivariate logistic regression analyses were performed to assess the independent risk factors for HT, with adjustment for potential confounders. Potential risk factors of HT including clinical characteristics, vascular disease history, blood biomarkers, intravenous thrombolysis, and treatment time were selected from baseline characteristics as variables in univariate logistic regression analysis. After adjusting for potential confounders, including sex, age, history of smoking and drinking, disease history of hypertension, diabetes mellitus, stroke, coronary artery disease, and atrial fibrillation, all the other factors were selected to perform multivariate logistic regression analysis. Receiver operating characteristic (ROC) curve analyses were performed to investigate the HT prediction efficacy of combinations of binary variates. The cutoff values for binary variates were determined by Youden's index (J) = (Sensitivity + specificity – 1). The point corresponding to the maximum Youden's index was considered a cutoff value. Statistical analyses were performed using SPSS software (ver. 22.0; IBM Corp., Armonk, NY, USA). *p*-values <0.05 were considered statistically significant.

RESULTS

Baseline Characteristics and Clinical Outcome of the Study Population

Ultimately, this study enrolled 135 AIS patients treated with MT following reperfusion. HT was diagnosed in 49 patients (36.3%) within 24 h after MT. **Table 1** compared the baseline characteristics and clinical outcome of the HT and non-HT groups. The patients with HT had significantly lower fibrinogen levels ($p = 0.003$) and platelet counts ($p = 0.006$), but there were no group differences in the other baseline characteristics and clinical outcome. **Table 2** compared the baseline characteristics and clinical outcome of the HI and PH subgroups. Among the 49 HT patients, 29 patients were diagnosed as HI and 20 patients as PH. There was no significant difference in baseline characteristics of the HI and PH groups. But the clinical outcome of the PH group was significantly worse than that of the HI group.

TABLE 1 | Baseline characteristics and clinical outcome of the study population.

Characteristics	Non-HT group $n = 86$	HT group $n = 49$	*p*-value
Sex (male), *n* (%)	54 (62.8%)	27 (55.1%)	0.488
Age, year, median (IQR)	65.0 (58.0–74.8)	71.0 (62.0–79.0)	0.068
Smoking, *n* (%)	29 (33.7%)	10 (20.4%)	0.149
Drinking, *n* (%)	16 (18.6%)	7 (14.3%)	0.686
Hypertension, *n* (%)	51 (59.3%)	31 (63.3%)	0.787
Diabetes mellitus, *n* (%)	25 (29.1%)	12 (24.5%)	0.709
History of stroke or TIA, *n* (%)	15 (17.4%)	5 (10.2%)	0.375
Coronary artery disease, *n* (%)	11 (12.8%)	10 (20.4%)	0.354
Atrial fibrillation, *n* (%)	35 (40.7%)	22 (44.9%)	0.769
TOAST, *n* (%)			
LAA	52 (60.5%)	27 (55.1%)	0.659
CE	25 (29.1%)	15 (30.6%)	
ODC	1 (1.2%)	0 (0%)	
SUE	8 (9.3%)	7 (14.3%)	
Baseline SBP, mmHg, median (IQR)	150 (132–167)	150 (132–162)	0.788
Baseline DBP, mmHg, median (IQR)	80.0 (71.5–90.0)	80.0 (74.5–95.0)	0.638
Baseline NIHSS, median (IQR)	15 (10–20)	14 (11–17)	0.683
Baseline ASPECTS, median (IQR)	9.0 (8.0–10.0)	8.0 (7.5–9.0)	0.074
Glucose, mmol/L, median (IQR)	8.20 (6.30–9.30)	7.50 (6.53–9.25)	0.342
Leukocyte, $\times 10^9$/L, median (IQR)	8.62 (6.15–11.20)	8.83 (7.50–10.60)	0.996
Platelet, $\times 10^9$/L, median (IQR)	207 (177–242)	169 (147–204)	**0.006***
Hemoglobin, g/L, median (IQR)	137 (125–148)	137 (131–148)	0.794
PT, s, median (IQR)	11.3 (10.7–11.9)	11.2 (10.5–12.0)	0.866
APTT, s, median (IQR)	25.6 (23.4–27.6)	25.8 (23.8–28.4)	0.427
INR, median (IQR)	0.99 (0.93–1.04)	0.97 (0.92–1.09)	0.952
Fibrinogen, g/L, median (IQR)	2.69 (2.24–3.22)	2.34 (1.88–2.84)	**0.003***
D-dimer, mg/L, median (IQR)	0.93 (0.42–2.44)	1.19 (0.51–2.74)	0.286
Creatinine, μmol/L, median (IQR)	73.0 (60.0–89.8)	77.0 (58.0–94.0)	0.682
Urea, mmol/L, median (IQR)	5.05 (4.33–6.38)	5.60 (4.70–7.10)	0.157
Uric acid, μmol/L, median (IQR)	328 (280–417)	321 (241–412)	0.414
Potassium, mmol/L, median (IQR)	3.79 (3.51–4.10)	3.80 (3.54–4.24)	0.605
Sodium, mmol/L, median (IQR)	140 (137–141)	140 (138–141)	0.579
Chloride, mmol/L, median (IQR)	104 (102–106)	104 (101–105)	0.426
Troponin, ng/ml, median (IQR)	0.01 (0–0.03)	0.01 (0.01–0.02)	0.884
BNP, pg/ml, median (IQR)	154 (26–346)	204 (61–342)	0.574
Intravenous thrombolysis, *n* (%)	33 (38.4%)	18 (36.7%)	1
Anticoagulant agent, *n* (%)	16 (18.6%)	13 (26.5%)	0.429
T1, h, median (IQR)	5.54 (4.06–7.56)	5.25 (3.83–7.25)	0.508
T2, h, median (IQR)	7.50 (5.67–9.15)	7.00 (5.58–8.58)	0.570
T2–T1, h, median (IQR)	1.83 (1.25–2.25)	1.50 (1.25–2.50)	0.570
3-month mRS (mRS ≥3), *n* (%)	52 (60.5%)	37 (75.5%)	0.113

HT, hemorrhagic transformation; IQR, interquartile range; TIA, transient ischemic attack; TOAST, Trial of Org 10172 in Acute Stroke Treatment; LAA, large-artery atherosclerosis; CE, cardioembolism; ODC, stroke of other determined cause; SUE, stroke of undetermined etiology; SBP, systolic blood pressure; DBP, diastolic blood pressure; NIHSS, National Institutes of Health Stroke Scale; ASPECTS, Alberta Stroke Program Early CT Score; PT, prothrombin time; APTT, activated partial thromboplastin time; INR, international normalized ratio; BNP, B-type natriuretic peptide; T1, time from symptom onset to puncture; T2, time from symptom onset to vascular recanalization; T2–T1, time of mechanical thrombectomy treatment; mRS, modified Rankin Scale.
Bold values indicate $p < 0.05$.*

TABLE 2 | Comparison of the baseline characteristics and clinical outcome according to the subcategorized groups of HT.

Characteristics	HI $n = 29$	PH $n = 20$	*p*-value
Sex (male), *n* (%)	19 (65.5%)	8 (40%)	0.141
Age, year, median (IQR)	70.0 (59.0–78.0)	73.5 (62.8–82.5)	0.395
Smoking, *n* (%)	8 (27.6%)	2 (10.0%)	0.254
Drinking, *n* (%)	5 (17.2%)	2 (10.0%)	0.767
Hypertension, *n* (%)	18 (62.1%)	13 (65.0%)	1
Diabetes mellitus, *n* (%)	6 (20.7%)	6 (30.0%)	0.684
History of stroke or TIA, *n* (%)	4 (13.8%)	1 (5.0%)	0.604
Coronary artery disease, *n* (%)	7 (24.1%)	3 (15.0%)	0.675
Atrial fibrillation, *n* (%)	11 (37.9%)	11 (55.0%)	0.374
TOAST, *n* (%)			
LAA	16 (55.2%)	11 (55.0%)	0.992
CE	9 (31.0%)	6 (30.0%)	
ODC	0 (0%)	0 (0%)	
SUE	4 (13.8%)	3 (15.0%)	
Baseline SBP, mmHg, median (IQR)	150 (131–160)	148 (131–170)	0.378
Baseline DBP, mmHg, median (IQR)	87.0 (78.0–95.8)	80.0 (71.0–90.0)	0.428
Baseline NIHSS, median (IQR)	13 (10–17)	14 (13–18)	0.556
Baseline ASPECTS, median (IQR)	10.0 (9.0–10.0)	9.0 (8.0–10.0)	0.122
Glucose, mmol/L, median (IQR)	7.45 (6.53–8.28)	7.95 (6.18–9.48)	0.462
Leukocyte, $\times 10^9$/L, median (IQR)	8.83 (7.84–10.40)	8.34 (5.48–11.86)	0.394
Platelet, $\times 10^9$/L, median (IQR)	171 (149–202)	167 (147–205)	0.527
Hemoglobin, g/L, median (IQR)	137 (132–148)	134 (125–150)	0.693
PT, s, median (IQR)	11.5 (10.9–12.1)	11.0 (10.0–12.3)	0.488
APTT, s, median (IQR)	25.8 (23.9–28.8)	25.9 (20.9–27.8)	0.352
INR, median (IQR)	1.00 (0.95–1.08)	0.94 (0.91–1.10)	0.379
Fibrinogen, g/L, median (IQR)	2.29 (1.86–2.78)	2.50 (2.00–2.98)	0.353
D-dimer, mg/L, median (IQR)	0.80 (0.42–2.07)	1.83 (1.02–6.42)	0.521
Creatinine, μmol/L, median (IQR)	84.0 (68.0–102.5)	64.5 (50.5–92.0)	0.394
Urea, mmol/L, median (IQR)	5.50 (4.50–7.00)	6.10 (5.00–7.65)	0.294
Uric acid, μmol/L, median (IQR)	321 (253–396)	323 (221–467)	0.380
Potassium, mmol/L, median (IQR)	3.82 (3.59–4.30)	3.66 (3.39–4.10)	0.527
Sodium, mmol/L, median (IQR)	140 (138–141)	140 (137–143)	0.255
Chloride, mmol/L, median (IQR)	103 (101–105)	105 (103–106)	0.495
Troponin, ng/ml, median (IQR)	0.01 (0–0.03)	0.01 (0.01–0.02)	0.754
BNP, pg/ml, median (IQR)	153 (31–382)	233 (130–301)	0.387
Intravenous thrombolysis, *n* (%)	9 (31.0%)	9 (45.0%)	0.487
Anticoagulant agent, *n* (%)	8 (27.6%)	5 (25.0%)	1
T1, h, median (IQR)	4.38 (3.75–7.15)	5.51 (4.08–7.31)	0.238
T2, h, median (IQR)	6.75 (5.33–8.83)	7.25 (6.21–8.69)	0.442
T2–T1, h, median (IQR)	1.60 (1.25–2.52)	1.33 (1.17–2.24)	0.466
3-month mRS (mRS ≥3), *n* (%)	17 (58.6%)	20 (100%)	**0.003***

HT, hemorrhagic transformation; PH, parenchymal hematoma; HI, hemorrhagic infarction; IQR, interquartile range; TIA, transient ischemic attack; TOAST, Trial of Org 10172 in Acute Stroke Treatment; LAA, large-artery atherosclerosis; CE, cardioembolism; ODC, stroke of other determined cause; SUE, stroke of undetermined etiology; SBP, systolic blood pressure; DBP, diastolic blood pressure; NIHSS, National Institutes of Health Stroke Scale; ASPECTS, Alberta Stroke Program Early CT Score; PT, prothrombin time; APTT, activated partial thromboplastin time; INR, international normalized ratio; BNP, B-type natriuretic peptide; T1, time from symptom onset to puncture; T2, time from symptom onset to vascular recanalization; T2–T1, time of mechanical thrombectomy treatment; mRS, modified Rankin Scale.
Bold values indicate $p < 0.05$.*

TABLE 3 | Univariate and multivariate logistic regression analyses of risk factors for HT in AIS patients with MT.

	Univariate		Multivariate	
Parameters	**cOR (95% CI)**	***p*-value**	**aOR (95% CI)**	***p*-value**
Sex (male vs. female)	1.38 (0.67–2.80)	0.381		
Age (age in years)	1.76 (1.01–3.05)	0.046		
Smoking	0.50 (0.22–1.15)	0.104		
Drinking	0.73 (0.28–1.92)	0.522		
Hypertension	0.85 (0.41–1.74)	0.650		
Diabetes mellitus	0.79 (0.36–1.76)	0.567		
History of stroke or TIA	0.54 (0.18–1.58)	0.260		
Coronary artery disease	1.75 (0.68–4.47)	0.244		
Atrial fibrillation	1.19 (0.58–2.41)	0.635		
Baseline SBP	0.95 (0.61–1.48)	0.811	0.86 (0.53–1.41)	0.559
Baseline DBP	1.12 (0.70–1.77)	0.637	1.11 (0.68–1.82)	0.671
Baseline NIHSS	0.80 (0.51–1.26)	0.337	0.81 (0.50–1.33)	0.408
Glucose	0.78 (0.51–1.19)	0.244	0.68 (0.40–1.17)	0.163
Leukocyte	1.03 (0.61–1.75)	0.910	1.14 (0.65–2.02)	0.640
Platelet	0.54 (0.32–0.90)	0.017	0.58 (0.33–0.99)	**0.048***
Hemoglobin	1.15 (0.73–1.81)	0.538	1.69 (0.92–3.11)	0.094
PT	0.97 (0.86–1.09)	0.570	0.94 (0.75–1.19)	0.620
APTT	1.11 (0.93–1.34)	0.251	1.10 (0.91–1.34)	0.333
INR	1.08 (0.77–1.51)	0.652	1.01 (0.71–1.44)	0.946
Fibrinogen	0.49 (0.29–0.82)	0.007	0.41 (0.23–0.72)	**0.002***
D-dimer	1.05 (0.94–1.17)	0.352	1.04 (0.96–1.13)	0.285
Creatinine	1.02 (0.70–1.49)	0.901	1.00 (0.66–1.51)	0.997
Urea	1.16 (0.80–1.68)	0.322	1.16 (0.80–1.68)	0.431
Uric acid	0.83 (0.50–1.39)	0.480	0.83 (0.48–1.45)	0.516
Potassium	1.16 (0.72–1.86)	0.552	1.18 (0.71–1.97)	0.515
Sodium	1.13 (0.79–1.61)	0.493	1.17 (0.81–1.70)	0.407
Chlorine	0.85 (0.57–1.27)	0.418	0.86 (0.56–1.32)	0.494
Troponin	0.94 (0.83–1.07)	0.361	0.90 (0.74–1.09)	0.289
BNP	1.02 (0.72–1.45)	0.908	0.73 (0.44–1.22)	0.229
Anticoagulant agent	1.53 (0.67–3.54)	0.315	1.45 (0.73–2.87)	0.235
Intravenous thrombolysis	0.93 (0.45–1.93)	0.850	1.52 (0.77, 2.98)	0.704
T1, h, median (IQR)	0.92 (0.63, 1.34)	0.676	0.90 (0.60, 1.35)	0.614
T2, h, median (IQR)	0.91 (0.63, 1.3)	0.596	0.89 (0.6, 1.32)	0.550
T2–T1, h, median (IQR)	0.87 (0.52, 1.47)	0.382	0.89 (0.51, 1.56)	0.691

HT, hemorrhagic transformation; AIS, acute ischemic stroke; MT, mechanical thrombectomy; cOR, crude odds ratio; aOR, adjusted odds ratio; CI, confidence interval; TIA, transient ischemic attack; SBP, systolic blood pressure; DBP, diastolic blood pressure; NIHSS, National Institutes of Health Stroke Scale; PT, prothrombin time; APTT, activated partial thromboplastin time; INR, international normalized ratio; BNP, B-type natriuretic peptide; T1, time from symptom onset to puncture; T2, time from symptom onset to vascular recanalization; T2–T1, time of mechanical thrombectomy treatment.
Bold values indicate $p < 0.05$.*

Risk Factors for Hemorrhagic Transformation in Acute Ischemic Stroke Patients Treated With Mechanical Thrombectomy

To identify the risk factors for HT in AIS patients after MT, we conducted univariate and multivariate logistic regression analyses (shown in **Table 3**). After adjusting for potential

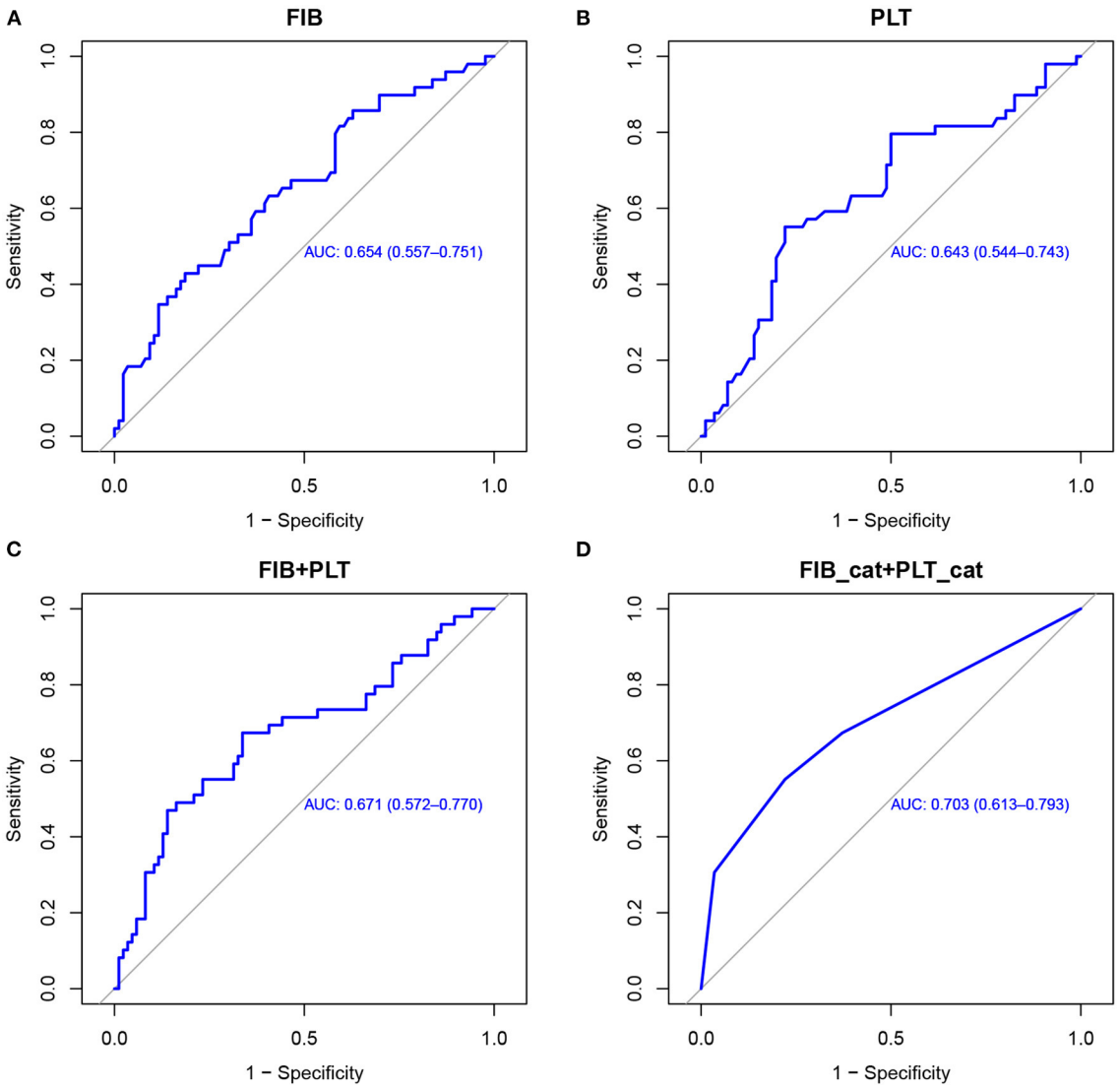

FIGURE 1 | ROC curves used to evaluate the predictive utility of risk factors for HT in AIS patients with MT. Utility of fibrinogen levels **(A)**, platelet counts **(B)**, fibrinogen combined with platelets **(C)**, and a combination of the binary variates fibrinogen (2.165 g/L) and platelets (171.5 × 10^9/L) **(D)** for predicting HT. ROC, receiver operating characteristic; HT, hemorrhagic transformation; AIS, acute ischemic stroke; MT, mechanical thrombectomy; FIB, fibrinogen; PLT, platelets.

confounders, including sex, age, and disease history, lower baseline fibrinogen level (OR, 0.41; 95% CI, 0.23–0.72; $p = 0.002$) and platelet count (OR, 0.58; 95% CI, 0.33–0.99; $p = 0.048$) were independently associated with higher odds of HT.

Predictors of Hemorrhagic Transformation in Acute Ischemic Stroke Patients Treated With Mechanical Thrombectomy

Following logistic regression analysis of HT, we performed ROC curve analysis to evaluate the predictive utility of fibrinogen and platelets. We found that the area under the curve (AUC) for fibrinogen was 0.654 (95% CI, 0.557–0.751), and the cutoff value was 2.165 g/L. The AUC for platelets was 0.643 (95% CI, 0.544–0.743), and the cutoff value was 171.5 × 10^9/L. The fibrinogen and platelet cutoffs together predicted HT with an AUC of 0.703, specificity of 77.9%, and sensitivity of 55.1%. The positive predictive value was 58.7%, and the negative predictive value was 75.3% (shown in **Figure 1**).

Figure 2 and **Table 4** showed the predictive power of the binary variates fibrinogen and platelets for HT after MT. Setting fibrinogen ≥2.165 g/L and platelets ≥171.5 × 10^9/L as references, the combination of fibrinogen ≥2.165 g/L and platelets ≥171.5 × 10^9/L predicted the lowest risk for HT, while the combination of fibrinogen <2.165 g/L and platelets <171.5 × 10^9/L was the strongest predictor of HT (OR, 23.17; 95% CI, 5.75–126.80; $p <$ 0.0001) (shown in **Figure 2**; **Table 4**).

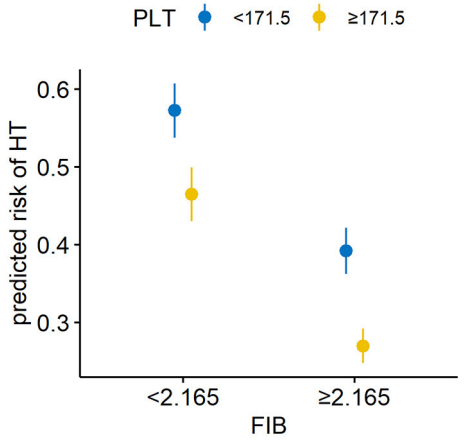

FIGURE 2 | Combined utility of the binary variates fibrinogen and platelets for predicting HT in AIS patients with MT. The combination of a fibrinogen level <2.165 g/L and platelet count <171.5 × 10^9/L was the strongest predictor of HT. HT, hemorrhagic transformation; AIS, acute ischemic stroke; MT, mechanical thrombectomy; FIB, fibrinogen; PLT, platelets.

TABLE 4 | Combined utility of the binary variates fibrinogen and platelets for predicting HT in AIS patients with MT.

Parameters	Total *N*	HT *n* (%)	OR (95% CI)	*p*-value
High fibrinogen (≥2.165 g/L)				
High platelet (≥171.5 × 10^9/L)	70	16 (22.86)	1 (Reference)	-
Low platelet (<171.5 × 10^9/L)	28	12 (42.86)	2.73 (0.97–7.83)	0.057
Low fibrinogen (<2.165 g/L)				
High platelet (≥171.5 × 10^9/L)	19	6 (31.58)	1.80 (0.53–5.71)	0.325
Low platelet (<171.5 × 10^9/L)	18	15 (83.33)	23.17 (5.75–126.80)	**<0.0001***

HT, hemorrhagic transformation; AIS, acute ischemic stroke; MT, mechanical thrombectomy; OR, odds ratio; CI, confidence interval.
Bold value indicates p < 0.05.*

DISCUSSION

In 2015, five randomized controlled trials demonstrated the superiority of MT for AIS caused by large-vessel occlusion in the anterior circulation (24). However, the incidence of HT after endovascular treatment varies from 46.0 to 49.5% (4, 8). Based on the radiological appearance, HT is roughly categorized as HI or PH (21). Symptomatic intracerebral hemorrhage (sICH) is defined as a PH2 hematoma with significant clinical deterioration caused by bleeding (25). The rate of sICH in the MT group was 7% in the DIFFUSE 3 trial and 6% in the DAWN trial (2, 3). In our study, HT occurred in 36.3% of the enrolled patients, similar to other studies (4, 8). We roughly categorize HT subtypes into HI and PH. However, we did not differentiate sICH from asymptomatic intracerebral hemorrhage or PH2 from other subtypes of HT since sICH and PH2 may have more clinical implications. Further study should identify more detailed classification to improve clinical utility.

An important finding of this study was the independent effects of decreased fibrinogen levels and platelet counts on hemorrhagic complications in AIS patients who underwent MT following reperfusion. Fibrinogen and platelets are important for hemostatic function, which indicates that there is a relationship between HT and coagulation/fibrinolysis disorder. Bleeding is more likely to occur in conjunction with a coagulation/fibrinolysis disorder; therefore, biomarkers of this system may serve as early predictors of HT incidence in AIS patients with MT (16).

Our study found that lower baseline fibrinogen levels were related to a higher risk of HT after MT. There are limited data on the relationship between fibrinogen and HT in the setting of MT, and most were obtained in the setting of thrombolysis. Wang et al. (17) found that fibrinogen <1.50 g/L was a risk factor for HT after thrombolysis. Conversely, in most studies, a higher baseline fibrinogen level was related to a higher likelihood of HT in AIS patients undergoing thrombolysis (14). Another study found that pre- and post-thrombolysis variation of fibrinogen >200 mg/dl was an independent predictor of sICH (26). Vandelli et al. (27) suggested that a decrease in post-thrombolysis fibrinogen levels of <2 g/L, or of ≥25%, was a risk factor for intracerebral hemorrhage. Yan et al. (28) found that an early decrease in fibrinogen levels was related to sICH after reperfusion therapy with thrombolysis, with or without endovascular thrombectomy. The above studies were based on thrombolysis treatments. Thrombolytic drugs themselves may affect fibrinogen levels (29). Our results indicated that lower baseline fibrinogen levels were associated with a higher risk of HT after MT, which may be attributed to the effect of thrombectomy. Lower preoperative fibrinogen plasma concentration was proven to be associated with excessive bleeding (usually defined as the amount of chest tube drainage after surgery) after cardiac operations in multiple studies (30–32). Fibrinogen is a key protein in the coagulation cascade and thus a potential biomarker for bleeding (33). Under normal pathological conditions, stable blood clot formation is the result of thrombin cleavage of fibrinogen into fibrin. As the amount of insoluble fibrin increases, factor XIII cross-links fibrin monomers forming the matrix for blood clot formation. During the coagulation cascade, fibrinogen concentration is depleted as it is cleaved into fibrin. A lower fibrinogen level may not ensure an appropriate coagulation during and after major surgical procedures (32). To our knowledge, this is the first study of the relationship between preoperative fibrinogen levels and HT in the setting of MT. The patients enrolled in our study underwent MT with or without thrombolysis. Therefore, future study should divide the patients receiving MT into thrombolysis and non-thrombolysis subgroups. It is important to obtain baseline fibrinogen levels and then monitor them during follow-up.

Our study also showed that lower baseline platelet counts were associated with a higher risk of HT after MT. The role of platelet count as a predictor of HT after thrombolysis has been investigated in studies with conflicting results. Some indicated that a lower platelet count does not significantly increase the risk

of HT (34, 35). On the contrary, a lower baseline platelet count was suggested to be associated with an increased risk of HT after thrombolysis in another study (18), and clinical guidelines from the American Heart Association/American Stroke Association published in 2018 did not recommend reperfusion therapy in patients with platelets $<100{,}000/mm^3$ (36). However, only a few studies have mentioned HT in the AIS patients treated with MT. Monch et al. (37) were the first ones to reveal that there was no clear association between initial thrombocytopenia (TP), a decline of platelet counts (DPC), and sICH. A recent study also showed no association between pre-procedural platelet count and sICH after thrombectomy (38). Thus, the results of the two studies are contradictory to ours. However, it is difficult to compare these studies due to variations in patient selection, study design, and complicated situation of thrombectomy procedure. Therefore, more research on platelet and HT in the setting of MT will be necessary. Also, considering that platelets have a high turnover during MT, therefore, follow-up of platelet count drop will be needed in future study. Platelets are small blood cells traditionally known for their role in hemostasis. Except for its hemostatic function, platelet also plays an important role in inflammation, angiogenesis, and controlled apoptosis following tissue damage. The term "platelet activation" is used to describe numerous processes, including changes in shape, upregulation of distinct surface molecules, protein synthesis from mRNA and exocytosis, and the release of granule contents. Upregulation of large receptor allows platelets to interact with almost every type of immune cell to mediate immune responses. Activated platelets also secrete a vast range of pro- and anti-inflammatory mediators. Platelets are an abundant source of growth factors, which recruit progenitor cells to the sites of injury to promote angiogenesis, specific regeneration, and tissue remodeling (39). FasL-induced apoptosis has been shown to serve as a controlled way to eliminate inflammation and prevent spreading of inflammatory responses within the eye (40). Blocking of FasL AND platelet depletion led to a decrease in apoptosis of neuronal tissue in models of stroke, suggesting that platelets are an important contributor to the prevention of uncontrolled cell death across tissues including the brain (41). Therefore, we supposed that platelet depletion may play an important role in HT in AIS undergoing MT by decreasing the "platelet activation" process, thus reducing the abilities of hemostasis, inflammatory response, angiogenesis, and tissue repair.

We also derived cutoff points for fibrinogen and platelets, which accurately predicted the risk of HT in AIS after MT. The specificity (77.9%) and sensitivity (55.1%) were best with a cutoff of <2.165 g/L for fibrinogen and $<171.5 \times 10^9$/L for platelets.

Some limitations of this study must be acknowledged. First, this was a single-center retrospective study with a relatively small sample size, and cause–effect relationships could not be inferred. Multicenter prospective studies are necessary to establish causality and provide more reliable long-term prognostic information. Second, our study roughly categorized HT subtypes into HI and PH; more detailed classification should be applied in future study. Third, due to the effect of fibrinogen depletion, further study is needed to obtain baseline and follow-up fibrinogen data. Fourth, since patients were treated between 2012 and 2018, processes and devices deployed after evidence in favor of MT published in 2015, the patients presumably contained heterogeneity. Therefore, future study will not include patients undergoing MT before 2015. Fifth, the relatively low AUC, specificity, sensitivity, and positive and negative predictive values of fibrinogen and platelet cutoffs together were detected, which may be due to the small sample size. Therefore, larger sample-size studies are needed to validate the result of the present study. Last but not least, we did not conduct further external validation of the model, which leaves this study remaining an exploratory approach on the descriptive level. The patients were enrolled independently from the two hospital districts of our department, which is under independent operation and management from the aspect of doctors, treatments, and laboratory equipment. Therefore, this study was a multicenter analysis theoretically to some extent. However, lacking external validation of the model is one of the greatest limitations of our present study. Therefore, further multicenter prospective research is needed to validate the model in this study.

CONCLUSION

Lower baseline fibrinogen levels and platelet counts are associated with HT in AIS patients with anterior circulation large-vessel occlusion after MT. The risk of HT after MT can be predicted by a fibrinogen level <2.165 g/L together with a platelet count of $<171.5 \times 10^9$/L. Platelet counts and fibrinogen levels as circulatory biomarkers are commonly checked before MT procedure in the emergency room. Therefore, it is feasible that the pre-procedural fibrinogen level and platelet counts may be used as biomarkers to identify patients with an increased risk of HT after MT in AIS patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Independent Ethics Committee of Shanghai Ninth People's Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JL conceived the study. JS designed the study and helped revise the manuscript. CL and HP analyzed all data and prepared the drafting of the article. YQ and PH contributed to the acquisition of clinical data. All authors contributed to the article and approved the submitted version.

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High Neutrophil Percentage-To-Albumin Ratio Can Predict Occurrence of Stroke-Associated Infection

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Purpose: Stroke-associated infection (SAI) is associated with adverse outcomes in patients with acute ischemic stroke (AIS). In this study, we aimed to evaluate the association between neutrophil percentage-to-albumin ratio (NPAR) and SAI occurrence in patients with AIS.

Methods: We retrospectively analyzed all AIS patients who were admitted to the Neurology ward of The Second Hospital of Tianjin Medical University from November 2018 to October 2020. The relationship between NPAR and SAI was analyzed by multivariable analysis. The receiver operating characteristic (ROC) curve was used to compare the predicted value of albumin, neutrophil percentage, neutrophil-to-lymphocyte ratio (NLR), and NPAR.

Results: We included 379 AIS patients out of which 51 (13.5%) developed SAI. The NPAR was independently associated with increased risk of SAI adjusting for confounders [adjusted odds ratio (aOR) = 10.52; 95% confidence interval (CI), 3.33–33.28; $P < 0.001$]. The optimal cutoff value of NPAR for predicting SAI incidence was 1.64, with sensitivity and specificity of 90.2 and 55.8%, respectively. The area under the curve (AUC) value of NPAR [0.771 (0.725–0.812)] was higher than that of albumin [0.640 (0.590–0.689)], neutrophil percentage [0.747 (0.700–0.790)], and NLR [0.736 (0.689–0.780)], though the statistical significance appeared only between NPAR and albumin.

Conclusions: We demonstrated that a higher NPAR could predict the occurrence of SAI. Thus, NPAR might be a more effective biomarker to predict SAI compared with albumin, neutrophil percentage, and NLR.

Keywords: stroke, infection, neutrophil, albumin, inflammation

INTRODUCTION

Stroke-associated infection (SAI) is one of the most common complications in patients with acute ischemic stroke (AIS) (1, 2). It has been reported that pneumonia and urinary tract infections are the most prevalent SAIs (3–5). SAI considerably increases disability and length of hospital stay for patients with AIS. Furthermore, it is one of the leading causes of death (6, 7). Early diagnosis and treatment are the best-known ways to reduce the SAI risk; hence, a simple and effective biomarker is needed to predict SAI.

Neutrophil percentage-to-albumin ratio (NPAR) is a novel indicator of systemic inflammation and infection. Several studies have shown that NPAR could be used as a prognostic indicator for patients with cardiogenic shock, myocardial infarction, acute kidney injury, and cancer (8–11). Additionally, it is well-known that high neutrophil percentage predicts bloodstream infection, while low albumin levels increase the susceptibility to infection complications. However, the relationship between NPAR and SAI is rarely reported to date. We aimed to explore the role of NPAR in predicting SAI in patients with AIS.

MATERIALS AND METHODS

Study Population

This was a retrospective study and approved by the Ethics Committee of The Second Hospital of Tianjin Medical University. All patients with AIS who were admitted to the Neurology ward of The Second Hospital of Tianjin Medical University from November 2018 to October 2020 were examined. The diagnosis of AIS was confirmed by computerized tomography (CT) or magnetic resonance imaging (MRI). The inclusion criteria were age $\geq$18 years and onset of symptoms $\leq$72 h. The exclusion criteria were patients having active infection on admission, patients with severe hepatic or renal diseases, those who recently underwent major trauma or surgery, cases with a history of malignant tumor, hematologic disease, or immunosuppressive treatments, or those having incomplete medical records. Active infection was defined as preexisting fever or suggestive symptoms including shortness of breath, cough, expectoration, and urinary tract symptoms.

Data Collection

We recorded all demographic and clinical data, including age, gender, previous history of stroke, hypertension, diabetes, smoking, atrial fibrillation, stroke severity on admission, occurrence of SAI, and laboratory examination values (blood cell counts and albumin levels) within 24 h of hospital admission. Stroke severity was assessed by the National Institutes of Health Stroke Scale (NIHSS). SAI was defined as any new infection occurring within 7 days of stroke onset. Signs of infection such as shortness of breath, cough, expectoration, urinary tract symptoms, and fever were examined by the treating physician as part of the daily ward round. Furthermore, diagnostic workups including laboratory and radiological examinations were also performed at the onset of symptoms. The diagnostic and treatment criteria for SAI were consistent with local clinical practice.

TABLE 1 | Baseline characteristics of AIS patients with SAI and non-SAI.

	Non-SAI (n = 328)	SAI (n = 51)	P
Age (years)	67 ± 12	75 ± 10	<0.001
Male, *n* (%)	222 (68%)	19 (37%)	<0.001
Smoke, *n* (%)	146 (45%)	13 (26%)	0.01
Hypertension, *n* (%)	274 (84%)	41 (80%)	0.577
Diabetes, *n* (%)	129 (39%)	20 (39%)	0.988
Previous stroke, *n* (%)	142 (43%)	21 (41%)	0.776
Atrial fibrillation, *n* (%)	29 (9%)	14 (28%)	<0.001
Initial NIHSS, median (IQR)	2 (1–4)	5 (1–12)	<0.001
Albumin (g/L)	40.4 ± 3.8	38.9 ± 3	0.007
Neutrophil percentage (%)	65.1 ± 9.3	73.6 ± 9.0	<0.001
NLR, median (IQR)	2.50 (1.95–3.48)	4.03 (2.79–6.02)	<0.001
NPAR, median (IQR)	1.59 (1.44–1.78)	1.91 (1.71–2.09)	<0.001

AIS, acute ischemic stroke; SAI, stroke associated infection; NIHSS, National Institute of Health Stroke Scale; NLR, neutrophil-to-lymphocyte ratio; NPAR, neutrophil percentage-to-albumin ratio; IQR, interquartile range.

Statistical Analyses

Statistical analyses were performed using SPSS 19.0 and MedCalc 15.2.2 software. For continuous variables with normal distributions, the data were presented as mean ± standard deviation (SD) and evaluated by independent samples t-test. For other distributions, median plus interquartile range (IQR) and the Mann-Whitney U-test were used. For categorical variables, the data were presented as frequency and percentage, and evaluated by chi-square test. Furthermore, multivariable logistic regression analysis was performed for the potential confounders (variables with $P < 0.05$ in the univariate results). The groups with high and low NPAR were compared by dichotomizing the cohort with median NPAR (1.64) to assess the basic characteristics of subjects with high NPAR. The area under the curve (AUC) of the receiver operating characteristic (ROC) curve was calculated to compare the predictive value of the albumin, neutrophil percentage, NLR, and NPAR. All variables with $P < 0.05$ were considered statistically significant.

RESULTS

This study enrolled 379 patients with AIS out of which 80 (21%) received intravenous thrombolysis. The mean patient age was 68 years and 241 (64%) cases were men. The median NIHSS and NPLR were 2 (1–5) and 1.64 (1.46–1.86), respectively. Out of 379 patients, 51 (13.5%) patients developed SAI, and 29 (57%) experienced fever over 38°C within 7 days after stroke onset. Of all patients with SAI, 34 patients had pneumonia, 12 patients developed urinary tract infections, and 5 patients had other infections.

A comparison between the SAI group (n = 51, 13.5%) and the non-SAI group (n = 328, 86.5%) revealed no significant difference in the history of stroke, hypertension, and diabetes mellitus. However, patients in the SAI group were found to

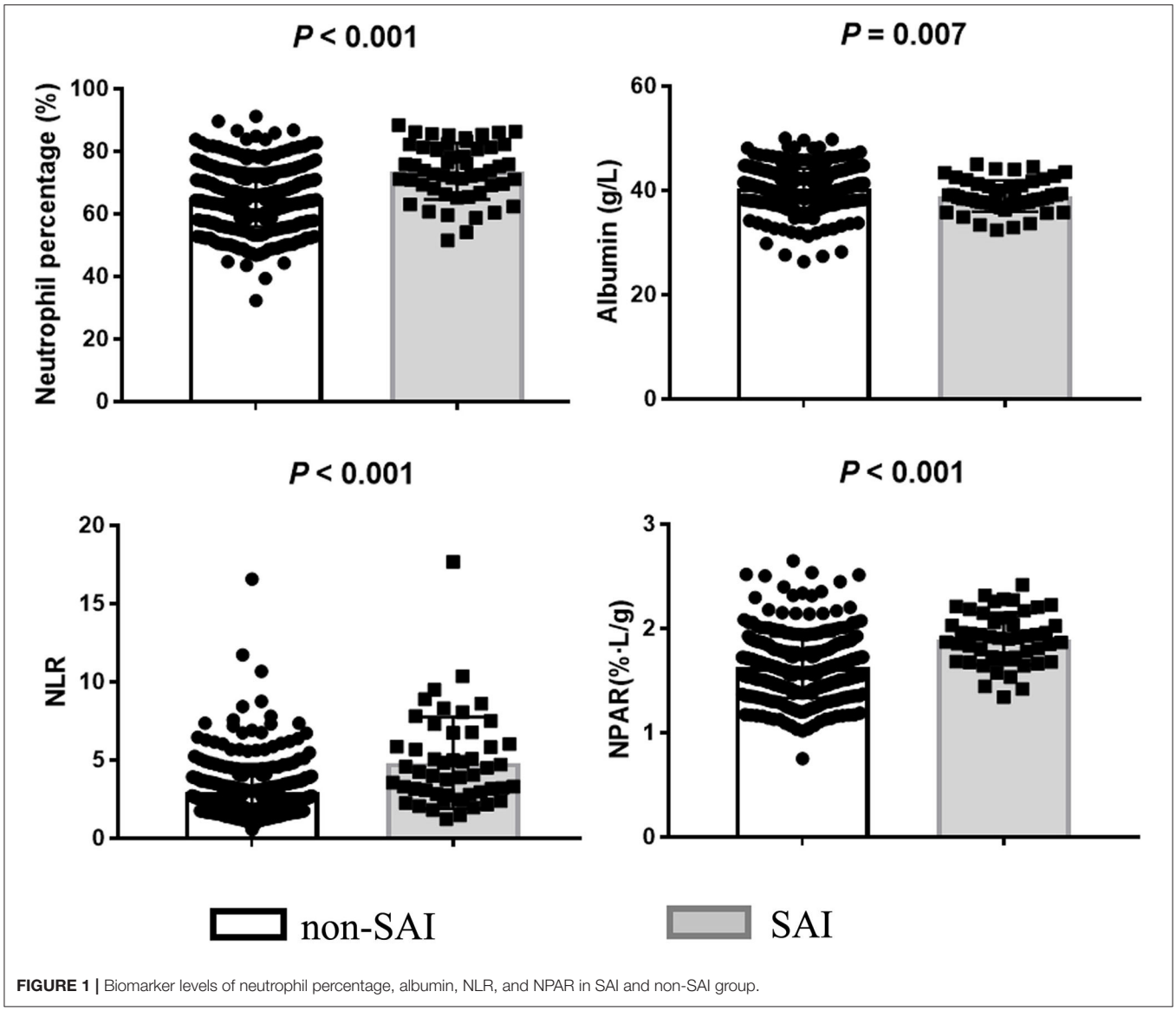

FIGURE 1 | Biomarker levels of neutrophil percentage, albumin, NLR, and NPAR in SAI and non-SAI group.

TABLE 2 | Multivariable analysis of possible predictors of SAI.

	Model 1		Model 2	
	OR (95% CI)	***P***	**OR (95% CI)**	***P***
Age	1.02 (0.99–1.05)	0.219	1.03 (1.00–1.06)	0.089
Male	0.36 (0.17–0.79)	0.01	—	—
Smoke	1.04 (0.45–2.39)	0.925	0.66 (0.31–1.39)	0.277
Initial NIHSS	1.11 (1.03–1.19)	0.005	1.11 (1.04–1.19)	0.002
Atrial fibrillation	1.43 (0.57–3.57)	0.444	1.34 (0.56–3.25)	0.511
NPAR	10.52 (3.33–33.28)	<0.001	9.78 (3.14–30.47)	<0.001

SAI, stroke associated infection; NIHSS, National Institute of Health Stroke Scale; NPAR, neutrophil percentage-to-albumin ratio; OR, odds ratio; CI, confidence interval. Model 1: adjusted for age, male, smoke, initial NIHSS, atrial fibrillation, and NPAR. Model 2: adjusted for age, smoke, initial NIHSS, atrial fibrillation, and NPAR.

be older and non-smoking and had a higher proportions of females, atrial fibrillation, and higher initial NIHSS and NLR levels compared to the non-SAI group. In addition, the SAI group presented a significantly higher level of NPAR than that of the non-SAI group [1.91 (1.71–2.09) vs. 1.59 (1.44–1.78); $P < 0.001$] (**Table 1**; **Figure 1**).

Multivariable logistic regression analysis showed that NPAR remained significant after adjusting for confounders [adjusted odds ratio (aOR) = 10.52; 95% confidence interval (CI), 3.33–33.28; $p < 0.001$]. Moreover, male patients (aOR = 0.36; 95% CI, 0.17–0.79; $P = 0.01$) and initial NIHSS (aOR = 1.11; 95% CI, 1.03–1.19; $P = 0.005$) were independently associated with the increased risk of SAI. When sex was excluded from this model, NPAR (aOR = 9.78; 95% CI, 3.14–30.47; $P < 0.001$) and NIHSS (aOR = 1.11; 95% CI, 1.04–1.19; $P = 0.002$) remained as independent predictors of SAI (**Table 2**).

A further comparison was conducted between the high and low NPAR groups. As shown in **Table 3**. The incidence of SAI was higher in the high NPAR group compared to the low NPAR group (23 vs. 3%; $P < 0.001$). In addition, patients in the high NPAR group were older and exhibited a higher proportion of female, hypertension, and atrial fibrillation, as well as higher levels of initial NIHSS and NLR than the low NPAR group.

ROC analysis showed the optimal cutoff value of NPAR for predicting SAI was 1.64 with sensitivity and specificity of 90.2 and 55.8%, respectively. While comparing the predictive power with other indicators, NPAR [0.771 (0.725–0.812)] demonstrated the highest AUC value than those of albumin [0.640 (0.590–0.689)], neutrophil percentage [0.747 (0.700–0.790)], and NLR [0.736 (0.689–0.780)]. However, the difference was significant only between NPAR and albumin ($P = 0.003$) (**Figure 2**).

TABLE 3 | Baseline characteristics of AIS patients with low and high NPARs.

	Low NPAR (NPAR ≤ 1.64)	High NPAR (NPAR ≥ 1.64)	*P*
No. of patients	189	189	
SAI, *n* (%)	6 (3%)	44 (23%)	<0.001
Age (years)	65 ± 12	72 ± 11	<0.001
Male, *n* (%)	130 (69%)	111 (59%)	0.042
Smoke, *n* (%)	92 (49%)	66 (35%)	0.007
Hypertension, *n* (%)	150 (79%)	165 (87%)	0.038
Diabetes, *n* (%)	77 (41%)	72 (38%)	0.599
Previous stroke, *n* (%)	79 (42%)	84 (44%)	0.604
Atrial fibrillation, *n* (%)	9 (5%)	34 (18%)	<0.001
Initial NIHSS, median (IQR)	2 (1–4)	3 (1–6)	0.04
Albumin, median (IQR)	41.7 (39.6–44.3)	38.6 (37.2–40.8)	<0.001
Neutrophil percentage (%)	59.8 ± 7.2	72.8 ± 7.1	<0.001
NLR, median (IQR)	2.07(1.59–2.50)	3.62(2.82–5.09)	<0.001

AIS, acute ischemic stroke; SAI, stroke associated infection; NIHSS, National Institute of Health Stroke Scale; NLR, neutrophil-to-lymphocyte ratio; NPAR, neutrophil percentage-to-albumin ratio; IQR, interquartile range.

DISCUSSION

To our knowledge, this was the first study to explore the diagnostic value of NPAR for SAI; we found that higher NPAR was significantly associated with the risk of SAI occurrence in patients with AIS. More importantly, NPAR may be a more effective biomarker for predicting SAI than albumin, neutrophil percentage, and NLR.

NPAR was a novel marker for systemic inflammation and infection. The elevated NPAR levels may be a result of increased neutrophil percentage and/or decreased albumin levels. In patients with AIS, neutrophils are the first cells to infiltrate the ischemic tissue and their count rises significantly within a few hours (12, 13). To date, a preponderance of data suggests that neutrophils promote blood-brain barrier disruption, cerebral edema, and brain injury (12) which can lead to an accelerated disruption of the homeostatic balance composed of the immune and nervous systems, thereby triggering a local inflammatory immune response and a systemic inflammatory response, including stroke-induced immunosuppression (14–16). Meanwhile, an increase in the peripheral neutrophil is associated with more severe stroke, larger infarct volume, and worse functional outcomes, all of which are strong risk factors for SAI (17–19). Currently, Deng et al. (20) demonstrated that elevated neutrophil was independently associated with increased risk of SAI in AIS patients treated with endovascular therapy. In addition, a ROC curve analysis performed by Nam et al. showed a high AUC value for neutrophils to predict stroke-associated pneumonia (SAP) (21).

As an indispensable substance in various physiological mechanisms, albumin has various functions, such as a major buffer, extracellular antioxidant, immunomodulator, detoxifier, and transporter in plasma (22, 23). Therefore, low serum albumin increases the susceptibility to infection complications. Moreover, hypoalbuminemia, a hallmark of malnutrition, can lead to impaired immune function, pulmonary edema, and fluid retention, thereby promoting the development of infection (24, 25). Additionally, Morotti et al. (26) demonstrated hypoalbuminemia was an independent predictor of pneumonia and sepsis in patients with acute intracerebral hemorrhage. Besides, Dziedzic et al. (27) found serum albumin level to be an independent predictor of SAP in patients with AIS.

Based on the above evidence, we first hypothesized and demonstrated that NPAR, the combination of albumin and neutrophils, showed a good predictive value for the occurrence of SAI. As an indicator that can be implemented even in some underdeveloped medical areas, NPAR is simple, inexpensive, and timely. More importantly, NPAR amplifies the predictive value of neutrophil percentage and albumin, especially when those two do not deviate significantly from the normal range, which often gets overlooked by clinicians. Apparently, NPAR combines the different mechanisms of neutrophil percentage and albumin levels to predict SAI occurrence and displays greater predictive power from the ROC curve. In this study, as shown in **Table 3**, the high NPAR group also exhibited higher initial NIHSS, as well as higher rates of hypertension and atrial fibrillation than the low NPAR group. As a result, patients with high NPAR might be more likely to develop SAI.

Consistent with previous studies (28, 29), our study showed 13.5% of patients diagnosed with SAI. Further, the AIS patients with high initial NIHSS were more likely to develop SAI, similar to previous studies (1, 21). However, we could not explain the reason for females having a higher risk of

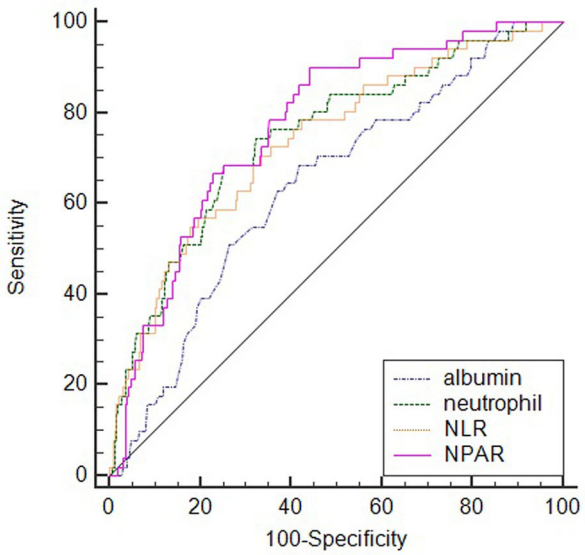

FIGURE 2 | Comparison of predictive value between NPAR and other indicators in the prediction of SAI.

developing SAI; besides, we could not rule out that this was a spurious association. When we excluded sex from the statistical model, the results of the analysis did not change significantly, and the aORs for NPAR and NIHSS remained similar to those obtained before. High sensitivity (90.2%) and relatively low specificity (55.8%) were observed when the overall diagnostic value was highest in our study. Therefore, the cutoff value of 1.64 would be more suitable for screening purposes and timely investigation of infection, while a combination of clinical symptoms, and laboratory and radiological examinations would be additionally required to initiate the treatment.

As a widely studied predictor in recent years, elevated NLR has been proved to be highly correlated with the occurrence of SAI or SAP events in patients with AIS (1, 21, 30). In this study, we confirmed this phenomenon, and the predictive value calculated from the ROC curve analysis was also consistent with previous studies (1, 30). While comparing these two indicators NPAR and NLR, we found that NPAR presented a higher predictive value, although the difference was not significant [0.771 (0.725–0.812) vs. 0.736 (0.689–0.780); $P = 0.1168$].

There were several limitations to this study. First, this was a single-center retrospective study and was therefore subjected to selection bias. Further, as there could be racial differences in susceptibility to the occurrence of hypoalbuminemia (31), our findings would need further validation for application in other places. Second, the timing of admission may lead to bias. Although we included AIS patients within 3 days of symptom onset, with nearly 60% of them within 1 day, we may still have missed some SAI events. Furthermore, the activated sympathetic nervous system after stroke mobilizes immune cells from peripheral reservoirs, resulting in an increased number of peripheral immune cells (32), and due to the differences in the time of admission after stroke, this may also poses a challenge to the comparability of NPAR among the patients. Third, due to incomplete data, we did not explore the relationship between NPAR and clinical outcomes in patients with SAI. Gong et al. (22) reported that high NPAR was significantly associated with an increased risk of death in patients with severe sepsis or septic shock. NPAR might indicate a predictive value for the prognosis of SAI. Fourth, although we have tried our best to control the bias with multivariable models, there could be still many other unknown factors influencing our results.

DATA AVAILABILITY STATEMENT

The original contributions generated for the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of The Second Hospital of Tianjin Medical University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

TW, XT, PL, and JW: collected, analyzed and interpreted the data, and draft the manuscript. HZ and YC: designed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Low Diastolic Blood Pressure Predicts Good Clinical Outcome in Patients With Cerebral Venous Thrombosis

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Background: Cerebral venous thrombosis (CVT) refers to a stroke subtype characterized by the disturbance of cerebral venous outflow caused by venous thrombosis. Previous studies have reported a range of factors that predict the prognosis of CVT. This study is aimed to find out whether systolic blood pressure (SBP) and diastolic blood pressure (DBP) are suitable as potential indicators of the severity and clinical outcome in CVT patients.

Methods: The CVT patients admitted to Xuanwu Hospital from January 2014 to December 2019 were enrolled. The severity of CVT was assessed by the National Institute of Health Stroke Scale (NIHSS) and intracranial pressure (ICP) at the time of admission. The modified Rankin score (mRS) was assessed at 6 months of follow-up.

Results: One hundred fifty-six CVT patients were enrolled with a mean age of 35.8 ± 12.8 years. A percentage of 55.8% of the CVT patients recruited were female, and 17.3% were either pregnant or in perinatal period. Headache was the most common symptom. SBP and DBP were not correlated with NIHSS at admission. Furthermore, SBP and DBP had no impact on the disturbance of consciousness, epilepsy, intracranial hemorrhage, and mental disorders. However, SBP and DBP were positively correlated with ICP at admission. SBP $>$ 129.5 mmHg and/or DBP $>$ 77.5 mmHg suggested the presence of intracranial hypertension (IH). Based on current results, SBP was not correlated with mRS at 6 months of follow-up. However, DBP was found to be positively correlated with mRS at 6 months of follow-up. DBP in CVT patients with good prognosis was significantly lower than in those with poor prognosis. DBP $>$ 79.5 mmHg was identified as a cutoff value to predict a poor clinical outcome. A higher mRS and a higher rate of poor clinical outcome were found in CVT patients with SBP $>$ 146 mmHg or DBP $>$ 79.5 mmHg compared to those with SBP $\leq$ 146 mmHg or DBP $\leq$ 79.5 mmHg.

Conclusion: SBP $>$ 129.5 mmHg and DBP $>$ 77.5 mmHg suggested the presence of IH in CVT patients. DBP $>$ 79.5 mmHg predicted a poor clinical outcome.

Keywords: blood pressure, intracranial hypertension, severity, prognosis, cerebral venous thrombosis (CVT)

INTRODUCTION

Cerebral venous thrombosis (CVT) refers to a stroke subtype characterized by the cerebral venous outflow disturbance caused by venous thrombosis (1). In recent studies, it has been demonstrated that the incidence of CVT is potentially higher than expected, reaching 1.32–1.57/100,000 people annually (2). The clinical manifestations of CVT are highly unpredictable (2). In severe CVT cases, patients suffered from disturbance of consciousness, new onset of epilepsy, intracranial hemorrhage, and mental disorders (3, 4).

It is reported that 13.4% of CVT patients had poor prognosis (5). Also, a series of studies have revealed that the following factors predicted poor clinical outcome: male, older age, an increase in National Institutes of Health Stroke Scale (NIHSS) $\geq$3 at admission, bilateral motor signs, malignancy, central nervous system infection, coma, mental disorders, deep cerebral venous thrombosis, hemorrhagic infarcts, and midline shift (5–7).

It is widely recognized that high blood pressure (BP) is a significant risk factor for arterial stroke and an indicator of poor prognosis in arterial stroke patients (8). However, there are few studies focusing on the role of blood pressure in CVT. The aim of this study is to investigate whether systolic blood pressure (SBP) and diastolic blood pressure (DBP) can be applied as potential indicators of severity and clinical outcome for CVT patients.

METHODS

Subject Recruitment

The CVT patients admitted to Xuanwu Hospital from January 2014 to December 2019 were enrolled. This prospective study was approved by the Xuanwu Hospital ethnics committee. The inclusion criterion was CVT confirmed by magnetic resonance venography (MRV), computed tomography venography (CTV), digital subtraction angiography (DSA), or high resolution-magnetic resonance imaging (HR-MRI).

Intracranial pressure (ICP) was detected by measuring CSF pressure with lumbar puncture. An ICP $>$ 250 mmH_2O was considered as intracranial hypertension (IH) (9). In order to evaluate the clinical outcome of CVT, the modified Rankin score (mRS) was assessed at 6 months of follow-up. mRS $\leq$ 2 and mRS $>$ 2 were treated as good clinical outcome and poor clinical outcome, respectively (10).

Statistical Analysis

SPSS Version 16.0 (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. The continuous data following Gaussian distribution were expressed as mean $\pm$ standard deviation and analyzed with independent *t*-test, while categorical data were expressed as a number (percentage) and processed using chi-square test. Pearson correlation coefficient and linear regression were used to predict the correlation between continuous variables. Logistic regression models were constructed using enter method. The cutoff points were calculated using receiver operating characteristic (ROC) curves. $p < 0.05$ was considered statistically significant.

TABLE 1 | Baseline demographic features and clinical characteristics of patients with CVT.

Characteristics	Enrolled subjects (*N* = 156)
Age (years)	35.8 ± 12.8
Female	87 (55.8%)
Pregnancy or perinatal period	27 (17.3%)
Co-morbid disease	
Hypertension	21 (13.5%)
Diabetes mellitus	7 (4.5%)
Hyperlipidemia	7 (4.5%)
Deep venous thrombosis	4 (2.6%)
Pulmonary embolism	4 (2.6%)
Systemic infectious disease	3 (1.9%)
Smoking	17 (10.9%)
Alcohol	17 (10.9%)
Clinical manifestations	
Headache	140 (89.7%)
Visual impairment	31 (19.9%)
Tinnitus	9 (5.8%)
Disturbance of consciousness	33 (21.2%)
New-onset epilepsy	54 (34.6%)
Limb weakness	52 (33.3%)
Blood pressure at admission	
Systolic pressure (mmHg)	122.8 ± 16.7
Diastolic pressure (mmHg)	77.6 ± 10.8

RESULTS

Baseline Demographic Features of CVT Patients

One hundred fifty-six CVT patients were recruited in this study, with a mean age of 35.8 $\pm$ 12.8 years. There were 87 (55.8%) female patients, and 27 (17.3%) patients were either pregnant or in the perinatal period. Twenty-one (13.5%) patients had hypertension, 7 (4.5%) had diabetes mellitus, 7 (4.5%) had hyperlipidemia, 4 (2.6%) had deep venous thrombosis, 4 (2.6%) had pulmonary embolism, and 3 (1.9%) had systemic infectious diseases. Seventeen (10.9%) patients had a history of smoking, and 17 (10.9%) patients had a history of alcohol. Headache (89.7%) was the most frequent symptom, followed by new-onset epilepsy (34.6%), limb weakness (33.3%), disturbance of consciousness (21.2%), visual impairment (19.9%), and tinnitus (5.8%). The average SBP and DBP were 122.8 $\pm$ 16.7 and 77.6 $\pm$ 10.8 mmHg, respectively (**Table 1**).

Blood Pressure Correlates With the Severity of CVT

NIHSS, clinical presentations, and ICP at admission were used to assess the severity of CVT. Our results showed that SBP and DBP were not correlated with NIHSS ($p = 0.107$; $p = 0.135$, **Figures 1A,B**). Results from the present study also revealed that SBP and DBP had no impact on the disturbance of consciousness, epilepsy, intracranial hemorrhage, and mental disorders (**Table 2**). However, both SBP and DBP were positively

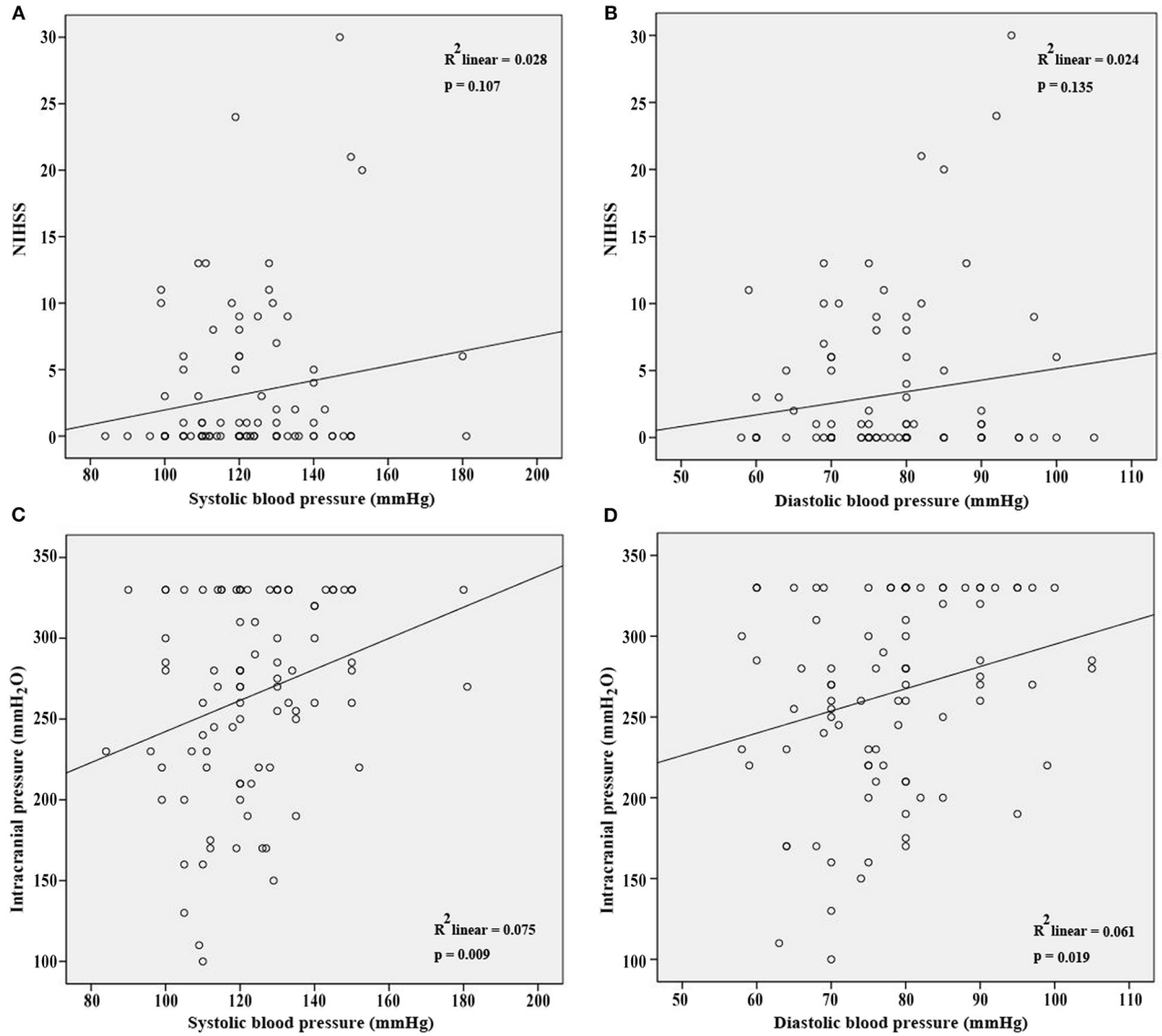

FIGURE 1 | SBP and DBP were not correlated with NIHSS **(A,B)** at admission. However, SBP and DBP were positively correlated with ICP at admission **(C,D)**.

correlated with ICP ($p = 0.009$; $p = 0.019$, **Figures 1C,D**). The ROC curve was used to identify the cutoff values of SBP and DBP. SBP > 129.5 mmHg (AUC = 0.7288, $p = 0.0004$, **Figure 2A**) and DBP > 77.5 mmHg (AUC = 0.6471, $p = 0.024$, **Figure 2B**) were identified as indicators for IH in CVT patients.

DBP Predicts the Prognosis of CVT

Subsequently, we aimed to evaluate the impact of BP on the prognosis of CVT. SBP was not correlated with mRS at 6 months of follow-up after adjusting for ICP and DBP ($p = 0.784$; $p = 0.159$, **Table 3**). Notably, DBP was positively correlated with mRS at 6 months of follow-up after adjusting for ICP and SBP ($p = 0.039$, **Table 3**).

No significant difference was found in SBP between the CVT patients with good prognosis and those with poor prognosis ($p = 0.222$, **Figure 3A**). DBP in the subjects with good prognosis was significantly lower compared with those with poor prognosis ($p = 0.046$, **Figure 3B**). SBP of 146 mmHg and DBP of 79.5 mmHg were identified as the cutoff values (**Figures 3C,D**). However, SBP 146 mmHg as a cutoff value exhibited low sensitivity and specificity (AUC = 0.5772, $p = 0.3830$, **Figure 3C**). DBP > 79.5 mmHg predicted a poor clinical outcome in CVT patients (AUC = 0.6733, $p = 0.0452$, **Figure 3D**). CVT patients with SBP > 146 mmHg or DBP > 79.5 mmHg had significantly higher mRS compared to those with SBP ≤ 146 mmHg or DBP ≤ 79.5 mmHg ($p = 0.002$, $p = 0.002$, **Figures 3E,F**). A higher rate of poor clinical outcome was found in CVT patients with SBP > 146 mmHg or DBP > 79.5 mmHg compared to those with SBP ≤ 146 mmHg or DBP ≤ 79.5 mmHg ($p = 0.008$, $p = 0.005$, **Table 4**).

DISCUSSION

A Turkish multicenter study reported that females are more susceptible to CVT (7). Sixty-eight percent of the CVT patients were female. Results from the present study reported a relatively low proportion of female. Only 55.8% of the CVT patients were female. Headache was the most frequent symptom based on results from this study. This finding is consistent with the previous reports (7, 11). The limitation of this study was the small sample size. For stronger evidence, a multicenter study recruiting CVT patients from 25 tertiary hospitals across China Mainland led by our research team is ongoing (NCT 03919305). Results from this multicenter study will be published later.

Our results also revealed that SBP and DBP were not correlated with NIHSS at admission. However, the results were not strong enough to draw a final conclusion that SBP and DBP were not correlated with the severity of CVT. NIHSS was designed to evaluate the severity of arterial stroke (12). It showed low sensitivity and specificity for CVT. For example, a CVT patient with headache and seizures was scored as 0. The severity of the clinical presentations was currently discussed more descriptively. Yet, there was no severity evaluation scale for CVT patients based on their clinical presentations. Thus, a modified NIHSS for venous stroke is required.

SBP and DBP were found to be positively correlated with ICP. It is suggested that higher SBP and DBP would ensure the perfusion pressure in the presence of IH among CVT patients (13). It seems contradictory that SBP and DBP were positively correlated with ICP, but had no impact on the disturbance of consciousness, epilepsy, intracranial hemorrhage, and mental disorders. Except for IH, inflammation as well as oxidative stress, apoptosis, glutamate excitotoxicity, and dysfunction of the blood–brain barrier were also involved in the pathogenesis of disturbance of consciousness, epilepsy, intracranial hemorrhage, and mental disorders (14–16). The increasing ICP level may not be the determining factor of these clinical manifestations.

TABLE 2 | Both SBP and DBP had no impact on disturbance of consciousness, epilepsy, intracranial hemorrhage and mental disorders.

Independent variables	Dependent variables	OR	*P*	Confidence interval	
				Lower	Upper
SBP	Conscious disturbance	0.979	0.131	0.953	1.006
	Epilepsy	1.013	0.266	0.990	1.036
	Intracranial hemorrhage	1.010	0.508	0.981	1.040
	Mental disorder	1.010	0.417	0.986	1.036
DBP	Conscious disturbance	0.989	0.578	0.951	1.029
	Epilepsy	1.013	0.465	0.978	1.049
	Intracranial hemorrhage	0.996	0.869	0.951	1.044
	Mental disorder	1.008	0.694	0.970	1.047

TABLE 3 | Neither SBP nor ICP was correlated with mRS at 6 months of follow-up.

Independent variables	Dependent variables	Coeff (B)	Std Coeff (β)	Significance (P)
mRS at 6 months of follow-up				
SBP	mRS	0.003	0.010	0.784
DBP		0.027	0.016	0.039*
ICP		0.015	0.011	0.159

*However, DBP was positively correlated with mRS at 6 months of follow-up. *$p < 0.05$.*

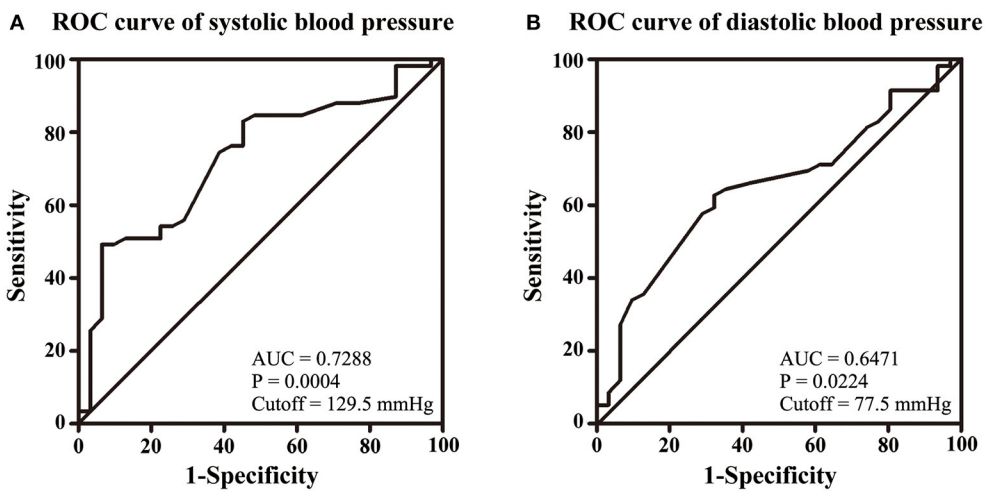

FIGURE 2 | Volumes of 129.5 mmHg of SBP **(A)** and 77.5 mmHg of DBP **(B)** were identified as cutoff values for IH.

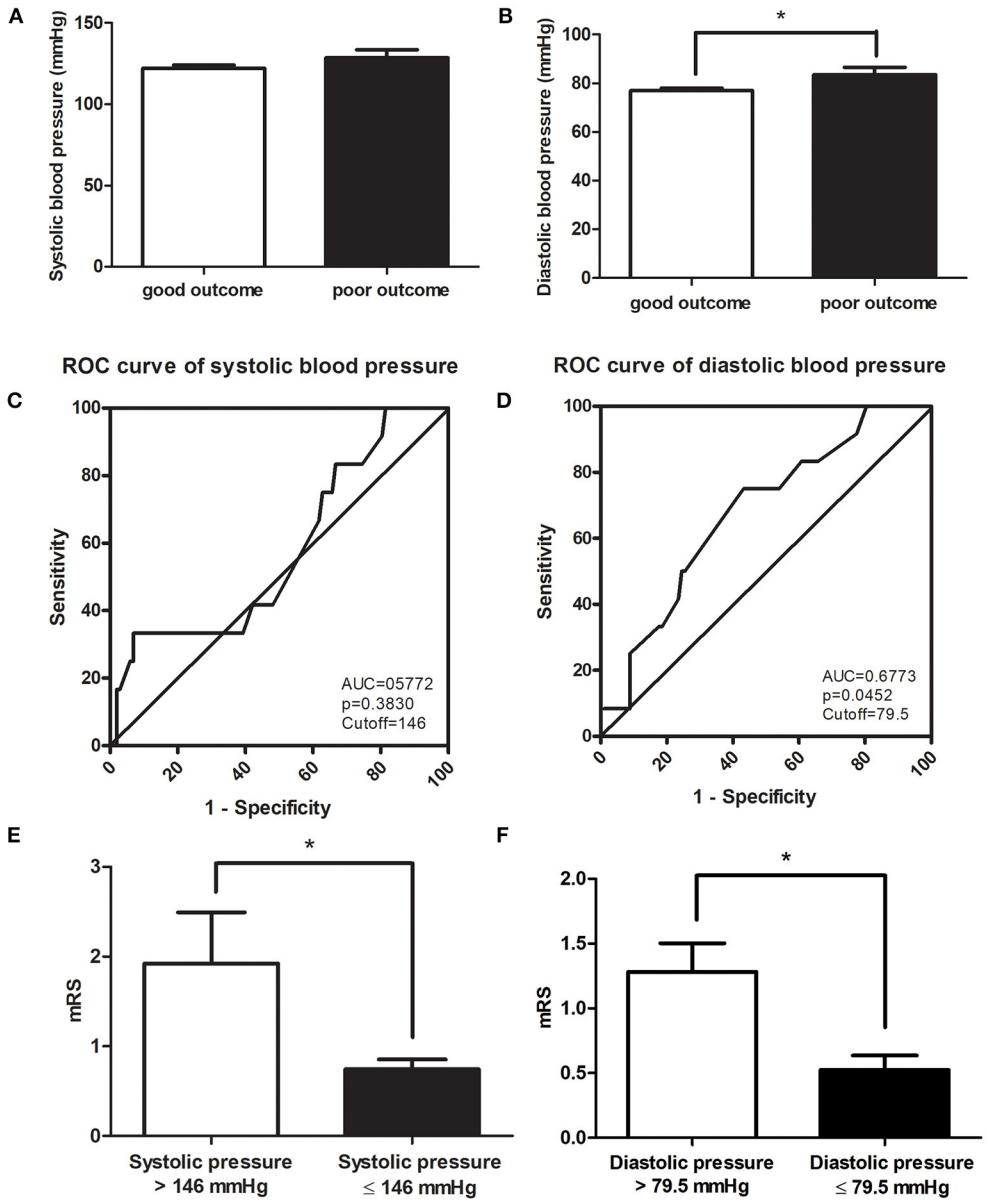

FIGURE 3 | No significant difference was found in SBP between CVT patients with good prognosis and those with poor prognosis **(A)**. DBP in CVT patients with good prognosis was significantly lower than in those with poor prognosis **(B)**. A volume of 146 mmHg of SBP as a cutoff value exhibited low sensitivity and specificity **(C)** whereas DBP < 79.5 mmHg predicted a good clinical outcome with high sensitivity and specificity **(D)**. CVT patients with SBP > 146 **(E)** or DBP > 79.5 mmHg **(F)** had significantly higher mRS compared to those with SBP ≤ 146 mmHg **(E)** or DBP ≤ 79.5 mmHg **(F)**. *$p < 0.05$.

In a study conducted by de Bruijn et al. (17), it was reported that CVT patients with IH did not suffer from poor prognosis. Our results also indicated that ICP was not correlated with mRS at 6 months of follow-up after adjusting for SBP and DBP. Although CVT patients with SBP > 146 mmHg had a higher rate of poor prognosis and higher mRS, a cutoff value of 146 mmHg exhibited low sensitivity and specificity. SBP was not correlated with mRS at 6 months of follow-up after adjusting for ICP and SBP. A higher rate of poor prognosis and higher mRS were also observed in CVT patients with DBP > 79.5 mmHg. DBP was positively correlated with mRS at 6 months of follow-up after adjusted for SBP and ICP. DBP > 79.5 mmHg suggested a poor prognosis with high sensitivity and specificity.

The explanations for this observation were as follows: first, increased DBP may be due to a markedly elevated ICP which results in aggravation of inflammation, oxidative stress, glutamate excitotoxicity, and dysfunction

TABLE 4 | A higher rate of poor clinical outcome was found in CVT patients with SBP > 146 mmHg or DBP > 79.5 mmHg compared to those with SBP ≤ 146 mmHg or DBP ≤ 79.5 mmHg.

		Good clinical outcome	Poor clinical outcome	Total	*P*
SBP	SBP > 146 mmHg	12	6	18	0.008*
	SBP ≤ 146 mmHg	126	12	138	
	Total	138	18	156	
DBP	DBP > 79.5 mmHg	58	14	72	0.005*
	SBP ≤ 79.5 mmHg	80	4	84	
	Total	138	18	156	

*$p < 0.05$.

of the blood–brain barrier (14–16). Second, a number of studies suggested that elevated venous pressure promoted the contraction of arteriolar smooth muscle, thus increasing the peripheral vascular resistance. This may further lead to elevated DBP (18, 19). A higher level of DBP may be correlated with an elevated central venous pressure which reduces the cerebral venous return. However, this hypothesis requires verification with further investigations.

CONCLUSIONS

Both SBP and DBP were positively correlated with ICP at admission. SBP > 129.5 mmHg and DBP > 77.5 mmHg suggested the presence of IH in CVT patients. DBP was positively correlated with mRS at 6 months of follow-up. DBP > 79.5 mmHg predicted a poor clinical outcome in CVT patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics board of Xuanwu Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

ML: contributed to study design and drafting the manuscript. LP: contributed to study design and data collection. XG: contributed to data analysis. JH: contributed to data collection. RM: contributed to acquisition of study funding, study design, and critical revision of the manuscript. XJ: contributed to data interpretation and critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Prognostic Significance of Admission Systemic Inflammation Response Index in Patients With Spontaneous Intracerebral Hemorrhage: A Propensity Score Matching Analysis

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Intracerebral hemorrhage (ICH) accounts for ∼15% of all strokes and is associated with high mortality and disability rates. The systemic inflammation response index (SIRI) is a novel systemic inflammatory marker based on peripheral neutrophil, monocyte, and lymphocyte counts. This study aimed to evaluate the prognostic significance of admission SIRI in patients with spontaneous ICH and compare its predictive ability with that of the neutrophil-to-lymphocyte ratio (NLR). This retrospective study was conducted based on a prospectively collected database of patients with ICH between June 2016 and January 2019. Propensity score matching (PSM) was conducted to adjust for potential imbalances in the clinical parameters. A total of 403 patients were included in the original cohort. The optimal SIRI cut-off value was 2.76. After 1:1 PSM based on potential confounding variables, a new cohort containing 262 patients was established for further analysis. In the original cohort, SIRI served as an independent predictor of 3-month functional outcome [odds ratio (OR), 1.302; 95% CI, 1.120–1.512; $p = 0.001$] and 1-month mortality (OR, 1.072; 95% CI, 1.020–1.126; $p = 0.006$), while NLR was independently associated with only 3-month functional outcomes (OR, 1.051; 95% CI, 1.004–1.100; $p = 0.031$) and not 1-month mortality. The same applied to the PSM cohort. Receiver operating characteristic analyses and predictive models indicated that in most instances, SIRI was superior to NLR and their components in predicting the outcomes of patients with ICH. Our study found that SIRI is determined to be an independent predictive indicator for ICH patients in 3-month functional outcomes and 1-month mortality. The prognostic predictive ability of SIRI was stronger than that of NLR.

Keywords: systemic inflammation response index, neutrophil to lymphocyte ratio, intracerebral hemorrhage, prognosis, propensity score matching

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INTRODUCTION

Intracerebral hemorrhage (ICH) is a life-threatening condition with a high mortality and disability rate and occurs due to spontaneous bleeding into the brain parenchyma, involving the ventricles and subarachnoid spaces in extreme circumstances. ICH accounts for ∼15% of all strokes (1). In terms of ICH, 75–85% of cases originate from the spontaneous rupture of small vessels damaged.

by chronic hypertension or amyloid angiopathy (2). The incidence of ICH is higher in male and elderly patients. Rapid CT after onset can be used to recognize almost all forms of acute ICH and help make optimal medical decisions within the shortest time. The global burden of ICH mainly results from inadequate management of chronic hypertension and other modifiable risk factors (3)

Growing evidence has indicated that inflammatory responses participate in the pathophysiological processes of brain injury after ICH, and inflammation is one of the crucial contributors to ICH-induced secondary brain injury (4). Leukocytes play an important role in immune response, cell migration, perihematomal edema formation, blood–brain barrier (BBB) integrity, and cell death after ICH (5, 6). Accumulating data have demonstrated that increased blood leukocyte count is associated with more serious disease and worse outcomes in ischemic and hemorrhagic strokes (7). Neutrophil-to-lymphocyte ratio (NLR), based on the coexistence of lymphopenia and leukocytosis in the initial inflammatory response, may be a useful peripheral biomarker for predicting the prognosis of stroke (8). Other peripheral inflammatory biomarkers, whose prognostic ability in ICH patients has also been confirmed, are systemic immune-inflammation index, NLR, and platelet-to-lymphocyte ratio (9, 10). Systemic inflammatory response syndrome, which is defined based on the changes in leukocyte and vital signs, is also associated with outcomes (11, 12).

The systemic inflammation response index (SIRI) is a novel systemic inflammatory marker based on peripheral neutrophil, monocyte, and lymphocyte counts. In previous studies, SIRI was found to be an independent prognostic indicator in various tumors (13–15). Therefore, this study aimed to evaluate the prognostic significance of admission SIRI in patients with spontaneous ICH and compare its prognostic ability with that of NLR.

MATERIALS AND METHODS

Study Design

This retrospective study was conducted based on a prospectively collected database of ICH patients at the Department of Neurosurgery of West China Hospital, Sichuan University between June 2016 and January 2019. All patients in this cohort were managed according to the latest guidelines for stroke, and their baseline clinical data were retrieved from the electronic medical record system of the West China Hospital (16).

The exclusion criteria were as follows: (1) age <18 years; (2) incomplete baseline clinical data; (3) ICH caused by a tumor, aneurysm, or arteriovenous malformation; (4) absence of CT angiography and follow-up CT within 24 h of admission; (5) a history of infectious diseases, cancers, rheumatic diseases, blood system diseases, or other diseases which evidently affect peripheral blood cells; (6) loss to follow-up.

Clinical Parameter Assessment

Clinical variables were retrieved from the electronic medical record system, including the following variables: (1) demographics: age of onset and sex; (2) clinical history: history of hypertension, diabetes mellitus, smoking, alcohol abuse, and stroke; (3) admission conditions: Glasgow Coma Scale (GCS) score, admission systolic blood pressure, diastolic blood pressure, and duration from onset to hospitalization; (4) ICH imaging characteristics: hematoma volume, location of hematoma, presence of intraventricular hematoma, and hematoma expansion (HE); (5) treatment; (6) routine blood tests. Notably, routine blood tests were conducted immediately after admission. SIRI was defined as neutrophil count × monocyte count/lymphocyte count, and NLR was defined as neutrophil count/lymphocyte count.

Patients were followed up every month after admission. The primary outcomes were 3-month functional outcomes and 1-month mortality rate. The modified Rankin Scale (mRS) was used to evaluate patients' functional outcomes at each follow-up. Patients who had been discharged were followed up by telephone. Good outcome was defined as an mRS score of 0–2, while a poor outcome was defined as an mRS score of 3–6 (17).

The volume of parenchymal hematoma was calculated on the initial CT scans using 3D Slicer (http://www.slicer.org), and manual segmentation was applied by two independent neurosurgeons (18). HE was defined as hematoma enlargement ≥6 ml or ≥33% within 24 h (19). Surgical interventions mainly included hematoma evacuation with craniotomy and external ventricular drainage.

Statistical Analysis

All statistical analyses were performed using SPSS software (version 22.0; IBM, Armonk, NY, USA) and R software (version 3.6.1). Continuous variables are presented as mean ± SD or median with interquartile range, while categorical variables are presented as frequency and percentage. Categorical variables were compared using the χ^2 or Fisher's exact test. Continuous variables that conformed to the normal distribution were compared using Student's *t*-test; otherwise, the Mann–Whitney *U*-test was employed. Logistic regression analyses were used to determine the influence of risk factors on outcomes in patients with ICH. Variables with $p < 0.1$ in univariate analysis were included in backward stepwise multivariate logistic regression. Receiver operating characteristic (ROC) analysis was conducted to assess the accuracy of the SIRI, NLR, and other markers for outcomes. The optimal cut-off value of SIRI was determined by calculating the maximum Youden index using ROC. DeLong's test was employed to compare the areas under the curve (AUC). Predictive models for outcomes were constituted by independent predictive indicators in multivariate logistic regression; Harrell's concordance index (C-index) and Akaike information criterion (AIC) were used to assess the predictive accuracy and model-fitting of predictive models, respectively. Higher C-index indicated better predictive accuracy, and lower

Abbreviations: ICH, intracerebral hemorrhage; BBB, blood–brain barrier; NLR, neutrophil-to-lymphocyte ratio; SIRI, systemic inflammation response index; GCS, Glasgow Coma Scale; mRS, modified Rankin Scale; HE, hematoma expansion; ROC, receiver operating characteristic; PSM, propensity score matching; OR, odds ratio; AUC, area under the curve.

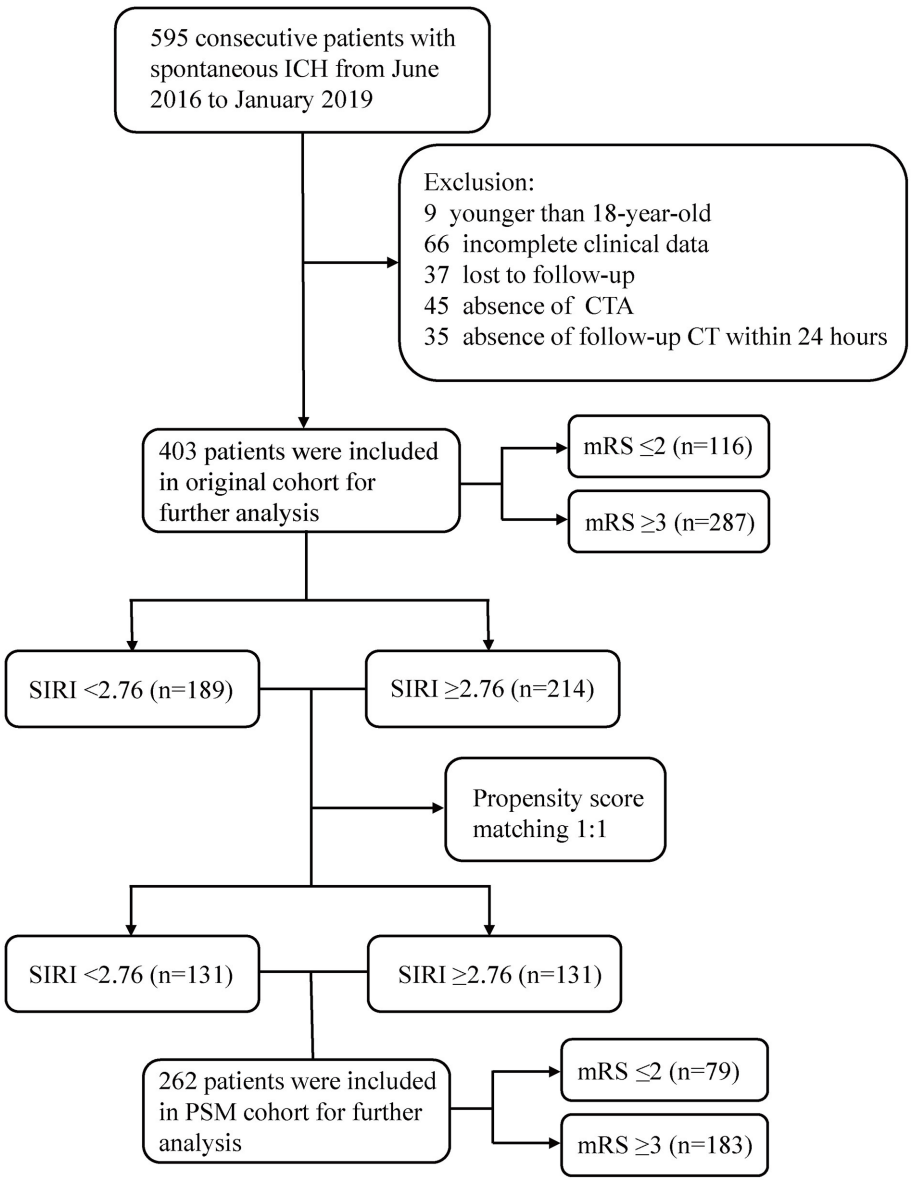

FIGURE 1 | Flow chart of the current study. ICH, intracerebral hemorrhage; CTA, computed tomography angiography; mRS, modified Rankin Scale; SIRI, systemic inflammation response index; PSM, propensity score matching.

AICs indicated superior model-fitting (20, 21). A two-sided $p < 0.05$ was considered statistically significant. Propensity score matching (PSM) was conducted to adjust for an imbalance of clinical parameters with a p-value of <0.1 in univariate analysis. These patients were matched 1:1 using the nearest-neighbor algorithm with a caliper width of 0.2 and without replacement.

Ethics

This study was approved by the Ethical Committee of Sichuan University (2013NO52) and conducted following the principles of the Declaration of Helsinki. All patients and their authorized trustees were informed and provided signed informed consent to use their clinical data for research purposes.

RESULTS

Baseline Clinical Characteristics

As shown in **Figure 1**, a total of 403 patients were included in the original cohort. The optimal cut-off value of SIRI was determined to be 2.76 in ROC analysis. Among the 403 patients, 189 patients had SIRI <2.76 and 214 had SIRI ≥2.76. After 1:1 PSM based on potential confounding variables, a new cohort containing 262 patients was established for further analysis.

TABLE 1 | Baseline characteristics of 403 patients with spontaneous ICH in original cohort.

Clinical variables	Total ($n = 403$)	3-month functional outcome			1-month mortality		
		Poor outcome ($n = 287$)	Good outcome ($n = 116$)	*P*-value	Death ($n = 84$)	Survival ($n = 319$)	*P*-value
Age (years)	58.56 ± 13.28	59.35 ± 13.31	56.60 ± 13.07	0.172	60.64 ± 13.90	58.01 ± 13.08	0.385
Male	276 (68.5)	196 (68.3)	80 (69.0)	0.895	56 (66.7)	220 (69.0)	0.687
GCS	13 (8, 15)	11 (7, 14)	15 (14, 15)	***<0.001***	7 (6, 9)	14 (11, 15)	***<0.001***
Admission SBP (mmHg)	167.34 ± 27.54	168.74 ± 28.55	163.87 ± 24.63	0.175	169.35 ± 30.84	166.81 ± 26.63	0.265
Admission DBP (mmHg)	98.10 ± 18.27	97.90 ± 19.11	98.61 ± 16.05	0.157	96.65 ± 20.12	98.49 ± 17.76	0.663
Duration from onset to hospitalization (h)	6 (4, 12)	6 (4, 10)	6 (4, 16)	0.395	5 (4, 7)	6 (4, 14)	***0.006***
History of hypertension	226 (56.1)	164 (57.1)	62 (53.4)	0.499	53 (63.1)	173 (54.2)	0.146
History of diabetes mellitus	25 (6.2)	21 (7.3)	4 (3.4)	0.145	3 (3.6)	22 (6.9)	0.262
Smoking	133 (33.0)	102 (35.5)	31 (26.7)	**0.089**	23 (27.4)	110 (34.5)	0.219
Alcohol abuse	127 (31.5)	95 (33.1)	32 (27.6)	0.281	21 (25.0)	106 (33.2)	0.149
Previous stroke	22 (5.5)	17 (5.9)	5 (4.3)	0.519	3 (3.6)	19 (6.0)	0.393
Antiplatelet or anticoagulation	30 (7.4)	18 (6.3)	12 (10.3)	0.159	5 (6.0)	25 (7.8)	0.293
Hematoma volume (ml)	18.36 (7.35, 36.33)	24.68 (9.18, 41.53)	10.19 (3.17, 20.51)	***<0.001***	25.99 (9.21, 45.36)	12.63 (7.05, 33.10)	***0.002***
Hematoma location							
Supratentorial	336 (83.4)	248 (86.4)	88 (75.9)	***0.010***	65 (77.4)	271 (85.0)	**0.098**
Infratentorial	67 (16.6)	39 (13.6)	28 (24.1)		19 (22.6)	48 (15.0)	
Presence of IVH	53 (13.2)	45 (15.7)	8 (6.9)	***0.018***	14 (16.7)	39 (12.2)	0.285
Presence of hematoma expansion	92 (22.8)	77 (26.8)	15 (12.9)	***0.003***	29 (34.5)	63 (19.7)	***0.004***
Treatment							
Surgical intervention	101 (25.1)	92 (32.1)	9 (7.8)	***<0.001***	24 (28.6)	77 (24.1)	0.405
Conservative treatment	302 (74.9)	195 (67.9)	107 (92.2)		60 (71.4)	242 (75.9)	
PLT (10^9/L)	153 (117, 203)	154 (114, 204)	154 (132, 202)	0.592	144 (112, 197)	158 (122, 206)	0.201
PT (s)	11.4 (10.9, 12.2)	11.4 (10.8, 12.2)	11.4 (10.9, 12.1)	0.396	11.3 (10.9, 12.4)	11.4 (10.8, 12.1)	0.463
APTT (s)	26.5 (23.7, 29.4)	26.1 (23.4, 28.5)	26.7 (24.4, 29.7)	0.701	25.2 (22.1, 28.5)	26.3 (24.0, 29.0)	0.240
INR	0.98 (0.93–1.05)	0.98 (0.93, 1.06)	0.99 (0.93, 1.04)	0.800	0.98 (0.93, 1.10)	0.98 (0.92, 1.05)	0.223
Neutrophil (10^9/L)	8.66 (6.19, 11.73)	9.68 (7.03, 12.98)	6.61 (4.88, 8.79)	***<0.001***	11.76 (8.66, 15.29)	8.00 (5.89, 10.52)	***<0.001***
Lymphocyte (10^9/L)	1.03 (0.72, 1.48)	0.98 (0.69, 1.41)	1.18 (0.82, 1.62)	***0.004***	0.93 (0.64, 1.37)	1.05 (0.76, 1.49)	0.131
Monocyte (10^9/L)	0.39 (0.27, 0.53)	0.42 (0.29, 0.56)	0.34 (0.23, 0.42)	***<0.001***	0.51 (0.41, 0.64)	0.36 (0.24, 0.49)	***<0.001***
NLR	8.63 (5.11, 13.96)	9.83 (6.23, 15.77)	5.71 (3.43, 9.58)	***<0.001***	12.16 (6.39, 21.04)	7.96 (4.66, 12.62)	***<0.001***
SIRI	2.87 (1.63, 5.56)	3.75 (2.10, 6.58)	1.87 (1.05, 2.89)	***<0.001***	5.68 (3.50, 10.13)	2.55 (1.50, 4.44)	***<0.001***

Data are expressed as n (%), mean ± SD, or median (25th, 75th quartile). Significant findings are expressed in bold and italic.
ICH, intracerebral hemorrhage; GCS, Glasgow Coma Scale; SBP, systolic blood pressure; DBP, diastolic blood pressure; IVH, intraventricular hematoma; PLT, platelet; PT, prothrombin time; APTT, activated partial thromboplastin time; INR, international normalized ratio; SIRI, systemic inflammation response index; NLR, neutrophil-to-lymphocyte ratio.

In the original cohort (**Table 1**), 287 patients had poor outcomes at 3 months with mRS score ≥3, while 116 patients had good outcomes with mRS score ≤2. Clinical variables including GCS score ($p < 0.001$), hematoma volume ($p < 0.001$), hematoma location ($p = 0.010$), presence of IVH ($p = 0.018$), presence of HE ($p = 0.003$), and treatment method ($p < 0.001$) were associated with 3-month functional outcomes. Meanwhile, neutrophil count ($p < 0.001$), lymphocyte count ($p = 0.004$), monocyte count ($p < 0.001$), NLR ($p < 0.001$), and SIRI ($p < 0.001$) were also associated with 3-month functional outcomes. Regarding 1-month mortality, 84 patients died within 30 days of admission, and 319 patients survived the first month. Among the clinical variables, GCS score ($p < 0.001$), duration from onset to hospitalization ($p = 0.006$), hematoma volume ($p = 0.002$), and presence of HE ($p = 0.004$) were associated with 1-month mortality. Peripheral blood markers, including neutrophil count ($p < 0.001$), monocyte count ($p < 0.001$), NLR ($p < 0.001$), and SIRI ($p < 0.001$), were significantly associated with 1-month mortality, whereas lymphocyte count ($p = 0.131$) was not associated with 1-month mortality.

The clinical characteristics of the PSM cohort are listed in **Table 2**, with a lower GCS score ($p < 0.001$), larger hematoma volume ($p < 0.001$), presence of IVH ($p = 0.037$), supratentorial hematoma ($p = 0.001$), and surgical interventions ($p = 0.001$) associated with unfavorable outcomes at 3 months after admission. In the group with 1-month mortality, a lower

TABLE 2 | Baseline characteristics of 262 patients with spontaneous ICH in PSM cohort.

Clinical variables	Total ($n = 262$)	3-month functional outcome			1-month mortality		
		Poor outcome ($n = 183$)	Good outcome ($n = 79$)	*P*-value	Death ($n = 42$)	Survival ($n = 220$)	*P*-value
Age (years)	58.77 ± 13.56	59.67 ± 13.42	56.67 ± 13.75	0.402	61.26 ± 14.82	58.29 ± 13.29	0.242
Male	177 (67.6)	124 (67.8)	53 (67.1)	0.915	28 (66.7)	149 (67.7)	0.893
GCS	13 (9, 15)	12 (8, 14)	15 (14, 15)	***<0.001***	8 (6, 11)	14 (11, 15)	***<0.001***
Admission SBP (mmHg)	167.17 ± 26.56	168.86 ± 27.25	163.24 ± 24.60	0.407	170.31 ± 27.05	166.57 ± 26.48	0.857
Admission DBP (mmHg)	97.56 ± 17.54	97.58 ± 17.86	97.49 ± 16.90	0.655	96.05 ± 18.81	97.85 ± 17.32	0.844
Duration from onset to hospitalization (h)	6 (4, 15)	6 (4, 12)	6 (4, 22)	0.242	5 (3, 8)	6 (4, 15)	***0.015***
History of hypertension	141 (53.8)	103 (56.3)	38 (48.1)	0.224	26 (61.9)	115 (52.3)	0.252
History of diabetes mellitus	14 (5.3)	13 (7.1)	1 (1.3)	0.054	1 (2.4)	13 (5.9)	0.352
Smoking	90 (34.4)	66 (36.1)	24 (30.4)	0.375	14 (33.3)	76 (34.5)	0.880
Alcohol abuse	88 (33.6)	67 (36.6)	21 (26.6)	0.115	13 (31.0)	75 (34.1)	0.694
Previous stroke	14 (5.3)	13 (7.1)	1 (1.3)	0.054	2 (4.8)	12 (5.5)	0.855
Antiplatelet or anticoagulation	21 (8.0)	13 (7.1)	8 (10.1)	0.409	3 (7.1)	18 (8.2)	0.821
Hematoma volume (ml)	20.10 (7.04, 35.15)	24.18 (9.61, 38.54)	10.13 (2.71, 24.02)	***<0.001***	24.87 (8.88, 39.99)	18.29 (7.00, 33.09)	0.136
Hematoma location							
Supratentorial	215 (82.1)	160 (87.4)	55 (69.6)	***0.001***	34 (81.0)	181 (82.3)	0.838
Infratentorial	47 (17.9)	23 (12.6)	24 (30.4)		8 (19.0)	39 (17.7)	
Presence of IVH	38 (14.5)	32 (17.5)	6 (7.6)	***0.037***	9 (21.4)	29 (13.2)	0.165
Presence of hematoma expansion	61 (23.3)	47 (25.7)	14 (17.7)	0.163	14 (33.3)	47 (21.4)	0.093
Treatment							
Surgical intervention	61 (23.3)	53 (29.0)	8 (10.1)	***0.001***	10 (23.8)	51 (23.2)	0.930
Conservative treatment	201 (76.7)	130 (71.0)	71 (89.9)		32 (76.2)	169 (76.8)	
PLT (10^9/L)	148 (117, 196)	147 (116, 195)	153 (123, 187)	0.525	136 (112, 196)	153 (120, 196)	0.171
PT (s)	11.4 (10.9, 12.2)	11.4 (10.8, 12.3)	11.5 (11.0, 12.2)	0.651	11.6 (10.9, 12.3)	11.4 (10.9, 12.2)	0.703
APTT (s)	26.2 (23.5, 29.2)	25.9 (23.4, 28.9)	26.5 (23.9, 29.3)	0.650	24.8 (22.5, 29.8)	26.2 (23.6, 29.1)	0.511
INR	0.98 (0.93, 1.05)	0.98 (0.92, 1.05)	1.00 (0.96, 1.05)	0.169	0.99 (0.94, 1.10)	0.98 (0.93, 1.05)	0.421
Neutrophil (10^9/L)	8.36 (6.37, 10.81)	9.06 (6.80, 11.35)	7.50 (5.49, 9.08)	***<0.001***	10.22 (7.19, 12.54)	8.19 (6.32, 10.39)	***0.017***
Lymphocyte (10^9/L)	1.05 (0.75, 1.49)	1.04 (0.71, 1.44)	1.13 (0.82, 1.57)	0.085	1.04 (0.70, 1.60)	1.06 (0.76, 1.48)	0.965
Monocyte (10^9/L)	0.38 (0.25, 0.49)	0.40 (0.28, 0.50)	0.34 (0.24, 0.46)	***0.044***	0.48 (0.40, 0.58)	0.36 (0.24, 0.47)	***<0.001***
NLR	8.18 (4.88, 12.67)	8.94 (5.91, 13.26)	6.41 (4.05, 9.95)	***<0.001***	10.31 (5.78, 14.86)	7.90 (4.87, 12.44)	0.271
SIRI	2.76 (1.63, 4.70)	2.99 (1.81, 5.20)	2.18 (1.30, 3.23)	***<0.001***	4.60 (1.95, 8.47)	2.62 (1.63, 4.15)	***0.003***

Data are expressed as n (%), mean ± SD, or median (25th, 75th quartile).

ICH, intracerebral hemorrhage; PSM, propensity score matching; GCS, Glasgow Coma Scale; SBP, systolic blood pressure; DBP, diastolic blood pressure; IVH, intraventricular hematoma; PLT, platelet; PT, prothrombin time; APTT, activated partial thromboplastin time; INR, international normalized ratio; SIRI, systemic inflammation response index; NLR, neutrophil-to-lymphocyte ratio. Significant findings are expressed in bold and italic.

GCS score ($p < 0.001$) and shorter duration from onset to hospitalization ($p = 0.015$) were directly related to death. Higher neutrophil count, monocyte count, and SIRI were associated with unfavorable outcomes in both groups. Higher NLR was significantly related to poor 3-month functional outcomes ($p < 0.001$) but not 1-month mortality ($p = 0.271$).

Association of SIRI With Outcomes

In the original cohort, multivariate logistic analysis (**Table 3**) revealed that the following factors all served as independent predictors for 3-month functional outcomes, including GCS score [odds ratio (OR), 0.686; 95% CI 0.606–0.776; $p < 0.001$), hematoma volume (OR, 1.022; 95% CI 1.002–1.042; $p = 0.027$), hematoma location (OR, 2.452; 95% CI 1.100–5.467; $p = 0.028$), treatment method (OR, 2.455; 95% CI 1.044–5.773; $p = 0.040$), lymphocyte count (OR, 0.469; 95% CI 0.290–0.758; $p = 0.002$), monocyte count (OR, 28.642; 95% CI 4.427–185.296; $p < 0.001$), NLR (OR, 1.051; 95% CI 1.004–1.100; $p = 0.031$), and SIRI (OR, 1.302; 95% CI 1.120–1.512; $p = 0.001$). As for 1-month mortality, GCS score (OR, 0.700; 95% CI 0.637–0.769; $p < 0.001$), hematoma volume (OR, 1.012; 95% CI 1.000–1.025; $p = 0.046$), monocyte count (OR, 3.734; 95% CI 1.283–10.869; $p = 0.016$), and SIRI (OR, 1.072; 95% CI 1.020–1.126; $p = 0.006$) were independent risk factors, but not NLR (OR, 1.021; 95% CI 0.987–1.057; $p = 0.225$).

As shown in **Table 4**, in the PSM cohort, GCS score (OR, 0.611; 95% CI 0.508–0.736; $p < 0.001$), hematoma volume (OR, 1.034; 95% CI 1.012–1.057; $p = 0.002$), neutrophil count (OR, 1.143; 95% CI 1.030–1.269; $p = 0.012$), NLR (OR, 1.076; 95% CI 1.016–1.139; $p = 0.012$), and SIRI (OR, 1.312; 95%

TABLE 3 | Multivariate logistic regression of included clinical variables for 3-month functional outcome and 1-month mortality in original cohort.

3-month functional outcome	OR	95% CI	*P*-value
GCS (per 1-point increase)	0.686	0.606–0.776	***<0.001***
Smoking (yes vs. no)	1.426	0.791–2.572	0.238
Hematoma volume (per 1-ml increase)	1.022	1.002–1.042	***0.027***
Hematoma location (supratentorial vs. infratentorial)	2.452	1.100–5.467	***0.028***
Presence of IVH (yes vs. no)	1.272	0.496–3.263	0.616
Presence of hematoma expansion (yes vs. no)	1.093	0.508–2.351	0.820
Treatment (surgical intervention vs. conservative treatment)	2.455	1.044–5.773	***0.040***
Neutrophil (per 1 × 10^9/L increase)	1.080	0.981–1.189	0.118
Lymphocyte (per 1 × 10^9/L increase)	0.469	0.290–0.758	***0.002***
Monocyte (per 1 × 10^9/L increase)	28.642	4.427–185.296	***<0.001***
NLR (per 1-point increase)	1.051	1.004–1.100	***0.031***
SIRI (per 1-point increase)	1.302	1.120–1.512	***0.001***
1-month mortality			
GCS (per 1-point increase)	0.700	0.637–0.769	***<0.001***
Duration from onset to hospitalization (per 1-h increase)	0.987	0.962–1.013	0.328
Hematoma volume (per 1-ml increase)	1.012	1.000–1.025	***0.046***
Hematoma location (supratentorial vs. infratentorial)	0.529	0.221–1.267	0.153
Presence of hematoma expansion (yes vs. no)	1.504	0.756–2.992	0.245
Neutrophil (per 1 × 10^9/L increase)	1.019	0.940–1.105	0.646
Monocyte (per 1 × 10^9/L increase)	3.734	1.283–10.869	***0.016***
NLR (per 1-point increase)	1.021	0.987–1.057	0.225
SIRI (per 1-point increase)	1.072	1.020–1.126	***0.006***

OR, odds ratio; GCS, Glasgow Coma Scale; IVH, intraventricular hematoma; NLR, neutrophil-to-lymphocyte ratio; SIRI, systemic inflammation response index. Significant findings are expressed in bold and italic.

CI 1.096–1.571; $p = 0.003$) were independently associated with 3-month functional outcomes. GCS score (OR, 0.684; 95% CI 0.607–0.770; $p < 0.001$), monocyte count (OR, 35.970; 95% CI, 4.490–288.130; $p = 0.001$), and SIRI (OR, 1.153; 95% CI 1.050–1.265; $p = 0.003$) were independently related to 1-month mortality. NLR was not included in the multivariate logistic analysis because the *p*-value in the univariate analysis did not match the criteria.

Predictive Ability of SIRI and NLR in Outcomes

ROC analysis was employed to determine and compare the predictive ability of SIRI and NLR in 3-month functional outcomes and 1-month mortality in patients with ICH (**Figure 2**, **Supplementary Figure 1**). In the original cohort, SIRI had a stronger predictive ability than NLR in 3-month functional outcome (**Figure 2A**, AUC 0.748 vs. 0.698; DeLong's test, $Z = 2.35$, $p = 0.019$) and 1-month mortality (**Figure 2B**, AUC 0.745 vs. 0.656; DeLong's test, $Z = 4.73$, $p < 0.001$). The same applied to the PSM cohort, where the predictive ability of SIRI was also better than that of NLR in 1-month mortality (**Figure 2D**, AUC 0.644 vs. 0.554; DeLong's test, $Z = 3.14$, $p = 0.002$). In 3-month functional outcome, although predictive ability of SIRI was superior to NLR, there was no statistical difference (**Figure 2C**, AUC 0.653 vs. 0.636; DeLong's test, $Z = 0.60$, $p = 0.550$).

Predictive models were conducted to further evaluate the predictive accuracy of the aforementioned markers (**Table 5**). Basic models consisted of independent predictive indicators other than peripheral blood markers. The results indicated that basic models with SIRI had highest C-index and lowest AIC in 3-month functional outcome in both original and PSM cohort, indicating the best predictive accuracy and model-fitting. With regard to 1-month mortality, the basic model with SIRI was superior to that with monocytes in the original cohort, but the result was opposite in PSM cohort.

DISCUSSION

In recent years, with the improvement of quality of life and medical conditions, excellent medical treatments, including medication and surgery, have been provided, which have a potent and direct impact on ICH morbidity and mortality (16). Multidisciplinary collaborations, such as between imaging, pathology, physiology, and neurosurgery, are needed to understand this condition and its underlying mechanism. In this study, we focused on the prognostic role of systemic inflammation biomarkers in peripheral blood in patients with spontaneous ICH.

Secondary damage due to ICH in the brain parenchyma induced by inflammatory cells and inflammatory cascades plays a crucial role in disease progression, thus affecting outcomes. Local inflammation adjacent to the primary injury could not be evaluated or measured directly, whereas systemic inflammation might reflect local inflammation in the peripheral blood system to some extent. Damage-associated molecular patterns, which are released by injured or dying neurons and cytokines during

TABLE 4 | Multivariate logistic regression of included clinical variables for 3-month functional outcome and 1-month mortality in PSM cohort.

3-month functional outcome	OR	95% CI	*P*-value
GCS (per 1-point increase)	0.611	0.508–0.736	***<0.001***
History of diabetes mellitus (yes vs. no)	2.521	0.272–23.410	0.416
Previous stroke (yes vs. no)	6.588	0.689–62.985	0.102
Hematoma volume (per 1-ml increase)	1.034	1.012–1.057	***0.002***
Hematoma location (supratentorial vs. infratentorial)	1.725	0.682–4.361	0.250
Presence of IVH (yes vs. no)	1.837	0.614–5.499	0.277
Treatment (surgical intervention vs. conservative treatment)	2.326	0.925–5.849	0.073
Neutrophil (per 1 × 10^9/L increase)	1.143	1.030–1.269	***0.012***
Monocyte (per 1 × 10^9/L increase)	1.853	0.258–13.333	0.540
NLR (per 1-point increase)	1.076	1.016–1.139	***0.012***
SIRI (per 1-point increase)	1.312	1.096–1.571	***0.003***
1-month mortality			
GCS (per 1-point increase)	0.684	0.607–0.770	***<0.001***
Duration from onset to hospitalization (per 1-h increase)	0.990	0.964–1.017	0.469
Presence of hematoma expansion (yes vs. no)	1.766	0.722–4.321	0.213
Neutrophil (per 1 × 10^9/L increase)	1.002	0.901–1.113	0.977
Monocyte (per 1 × 10^9/L increase)	35.970	4.490–288.130	***0.001***
SIRI (per 1-point increase)	1.153	1.050–1.265	***0.003***

OR, odds ratio; GCS, Glasgow Coma Scale; IVH, intraventricular hematoma; NLR, neutrophil-to-lymphocyte ratio; SIRI, systemic inflammation response index. Significant findings are expressed in bold and italic.

early injury, can gain access to the systemic circulation through the broken BBB or cerebrospinal fluid drainage system (22). In animal models of ischemic stroke, immuno-dysregulation after ischemic stroke includes upregulation of systemic inflammatory response. In animal models, a large ICH volume results in decreased leukocytes and lymphocytes and increased monocytes (7). In the same, higher leukocyte counts have been associated to hematoma growth and early neurological deterioration (8). Relevant evidence indicated that the peripheral cellular immune system changed dramatically in the immediate aftermath of ICH (23). Therefore, changes in specific inflammatory markers in the peripheral blood are an indicator of the severity of the primary injury, theoretically. In oncology, inflammatory markers from peripheral blood are used to predict tumor progression and prognosis (24).

We have introduced a novel systemic inflammatory marker SIRI in our study, which was first reported in pancreatic cancer in 2016 (25). Since SIRI and NLR have a great similarity in their components, their predictive abilities in prognosis are compared in this study. NLR has been widely used as an effective indicator and monitor in various diseases, but not limited to tumors, rheumatic diseases, cardiovascular diseases, and infectious diseases (26–29). It is a very sensitive but less specific hematologic parameter to measure stress, intensity of infection/inflammation, and severity of illness of various origin (30). It has also been determined to play a strong predictive role in prognosis for ICH and subarachnoid hemorrhage patients in previous studies (31, 32). Similar to most related studies, the results of this study indicate that NLR is an independent risk factor for 3-month functional outcomes measured by the mRS. Compared with NLR, SIRI is mainly reported in the field of cancer. Recent researches about SIRI in aneurysmal subarachnoid hemorrhage showed that higher level of SIRI served as an independent indicator of unfavorable clinical outcomes (33, 34). In our research, SIRI was superior to NLR in predicting 3-month functional outcomes and has significant advantages in predicting 1-month mortality. However, NLR did not serve as an independent risk factor for 1-month mortality in ICH patients in our study.

Monocytes are mononuclear myeloid cells that originate from the bone marrow and circulate within the bloodstream (35). Like neutrophils, monocyte recruitment in circulation and injured tissues is a key feature of inflammation (36). A previous study has shown that a higher monocyte count on admission is an independent predictor of HE (37). In a study by Walsh et al., absolute monocyte count was independently associated with 30-day case fatality in 240 adult ICH patients, which is consistent with their previous study and our current study (38, 39). In a previous study by Mackey et al., elevated monocyte count was also an independent risk factor for 30-day case fatality (40). In the current study, we also found that monocyte count also served as independent prognostic predictors in 3-month functional outcome and 1-month mortality in the original cohort, and presented an excellent predictive ability in 1-month mortality in the PSM cohort. In consideration of the prognostic ability of monocytes in ICH patients, this could partly explain why the combination of monocyte and NLR gains predictive ability in outcomes.

From another perspective, stability of prognostic capacity in single component including neutrophil, lymphocyte, and monocyte was inferior to SIRI according to the results from multivariate analysis, ROC analyses, and predictive models. In

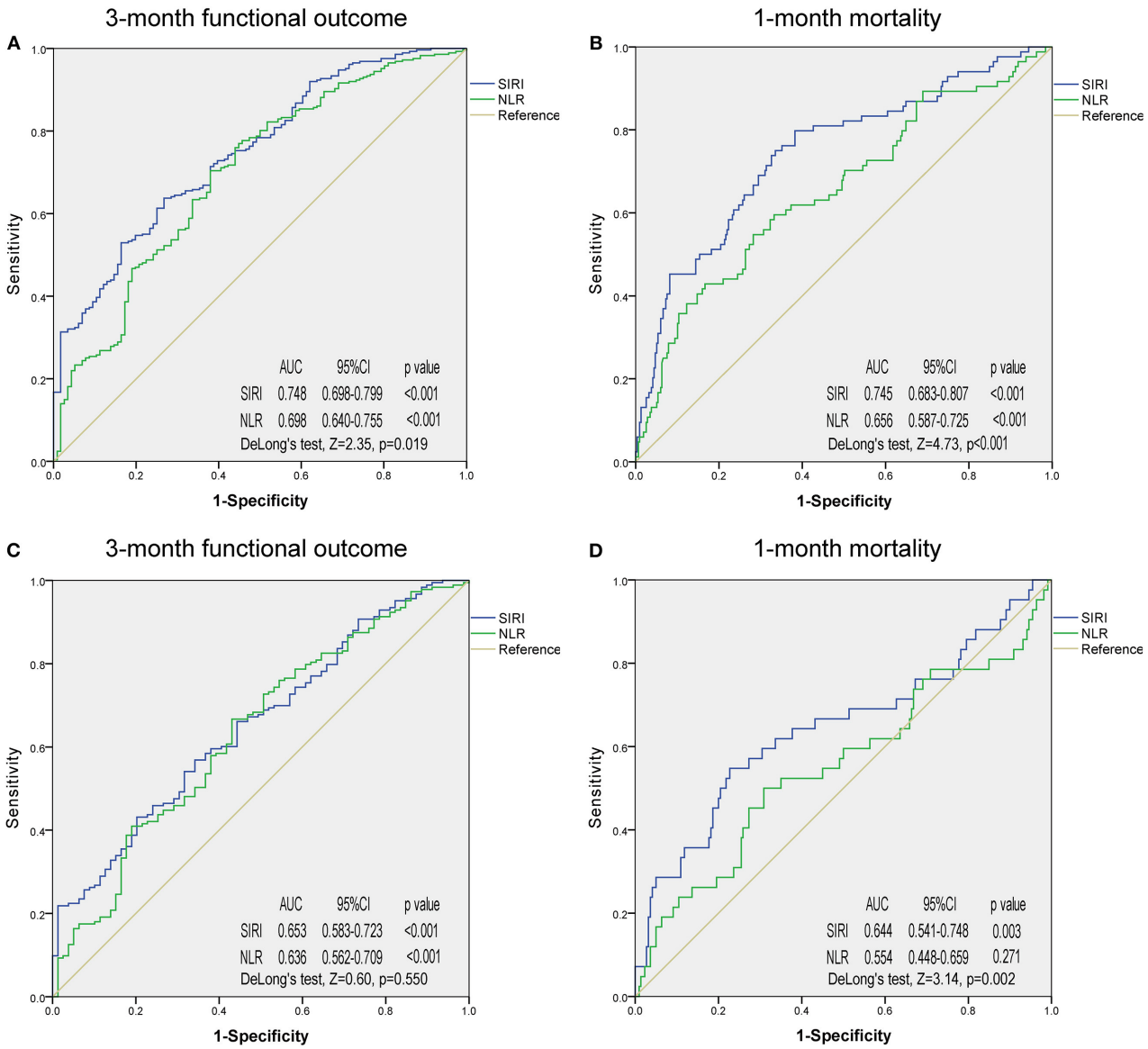

FIGURE 2 | Receiver operating characteristic curves of systemic inflammation response index and neutrophil-to-lymphocyte ratio for predicting 3-month functional outcome and 1-month mortality in the original cohort **(A,B)** and propensity score matching cohort **(C,D)**. SIRI, systemic inflammation response index; NLR, neutrophil-to-lymphocyte ratio; AUC, area under the curve.

sum, the ability to mirror the extent of inflammation corresponds to the ability to predict prognosis. For peripheral blood-relevant inflammatory markers, diversity compound modes are worth trying and easily realized, which might improve the predictive ability in specific diseases.

In fact, inflammation was not only a prognostic indicator for ICH patients but also a crucial therapeutic target based on the theory that cellular and molecular components of inflammation are involved in post-hemorrhagic secondary brain injury (41). Although the progression of developing specific therapeutic targets remains challenging, markers such as NOD-like receptor family, pyrin domain-containing 3 (NLRP3), C–C chemokine receptor type 1 (CCR1), and Toll-like receptor 4 (TLR4) are proven effective in intervening the progression of ICH-related inflammation (42–44).

There are several limitations to this study. First, follow-up blood tests at each follow-up time point were absent in this study due to incomplete baseline clinical data. For various reasons, it was inconvenient and difficult for some patients to have blood tests regularly, especially when they were not in the hospital. Second, the sample size was not large enough to be divided into training and validation cohorts for further verification. Third, more complicated and comprehensive prognostic patterns are needed to evaluate the prognosis of

TABLE 5 | Predictive models for predicting primary outcomes of ICH patients.

Original cohort	3-month functional outcome		Original cohort	1-month mortality	
Predictive models	**C-index**	**AIC**	**Predictive models**	**C-index**	**AIC**
Basic model[§]	0.8551	352.3467	Basic model[†]	0.8573	292.8231
Basic model + lymphocyte	0.8568	352.2308	Basic model + monocyte	0.8636	288.2819
Basic model + monocyte	0.8613	347.1597	Basic model + SIRI	0.8668	285.9310
Basic model + NLR	0.8606	349.0812			
Basic model + SIRI	0.8709	335.6420			
PSM cohort	**3-month functional outcome**		**PSM cohort**	**1-month mortality**	
Predictive models	**C-index**	**AIC**	**Predictive models**	**C-index**	**AIC**
Basic model[‡]	0.8400	244.9730	Basic model*	0.8162	181.2230
Basic model + neutrophil	0.8480	239.7489	Basic model + monocyte	0.8421	171.7294
Basic model + NLR	0.8520	239.9733	Basic model + SIRI	0.8339	173.6463
Basic model + SIRI	0.8570	232.8056			

[§]*Basic model: GCS, hematoma volume, hematoma location, treatment.*
[†] *Basic model: GCS, hematoma volume.*
[‡]*Basic model: GCS, hematoma volume.*
**Basic model: GCS.*
ICH, intracerebral hemorrhage; GCS, Glasgow Coma Scale; C-index, Harrell's concordance index; AIC, Akaike information criterion; NLR, neutrophil-to-lymphocyte ratio; SIRI, systemic inflammation response index.

ICH patients in various aspects, including cognitive function and quality of life. Fourth, not all patients were admitted to hospital within 24 h after onset. Although these patients were in the minority, this might induce unknown bias in laboratory results. Fifth, some occult infections cannot be diagnosed at an early stage by using clinical and laboratory criteria; this might also create bias. Finally, this analysis was conducted in a single institution; therefore, the results should be verified using multi-center data.

CONCLUSION

To our knowledge, this is the first study focusing on the prognostic significance of admission SIRI in patients with spontaneous ICH. In this study, SIRI was determined to be an independent predictive indicator for ICH patients in both 3-month functional outcomes and 1-month mortality. Furthermore, its prognostic predictive ability is better than that of NLR. In the near future, multi-center collabora tion is needed to further verify the results and illuminate the underlying mechanism.

DATA AVAILABILITY STATEMENT

The datasets for this study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Sichuan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JZ and JL: study design. JL and YY: data acquisition and writing—original draft. JL, XL, and JZ: statistical analysis. JL and ZY: result interpretation. JZ and HL: writing—review and editing. JZ: funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.718032/full#supplementary-material

Supplementary Figure 1 | Receiver operating characteristic curves of inflammatory markers for predicting 3-month functional outcome and 1-month mortality in the original cohort **(A,B)** and propensity score matching cohort **(C,D)**. SIRI, systemic inflammation response index; NLR, neutrophil-to-lymphocyte ratio; AUC, area under the curve.

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Evolution of Blood-Brain Barrier Permeability in Subacute Ischemic Stroke and Associations With Serum Biomarkers and Functional Outcome

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Background and Purpose: In the setting of acute ischemic stroke, increased blood-brain barrier permeability (BBBP) as a sign of injury is believed to be associated with increased risk of poor outcome. Pre-clinical studies show that selected serum biomarkers including C-reactive protein (CRP), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNFα), matrix metallopeptidases (MMP), and vascular endothelial growth factors (VEGFs) may play a role in BBBP post-stroke. In the subacute phase of stroke, increased BBBP may also be caused by regenerative mechanisms such as vascular remodeling and therefore may improve functional recovery. Our aim was to investigate the evolution of BBBP in ischemic stroke using contrast-enhanced (CE) magnetic resonance imaging (MRI) and to analyze potential associations with blood-derived biomarkers as well as functional recovery in subacute ischemic stroke patients.

Methods: This is an exploratory analysis of subacute ischemic stroke patients enrolled in the *BAPTISe* study nested within the randomized controlled *PHYS-STROKE* trial (interventions: 4 weeks of aerobic fitness training vs. relaxation). Patients with at least one CE-MRI before (v1) or after (v2) the intervention were eligible for this analysis. The prevalence of increased BBBP was visually assessed on T1-weighted MR-images based on extent of contrast-agent enhancement within the ischemic lesion. The intensity of increased BBBP was assessed semi-quantitatively by normalizing the mean voxel intensity within the region of interest (ROI) to the contralateral hemisphere ("normalized CE-ROI"). Selected serum biomarkers (high-sensitive CRP, IL-6, TNF-α, MMP-9, and VEGF) at v1 (before intervention) were analyzed as continuous and dichotomized

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variables defined by laboratory cut-off levels. Functional outcome was assessed at 6 months after stroke using the modified Rankin Scale (mRS).

Results: Ninety-three patients with a median baseline NIHSS of 9 [IQR 6–12] were included into the analysis. The median time to v1 MRI was 30 days [IQR 18–37], and the median lesion volume on v1 MRI was 4 ml [IQR 1.2–23.4]. Seventy patients (80%) had increased BBBP visible on v1 MRI. After the trial intervention, increased BBBP was still detectable in 52 patients (74%) on v2 MRI. The median time to v2 MRI was 56 days [IQR 46–67]. The presence of increased BBBP on v1 MRI was associated with larger lesion volumes and more severe strokes. Aerobic fitness training did not influence the increase of BBBP evaluated at v2. In linear mixed models, the time from stroke onset to MRI was inversely associated with normalized CE-ROI (coefficient −0.002, Standard Error 0.007, $p < 0.01$). Selected serum biomarkers were not associated with the presence or evolution of increased BBBP. Multivariable regression analysis did not identify the occurrence or evolution of increased BBBP as an independent predictor of favorable functional outcome post-stroke.

Conclusion: In patients with moderate-to-severe subacute stroke, three out of four patients demonstrated increased BBB permeability, which decreased over time. The presence of increased BBBP was associated with larger lesion volumes and more severe strokes. We could not detect an association between selected serum biomarkers of inflammation and an increased BBBP in this cohort. No clear association with favorable functional outcome was observed.

Trial registration: NCT01954797.

Keywords: ischemic stroke, subacute, biomarkers, blood-brain barrier, functional outcome

INTRODUCTION

Increased blood-brain barrier permeability (BBBP) is frequently observed after ischemic stroke and can be a sign of acute injury. In the subacute setting, increased BBBP may be a result of recovery mechanisms including neuroprotective inflammation and angiogenesis. Bernardo-Castro et al. and Yang et al. previously summarized the main pathophysiological processes likely underlying the evolution of BBBP in subacute stages post-stroke (1, 2). Directly following an acute vessel occlusion, oxidative stress and subsequent inflammation lead to early impairment of the blood-brain barrier (BBB). Subsequent reperfusion and endothelial damage lead to further BBB impairment (3) and can last up to weeks following the index event (4). The underlying pathophysiology of increased BBBP is highly complex (5, 6) and may play a crucial role in tissue recovery and outcome post-stroke. However, the exact time course and role of selected biomarkers in increased BBBP following ischemia remain poorly understood.

In the acute phase, both the ischemic event and subsequent reperfusion can result in oxidative stress and neuro-inflammation of the brain (1). The inflammatory processes result in increased cytokine levels such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNFα), and the acute phase reactant C-reactive protein (CRP) (7–9). Preclinical studies suggest that selected pro-inflammatory proteins and proteolytic enzymes may play a causal role in increased BBBP by negatively modifying tissue recovery following cerebral ischemia (10–13). Clinical imaging studies in stroke patients found high matrix metallopeptidases-9 (MMP-9) blood levels to be associated with secondary brain damage in the hyper-acute and acute stage after an ischemic stroke (14, 15). Animal studies with histological assessment of rat brains suggest that high MMP-9 expression in histological samples have a negative effect on BBB function and ultimately post-stroke recovery (16). In the acute setting of an ischemic stroke, the upregulation of vascular endothelial growth factor (VEGF) was observed to influence the BBB integrity by increasing para-cellular permeability (17, 18). Moreover, using magnetic resonance imaging (MRI) of rodent brains, Zhang et al. could show that intravenous application of VEGF may enhance angiogenesis in the ischemic penumbra during the subacute phase of an IS and hence potentially modify outcome (19).

Stroke rehabilitation studies hypothesize that through the reduction of oxidative stress and anti-inflammatory processes, aerobic fitness training may improve BBB integrity (20). In line with this hypothesis, previous studies suggest that physical training post-stroke may promote synaptic plasticity and enhance neurogenesis and angiogenesis, leading to better functional recovery (21–23).

Both the underlying mechanisms of increased vascular remodeling and angiogenesis were observed to be associated with better functional recovery following ischemia in rodent

studies (24). Previously published clinical studies using imaging techniques (including MRI and single photon emission computed tomography) found that increased BBBP in hyperacute and acute phases of ischemic stroke was associated with poor functional outcome (14, 25, 26). There are still no clinical studies published investigating potential associations of increased BBBP and functional recovery in subacute ischemic stroke patients. However, a comprehensive analysis of how selected blood serum biomarkers and aerobic physical training may affect BBBP post-stroke in the clinical setting is still lacking.

In this study, we aimed to assess the evolution of BBBP in subacute ischemic stroke assessed via contrast-enhanced MRI using the prospective *BAPTISe* study ("Biomarkers and Perfusion—Training-Induced changes after Stroke") that accompanied the randomized controlled stroke rehabilitation trial *PHYS-STROKE* ("Physical Fitness Training in Patients with Subacute Stroke"). In this exploratory analysis, we evaluated underlying associations of increased BBBP with selected blood biomarkers, exposure to early aerobic fitness training as well as long-term functional recovery after stroke.

MATERIALS AND METHODS

Study Design

All patients were enrolled in the prospective, observational *BAPTISe* study (27) nested within the multicenter, randomized-controlled *PHYS-STROKE* trial (28). In *PHYS-STROKE,* 200 subacute stroke patients were randomized to a 4-week intervention of aerobic bodyweight supported, treadmill-based physical fitness training vs. relaxation sessions. All participants provided written informed consent to participate in this study. The study was approved by the institutional review board of Charité–Universitätsmedizin Berlin (EA1/138/13). The trial did not show a significant difference in the co-primary efficacy endpoints: maximal walking speed and Barthel Index at 3 months after stroke. A detailed description of the trial intervention, outcome assessments, and the main analyses of the *PHYS-STROKE* trial were reported previously (29).

All patients enrolled in *BAPTISe* had a subacute ischemic stroke (5–45 days after stroke onset) and received cerebral MRI before and after the trial intervention. The time of stroke onset for each patient was documented based on written reports from the primary treating stroke unit and confirmed by patients and/or relatives. The main inclusion criteria of the *BAPTISe* study are listed in **Supplementary Table 1**. Patients who received at least one MRI with contrast agent application were eligible for this analysis. Inclusion and exclusion criteria are depicted in the study flow chart (**Figure 1**).

Clinical and Blood Biomarker Assessment

Patient demographics including medical history, treatment with systemic thrombolysis, Trial of ORG 10172 in Acute Stroke Treatment (TOAST) classification, and National Institute of Health Stroke Scale (NIHSS) at the primary treating stroke unit were documented. The NIHSS was additionally assessed at each MRI visit of the *BAPTISe* study before (v1) and after (v2) the intervention. Clinical follow-up took place at 3 and 6 months after stroke. To assess long-term functional outcome, we used the modified Rankin Scale (mRS) at 6 months post-stroke. A favorable outcome was defined as an mRS score of 0–2. In additional exploratory analyses, we defined an independent functional outcome at an mRS of 3, which reflects the median split of our cohort.

The following blood biomarkers were assessed prior to the start of the trial intervention at v1: high sensitive C-reactive protein (hs-CRP), IL-6, TNF-α, MMP-9, and VEGF. The biomarkers hs-CRP, TNF-α, and IL-6 were directly measured by solid-phase, chemiluminescent immunometric assays (IMMULITE® 1000, Siemens Healthcare Diagnostics) within 6 h after blood draw. Both MMP-9 and VEGF were analyzed from a subsample of patients by enzyme-linked immunosorbent assay (ELISA) in serum samples after being frozen at −80°C. The following laboratory cut-off levels were used to define elevated serum levels: hs-CRP ≥3.0 mg/L, IL-6 ≥3.6 pg/ml, TNF-α ≥8.1 pg/ml, VEGF ≥991 pg/ml, and MMP-9 ≥1,279 ng/ml.

All MRI examinations before (v1) and after (v2) the trial intervention were performed on a 3-Tesla MRI scanner (TIM Trio; Siemens AG, Erlangen, Germany). The MRI protocol of *BAPTISe* was previously published (27). Increased permeability of the BBB was assessed on T1-weighted images following intravenous administration of contrast agent (0.13 ml/kg body weight of Gadolinium).

Qualitative and Semi-quantitative Assessment of Increased BBBP

The presence of increased BBBP was qualitatively assessed and defined as a visual contrast-agent enhancement (CE) within the ischemic lesion on T1-weighted sequences (30–32). First the infarcted area was identified on diffusion weighted imaging (DWI; b1000), as presented in **Figure 2A**. Corresponding, T1-weighted sequences after contrast agent application were analyzed and assessed for CE within the area of diffusion restriction as presented in **Figure 2B**. The region with CE was defined as the region of interest (CE-ROI) and manually delineated by one experienced rater (S.M.). The CE-ROI was subsequently mirrored to the contralateral healthy hemisphere (mirrored ROI, see **Figures 2C,D**).

The intensity of increased BBBP was assessed semi-quantitatively by normalizing the mean voxel intensity of the CE-ROI to the mean voxel intensity of the mirrored ROI and defined as the normalized CE-ROI as depicted in **Figure 2**.

Subtraction Imaging: Evolution of BBBP

For visualization and qualitative evaluation of the evolution of BBBP within one subject, we used T1-weighted subtraction images. T1-weighted images before and after contrast agent application (pre- and post-contrast agent) injection were aligned and co-registered at v1 and v2 with the previously described ROI using FSL (FMRIB Software Library v6.0; https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/Fslutils) and ANTs (Advanced Normalization Tools; https://stnava.github.io/ANTs/) software. First, the pre—post-contrast agent image at v1 was subtracted from the pre-post-contrast agent image at v2. Subsequently, the evolution of

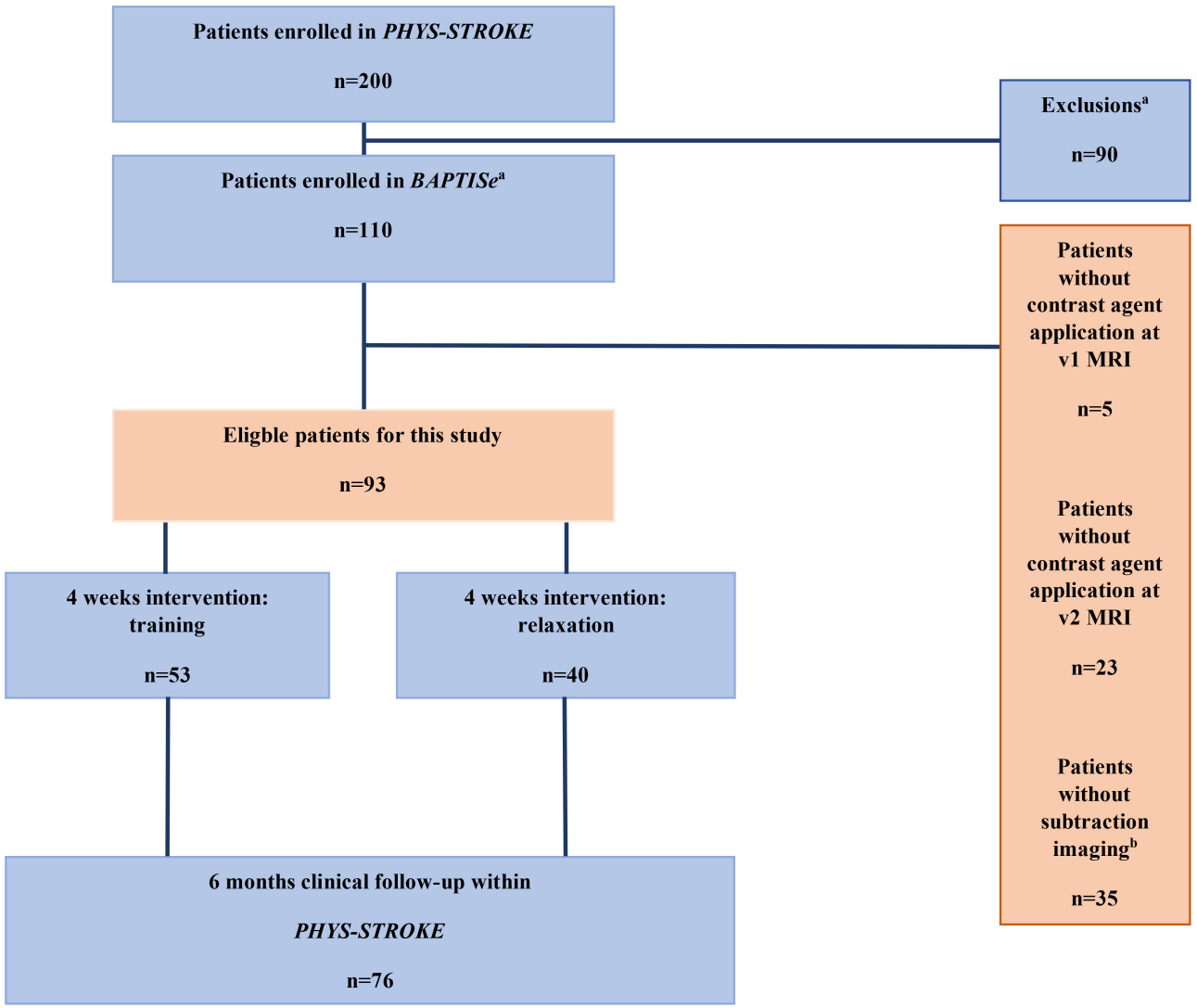

FIGURE 1 | Study flow chart. [a]Exclusion criteria for *BAPTISe* study are listed in **Supplementary Table 1**. [b]Exclusions due to complex post-processing steps.

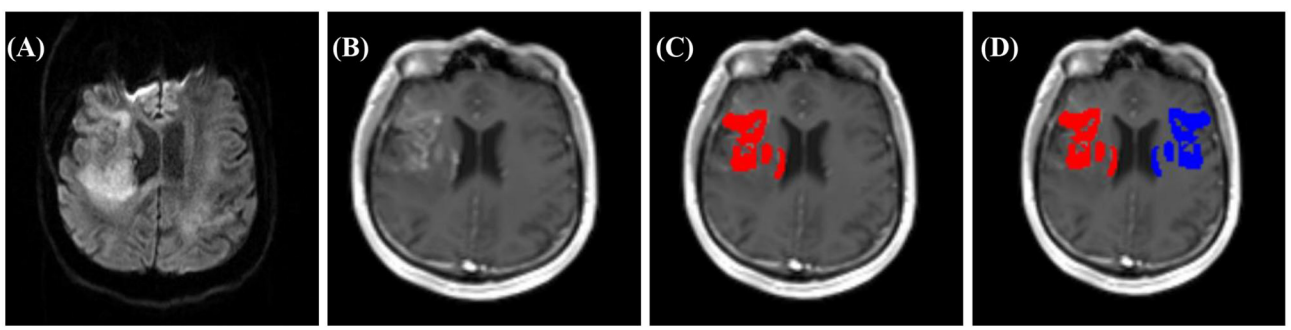

FIGURE 2 | Example of BBBP assessment in a patient with subacute right middle cerebral artery infarction. **(A)** DWI b1000 for the detection of the infarcted area, **(B–D)** T1 post-CA sequence: **(B)** with contrast-agent enhancement (CE), **(C)** delineation of CE within ischemic lesion (CE-ROI red), with **(D)** corresponding mirrored ROI (blue).

BBBP was qualitatively assessed on the resulting final subtraction images: a visible hyper-intense signal was defined as an increase of the BBBP and a hypo-intense signal as a decrease of BBBP over time. The absence of intensity changes was categorized as an unchanged BBBP.

Statistical Analysis

Continuous variables with skewed distribution are reported as a median with interquartile range (IQR); those with normal distribution are reported as means with standard deviations (SDs). We used the Student's *t*-test and Wilcoxon–Mann Whitney–*U*-test to evaluate the difference between groups of continuous variables. Categorical variables are presented as frequency and percentage. We compared categorical variables by using the chi-squared test.

In order to assess parameters associated with increased BBBP intensity on v1 and v2 MRIs, we performed linear mixed-model analyses with normalized lesion-CE as the dependent variable. The subject was included as a random effect. Fixed effects included time points of MRI (v1 vs. v2), time to MRI in days, and intervention group. In a second linear mixed-model analysis, the model was adjusted for parameters that reached significance ($p < 0.05$) in univariate analysis.

Furthermore, we performed logistic regression analysis to evaluate the association between increased BBBP and patient characteristics and blood biomarkers. The prevalence and evolution of increased BBBP were analyzed as dependent variables. We assessed the association of both the prevalence and evolution of increased BBBP and the dependent variable of favorable outcome at 6 months after stroke using multivariable logistic regression analyses: models were adjusted for age, sex, and variables that reached a significance of $p \leq 0.1$ in univariate analyses.

Due to the exploratory approach of this study, we did not correct for multiple testing. The significance level was defined as $p \leq 0.05$. All *p*-values constitute exploratory research and do not allow for confirmatory generalization of results. All statistical analyses were performed using SPSS Version 25 for Windows (SPSS Inc.) and Stata/IC 14.1 for Windows (StataCorp LP).

RESULTS

Prevalence, Intensity, and Evolution of Increased BBBP

Of 110 patients included in the *BAPTISe* study, 85% ($n = 93$) met the criteria of at least one MRI-scan with contrast agent application. Thirty-eight percent of the participants were women, the mean age was 68.5 years (*SD* 11.3), and the median acute NIHSS assessed in the primary treating stroke unit was 9 [IQR 6–12]. The median time to v1 MRI was 30 days [IQR 18–37], the median lesion volume at v1 was 4.3 ml [IQR 1.2–23.4], and the median NIHSS at v1 was 4 [IQR 3–9]. At v2, the median time to MRI was 60 days [IQR 46–70], the median lesion volume was 3.0 ml [IQR 0.9–26.1], and the median NIHSS was 3 [IQR 2–5]. Patient characteristics of the entire cohort are described in **Table 1**.

TABLE 1 | Patient demographics of the study cohort.

	Total ($n = 93$)
Age, mean (SD)	68.5 (11.3)
Female sex, % (*n*)	38 (35)
Cigarette smoking, % (*n*)	36 (33)
Hypertension, % (*n*)	82 (77)
DM, % (*n*)	29 (27)
Atrial fibrillation, % (*n*)	18 (17)
HLP,% (*n*)	52 (48)
i.v. thrombolysis, % (*n*)	32 (30)
Aerobic fitness training, % (*n*)	57 (53)
TOAST criteria	
Large artery atherosclerosis, % (*n*)	32 (30)
Cardioembolic, % (*n*)	28 (26)
Microangiopathic, % (*n*)	20 (19)
Others, % (*n*)	5 (5)
Undefined, % (*n*)	14 (13)
Lesion volume (mL)	
v1, median (IQR)	4.0 (1.2–23.4)
v2, median (IQR)	3.0 (0.9–26.1)
NIHSS	
Stroke Unit, median (IQR)	9 (6–12)
v1, median (IQR)	4 (3–9)
v2, median (IQR)	3 (2–5)
Time to MRI in days	
v1, median (IQR)	30 (18–37)
v2, median (IQR)	60 (46–70)

DM, diabetes mellitus; HLP, hyperlipoproteinemia; i.v., intravenous; TOAST, Trial of ORG 10172 in Acute Stroke Treatment classification; NIHSS, National Institute of Health Stroke Scale.

Eighty-eight patients (95%) underwent the v1 MRI with contrast agent application, compared to 70 patients at v2 (75%). Only 61% ($n = 65$) underwent an MRI with contrast agent application at both time points. Patient demographics of the analyzed cohort stratified by presence of increased BBBP at v1 and v2 are listed in **Table 2**.

By visual assessment, increased BBBP on v1 MRI was observed in 80% of patients ($n = 70$), compared to 74% ($n = 52$) on v2 MRI. In patients with increased BBBP at v1, the median time to MRI was 26 days [IQR17–35]; in patients without an increased BBBP at v1, the median time to MRI was 34 days [IQR17–44]. In patients with increased BBBP at v2, the median time to MRI was 56 days [IQR 46–67.25]; in patients without an increased BBBP at v2, the median time to MRI was 63 days [IQR 40–72]. The majority of participants (84%, $n = 78$) demonstrated an increased BBBP on MRI at any time point, i.e., either at v1 or at v2 or at both time points. Only 16% ($n = 15$) of patients demonstrated no visible BBBP at all. Characteristics of patients stratified by presence of increased BBBP at any time vs. no increased BBBP at all are listed in **Supplementary Table 2**.

In final subtraction imaging across time points, the majority of cases (78%, $n = 45$) experienced a decrease in BBBP over

TABLE 2 | Patient demographics stratified by increased blood-brain barrier permeability (BBBP) at MRI before (v1) or after (v2) intervention.

	Increased BBBP at v1		*p*-value	Increased BBBP at v2		*p*-value
	Yes ($n = 70$)	No ($n = 18$)		Yes ($n = 52$)	No ($n = 18$)	
Age, mean (SD)	68.1 (11.4)	69 (11.0)	0.9[a]	67.4 (11.1)	70.4 (11.8)	0.6[a]
Female sex, % (*n*)	40 (28)	33 (6)	0.6[b]	39 (20)	22 (4)	0.2[b]
Cigarette smoking, % (*n*)	36 (25)	44 (8)	0.2[b]	35 (18)	22 (4)	0.2[b]
Hypertension, % (*n*)	87 (61)	72 (13)	0.1[b]	83 (43)	83 (15)	1.0[b]
DM, % (*n*)	27 (19)	28 (5)	0.3[b]	23 (12)	44 (8)	0.2[b]
Atrial fibrillation, % (*n*)	16 (11)	22 (4)	0.4[b]	14 (7)	22 (4)	0.4[b]
HLP,% (*n*)	47 (33)	72 (13)	0.1[b]	42 (22)	72 (13)	0.1[b]
i.v. thrombolysis, % (*n*)	36 (25)	17 (3)	0.6[b]	35 (18)	17 (3)	0.2[b]
Aerobic fitness training, % (*n*)	54 (38)	67 (12)	0.6[b]	58 (30)	56 (10)	1.0[b]
TOAST criteria			0.7[b]			0.4[b]
Large artery atherosclerosis, % (*n*)	33 (23)	28 (5)		31 (16)	33 (6)	
Cardioembolic, % (*n*)	27 (19)	28 (5)		27 (14)	28 (5)	
Microangiopathic, % (*n*)	20 (14)	28 (5)		25 (13)	17 (3)	
Others, % (*n*)	6 (4)	0 (0)		10 (5)	0 (0)	
Undefined, % (*n*)	14 (10)	17 (3)		8 (4)	22 (4)	
Lesion volume (mL)						
v1, median (IQR)	4 (1.7–26.1)	1 (0.4–7.1)	*0.01*[c]	5 (1.4–26.1)	2 (0.4–7.2)	*0.03*[c]
v2, median (IQR)	4 (0.9–27.8)	2 (0.3–10.0)	0.1[c]	5 (1.2–32.8)	1 (0.3–7.4)	*0.02*[c]
NIHSS						
Stroke Unit, median (IQR)	9 (6–13)	9.5 (5–11)	0.5[c]	10 (6–12)	9 (5–11.25)	0.6[c]
v1, median (IQR)	5 (3–9)	3 (1.75–5.25)	*0.03*[c]	5 (3–9)	3 (2–4.25)	*0.02*[c]
v2, median (IQR)	3.5 (2–5)	2 (1–4.75)	*0.07*[c]	4 (2–6)	2 (1–4)	*0.03*[c]
Time to MRI in days						
v1, median (IQR)	26 (17–35)	34 (16.8–43.5)	0.1[c]	27 (19–35)	30 (12.5–44)	0.9[c]
v2, median (IQR)	57 (46–67)	64.5 (47.5–73.5)	0.3[c]	56 (46–67.25)	62.5 (39.5–72.25)	0.8[c]

DM, diabetes mellitus; HLP, hypolipoproteinemia; i.v., intravenous; TOAST, Trial of ORG 10172 in Acute Stroke Treatment classification; NIHSS, National Institute of Health Stroke Scale.
[a] *t-test.*
[b] *Chi²-test.*
[c] *Mann–Whitney U-Test.*
Italic values emphasize the values that reached a significance levels/p-value ≤0.05.

time. In only three cases, an increase was detectable. Ten cases of BBBP increase remained unchanged over time. Examples of BBBP increase and decrease detection are presented in **Supplementary Figures 1, 2**.

Patient Characteristics and BBBP

Visually assessed presence of increased BBBP at v1 was associated with larger median lesion volumes (4.4 ml [IQR 1.7–26.1] vs. 1.0 ml [IQR 0.4–7.1]; $p = 0.01$) and more severe stroke assessed by median NIHSS scores at v1 (5 [IQR 3–9] vs. 3 [IQR 2–5]; $p = 0.03$) in univariate analyses (**Table 2**). At v2, we could observe a similar association of visibly increased BBBP and larger median lesion volumes (4.7 ml [IQR 1.2–32.8] vs. 1.4 ml [IQR 0.3–7.4]; $p = 0.02$) and more severe strokes assessed by median NIHSS scores (4 [IQR 2–6] vs. 2 [IQR 1–4]; $p = 0.03$). There was no association between the presence of qualitatively increased BBBP and intravenous thrombolysis at v1 [36% ($n = 25$) vs. 17% ($n = 3$); $p = 0.6$] or v2 [35% ($n = 18$) vs. 17% ($n = 3$); $p = 0.2$, see **Table 2**]. Additionally, occurrence rates of increased BBBP were not significantly higher in the trial intervention group of aerobic fitness training at v2 (increased BBBP 58 vs. no increased BBBP 56%, see **Table 2**).

Further, increased BBBP was more frequently detected in females, both on v1 and v2. In additional exploratory *post-hoc* analysis, no independent association of female sex and increased BBBP at both time points could be observed (see **Table 3**). Here, prior history of hyperlipoproteinemia was observed to modify the risk of increased BBBP at v2 (OR 0.3, 95% CI 0.1–1.0; $p = 0.04$, see **Table 3**). After adjusting for pre-existing statin medication as secondary prevention post-stroke, pre-diagnosis of HLP was no longer significantly associated with lower risk of increased BBBP at v2 (OR 0.3 95% CI 0.1–1.0, $p = 0.07$).

In adjusted linear mixed models for the semi-quantitatively assessed intensity of increased BBBP (presented in **Table 4**), only the time from stroke onset to MRI was identified as a main effect and was inversely associated with increased BBBP [coefficient −0.002; Standard Error (SE) 0.007, $p < 0.01$]. Age, sex, and arterial hypertension were not identified as modifying factors.

TABLE 3 | Multivariate regression analyses of factors associated with presence of increased BBBP before (v1) and after (v2) intervention.

	Odds ratio (95% CI)	*p*-value
Presence of increased BBBP v1		
Female sex	1.3 (0.4–5.0)	0.7
Age	1.0 (0.9–1.0)	0.5
Arterial hypertension	6.0 (0.9–39.8)	0.1
Hyperlipoproteinemia	0.4 (0.1–1.5)	0.2
Lesion volume at v1	1.0 (1.0–1.1)	0.3
NIHSS at v1	1.1 (0.9–1.3)	0.5
Presence of increased BBBP v2		
Female sex	2.5 (0.7–9.8)	0.2
Age	1.0 (0.9–1.0)	0.4
Arterial hypertension	1.1 (0.2–7.2)	0.9
Hyperlipoproteinemia	0.3 (0.1–1.0)	*0.04*
Lesion volume at v2	1.0 (1.0–1.1)	0.4
NIHSS at v2	1.2 (0.9–1.6)	0.4

Adjustment for variables which reached a significance level of $p \leq 0.05$ in univariate analyses (lesion volume and severity of stroke based on NIHSS) and variables known from literature to influence the BBBP (age, sex, arterial hypertension and hyperlipoproteinemia). NIHSS, National Institute of Health Stroke Scale.
Italic values emphasize the values that reached a significance levels/p-value ≤0.05.

TABLE 4 | Linear mixed models for normalized contrast enhancement within region of interest (normalized CE-ROI) at MRI before (v1) and after (v2) intervention.

Dependent variable: normalized CE-ROI			
	Fixed-effects		
	Coefficient	**Std. Error**	***p*-value**
Time to MRI in days	−0.002	0.001	*0.001*
Time point of MRI (v1 vs. v2)	−0.009	0.026	0.724
Intervention group (training)	0.006	0.18	0.735
	Random-effects		
	Estimate	**Std. Error**	**95% CI**
Subject ID	0.044	0.018	0.020 – 0.096
Dependent variable: *adjusted normalized CE-ROI			
	Fixed-effects		
	Coefficient	**Std. Error**	***p*-value**
Time to MRI in days	−0.002	0.001	*0.001*
Time point of MRI (v1 vs. v2)	−0.011	0.026	0.672
Intervention group (training)	0.005	0.018	0.776
Arterial hypertension	−0.028	0.026	0.297
Age	<0.001	0.001	0.945
Male sex	−0.006	0.018	0.761
	Random-effects		
	Estimate	**Std. Error**	**95% CI**
Subject ID	0.039	0.021	0.014–0.111

**Adjusted for age, sex, and hypertension.*
Italic values emphasize the values that reached a significance levels/p-value ≤0.05.

Exploratory analysis showed that pre-existing atrial fibrillation was more frequently found in patients with persisting BBBP assessed by subtraction imaging (39 vs. 9%; $p < 0.001$). Concerning the trial intervention, we did not find differences between persisting and decreasing BBBP over time (54 vs. 58%, $p = 0.8$). **Supplementary Table 3** summarizes the patient demographics stratified by evolution of BBBP at follow-up.

TABLE 5 | Multivariate regression analyses of elevated low-density lipoprotein (LDL) levels before (v1) intervention with presence of increased BBBP before intervention and during follow-up.

	Crude OR (95% CI)	*p*-value	Adjusted* OR (95%CI)	*p*-value
Presence of increased BBBP v1				
High LDL v1	2.8 (1.0–8.4)	0.06	2.1 (0.5–8.5)	0.3
Presence of increased BBBP v2				
High LDL v1	2.9 (0.9–9.1)	0.07	4.4 (1.0–19.4)	*<0.03*
Presence of increased BBBP at any time				
High LDL v1	4.8 (1.5–15.4)	*<0.01*	4.9 (1.1–22.2)	*<0.02*
Increase/unchanged BBBP				
High LDL v1	0.2 (0.02–1.7)	0.1	0.2 (0.02–1.9)	0.2

**Adjusted for female sex, age, arterial hypertension, HLP, lesion volume, and NIHSS. HLP, hyperlipoproteinemia; LDL, low-density lipoprotein; NIHSS, National Institute of Stroke Scale; OR, odds ratio; CI, confidence interval.*
Italic values emphasize the values that reached a significance levels/p-value ≤0.05.

Serum Biomarkers and BBBP

Due to the observation that hyperlipoproteinemia might influence the BBBP, we performed additional *post-hoc* analyses on low-density lipoprotein (LDL) and high-density lipoprotein (HDL): in univariate analysis, we observed a significant association between high LDL levels defined by laboratory cut-offs and an increased BBBP at v1 (78 vs. 56%; $p = 0.01$). Moreover, we found LDL at v1 as continuous (2.2 mmol/L [IQR 1.8–2.5] vs. 1.6 mmol/L [IQR 1.2–2.3]; $p = 0.02$) and dichotomized (76 vs. 40%; $p < 0.01$) variable to be associated with an increased BBBP at any time (see **Supplementary Table 4**). In multivariate analysis adjusted for age, sex, arterial hypertension, HLP, lesion volume, and stroke severity, high LDL levels were still significantly associated with the presence of increased BBBP at v2 (OR 4.4 95% CI 1.0–19.4, $p = 0.03$) and at any time (OR 4.9 95% CI 1.1–22.2, $p = 0.02$; see **Table 5**).

High-sensitive CRP was available in 89 patients (96%), whereas TNFα and IL-6 values were available in 88 patients (95%) and 91 patients (98%), respectively. Levels of MMP-9 and VEGF were only available in a minority of blood samples (both $n = 36$, 39%). No differences between the levels of serum biomarkers (hs-CRP, TNFα, IL-6, VEGF, and MMP-9) as continuous and dichotomized variables before the intervention (v1) were observed in patients with or without increased BBBP neither at v1 nor at v2 (**Table 6**). Further, serum biomarker levels did not differ between patients with presence of increased BBBP at any time point and patients without increased BBBP at all (**Supplementary Table 5**). Moreover, there were no significant differences between the selected blood-derived biomarkers at v1 in patients with an increase of/unchanged BBBP and those with a decrease of BBBP over time (**Supplementary Table 6**).

Differences in baseline characteristics between patients with and without elevated biomarkers levels are presented in **Supplementary Table 7**. As depicted in **Supplementary Table 8**,

TABLE 6 | Serum biomarkers before (v1) intervention in patients with or without increased BBBP before and after (v2) intervention.

	Total	Increased BBBP v1		*p*-value	Increased BBBP v2		*p*-value
		Yes (*n* = 70)	No (*n* = 18)		Yes (*n* = 52)	No (*n* = 18)	
hsCRP mg/L, median (IQR)	4.8 (1.2–10.6)	2.6 (1.1–7.8)	1.6 (0.5–8.5)	0.4[a]	5.2 (1.7–10.8)	4.9 (0.9–17.6)	0.6[a]
TNFα pg/mL, median (IQR)	8.2 (6.6–9.7)	8.1 (6.6–10.8)	7.6 (6.1–8.6)	0.2[a]	7.7 (6.1–9.6)	7.6 (6.1–8.5)	0.5[a]
IL-6 pg/mL, median (IQR)	3.6 (2.4–6.1)	3.5 (2.4–5.9)	3.7 (1.9–8.6)	0.8[a]	3.6 (2.4–5.3)	3.4 (2.1–7.6)	1.0[a]
VEGF pg/mL, median (IQR)	718 (433–1,070)	718 (436–1,025)	855 (549–1,397)	0.3[a]	762 (433–1,070)	663 (341–1,203)	0.7[a]
MMP–9 ng/mL, median (IQR)	1,050 (846–1,318)	955 (836–1,316)	1,294 (870–1,477)	0.3[a]	1,092 (864–1,344)	942 (682–1,294)	0.6[a]
High hsCRP,% (*n*)	59.1 (55)	60 (42)	56 (10)	0.9[b]	64 (33)	56 (10)	0.7[b]
High TNFα, % (*n*)	48 (45)	49 (34)	33 (6)	*0.05*[b]	46 (24)	39 (7)	0.3[b]
High IL–6, % (*n*)	43 (40)	41 (29)	44 (8)	0.8[b]	44 (23)	50 (9)	0.7[b]
High VEGF, % (*n*)	13 (12)	11 (8)	17 (3)	0.8[b]	15 (8)	11 (2)	0.6[b]
High MMP-9, % (*n*)	13 (12)	11 (8)	22 (4)	0.2[b]	14 (7)	11 (2)	1.0[b]

[a]*Mann–Whitney U-Test.*
[b]*Chi^2-test.*
Italic values emphasize the values that reached a significance levels/p-value ≤0.05.

TABLE 7 | Multivariate regression analyses of increased BBBP before intervention and during follow up and favorable functional outcome (mRS<3).

Modified Rankin Scale: favorable outcome 6 months		
	Crude OR (95% CI)	Adjusted OR (95%CI)
Presence of increased BBBP v1	1.1 (0.8–1.4)	0.9 (0.6–1.4)[a]
Presence of increased BBBP v2	1.0 (0.9–1.2)	0.9 (0.7–1.2)[b]
Increased BBBP at any time	0.7 (0.2–2.2)	0.2 (0.02–1.3)[c]
Increase/unchanged BBBP	1.1 (1.0–1.3)	1.1 (1.0–1.3)[d]

[a]*Adjusted for sex, age, and variables that reached a significance of ≤0.1 in univariate analysis (aHT arterial hypertension, HLP hyperlipoproteinemia, NIHSS National Institute of Health Stroke Scale at v1, lesion volume at v1, time to v1 MRI in days).*
[b]*Adjusted for sex, age, and variables that reached a significance of ≤0.1 in univariate analysis (HLP hyperlipoproteinemia, NIHSS National Institute of Health Stroke Scale at v1, lesion volume at v1).*
[c]*Adjusted for sex, age, and variables that reached a significance of ≤0.1 in univariate analysis (HLP hyperlipoproteinemia, NIHSS National Institute of Health Stroke Scale at v1, lesion volume at v1, time to v1 MRI in days).*
[d]*Adjusted for sex, age, and variables that reached a significance of ≤0.1 in univariate analysis (atrial fibrillation).*
OR, odds ratio; CI, confidence interval.

no association was observed between levels of serum biomarkers at v1 and BBB permeability at v1 or at v2 following adjustment for selected cerebrovascular risk factors.

BBBP and Functional Outcome

At 6 months follow-up, 41% ($n = 31$) of patients had a favorable functional outcome (mRS 0–2) and 59% ($n = 45$) had an mRS ≥3. Neither the presence of increased BBBP on v1 (adjusted OR 0.9, 95% CI 0.6–1.4) nor the evolution of increased permeability (adjusted OR 1.1, 95% CI 1.0–1.3) was associated with favorable outcome 6 months after stroke (**Table 7**).

In *post-hoc* multivariate regression analyses using the median split (mRS 0–3: 68%, $n = 52$ vs. mRS 4–6: 32%, $n = 24$), we observed that a persisting BBBP in subtracted images was associated with worse functional outcome in patients at 6 months post-stroke (adjusted OR 1.2, 95% CI 1.0–1.4; $p = 0.02$).

DISCUSSION

In this exploratory analysis of the *BAPTISe* study, we observed that increased BBBP evaluated on contrast-enhanced MRI following moderate-to-severe ischemic stroke was detectable in approximately three out of four cases in early subacute phase of stroke. The presence and intensity of BBBP decreased over time; however, it remained detectable up to 2 months reflecting persisting BBB changes throughout recovery processes in the subacute phase post-stroke. Furthermore, we could demonstrate an association of an increased BBBP with larger lesion volumes and more severe strokes both before and after

the trial intervention. To the best of our knowledge, this is the first clinical study to investigate the effect of increased BBBP on long-term outcome in subacute stroke patients.

Although increased BBBP assessed via contrast-enhanced MRI is frequently observed in the hyper-acute and acute phase following ischemia (3, 4, 33–36), the dynamic in later stages is far less understood. Several animal studies could demonstrate that an increased BBBP is still detectable up to 3 weeks following an ischemic event (3, 4). Previous clinical studies support the theory that BBBP may persist into subacute stages in some cases (33, 35, 36). In this study, we were able to observe that most stroke patients still demonstrated an increased BBBP of the ischemic lesion visible on contrast-enhanced MRI at 2 months after the acute event.

BBB dynamics are believed to be diverse and likely influenced by stroke severity (34). In our analyses, stroke severity defined by NIHSS was associated with the presence of an increased BBBP. This has not yet been observed in previous clinical BBBP studies (16–18); albeit BBBP was assessed using differing methodologies including CSF fluid-serum albumin ratios or CT-perfusion imaging in these studies. Moreover, in the current study, serially performed MRI allowed an evaluation of BBBP evolution over time. We observed that BBBP tended to decrease over time in this patient cohort. MR-based detection of increased BBBP might help to understand underlying regeneration mechanisms such as vascular remodeling and possible associations with functional outcome (24).

Whereas, previous studies have suggested that selected cytokines such as TNFα and IL-6 may serve as surrogate markers of increased BBBP (7, 8), we found no robust associations between selected pro-inflammatory biomarkers and BBB integrity in this patient cohort derived from a randomized-controlled stroke rehabilitation trial. Moreover, the levels of selected biomarkers representing pathophysiological mechanisms such as enzymatic proteolysis (MMP-9) and vascular remodeling (VEGF) did not differ between patients with and without increased BBBP.

One of our primary aims was to determine whether blood biomarkers could serve as surrogate markers of the presence and evolution of increased BBBP in subacute stroke. A handful of previously published studies described underlying associations between selected serum biomarkers and BBBP. For example, it has been shown that IL-6 increases BBB dysfunction in rodent models (7). Interestingly, it has been demonstrated that the inhibition of VEGF signaling diminishes the BBB impairment (37). This phenomenon was only observed in mice with pre-existing diabetes. However, in the current exploratory study, we found no robust associations between TNFα, hsCRP, IL-6, VEGF, and MMP-9 levels at v1 and the prevalence or evolution of BBBP in this cohort of subacute stroke patients.

We observed a higher rate of increased BBBP in patients with high LDL-cholesterol levels at inclusion (**Table 5** and **Supplementary Table 4**). Our findings support the observation that elevated LDL levels influence the occurrence of an increased BBBP in the subacute stages of ischemic stroke. Cholesterol levels might influence BBB integrity through activation of inflammation and oxidative stress as described previously (38). Interestingly, previous clinical studies found that low LDL levels and low total cholesterol levels to be associated with higher rates of hemorrhagic transformation (39–41). Whether there is a causal connection between the effects of cholesterol levels on BBB integrity and increased risk of hemorrhagic transformation post-stroke should be investigated in detail in future, independent analyses.

Of note, preclinical studies have reported contradicting results. For example, Kalayci et al. (42) suggested that hypercholesterolemia might have a positive effect on the BBB by increasing the expression of tight junction proteins and thereby possibly decreasing paracellular permeability in rodents. Since the increased BBBP in the subacute stage of the ischemic event also represents regenerative processes, further studies on the lipoprotein's influence in the subacute setting may be of interest. Nevertheless, a comprehensive analysis in a larger independent cohort of stroke patients is warranted to support or refute the observations reported here.

Of note, due to the advantages and disadvantages of single biomarkers, there may be additional value of a combination of selected biomarkers for the prognostic value of increased BBBP. In their clinical study, Tu et al. (43) proclaimed that a panel of neuroendocrine biomarkers predicts functional outcome more efficiently than the NIHSS or single biomarkers alone, suggesting that certain biomarker panels may help in the early evaluation of stroke.

Previous studies suggest that the application of intravenous thrombolysis in the setting of acute stroke may contribute to BBB alteration due to its potential neurovascular toxicity and effect on neuro-inflammation following ischemia (44). However, we found no correlation between increased BBBP and intravenous thrombolysis in the current analysis.

The effect of training on the BBB is still under investigation; previous studies have implied that physical training might reduce oxidative stress and inflammatory processes and thereby strengthen the BBB integrity (20). In our study, a 4-week aerobic fitness training intervention was not identified as a modifier of BBBP over time (**Table 2**). These results are in line with the co-primary efficacy endpoints of the *PHYS-STROKE* trial. Here, no associations of the maximum walking speed and Barthel Index at 3 months post-stroke and the intervention were observed (29).

Assuming that BBBP affects the regenerative processes following ischemic tissue damage on a molecular basis, one might expect that long-lasting BBBP post-stroke may modify functional recovery (1). Previous studies that used imaging techniques as well as cerebrospinal fluid/serum albumin ratios to quantify the BBBP found that BBB alteration in the acute setting influenced patients' long-term outcome (14, 25, 26). Although in the current analysis, increased BBBP in subacute stages post-stroke were not associated with a favorable functional long-term outcome (mRS <3), we observed an association between a persisting BBBP and worse functional outcome (mRS >3). However, these findings should be validated in larger, independent cohort analyses. Detection of persisting BBBP in subacute stages could, for example, guide individual rehabilitation strategies (45, 46) if the studies find prognostic value in prolonged BBBP. The

identification of a surrogate marker of BBBP (i.e., via increase in selected serum biomarkers) would provide an accessible tool to easily assess BBBP in the clinical setting and subsequently guide further therapeutic procedures.

In summary, this is the first study to show increased BBBP on contrast-enhanced MRI through early and late subacute stages of stroke, which takes us closer to understanding the complex regeneration and recovery processes taking place after ischemia (24, 32). A deeper knowledge of the prognostic value of patient-individual BBBP dynamics in subacute stages may guide therapeutic and rehabilitation strategies in the future.

LIMITATIONS

This analysis has several limitations that warrant discussion. First, this is an exploratory analysis as part of a randomized-controlled stroke rehabilitation trial. Therefore, the existing MRI protocol was not explicitly designed for BBB evaluation. However, dynamic contrast enhanced-MRI is the most well-established method for the visualization and quantification of BBBP (30, 47, 48) and contrast-enhanced MRI is a reliable method to assess BBB integrity and has been successfully applied in previous clinical studies (5, 31). Of note, the time point of MR-imaging at v1 and v2 was inhomogeneous, causing overlap, and limiting comparability across individuals with differing imaging time points. Furthermore, we used subtraction maps to show increased BBBP changes within one subject over time (49, 50); the limitations of this technique include complex post-processing steps and the final visual evaluation of the subtraction images, which is subject to rater-bias. Lastly, the number of participants who received a contrast agent application at both v1 and v2 MRI time points was low, minimizing the sample size considerably and increasing the risk of Type II errors. Concerning the blood-based biomarkers, the total numbers of MMP9 and VEGF measurements were likewise considerably low, which also limits the statistical power of this analysis. Of note, there are several well-known biomarkers of inflammation influencing the BBBP in subacute stroke such as IL-1beta and IL-8 (51, 52). Although these cytokines were not included in the current analysis, they could add diagnostic value in further analyses.

CONCLUSION

The permeability of the blood-brain barrier assessed on contrast-enhanced MRI decreases during the subacute phase of ischemic stroke but remains detectable for up to 2 months in three out of four patients after moderate-to-severe ischemic stroke. The presence of increased BBBP is associated with more severe strokes and larger infarct volumes. We did not observe a relation between the presence of increased BBBP and exposure to aerobic fitness training. In our cohort, we could not detect an association between the selected serum biomarkers (hs-CRP, TNFα, IL-6, MMP-9, and VEGF) and the phenomenon of an increased BBBP. No clear associations were observed between an increased BBBP and functional outcome at 6 months post-stroke; hence, the long-term prognostic value of the phenomenon in subacute phases of an ischemic stroke remains unclear and warrants further investigation in independent cohorts.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional review board of Charité-Universitätsmedizin Berlin (EA1/137/13 and EA1/138/13). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AF, MEb, AN, and TR conceived or designed the study and supervised the study. SM was responsible for analyzing the data and drafted the manuscript. AK and SP contributed in statistical analysis. AF, MEb, and MEn obtained funding. AN attests that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted. All authors critically revised the manuscript for important intellectual content and gave final approval of the version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.730923/full#supplementary-material

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Hemorrhagic Stroke Induces a Time-Dependent Upregulation of miR-150-5p and miR-181b-5p in the Bloodstream

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To date, the only effective pharmacological treatment for ischemic stroke is limited to the clinical use of recombinant tissue plasminogen activator (rtPA), although endovascular therapy has also emerged as an effective treatment for acute ischemic stroke. Unfortunately, the benefit of this treatment is limited to a 4.5-h time window. Most importantly, the use of rtPA is contraindicated in the case of hemorrhagic stroke. Therefore, the identification of a reliable biomarker to distinguish hemorrhagic from ischemic stroke could provide several advantages, including an earlier diagnosis, a better treatment, and a faster decision on ruling out hemorrhage so that tPA may be administered earlier. microRNAs (miRNAs) are stable non-coding RNAs crucially involved in the downregulation of gene expression *via* mRNA cleavage or translational repression. In the present paper, taking advantage of three preclinical animal models of stroke, we compared the miRNA blood levels of animals subjected to permanent or transient middle cerebral artery occlusion (MCAO) or to collagenase-induced hemorrhagic stroke. Preliminarily, we examined the rat miRNome in the brain tissue of ischemic and sham-operated rats; then, we selected those miRNAs whose expression was significantly modulated after stroke to create a list of miRNAs potentially involved in stroke damage. These selected miRNAs were then evaluated at different time intervals in the blood of rats subjected to permanent or transient focal ischemia or to hemorrhagic stroke. We found that four miRNAs—miR-16-5p, miR-101a-3p, miR-218-5p, and miR-27b-3p—were significantly upregulated in the plasma of rats 3 h after permanent MCAO, whereas four other different miRNAs—miR-150-5p, let-7b-5p, let-7c-5p, and miR-181b-5p—were selectively upregulated by collagenase-induced hemorrhagic stroke. Collectively, our study identified some selective miRNAs expressed in the plasma of hemorrhagic rats and pointed out the importance of a precise time point measurement to render more reliable the use of miRNAs as stroke biomarkers.

Keywords: microRNA, stroke hemorrhagic, biomarker (BM), rat, blood

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INTRODUCTION

Cerebral ischemia results from the interruption of blood flow to a brain region caused by two possible events: a hemorrhagic break or an ischemic occlusion of a cerebral vessel (1, 2). Hemorrhagic stroke accounts for 15% of all strokes, whereas ischemic stroke accounts for 85% of cases. According to the Global Burden of Disease Study, performed from 1990 to 2013, stroke is the second main cause of death (representing 11.8% of all deaths worldwide) and the third leading cause of disability-adjusted life years worldwide (3–6).

To date, the only effective pharmacological treatment for ischemic stroke is limited to the use of recombinant tissue plasminogen activator (rtPA) (7), although endovascular therapy has also emerged as an effective treatment for acute ischemic stroke (8). By contrast, the emergency treatment of hemorrhagic stroke focuses on controlling bleeding and reducing pressure in the brain (1, 2). Unfortunately, the benefit of rtPA treatment for ischemic stroke is time dependent, limited to a 4.5-h therapeutic time window, a largely recognized useful time for penumbra restoring; therefore, the majority of patients cannot benefit from this therapy (9, 10) due to the longer average time needed to carry out an effective diagnosis and therapeutic directioning (11). Most importantly, the use of rt-PA is contraindicated in the case of hemorrhagic stroke, as it would worsen the hemorrhage.

Modern neuroimaging tools, such as computed tomography (CT) or magnetic resonance imaging (MRI), are now used to diagnose a stroke and identify its subtype (12, 13). However, some hospitals do not yet provide MRI and CT service, and many patients cannot benefit from these techniques in the diagnostic process. Moreover, the time required to reach the medical center and to prepare the patients for imaging tests often does not match with the urgency of an early diagnosis for an immediate therapy. In addition, the application of these imaging tools weighs on stroke care costs (14). For all these reasons, the search of biomarkers is critically important to speed up the diagnosis of stroke and selectively distinguish cerebral ischemia from hemorrhagic damage in order to ensure therapeutic interventions in a very short time frame from the onset of the disease.

microRNAs (miRNAs) are evolutionarily conserved non-coding RNAs consisting of 20–22 nucleotides (15), crucially involved in the downregulation of gene expression *via* mRNA cleavage or translational repression (16). Over the last 10 years, the role of miRNAs in stroke has been widely discussed and evaluated, focusing attention on the regulation of stroke risk factors (17) and the mechanisms activated and elicited by the ischemic insult (18).

Abbreviations: rtPA, recombinant tissue plasminogen activator; MCAO, middle cerebral artery occlusion; pMCAO, permanent middle cerebral artery occlusion; tMCAO, transient middle cerebral artery occlusion; ICH, intracerebral collagenase-induced hemorrhage; miRNA, microRNA; DALYs, disability-adjusted life years; CT, computed tomography; MRI, magnetic resonance imaging; HDL, high-density lipoproteins; CBF, cerebral blood flow; PRC2, polycomb repressive complex 2; EZH2, enhancer of zeste homolog 2; H3K27me3, tri-methylation of histone 3 at lysine 27; NCX1, sodium/calcium exchanger 1; ZnT6, zinc transporter 6; TRPM7, transient receptor potential cation channel subfamily M, 7; ASIC1, acid-sensing ionic channel 1.

For the first time, in the last decade, miRNAs have been observed outside the cells, including in various body fluids (19). Several release mechanisms have been hypothesized: one of these consists in microRNA release by cells through microvesicles that originate by outward budding and fission of the plasma membrane (20). Moreover, a specific type of vesicle with a characteristic process of biogenesis, called exosomes, is shown to be enriched of miRNA and involved in the phenomena of cell-to-cell communication (21). Alternative mechanisms of miRNA transport concern the activity of apoptotic bodies, formed during the programmed cell death, and high-density lipoproteins. In this scenario, circulating microRNAs become important mediators of cell communication by altering the gene expression of recipient cells, and the modification of their release in biofluids reflects the expression changes occurring in the origin cells (22).

Whatever the origin of miRNA is, the presence of microRNA in blood and the ability to measure their levels in a non-invasive way have opened new doors in the search for peripheral biomarkers for the diagnosis and prognosis of diseases such as brain ischemia. Indeed the expression levels of miRNAs in blood are reproducible and indicative of several diseases (23). Since the recommended therapeutic window is very limited, the biomarkers for stroke have the potential to expedite diagnosis and the institution of treatment.

In the present comparative report, the plasma microRNA levels were assessed in both rat models of ischemic and hemorrhagic stroke in order to characterize a specific signature of microRNA potentially useful to distinguish among stroke subtypes.

METHODS

Animals

Male Sprague–Dawley rats (Charles River), weighing 200–250 g, were housed under diurnal lighting conditions (12 h darkness/light) and in a conditioned room (23°C). The experiments were performed according to the international guidelines for animal research and approved by the Animal Care Committee of "Federico II," University of Naples, Italy. The animals, during any surgical or invasive procedure, were anesthetized using a mixture of oxygen and sevoflurane at 3.5% (Medical Oxygen Concentrator LFY-I-5A), and the rectal temperature was maintained at 37 ± 0.5°C with a heat-controlled mat (Harvard Apparatus). The rats were randomly assigned into experimental and control groups.

Among the 55 animals used for the present study, seven have been excluded from the statistical analysis. In particular, two died during the surgical procedures of hemorrhage induction, and five were excluded for lack of achievement of stroke models.

Permanent and Transient Focal Ischemia

Stroke was induced by middle cerebral artery occlusion (MCAO) by introducing a suture filament into the internal carotid artery until the middle cerebral artery (24), modified and readapted in our laboratory (25). Briefly, under an operating stereomicroscope (Nikon SMZ800, Nikon Instruments, Florence, Italy), right carotid bifurcation was identified and exposed by using surgical

pincers (Dumont #7, FST), and the external carotid artery near the bifurcation was cut and electrocauterized to create a stump on the artery. A silicon-coated nylon filament (Doccol, CA, USA) was inserted through the stump and gently advanced 19 mm into the right internal carotid artery in order to reach the origin of the middle cerebral artery. All animals were sacrificed after 24 h from MCAO. In the model of transient ischemia, the filament was removed after 100 min, and the animals were sacrificed after 3 and 24 h from reperfusion.

Intracerebral Collagenase-Induced Hemorrhage

Intracerebral hemorrhagic stroke was induced in rats as previously described (26, 27) and adapted in our laboratory. Briefly, using a stereotaxic apparatus, the anesthetized rats were injected with 0.5 U collagenase 2-μl volume dissolved in phosphate-buffered saline (collagenase type VII from Sigma-Aldrich, catalog number C2399) through a Hamilton syringe (26-gauge needle, 10-μl volume) in a burr hole into the right striatum (3 mm lateral to midline, 0.5 mm anterior to bregma, and 5.2 mm below the surface of the skull) over 5 min. The syringe was kept in place for 10 min and removed over 5 min. The animals were sacrificed after 24 h from collagenase injection.

Monitoring of Blood Gas Concentration and Cerebral Blood Flow

A catheter was inserted into the femoral artery to measure the arterial blood gases before and after ischemia (Rapid lab 860; Chiron Diagnostic, Medfield, MA, USA). Induction of ischemia was confirmed by monitoring the regional cerebral blood flow (CBF) in the area of the right MCA through a disposable microtip fiber optic probe (diameter, 0.5 mm) applied on the right temporo-parietal region of the skull, connected through a Master Probe to a laser Doppler computerized main unit (PF5001; Periflux system, 5000, Perimed AB, Järfälla, Sweden), and analyzed using PSW Perisoft 2.5 (28). CBF monitoring was continued up to 30 min after the end of the surgical procedure once the occurrence of reperfusion was verified.

microRNA Expression Profiling by Microarray

Brain regions corresponding to the ischemic core and penumbra were dissected from rats subjected to sham surgery and transient middle cerebral artery occlusion (tMCAO). Total RNA from brain tissues was extracted with Trizol, following the instruction of the supplier (TRI Reagent®-Sigma), and RNA quality was assessed using a Thermo Scientific™ NanoDrop™ One Microvolume UV–vis spectrophotometer. The RNA samples were sent to LC Sciences (Houston, Texas, USA), a global biotechnology company which performed a miRNA microarray analysis, including separation, quality control, labeling, hybridization, and scanning. The array contained 810 mature rat miRNA probes for the whole *Rattus norvegicus* miRNome based on a database of published miRNA sequences and annotation (Sanger miRbase Release 21.0). In detail, hybridization was performed on a μParaflo microfluidic chip using a micro-circulation pump (Atactic Technologies, Inc., Houston, TX, USA). After the hybridization, the chips were washed, and then the fluorescence data images were collected using a laser scanner (GenePix 4000B; Molecular Devices, LLC, Sunnyvale, CA, USA) and digitized using Array-Pro image analysis software (Media Cybernetics, Inc., Rockville, MD, USA). The chips were scanned at a pixel size of 10 μM with Cy3 Gain and Cy5 Gain at 460 and 470 nm scanning, respectively. The data were analyzed by first subtracting the background and then normalizing the signals using a locally weighted regression filter.

The datasets presented in this study can be found in the online GEO dataset repositories.

A significantly different expression pattern between tMCAO and sham-operated groups was obtained by microarray analysis, as shown in the volcano plot graph (**Figure 1**, at the top). A clustered heat map was then extrapolated, showing a colorful illustration of miRNA expression profiles across three animals for each experimental condition (**Figure 1**, at the bottom).

Plasma Sample Collection

Blood samples were withdrawn from the tail vein of anesthetized rats before the ischemia induction and at different time intervals from reperfusion by using a 1-ml syringe with a 23-G needle and collected in BD Vacutainer tubes (K3 EDTA 5.4 mg). To separate the plasma, the blood samples were centrifuged in the same collecting tubes at 1,500 × *g* (2,900 rpm) for 8 min at room temperature in a ALC PK 120 centrifuge. The supernatant plasma was transferred to sterile tube and centrifuged in an Eppendorf centrifuge at 11,000 rpm to purify the sample from any cellular residues. Prior to RNA extraction, the absorbance at 415 nm of a 50-μl aliquot for each sample was measured in a Bio-rad Microplate Reader to evaluate the presence of free hemoglobin because of a previous hemolytic process. The data reported in the literature suggest working on plasma samples with absorbance values below 1.0 OD. This restriction is necessary in order to obtain an evaluation of microRNAs that are present in exosome or free in plasma and released after ischemic lesion, excluding those present in the red and white blood cells and released following hemolysis.

microRNA Isolation and Assessment From Brain and Plasma Samples

RNA samples were prepared from the ischemic core, corresponding to the striatum, and penumbra region, corresponding to the temporo-parietal brain surrounding cortex. These brain regions were dissected from rats subjected, respectively, to sham surgery and to tMCAO. The sham-operated rats are, in fact, healthy animals. This experimental group has been chosen in order to compare the miRNA expression to that of a control group that underwent similar conditions in terms of anesthesia and rat manipulation. Total RNA from brain tissues was extracted with Trizol (TRI Reagent®-Sigma) following the instruction of the supplier, and RNA quality was assessed using a Thermo Scientific™ NanoDrop™ One Microvolume UV–vis spectrophotometer.

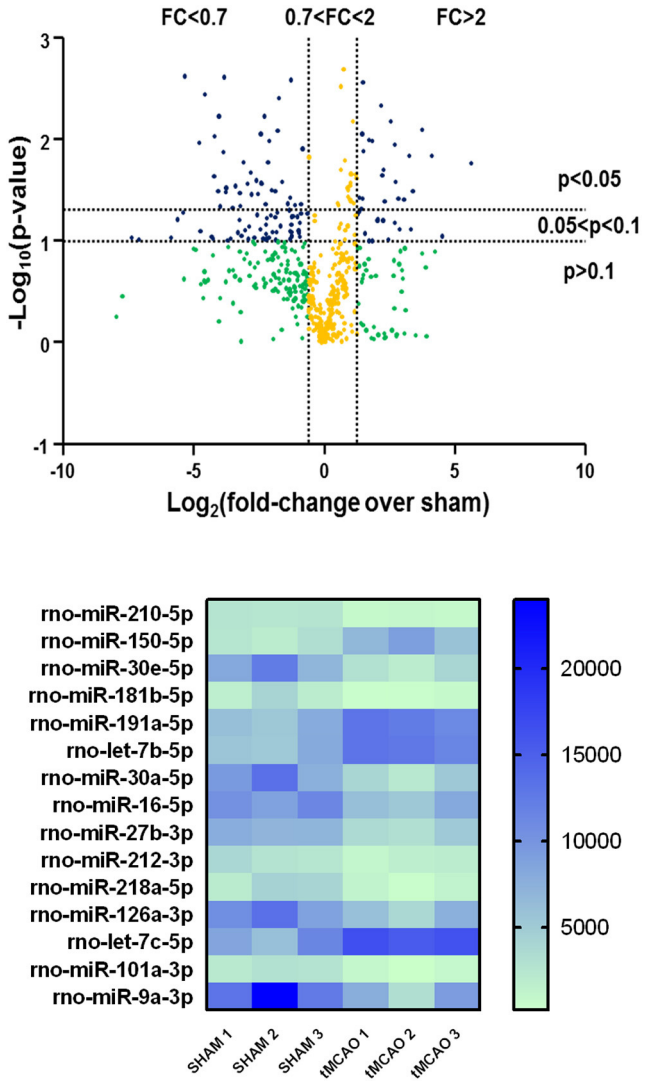

FIGURE 1 | Volcano plot analysis of microRNA expression profiles in the whole ischemic area from sham and transient middle cerebral artery occlusion (tMCAO) animals. Volcano plot analysis in the higher side of the panel shows the miRNA expression changes after tMCAO induction according to a microarray analysis. The y-axis corresponds to the negative $\log_{10}$ (*p*-value), and the x-axis displays the $\log_2$ (fold change) value. The blue dots represent the miRNAs of the tMCAO group with variations of fold change more than 2 or <0.7 compared to the sham group, with a $p < 0.1$. The yellow dots belong to miRNAs whose expression changes were less relevant, with fold change values between 0.7 and 2. The green dots comprise miRNAs with high fold change values but statistically not significant ($p > 0.1$). A heat map of the 15 microRNAs selected from a microarray analysis is shown in the lower side of the panel. The signal intensity values for each significantly expressed miRNA of each sample are reported ($p < 0.05$) in color-coded blocks, according to the colorimetric scale at the right side of the panel.

The expression levels of 15 microRNAs selected from the microarray analysis performed on ischemic brain were singularly evaluated by real-time PCR in plasma of rats subjected to permanent or transient focal cerebral ischemia or to intracerebral collagenase-induced hemorrhage (ICH) at different duration times (0.5, 3, and 9 h from ischemic stroke induction).

miRNA isolation from plasma samples was performed with miRNeasy Serum/Plasma Kit (Qiagen) according to the protocol of the manufacturer. For miRNA analysis in the plasma samples, not specific concentration but precise volumes (5 μl) of RNA were retrotranscribed by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and Taqman probes (Thermo Fisher Scientific), following TaqMan Small RNA Assays Protocol (16°C for 30 min, 42°C for 30 min, and 85°C for 5 min). Quantitative real-time polymerase chain reaction was performed with TaqMan Universal PCR Master Mix II (Applied Biosystems) in a 7500 Fast Real-Time PCR System (AB Applied Biosystems). The cDNA samples were amplified simultaneously in triplicate in one assay run, following the protocol for Taqman assays: 50°C for 2 min, 95°C for 10 min, 40 cycles of amplification of 95°C for 15 s, and 60°C for 1 min. The results were analyzed and exported with 7500 Fast System SDS Software.

The TaqMan probes used are the following: mmu-miR-210* (ID: 462444_mat), hsa-miR-150 (ID: 000473), hsa-miR-30e (ID: 002223), hsa-miR-181b (ID: 001098), hsa-miR-191 (ID: 002299), hsa-let-7b (ID: 002619), hsa-miR-30a-5p (ID: 000417), hsa-miR-16 (ID: 000391), hsa-miR-27b (ID: 000409), mmu-miR-212 (ID: 002551), hsa-miR-218 (ID: 000521), hsa-miR-126 (ID: 002228), hsa-let-7c (ID: 000379), hsa-miR-101 (ID: 002253), hsa-miR-9* (ID: 002231), and miRNA Control Assay U6 snRNA (ID: 001973).

Information on microRNA-Gene-Disease Ontology Interactions

The extensive file of predicted or verified targets of all aberrantly expressed microRNAs in plasma from rats subjected to brain ischemia of different entity, permanent or transient, or origin, hemorrhagic or occlusive, indicates that a large group of genes may be potentially dysregulated since the prenatal period of life. However, for the present study, the attention has been mainly focused on those genes coding plasma membrane proteins controlling ion influx or efflux, whose regulation has been investigated and acknowledged in stroke mechanisms and in several neuroprotective approaches (**Supplementary Table 1**). Four different web servers, each one based on a specific algorithm, were used to predict the targets of those microRNAs that have been found to be dysregulated in plasma from rats subjected to brain ischemia of different entity, permanent or transient, or pathophysiology, hemorrhagic or occlusive.

TargetScan is a computational method to predict the targets of conserved vertebrate miRNAs, integrating the model of miRNA–mRNA interaction on the basis of thermodynamics and sequence alignment analysis between miRNA binding sites among different species (29). The latest updated 7.2 version examined the function of non-canonical binding sites identified in recent studies and considers 14 different features of the microRNA, microRNA site, or mRNA to predict those sites within mRNAs that are most effectively targeted by microRNAs (30).

MiRDB is an online database for miRNA target prediction and functional annotations (31). All targets were predicted by a bioinformatics tool, MirTarget, which was developed by analyzing thousands of miRNA-target interactions from high-throughput sequencing experiments (32).

PicTar uses the criteria of co-expression in space and time of miRNAs and their targets through combinations of different microRNAs (33). This algorithm requires that the binding stability of the putative miRNA-target interaction, measured by thermodynamic binding energy, is higher than a specified threshold.

miRmap is an open source software library which employs thermodynamic, evolutionary, probabilistic, or sequence-based features (34), making it currently the most comprehensive miRNA target prediction resource. Only miRNA-mRNA interactions with a miRmap score above 70 were considered for the present study.

Statistical Analysis

As regard the microarray experiments, Student's *t*-test analysis was conducted for individual comparisons between the two experimental groups. The false discovery rate was $P < 0.05$ and served as the cut-off criteria. The data were $\log_2$ transformed and median centered by Cluster 3.0 software (Informer Technologies Inc., Los Angeles, CA, USA). Real-time PCR results are expressed as fold change ($2^{-\Delta\Delta Ct}$) compared to the control group set to 1, following the instructions provided by the literature (35). Briefly, the difference between the Ct values of the gene of interest and the internal control (ΔCt) is calculated for both control sample and target sample. Then, the difference between the ΔCt of the target sample and the control sample ($\Delta\Delta$Ct) is calculated. The fold change of gene expression of target samples compared to the control sample is calculated as $2^{-\Delta\Delta Ct}$. Values are expressed as means $\pm$ standard deviation. Statistical analysis was performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) using one-way analysis of variance followed by Newman–Keuls *post-hoc* test for groups of more than two. Statistical significance was accepted at 95% confidence level ($p < 0.05$).

RESULTS

Circulating miR-16-5p, miR-101a-3p, miR-218a-5p, and miR-27b-3p Are Upregulated in the Plasma of Rats 3 h After MCAO Onset

Among the 15 microRNAs selected from the microarray analysis, 11 miRNAs showed no significant difference in plasma samples at all times assessed after cerebral ischemia compared to the control group (**Figures 2A–C,E–J,L,O**). By contrast, miR-16-5p, miR-101a-3p, miR-218a-5p, and miR-27b-3p resulted to be upregulated in plasma samples withdrawn up to 3 h of permanent middle cerebral artery occlusion (pMCAO) (**Figures 2D,K,M,N**). These miRNAs did not display an overexpression after 9 h of permanent ischemia.

Circulating miR-150-5p, let-7b-5p, let-7c-5p, and miR-181b-5p Are Selective Biomarkers of Hemorrhagic Stroke

Altered miRNAs in the plasma of rats subjected to ischemic stroke were evaluated also in plasma from ICH rats (**Figure 3**); among them, miR-16-5p and miR-27b-3p significantly increased 30 min after hemorrhagic stroke, but the levels were already restored at 3 h (**Figures 3D,N**). Conversely, miR-101a-3p and miR-218a-5p did not show expression changes at any time evaluated (**Figures 3K,M**). Moreover, some microRNAs whose plasma levels were not modulated by ischemic stroke displayed upregulation after ICH. Indeed let-7c-5p was upregulated only after 30 min from the onset of hemorrhagic stroke (**Figure 3G**), whereas the let-7b-5p, miR-150-5p, and miR-181b-5p levels significantly increased up to 9 h (**Figures 3E,F,H**).

Circulating miR-16-5p and miR-101a-3p Expression in the Plasma of Ischemic Rats Is Affected by Reperfusion

Reperfusion affected the expression of miR-16-5p, miR-101a-3p, and miR-27b-3p. In particular, in the plasma of rats subjected to tMCAO, the upregulation of miR-16 and miR-101a-3p was delayed compared to the expression levels assessed after pMCAO, showing a statistical increment after 9 h from reperfusion (**Figure 4**). By contrast, miR-218 and miR-27b resulted to be not modified by tMCAO compared to the control group (**Figure 4**). Furthermore, miR-30a, let-7b, let-7c, miR-212, and miR-9, whose expression was not modulated by permanent ischemia, were upregulated after reperfusion (**Figures 4A,E,G,L,O**).

Information on microRNA–Gene–Disease Ontology Interactions

microRNA–target interaction analysis showed that, among the proteins involved in the control of ionic homeostasis, miR-150-5p activity has been linked, among others, to the control of immune response after stroke. In addition, we identified sodium/calcium exchanger, NCX1, and zinc transporter, ZnT6, and transient receptor potential cation channel, TRPM7, as putative additional targets. All these membrane proteins are implied in cellular ionic homeostasis, and their activity has been strongly linked to stroke pathophysiology (36–38). Similarly, among miR-180 targets linked to stroke pathophysiology, the membrane channel acid sensing ionic channel, ASIC1, and the Na+/H+ exchanger it should be mentioned (**Tables 1**, **2**).

DISCUSSION

The present paper, taking advantage of three preclinical animal models of stroke, compared the miRNA blood levels of animals subjected to permanent brain ischemia, transient ischemia, and hemorrhagic stroke at differential time intervals from stroke onset. In particular, starting from a miRNome analysis carried out in the brain tissue of ischemic animals, a list of candidate miRNAs involved in ischemic brain damage was generated. The levels of expression of these miRNAs were then evaluated in the

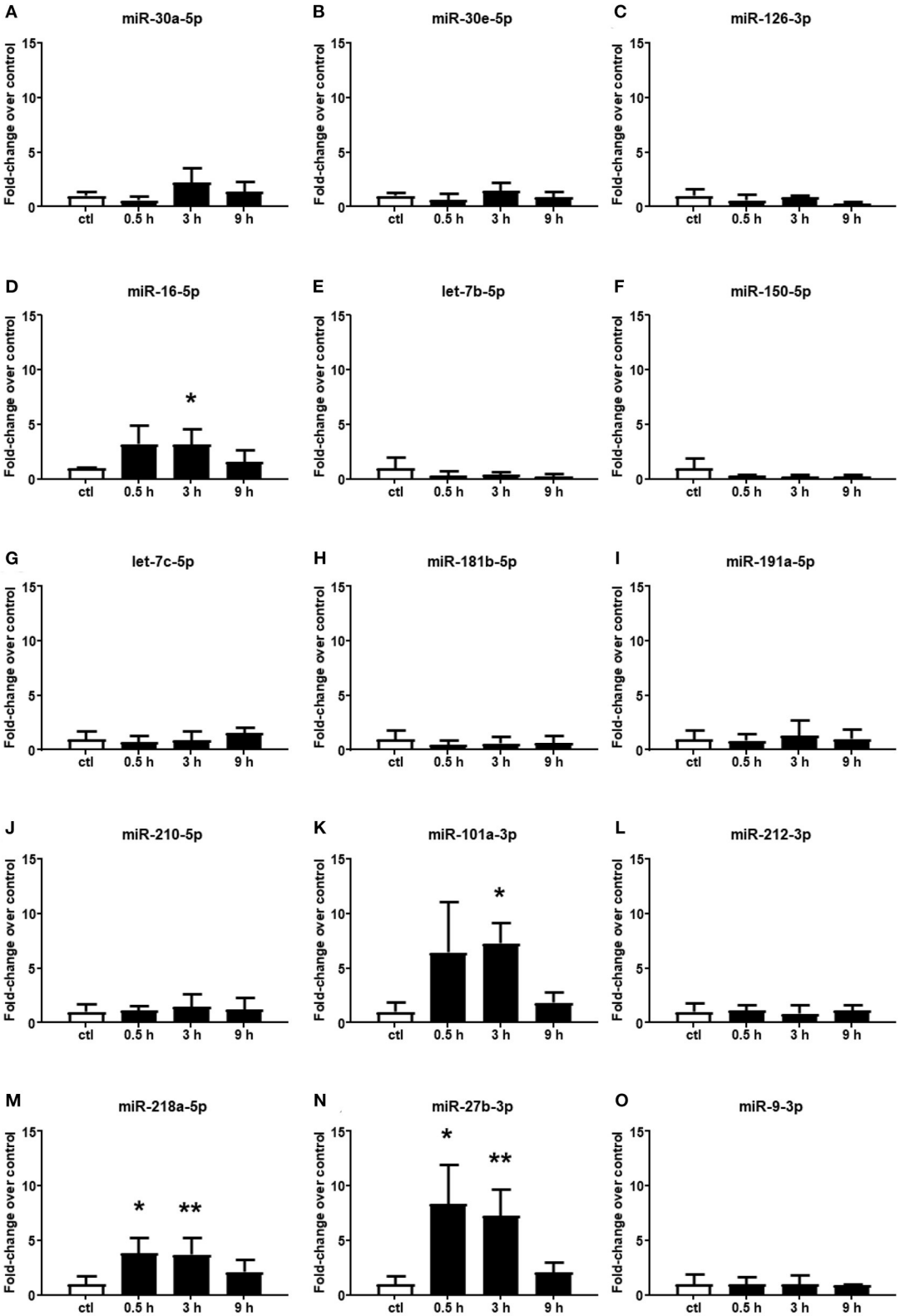

FIGURE 2 | Plasma microRNA expression analysis after permanent middle cerebral artery occlusion by real-time PCR. The microRNA levels analyzed by real-time PCR in plasma samples withdrawn from the tail vein of rats subjected to permanent middle cerebral artery occlusion are expressed as fold change over the control group. **(A–O)** indicate miRNAs evaluated. Each column represents the mean ± SD. The results of the microRNA expression were normalized with respect to U6 snRNA as the internal control. $n = 3$ or 4 per group. $^{*}p < 0.05$ vs. control group. $^{**}p < 0.01$ vs. control group.

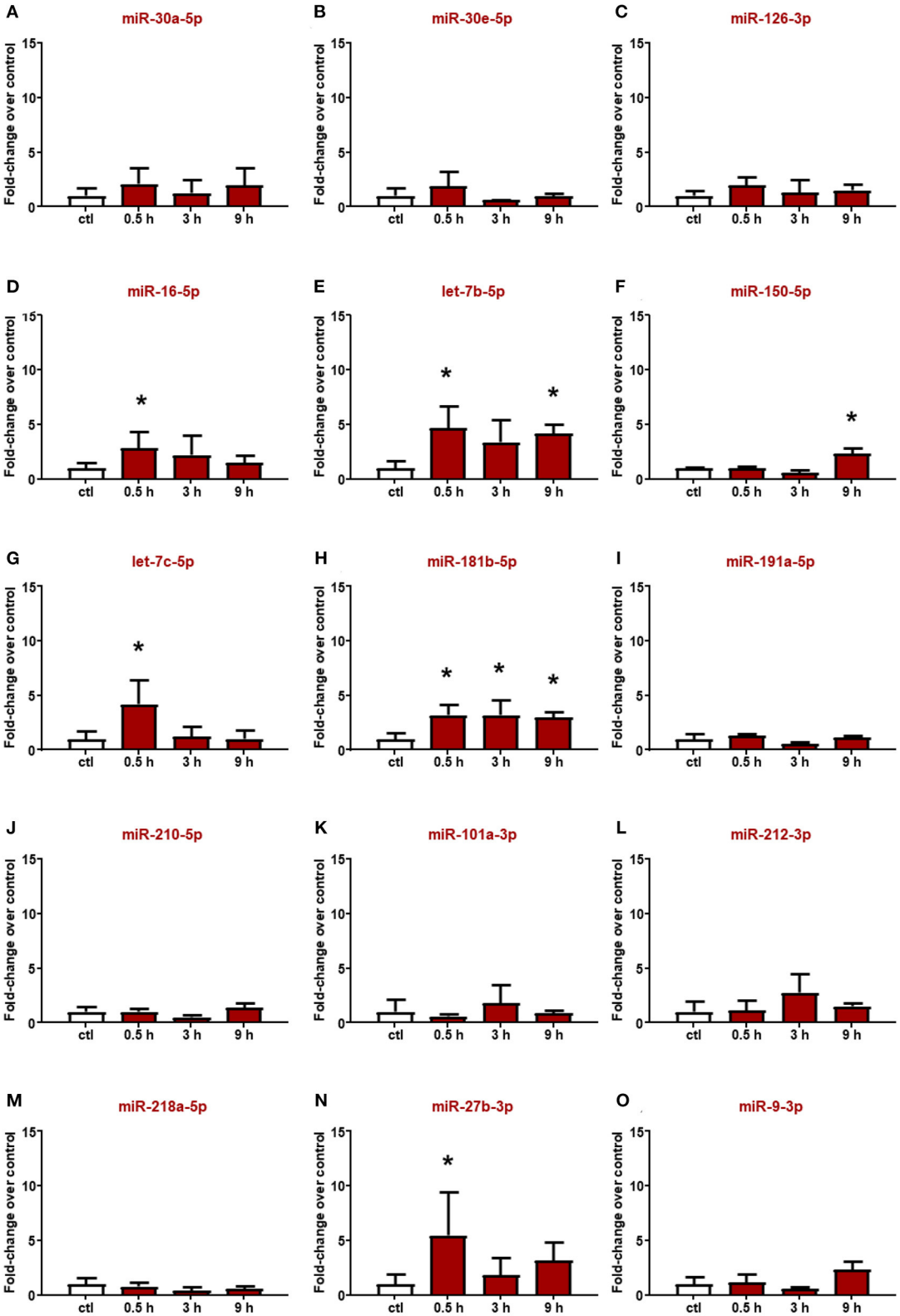

FIGURE 3 | Plasma microRNA expression analysis after intracerebral hemorrhage by real-time PCR. The microRNA levels analyzed by real-time PCR in plasma samples withdrawn from the tail vein of rats subjected to intracerebral hemorrhagic stroke are expressed as fold change over the control group. **(A–O)** indicate miRNAs evaluated. Each column represents the mean ± SD. The results of the microRNA expression were normalized with respect to U6 snRNA as internal control. $n = 5$ or 6 per group. $^*p < 0.05$ vs. control group.

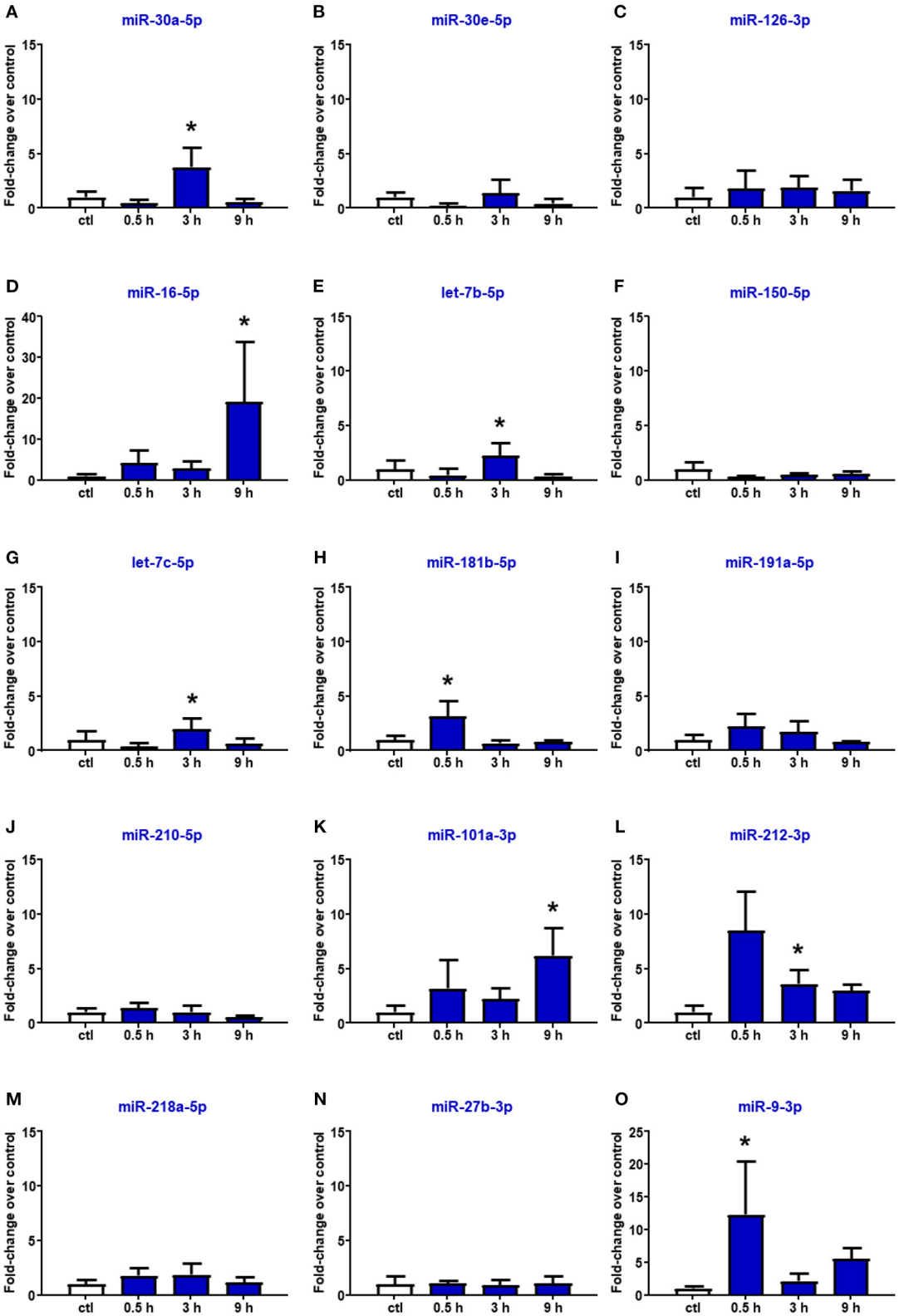

FIGURE 4 | Plasma microRNA expression analysis after transient middle cerebral artery occlusion by real-time PCR. microRNA levels analyzed by real-time PCR in plasma samples withdrawn from the tail vein of rats subjected to tMCAO are expressed as fold change over the control group. **(A–O)** indicate miRNAs evaluated. Each column represents the mean ± SD. The results of the microRNA expression were normalized with respect to U6 snRNA as internal control. $n = 4$ or 5 per group. *$p < 0.05$ vs. control group.

TABLE 1 | List of predicted genes as potential targets of circulating microRNAs modulated by ischemic stroke.

	Sequence	Predicted target
miR-16-5p	5′-UAGCAGCACGUAAAUAUUGGCG-3′	ATP2B2 (ATPase Plasma Membrane Ca^{2+} Transporting 2, PMCA2) SLC9A6 (sodium/hydrogen exchanger 6; NHE6) SLC9A8 (sodium/hydrogen exchanger 8; NHE8) SLC12A2 (sodium-potassium-chloride cotransporter 1, NKCC1) SLC24A3 (sodium/potassium/calcium exchanger 3; NCKX3) SLC30A8 (zinc transporter, ZnT-8) SLC39A9 (zinc transporter, ZIP-9) SLC39A10 (zinc transporter, ZIP-10)
miR-101a-3p	5′-UACAGUACUGUGAUAACUGAA-3′	ATP2B2 (ATPase Plasma Membrane Ca^{2+} Transporting 2, PMCA2) SLC12A2 (sodium-potassium-chloride cotransporter 1, NKCC1) SLC30A6 (zinc transporter, ZnT6) SLC30A7 (zinc transporter, ZnT-7) SLC39A10 (zinc transporter, ZIP-10)
miR-218a-5p	5′-UUGUGCUUGAUCUAACCAUGU-3′	ASIC1 (acid-sensing ion channel 1, ASIC1) SLC12A2 (sodium-potassium-chloride cotransporter 1, NKCC1) SLC24A4 (sodium/potassium/calcium exchanger 4; NCKX4) SLC39A1 (zinc transporter, ZIP-1)
miR-27b-3p	5′-UUCACAGUGGCUAAGUUCUGC-3′	ASIC1 (acid-sensing ion channel 1, ASIC1) ATP2B1 (ATPase Plasma Membrane Ca^{2+} Transporting 1, PMCA1) SLC9A4 (sodium/hydrogen exchanger 4; NHE4) SLC9A7 (sodium/hydrogen exchanger 7; NHE7) SLC24A1 (sodium/potassium/calcium exchanger 1; NCKX1) SLC24A4 (sodium/potassium/calcium exchanger 4; NCKX4) SLC30A7 (zinc transporter, ZnT-7) SLC39A11 (zinc transporter, ZIP-11) SLC39A13 (zinc transporter, ZIP-13)

Only the predicted targets involved in the mechanisms of ionic homeostasis regulation proposed from at least two of the four databases applied are shown.

blood of animals subjected to ischemic stroke and compared to those of rats exposed to hemorrhagic stroke.

Our study examined the expression of 548 mirRNAs in the brain tissue of ischemic and sham-operated rats and selected those miRNAs whose expression was increased or reduced after stroke. Therefore, based on the analysis of miRNome in ischemic brain tissue, it has been possible to create a list of miRNAs potentially involved in stroke damage. Since it has been previously demonstrated that the levels of expression of miRNAs in ischemic brain tissue may correlate with those present in the plasma (39), these miRNAs were then evaluated at different time intervals in the blood of rats subjected to ischemic or hemorrhagic stroke. From a first examination that allowed us to narrow our research field on 15 circulating miRNAs examined, four miRNAs—miR-16-5p, miR-101a-3p, miR-218-5p, and miR-27b-3p—were found to be significantly upregulated in the plasma of rats 3 h after pMCAO, whereas four miRNAs—miR-150-5p, let-7b-5p, let 7c-5p, and miR-181b-5p—were selectively upregulated in the plasma of rats subjected to ICH.

These results are of particular relevance since, for the first time, we compared the miRNA levels in the plasma of ischemic animals at different time intervals after the ischemic insult. The choice of the time point examined reflects the time window of intervention in patients affected by stroke. Having a hypothetical biomarker expressed after 6 h from stroke onset is not very helpful in order to properly treat the patient. The time-dependent expression of miRNAs in plasma underlines the importance of a precise timing when miRNAs are considered as prognostic and diagnostic tools. In fact, due to the high stability in biological fluids and the reproducibility of detection, circulating miRNAs have been proposed as new non-invasive biomarkers for the diagnosis of many neurodegenerative disorders, including stroke (40–42).

On the other hand, the importance to have a tool for a rapid diagnosis of stroke represents a medical need since the diagnosis of stroke is remarkably time-consuming, being mainly dependent upon examination by a clinical care provider and by means of various neuroimaging techniques. This way of proceeding leads to fewer possibilities of starting an effective recanalizing therapy in stroke patients within the defined time interval of 4.5 h. Therefore, much effort must be made in order to improve the diagnostic decision-making process. In this direction, the

TABLE 2 | List of predicted genes as potential targets of circulating microRNAs modulated by hemorrhagic stroke.

microRNA	Sequence	Predicted target
miR-150-5p	5′-UCUCCCAACCCUUGUACCAGUG-3′	SLC8A1 (sodium/calcium exchanger 1, NCX1) SLC30A5 (zinc transporter, ZnT-5) TRPM7 (Transient Receptor Potential Cation Channel M, 7)
let-7b-5p	5′-UGAGGUAGUAGGUUGUGUGGUU-3′	ATP2A2 (ATPase Sarcoplasmic/Endoplasmic Reticulum Ca^{2+} Transporting 2, SERCA2) ATP2B4 (ATPase Plasma Membrane Ca2+ Transporting 4, PMCA4) SLC8A2 (sodium/calcium exchanger 2, NCX2) SLC9A9 (sodium/hydrogen exchanger 9; NHE9) SLC30A4 (zinc transporter, ZnT-4) TRPM6 (Transient Receptor Potential Cation Channel M, 6)
miR-181b-5p	5′-AACAUUCAUUGCUGUCGGUGGGU-3′	ASIC1 (acid-sensing ion channel 1, ASIC1) ATP2A2 (ATPase Sarcoplasmic/Endoplasmic Reticulum Ca^{2+} Transporting 2, SERCA2) ATP2B1 (ATPase Plasma Membrane Ca^{2+} Transporting 1, PMCA1) ATP2B2 (ATPase Plasma Membrane Ca^{2+} Transporting 2, PMCA2) SLC9A6 (sodium/hydrogen exchanger 6; NHE6) SLC12A5 (potassium-chloride cotransporter 2, KCC2) TRPM3 (Transient Receptor Potential Cation Channel M, 3)
let-7c-5p	5′-UGAGGUAGUAGGUUGUAUGGUU-3′	ATP2A2 (ATPase Sarcoplasmic/Endoplasmic Reticulum Ca^{2+} Transporting 2, SERCA2) SLC8A2 (sodium/calcium exchanger 2, NCX2) SLC9A9 (sodium/hydrogen exchanger 9; NHE9) SLC12A9 (cation-chloride cotransporter 6, CCC6) SLC30A4 (zinc transporter, ZnT-4) TRPM6 (Transient Receptor Potential Cation Channel M, 6)

Only the predicted targets involved in the mechanisms of ionic homeostasis regulation proposed from at least two of the four databases applied are shown.

possibility to rely on an easily detectable circulating biomarker able to distinguish between ischemic and hemorrhagic stroke could be of great help for the clinicians. In this context, the role of miRNA is a developing promising field, with growing interest in their potential application as a biomarker for the rapid diagnosis and prognosis of brain ischemia as well as for the development of therapeutic agents (18, 43). Different patient-based studies have already reported some changes in the circulatory expression of miRNA during cerebral ischemia (44).

As anticipated above, miRNAs are present in human plasma or in serum in a remarkably stable form and represent potentially informative biomarkers for a range of diseases. Chen et al. demonstrated that plasma miRNAs are stable and protected against RNases as well as other prohibitive conditions, including boiling, low/high pH, extended storage, and repetitive freezing/thawing cycles (45). The expression levels of miRNAs in blood have been found to be reproducible and indicative of the disease state. Although the mechanism of how miRNAs are released into the circulation is unclear, their presence in the circulation and association with diverse pathophysiological states are now generally accepted. Circulating miRNAs in plasma are altered both qualitatively and quantitatively in a variety of conditions, including different tumor types, cardiovascular diseases, stroke, and neurodegenerative diseases (46).

It is widely believed that miRNAs released from damaged cells or circulating cells lead to increased serum miRNA expression (47). The present study reports unique circulating miRNA expression profiles following cerebral ischemia in adult male rats. In fact, to investigate the time dependency on the expression of stroke-related miRNAs, three time points were evaluated: 0.5, 3, and 9 h. Among the 15 miRNAs considered, miR-101a-3p emerges as the most promising ischemic stroke biomarker for further future evaluation. The levels of expression of miR-101a-3p increase at early time intervals, 0.5 and 3 h, after permanent ischemia induction and at a later time interval, 9 h, after transient ischemia. No changes were detected after hemorrhagic stroke.

As for hemorrhagic stroke, the most promising diagnostic candidate miRNAs are miR-150-5p and miR-181-5p, whose levels of expression in the blood of hemorrhagic rats are selectively upregulated starting from 30 min after hemorrhagic stroke induction.

From *in silico* analysis data and from data that already appeared in the scientific literature, it is possible to link the role of these three miRNAs to stroke pathophysiology.

In fact, miR-101a-3p has one of its major targets in polycomb repressive complex 2, a multi-protein complex including histone methyltransferase enhancer of zeste homolog 2 (48, 49), which mediates gene silencing *via* the tri-methylation of histone 3 at lysine 27 (H3K27me3) (50, 51).

As for miR-150-5p, its activity has been linked, among others, to the control of immune response after stroke. In addition, we identified sodium/calcium exchanger, NCX1, zinc transporter,

ZnT6, and transient receptor potential cation channel, TRPM7, as putative additional targets. All these membrane proteins are implied in cellular ionic homeostasis, and their activity has been strongly linked to stroke pathophysiology (36–38). Similarly, among miR-180 targets linked to stroke pathophysiology, the membrane channel acid sensing ionic channel, ASIC1, and the Na^+/H^+ exchanger should be mentioned (52, 53).

Collectively, our study identified some peculiar miRNAs expressed in the plasma of hemorrhagic rats and pointed out the importance of a precise time point definition in order to render the use of miRNAs as stroke biomarkers more reliable.

LIMITATIONS

Future studies should include a larger set of animals, eventually of both genders, affected by comorbidities, and at different ages. Furthermore, it is important to underline the need to speed up all those technical procedures capable of detecting miRNAs in the shortest and simplest possible way, i.e., by setting up innovative sensors capable of measuring miRNAs in a few seconds, to avoid running into the same problems currently present with the use of CT and MRI to make a differentiated diagnosis of ischemic and hemorrhagic stroke.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: GEO Submission (GSE184975) [NCBI tracking system #22375569].

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical Committee of University of Naples Federico II.

AUTHOR CONTRIBUTIONS

GP, LA, and PC: conception and design of the study. PC, OC, and MT: acquisition and analysis of data. PC, OC, MT, AV, GP, LA, and MT: drafting a significant portion of the manuscript, tables, and figures. PC and MT evaluated expression of miRNA in blood samples. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.736474/full#supplementary-material

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A Combined Clinical and Serum Biomarker-Based Approach May Allow Early Differentiation Between Patients With Minor Stroke and Transient Ischemic Attack as Well as Mid-term Prognostication

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Background: Early differentiation between transient ischemic attack (TIA) and minor ischemic stroke (MIS) impacts on the patient's individual diagnostic work-up and treatment. Furthermore, estimations regarding persisting impairments after MIS are essential to guide rehabilitation programs. This study evaluated a combined clinical- and serum biomarker-based approach for the differentiation between TIA and MIS as well as the mid-term prognostication of the functional outcome, which is applicable within the first 24 h after symptom onset.

Methods: Prospectively collected data were used for a retrospective analysis including the neurological deficit at admission (National Institutes of Health Stroke Scale, NIHSS) and the following serum biomarkers covering different pathophysiological aspects of stroke: Coagulation (fibrinogen, antithrombin), inflammation (C reactive protein), neuronal damage in the cellular [neuron specific enolase], and the extracellular compartment [matrix metalloproteinase-9, hyaluronic acid]. Further, cerebral magnetic resonance imaging was performed at baseline and day 7, while functional outcome was evaluated with the modified Rankin Scale (mRS) after 3, 6, and 12 months.

Results: Based on data from 96 patients (age 64 ± 14 years), 23 TIA patients (NIHSS 0.6 ± 1.1) were compared with 73 MIS patients (NIHSS 2.4 ± 2.0). In a binary logistic regression analysis, the combination of NIHSS and serum biomarkers differentiated MIS from TIA with a sensitivity of 91.8% and a specificity of 60.9% [area under the curve (AUC) 0.84]. In patients with NIHSS 0 at admission, this panel resulted in a still acceptable sensitivity of 81.3% (specificity 71.4%, AUC 0.69) for the differentiation between MIS ($n = 16$) and TIA ($n = 14$). By adding age, remarkable sensitivities of 98.4, 100, and 98.2% for the prediction of an excellent outcome (mRS 0 or 1) were achieved with respect to time

points investigated within the 1-year follow-up. However, the specificity was moderate and decreased over time (83.3, 70, 58.3%; AUC 0.96, 0.92, 0.91).

Conclusion: This pilot study provides evidence that the NIHSS combined with selected serum biomarkers covering pathophysiological aspects of stroke may represent a useful tool to differentiate between MIS and TIA within 24 h after symptom onset. Further, this approach may accurately predict the mid-term outcome in minor stroke patients, which might help to allocate rehabilitative resources.

Keywords: biomarker panel, minor ischemic stroke, transient ischemic attack, prognostication, functional impairment

INTRODUCTION

Time-sensitive diagnosis of ischemic stroke is essential for patients not only to allow decision making regarding acute treatment, but also to guide the individual diagnostic workup (1). Especially in patients presenting with minor or short-lasting neurological deficits, the differentiation between minor ischemic stroke (MIS) (2) and transient ischemic attack (TIA) is rather challenging. Furthermore, knowledge on the individual diagnoses is essential to initiate rehabilitative programs early after the event with the intention to reduce stroke-related sequelae as best as possible.

According to the widely applied definitions, cerebral magnetic resonance imaging (MRI) is mandatory in these patients to detect neuronal damage in terms of an ischemic lesion (3). However, computed tomography is routinely used as first cerebral imaging method in many countries, since it offers all necessary information for acute treatment decisions. Although a more accurate diagnosis may arise from MRI especially in early phases, access to this technique is typically limited due to costs and availability.

In addition to cerebral imaging, serum biomarkers have been discussed as an option to differentiate between ischemic stroke and TIA (4). So far, many serum biomarkers were examined in the (hyper)acute phase of ischemic stroke with the intention to guide acute treatment decisions like systemic thrombolysis by reliably differentiating ischemic from hemorrhagic stroke (5, 6). However, serum biomarkers alone or in combination have often demonstrated an only moderate to good sensitivity for the differentiation between ischemia and hemorrhage (7). In a more general perspective, the etiology of ischemic stroke is known to be rather complex, ranging for example from cardio-embolic sources, to carotid artery or small vessel disease (8), to rare causes like spontaneous cerebral artery dissection (9), or tumor-associated hypercoagulability (10). Thus, it seems plausible that a single serum biomarker or even a panel that focus on one mechanism cannot cover the variety of aspects involved in stroke pathophysiology.

Furthermore, most biomarker approaches addressed only single scenarios like the differentiation between ischemic and hemorrhagic stroke [e.g., (5)], the prediction of stroke associated complications (11), or the prognostication of functional outcome after ischemic stroke (12). A biomarker-based approach that covers multiple of these scenarios would be easy to use and resource-effective, which would facilitate its translation and acceptance into daily clinical practice.

Considering different pathophysiological aspects of stroke, this study aimed to evaluate a combined clinical- and serum biomarker-based approach within the first 24 h after symptom onset for the differentiation between TIA and MIS and for the prognostication of the functional outcome in MIS patients in a follow-up period of 12 months.

Inclusion criteria
- Age between 18 and 85 years
- acute onset of a focal neurological deficit which is attributed to the territory of the right or left middle cerebral artery
- NIHSS between 0 - 20 points
- pre modified Rankin scale 0 - 2
- informed written or oral (under witness) consent

Exclusion criteria
- soporous or comatose patients
- intravenous or intraarterial thrombolysis
- history of ischaemic or hemorrhagic stroke
- any cancer
- history of depression, anxiety disorder, or any other relevant mental illness
- dementia
- chronic degenerative disorder of the central nervous system
- pregnant or breast feeding women
- epileptic seizure at symptom onset
- instable angina pectoris, myocardial infarction, endocarditis, clinically relevant kidney-, liver-, or pulmonary failure
- acute arthritis or chronic polyarthritis
- known or supposed vasculitis or collagenosis
- acute severe infection or chronic infection
- patients that can not be followed-up for 1 year
- contraindication for cerebral MR imaging
- cerebral MR imaging contraindications:
  - * intracranial hemorrhage
  - * cerebral sinus- / venous thrombosis
  - * intracerebral neoplasia
  - * (suspicion of) inflammatory / infectious disorder of the central nervous system

FIGURE 1 | In- and exclusion criteria of the study population in detail.

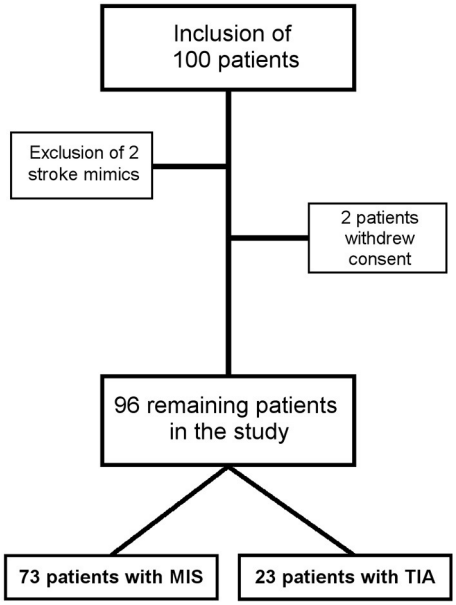

FIGURE 2 | Flow chart for the study. Minor ischemic stroke MIS, transient ischemic attack TIA.

TABLE 1 | Baseline demographic and clinical data of patients with transient ischemic attack and minor ischemic stroke.

	Patients with MIS ($n = 73$)	Patients with TIA ($n = 23$)	p
Age in years	64.6 ± 12.8	63.4 ± 16.3	0.919
Female/Male	34/39	11/12	
NIHSS at admission	2.4 ± 2.0	0.6 ± 1.1	<0.001#
Pre-mRS	0.1 ± 0.3	0.3 ± 0.7	0.404
mRS at admission	1.6 ± 1.3	0.6 ± 0.8	0.002#
mRS at 3 months	0.9 ± 1.1	–	–
mRS at 6 months	0.9 ± 1.0	–	–
mRS at 12 months	0.9 ± 1.2	–	–
Arterial hypertension	55 (75.3 %)	16 (69.6 %)	0.582*
Diabetes mellitus	11 (15.1 %)	4 (17.4 %)	0.789*
Current smoking	16 (22.0 %)	3 (13.0 %)	0.352*
Hyperlipidemia	22 (30.1 %)	8 (34.8 %)	0.675*

MIS, minor ischemic stroke; TIA, transient ischemic attack; mRS, modified Rankin Scale.
#Mann-Whitney U test.
**Chi square test.*

TABLE 2 | Etiologies of patients with minor ischemic stroke and transient ischemic attack.

Etiology	Patients with MIS ($n = 73$)	Patients with TIA ($n = 23$)	p
Carotid artery disease with at most moderate stenosis (<70% NASCET)	41 (56.2%)	1 (4.3%)	<0.001
Carotid artery disease with high grade stenosis (≥70%)	7 (9.6%)	4 (17.4%)	0.301
Cardio-embolic	15 (20.5%)	1 (4.3%)	0.069
Small vessel disease	3 (4.1%)	1 (4.3%)	0.960
Spontaneous cervical artery dissection	3 (4.1%)	0	–
Cryptogenic	4 (5.5%)	16 (69.6%)	<0.001

Groups were compared using chi square test. MIS, minor ischemic stroke; TIA, transient ischemic attack.

METHODS

Study Design and Content

This work used data from a prospective observational study, which complies with the guidelines for human studies and had been approved by the local ethic committee of the University of Leipzig (approval number 198-08). In- and exclusion criteria are shown in detail in **Figure 1**. All participants have given informed consent, either in a written or witnessed oral form. Patients were enrolled between 11/2008 and 09/2010 at the Stroke Unit of the Department of Neurology at the University Hospital Leipzig within the first 24 h after symptom onset. Information about symptom onset or last seen well were given by the patients themselves. Patients with evidence for hemorrhage on initial cerebral imaging were excluded.

The pre-hospital functional status and the functional status at admission as well as at months 3, 6, and 12 were assessed by the modified Rankin Scale (pre-mRS, mRS at admission, etc.). The National Institute of Stroke Scale (NIHSS) was used to assess the severity of neurological symptoms at admission. The assessors of the mRS during the follow-up period were unaware of the results of the clinical and para-clinical (imaging, laboratory) baseline examinations.

Blood samples were collected at study enrollment by venipuncture (EDTA, serum and citrate blood, S-Monovette®, Sarstedt AG&Co, Germany) and the following laboratory parameters were measured: leukocyte and platelet count (automated hematology analyzer XE-2100, Sysmex Europe, Germany), C reactive protein (CRP) (latex-enhanced immunoturbidimetric test, cobas® analyzer, Roche Diagnostics, Germany), interleukin 6 (IL-6), neuron specific enolase (NSE) (electrochemiluminescence immunoassay, cobas®), procalcitonine (PCT) (Immunofluorescent assay, Kryptor Immunoanalyzer, Brahms AG, Germany), D-Dimer (latex-enhanced immunoturbidimetric test, BCS® coagulation analyzer, Siemens, Germany), activated partial thromboplastin time (aPTT), fibrinogen, prothrombin time (clotting-based; PT, given as activity percentage based on the Quick method involving a standard curve based on dilutions of normal plasma, BCS®), and antithrombin (chromogenic, BCS®).

Only CE-IVD-certified laboratory tests approved for diagnostic use were applied, and all analytical procedures were performed according to the manufacturer's instruction. Analysis was performed in the Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics, University Hospital Leipzig.

The serum levels of matrix metalloproteinase-9 (MMP-9), tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), and hyaluronic acid were measured by certified enzyme-linked

TABLE 3 | Comparison of biomarkers between patients with minor ischemic stroke and transient ischemic attack.

	Patients with MIS ($n = 73$)	Patients with TIA ($n = 23$)	p
Coagulation System			
Fibrinogen (g/L)	3.3 ± 0.9	2.8 ± 0.7	0.018
	20 (27.4%)	1 (4.3%)	0.020#
D-Dimer (mg/L)	0.98	0.70	0.644
	25th percentile: 0.33	25th percentile: 0.22	
	75th percentile: 0.81	75th percentile: 1.08	
	37 (50.7%)	10 (43.5%)	0.547#
Antithrombin (%)	92.7 ± 11.6	95.6 ± 10.2	0.259
	3 (4.1%)	0 (0%)	–
Thrombocyte count (10^9/L)	232 ± 63	214 ± 60	0.313
	15 (20.5%)	3 (13%)	0.421#
aPTT (s)	31.3 ± 4.2	30.0 ± 3.8	0.166
	7 (9.6%)	1 (4.3%)	0.428#
Prothrombin time (%)	99 ± 19	104 ± 10	0.317
Inflammation			
Leucocyte count (10^9/L)	7.9 ± 2.5	8.0 ± 2.0	0.356
	20 (27.4%)	6 (26.1%)	0.902#
Interleukin 6 (pg/ml)	10.2 ± 10.2	6.9 ± 6.7	0.051
	38 (52.1%)	7 (30.4%)	0.070#
CRP (mg/L)	5.4	3.0	0.237
	25th percentile: 1.2	25th percentile: 1.0	
	75th percentile: 5.2	75th percentile: 3.7	
	19 (26.0%)	4 (17.4%)	0.397#
PCT (ng/ml)	0.06 ± 0.03	0.09 ± 0.09	0.930
	46 (63.0%)	18 (78.3%)	0.176#
Neuronal Damage			
NSE (ng/ml)	26.7 ± 13.0	21.9 ± 6.7	0.038
	64 (87.7%)	17 (73.9%)	0.113#
Markers of the Extracellular Compartment			
MMP-9	232 ± 158	251 ± 179	0.776
TIMP-1	245 ± 159	212 ± 171	0.189
Hyaluronic acid	96.1 ± 56.6	110.6 ± 76.1	0.533

Non-parametric testing (Mann-Whitney U test) was applied for intergroup comparison with correction for multiple testing (Bonferroni-Holm correction), resulting in a corrected significance level of p = 0.0036. Number and percentage of values outside the local laboratory reference intervals are given in the second row for the respective parameter with statistical significance being tested between groups with chi square test (indicated by #). MIS, minor ischemic stroke; TIA, transient ischemic attack; aPTT, activated partial thromboplastin time; CRP, C reactive protein; PCT, procalcitonin; NSE, neuron specific enolase; MMP-9, matrix metalloproteinase-9; TIMP-1, tissue inhibitor of matrix metalloproteinase 1.

immunosorbent assays (designed by Cloud-Clone Corp., Houston, United States of America; assembled by Uscn Life Science Inc., Wuhan, People's Republic of China).

All study participants underwent cerebral 1.5 Tesla MRI upon enrollment in the study and at day 7 ± 1. Based on cerebral MRI and duration of symptoms, patients were classified to either an ischemic stroke (evidence of an acute ischemic lesion in the diffusion weighted imaging sequence in at least one MRI *and/or* a symptom duration of more than 24 h) or a TIA (no evidence of an acute lesion in the diffusion weighted imaging sequence of both MRI *and* a symptom duration of <24 h), according to Sacco et al. (3).

Statistical Analysis

For statistical calculations the IBM SPSS Statistics Package Version 25 (IBM Corp., Armonk, NY, USA) was used. After descriptive analyses, statistical significance between groups was assessed by chi square test for categorical variables and by Mann-Whitney U test for interval-scaled parameters. Bonferroni-Holm correction was applied to consider multiple testing. Based on pathophysiological considerations (7) we calculated forward logistic regression analyses with MIS vs. TIA and excellent (mRS 0–1) vs. non-excellent (mRS ≥ 2) functional outcome after 3, 6, and 12 months as dependent variables and different combinations of clinical data (NIHSS, age) and laboratory parameters (with at least one parameter from each of the four domains) as predictor variables to obtain the sensitivity, specificity, and area under the curve (AUC) of the applied models.

RESULTS

Data from 100 patients were used for the study. Two patients with stroke mimics (one patient with a peripheral facial nerve paresis, one patient with meningitis) and two patients who withdraw their consent were excluded (**Figure 2**). Thus, data from 96 patients (mean age 64 ± 14 years) were included in the final analysis, while 23 TIA patients (NIHSS at admission 0.6 ± 1.1) were compared with 73 MIS patients (NIHSS at admission 2.4 ± 2.0). Detailed baseline demographic and clinical data of patients are shown in **Table 1** and, with the exception of a higher NIHSS and a higher mRS at admission for patients with MIS, were almost similar between patients with MIS and patients with TIA. Etiology of MIS or TIA is shown in **Table 2**; while carotid artery disease with at most moderate stenosis (<70% NASCET) was more prevalent in patients with MIS, patients with TIA were diagnosed more often as of cryptogenic etiology. Laboratory panel parameters are given in **Table 3** as mean values and as the number and percentage of values outside the local laboratory reference intervals. Briefly, we found no significant differences between both groups including all laboratory parameters.

In a binary logistic regression analysis with ischemic stroke or TIA as the dependent variable the combination of NIHSS at admission, fibrinogen, antithrombin, CRP, MMP-9, hyaluronic acid, and NSE was found to be best associated with MIS, resulting in a sensitivity of 91.8% and a specificity of 60.9% (AUC 0.84, 95% confidence interval 0.74–0.94) (**Figure 3A**). An increased NIHSS at admission doubled the risk for the patient to have suffered an ischemic stroke (odd ratio 2.0; confidence interval 1.3–3.3; **Table 4**). Selecting any biomarker of inflammation while excluding the others did not change sensitivity of this model relevantly: CRP 91.7%, IL-6 88.7%, PCT 93.1%, and leucocyte count 93.0%, while specificity was also comparable: CRP 60.9%, IL-6 52.2%, PCT 60.9%, and leucocyte count 56.5%. Focusing only on patients with complete recovery of neurological symptoms (NIHSS 0) upon admission to the stroke unit, this panel resulted in a still acceptable sensitivity of 81.3% and a

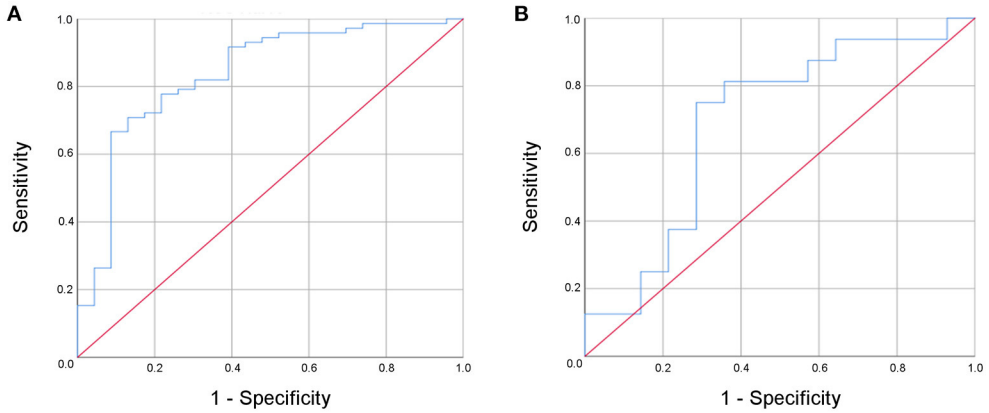

FIGURE 3 | Receiver operated curve (ROC) analysis of the multi-modal biomarker panel for the differentiation between minor ischemic stroke and transient ischemic attack for all patients **(A)** and for patients with complete recovery upon admission to the stroke unit **(B)**.

specificity of 71.4% (AUC 0.69, 95% confidence interval 0.49–0.89) for the differentiation between MIS ($n = 16$) and TIA ($n = 14$) (**Figure 3B**).

By adding age, this multi-dimensional approach yielded remarkable sensitivities of 98.4, 100, and 98.2% with respect to the time points investigated within the first 12 months after the event for predicting an excellent outcome (mRS 0 or 1) (**Figure 4**). However, the specificity was moderate and decreased over time (83.3, 70, and 58.3%; AUC 0.96 (95% confidence interval 0.88–1.0), 0.92 (95% confidence interval 0.80–1.0), 0.91 (95% confidence interval 0.80–1.0).

TABLE 4 | Odds ratios with confidence intervals for the NIHSS score and each laboratory parameter that was included into the model to differentiate between patients with minor ischemic stroke and transient ischemic attack within 24 h after symptom onset.

	Odds ratio	Confidence interval
NIHSS	2.04	1.27–3.29
Fibrinogen	2.28	0.77–6.76
Antithrombin	0.98	0.93–1.04
CRP	0.97	0.84–1.13
NSE	1.06	0.98–1.16
MMP-9	1.0	1.0–1.0
Hyaluronic acid	1.0	0.99–1.01

CRP, C reactive protein; NSE, neuron specific enolase; MMP-9, matrix metalloproteinase-9; NIHSS, National Institute of Health Stroke Scale.

DISCUSSION

This pilot study provides robust evidence that a combined clinical- and serum biomarker-based approach, which covers different pathophysiological aspects of stroke and is obtained early after the event, might help to differentiate between MIS and TIA. This is of interest, as early differentiation appears difficult in clinical practice and usually depends on an additional cerebral MRI. Moreover, the same approach was shown to accurately predict an excellent mid-term outcome in patients suffering from MIS.

Although many serum biomarkers and their combinations have been evaluated in the setting of an acute ischemic stroke, a "troponin of the brain," i.e., a highly sensitive and specific serum biomarker indicating an acute ischemic damage, is still lacking. Hence, the diagnosis of an ischemic stroke is currently linked to the detection of a persistent and not only transient neuronal damage either by cerebral imaging in terms of an ischemic lesion or by persisting neurological symptoms for more than 24 h (3). Besides the differentiation between ischemic and hemorrhagic stroke within the (hyper)acute phase of stroke (5, 6), serum biomarkers might also be helpful in predicting complications of stroke like pneumonia (11, 13), in functional prognostication (12, 14, 15), as well as in the allocation of diagnostic and rehabilitative resources. However, so far, no single biomarker or panel of biomarkers succeeded translation from bench to bedside, i.e., to facilitate diagnosis, treatment, or prognostication (7). Furthermore, highly elaborated multistep approaches in large samples have failed (16–18). One reason for this failure might be the variety of stroke etiologies and the complex pathophysiological mechanisms occurring during early stages after the event (8, 19).

Consequently, recent reviews discussed the potential use of biomarker panels that would cover several relevant pathophysiological aspects of stroke. In their review, Baez et al. proposed different combinations of biomarkers comprising cellular (glial or neuronal) components, extracellular components, and the coagulation system (20). Here, we combined clinical data with serum biomarkers involving the coagulation system, inflammation, neuronal damage, and the extracellular compartment. In our study the combination of the NIHSS, fibrinogen, antithrombin, CRP, MMP-9, hyaluronic acid, and NSE within 24 h after symptom onset was found to be associated with MIS, resulting in an accuracy of 0.84. Further, in the subgroup of patients presenting with a complete recovery of neurological symptoms at admission to the stroke unit, this combined approach still resulted in an accuracy of 0.69.

Remarkably, by adding age, the same multi-modal approach accurately predicted an excellent mid-term outcome in patients

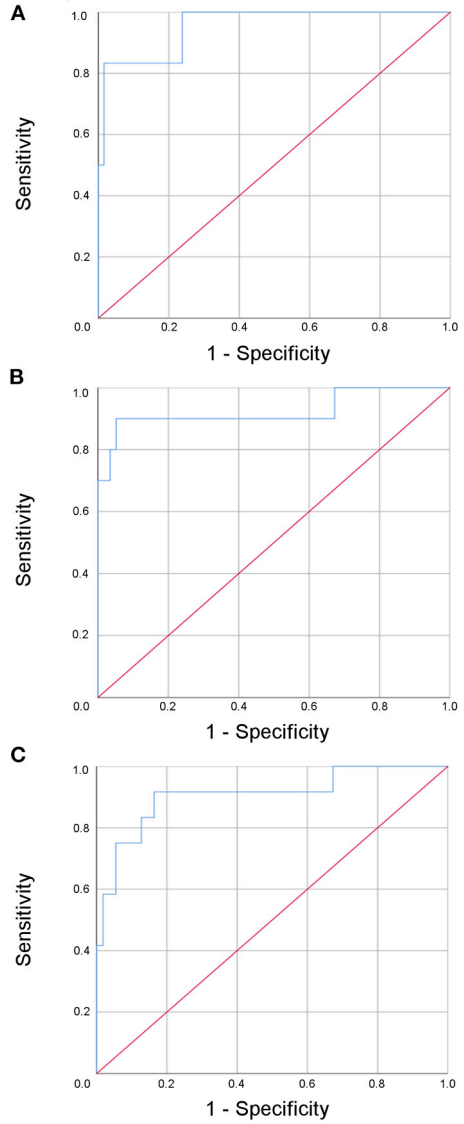

FIGURE 4 | Receiver operated curve (ROC) analysis of the multi-modal biomarker panel for the prediction of an excellent outcome after three **(A)**, six **(B)**, and twelve **(C)** months for patients with minor ischemic stroke.

with MIS, too. Therefore, this proposed approach might facilitate further decisions in the diagnostic work-up (e.g., the need and timing of MRI, or the length of monitoring on a stroke unit) and in allocation to rehabilitation.

So far, biomarkers were optimized with respect to different scenarios (7, 14). Thus, the composition of biomarker panels does not only greatly vary within the same scenario (e.g., the differentiation between ischemic and hemorrhagic stroke) but even more between different scenarios. Hence, the here applied clinical- and biomarker-based approach would be a pragmatic compromise for two relevant scenarios. In particular, this multi-modal biomarker approach might be cost-effective in settings where cerebral MRI is not available in time or causes of great costs. Of course, acute treatment decisions like systemic thrombolysis will still be based on cerebral imaging as for instance CT, which is widely available and sufficient to rule out an intracranial bleeding (1). However, in clinical routine, the current diagnostic work-up of patients suffering from a focal neurological deficit with sudden onset do not depend on a confirmed ischemic stroke by cerebral MRI or a symptom duration of more than 24 h. Due to the high sensitivity of the here described combined clinical and serum biomarker-based approach, a future paradigm might allow the diagnosis of a biomarker-positive ischemic stroke while the confirmatory cerebral MRI could be omitted. Furthermore, the same biomarker panel might support decisions concerning the allocation of patients with MIS to rehabilitation at all, as well as to the timing and to the kind of rehabilitation (early vs. delayed, in- vs. out-patient rehabilitation).

This study has some limitations. First, although data collection was performed in a prospective manner, this study followed a retrospective data analyses, which limits generalization of the findings. Second, due to the relatively small sample size, the combined clinical- and serum biomarker-based approach needs to be confirmed in a larger cohort of patients with MIS and TIA, preferably with the use of validation groups. However, statistical analyses without pre-specified parameters carry the inherent risk for an over-optimization of the statistical model, and, therefore, an overestimating effect size. Noteworthy, validation studies can yield less accurate results than the initial study (16, 18). Third, blood samples were collected at a single time point within the first 24 h after symptom onset. Release kinetics of biomarkers may naturally differ, especially within the acute phase of stroke. For example, levels of NSE were found to demonstrate a biphasic course with a first rise within 3 h followed by a decrease and secondary increase until day 5 (21). Varying levels within the first 25 h were also described for MMP-9 and TIMP-1 in experimental stroke (22). Thus, a smaller time window for the collection of blood samples, blood sampling at specified time points, or repeated collections might increase accuracy. Further, future studies may also include novel biomarkers as for instance neurofilament light chain, which has shown an association with the long-term outcome after stroke (23), while its relevance in the acute and short-term course is still pending (24). Fourth, in MIS patients the etiology of the event was very heterogeneous and the relatively small sample size excluded further subgroup analyses. As a rule of thumb, there should be at least 20 patients for every predictor variable in a binary logistic regression analysis which would have resulted in the inclusion of at least 140 patients in this study. Thus, this study might have been underpowered.

On the other side, one strength of this study is the in-depth characterization of patients with TIA based on two negative MRI examination at the time point of study inclusion and day 7. Moreover, we addressed two relevant scenarios with the same clinical- and biomarker-based approach.

CONCLUSION

This pilot study provides evidence that the NIHSS together with a multi-modal panel of serum biomarkers covering pathophysiological aspects of stroke represents a promising

tool to differentiate between patients with MIS and TIA within the first 24 h after symptom onset. Furthermore, only by adding age, the same approach accurately predicted an excellent mid-term outcome in patients with MIS. Assuming that these findings can be confirmed in larger cohorts of stroke patients, the emerging paradigm of a biomarker-positive ischemic stroke might allow a more focused diagnostic workup and early planning of rehabilitative programs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon reasonable request.

ETHICS STATEMENT

The study complies with the guidelines for human studies and had been approved by the Local Ethics Committee of the University of Leipzig (approval number 198-08). All participants have given informed consent, either in a written or witnessed oral form.

AUTHOR CONTRIBUTIONS

DM and CH conceived the underlying investigation, while JP conducted the part resulting in the present study. DM, CH, MF, JP, and KK collected data. KH and MK-L contributed to data analysis. JP and DM performed data analyses and wrote the paper. All authors provided critical revisions to the manuscript.

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The Dynamic of Extracellular Vesicles in Patients With Subacute Stroke: Results of the "Biomarkers and Perfusion—Training-Induced Changes After Stroke" (*BAPTISe*) Study

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Objective: Extracellular vesicles (EV) are sub-1 μm bilayer lipid coated particles and have been shown play a role in long-term cardiovascular outcome after ischemic stroke. However, the dynamic change of EV after stroke and their implications for functional outcome have not yet been elucidated.

Methods: Serial blood samples from 110 subacute ischemic stroke patients enrolled in the prospective *BAPTISe* study were analyzed. All patients participated in the *PHYS-STROKE* trial and received 4-week aerobic training or relaxation sessions. Levels of endothelial-derived (EnV: Annexin V+, CD45–, CD41–, CD31+/CD144+/CD146+), leukocyte-derived (LV: Annexin V+, CD45+, CD41–), monocytic-derived (MoV: Annexin V+, CD41–, CD14+), neuronal-derived (NV: Annexin V+, CD41–, CD45–, CD31–, CD144–, CD146–, CD56+/CD171+/CD271+), and platelet-derived (PV: Annexin V+, CD41+) EV were assessed via fluorescence-activated cell sorting before and after the trial intervention. The levels of EV at baseline were dichotomized at the 75th percentile, with the EV levels at baseline above the 75th percentile classified as "high" otherwise as "low." The dynamic of EV was classified based on the difference between baseline and post intervention, defining increases above the 75th percentile as "high increase" otherwise as "low increase." Associations of baseline levels and change in EV concentrations with Barthel Index (BI) and cardiovascular events in the first 6 months post-stroke were analyzed using mixed model regression analyses and cox regression.

Results: Both before and after intervention PV formed the largest population of vesicles followed by NV and EnV. In mixed-model regression analyses, low NV [−8.57 (95%

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CI −15.53 to −1.57)] and low PV [−6.97 (95% CI −13.92 to −0.01)] at baseline were associated with lower BI in the first 6 months post-stroke. Patients with low increase in NV [8.69 (95% CI 2.08–15.34)] and LV [6.82 (95% CI 0.25–13.4)] were associated with reduced BI in the first 6 months post-stroke. Neither baseline vesicles nor their dynamic were associated with recurrent cardiovascular events.

Conclusion: This is the first report analyzing the concentration and the dynamic of EV regarding associations with functional outcome in patients with subacute stroke. Lower levels of PV and NV at baseline were associated with a worse functional outcome in the first 6 months post-stroke. Furthermore, an increase in NV and LV over time was associated with worse BI in the first 6 months post-stroke. Further investigation of the relationship between EV and their dynamic with functional outcome post-stroke are warranted.

Clinical Trial Registration: clinicaltrials.gov/, identifier: NCT01954797.

Keywords: stroke, biomarker, extracellular vesicle (EV), subacute stroke, functional recovery

INTRODUCTION

Stroke is the most frequent neurological cause for disability-adjusted life years (DALYs) worldwide (1). Long-lasting impairment in a patient's daily life is common after stroke. However, it is still difficult for clinicians to predict each patient's recovery potential and influencing factors (2). Clinical features account only for a portion of the predictable outcome (3, 4). Novel biomarkers are required aiming to adequately predict post-stroke recovery. These new biomarkers might also enable us to better understand the biological processes leading to clinical heterogeneity in stroke patients (5, 6).

The family of extracellular vesicles (EV) is a heterogeneous group of particles, formed by outward budding or exocytosis of the origin cell. Their diameter ranges between 100 nm and 1 μm. The outer membrane is formed by a lipid bilayer membrane that encloses the cargo of the respective particle (7). The concentration and differentiation of EV are potential biomarkers with a prognostic value. The association between EV and coronary artery disease is well-described, and studies have demonstrated involvement of EV in coagulation, inflammation, immune regulation, and angiogenesis, factors which also play a role in the pathomechanisms of stroke (8, 9).

Simak et al. showed in a cohort of 41 patients that levels of endothelial EV (EnV) after acute ischemic stroke are increased compared to a control group and correlate with stroke lesion size. Furthermore, higher levels of EnV in acute stroke were found in patients with moderate to severe stroke compared to mild stroke. In addition, high levels of EnV in acute stroke were associated with a lower Barthel Index (BI) at discharge (10). Jung et al. demonstrated an association of EnV to the national institute of health stroke scale (NIHSS) in acute stroke (11). Not only are EV potential biomarkers in the acute phase after stroke but, as Huo et al. demonstrated in a prospective cohort of 571 stroke patients, they also correlate with the number of recurrent cardiovascular events. They showed that high levels of EnV, leukocyte-derived EV (LV), and platelet-derived EV (PV) were associated with a worse cardiovascular outcome within 3 years (12). Levels of EV are not only increased in acute but also in subacute stroke as Lundström et al. demonstrated in a case–control study with healthy individuals. They also showed an association between increased tissue-factor bearing vesicles in acute stroke and cardiovascular events during follow up (13). Li and Qin demonstrated that EnV are also weakly correlated to the subtype of stroke in the Oxfordshire Community Stroke Project (OCSP) classification system (14). In a cohort of patients with coronary artery disease (CAD), van Craenbrook et al. demonstrated that over the course of 12 weeks aerobic exercise did not lead to an increase in EnV. However, patients with lower baseline concentrations of EnV showed a better functional response to the training sessions (15). Most of these studies investigated the levels of EV in the acute phase after stroke. The associations of dynamic changes in vesicles in subacute stroke and patient outcome have not been investigated in previous trials. Thus, we aimed to investigate the relationship between EV levels in subacute stroke and their dynamic changes with functional recovery and major vascular events up to 6 months after stroke.

METHODS

The *BAPTISe*-Study

The "Biomarkers and Perfusion—Training-Induced changes after Stroke" study (*BAPTISe*, clinicaltrials.gov identifier: NCT01954797) is a prospective, endpoint-blinded longitudinal observational substudy of a randomized controlled trial named "Physical Fitness Training in Patients with Subacute Stroke" (*PHYS-STROKE*, clinicaltrials.gov identifier: NCT01953549). For further details we refer to the previously published study protocols (16, 17). Inclusion criteria for *BAPTISe* can be found in **Supplementary Table 1**. Here, we briefly summarize *PHYS-STROKE*: 200 patients with subacute and moderate to severe stroke were randomized to a fitness group performing 25 min of treadmill-based, aerobic fitness training five times a week for 4 weeks, or a relaxation group, performing muscle relaxation

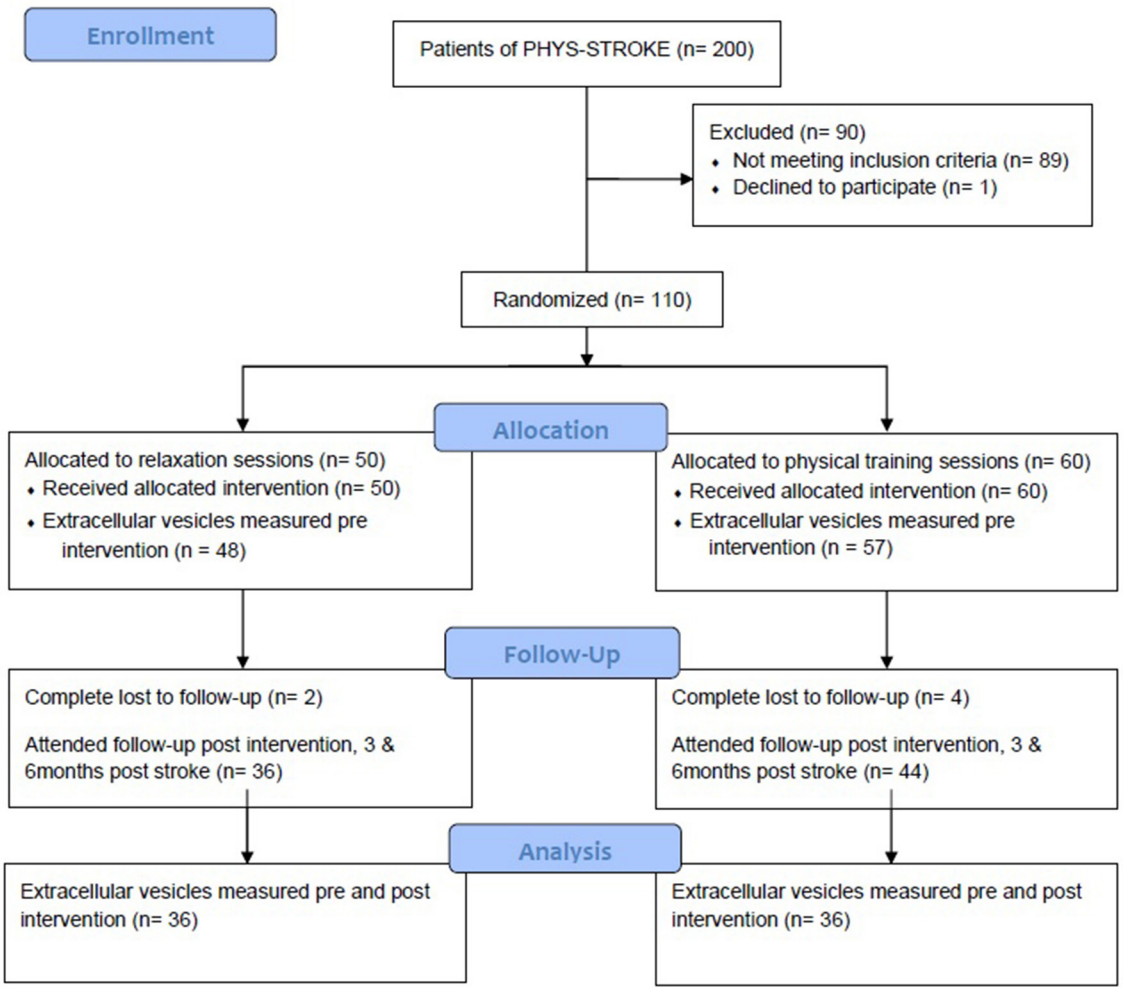

FIGURE 1 | Consort flow diagram for the BAPTISe trial.

with the same duration and frequency. The trial did not a show a significant difference in the co-primary efficacy endpoints, maximal walking speed, and Barthel Index (BI) at 3 months post stroke, but a higher rate of serious adverse events in the fitness group; 110 patients of the *PHYS-STROKE* cohort participated in *BAPTISe*. All patients in *BAPTISE* suffered from subacute ischemic stroke (5–45 days after stroke onset), scored a BI of <65 points, and received cerebral MRI (18). The intervention was started when the patients were clinically able to perform the exercise demanded by their intervention arm.

Levels of EV were measured before and after the intervention, which the patients underwent as part of *PHYS-STROKE*. The BI, a numerical score ranging from 0 to 100 ranking the patient higher for independence in activities of daily life, was evaluated pre-intervention, post-intervention, 3 months post-stroke, and 6 months post-stroke (see also **Figure 1**).

For this analysis, the BI, as a measure for functional outcome, was used as the primary outcome parameter. Our secondary outcome is the combined occurrence of major cardiovascular events being defined as ventricular flutter or fibrillation, myocardial infarction, recurrent stroke, or transient ischemic attack or death.

Blood Draws and Measurement of Extracellular Microvesicles

We had drawn the blood samples shortly before the first day of intervention and shortly after the last day of the intervention by a study nurse. Blood samples of 4.5 ml were drawn and immediately centrifuged at $1{,}500\times g$ for 15 min and $13{,}500\times g$ for 5 min to retrieve platelet-free plasma and were subsequently stored at −80°C. The methodology for measurement of EV has been published previously (12). To summarize the essentials, quantification of EV concentrations was done using fluorescence-activated cell sorting (FACS). We used Attune Nxt acoustic focusing cytometer (Thermo Fisher Scientific, Carlsbad, CA, USA) with red (637 nm), blue (488 nm), and violet (405 nm) lasers to perform FACS. Our gating was performed with Kaluza Software (version 1.5; Beckman and Coulter, Brea, CA, USA).

TABLE 1 | Definition of subpopulations of extracellular vesicles based on the surface markers.

Origin cell	Surface markers
All extracellular vesicles	Annexin V (AV)+
Platelet-derived EV	AV+ CD41+
Leukocyte-derived EV	AV+ CD45+
Monocyte-derived EV	AV+ CD45+ CD14+
Endothelial-derived EV	AV+ CD45– CD144+/CD146+/CD31+
Neuronal-derived EV	AV+ CD45– CD144– CD146– CD31– CD56+/CD171+/CD271+

TABLE 2 | Baseline characteristics of the full *BAPTISe* population.

Variable	Baseline cohort ($n = 110$)
Age in years (median, IQR)	69, 60–78.75
Sex (female, %)	45, 40.9
NIHSS at admission (median, IQR)	9, 6–12.75
Barthel Index at admission (median, IQR)	50, 35–60
Platelet vesicles pre-intervention in Vesicles/μl (mean ± standard deviation)	616.09 ± 933.42
Neuronal vesicles pre-intervention in Vesicles/μl (mean ± standard deviation)	56.91 ± 69.42
Endothelial vesicles pre-intervention in Vesicles/μl (mean ± standard deviation)	47.84 ± 53.62
Leukocyte vesicles pre-intervention in Vesicles/μl (mean ± standard deviation)	17.1 ± 25.83
Monocyte vesicles pre-intervention in Vesicles/μl (mean ± standard deviation)	3.24 ± 6.23

All EV were defined as Annexin V-binding particles and with a diameter below 1 μm. To define populations of EV we applied sequential gating to cell-surface specific markers (see **Table 1**).

Statistical Analysis

Patients were dichotomized based on baseline EV levels, and dynamics in EV levels were defined as the difference between pre-intervention EV levels and post intervention EV-levels. In both cases, the cut-off was defined as the 75th percentile, to differentiate between high or low levels at baseline and high or low change in EVs. We used a mixed model approach for the change of BI depending upon EV levels and EV dynamics adjusting for BI pre-intervention, age, sex, NIHSS at baseline, and stroke etiology classified by TOAST (19). To model the occurrence of major cardiovascular events, we performed Cox proportional hazard analyses with adjustment for age, sex, NIHSS at baseline, and stroke etiology. The statistical analyses were predefined in an analysis plan. All analyses were performed using "R: A Language and Environment for Statistical Computing" version 3.6.2. For modeling the "lme4" package was used in version 1.1.23.

RESULTS

One hundred ten patients of the *PHYS-STROKE* study were included in the BAPTISe cohort and had levels of EV determined at least once. Concentrations of EV prior to intervention were obtained in 105 patients. In 82 patients EV were evaluated twice (before and after the trial intervention).

Baseline characteristics including levels of EV of all patients are shown in **Table 2**. Our patient collective has a median age of 69, consists of ∼41% females, and presented with a mean NIHSS of 9 on admission. There were no differences in BI or NIHSS at baseline regarding baseline EV-populations detected by Student's *t*-test (see **Supplementary Table 2**). The largest population of EV at baseline was formed by PV with a mean of 616.1/μl, followed by NV (mean: 56.9/μl), EnV (mean: 47.8/μl), LV (mean: 17.1/μl), and monocyte-derived EV (MoV) (mean: 3.2/μl) (see also **Figure 2**). After the intervention, the vesicle populations were unchanged in order and magnitude with PV forming the largest population (mean: 505.8/μl), followed by NV (mean: 52.8/μl), EnV (mean: 45.8/μl), LV (mean: 16.6/μl), and MoV (mean: 2.8/μl). Student's *t*-test did not reveal any difference between the distributions of vesicle concentration at baseline and at follow-up in each EV subtype (see **Supplementary Figure 1**). In addition, neither at baseline nor at follow-up, Students *t*-test detects any sex-related or intervention related differences in each vesicle population. We also did not find a correlation between NIHSS at admission and EV concentrations, neither at baseline nor at follow-up. We could also not detect an association of concentration in EV at baseline and time since stroke onset.

The average Barthel Index showed an increase from 50 at baseline, up to a median of 90 at 6 months post-stroke (see also **Supplementary Figure 2**). We recorded 24 major cardiovascular events with a median time to event of 60 days in 37.4 person-years of follow-up (see also **Table 3**).

Using mixed models with the BI in the first 6 months post-stroke as dependent and EV levels as independent variable, low PV levels and low NV levels at baseline were associated with lower BI values in the first 6 months post-stroke. For low PV at baseline, we observed a change in BI of −6.97 (95% CI: −15.53 to −1.6) and for low NV at baseline, a change in BI of −8.57 (95% CI: −13.92 to −0.01) in the first 6 months post-stroke (see **Figure 3**). Furthermore, we observed an association of an increase in LV and NV and a reduced BI in the first 6 months post-stroke, meaning that in patients that did not show an increase in LV, the BI in the 6 months post-stroke was higher by 6.82 (95% CI: 0.25–13.4). Patients which did not increase in NV showed an increase in BI in the 6 months post-stroke by 8.7 (95% CI: 2.1–15.34, see **Figure 4**). In **Table 4**, the effect of each vesicle at baseline and their dynamic on the BI can be found.

DISCUSSION

Our study showed that lower levels of NV and PV at baseline are associated with poor functional outcome in the first 6 months post-stroke. Furthermore, we could demonstrate an association in the patients with the largest increase in LV and NV in the subacute phase after stroke and a decreased BI in the first 6

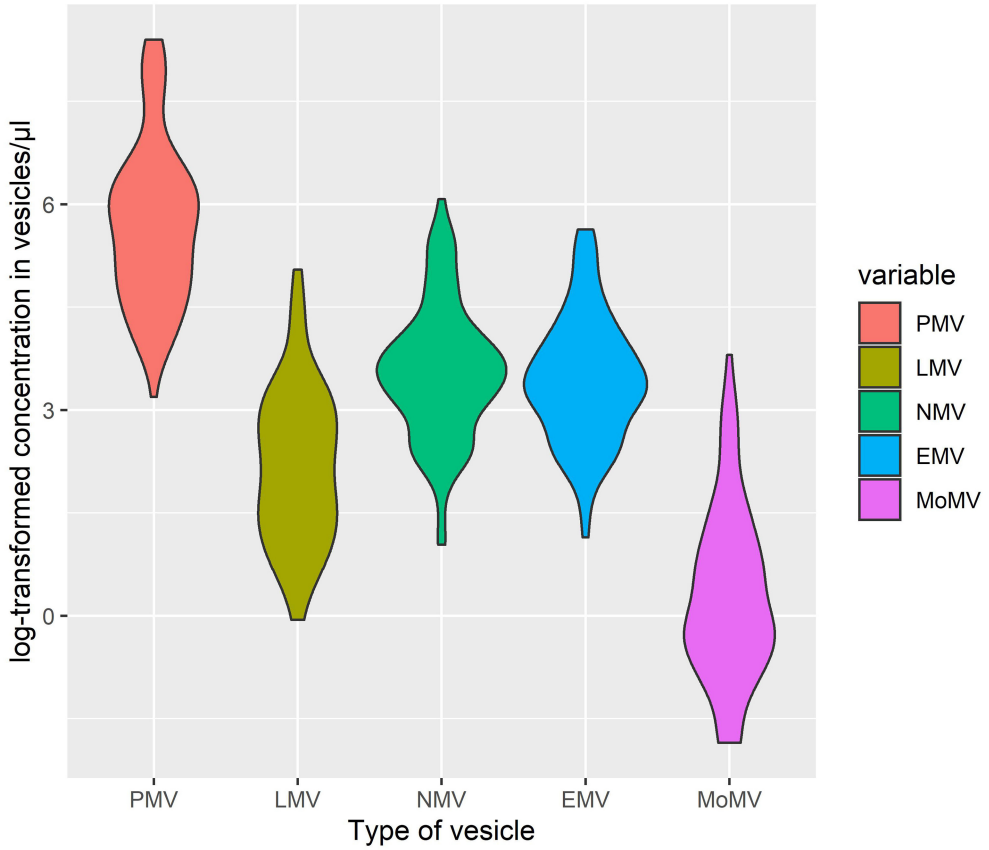

FIGURE 2 | Violin-plot of log-transformed concentrations of extracellular vesicles subpopulations at baseline. PV, platelet-derived vesicles; LV, leukocyte-derived vesicles; NV, neuronal-derived vesicles; EnV, endothelial-derived vesicles; MoV, monocytal-derived vesicles.

TABLE 3 | Functional outcome parameters (BI, Barthel Index; IQR, interquartile range).

BI pre-intervention (median, IQR)	50, 35–60
BI post-intervention (median, IQR)	75, 55–90
BI at 3-months follow up (median, IQR)	80, 65–95
BI at 6-months follow up (median, IQR)	90, 70–100
Number of major events	24
Time to major events in days (median, IQR)	60, 41.5–98.25

months post-stroke. Neither the baseline levels of EV nor their change showed an association to cardiovascular risk and death 6 months post-stroke.

A previous systematic review by Wang and colleagues showed that various populations of EV are increased after ischemic stroke (20). One study could also demonstrate that EnV-levels assessed in the first 7 days post stroke were associated with stroke severity (14). In our report, we could not link baseline levels of EV to NIHSS or BI on admission (see **Supplementary Table 2**). However, Li and Qin detected differences in EV concentration in patients with NIHSS <5 and ≥ 5. Our patients, on the contrary, presented with a median NIHSS of 9 suffering from a more severe clinical syndrome. One possible interpretation would be that EV in acute stroke increase up to a certain threshold based on stroke severity and reach a ceiling effect.

We detected an association of low NV, PV, and EnV levels at baseline with a lower BI during the first 6 months post-stroke. In contrast, Simak and colleagues showed that in acute stroke, high levels of EnV are associated with a worse BI at hospital discharge (10). However, there are two key differences in the trials. In *BAPTISe*, we evaluated levels of EV in subacute stroke, ranging from day 5–45 post-stroke, whilst Simak et al. evaluated the levels of EV far earlier, with a median time of 37 h after symptom onset. Therefore, we are comparing the EV of patients in subacute stroke with patients in acute stroke. Furthermore, we evaluated BI at multiple predefined time-points with longer follow-up and not at hospital discharge. This reduces bias quite a bit since hospital discharge typically happens after clinical improvement in the patient which most likely is accompanied by improvement in BI. We also want to stress the point that the EnV were differently defined in the studies leading to measurement and comparison of different EV-populations. Furthermore, we want to emphasize that also in the same vesicle populations, paradoxical observations can be noted (21). In contrast to Jung et al. we could not demonstrate an association between EnV and NIHSS at admission (11). However, they obtained all their

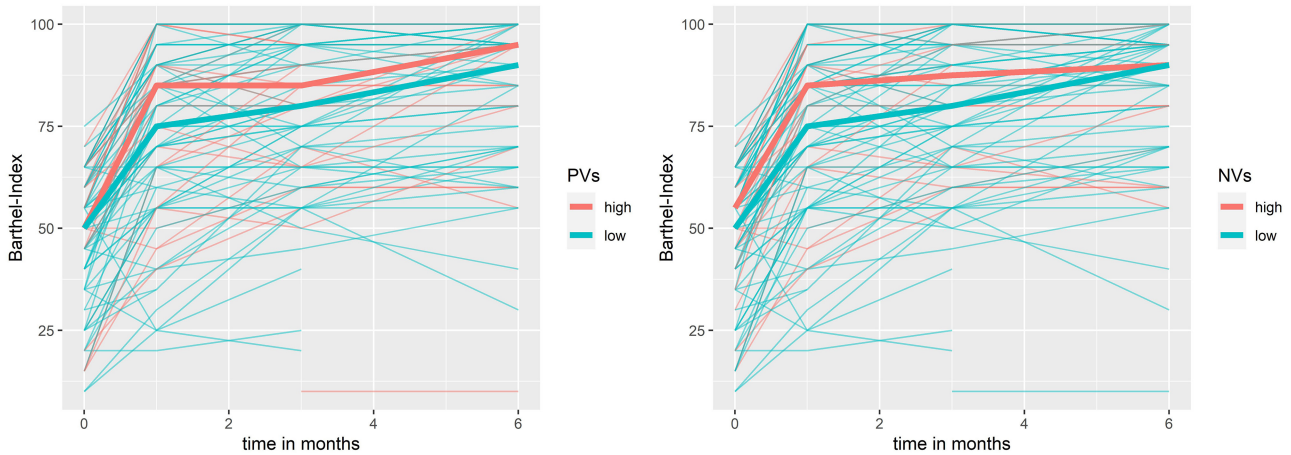

FIGURE 3 | Barthel Index in the first 6 months post stroke by neuronal- and platelet-derived extracellular vesicles at baseline. bold line = median; NV, neuronal-derived vesicles at baseline; PV, platelet-derived vesicles at baseline.

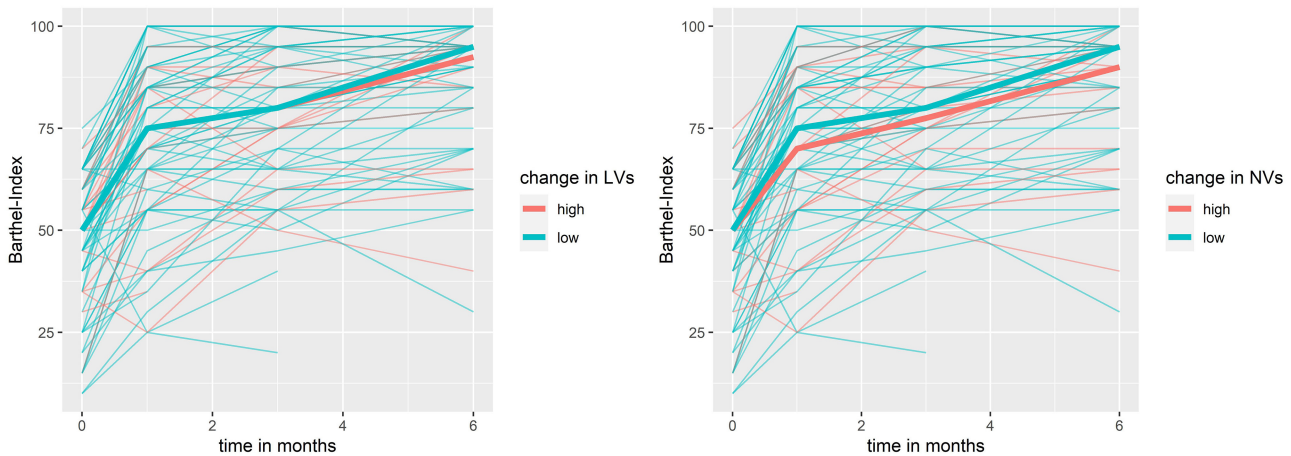

FIGURE 4 | Barthel-Index in the first 6 months post stroke by dynamic in neuronal- and leukocyte-derived extracellular vesicles. bold line = median; NV, neuronal-derived vesicles; LV, leukocyte-derived vesicles.

data on vesicles in the first 6 days post stroke in contrast to our 5–45 days post stroke. As the colleagues also demonstrated in their paper, the concentrations of EnV in their trial highly are dependent on the time since symptom onset, with concentrations being negatively correlated to the time since onset. Since such a correlation could not be found in our patients with subacute stroke, the concentration of EnV might reach a plateau. This is also underlined by the findings of Chiva–Blanch, in which they also did not find a change in EnV concentrations in subacute stroke (22).

In previous trials, PV in acute stroke were linked to small and large vessel occlusion (23, 24). Elevation of PV has been shown to be associated with a thrombotic state and activated platelets (25). However, we find that elevated levels of PV, in subacute stroke, are associated with a better BI post stroke. As has been shown, PV can also exhibit anticoagulatory abilities (26). Further research is needed to understand the underlying mechanisms of PV, especially in explaining the dichotomous effects they can exhibit. For future research, the comparison of tissue factor-positive and -negative PV populations promises interesting findings, as already demonstrated by Lundström et al. (13).

There are only few studies investigating the role of EV as biomarkers for long-term outcome in stroke patients. Huo and colleagues investigated EV levels in a cohort of 621 patients in acute stroke (the acute phase defined as the first week after stroke). They compared patients based on quartiles of EV levels and found an association of high EnV and LV levels and cardiovascular events or death over a follow-up of 3 years (12). Lee and colleagues on the other hand investigated EV

TABLE 4 | Adjusted effect on the Barthel Index and the 95% Confidence interval of each vesicle at baseline and their dynamic from the mixed model.

Vesicle type	Estimate of change in barthel index progression	95% Confidence interval
Endothelial vesicles low	−6.97	−13.98 to 0.07
Leukocyte vesicles low	−4.35	−11.41 to 2.69
Monocyte vesicles low	−3.91	−11.14 to 3.34
Neuronal vesicles low	−8.57*	−15.53 to −1.6
Platelet vesicles low	−6.97*	−13.92 to −0.01
Endothelial vesicles low increase	6.77	−0.03 to 13.59
Leucocyte vesicles low increase	6.82*	0.25–13.4
Monocyte vesicles low increase	2.85	−3.82 to 9.46
Neuronal vesicles low increase	8.7*	2.1–15.34
Platelet vesicles low increase	4.58	−2.43 to 11.57

$p < 0.05$, adjusted for age, sex, baseline NIHSS, and TOAST.

TABLE 5 | Adjusted proportional hazard ratio of cox regression and 95% confidence interval for vesicles at baseline and their dynamic for the occurrence of major cardiovascular events.

Vesicle type	Hazard ratio estimate	95% Confidence interval
Endothelial vesicles low	0.54	0.18–1.58
Leukocyte vesicles low	0.89	0.3–2.61
Monocyte vesicles low	0.89	0.3–2.68
Neuronal vesicles low	0.48	0.17–1.35
Platelet vesicles low	0.96	0.33–2.79
Endothelial vesicles low increase	1.55	0.28–8.68
Leucocyte vesicles low increase	1.49	0.28–8.01
Monocyte vesicles low increase	1.02	0.2–5.16
Neuronal vesicles low increase	1.44	0.27–7.77
Platelet vesicles low increase	4.85	0.54–43.61

Adjusted for age, sex, baseline NIHSS, and TOAST.

levels in 298 patients in subacute stroke, defined as stroke in the last 3 months. They dichotomized their cohort for EnV levels at the 50th percentile and could also find an association of cardiovascular events or recurrent stroke over the course of 3 years (27). We were not able to replicate these findings in our trial. This is likely attributable to the smaller sample size and a way shorter follow-up time of 6 months compared to the two other studies. In addition, Lundström and colleagues showed the association of phosphatidyl-serine-negative PV which were also associated with recurrent ischemic stroke or myocardial infarction (13). This is a vesicle population, which we did not measure. However, this emphasizes the broad spectrum of EV types and possible mechanisms influencing the patients' follow-up.

All estimated Cox proportional hazards ratios and their 95% confidence intervals are listed in **Table 5**. The full presentation of corresponding Kaplan–Meier plots can be found in **Supplementary Figures 3, 4**.

It has been demonstrated that CD34+, CD56+/Annexin V+ EV, labeled neural progenitor cell-derived vesicles, are also increased over a prolonged period post-stroke compared to patients matched for cardiovascular risk (22), which the authors interpreted as an indicator for breakdown of blood–brain barrier. The increase in LV could be interpreted as an inflammatory state (21), which has been associated with worse outcome in stroke (28). It has to be noted that we gated our NV differently and used surface markers that are associated with neuronal cells. Furthermore, we excluded all EV bearing typical surface antigens for other cell-types. However, it is not clear if the measured EV truly originate from neuronal cells, especially since the density of surface proteins on EV is different from their origin cell (29). Therefore, we want to emphasize that the quantification of NV is still experimental and not fully validated. However, it should be the goal of further research to validate the NV and investigate their role in stroke and other neurological diseases.

One of the main limitations of our study is the relatively short follow-up time. In addition, there are always subpopulations of EV, which were not investigated such as tissue-factor bearing vesicles or phosphatidyl-serine negative vesicles. A correction for confounding agents such as anticoagulation has also not been done. Also, the evaluation of NV in patient samples is still experimental and not validated. It is not clear if the measured EV are generated in the central nervous system and pass through the blood–brain barrier or are generated in the periphery. Furthermore, we are observing wide confidence intervals, giving our results less precision. A methodological weakness in our study is the single use of FACS for quantification and differentiation of EV and the omission of complementary resources. One should also note that even though our patients received two different interventions, no single-arm analysis was performed. This is mainly due to *PHYS-STROKE* not finding any effect of the intervention on BI but also due to resulting loss of power (18). It is, however, not clear if the intervention might have altered the results, e.g., having an effect on vesicle concentrations or modifying the event-rate for cardiovascular events. Therefore, we want to emphasize that in future research, different interventions and their effect on EV should be evaluated. As a final remark, we want to stress that in our study, no control group is given, which would give more context to the baseline levels and the dynamic of our EV.

The main strength on the other hand is sampling EV at two time points and having multiple follow-up visits regarding functional capacity of our patients at predefined time points. We were the first to link changes in vesicle population to functional outcome. In our study, we showed that both an increase in LV, which might be interpreted as in increase in inflammation, and an increase in NV are associated with a worse BI at follow-up. However, further studies are needed to investigate the mechanisms at place and to validate cut-offs for vesicles as prognostic tools. After better understanding EV,

they might also be used as a therapeutic agent or target in the future.

DATA AVAILABILITY STATEMENT

The pseudonymized raw data will be made available upon reasonable request to the authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Commission of the Charité, EA1/137/13. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AF, MEb, and AN conceived or designed and supervised the study. SH and NK designed the protocol for measuring vesicles. RJ drafted the manuscript and responsible for analyzing the data. SP contributed to statistical analysis. AF, MEb, and MEn obtained funding. All authors critically revised the manuscript for important intellectual content and gave final approval of the version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.731013/full#supplementary-material

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Oxidized Albumin and Cartilage Acidic Protein-1 as Blood Biomarkers to Predict Ischemic Stroke Outcomes

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Background: There is high demand for blood biomarkers that reflect the therapeutic response or predict the outcomes of patients with acute ischemic stroke (AIS); however, few biomarkers have been evidentially verified to date. This study evaluated two proteins, oxidized albumin (OxHSA) and cartilage acidic protein-1 (CRTAC1), as potential prognostic markers of AIS.

Methods: The ratio of OxHSA to normal albumin (%OxHSA) and the level of CRTAC1 in the sera of 74 AIS patients were analyzed on admission (day 0), and at 1 and 7 days after admission. AIS patients were divided into two groups according to their modified Rankin Scale (mRS) at 3 months after discharge: the low-mRS (mRS < 2) group included 48 patients and the high-mRS (mRS ≥ 2) group included 26 patients. The differences in %OxHSA and CRTAC1 between the two groups on days 0, 1, and 7 were evaluated.

Results: The mean %OxHSA values of the high-mRS group on days 0, 1, and 7 were significantly higher than those of the low-mRS group ($p < 0.05$). The CRTAC1 levels continuously increased from day 0 to day 7, and those of the high-mRS group were significantly higher than those of the low-mRS group on day 7 ($p < 0.05$).

Conclusions: These results suggest that higher %OxHSA and CRTAC1 are associated with poor outcomes in AIS patients. An index that combines %OxHSA and CRTAC1 can accurately predict the outcomes of AIS patients.

Keywords: acute ischemic stroke, cartilage acidic protein-1, oxidative stress, oxidized albumin, mass spectrometry, LC-MS/MS, biomarker (BM)

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INTRODUCTION

Acute ischemic stroke (AIS) caused by a thrombus is one of the most lethal and physical disabling cerebrovascular diseases (1). To minimize cellular necrosis and improve the outcomes of AIS patients, preventing further generation of reactive oxygen species (ROS) in the penumbra is an important issue (2). Edaravone, a free radical scavenger, plays an important role in removing ROS radicals and reduces the risk of further destruction of neuronal networks (3). However, administration of too much edaravone causes serious renal failure, so the administered dose needs to be strictly controlled. We hypothesized that if the redox state of AIS patients could be monitored

correctly, we could provide optimal, individualized doses of radical scavengers, which should improve patient outcomes.

Several studies have investigated the relationship between the outcomes of AIS patients and oxidative stress, aiming to verify biomarkers that can predict the outcomes using non-invasively obtained specimens, such as urine or blood (4–11). However, the experimental evidence regarding oxidative stress in humans remains lacking, mainly because it has not been sufficiently verified whether these biomarkers reflect actual oxidative stress (12).

Human serum albumin includes 17 disulfides and one unpaired thiol residue (Cys 34), and this unpaired thiol generates a disulfide bond with a free cysteine in plasma during periods of increased oxidative stress, yielding oxidized albumin (OxHSA) (13). Although the ratio of the oxidized form to the reduced form in healthy individuals (%OxHSA) is <40%, it increases to more than 50% in patients with conditions that expose them to high levels of oxidative stress, e.g., diabetes (14, 15), cardiovascular disease (16), Parkinson's disease (17), or liver cirrhosis (18). Since albumin is a major protein in plasma (3.5–5.5 g/dL), %OxHSA is considered to be the most reliable indicator of the whole-body redox state. Some studies have evaluated %OxHSA as an AIS biomarker. Moon et al. revealed that %OxHSA was elevated in the cerebrospinal fluid of AIS patients (19).

Recent studies have revealed that not only reducing oxidative stress, but also restoring nervous system function in the penumbra affects the outcomes of AIS patients. AIS patients often exhibit continued functional recovery for many years after their initial injury (20), which has also been observed in animal stroke models (21–23). Cartilage acidic protein-1 (CRTAC1) is a plasma protein that binds to the Nogo receptor-1 (NgR1) in the brain (24–26). Nogo inhibits neural regeneration, so binding of CRTAC1 to NgR1 blocks the interaction between the receptor and Nogo, thereby promoting axon growth. Takase et al. revealed that the CRTAC1 (LOTUS) in blood contributes to promote nerve regeneration in mice overexpressing CRTAC1 (26). We hypothesized that the plasma level of CRTAC1 changes depending on the degree of AIS, and may affect AIS patient outcomes. Although there are currently no medications for regenerating the nervous system, plasma levels of CRTAC1 may provide scientists clues toward developing new medications or treatments for AIS.

In this study, we aimed to evaluate two biomarkers, OxHSA and CRTAC1, as potential prognostic biomarkers in AIS patients.

MATERIALS AND METHODS

Participants

This study prospectively recruited patients with AIS from March 2017 to February 2018, to search for potential prognostic biomarkers. Institutional Review Board (IRB) approval was obtained from both the National Hospital Organization Kyushu Medical Center (IRB registration number, 16C132) and LSI Medience Corporation (MS/Shimura 16-22) for use of the patients' clinical information and plasma samples.

Diagnosis of AIS

Consecutive patients ($n = 74$) were enrolled who were admitted to the National Hospital Organization Kyushu Medical Center within 24 h after the symptoms of AIS were recognized. The number of participants was calculated by the estimated difference of %OxHSA between two groups (poor and mild outcomes, 2%), alpha-error (0.05), beta-error (0.2), and the standard deviation of %OxHSA obtained from validation (2.7%). AIS diagnosed by brain imaging, including computed tomography (CT) and magnetic resonance imaging (MRI), in all patients was classified as atherothrombotic brain (ATBI), cardioembolic (CE) or lacunar (LAC) infarction, and unclassified (UC) based upon the diagnostic criteria of the Classification of Cerebrovascular Disease III proposed by the National Institute of Neurological Disorders and Stroke (27), and of the Trial of Org 10172 in Acute Stroke Treatment (TOAST) study (28). Patients with serious renal, liver, respiratory, or cardiac conditions, and patients with infectious diseases, Parkinson's disease, schizophrenia, severe dementia, or severe cognitive impairment (mRS $\geq$ 4) at stroke onset were excluded from the present study. Informed consent was obtained from all patients who participated in the study. All patients underwent full physical and neurological examinations to obtain NIH stroke scale and mRS scores on admission and, at discharge. The mRS score was obtained again at the 3-month follow-up. Vascular risk factors, medical history, and smoking status were also recorded. Laboratory blood tests related to liver functions, lipid metabolism, and renal function were performed on admission. CRTAC1 and %OxHSA were analyzed at days 0 (on admission), 1, 7, and 14; however, data from day 14 were excluded because more than 40% of the patients were discharged from the hospital before then.

Blood Sampling for %OxHSA and CRTAC1

Albumin in plasma rapidly binds to free cysteine and generates OxHSA after blood sampling, so preventing further oxidation of albumin is crucial. Kubota et al. discovered that addition of an acid (0.5 M citrate buffer, pH 4.2) to plasma immediately after blood sampling prevented further oxidation (29). To avoid the need for adding citrate buffer to separated plasma, thereby enhancing the ease of use of the test, we developed a new vacuum blood collection tube containing 0.5 mol/L citrate buffer (pH 4.2) in cooperation with NIPRO Corporation (Osaka, Japan). Approximately 2 mL of whole blood was collected from AIS patients using this vacuum sampling tube. Each blood sample was centrifuged (2000 g, 4°C for 15 min,), and the plasma was either transferred to a 1-mL microtube within 4 h after blood sampling, or the sampling tubes with blood cells were stored at 4°C and the plasma separated within 24 h. Separated plasma was stored at −80°C and %OxHSA was analyzed within 3 months.

Abbreviations: %OxHSA, Ratio of oxidized albumin to total albumin; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; AIS, acute ischemic stroke; CRTAC1, cartilage acidic protein-1; LC-MS, liquid chromatography–mass spectrometry; mRS, modified Rankin Scale; NgR1, nogo receptor-1; OxHSA, oxidized human serum albumin; PCA, principal component analysis; ROS, reactive oxygen species.

%OxHSA Analysis

Plasma samples (25 μL) were diluted with 1 mL of 50 mM phosphate buffer (pH 6.0). The diluted sample was subsequently applied to a Bond Elute C18 EWP column, followed by washing with 1 mL of solvent A (0.1% formic acid, 9.9% acetonitrile and 90% water [%v/v]). Albumin was then eluted using 1 mL of solvent B (0.1% formic acid, 9.9% water and 90% acetonitrile [%v/v]). Liquid chromatography-mass spectrometry (LC-MS) data were acquired using a liquid chromatography system (Agilent HP1200, Agilent Technologies, Palo Alto, CA) coupled with an electrospray ionization quadrupole time-of-flight mass spectrometer (Agilent 6520, Agilent Technologies). The liquid chromatograph and mass spectrometer were linked with a stainless steel microtube (0.1-mm internal diameter, 1-m length), and no column was used. The mobile phase was 0.1% formic acid and 40% acetonitrile in water (%v/v), and albumin was eluted at a flow rate of 50 μL/min at room temperature under isocratic conditions. The mass spectrometer was operated in the positive mode with a capillary voltage of 4000 V. The nebulizing gas pressure was 20 psi and the dry gas flow was 5 L/min at 325°C. The mass range was set from m/z 800 to 3000. The injection volume was 2 μL, and the total measurement time was 3.0 min. The LC-MS raw spectrum was converted to CSV format using Mass Hunter Export (Agilent Technologies) and deconvoluted using Excel VBA (Excel 2010, Microsoft, Redmond, WA) software.

CRTAC1 Analysis

The plasma concentration of CRTAC1 was analyzed using a human CRTAC1 ELISA kit (CUSABIO, Houston, TX) according to the manufacturer's instructions.

Principal Component Analysis

Principal component analysis (PCA) was performed for CRTAC1 (days 0, 1, and 7), %OxHSA (days 0, 1, and 7), and laboratory biochemistry tests on admission using SIMCA software (version 13.0.3; Umetrics, Umeå, Sweden). The main purpose for conducting PCA was to condense the large amount of variable information into a smaller set of new composite dimensions with a minimum loss of information, and to discover underlying characteristics or relationships in the large dataset.

Statistical Analysis

The mRS-predictive index (mRS-PI) were obtained by the following procedure. First, the values of d-dimer, %OxHSA and CRTAC1 are preliminarily normalized by unit variance and zero mean centering, then the coefficients for the equation were optimized by sequential quadratic programming provided by Excel Solver to minimize *p*-value of student *t*-test between two groups.

RESULTS

Demographic Characteristics

The 74 AIS patients were divided into two groups based on their mRS score at 3 months after discharge. The low-mRS group included 48 patients with an mRS score of 0 or 1, and the high-mRS group included 26 patients with an mRS score ≥2. The cutoff was determined according to whether the patients had impairments. The characteristics of the patients are summarized in **Table 1**.

TABLE 1 | Characteristics of the patients.

Features		mRS < 2 (*n* = 48)	mRS ≥ 2 (*n* = 26)	*p*-value
Age (years)		70.4 (±14.2)	74.2 (±10.7)	0.20
Gender	Male (%)	32 (66.7%)	13 (50.0%)	
Stroke subtypes	ATBI	5	3	
	LAC	11	4	
	CE	8	7	
	UC	24	12	
mRS 3-months	0	26	-	
post-discharge	1	22	-	
	2	-	6	
	3	-	10	
	4	-	9	
	>5	-	1	
NIHSS	Admission	1.46 (±1.47)	4.92 (±4.74)	0.0011
Missing data	OxHSA	2	0	
	CRTAC1	3	0	
	Laboratory test	4	0	

OxHSA, oxidized albumin; CRTAC1, cartilage acidic protein-1; ATBI, atherothrombotic brain infarction; LAC, lacunar infarction; CE, cardioembolic infarction; UC, unclassified infarction.

Validating the New Vacuum Blood Collection Tube for %OxHSA

We obtained blood samples using the newly developed tube, and then left the collected samples at room temperature for 2 and 4 h, or at 4°C for 1, 3, and 10 days, without separating blood cells. We also stored the separated plasma at −20°C or −80°C for 1 and 3 months. We compared the %OxHSA of those samples to that of the plasma that was immediately separated after blood sampling. As a result. The validation tests demonstrated that %OxHSA was stable (relative error ≤ 5%) when the collection tube containing blood was left at room temperature for up to 4 h, or at 4°C for up to 10 days. After plasma separation, %OxHSA was stable at −80°C for 3 months; however, it was not stable at −20°C for ≤ 1 month (**Supplementary Table I**). Considering these results, we ensured that each blood sample was centrifuged and the plasma transferred to a microtube within 4 h after blood sampling. Alternatively, blood sampling tubes containing blood cells were stored in a refrigerator at 4°C and the plasma was separated within 24 h. Separated plasma was stored at −80°C and %OxHSA was analyzed within 3 months. We further evaluated the precision, accuracy, and robustness of the mass spectrometry analysis (**Supplementary Table II**). Both within-day and between-day reproducibility, and dilution, freeze–thaw,

TABLE 2 | Laboratory test results on admission for the low-mRS and high-mRS score groups.

Biomarker	mRS < 2 ($n = 48$) Ave. (St. Dev.)	mRS ≥ 2 ($n = 26$) Ave. (St. Dev.)	*p*-value
Systolic arterial pressure (mmHg)	154.8 (±26.6)	160.3 (±29.2)	0.767
Diastolic pressure (mmHg)	84.2 (±15.6)	88.8 (±19.3)	0.807
White blood cells	6617 (±1805)	7308 (±1919)	0.264
Red blood cells	461.1 (±72.1)	461.5 (±85.8)	0.891
Hematocrit (vol%)	40.3 (±7.8)	41.6 (±6.2)	0.735
Hemoglobin (g/dL)	14.2 (±2.1)	14.1 (±2.3)	0.818
Platelet ($\times 10^4/\mu$l)	22.5 (±7.7)	21.2 (±6.3)	0.702
AST (U/L)	25.4 (±8.4)	26.7 (±9.9)	0.429
ALT (U/L)	24.2 (±18.0)	23.3 (±18.2)	0.580
LDH (U/L)	228.8 (±58.5)	258.0 (±85.5)	0.399
ALP (U/L)	249.9 (±70.1)	248.2 (±86.2)	0.957
γ-GTP (U/L)	49.5 (±42.3)	34.2 (±35.7)	0.554
T-Bil (mg/dL)	0.683 (±0.439)	0.738 (±0.381)	0.372
Total cholesterol (mg/dL)	198.7 (±54.0)	197.9 (±36.5)	0.414
LDL-cholesterol (mg/dL)	110.5 (±38.3)	112.3 (±31.2)	0.456
HDL-cholesterol (mg/dL)	49.7 (±11.0)	55.2 (±17.8)	0.743
Triglycerides (mg/dL)	185.9 (±267.5)	127.3 (±62.4)	0.272
TP (g/dL)	7.05 (±0.52)	7.03 (±0.56)	0.875
Albumin (g/dL)	4.13 (±0.34)	4.04 (±0.40)	0.176
CPK (U/L)	100.1 (±59.4)	95.2 (±51.2)	0.397
BUN (mg/dL)	16.8 (±5.3)	17.5 (±7.6)	0.855
Cr (mg/dL)	1.12 (±1.48)	0.85 (±0.33)	0.459
eGFR (ml/min/1.73 m^2)	68.5 (±25.8)	66.5 (±23.0)	0.653
Urinary acid (mg/dL)	6.07 (±1.62)	5.62 (±1.29)	0.999
FBS (mg/dL)	113.9 (±54.4)	108.1 (±37.2)	0.832
BS (mg/dL)	149.0 (±112.0)	132.3 (±76.1)	0.656
HbA1c (%)	6.19 (±2.39)	5.79 (±1.70)	0.684
CRP (mg/dL)	0.26 (±0.25)	1.24 (±3.22)	0.123
PT-INR	1.08 (±0.24)	1.01 (±0.08)	0.101
APTT (seconds)	29.7 (±3.7)	29.1 (±2.6)	0.379
Fibrinogen (mg/dL)	307.0 (±65.3)	311.2 (±85.4)	0.829
D-dimer (μg/mL)	0.97 (±1.2)	3.30 (±4.99)	*0.027

*mRS, modified Rankin scale; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; γ-GTP, gamma-glutamyltransferase; T-Bil, total bilirubin; LDL-cholesterol, low-density lipoprotein cholesterol; HDL-cholesterol, high-density lipoprotein cholesterol; TP, total protein; CPK, creatine phosphokinase; BUN, blood urea nitrogen; Cr, creatinine; eGFR, estimated glomerular filtration rate; FBS, fasting blood sugar; BS, blood sugar; HbA1c, hemoglobin A1c; CRP, C-reactive protein; PT-INR, prothrombin time-international normalized ratio; APTT, activated partial thromboplastin time. *$p < 0.05$.*

and short-period stability were acceptable (relative error and coefficient of variance % ≤5%).

Relationship Between Outcome of AIS Patients and Laboratory Test Results on Admission

Table 2 shows the means and the standard deviations of the laboratory test results related to vascular risk (blood pressure), liver function (AST, ALT, LDH, ALP, γ-GTP, T-Bil, platelets, albumin, and PT-INR), lipid metabolism (total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides), sugar metabolism (BS, FBS, and HbA1c), and renal function (BUN, Cr, urinary acid, and eGFR) on admission. Comparisons of the low- and high-mRS groups revealed no significant differences in these values between the two groups (Student's *t*-test, $p > 0.1$). Only d-dimer was markedly elevated in the high-mRS group ($p < 0.05$) compared to the low-mRS group.

Change in %OxHSA and CRTAC1 Levels From Admission to Day 7

Figure 1 shows the changes in %OxHSA and CRTAC1 levels from admission to day 7. %OxHSA decreased in 48 (67%) of 72 patients (2 with missing data) over the 7-day period. The average %OxHSA on admission (41.1%) decreased slightly to 40.1% on day 7 ($p = 0.15$, **Figure 1A**). **Supplementary Figure I** shows the typical pattern of changes in %OxHSA during hospitalization for a 73-year-old man in the high-mRS group. On the other hand, increases in CRTAC1 were observed in 58 (82%) of 71 patients (3 with missing data), and the average CRTAC1 value on admission (187.5 ng/mL) was elevated significantly to 276.1 ng/mL on day 7 ($p < 10^{-5}$, **Figure 1B**). The putative functions of OxHSA and CRTAC1 are illustrated in **Figure 1C**. These results suggested that OxHSA increases with AIS and gradually decreases after commencement of treatment. On the other hand, CRTAC1 starts to increase after the onset of AIS, to restore nervous system function.

Principal Component Analysis

PCA was performed to understand the distribution of patients in the low- and high-mRS groups, the similarity and dissimilarity of biomarker expression patterns, and biomarker contributions to the mRS score. **Figure 2A** shows the PCA score plot expressing the distribution of patients in both the low- and high-mRS groups. The distribution of patients in the high-mRS group (**Figure 2A**, red) was slightly shifted up and to the left relative to that of patients in the low-mRS group (**Figure 2A**, blue). **Figure 2B** shows the loading plot, which indicates the similarity and dissimilarity of the biomarker expression patterns. Red blood cells, hematocrit, and hemoglobin were located in an adjacent area in the upper-right corner, which suggests that the expression patterns of these indicators among AIS patients are relatively similar. The axes of the score and loading plots were interlocked to allow comparison of the two plots; thus, the biomarkers located in the upper-left side in the loading plot (**Figure 2B**) were elevated in the high-mRS group. CRTAC1, %OxHSA, and d-dimer were located in an adjacent area in the upper-left corner of the plot, which suggests that their expression patterns are similar, and that they tended to be increased in the high-mRS group. The complete score and loading information are described in **Supplementary Tables III**, **IV**.

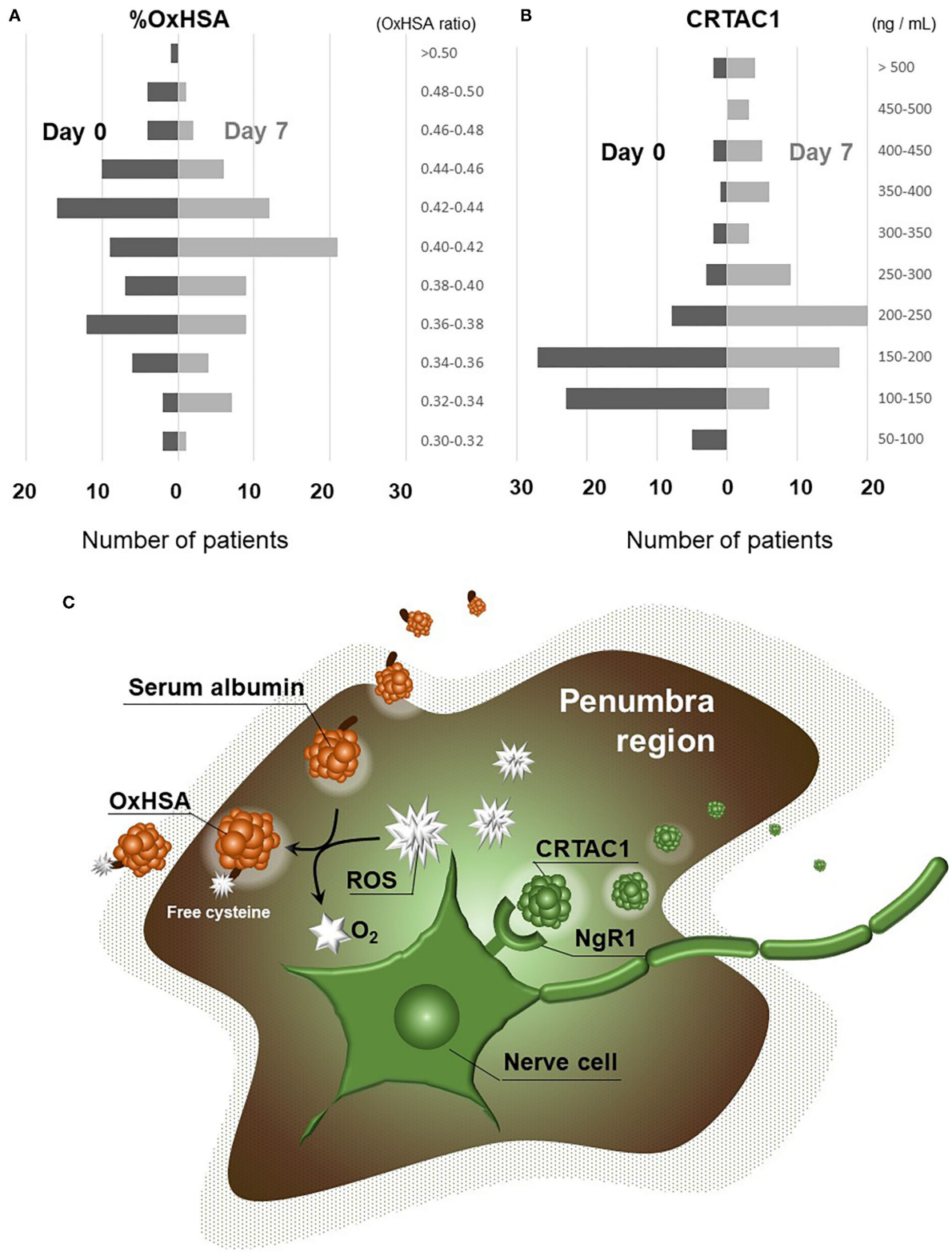

FIGURE 1 | Changes in %OxHSA and CRTAC1 from day 0 to day 7. **(A)** Histogram showing the levels of %OxHSA classified into 0.02-unit (2%) increments is displayed on the left (day 0) with black bars, and on the right (day 7) with gray bars. **(B)** Histogram with levels of CRTAC1 classified into 50-ng/mL increments is displayed on the left (day 0) with black bars, and on the right (day 7) with gray bars. **(C)** An illustration of OxHSA and CRTAC1 functions in the penumbra. OxHSA, oxidized albumin; CRTAC1, cartilage acidic protein-1.

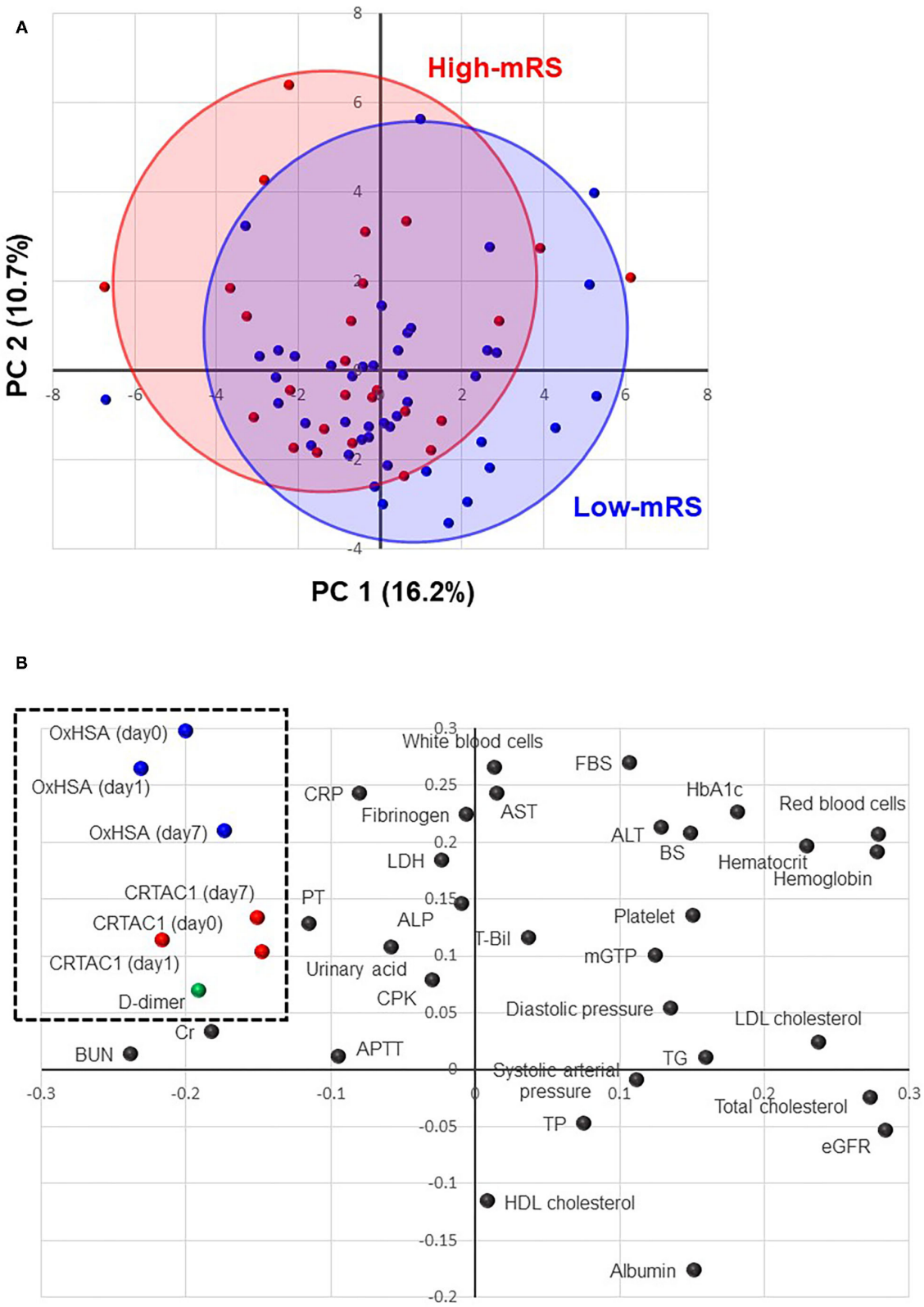

FIGURE 2 | Principal component analysis (PCA). **(A)** A score plot of the first and second components of PCA analysis. Red solid circles indicate high-mRS patients, and the red oval shows the distribution of the high-mRS group. Blue solid circles indicate low-mRS patients, and the blue oval shows the distribution of the low-mRS group. **(B)** A loading plot of the first and second PCA components: each dot represents biomarkers, such as %OxHSA, CRTAC1, and laboratory test results. The biomarkers located in the upper-left corner (enclosed with a dotted-line square) are elevated in the high-mRS group. mRS, modified Rankin scale; OxHSA, oxidized albumin; CRTAC1, cartilage acidic protein-1.

TABLE 3 | %OxHSA and CRTAC1 levels in the low-mRS and high-mRS score groups over a 7-day period.

Sampling	Biomarker	mRS < 2 ($n = 48$) Ave. (95% CI)	mRS ≥ 2 ($n = 26$) Ave. (95% CI)	*p*-value
Day 0	%OxHSA	40.2 (38.9–41.4)	42.7 (40.8–44.5)	*0.027
	CRTAC1 (ng/mL)	171.4 (14.4–195.4)	216.6 (175.0–258.3)	0.061
Day 1	%OxHSA	39.0 (37.8–40.1)	41.6 (39.9–43.3)	*0.013
	CRTAC1 (ng/mL)	246.5 (208.5–284.6)	316.3 (250.2–382.5)	0.068
Day 7	%OxHSA	39.1 (38.0–40.3)	41.7 (40.2–43.1)	**0.007
	CRTAC1 (ng/mL)	246.7 (217.1–276.4)	328.2 (271.6–384.7)	*0.013
Day 1–7	Δ OxHSA (%)	−1.1 (−2.0 to −0.2)	−1.0 (−2.5 to −0.6)	0.870
	Δ CRTAC1 (ng/mL)	77.0 (52.6–101.5)	111.5 (56.0–167.0)	0.251

*OxHSA, oxidized albumin; CRTAC1, cartilage acidic protein-1. *$p < 0.05$; **$p < 0.01$.*

Significant Differences in CRTAC1 and %OxHSA Between Low- and High-MRS Groups

Comparisons of CRTAC1 and %OxHSA on days 0, 1, and 7 between the low-mRS and high-mRS groups revealed that %OxHSA in the high-mRS group was significantly elevated at each time point compared to that of the low-mRS group (**Table 3**; **Figure 3A**). The CRTAC1 level in the high-mRS group was markedly increased compared to that of the low-mRS group, only on day 7 (**Table 3**; **Figure 3B**). As shown in **Table 2**, the level of d-dimer in the high-mRS group was also increased compared to that in the low-mRS group on admission (**Figure 3C**). The correlations between %OxHSA, CRTAC1, and d-dimer were positive but not strong ($r = 0.25$, 0.12, and 0.22 between d-dimer and %OxHSA, %OxHSA and CRTAC1, and CRTAC1 and d-dimer, respectively), i.e., the relationships between these three markers were complementary (**Supplementary Figure II**). The mRS-predictive index (mRS-PI), defined by the following equation, showed a significant increase in the high-mRS group ($p = 0.0004$, **Figure 3D**):

$$mRS - PI = 0.23 \times (d - dimer) + 0.44 \times (\%OxHSA) + 0.33 \times (CRTAC1),$$

where the values of d-dimer, %OxHSA and CRTAC1 are preliminarily normalized by unit variance and zero mean centering. The coefficients for this equation were optimized by sequential quadratic programming, an iterative method for constrained non-linear optimization, provided by Excel Solver.

DISCUSSION

In the present study, we provided evidence of the utility of two biomarkers, %OxHSA and CRTAC1, for predicting the outcomes of AIS patients. The levels of %OxHSA in patients with poor outcomes were much higher than those of patients with better outcomes. This suggests that markedly more ROS are generated in the ischemic brain of patients with poor outcomes than in those of patients with better outcomes, and may imply that %OxHSA was relatively proportional to the amount of ROS generated. However, further research will be needed because the increase in OxHSA during ischemic stroke may be related to other physiological stress.

A study by Khatri et al. found that administering albumin within 2 h of ischemia onset improved outcomes at 3 months (30), which suggests that albumin plays a role in maintaining the whole-body reductive conditions and removes ROS by self-oxidation. The results of Khatri et al.'s study also imply that %OxHSA is not only a viable biomarker, but also an albumin treatment indicator.

To the best of our knowledge, Rael et al.'s report is the only study to date to have demonstrated the relationship between %OxHSA in blood and the outcomes of AIS patients (31). Their study revealed a slight negative correlation between mRS and %OxHSA at discharge ($r = -0.17$, $p = 0.08$), where patients with higher %OxHSA showed relatively better outcomes. Since this result was contrary to our own, we investigated whether the discordant results may have arisen from differences in the criteria used for group assignment (the mRS cutoff score was 3 in Rael et al.'s study, but 2 in our study), or from the timing of determining mRS scores (values at discharge from the hospital were employed in Rael et al.'s study, whereas those at 3 months after discharge were used in our study). We thus reassigned patients in our study into two groups according to Rael's criteria, and repeated the comparison. We found that %OxHSA in the high-mRS group (≥3 at discharge) was significantly elevated compared to that in the low-mRS group (<3 at discharge) ($p < 0.05$); thus, we determined that our conclusion was not strongly affected by the cutoff of mRS (2 or 3) or the timing of determining mRS scores (at discharge or at 3 months after discharge). We supposed that another reason for the differences in the findings of these two studies may be the difference in blood sample collection. Albumin is susceptible to an oxidant atmosphere, and is easily oxidized after blood sampling if the plasma is not kept in an acidic state. However, the efficacy, accuracy, and reliability of our %OxHSA test for AIS should be verified in a larger cohort study.

We revealed that the level of CRTAC1 in blood increases after AIS onset and that the level on day 7 after onset is strongly associated with the outcomes of AIS patients.

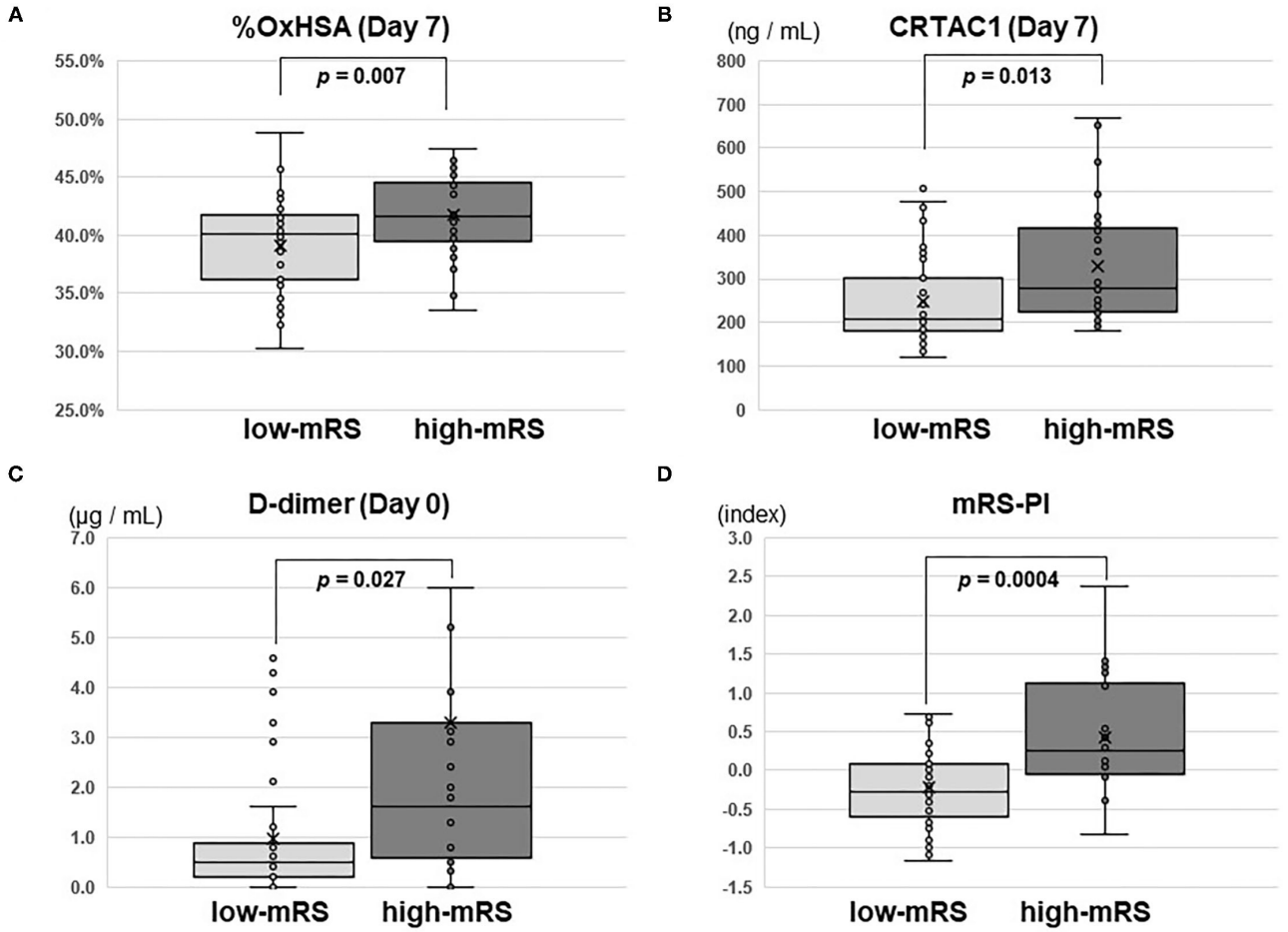

FIGURE 3 | Box-whisker plots of %OxHSA, CRTAC1, D-dimer, and mRS-PI. Box-whisker plots of **(A)** %OxHSA (day 7), **(B)** CRTAC1 (day 7), **(C)** D-dimer (day 0), and **(D)** mRS-PI. Student's *t*-test *p*-values for comparisons between low-mRS and high-mRS groups are shown. OxHSA, oxidized albumin; CRTAC1, cartilage acidic protein-1; mRS, modified Rankin square; PI, predictive index.

Takase et al. revealed that overexpression of CRTAC1 in an ischemic rat model improved the neurological score significantly, with CRTAC1 inhibiting NgR1 signaling to allow regeneration of the damaged nervous system (26). Considering this positive effect of CRTAC1 on ischemic brain damage, it is plausible that high plasma CRTAC1 levels yield positive effects on the outcomes of AIS patients. However, we found the opposite result, which suggested that the amount of CRTAC1 secreted is proportional to the degree of ischemic damage. Severe ischemic damage may result in rapid and excess supply of CRTAC1 to the brain; however due to the brain's limited ability to repair itself, or insufficient supply to the damaged area, the concentration in blood remains high for a certain period of time following the ischemic injury.

It was not surprising that the level of d-dimer on admission was elevated in the high-mRS group. In the study of 2,479 patients, Zhang et al. revealed that higher d-dimer levels within 24 h after stroke onset were associated with poor functional outcome at 90 days (32). Since the correlation among the three markers was weak, i.e., they have complementary relationships, we created a combined index involving all three markers, which increased the accuracy of predicting the outcomes of AIS patients ($p = 0.0004$).

To apply these markers for the prediction of the outcomes of patients following AIS in clinical practice, several issues need to be overcome. First, since the number of enrolled patients was low in this study, it is necessary to verify the efficacy of these biomarkers in a large number of AIS patients as well as healthy persons. Second, an interventional trial, such as increasing the amount of edaravone administration to high-risk patients should be conducted. Third, CRTAC1 splice variants CRTAC-1A and−1B should ideally be distinguished, as only CRTAC1-B is thought to have NgR1 inhibitory activity, and a more selective assay may increase the sensitivity and specificity of the test.

CONCLUSIONS

We provided insight into predicting the outcomes of AIS patients using two biomarkers, %OxHSA and CRTAC1. Higher %OxHSA and CRTAC1 are associated with worse outcomes. An index that combines %OxHSA, CRTAC1, and d-dimer can accurately predict the outcomes of AIS patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of National Hospital Organization Kyushu Medical Center (IRB registration number, 16C132) Institutional Review Board of LSI Medience Corporation (MS/Shimura 16-22). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TK designed this work and revised the manuscript. KT provided the conception of the work and wrote the manuscript. CH did data analysis and interpretation. TM, KM, JJ, and MY collected and analyzed the patent information. YO approved final version to be published. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.686555/full#supplementary-material

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Conflict of Interest: KT and CH are employed by LSI Medience Corporation, which provides the commercially available %OxHSA and CRTAC1 tests. KT is also employed by Kyushu Pro Search Limited Liability Partnership. The authors declare that this study received funding from LSI Medience Corporation. The funder had the following involvement in the study: the study design, data collection and analysis, decision to publish, and preparation of the manuscript.

The remaining authors declare that the 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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BRIEF RESEARCH REPORT
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Circulating Soluble CD163: A Potential Predictor for the Functional Outcome of Acute Ischemic Stroke

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Background: CD163 is a transmembrane glycoprotein receptor expressed on innate immune cells that sheds from the cell membrane and circulates as a soluble form (sCD163). This study aimed to investigate the circulating levels and clinical relevance of soluble CD163 (sCD163) in acute ischemic stroke (AIS).

Methods: This study recruited 300 patients with AIS and 78 healthy controls. The patients were followed up for 1 month to observe the functional outcomes. The neurological functions of the patients were assessed using the NIH Stroke Scale (NIHSS) and the modified Rankin Scale (mRS). The plasma concentrations of sCD163 at the baseline (patient admission) were determined by ELISA.

Results: We found that patients with AIS had significantly higher plasma sCD163 concentrations than the healthy control. Patients with high sCD163 concentrations had better functional outcomes than patients with low sCD163 concentrations. The plasma sCD163 concentrations were positively associated with the NIHSS scores and infarction volume at the baseline. The plasma sCD163 was positively associated with the improvement of the NIHSS scores but was negatively associated with the risk of poor functional outcomes during follow-up.

Conclusions: These findings indicate that circulating sCD163 is a potential biomarker that is associated with disease severity and the functional outcome of AIS.

Keywords: acute ischemic stroke, biomarker, soluble CD163, short-term, functional outcome

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INTRODUCTION

Stroke is one of the leading causes of death and disability worldwide (1). Biomarkers with the potential in identifying patients with a risk of having poor clinical outcomes are critical for aggressive monitoring and therapeutic interventions in these subjects. A panel of blood-based biomarkers is suggested to be predictive for the severity and prognosis of acute ischemic stroke (AIS) (2, 3).

The plasma membrane glycoprotein receptor CD163 is a member of the scavenger receptor cysteine-rich (SRCR) superfamily class B that is mostly expressed on monocytes and macrophages. CD163 could shed from cell membranes to

release soluble CD163 (sCD163) upon stimulation by inflammatory stimuli (4). sCD163 has been suggested to be biomarkers of many diseases, such as infectious diseases (5), tumors (6), and autoimmune diseases (7). sCD163 is increased in patients with intracranial hemorrhage and is associated with the improvement of neurological functions by promoting hematoma absorption (8). AIS involves local immune responses that encompass brain resident microglia and monocytes infiltrating from the circulation (9). The CD163 induced anti-inflammatory effects of monocytic cells are suggested to be involved in the pathogenesis of AIS (10). Animal studies have demonstrated that CD163 is upregulated following AIS (11). However, the changes of circulating sCD163 in patients with AIS are unknown. Therefore, this study aims to investigate the levels and clinical relevance of sCD163 in patients with AIS.

MATERIALS AND METHODS

Subjects

Patients with their first AIS (to exclude the effects of previous AIS events on circulating sCD163 concentrations) who visited the Department of Neurology, Chongqing General Hospital, University of Chinese Academy of Sciences during January 1, 2019, and January 31, 2020, were screened. The inclusion criteria included: (1) patients with newly onset AIS; (2) who visited the hospital within 24 h after symptom onset; (3) who are willing to participate. Seventy-eight age and sex-matched healthy subjects were recruited as controls from the healthy examination center of the same hospital. Subjects with diseases that might influence the circulating sCD163 levels were excluded from participation. Therefore, subjects were excluded if they have: (1) co-existing infections; (2) any types of tumors; (3) any types of autoimmune diseases; (4) other conditions that may influence the blood sCD163 levels, such as cirrhosis and severe inflammatory diseases; (5) declined to participate in this study. The patients were screened for eligibility for participation right after admission. However, although diabetes mellitus may contribute to the alteration of sCD163 (12), patients with diabetes mellitus were not excluded as it is a significant risk factor of AIS. Finally, 300 patients and 78 healthy controls were recruited in this study after excluding the subjects who failed for inclusion. Written informed consents for participation in this study and blood sampling were obtained from the subjects or their legal relatives. This study conformed with the principles of the Declaration of Helsinki and was approved by the investigational review board of the Chongqing General Hospital, University of Chinese Academy of Sciences.

Clinical Evaluation

At baseline (time of patient admission), the demographic information such as age, sex, and body mass index (BMI), pre-stroke medical history including oral antiplatelet or anticoagulants drug use, comorbidities including hypertension, diabetes mellitus, hypercholesteremia, and atrial fibrillation were collected and assessed right after admission. AIS was diagnosed according to the WHO Multinational Monitoring of Trends and Determinants in Cardiovascular Disease (WHO-MONICA) criteria with verification by MRI performed within 12 h after admission. The neurological deficits were examined with the National Institutes of Health Stroke Scale (NIHSS) upon admission (13), performed by a certified stroke neurologist. The AIS subtype was determined with the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria (14).

Patients' Follow-Up

The patients were followed up for 1 month since admission for the observation of functional outcomes. The primary endpoint was neurological functions 1 month since admission. The functional outcomes were assessed using NIHSS and the modified Rankin Scale (mRS) (15), which were blinded to the plasma sCD163 concentrations.

Blood Sampling and Measurement of CD163

Blood sampling was conducted right after patient admission before they receive any medical interventions. The blood was centrifuged right after collection at 20°C, spun at 2,000 g for 10 min, and stored at −80°C for biochemical analysis. The plasma sCD163 levels were determined using a human CD163 ELISA kit (Abcam, USA) according to the manufactural instructions. To preserve the linearity of the assays, samples containing high concentrations of sCD163 were diluted with an appropriate amount of calibrator diluent. The minimum detectable dose of sCD163 was 1.377 ng/ml, which was significantly lower than the sCD163 concentrations of the subjects in this study. Each test was conducted in duplicates and the means were used for statistical analysis.

Statistical Analysis

If continuous variables were normally distributed, an independent *t*-test was used, and if not, a Mann-Whitney *U*-test was used. Two-sample tests of proportions were used to compare the proportions for the categorical variables. The comparisons of means among groups were conducted using one-way ANOVA and the comparisons of the rate of NIHSS change during the follow-up between groups were conducted using two-way ANOVA. Spearman correlation analyses were conducted to assess the association between plasma sCD163 concentrations and NIHSS or infarction volume. A linear regression model was utilized to investigate the association between the plasma sCD163 levels and functional improvement (as indicated by the change of NIHSS during follow-up). A logistical regression model was utilized to evaluate the risk factors of poor functional outcome (as indicated by mRS$\geq$4) during follow-up. We first fitted univariate models with a single candidate variable at one time. The potential risk factors as determined by $p < 0.05$ were included in the final multivariate regression model. Statistical analyses were conducted using SPSS statistical package version 24 (IBM SPSS Statistics for Windows, Armonk, NY, USA).

RESULTS

Demographic Characteristics of Subjects

Three-hundred-nine patients with AIS and 99 healthy controls were screened for eligibility for participation in this study. Two-hundred-nine patients failed screening due to the following reasons: 176 patients declined to participate, 2 patients deceased during hospitalization, 11 patients had co-existing infections, 13 patients had previously diagnosed tumors, and 7 patients had autoimmune diseases. Twenty-one healthy controls declined to participate in this study. Finally, 300 AIS patients and 78 healthy controls participated in this study (**Figure 1**).

The patients were further divided into the high and low plasma sCD163 groups, respectively, according to their plasma sCD163 concentrations. The patients had significantly higher median BMI than the healthy controls. No significant differences in the mean age, percentages of males, frequencies of subjects with a history of smoking, antiplatelet drug use, antithrombotic drug use, a family history of stroke, hypertension, diabetes mellitus, hypercholesteremia, and atrial fibrillation between the patients and controls were observed. The high sCD163 group had a significantly higher proportion of males, a higher median diffusion-weighted imaging (DWI) hyperintensity volume, higher NIHSS scores at baseline, and higher frequencies of lacunar-type stroke than the low sCD163 group. However, there was no difference in the mean age, median BMI, frequency of smoking, antiplatelet drug use, antithrombotic drug use, family history of stroke, comorbidities including hypertension, diabetes mellitus, hypercholesteremia, and atrial fibrillation between the two groups. Besides, the median DWI hyperintensity volume, the median NIHSS and mRS scores at follow-up, frequencies of atherothrombotic, cardioembolic, and unknown type stroke, frequencies of hemorrhagic transformation, and recurrent AIS during follow-up were not significantly different between the high and low sCD163 groups (**Table 1**).

Plasma Concentrations and Clinical Relevance of sCD163 in AIS

We first compared the sCD163 concentrations between the patients with AIS and the control. We found that patients with AIS had significantly higher plasma sCD163 concentrations than the control (mean ± SD: 618.1 ± 292.0 ng/ml vs. 408.8 ± 157.5 ng/ml, $p < 0.001$) (**Figure 2A**). The high sCD163 group had better functional improvement as indicated by the change of NIHSS during follow-up than the low sCD163 group ($p < 0.001$) (**Figure 2B**). The plasma sCD163 concentrations were positively associated with the NIHSS at baseline ($\gamma = 0.609$, $p < 0.001$) and the DWI hyperintensity volume ($\gamma = 0.509$, $p < 0.001$) (**Figures 2C,D**). However, plasma sCD163 concentrations were not significantly associated with NIHSS at follow-up ($\gamma = 0.049$, $p = 0.401$) (**Figure 2E**). Patients with an mRS = 3 had significantly higher sCD163 concentrations than patients with an mRS = 1 ($p < 0.001$), 2 ($p < 0.001$), 4 ($p < 0.001$), and 5 ($p < 0.001$) (**Figure 2F**). Plasma sCD163 concentrations were positively associated with peripheral monocyte count, but no significance had been achieved ($\gamma = 0.108$, $p = 0.062$) (**Figure 2G**).

Factors Associated With Plasma sCD163 Levels

We utilized a linear regression model to investigate potential factors that were associated with plasma sCD163 levels. We found that hypercholesteremia and DWI hyperintensity volume were significantly associated with plasma sCD163 levels. These two factors along with diabetes mellitus, which is well-validated to be associated with sCD163 levels, were excluded from subsequent analysis (**Table 2**).

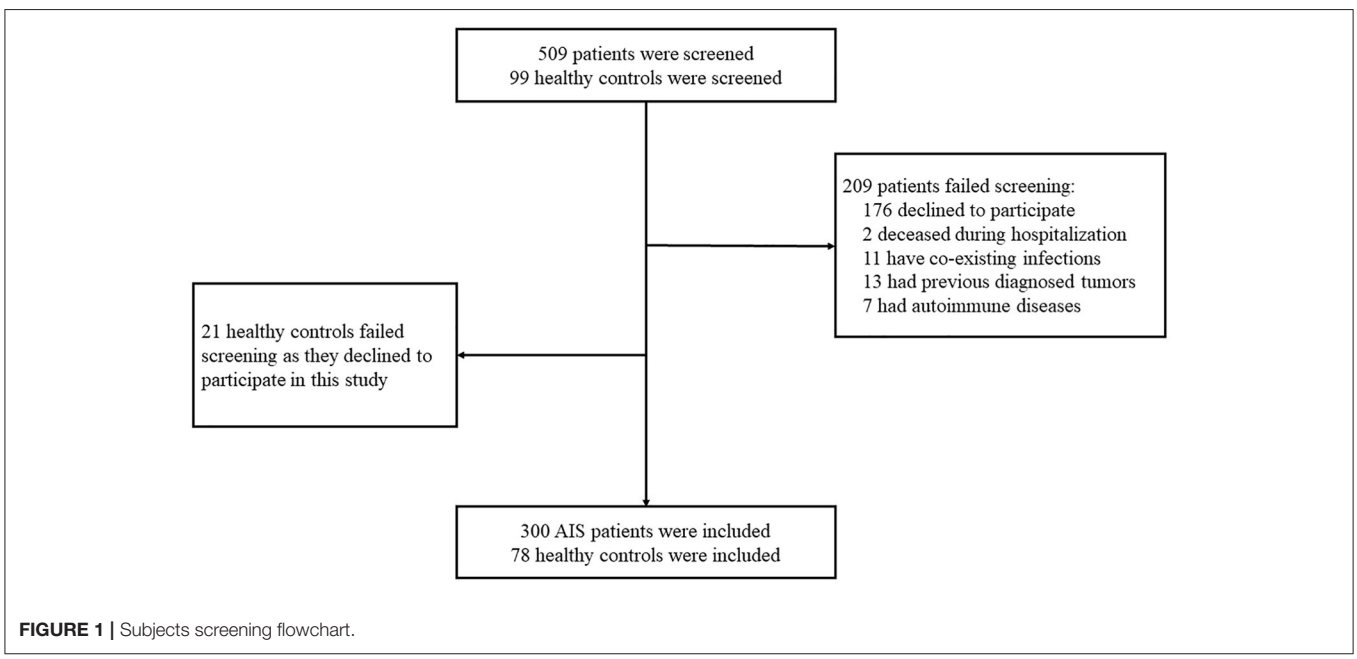

FIGURE 1 | Subjects screening flowchart.

TABLE 1 | Demographic data of subjects.

Variables	Control (n =78)	Low sCD163 group (n = 150)	High sCD163 group (n = 150)	P-value Control vs. patients	P-value Low vs. High
Age, Mean (SD)	62.86 (10.89)	64.83 (9.20)	65.16 (9.34)	0.082	0.756[a]
Male, No. (%)	48 (61.54)	95 (63.33)	75 (50.00)	0.520	**0.027**[b]
BMI, Median (IQR)	23.85 (22.28, 25.33)	24.68 (23.14, 25.76)	24.19 (22.96, 25.37)	**0.029**	0.086[c]
Smoking history, No. (%)	7 (8.97)	11 (7.33)	16 (10.67)	0.437	0.420[b]
Antiplatelet drug use, No. (%)	9 (11.54)	21 (14.00)	19 (12.67)	0.850	0.865[b]
Antithrombotic drug use, No. (%)	4 (5.13)	13 (8.67)	6 (4.00)	1.000	0.153[b]
Family history of stroke, No. (%)	3 (3.85)	9 (6.00)	9 (6.00)	0.587	1.000[b]
Comorbidities					
Hypertension, No. (%)	21 (26.92)	52 (34.67)	51 (34.00)	0.226	1.000[b]
Diabetes Mellitus, No. (%)	13 (16.67)	26 (17.33)	23 (15.33)	1.000	0.755[b]
Hypercholesteremia, No. (%)	6 (7.69)	9 (6.00)	19 (12.67)	0.825	0.072[b]
Arial fibrillation, No. (%)	7 (8.97)	13 (8.67)	6 (4.00)	0.451	0.153[b]
DWI hyperintensity volume, ml (SD)	NA	24.56 (8.86)	32.36 (7.53)	NA	**0.000**[a]
NIHSS at baseline, Median (IQR)	NA	8 (3, 13)	15 (11.75, 19)	NA	**0.000**[c]
NIHSS at follow-up, Median (IQR)	NA	4 (1, 9)	6 (3, 7)	NA	0.595[c]
mRS at follow-up, Median (IQR)					
Stroke etiology					
Atherothrombotic, No. (%)	NA	122 (81.33)	131 (87.33)	NA	0.204[b]
Cardioembolic, No. (%)	NA	13 (8.67)	6 (4.00)	NA	0.153[b]
Lacunar, No. (%)	NA	13 (8.67)	4 (2.67)	NA	**0.043**[b]
Unknown, No. (%)	NA	2 (1.33)	9 (6.00)	NA	0.061[b]
Complication					
Hemorrhagic transformation, No. (%)	NA	3 (2.00)	7 (4.67)	NA	0.335[b]
Recurrent AIS, No. (%)	NA	4 (2.67)	1 (0.67)	NA	0.371[b]
rtPA treatment, No. (%)	NA	13 (8.67)	16 (10.67)	NA	0.697[b]
mRS scores, Median (IQR)	NA	2 (0, 4)	3 (2, 3)	NA	0.641[c]

IQR, Inter-Quartile Range; BMI, Body Mass Index; TICS, Telephone Interview of Cognitive Status 40; NIHSS, National Institutes of Health Stroke Scale; NA, not applicable.
[a]Unpaired t-test; [b]Pearson χ^2-test; [c]Mann-Whitney U-test. Bold values represents statistically significant.

Association Between Plasma sCD163 Levels and Functional Improvement During Follow-Up

We first utilized a linear regression model to investigate the association between plasma sCD163 levels and functional improvement during follow-up as indicated by the decrease of NIHSS. Factors that are potentially associated with sCD163 levels were excluded from the model, including hypercholesteremia, DWI hyperintensity volume, and diabetes mellitus. Male sex and higher sCD163 concentrations were found to be positively associated with functional improvement during follow-up (**Table 3**).

Association Between Plasma sCD163 Concentrations and Poor Functional Outcome During Follow-Up

We next utilized a logistical regression model to investigate the association between plasma sCD163 concentrations and poor functional outcome during follow-up as indicated by mRS $\geq$ 4. In the univariate analyses, stroke type was found to be associated with poor functional outcomes during follow-up. Higher sCD163 concentrations were found to be protective factors of poor functional outcome during follow-up, and these associations remained significant in the multivariate analyses (**Table 4**).

DISCUSSION

In this study, we investigated the levels and clinical relevance of sCD163 in AIS. We found that patients with AIS had increased circulating sCD163 concentrations. sCD163 concentrations were associated with the severity and prognosis of AIS. Specifically, sCD163 was positively associated with the improvement of neurological functions but negatively associated with the risk of poor prognosis in AIS during follow-up.

Previous studies suggest that the AIS-associated inflammatory component is partly driven by the myeloid immune compartment, including microglia, peripheral monocytes, and macrophages (16). Numerous studies identified alterations in immune biomarkers in the cerebral spinal fluid (CSF) and

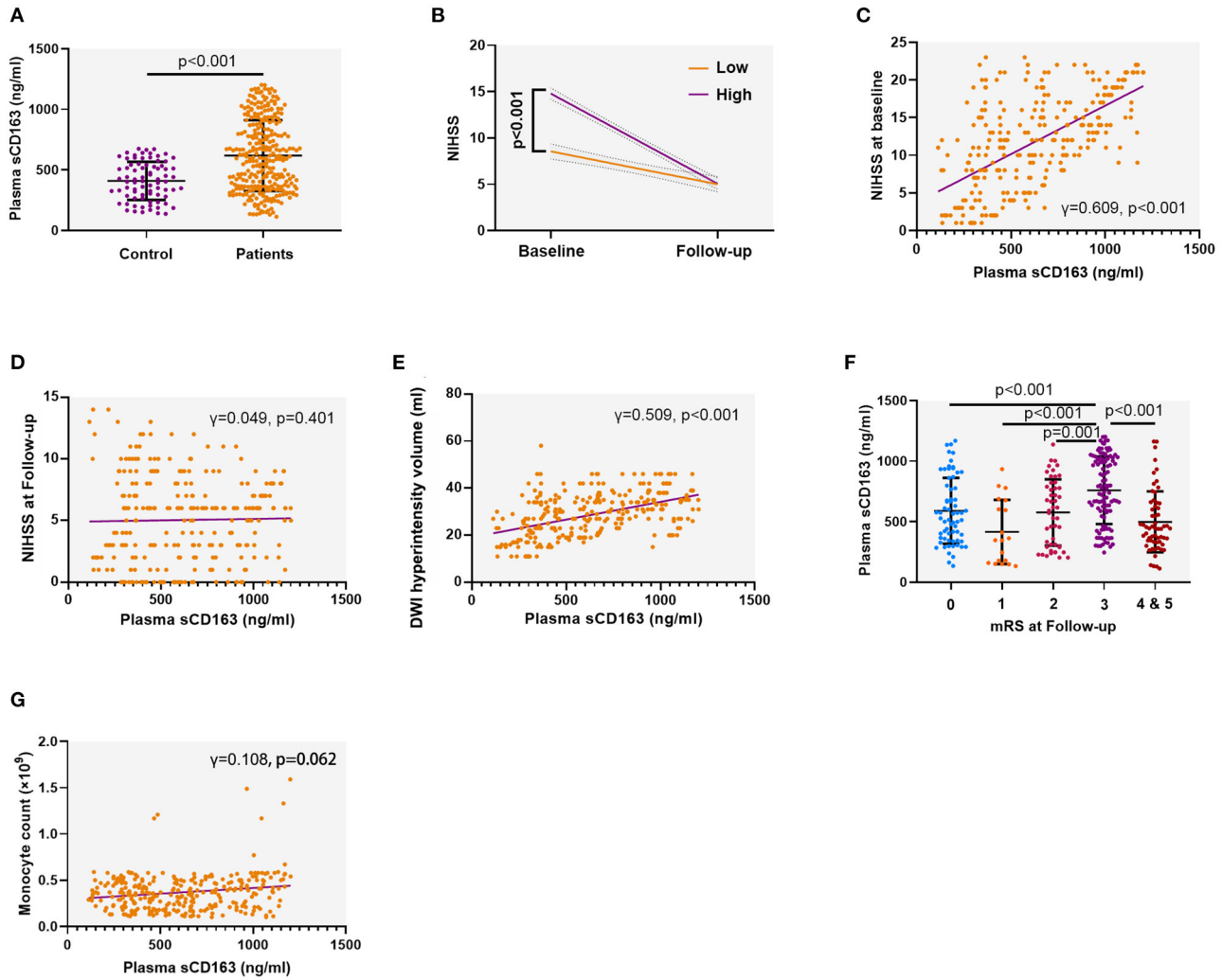

FIGURE 2 | Plasma soluble CD163 (sCD163) is increased in patients with acute ischemic stroke (AIS) and associated with disease severity. **(A)** Plasma sCD163 concentrations in AIS patients and control. Independent sample *t*-test. **(B)** The trajectory of neurological function as indicated by the change of the National Institutes of Health Stroke Scale (NIHSS) during follow-up. Two-way ANOVA. **(C)** Association between plasma sCD163 concentrations and NIHSS at baseline. Spearman correlation analysis. **(D)** Association between plasma sCD163 concentrations and NIHSS at follow-up. Spearman correlation analysis. **(E,F)** Association between plasma sCD163 concentrations and diffusion-weighted imaging (DWI) hyperintensity volume. **(G)** Association between plasma sCD163 concentrations and circulating monocyte count. DWI, Diffusion-weighted imaging. Spearman correlation analysis.

blood that are associated with the severity and prognosis of AIS (17). However, few of these biomarkers are cell type-specific and thus provide limited information on cellular relevance in the pathogenesis of AIS. Here, we show that plasma sCD163, a monocyte/macrophage-specific biomarker, was increased in AIS, hence suggesting increasing monocytic activation after AIS. sCD163 was associated with the disease severity as reflected by the NIHSS and infarction volume at the baseline. In previous studies, sCD163 is suggested to be increased in a panel of neurological diseases, such as intracranial hemorrhage (18), Parkinson's disease (19), Alzheimer's disease (20), and multiple sclerosis (21). These diseases without exception involve monocytic activation. Therefore, we suppose that the increase in sCD163 levels after AIS is indicative of monocytic activation, which is a well-documented phenomenon in AIS (22). This notion is further supported by our findings that plasma sCD163 concentrations were associated with peripheral monocyte count, although no significance had been achieved. Monocyte activation may attenuate with the diminishment of the post-AIS inflammation and the recovery of the disease (10), which is supported by the loss of association between plasma sCD163 concentrations and NIHSS at follow-up when neurological functions had been significantly improved.

Ischemic stroke causes local inflammation, which involves both the activation of resident microglia and infiltrating of

TABLE 2 | A linear regression model to investigate potential factors associated with plasma sCD163 levels.

Variables	β unadjusted	S.E.	β adjusted	*P*-value
Constant	264.512	283.288		0.351
Age	−0.241	1.611	−0.008	0.881
Male	−56.423	33.021	−0.096	0.089
BMI	−2.757	10.833	−0.014	0.799
Hypertension	5.681	32.151	0.009	0.860
Diabetes mellitus	−66.343	40.596	−0.084	0.103
Hypercholesteremia	**116.374**	**52.105**	**0.116**	**0.026**
Smoking history	19.139	58.664	0.019	0.744
Family history of stroke	37.071	70.546	0.030	0.600
Stroke type	19.856	21.462	0.050	0.356
DWI hyperintensity Volume	**15.328**	**1.709**	**0.477**	**0.000**
Antiplatelet drug use	43.171	44.491	0.050	0.333
Antithrombotic drug use	−81.982	63.849	−0.068	0.200

Dependent variable: sCD163 concentrations. Bold values represents statistically significant.

TABLE 3 | A linear regression model to evaluate the association between plasma sCD163 levels and functional improvement during follow–up.

Variables	β unadjusted	S.E.	β adjusted	*P*-value
Constant	2.177	3.616		0.548
Age	−0.015	0.021	−0.029	0.457
Male	**−1.885**	**0.430**	**−0.186**	**0.000**
BMI	−0.039	0.141	−0.012	0.784
Hypertension	0.152	0.417	0.014	0.717
Smoking history	−0.654	0.758	−0.037	0.389
Family history of stroke	.001	0.917	0.000	0.999
Stroke type	0.018	0.274	0.003	0.947
Antiplatelet drug use	0.491	0.571	0.033	0.391
Antithrombotic drug use	−0.432	0.829	−0.021	0.603
sCD163 concentrations	**0.012**	**0.001**	**0.698**	**0.000**

Dependent variable: Functional improvement as indicated by the change of NIHSS during follow–up (NIHSS at baseline−NIHSS at follow–up). Factors that are potentially associated with sCD163 levels were excluded from the model. Bold values represents statistically significant.

peripheral immune cells, including monocytes. Blocking monocyte recruitment post-AIS abolishes long-term neurological recovery and decreases the tissue expression of anti-inflammatory factors including CD163 (10). In a previous study, post-mortem brain specimens from patients with AIS showed the time-dependent accumulation of CD163+ monocytes in the ischemic parenchyma (23), indicating that the increase of CD163 expression post-AIS might be a physical protective mechanism against ischemia-associated neuronal damage. In accordance with this speculation, we found that patients with high baseline sCD163 levels have better improvement of neurological functions than those with low baseline sCD163 levels. Furthermore, we found in the regression analyses that sCD163 levels were positively associated with the improvement of neurological functions but negatively associated with the risk of poor clinical outcomes, further supporting the hypothesis that monocyte activation may serve as a protective mechanism of neuronal injury repairment post-AIS.

There are several limitations of this study. First, there is a phenomenon in this study that could not be reasonably explained that sCD163 concentrations were highest in patients with an mRS score = 3 but were relatively low in patients with an mRS score = 4 and 5. This inconsistency may limit the confidence of the present study, thus, further investigations with larger sample sizes are needed to address a more solid conclusion. Second, the novelty of this study is limited by previous findings that sCD163 levels are increased after AIS onset. Actually, previous studies demonstrate that CD163 in the brain showed a dynamic change with a peak level at the 3rd day after AIS in animal models (10). Furthermore, we did not investigate the dynamic change of sCD163 post-AIS, which is of importance to interpret the role of CD163 in the pathogenesis of AIS from a clinical perspective. Therefore, further studies are needed to investigate the change of sCD163 in different stages of AIS. Moreover, the exact time of the blood draw for sCD163 levels was not consistent as far as time from stroke symptom onset in patients in the study. Most importantly, the data presented is correlative and only demonstrates a potential

TABLE 4 | A logistic regression model to evaluate risk factors of poor functional outcome during follow-up.

Variables	Univariable ORs (95%CI)	*P* value	Multivariable ORs (95%CI)	*P*-value
Age	0.987 (0.957, 1.018)	0.394		
Sex, male vs. female	1.413 (0.789, 2.533)	0.245		
BMI	1.044 (0.865, 1.260)	0.656		
Hypertension, yes vs. no	0.782 (0.424, 1.442)	0.430		
Diabetes mellitus, yes vs. no	0.509 (0.206, 1.259)	0.144		
Hypercholesteremia, yes vs. no	0.858 (0.312, 2.358)	0.766		
Smoking history, yes vs. no	0.737 (0.208, 2.616)	0.637		
Family history of stroke, yes vs. no	1.159 (0.446, 3.011)	0.762		
Stroke type	0.456 (0.225, 0.925)	**0.030**	**0.392 (0.184, 0.835)**	**0.015**
Antiplatelet drug use, yes vs. no	0.532 (0.199, 1.423)	0.209		
Antithrombotic drug use, yes vs. no	0.737 (0.208, 2.616)	0.637		
sCD163 concentrations, high vs. low	0.351 (0.191, 0.644)	**0.001**	**0.998 (0.997, 0.999)**	**0.000**

Dependent variable: Poor outcome as indicated by mRS = 4 or 5 at follow-up. Factors that are potentially associated with sCD163 levels were excluded from the model. Bold values represents statistically significant.

relationship between functional outcomes following AIS and increased plasma levels of CD163, which might be a true phenomenon but also unrelated to disease severity and functional outcomes. This study lacks experimental evidence to support the interpretations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This study was approved by the investigational review board of the Chongqing General Hospital, University of Chinese Academy of Sciences. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZC and HS designed the study and drafted the manuscript. HS, XZ, JM, and ZL collected the samples and analyzed the data. YQ and LF supervised the project. YZ and LF were responsible for the clinical assessment of subjects. All authors contributed to the article and approved the submitted version.

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Proteomics-Based Approach to Identify Novel Blood Biomarker Candidates for Differentiating Intracerebral Hemorrhage From Ischemic Stroke—A Pilot Study

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Background: A reliable distinction between ischemic stroke (IS) and intracerebral hemorrhage (ICH) is required for diagnosis-specific treatment and effective secondary prevention in patients with stroke. However, in resource-limited settings brain imaging, which is the current diagnostic gold standard for this purpose, is not always available in time. Hence, an easily accessible and broadly applicable blood biomarker-based diagnostic test differing stroke subtypes would be desirable. Using an explorative proteomics approach, this pilot study aimed to identify novel blood biomarker candidates for distinguishing IS from ICH.

Material and Methods: Plasma samples from patients with IS and ICH were drawn during hospitalization and were analyzed by using liquid chromatography/mass spectrometry. Proteins were identified using the human reference proteome database UniProtKB, and label-free quantification (LFQ) data were further analyzed using bioinformatic tools.

Results: Plasma specimens of three patients with IS and four patients with ICH with a median National Institute of Health Stroke Scale (NIHSS) of 12 [interquartile range (IQR) 10.5–18.5] as well as serum samples from two healthy volunteers were analyzed. Among 495 identified protein groups, a total of 368 protein groups exhibited enough data points to be entered into quantitative analysis. Of the remaining 22 top-listed proteins, a significant difference between IS and ICH was found for Carboxypeptidase N subunit 2 (CPN2), Coagulation factor XII (FXII), Plasminogen, Mannan-binding lectin serine protease 1, Serum amyloid P-component, Paraoxonase 1, Carbonic anhydrase 1, Fibulin-1, and Granulins.

Discussion: In this exploratory proteomics-based pilot study, nine candidate biomarkers for differentiation of IS and ICH were identified. The proteins belong to the immune system, the coagulation cascade, and the apoptosis system, respectively. Further investigations in larger cohorts of patients with stroke using additional

biochemical analysis methods, such as ELISA or Western Blotting are now necessary to validate these markers, and to characterize diagnostic accuracy with regard to the development of a point-of-care-system for use in resource-limited areas.

Keywords: mass spectrometry, blood, biomarker, differentiation, ischemic stroke, intracerebral hemorrhage

INTRODUCTION

In recent years, treatment options for patients with ischemic stroke (IS) have largely expanded. Subsequent to the broad implementation of intravenous thrombolysis, mechanical thrombectomy has now become the standard of care for patients with intracranial large vessel occlusion (1–5). Moreover, multimodal CT- and MR-imaging techniques allow the application of recanalizing treatment strategies even in extended time windows (6–9). In metropolitan areas, mobile stroke units have been released to apply thrombolysis already in the preclinical setting with the shortest possible delays after symptom onset (10, 11).

In contrast to these "high-tech" advances to stand by in many high-income countries, low- to middle-income countries still face immense shortcomings in medical resources. This weighs heavily as these countries have to carry the majority of the global burden of stroke (12). Regarding brain imaging, some countries have only one CT unit available per 1 million inhabitants (13). Hence, the stratification into IS and intracerebral hemorrhage (ICH) is not possible at all or only after long transports and transfer delays (14). This prevents acute target-orientated stroke treatment, but also from the timely initiation of diagnosis-specific secondary prevention (i.e., platelet inhibitors in patients with IS). However, the effect in reducing recurrent stroke for platelet inhibition is highest within the first weeks after the initial event (15).

Thus, in resource-limited areas, an inexpensive and easy-to-use stratification tool to substitute CT-imaging before the initiation of secondary prevention would be desirable. Here recent research on blood-based brain biomarkers has revealed interesting results. Glial fibrillary acidic protein (GFAP) has been characterized in several prospective studies as a biomarker of ICH. However, it reliably distinguishes ICH from IS only within 6 h of symptom onset. Its potential use has been demonstrated in an Indian trial, too (16–21). On the other side, comparable markers of IS have not been identified so far (22–24).

This pilot study aimed to identify candidate biomarkers suitable to differentiate IS and ICH within the first days after symptom onset. Hence, in an exploratory approach, the entire plasma/serum proteome was screened through mass spectrometry (MS) techniques. Ensuing, an extensive literature search was performed, to identify the relevant publications focusing on the diagnostic value of the candidate markers in acute stroke.

MATERIALS AND METHODS

Study Design

For this explorative pilot study, we targeted to compare the two "prototypes" of stroke, i.e., patients with IS in the middle cerebral artery (MCA) territory and patients with ICH in the basal ganglia or the thalamus ("deep") as well as in the parietal or temporal lobes ("lobar"). Both conditions typically present as a classical stroke syndrome, and differentiation between the entities solely based on clinical examination alone is usually not possible. To presume a considerable amount of brain tissue damage with release of brain proteins in the bloodstream, only patients with infarctions affecting at least one-third of MCA territory and only patients with hematoma volumes higher than 20 ml were included. For doing so, we screened plasma samples collected in the context of a prior prospective study on GFAP levels in neurological diseases performed in our center for these criteria (25). In total, plasma samples of three patients with IS and of four patients with ICH, who met the above criteria, were randomly chosen among the available samples. Ultimately, the cohort was enriched by serum samples from two healthy controls.

The Ethics Committee of the Goethe University Frankfurt am Main, Germany approved the protocols of the previous and the current study. The studies were conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from each patient, or if applicable of the next-of-a-kin.

Blood Sampling

According to the study protocol, 1 ml of ethylenediaminetetraacetic acid (EDTA)-plasma was collected during hospitalization at variable time points after stroke symptom onset and transferred into an Eppendorf tube (25). Within 60 min after blood draw, the samples were centrifugated at 10,000 g for 4 min, and the supernatant was immediately frozen and stored at −25°C; for long-term storing, the samples were transferred to −80°C freezers. Processing and storage of the serum samples of the two healthy controls were done in the same way.

Abbreviations: AGC, automatic gain control; APCS, serum amyloid P-component; BBB, blood brain barrier; CA1, carbonic anhydrase 1; CPN2, carboxypeptidase N subunit 2; CT, computed tomography; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; f, female; FDR, false discovery rate; Fig., figure; FXII, factor XII; FBLN1, fibulin-1; GdmCl, guanidinium hydrochloride; GFAP, glial fibrillary acidic protein; GRN, granulins; HCl, hydrochloric acid; ICH, intracerebral hemorrhage; IQR, interquartile range; IS, ischemic stroke; LC-MS, liquid chromatography mass spectrometry; LFQ, label free quantification; m, male; MASP1, Mannan-binding lectin serine protease 1; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; MI, myocardial infarction; MR, magnetic resonance; MS, mass spectrometry; m/z, mass-to-charge ratio; NIHSS, National Institutes of Health Stroke Scale; PLG, plasminogen; PON1, paraoxonase 1; Ref., references; SAH, subarachnoid hemorrhage; Supp., supplementary; TAFI, thrombin-activated fibrinolysis inhibitor; TBI, traumatic brain injury; TCEP, tris-carboxyethylphosphine; TRIS, tris(hydroxymethyl)aminomethane.

Mass Spectrometry

The protein content was determined by using the method of Lowry (26). For this, 200 μg of plasma/serum proteins were diluted to a final volume of 20 μl with 6 M GdmCl, 50 mM tris(hydroxymethyl)aminomethane (TRIS)/HCl, pH 8.5, 10 mM tris-carboxyethylphosphine (TCEP), and incubated at 95°C for 5 min. Reduced thiols were alkylated with 40 mM chloroacetamide and the samples were diluted with 25 mM TRIS/HCl, pH 8.5, 10% acetonitrile to obtain a final GdmCl concentration of 0.6 M. The proteins were digested with 2 μg trypsin (sequencing grade, Promega, WI, USA) overnight at 37°C under gentle agitation. Digestion was stopped by adding trifluoroacetic acid to a final concentration of 0.5%. The tryptic peptides were cleaned through reversed phase chromatography with C18 material (3M Empore™ SPE Extraction Disks) (27), dried in microtiter plates, and resolved in 1% acetonitrile and 0.1% formic acid before peptide identification.

Liquid chromatography/mass spectrometry (LC/MS) was performed on Thermo Scientific™ Q Exactive Plus equipped with an ultra-high-performance liquid chromatography unit (Thermo Scientific Dionex Ultimate 3000, Thermo Fisher Scientific, MA, USA) and a Nanospray Flex Ion-Source (Thermo Fisher Scientific, MA, USA). The peptides were eluted from the trap column by a continuously increasing concentration of organic solvent (4–50% acetonitrile and 0.1% formic acid) over 90 min at a flow rate of 250 nl/min and then, separated on an analytical column (with 2.4 μm Reprosil C18 resin from Dr. Maisch GmbH in-house packed picotip emitter tip with diameter 100 μm, 15 cm from New Objectives). The peptides were then ionized (2.6 kV) in the ion source and sprayed into the mass spectrometer. MS data were recorded by data dependent acquisition. The full MS scan range was 300–2,000 m/z with a resolution of 70,000, and an automatic gain control (AGC) value of 3×10^6 total ion counts with a maximal ion injection time of 160 ms. Only higher charged ions (2+) were selected for MS/MS scans with a resolution of 17,500, an isolation window of 2 m/z, and an AGC value set to 10^5 ions with a maximal ion injection time of 150 ms. MS-Data were acquired in profile mode, MS/MS data in Centroid mode. Each patient was measured one time. The two control donors were measured in technical triplicates and quadruplicates, respectively. All samples were measured consecutively with the same instrumental setup (identical analytical column, buffers, and mass calibration).

MS Data Analysis

Mass spectrometry data were analyzed by MaxQuant (Max-Planck-Institute of Biochemistry, Martinsried, Germany) (v1.5.3.30) using default settings (28). Proteins were identified using the human reference proteome database UniProtKB with 71,567 entries, released in July 2017. The enzyme specificity was set to Trypsin. Acetylation (+42.01) at N-terminus, deamidation of N and Q (+0.98), and oxidation of methionine (+15.99) were selected as variable modifications and carbamidomethylation (+57.02) as a fixed modification on cysteines. False discovery rate (FDR) was calculated using the reverse decoy database implemented in MaxQuant. FDR was 1% for the identification of protein and peptides. Label-free quantification (LFQ) data were further analyzed using the bioinformatics tool Perseus (Max-Planck-Institute of Biochemistry, Martinsried, Germany) (v1.5.6.0) (29).

Contaminants from the internal MaxQuant list, only identified by site and reverse hits were removed from the initial protein ID list. The patients were grouped into ICH ($n = 4$) and IS ($n = 3$), the control group contains all the replicates ($n = 7$) of the two donors. Identified proteins were filtered to at least three valid values in one group. Missing values were replaced by the lowest value of the data set. A two-tailed Student's *t*-test was used to examine the levels of significance.

In addition, the statistics, correlations, and heat maps were created with Perseus as well. Other diagrams were created by using GraphPad Prism 8 (v.8.0.2) and an online Webtool from Bioinformatics and Evolutionary Genomics (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Review of the Literature

After identifying the biomarker candidates, we performed a structured literature search to identify relevant publications focusing on the diagnostic value of these markers in acute stroke. For doing so, a PubMed search was performed with the following search terms: the protein's name, its aliases, and its abbreviations according to UniProtKB (release 2021_01) linked ("AND") to "stroke, apoplexy, ischemic stroke, ischaemic stroke, intracerebral hemorrhage or intracerebral haemorrhage." Only studies published before August 2021 were included. Identified reviews were screened for primary sources. The results were filtered by the first author (DM) of this manuscript after a review of the title and abstract of the manuscripts. Publications relevant to the context of the present investigation were finally selected for evaluation. Please see the flow diagram (**Figure 3**) illustrating the database search for the review of the literature. The exact search terms are listed in the **Supplementary Materials** (**Supplementary Table 5**).

RESULTS

Mass spectrometry analysis comprised plasma samples of three patients with MCA infarction, four patients with ICH, and serum samples of two healthy controls (as outlined above in more detail). The baseline characteristics of the study population are depicted in **Table 1**.

Among the 495 identified protein groups, a total of 368 protein groups exhibited enough data points to be entered into quantitative analysis. Noticeably more proteins are expected to be present in human blood samples, however, proteins with low expression are strongly underrepresented in such analyses. Albumin, immunoglobulins, and transport proteins make up a large part and are increasingly represented in peptide analysis. Therefore, the tryptic peptides were separated by cation exchange chromatography. The serum/plasma amount of thus identified proteins is comparable with those reported in the literature (30). To validate the quality of data, the total results of all proteins were compared with those of all samples (**Supplementary Figure 1**). The determined correlation coefficients (> 0.88) show that the

TABLE 1 | The baseline characteristics of the study subjects.

Diagnosis	Sex	Age (years)	NIHSS	Time (days) between symptom onset and blood withdrawal	Protein content of the sample (mg/ml)
Control	m	24	–	–	76.9
Control	m	27	–	–	69.5
IS	m	75	11	12 days	67.2
IS	m	69	10	8 days	60.8
IS	m	42	23	15 days	62.6
ICH	f	59	4	9 days	50.6
ICH	f	79	19	3 days	66.2
ICH	m	89	12	2 days	74.0
ICH	m	75	18	3.5 h	63.8

IS, ischemic stroke; ICH, intracerebral hemorrhage; NIHSS, National Institute of Health Stroke Scale.

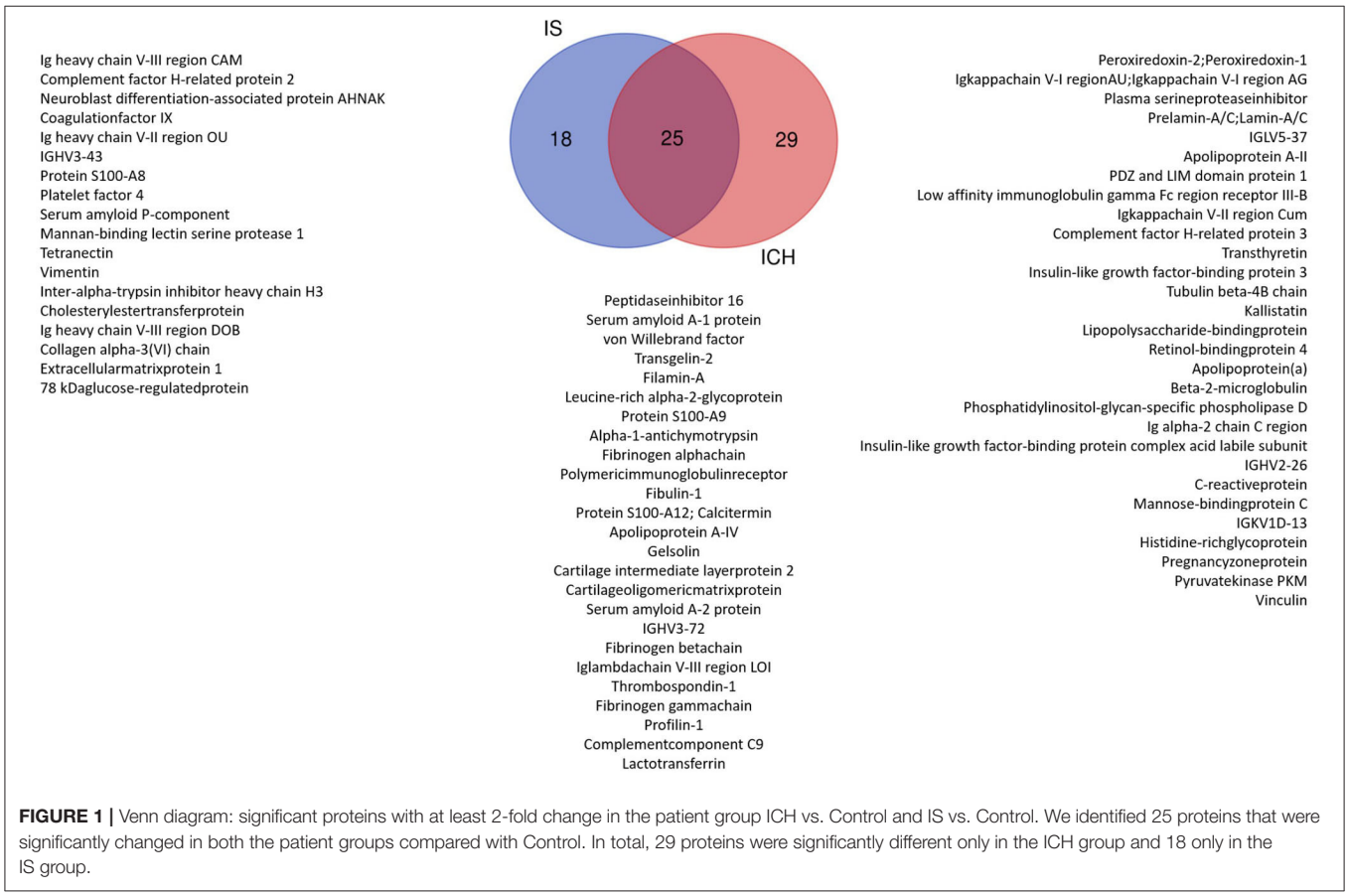

FIGURE 1 | Venn diagram: significant proteins with at least 2-fold change in the patient group ICH vs. Control and IS vs. Control. We identified 25 proteins that were significantly changed in both the patient groups compared with Control. In total, 29 proteins were significantly different only in the ICH group and 18 only in the IS group.

mass spectrometric analysis provides very homogeneous data (**Supplementary Figure 2**).

Taking into account these 368 proteins, we then compared the amount of each protein in the patients group (P) as a whole (IS and ICH together) with the healthy (C) controls. Here, 91 proteins could be identified with significantly different amounts (P vs. C) (as shown in **Supplementary Table 1**; **Supplementary Figure 3**). In the next step, we compared the number of proteins in each disease entity, IS respectively ICH, with the healthy controls (C) (as shown in **Supplementary Tables 2**, **3**). To create a top list of potential promising biomarkers, only proteins with significant differences with a fold change of at least ±2 between the groups (IS vs. C and ICH vs. C) were selected. Here, 25 proteins could be found both in the IS as well as in the ICH group with significant differences in abundance to the healthy controls, 18 individual proteins were differentially expressed only in the patients with IS, and 29 individual proteins only in the patients with ICH (as shown in **Figure 1**, Venn diagram).

Since the primary aim of the study was to identify differentially expressed proteins in patients with symptoms of stroke having either ICH or IS (and not necessarily in comparison with controls), we then screened for differences between ICH and IS, irrespective of differences to the control group and independent of the fold change, starting again from the original 368 proteins with enough data points (**Supplementary Table 4**). Here, 21 additional proteins could be identified (not part of the Venn diagram). After eliminating general structural and functional proteins, such as transport proteins, myosin chains, hemoglobin subunits, and components of immunoglobulins which are unsuitable as biomarkers due to their ubiquitous abundance, according to the literature (31, 32), 22 proteins remained and listed in **Table 2**.

Candidate Proteins for Differentiating IS and ICH

Focusing on significant differences between the patients with ICH and IS, **Figure 2** shows the nine most promising candidate proteins from the top list. Carboxypeptidase N subunit 2 (CPN2) and the coagulation factor XII (FXII) showed strongly increased ($p < 0.01$) protein amount in the patients with IS as compared with the patients with ICH. Both proteins play a role in the kinin-kallikrein-system and are involved in thrombus formation (33–36). Significantly increased ($p < 0.05$) protein amount in IS as compared to ICH patients were found for plasminogen (PLG), a central regulator in the fibrinolytic system, and Mannan-binding lectin serine protease 1 (MASP1), which was attributed a role in the blood clotting system by its thrombin-like activity (37, 38). Other proteins with a considerably increased amount of protein in IS in comparison to ICH patients, whose functionality is mainly not related to the blood coagulation system, were Amyloid P-component (APCS), Paraoxonase 1 (PON1), and Carbonic anhydrase 1 (CA1). Vice versa, only two proteins showed higher values in ICH patients as compared to IS patients [Fibulin-1 (FBLN1) and Granulines (GRN)].

Review of the Literature and Integrative Evaluation

After identifying the above outlined biomarker candidates, we reviewed the literature to figure out which proteins have already been investigated in human or animal studies relating to stroke and to integrate the current knowledge with our findings (as shown in **Table 3**; **Figure 3**). Overall, these studies analyzed patients with IS and ICH compared with controls, but not IS and ICH patients compared with each other. Moreover, the largest proportion of the available and identified studies analyzed serum and not plasma samples.

Serum levels of CPN2 were not investigated in patients with stroke, but increased concentrations were found in patients with acute myocardial infarction (41). For FXII, studies showed high concentrations in patients with chronic cerebrovascular diseases (42), and FXII inhibition, as well as FXII deficiency improved outcome in experimental IS (45–49). In our study, FXII-levels were decreased in plasma of the patients with ICH compared with both controls and IS. For PLG, increased as well as decreased plasma levels in IS compared with controls have been described in the literature (50–53). We did not find differences between IS patients and controls, but ICH patients had significantly lower PLG protein amounts. MASP1 concentration was increased in myocardial infarction, however, in IS was reported to be either increased or decreased, whereas in ICH and SAH, lower levels were found (56–58). In our study, we detected higher MASP1 protein amount in IS than in healthy controls as well as in ICH. Consistent with our findings, one study showed higher APCS values in ICH than in controls (61). However, we additionally found higher values in IS compared to ICH patients. Among the identified proteins, PON1 seems to be best validated in human stroke patients, especially for several gene polymorphisms leading to higher susceptibility for IS. For PON1, reduced serum levels are described in IS (64–67, 69, 116). We did not find significant differences between IS and controls but within the patient groups elevated PON1 concentrations were found in IS compared to ICH. CA1 was found to be released from erythrocytes due to ICH in animal experiments but studies to evaluate blood levels of CA1 in patients with stroke are not available (101, 102). FBLN1 showed reduced serum concentrations in myocardial infarction (106). Comparable data for IS—except for the certain subgroup of IS caused by cervical artery dissection (107)—could not be identified. GRN was reported to be increased in the serum of patients with IS (108). It is expressed in the microglia in ischemic tissue (110–113). Increased GRN levels have not been confirmed in IS patients in our study.

Most Promising Biomarker Candidates

Based on our data, we suggest that CPN2, FXII, PLG, MASP1, APCS, PON1, and CA1 for IS and FBLN1 and GRN for ICH should be further scientifically pursued as potential stroke blood biomarkers.

DISCUSSION

This exploratory pilot study identified nine proteins by means of mass spectrometry which showed different protein abundance in patients with IS and ICH. These nine proteins alone or in combination may now be evaluated in future prospective studies regarding their diagnostic accuracy to discriminate between those two subtypes of stroke. Moreover, detailed analyses of release kinetics of these markers after IS and ICH onset are mandatory.

At a closer look at the (patho-)physiological function of the identified proteins, it becomes apparent that all proteins, except APCS1 and CA1, are involved in the immune and/or coagulation system. This is not surprising because of previous findings on the pathophysiology of IS described as a "thrombo-inflammatory" disease (36, 117). The individual components of both systems influence each other. Besides its involvement in the coagulation cascade FXIIa activates the kinin-kallikrein system, which mediates, i.e., the activation of PLG and carboxypeptidases (35). Moreover, CPN seems to be involved in the kinin-kallikrein

TABLE 2 | Protein "top list."

Protein	P vs. C	IS vs. C	ICH vs. C	IS vs. ICH
Carboxypeptidase N subunit 2	ns	**	ns	**
Coagulationfactor XII	**	ns	***	**
Plasminogen	**	ns	***	*
Mannan-binding lectin serine protease 1	ns	*	ns	*
Serum amyloid P-component	**	***	*	*
Paraoxonase 1	ns	ns	ns	*
Carbonicanhydrase 1	ns	ns	ns	*
Fibulin-1	ns	*	ns	*
Granulins	ns	ns	ns	*
Inter-alpha-trypsin inhibitor heavy chain H3	**	*	***	ns
Coagulationfactor IX	***	**	**	ns
Protein S100-A8	*	*	ns	ns
Platelet factor 4	ns	*	ns	ns
C-reactive protein	**	ns	**	ns
Lipopolysaccharide-binding protein	*	ns	**	ns
Mannose-binding protein C	ns	ns	*	ns
Beta-2-microglobulin	*	ns	**	ns
PDZ and LIM domain protein 1	ns	ns	*	ns
Tubulin beta-4B chain	*	ns	*	ns
Pregnancy zone protein	**	ns	**	ns
Lamin-A/C	ns	ns	*	ns
Vinculin	*	ns	*	ns

*IS, ischemic stroke; ICH, intracerebral hemorrhage; C, healthy control; P, group of ICH + IS patients. ns (non-significant) $p \geq 0.05$, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; gray shaded are those proteins without significant difference between IS and ICH.*

system and is able to reduce the cell binding capacity for PLG (33, 34, 37, 118). However, CPN itself is activated by plasmin as a negative feedback mechanism, ultimately leading to increased antifibrinolytic activity (119, 120). CPN, as well as FBLN1, have also been detected in fibrin clots (39, 106). By interaction of FBLN1 exposed after vascular injury with plasma fibrinogen, a linkage to a platelet integrin is formed and results in the formation of a platelet plug (105). CPN in the fibrin clot seems to act as a fibrinolysis inhibitor and belongs to the same family of zinc metalloproteinases as thrombin-activated fibrinolysis inhibitors (TAFI) (121). In turn, MASP1, traditionally attributed to the complement system, should interact with TAFIs, is conversely activated itself by activated platelets and fibrin and leads to fibrin clot formation and activation of thrombin and platelets, which seems to be essential for obstructive thrombosis at least in a mouse model of arterial injury (38, 60, 122). The formation of reactive oxygen species (ROS) in the context of ischemia/reperfusion processes with the influence of antioxidant components is reflected in the altered activity of associated proteins, such as PON1 (123, 124). Furthermore, the release of pro-inflammatory factors during IS leads to the activation of neuroprotective factors, such as GRN especially in viable neurons and endothelial cells in the ischemic penumbra (110, 112).

APCS and CA1 are not directly involved in coagulation or inflammatory pathways. APCS binds to apoptotic cells, is involved in chromatin degradation, and acts toxic to cerebral neurons (125, 126). CA1 is one of the 14 isoforms of carbonic anhydrases and occurs mainly in the cytosol of erythrocytes. In the context of ICH, erythrocyte lysis occurs around the hematoma, causing the release of iron and CA1 and subsequently increased tissue damage through edema formation and neuronal cell death. In addition, extracellular CA1 should promote the destruction of the blood-brain barrier by activating the kinin-kallikrein system (101, 127).

From a clinical point of view, until now no single biomarker identified in the context of stroke is suitable to certainly distinguish IS from ICH (21–24, 128). The most promising results so far have been published for GFAP. However, the different release kinetics of GFAP in IS and ICH exist only within the first 6 h after symptom onset (18, 129, 130). Thus, this protein is likely not helpful to differentiate strokes at any time point after symptom onset in resource limited settings. Moreover, GFAP release is strongly linked to the extent of damage to astroglial tissue. Thus, smaller ICH or expanding ICH may not always present with increased blood concentrations. More likely for this purpose, a combination of several markers may be favorable (131, 132). However, ischemic stroke is a heterogeneous disease comprising patients with large territorial infarctions and small lacunar strokes, as well as a large diversity of underlying etiologies. This makes it difficult to identify a biomarker panel that copes with all the facets of ischemic stroke. Interestingly, most of the

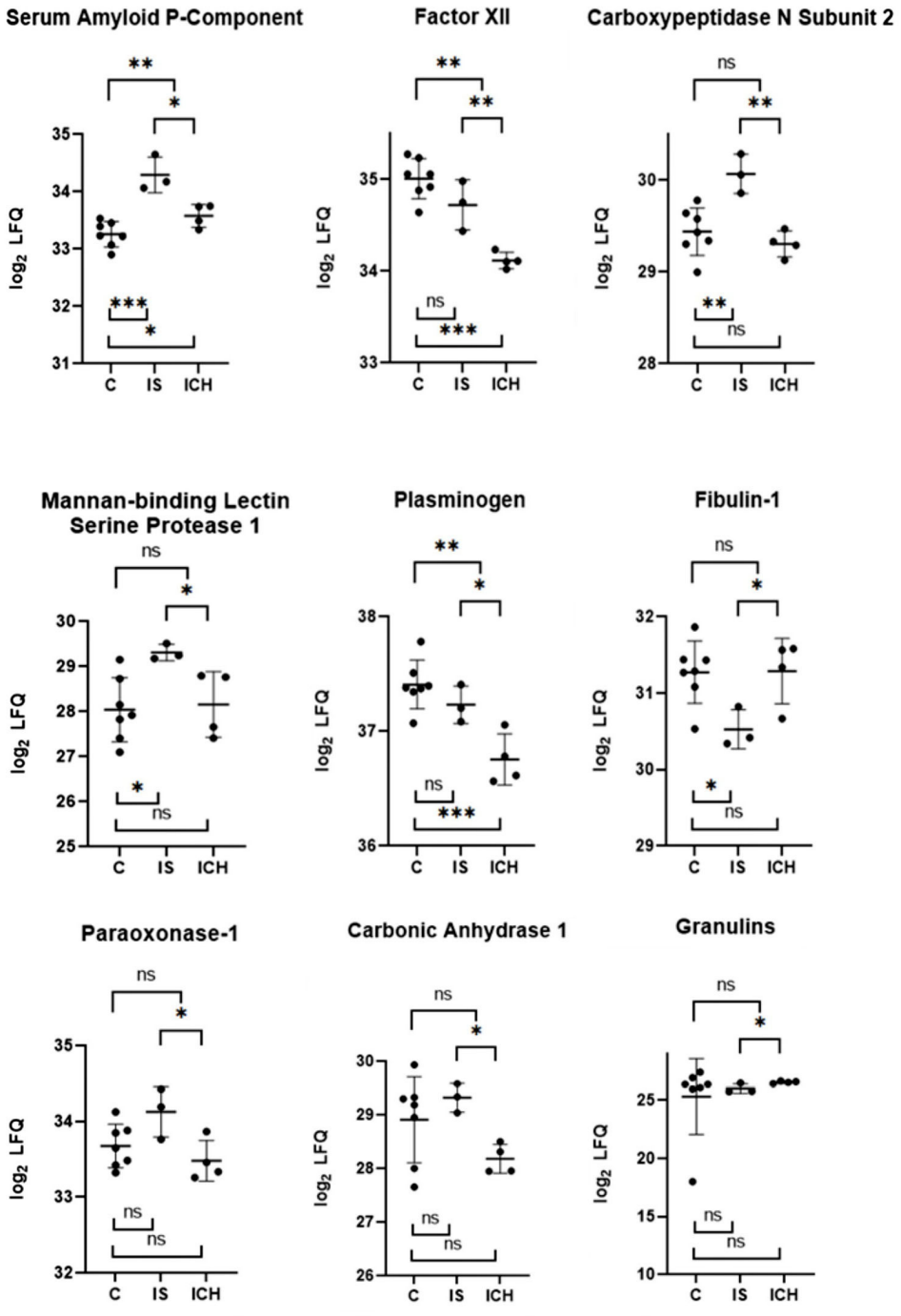

FIGURE 2 | Selected proteins from the "top list" with the most significant differences within each group. The *y*-axis is transformed logarithmically. The scatter plots show the measured values with mean value and SD. Above the scatter plots, the differences between IS and ICH, respectively between the patients and control group are shown. Below the scatter plots, the differences between IS and control group are shown, as well as between ICH and control group. ns $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

proteins identified in our pilot study play pivotal roles in the immune and coagulation system. Thus, it is likely that they are directly involved in the pathophysiology of stroke and are not just an epiphenomenon. They are interesting candidates that add to the existing portfolio of potential biomarkers in stroke.

A shortcoming of this explorative pilot study is the very limited sample size. Reconfirmation of the core findings

TABLE 3 | Literature search.

Protein	Species	Methodology	Collective	Summary of findings	References
CPN2	Human	Mass spectrometry	Healthy	CPN is a component of fibrin-clots	(39, 40)
		Western blot			
	Human	Spectrophotometry	MI	Elevated CPN serum levels are detected in acute MI	(41)
FXII	Human	ELISA	IS and chronic cerebrovascular diseases	**Patients with chronic cerebrovascular disease show higher FXII levels in serum than patients with IS**	(42)
	Human	Medical hypothesis	IS	Via raised epinephrin levels due to chronic stress platelets activate pre-bound FXII which leads to hypercoagulability and together with essential hypertension favor atherosclerosis and ultimately IS	(43)
	Human	Case-control-study	IS	A certain gene polymorphism is a risk factor for IS	(44)
	Rat and Mouse	Neurological performance test	MCAO	Pharmacological inhibition of FXII reduces extent of infarction and improves neurological outcome after ischemia/reperfusion	(45, 46)
		Histopathology			
	Mouse	Histopathology	MCAO	FXII is essential for thrombus formation	(47–49)
		Immunofluorescence			
		Neurological performance test	Model of thrombo-embolism	Deficiency or inhibition of FXII protects from ischemic brain injury	
		MR			
PLG	Human	Mass spectrometry	IS	**IS patients show higher plasminogen blood levels than healthy subjects**	(50–52)
		Coagulation assay			
	Human	Chromozym assay	IS	**IS patients show lower plasminogen activity compared to healthy subjects**	(53)
	Human	Bioinformatical database research	IS	PLG was identified as critical protein for all subtypes of IS	(54)
	Mouse	Histopathology	Model of thrombo-embolism	Higher PLG levels attenuate brain infarction, endogenous fibrinolysis, microvascular thrombosis, inflammation, and BBB breakdown	(55)
		Immunofluorescence			
MASP-1	Human	Immunofluorimetry	IS and MI	**MASP1 shows higher levels in MI and lower levels in IS compared to controls**	(56)
	Human	Immunofluorescence	IS	**MASP-1 activity in IS patients is higher than in healthy subjects**	(57)
	Human	Immunofluorimetry	ICH and SAH	**MASP-1 levels decreased significantly in ICH and SAH patients during 24 h after symptom onset**	(58)
	Human	Immunofluorimetry	SAH	Cerebral blood concentration of MASP-1 is lower than in peripheral blood	(59)
	Mouse	Immunofluorescence	($FeCl_3$)-induced arterial thrombosis	MASP-1 has thrombin-like activity and is a significant regulator of thrombus formation *in vitro* and *in vivo*	(60)
		Immunostaining			
APCS	Human	Mass spectrometry	ICH	**Plasma APCS is higher in ICH than in healthy controls**	(61)
		Western Blot			
	Human	ELISA	Cardiovascular diseases	Increased APCS serum levels in the elderly are associated with angina pectoris and myocardial infarction but not with stroke	(62)
	Human	Mass spectrometry	Healthy	APCS is a component of fibrin-clots	(40)
	Human	Multiplex assay	IS	Increases in plasma levels of APCS are associated with worse clinical outcomes after IS	(63)
PON1	Human	ELISA	IS	**Serum PON1 activity is reduced in IS patients compared to controls**	(64–68)
		Spectrophotometry			
	Human	Multiplex assay	IS and ICH	**Serum PON1 activity is lower in IS patients than in ICH and controls**	(69)

(Continued)

TABLE 3 | Continued

Protein	Species	Methodology	Collective	Summary of findings	References
	Human	Spectrophotometry	IS	PON-activity affects the outcome after IS	(70, 71)
	Human	Genetic engineering	IS	Particular gene polymorphisms (above all Q192R and L55M but also less common variants) and potentially the related enzyme activity raise the susceptibility for IS	(72–100)
CA1	Rat	Western Blot	ICH model	Erythrocyte lysis due to ICH may lead to CA release with tissue damaging and edema formation; Inhibition of CA reduces brain damage after ICH	(101)
	Rat	Mass spectrometry	ICH model	CA1 is upregulated in an ICH model compared to sham	(102)
	Human	Mass spectrometry	TBI & SAH	CA1 is elevated in CSF of TBI and SAH compared to controls, but no difference could be identified between TBI and SAH	(103)
FBLN1	Human	Mass spectrometry	IS	Serum FBLN1 is higher in a monozygotic twin suffering from IS	(104)
	Human	ELISA	Healthy	FBLN1 binds to Fibrinogen and is incorporated in Fibrin clots	(105)
	Human	Histopathology	Coronary heart disease	FBLN1 was detected in coronary atherosclerotic lesions and patients with unstable angina pectoris and acute MI show lower FBLN1 serum levels compared to controls	(106)
		Immunofluorescence			
	Human	Mass spectrometry	Cervical artery dissection-IS vs. Non-cervical artery dissection-IS	FBLN1 is significantly upregulated in IS due to cervical artery dissection compared to non-cervical artery dissection	(107)
		ELISA			
PGRN/GRN	Human	ELISA	IS	**Serum Progranulin levels are increased in IS compared to healthy controls**	(108)
	Human	ELISA	IS	GRN concentration affects outcome after IS	(109)
		Neurological performance test			
	Rat	Histopathology	Transient acute focal cerebral ischemia	Increased levels of PGRN expression in microglia within the ischemic core, increased levels of PGRN expression in viable neurons, induction of PGRN expression in endothelial cells within the ischemic penumbra	(110)
		Immunofluorescence			
	Rat	Histopathology	Transient acute focal cerebral ischemia	PGRN overexpression and artificial administration reduce cerebral infarction volume, edema, suppress hemorrhagic transformation and improve functional outcome	(110–114)
		Immunofluorescence			
	Mouse	Western Blot	MCAO		
	Mouse	Flow cytometry	MCAO	PGRN deficiency in mice leads to early BBB disruption and increased areas of hemorrhage in the ischemic territory	(115)
		Western Blot			
		Histopathology			
		Immunofluorescence			

Investigation of protein levels in patients with stroke is highlighted in bold letters.

in larger patient cohorts is mandatory. In addition, other detection methods, such as ELISA or Western Blotting need to be applied to verify the proteins identified by mass spectrometry.

Furthermore, plasma samples were used for the primary comparison between IS and ICH. The control group, however, consisted of serum samples, thereby increasing the heterogeneity of the study.

Another limitation with the risk of a possible selection bias is the imbalance of baseline variables (such as age and sex) between the diagnosis groups. However, we focused on comparing "prototype" strokes as described above, and other exploratory studies based on the mass spectrometry techniques were designed in a similar way, nevertheless allowing the successful identification of novel biomarker candidates (133–135).

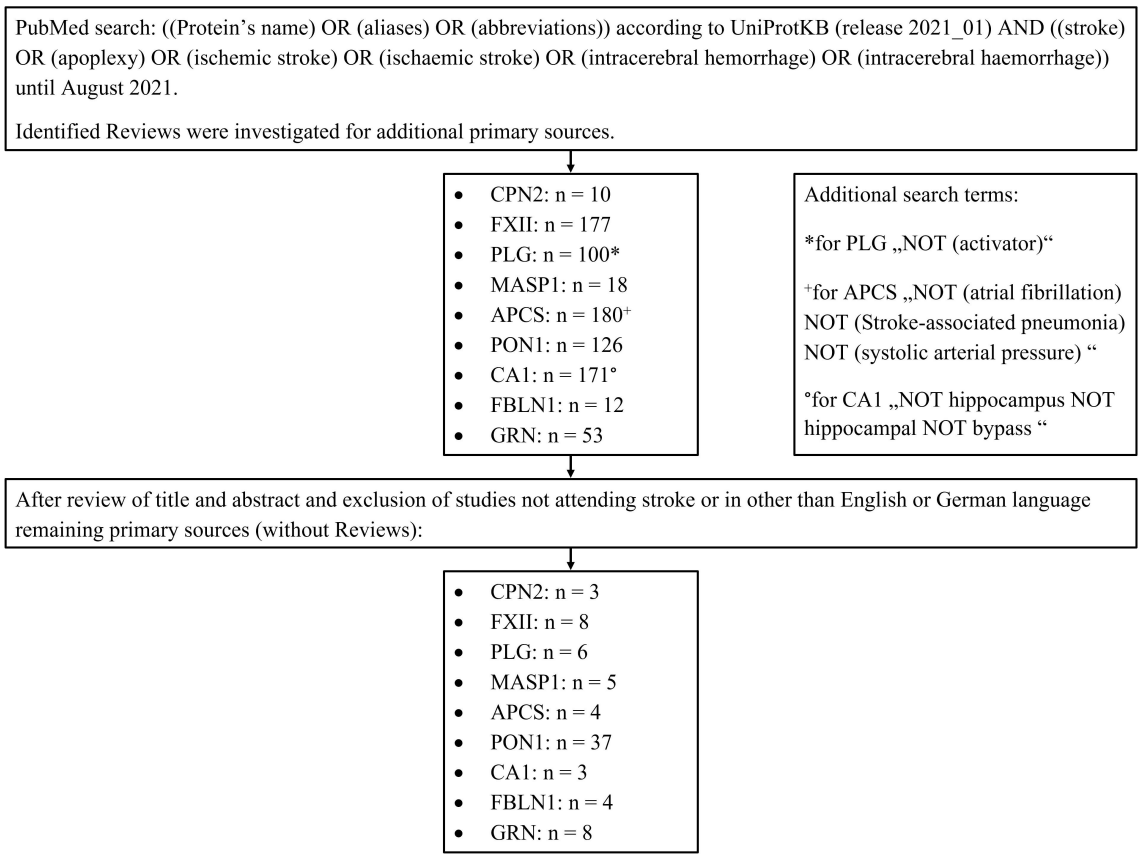

FIGURE 3 | Flow diagram illustrating the database search for review of the literature.

In summary, in this exploratory proteomics-based study, nine candidate blood biomarkers for differentiation of IS and ICH were identified. The proteins belong to the immune system, the coagulation cascade, and the apoptosis system, respectively. Due to the exploratory nature of the study, further investigations in independent, well-matched, and large-scaled cohorts of stroke patients are now necessary to validate these markers, and to characterize diagnostic accuracy with regard to the development of a point-of-care-system differentiating IS and ICH in resource-limited areas.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Goethe University Frankfurt am Main. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DM and IW: acquisition, analysis and interpretation of data, and drafting the work or revising it critically for important intellectual content. SL and CF: substantial contributions to the conception or design of the work and drafting the work or revising it critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.713124/full#supplementary-material

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