HEMOSTASIS AND STROKE

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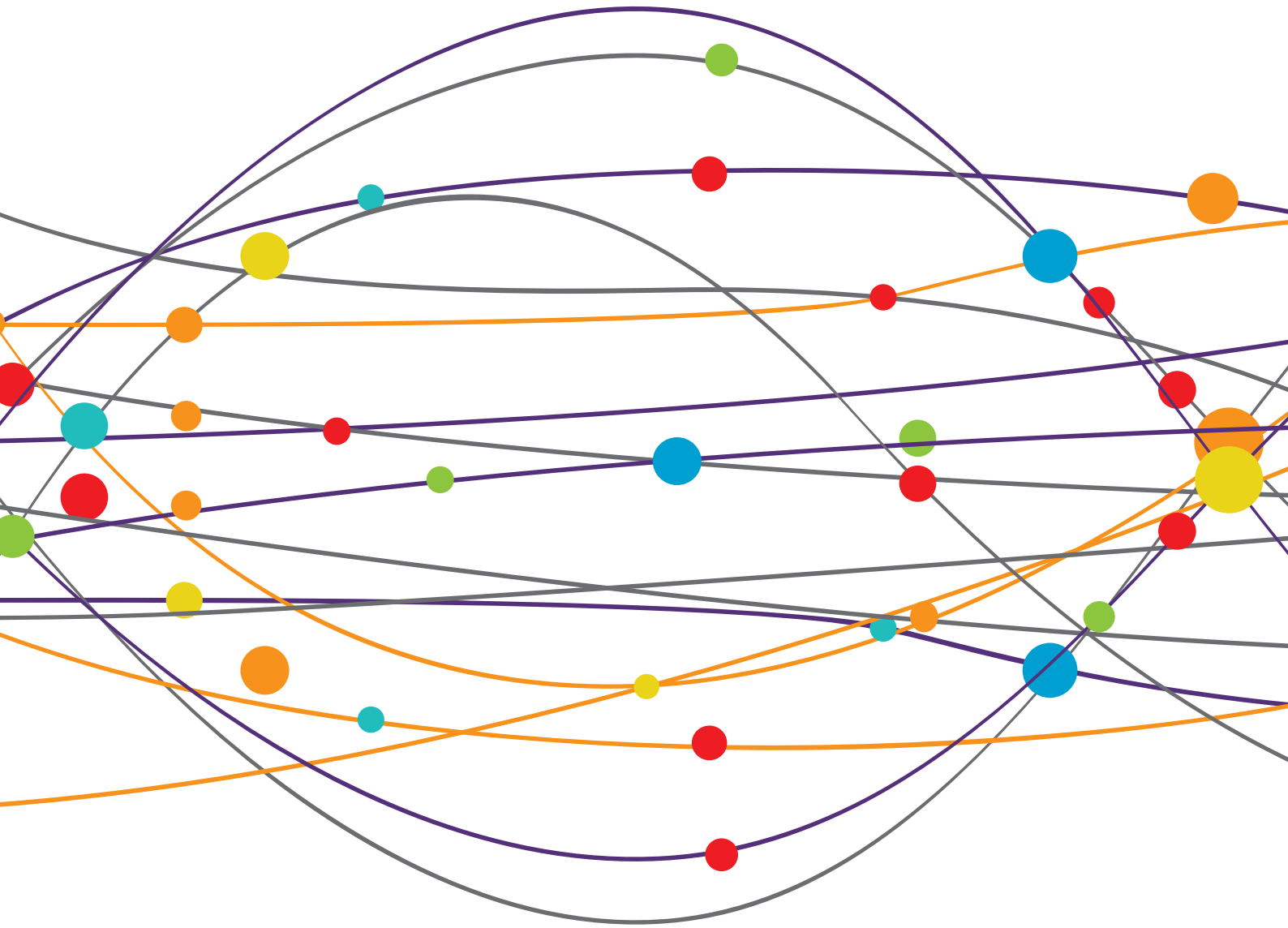

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HEMOSTASIS AND STROKE

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Editorial: Hemostasis and Stroke

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Editorial on the Research Topic

Hemostasis and Stroke

Stroke is the leading cause of death and permanent disability worldwide (1, 2). Understanding the hemostasis system in stroke presents an exciting and relatively new area of research with potential applications in stroke therapy (3–5). Better understanding of the alterations of the hemostasis system, particularly its counterplay with inflammation and vascular biology has tremendous potentials in stroke management. In this Special Issue, original research articles and reviews focus on most recent advances on hemostasis, thrombosis or vascular biology related to all areas of stroke research.

Hemorrhagic transformation (HT) after ischemic stroke is a common and potentially severe complication (6). HT carries a high risk of clinical deterioration and is associated with poor outcomes and mortality (7). Early recognition or prediction of HT would be an important aid for the appropriate management of AIS patients. Due to the importance of this topic, it is not surprising that a series of papers in this Special Issue aimed to improve our understanding of HT. In the research paper by He et al. the authors investigated whether an easily accessible and rapid screening tool, the neutrophil-platelet ratio (NPR) could predict HT in patients with AIS. In theory, NPR shows its advantage in revealing information about the crosstalk between inflammation and hemostasis. Based on a cohort of 279 stroke patients, the authors conclude that high NPR (>39.9) was independently associated with the increased risk of HT, particularly that of parenchymal hematoma in AIS (OR = 2.00, 95%CI: 1.041–3.843, $p = 0.037$). Not only low platelet count, but impaired platelet function may also increase the risk of HT. Rapid testing of platelet count is a well-established standard in the treatment of AIS and patients with low platelet counts are excluded from recombinant tissue plasminogen activator (rt-PA) treatment due to the significantly increased risk of HT (8). Although current guidelines do not recommend it, whether platelet function testing could be used to guide treatment decision during the acute phase of AIS is unknown (9). In the study by Lieschke et al. the authors aimed to demonstrate the feasibility of incorporating flow cytometry-based platelet function testing in a mouse model of dual antiplatelet therapy followed by ischemic stroke and rt-PA infusion (Yuan et al.). In addition to monitoring the efficacy of antiplatelet therapy, they sought to investigate the impact of platelet function in the development of HT. Their results suggest that reduced platelet activation is indicative of an increased risk for HT following experimental stroke and rt-PA treatment. Another important factor that might contribute to HT in AIS patients could be the impaired hemostasis balance due to liver fibrosis. The paper by Yuan et al. highlights that although subclinical liver fibrosis or steatosis may not be rare in patients with stroke, data are lacking regarding the association between liver

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fibrosis and HT for patients with AIS (10). In their single center retrospective study, 185 consecutive patients with HT and 199 age- and sex-matched AIS patients without HT were enrolled, and the extent of liver fibrosis was assessed using a validated fibrosis index (FIB-4). After adjustments for potential confounders, the authors concluded that AIS patients with a high FIB-4 score had a 3.461-fold risk (95%CI: 1.404–8.531) of HT as compared to patients with low FIB-4 score. HT may be a rare but severe complication of adult ischemic Moyamoya disease (MMD) (11). In the research paper by Lu et al. the authors investigated the differences of clinical and radiological features between adult ischemic MMD patients with and without HT and studied clinical outcomes. Their data suggest that normal cerebral perfusion may be a risk factor associated with HT in adult ischemic MMD. As expected, HT was strongly associated with increased disability rates and mortality in the investigated cohort of patients.

HT following AIS may be facilitated by rt-PA therapy. rt-PA has been the mainstay of therapeutic thrombolysis in AIS, however, in ∼6–8% of treated patients, potentially devastating intracerebral hemorrhage may occur (12). Moreover, rt-PA may have other effects on the central nervous system by modulating the blood brain barrier (BBB) permeability and influencing neuroinflammatory processes (13). These include the complement system that has received much attention in CNS disorders in recent years. While the role of the complement's cascade in neuroinflammation, neurodegenerative disease, and CNS injury is well-recognized, its role in rt-PA mediated neuroinflammation and stroke has not been fully explored as yet. Tenecteplase (TNK-tPA) is a challenging alternative of rt-PA, developed over 25 years ago to circumvent the short half-life of t-PA (14). Parallel studies on TNK-tPA is important in studies attempting to investigate the efficacy and safety of rt-PA. In their original work, Keragala et al. demonstrate that inhibition of the complement C5a-C5R1 interaction reduces the ability of rt-PA and TNK-r-PA to increase BBB permeability, which may offer novel means to improve the safety profile of thrombolytic therapy for patients with AIS.

HT and the increase of BBB permeability is not the only concern in patients receiving thrombolytic agents. Unfortunately, the majority of patients who receive thrombolysis using rt-PA fail to recanalize and lack neurological improvement (15). In the study protocol described by Lilicrap et al., the authors hypothesize that individual plasmin potential, as measured by *in vitro* response to rt-PA, may serve as a biomarker of rt-PA response and patients with greater plasmin response are more likely to recanalize early (Lillicrap et al.). Their study will be based on historical samples from the Barcelona Stroke Thrombolysis Biobank, comprised of 350 pre-thrombolysis plasma samples from AIS patients who received serial transcranial Doppler measurements before and after thrombolysis. The primary outcome of the study will be time to recanalization detected by TCD (defined as TIBI $\geq$ 4). Results of this study may have important implications for the clinical practice: if association between proteolytic response to rt-PA and recanalization is confirmed, future clinical treatment may customize thrombolytic therapy to maximize outcomes and minimize adverse effects for individual patients.

Interestingly, our knowledge today is still limited about hemostasis alterations increasing the risk of stroke. In this Special Issue, informative case reports are published on inherited and acquired thrombophilic risk factors associated with stroke (Watanabe et al. and Huseynov et al.). Cases of cyclosporine A induced intracranial thrombotic complications and a systematic review of the literature is provided by Song et al., reviewing articles on cyclosporine A- related thrombotic events and summarizing features of clinical characteristics and neuroimaging findings in drug-induced cerebral venous thrombosis. Significant sex-differences in risk factors for transient ischemic attack (TIA) were found and published in this Special Issue by Wang et al. by screening a high-risk population of >230,000 residents in eastern China. This original work highlights that improving the understanding of sex differences in the prevalence of stroke risk factors is necessary to develop strategies to reduce stroke incidence and mortality. The research paper by Fang et al. aimed to investigate the association between intracranial atherosclerotic stenosis (ICAS) and the severity of white matter changes (WMC). Their work may improve our understanding regarding how the presence of ICAS would influence the progression of WMC, which could potentially provide future therapeutic opportunities for prevention.

In the past few years, new perspectives were opened in the field of thrombosis and vascular biology, when mechanical thrombectomy has permitted the histological analysis of retrieved thrombi of AIS patients. In depth characterization of the structure of ischemic stroke thrombi may serve as an important tool to provide useful insights on AIS pathomechanisms and treatment failure (16). In this Issue, two relevant papers were published assessing cellular, hemostatic and protein components of AIS thrombi. In their original research paper, Dargazanli et al. resolved the proteomes of cardioembolic and atherothrombotic cerebrovascular human thrombi and applied an artificial intelligence routine to examine protein signature between the two selected groups. Marta-Enguita et al. performed histological analysis of different hemostatic parameters including some key proteins, potentially determining thrombus stability that have not been investigated as yet: thrombin activatable fibrinolysis inhibitor (TAFI) and matrix metalloproteinase 10 (MMP-10). The authors report that histological composition and distribution of different thrombi hemostasis components have prognostic implications, and it could potentially have an impact on the strategies to guide personalized therapies for stroke patients.

Hemorrhagic stroke accounts for ∼10–15% of all strokes and results in a higher rate of mortality as compared to ischemic strokes (17). In the IRONHEART study, published as a study protocol by Árokszállási et al.. and an original research paper by Orbán-Kálmándi et al. the authors aimed to test whether various hemostasis parameters may predict the outcome of non-traumatic intracerebral hemorrhage (ICH). Their results show that a modified clot lysis assay, that incorporates the effect of neutrophil extracellular traps correlated with the estimated bleeding volumes in patients with ICH and might serve as

a useful tool to predict ICH outcomes. It is known that about 10% of intracerebral neoplastic lesions initially present as spontaneous ICH (18). In the work of Nawabi et al., the authors evaluated the potential of a machine learning-based prediction of etiology for acute ICHs based on quantitative radiomic image features extracted from initial non-contrast-enhanced tomography (NECT) brain scans. The quantitative evaluation of acute NECT images in a machine learning algorithm provided high discriminatory power in predicting non-neoplastic vs. neoplastic ICH, thus the authors suggest that using this approach in the clinical routine might improve patient care. Treatment options in ICH are often challenging due to the high mortality of this disorder (19). The paper by Apostolaki-Hansson et al. investigated whether the effect of oral anticoagulant (OAC) treatment reversal is beneficial in patients with ICH. They compared 90-day survival and outcome in patients with OAC-ICH who received OAC reversal therapy and those who did not using the database of The Swedish Stroke Register. Their results showed that patients receiving OAC-reversal treatment had an improved 90-day mortality outcome as compared to those not receiving treatment. Their results warrant larger studies to determine which patient groups are likely to benefit from reversal therapy. Another paper by Zhou et al. tested whether minimally invasive surgery or conservative treatment is more beneficial for patients with ICH. In their review paper, trial sequential analysis was applied on data from randomized trials to answer this question. They conclude that minimally invasive surgery is more effective than conservative treatment in patients with ICH in reducing morbidity and mortality. Repeating a clinical trial with similar devices, design and outcomes is unlikely to change current evidence.

We are confident that the papers in this Special Issue will be of interest and relevance to those involved in the experimental and clinical fields related to stroke and hemostasis. We hope that the published articles will provide ideas and inspiration to those dedicated to understanding the risk factors, pathophysiology and treatment outcomes of stroke in order to better diagnose, treat or prevent this devastating disorder in the future.

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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REFERENCES

1. Raskob GE, Angchaisuksiri P, Blanco AN, Buller H, Gallus A, Hunt BJ, et al. Thrombosis: a major contributor to global disease burden. *Arterioscler Thromb Vasc Biol.* (2014) 34:2363–71. doi: 10.1161/ATVBAHA.114.304488
2. Donkor ES. Stroke in the 21(st) century: a snapshot of the burden, epidemiology, and quality of life. *Stroke Res Treat.* (2018) 2018:3238165. doi: 10.1155/2018/3238165
3. Bagoly Z, Szegedi I, Kalmandi R, Toth NK, Csiba L. Markers of coagulation and fibrinolysis predicting the outcome of acute ischemic stroke thrombolysis treatment: a review of the literature. *Front Neurol.* (2019) 10:513. doi: 10.3389/fneur.2019.00513
4. Henderson SJ, Weitz JI, Kim PY. Fibrinolysis: strategies to enhance the treatment of acute ischemic stroke. *J Thromb Haemost.* (2018) 16:1932–40. doi: 10.1111/jth.14215
5. Thiebaut AM, Gauberti M, Ali C, Martinez De Lizarrondo S, Vivien D, Yepes M, et al. The role of plasminogen activators in stroke treatment: fibrinolysis and beyond. *Lancet Neurol.* (2018) 17:1121–32. doi: 10.1016/S1474-4422(18)30323-5
6. Jickling GC, Liu D, Stamova B, Ander BP, Zhan X, Lu A, et al. Hemorrhagic transformation after ischemic stroke in animals and humans. *J Cereb Blood Flow Metab.* (2014) 34:185–99. doi: 10.1038/jcbfm.2013.203
7. Yaghi S, Willey JZ, Cucchiara B, Goldstein JN, Gonzales NR, Khatri P, et al. Treatment and outcome of hemorrhagic transformation after intravenous alteplase in acute ischemic stroke: a scientific statement for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke.* (2017) 48:e343–61. doi: 10.1161/STR.0000000000000152
8. Gensicke H, Al Sultan AS, Strbian D, Hametner C, Zinkstok SM, Moulin S, et al. Intravenous thrombolysis and platelet count. *Neurology.* (2018) 90:e690–7. doi: 10.1212/WNL.0000000000004982
9. Powers WJ, Rabinstein AA, Ackerson T, Adeoye OM, Bambakidis NC, Becker K, et al. 2018 guidelines for the early management of patients with acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke.* (2018) 49:e46–110. doi: 10.1161/STR.0000000000000163
10. Berge E, Whiteley W, Audebert H, De Marchis GM, Fonseca AC, Padiglioni C, et al. European Stroke Organisation (ESO) guidelines on intravenous thrombolysis for acute ischaemic stroke. *Eur Stroke J.* (2021) 6:I–LXII. doi: 10.1177/2396987321989865
11. Scott RM, Smith ER. Moyamoya disease and moyamoya syndrome. *N Engl J Med.* (2009) 360:1226–37. doi: 10.1056/NEJMra0804622
12. Emberson J, Lees KR, Lyden P, Blackwell L, Albers G, Bluhmki E, et al. Effect of treatment delay, age, and stroke severity on the effects of intravenous thrombolysis with alteplase for acute ischaemic stroke: a meta-analysis of individual patient data from randomised trials. *Lancet.* (2014) 384:1929–35. doi: 10.1016/S0140-6736(14)60584-5
13. Niego B, Medcalf RL. Plasmin-dependent modulation of the blood-brain barrier: a major consideration during tPA-induced thrombolysis? *J Cereb Blood Flow Metab.* (2014) 34:1283–96. doi: 10.1038/jcbfm.2014.99
14. Warach SJ, Dula AN, Milling TJ Jr. Tenecteplase thrombolysis for acute ischemic stroke. *Stroke.* (2020) 51:3440–51. doi: 10.1161/STROKEAHA.120.029749
15. Bhatia R, Hill MD, Shobha N, Menon B, Bal S, Kochar P, et al. Low rates of acute recanalization with intravenous recombinant tissue plasminogen activator in ischemic stroke: real-world experience and a call for action. *Stroke.* (2010) 41:2254–8. doi: 10.1161/STROKEAHA.110.592535
16. Brinjikji W, Duffy S, Burrows A, Hacke W, Liebeskind D, Majoie C, et al. Correlation of imaging and histopathology of thrombi in acute ischemic stroke with etiology and outcome: a systematic review. *J Neurointerv Surg.* (2017) 9:529–34. doi: 10.1136/neurintsurg-2016-012391
17. Pinho J, Costa AS, Araujo JM, Amorim JM, Ferreira C. Intracerebral hemorrhage outcome: a comprehensive update.

J Neurol Sci. (2019) 398:54–66. doi: 10.1016/j.jns.2019.01.013

18. Choi YS, Rim TH, Ahn SS, Lee SK. Discrimination of tumorous intracerebral hemorrhage from benign causes using CT densitometry. *Am J Neuroradiol.* (2015) 36:886–92. doi: 10.3174/ajnr.A4233
19. Dastur CK Yu W. Current management of spontaneous intracerebral haemorrhage. *Stroke Vasc Neurol.* (2017) 2:21–9. doi: 10.1136/svn-2016-000047

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High Neutrophil-to-Platelet Ratio Is Associated With Hemorrhagic Transformation in Patients With Acute Ischemic Stroke

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Background: Hemorrhagic transformation (HT) is a complication that may cause neurological deterioration in patients with acute ischemic stroke. Both neutrophil and platelet have been associated with the stroke progression. The aim of this study was to explore the relationship between neutrophil-to-platelet ratio (NPR) and HT after acute ischemic stroke.

Methods: A total of 279 stroke patients with HT were consecutively recruited. HT was diagnosed using magnetic resonance imaging (MRI) or computed tomography (CT) and classified into hemorrhagic infarction (HI) and parenchymal hematoma (PH). Blood samples for neutrophil and platelet counts were obtained at admission. Meanwhile, 270 age- and gender-matched controls without HT were included for comparison.

Results: Among the patients with HT, 131 patients had PH and 148 patients had HI. NPR was higher in patients with PH than those with HI or non-HT [36.8 (23.7–49.2) vs. 26.6 (17.9–38.3) vs. 19.1 (14.8–24.8), $P < 0.001$]. After adjustment for potential confounders, high NPR remained independently associated with the increased risk of HT (OR = 2.000, 95% CI: 1.041–3.843, $P = 0.037$). NPR (>39.9) was independently associated with PH (OR = 2.641, 95% CI: 1.308–5.342, $P = 0.007$).

Conclusions: High NPR was associated with the increased risk of HT especially PH in patients with acute ischemic stroke.

Keywords: neutrophil-to-platelet ratio, stroke, hemorrhagic transformation, parenchymal hematoma, outcome

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INTRODUCTION

Acute ischemic stroke is among the leading causes of mortality and long-term morbidity throughout the world (1). Hemorrhagic transformation (HT) is a common and serious complication after acute ischemic stroke (2). Patients with HT were vulnerable to experience neurological deterioration, worse functional outcome and increased mortality (3). Moreover, HT is categorized into parenchymal hematoma (PH) and hemorrhagic infarction (HI) based on the radiological appearance (4). A large population-based prospective study revealed that PH rather than HI was an independent risk factor for death or disability (5). Therefore, early recognition of HT especially PH is essential for the appropriate management and better prognosis in patients with acute ischemic stroke.

Neutrophil, the key role in the innate immune response, has been found to be associated with ischemic stroke and HT (6, 7). One study comparing the prognostic value of different inflammatory factors indicated that neutrophil was a more sensitive indicator for cardiovascular mortality compared with other subsets of leukocyte and C-reactive protein (8). The activation of neutrophil after stroke could contribute to large infarct volume, blood-brain barrier (BBB) disruption and HT (9). Higher counts of neutrophil after ischemic stroke were associated with symptomatic intracranial hemorrhage and worse functional outcome (10). Moreover, the reduction in brain neutrophil recruitment through the inhibition of nod-like receptor protein 3 (NLRP3) could preserve the integrity of BBB and attenuate HT (11).

Platelet has been considered as a necessary factor against HT following ischemia/reperfusion because of its hemostatic function (12). The decrease in platelet count after ischemic stroke, caused by hemodilution of excessive fluid replacement (13), was correlated with the high rates of HT and even symptomatic HT (14, 15). Except for its hemostatic function, platelet also plays an important role in the inflammatory response (16). The interaction between neutrophil and platelet was involved in the process of vascular injury after ischemic stroke (16). Platelet could facilitate neutrophil recruitment and extravasation into brain parenchyma after stroke, and the amount of infiltrated neutrophil was correlated with stroke progression (17, 18). Moreover, high neutrophil-platelet complex formation may increase the risk of stroke in patients with symptomatic carotid stenosis (19). Several studies found that the activation and aggregation of neutrophil and platelet within cerebral microvessels resulted in vascular inflammation and BBB dysfunction following ischemic-reperfusion injury (20, 21).

Increased neutrophil count and decreased platelet count may be associated with poor functional outcome in patients with HT (22, 23). A recent study found that high neutrophil-to-platelet ratio (NPR) was associated with long-term poor outcome in patients with acute ischemic stroke (24). Indeed, NPR shows its advantage in revealing information about the crosstalk between inflammation and hemostasis, and has been suggested as a useful and rapid screening tool to assess systemic inflammation in infective endocarditis (25). However, to date, no study has investigated the relationship between NPR and HT after ischemic stroke. The present study was designed to explore whether high NPR was associated with HT especially PH in patients with acute ischemic stroke.

MATERIALS AND METHODS

Subjects

This was a retrospective study of HT patients who had been consecutively admitted to the Stroke Unit at the First Affiliated Hospital of Wenzhou Medical University between October 2011 and September 2018. The exclusion criteria were as follows: (1) acute infection within 2 weeks before admission or chronic infection; and (2) cancer, severe hepatic or renal diseases. Meanwhile, 270 age- and gender-matched controls without HT were included for comparison.

The study was approved by the ethics committee of the First Affiliated Hospital of Wenzhou Medical University, and the protocol followed the local ethics criteria for human research. Although written informed consent was not obtained for this study because of the retrospective design, it was obtained for data collection from our stroke registry.

Clinical and Radiological Variables

Data were collected containing demographics (age and gender), risk factors (hypertension, diabetes mellitus, coronary heart disease, atrial fibrillation, history of stroke, and cigarette smoking), systolic blood pressure, diastolic blood pressure, the Trial of ORG10172 in the Acute Stroke Treatment (TOAST) classification and treatment in hospital (thrombolysis, antiplatelet and anticoagulation). The stroke severity was assessed by the National Institutes of Health Stroke Scale (NIHSS) at admission (26). Functional outcome was assessed by the Barthel Index (BI) at discharge, and the BI < 60 was defined as poor functional outcome (27).

All patients underwent brain Computed Tomography (CT)/Magnetic Resonance Imaging (MRI) scans at admission, at day 4 (± 2) and at any clinical worsening. HT was diagnosed using follow-up CT/MRI scans, and classified into PH and HI according to the recommendations of European Cooperative Acute Stroke Study (ECASS) II classification (28). PH was defined as hemorrhage with a mass effect. HT was determined separately by two neurologists blinded to clinical data, and the third was consulted when a divergence occurred. Moreover, the infarct location was divided into two groups: anterior circulation included frontal, parietal, lateral temporal cortical and subcortical regions, internal capsule, and basal ganglia; posterior circulation included brainstem, cerebellum, thalamus, medial temporal, and occipital regions (29). The infarct size was categorized as follows: less than one-half of a lobe was classified as small infarct volume, and more than one-half of a lobe was classified as large infarct volume (30).

Laboratory Test

Blood samples were collected at admission in the Department of Emergency of our hospital and were obtained from the antecubital vein. The counts of leukocyte, neutrophil, lymphocyte, monocyte and platelet were obtained. The NPR was calculated by neutrophil count ($\times 10^9$L) $\times$1000/platelet count ($\times 10^9$/L). NPR was further divided into tertiles (tertile 1, tertile 2, tertile 3) in all patients and HT patients, respectively. The leukocyte-platelet ratio was calculated by leukocyte count ($\times 10^9$L) $\times$1000/platelet count ($\times 10^9$/L). The lymphocyte-platelet ratio was calculated by lymphocyte count ($\times 10^9$L) $\times$1000/platelet count ($\times 10^9$/L). The monocyte-platelet ratio was calculated by monocyte count ($\times 10^9$L) $\times$1000/platelet count ($\times 10^9$/L).

Statistical Analysis

All patients were divided into HT and non-HT, and the HT group was further divided into PH and HI. The data were displayed as mean (standard deviation, SD) or median (interquartile range, IQR) for the continuous variables and percentages for

the categorical variables. Student's *t*-test, analysis of variance (ANOVA) or Mann–Whitney U-test were applied for continuous variables, while the Chi-squared test was applied for proportions. On the one hand, receiver-operating characteristic (ROC) curves analysis were used to determine diagnostic accuracy of HT, and the cut-off values of NPR were calculated according to the Youden index. The area under the ROC curve (AUC) was considered as a critical diagnostic index. One the other hand, the levels of NPR were analyzed according to the degree of HT, while the degree of HT was also compared according to the NPR tertiles. Furthermore, multiple logistic regression analysis was used to evaluate whether NPR was associated with the incidence of HT and PH. For HT, model 1 was adjusted for age and sex; model 2 was adjusted for the variables in model 1 plus the factors that had already been established as predictors of HT (diabetes mellitus, atrial fibrillation, systolic blood pressure, large infarct volume, baseline NIHSS, anticoagulant and thrombolysis); and model 3 was adjusted for all the variables in model 2 plus the factors that significantly differed between the HT groups on the univariate analysis (coronary heart disease, anterior circulation and antiplatelet). For PH, model 1 was adjusted for age and sex; and model 2 was adjusted for the variables in model 1 plus the factors that had already been established as predictors of HT and that significantly differed between the outcome groups on the univariate analysis (diabetes mellitus, atrial fibrillation, systolic blood pressure, large infarct volume, baseline NIHSS, anticoagulant and thrombolysis). Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. $P < 0.05$ at two-tailed was considered statistically significant. All statistical analyses were performed on SPSS for Windows, version 23.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Characteristics of Patients With HT/PH

This study enrolled 549 patients: 375 men (68.3%) and 174 women (31.7%). Their mean age was 69.0 ± 12.3 years. Among the 279 patients with HT, 131 patients had PH and 148 patients

TABLE 1 | Differences of the characteristics according to the subcategorized groups of HT.

	All patients ($n = 549$)			Patients with HT ($n = 279$)		
	No HT ($n = 270$)	**HT ($n = 279$)**	***P*-value**	**HI ($n = 148$)**	**PH ($n = 131$)**	***P*-value**
Demographics						
Age (y), mean ± SD	69.1 ± 12.1	68.9 ± 12.6	0.815	69.3 ± 11.8	68.4 ± 13.4	0.548
Male, *n* (%)	181 (67.0)	85 (30.5)	0.530	103 (69.6)	91 (69.5)	0.981
Risk factors, *n* (%)						
Hypertension	189 (70.0)	177 (63.4)	0.103	99 (66.9)	78 (59.5)	0.203
Diabetes mellitus	75 (27.8)	69 (24.7)	0.417	35 (23.6)	34 (26.0)	0.656
Coronary heart disease	14 (5.2)	32 (11.5)	0.008*	18 (12.2)	14 (10.7)	0.700
Atrial fibrillation	26 (9.7)	104 (37.3)	<0.001**	43 (29.1)	61 (46.6)	0.003*
History of stroke	31 (11.5)	40 (14.3)	0.319	25 (16.9)	15 (11.5)	0.196
Cigarette smoking	115 (42.6)	122 (43.7)	0.788	68 (45.9)	54 (41.2)	0.427
SBP (mmHg), mean ± SD	158 ± 23	149 ± 22	<0.001**	152 ± 22	146 ± 22	0.021*
DBP (mmHg), mean ± SD	82 ± 14	83 ± 14	0.546	83 ± 14	82 ± 14	0.505
Baseline NIHSS, median (IQR)	3 (1–5)	10 (5–13)	<0.001**	7 (3–12)	11 (7–14)	<0.001**
Stroke mechanisms, *n* (%)			<0.001**			0.002*
Large-artery atherosclerosis	245 (90.7)	157 (56.3)		97 (65.5)	60 (45.8)	
Cardioembolism	20 (7.4)	118 (42.3)		49 (33.1)	69 (52.7)	
Others	5 (1.9)	4 (1.4)		2 (1.4)	2 (1.5)	
Infarct location, *n* (%)						
Anterior circulation	183 (67.8)	257 (92.1)	<0.001**	133 (89.9)	124 (94.7)	0.138
Posterior circulation	126 (46.7)	117 (41.9)	0.265	68 (45.9)	49 (37.4)	0.149
Large infarct volume, n (%)	5 (1.9)	117 (41.9)	<0.001**	51 (34.5)	66 (50.4)	0.007*
Treatment in hospital, *n* (%)						
Thrombolysis	5 (1.9)	20 (7.2)	0.003*	7 (4.7)	13 (9.9)	0.093
Anticoagulant	26 (9.7)	84 (30.1)	<0.001**	38 (25.7)	46 (35.1)	0.086
Antiplatelet	248 (92.9)	154 (55.2)	<0.001**	86 (58.1)	68 (51.9)	0.299
Neutrophil count, mean ± SD	4.2 ± 1.4	6.3 ± 3.3	<0.001**	5.7 ± 3.0	7.0 ± 3.6	0.001
Platelet count, mean ± SD	207.0 ± 51.2	198.5 ± 67.3	0.096	203.5 ± 66.7	190.0 ± 63.4	0.085
NPR, median (IQR)	19.1 (14.8–24.8)	30.1 (20.5–44.7)	<0.001**	26.6 (17.9–38.3)	36.8 (23.7–49.2)	<0.001**

*HT, hemorrhagic transformation; HI, hemorrhagic infarct; PH, parenchymal hematoma; SD, standard deviation; SBP, systolic blood pressure; DBP, diastolic blood pressure; IQR, interquartile range; NPR, neutrophil-to-platelet ratio; *P < 0.05; **P < 0.001.*

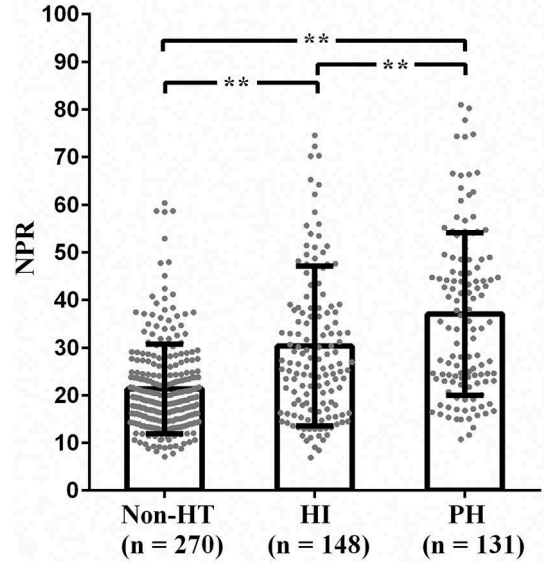

FIGURE 1 | The levels of NPR in the subcategorized groups of HT. (1) The NPR was higher in patients with HI than those with non-HT [26.6 (17.9–38.3) vs. 19.1 (14.8–24.8), $P < 0.001$]; (2) The NPR was higher in patients with PH than those with non-HT [36.8 (23.7–49.2) vs. 19.1 (14.8–24.8), $P < 0.001$]; (3) The NPR was higher in patients with PH than those with HI [36.8 (23.7–49.2) vs. 26.6 (17.9–38.3)], $P < 0.001$). NPR, neutrophil-to-platelet ratio; HT, hemorrhagic transformation; HI, hemorrhagic infarct; PH, parenchymal hematoma; $^{**}P < 0.001$.

had HI. Clinical characteristics in patients with HT/PH and those without are summarized in **Table 1**. Patients with HT/PH were more likely to have atrial fibrillation, high baseline NIHSS and large infarct volume. The therapy of thrombolysis, anticoagulant and antiplatelet were more frequently observed in patients with HT than those without HT. Patients with PH had higher proportion of poor functional outcome compared with those with HI.

Neutrophil count was significantly higher in the patients with PH than those with HI or non-HT [7.0 ± 3.6 vs. 5.7 ± 3.0 vs. 4.2 ± 1.5, $P < 0.001$]. Platelet count was significantly lower in the patients with PH than those with HI or non-HT [190.3 ± 63.2 vs. 205.8 ± 70.2 vs. 207.0 ± 51.2, $P = 0.025$]. According to the ROC curves, with an AUC of 0.733, NPR showed a greater discriminatory ability compared with neutrophil count [AUC 0.727, 95% CI (0.685–0.769), $P < 0.001$] and platelet count [AUC 0.570, 95% CI (0.521–0.617), $P = 0.005$] (**Supplementary Figure 1**). With an AUC of 0.733, NPR also showed a greater discriminatory ability compared with leukocyte-platelet ratio [AUC 0.695, 95% CI (0.651–0.740), $P < 0.001$], lymphocyte-platelet ratio [AUC 0.355, 95% CI (0.308–0.401), $P < 0.001$], and monocyte-platelet ratio [AUC 0.664, 95% CI (0.618–0.710), $P < 0.001$] (**Supplementary Figure 2**).

Association Between NPR and HT/PH

NPR was significantly higher in the patients with PH than those with HI or non-HT [36.8 (23.7–49.2) vs. 26.6 (17.9–38.3) vs. 19.1 (14.8–24.8), $P < 0.001$] (**Figure 1**). Furthermore, all patients were divided into three subgroups according to tertiles of NPR levels (tertile 1, <18.4; tertile 2, 18.4–29.1; and tertile 3, >29.1). Patients with high NPR had a higher incidence of HT compared with those with middle or low NPR, respectively (77.2% vs. 42.3% vs. 32.8%; $P < 0.001$) (**Figure 2A**). With all patients taken as a whole, the incidence of HT taken as a dependent variable and low NPR taken as the reference used for NPR in the logistic analysis. Highest NPR was associated with the incidence of HT after adjustment for variables with clinical significance (model 2: OR = 2.503, 95% CI: 1.381–4.537, $P = 0.002$). The association remained significant after adjusting for factors that significantly differed between the HT groups on the univariate analysis (model 3: OR = 2.000, 95% CI: 1.041–3.843, $P = 0.037$). Atrial fibrillation, large infarct volume, baseline NIHSS and anterior circulation were also risk factors of HT (**Table 2**). According to the ROC curves, the optimal cut-off value for NPR as a diagnostic marker of HT was 24.24, with a sensitivity of 65.9% and a specificity of 72.6% [AUC 0.733, 95% CI (0.691–0.775), $P < 0.001$] (**Figure 3**). The NPR above the cut-off (24.24) remained independently associated with HT after adjustment for confounding variables (OR 2.069, 95% CI 1.195–3.584, $P = 0.009$).

When the HT patients were classified into tertiles of NPR levels (tertile 1, <23.9; tertile 2, 23.9–39.9; and tertile 3, >39.9), HT patients with high NPR had a higher incidence of PH compared with those with middle or low NPR, respectively (66.0% vs. 39.1% vs. 35.5%; $P < 0.001$) (**Figure 2B**). With HT patients taken as a whole, the incidence of PH taken as a dependent variable and low NPR taken as the reference used for NPR in the logistic analysis. Highest NPR remained independently associated with the incidence of PH (model 2: OR = 2.641, 95% CI: 1.308–5.342, $P = 0.007$), after adjustment for confounding factors. Baseline NIHSS was also a risk factor of PH (**Table 3**). According to the ROC curve, the optimal cut-off value for NPR as a diagnostic marker of PH was 39.40, with a sensitivity of 48.1% and a specificity of 78.4% [AUC 0.641, 95% CI (0.577–0.706), $P < 0.001$] (**Figure 3**). The NPR above the cut-off (39.40) remained independently associated with PH after adjustment for confounding variables (OR 2.724, 95% CI 1.497–4.959, $P = 0.001$). Moreover, similar conclusion was attained when the NPR level was continuous variable in the logistic regression analysis [HT (OR = 1.033 95% CI, 1.009–1.057, $P = 0.006$) and PH (OR = 1.020, 95% CI, 1.004–1.036, $P = 0.013$)].

DISCUSSION

To the best of our knowledge, this is the first study to explore the association between NPR and HT in patients with acute ischemic stroke. The present study indicated that high NPR was associated with an increased risk of HT after acute ischemic stroke. Moreover, we also found that HT patients with higher NPR were more likely to develop PH.

Accumulating studies have indicated that HT was correlated with ischemia/reperfusion injury, which was mainly attributed to BBB disruption, hemostatic dysfunction, oxidative stress and inflammation (31). One study investigating the relationship

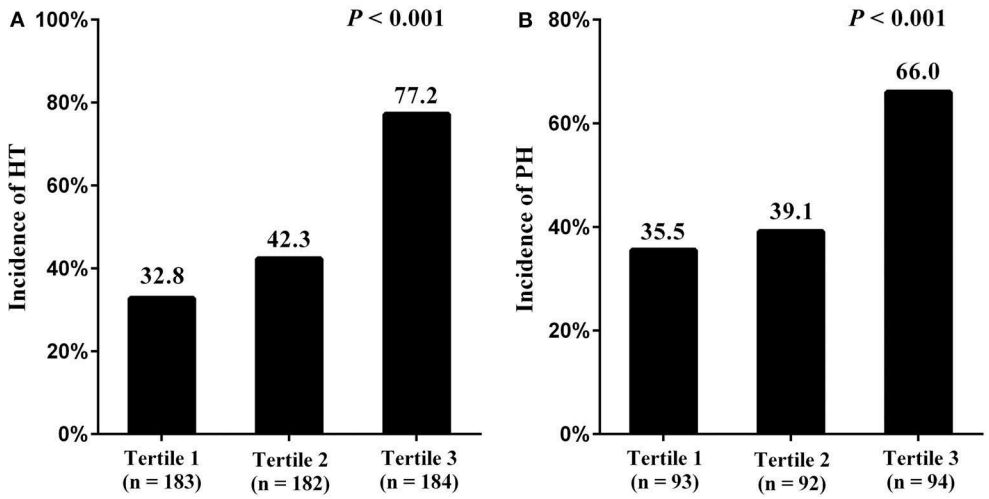

FIGURE 2 | Incidence of HT and PH stratified by NPR tertiles [NPR tertiles in all patients: tertile 1, <18.4; tertile 2, 18.4–29.1; tertile 3, >29.1 **(A)**. NPR tertiles in patients with HT: tertile 1, <23.9; tertile 2, 23.9–39.9; tertile 3, >39.9 **(B)**]. NPR, neutrophil-to-platelet ratio; HT, hemorrhagic transformation; PH, parenchymal hematoma.

TABLE 2 | Factors associated with HT by multivariate logistic regression analysis.

	Model 1		Model 2		Model 3	
	OR (95% CI)	***P*-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**	***P*-value**
NPR						
Tertile 1	Reference		Reference		Reference	
Tertile 2	1.521 (0.991–2.335)	0.055	0.946 (1.299–1.812)	0.837	1.017 (0.575–1.800)	0.953
Tertile 3	7.245 (4.525–11.600)	<0.001	7.839 (2.842–4.301)	0.005	2.000 (1.041–3.843)	0.037
Atrial fibrillation			2.627 (1.353–5.102)	0.004	2.514 (1.242–5.089)	0.010
Large infarct volume			7.839 (2.842–21.625)	<0.001	4.810 (1.679–13.780)	0.003
Baseline NIHSS			1.249 (1.175–1.329)	<0.001	1.256 (1.176–1.343)	<0.001
Anterior circulation					4.133 (2.068–8.261)	<0.001
Antiplatelet					0.213 (0.109–0.417)	<0.001

Model 1 was adjusted for age and gender. Model 2 was adjusted for the variables in model 1 plus the factors that had already been established as predictors of HT, including diabetes mellitus, atrial fibrillation, systolic blood pressure, large infarct volume, baseline NIHSS, anticoagulant and thrombolysis. Model 3 was adjusted for all the variables in model 2 plus the factors that significantly differed between the HT groups on the univariate analysis, including coronary heart disease, anterior circulation and antiplatelet. NPR, neutrophil-to-platelet ratio; HT, hemorrhagic transformation; OR, odd ratio; CI, confidence interval; NIHSS, National Institutes of Health Stroke Scale.

between BBB permeability and degree of HT found that higher BBB permeability may contribute to major intracranial bleeding in patients with acute ischemic stroke (32). Another neuroimaging study found that mild BBB disruption in ischemic brain was reversible due to the early reperfusion, while severe BBB disruption after sustained ischemia may increase the risk of HT and PH (33). Moreover, the increase in matrix metalloproteinase-9 (MMP-9) during the inflammatory phase of ischemic stroke was related to HT, and a 24 h peak of MMP-9 occurred before PH (34). The inhibition of MMP-9 expression with baicalin could maintain the BBB integrity, attenuate thrombolysis-induced HT and improve the prognosis in patients with acute ischemic stroke (35).

Several studies have indicated that neutrophil played an important role in the BBB disruption after stroke (22, 36). Neutrophils could release pro-inflammatory factors, reactive oxygen species and proteolytic enzymes, leading to BBB disruption and brain injury (9, 37). Previous studies found that neutrophil was the major source of MMP-9 acting on the BBB, which may result in symptomatic HT and poor outcome after ischemic stroke (10, 38). Higher neutrophil count was correlated with higher intracerebral hemorrhage volume at admission (39). The suppression of neutrophil recruitment was found to reduce hemorrhage volume and attenuate the severity of HT (11). While one study showed that low neutrophil count was associated with an increased risk of hematoma expansion during the hyperacute phase of intracerebral hemorrhage (40). One explanation for the contradictory findings may be that the effect of neutrophil on the vascular injury may be mediated by platelet, and the role of neutrophil-platelet interaction in the vascular inflammation may be varied during the different phase of intracerebral hemorrhage (41). The interaction between neutrophil and platelet could enhance the reactive oxygen species generation and exasperate the vascular injury (42), despite of the procoagulant properties

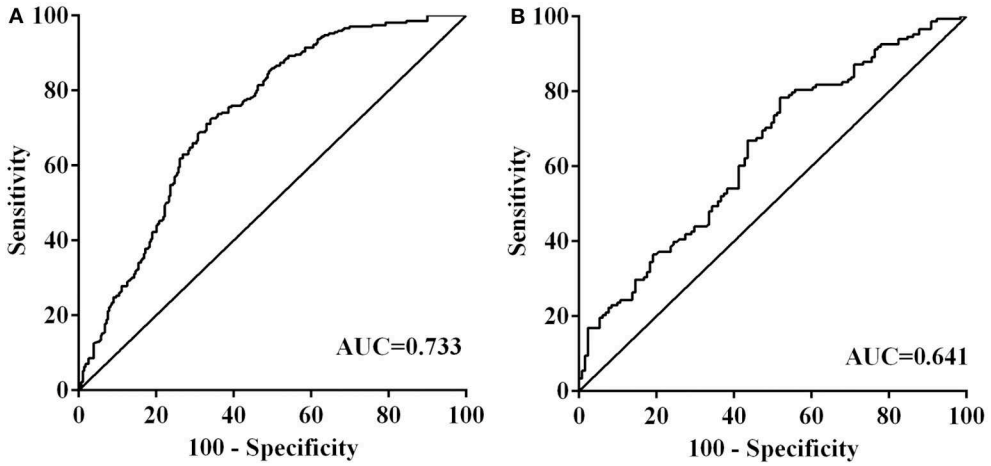

FIGURE 3 | Receiver operator characteristic analysis of neutrophil-to-platelet ratio for predicting hemorrhagic transformation **(A)** and parenchymal hematoma **(B)**. AUC, area under the curve.

TABLE 3 | Factors associated with PH by multivariate logistic regression analysis.

	Model 1		Model 2	
	OR (95%CI)	***P*-value**	**OR (95%CI)**	***P*-value**
NPR				
Tertile 1	Reference		Reference	
Tertile 2	1.249 (0.680–2.293)	0.474	0.975 (0.512–1.855)	0.938
Tertile 3	3.971 (2.121–7.437)	<0.001	2.641 (1.308–5.342)	0.007
Atrial fibrillation			1.492 (0.844–2.638)	0.169
Large infarct volume			1.073 (0.607–1.899)	0.808
Baseline NIHSS			1.074 (1.016–1.135)	0.011

Model 1 was adjusted for age and gender. Model 2 was adjusted for the variables in model 1 plus the factors that had already been established as predictors of HT and that significantly differed between the outcome groups on the univariate analysis, including diabetes mellitus, atrial fibrillation, systolic blood pressure, large infarct volume, baseline NIHSS, anticoagulant and thrombolysis. NPR, neutrophil-to-platelet ratio; PH, parenchymal hematoma; OR, odd ratio; CI, confidence interval; NIHSS, National Institutes of Health Stroke Scale.

of the activated neutrophils (43, 44). Moreover, platelet serves as a major contributor of several pro-inflammatory factors like tumor necrosis factor-α (41), which contributed to the increase in activated neutrophils and neutrophil-platelet aggregates (45). An animal study found that the depletion of platelet reduced the neutrophil recruitment and vascular inflammation after the occlusion of the middle cerebral artery (46).

In addition, a review of neutrophil-platelet interactions revealed that activated platelets were involved in the release of inflammatory mediators, neutrophil accumulation and the increase in vascular permeability (16). Platelet-endothelial interactions might prevent or heal neutrophil-induced vascular injury through the local release of soluble vasculoprotective factors (47). Hemostatic function of platelets was also attributed to the formation of plugs and clotting at the site of vessel injury (48), which helped to maintain the BBB integrity (49). Systemic inflammation was often accompanied by low platelet count, and consumption of platelet may be caused by the immunological process in the circulation (41). Therefore, it can be inferred that patients with higher NPR may have more severe disruption of BBB, leading to higher incidence of HT and PH. The better comprehension of neutrophil-platelet interactions may pave the way to promote neuroprotection and vascular repair in response to systematic inflammation after ischemic stroke (16).

In the present study, patients with PH had greater stroke severity and worse functional outcome compared with those with HI. A recent study supporting our findings showed that PH rather than HI was independently associated with poor functional outcome after thrombectomy in patients with acute large vessel occlusion (50). Some studies even indicated that patients with HI had a better functional outcome than those without HT because HI was a sign of early revascularization and better reperfusion (51, 52). Consistent with previous studies, we found that atrial fibrillation and large infarct volume were risk factors of HT (2). Moreover, we also found that anterior circulation was associated with HT. One possible reason for this phenomenon may be that patients with anterior circulation stroke were more likely to have greater stroke severity, atrial fibrillation and vessel occlusion, which were correlated with HT (2, 53).

There are some limitations in this study. First, the NPR was recorded only once, and it is necessary to investigate the association of dynamic changes in NPR after stroke with HT. Second, we did not explore the mechanisms underlying the influence of neutrophil and platelet on the BBB disruption in animal models, which should be conducted in our future work. Third, the functional outcome in stroke patients was assessed by BI, and our prospective studies will be conducted to better assess the functional outcome by modified Rankin scale (mRS). Fourth, the infarct size was taken as categorical variables in the analysis, and it is better to estimate the infarct size using the Alberta Stroke Program Early CT Score (ASPECTS) system by trained radiologists. Finally, considering the role of inflammation in the HT is still complex, more studies should be taken to further and better record more inflammatory index in the future.

CONCLUSIONS

The present study demonstrated that high NPR was associated with the risk of HT and PH in patients with acute ischemic stroke. These findings may help clinicians to identify which stroke patients are at high risk of HT especially PH, and thus conduct appropriate therapy and CT scan in this population. Considering the limitations in the present study, this relationship needs further investigations in future.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the corresponding author, without undue reservation, to any qualified researcher upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The First 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

WH and JH designed the project. WH did the statistical analyses and wrote the manuscript draft. WH, YR, CY, QC, HCheng, YZ, YC, GH, and HChen screened and extracted data. All authors have made an intellectual contribution to the manuscript and approved the 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.2019.01310/full#supplementary-material

REFERENCES

1. Hasan TF, Rabinstein AA, Middlebrooks EH, Haranhalli N, Silliman SL, Meschia JF, et al. Diagnosis and management of acute ischemic stroke. *Mayo Clin Proc*. (2018) 93:523–38. doi: 10.1016/j.mayocp.2018.02.013
2. Jickling GC, Liu DZ, Stamova B, Ander BP, Zhan XH, Lu AG, et al. Hemorrhagic transformation after ischemic stroke in animals and humans. *J Cereb Blood Flow Metab*. (2014) 34:185–99. doi: 10.1038/jcbfm.2013.203
3. Yaghi S, Willey JZ, Cucchiara B, Goldstein JN, Gonzales NR, Khatri P, et al. Treatment and outcome of hemorrhagic transformation after intravenous alteplase in acute ischemic stroke: a scientific statement for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke*. (2017) 48:E343–61. doi: 10.1161/STR.0000000000000152
4. Guenego A, Lecler A, Raymond J, Sabben C, Khoury N, Premat K, et al. Hemorrhagic transformation after stroke: inter- and intrarater agreement. *Eur J Neurol*. (2019) 26:476–82. doi: 10.1111/ene.13859
5. Paciaroni M, Agnelli G, Corea F, Ageno W, Alberti A, Lanari A, et al. Early hemorrhagic transformation of brain infarction: rate, predictive factors, and influence on clinical outcome - results of a prospective multicenter study. *Stroke*. (2008) 39:2249–56. doi: 10.1161/STROKEAHA.107.510321
6. Bonaventura A, Liberale L, Vecchie A, Casula M, Carbone F, Dallegri F, et al. Update on inflammatory biomarkers and treatments in ischemic stroke. *Int J Mol Sci*. (2016) 17:53. doi: 10.3390/ijms17121967
7. Ritzel RM, Lai YJ, Crapser JD, Patel AR, Schrecengost A, Grenier JM, et al. Aging alters the immunological response to ischemic stroke. *Acta Neuropathol*. (2018) 136:89–110. doi: 10.1007/s00401-018-1859-2
8. Hartaigh BO, Bosch JA, Thomas GN, Lord JM, Pilz S, Loerbroks A, et al. Which leukocyte subsets predict cardiovascular mortality? From the LUdwigshafen RIsk and Cardiovascular Health (LURIC) Study. *Atherosclerosis*. (2012) 224:161–9. doi: 10.1016/j.atherosclerosis.2012.04.012
9. Jickling GC, Liu DZ, Ander BP, Stamova B, Zhan XH, Sharp FR. Targeting neutrophils in ischemic stroke: translational insights from experimental studies. *J Cereb Blood Flow Metab*. (2015) 35:888–901. doi: 10.1038/jcbfm.2015.45
10. Maestrini I, Strbian D, Gautier S, Haapaniemi E, Moulin S, Sairanen T, et al. Higher neutrophil counts before thrombolysis for cerebral ischemia predict worse outcomes. *Neurology*. (2015) 85:1408–16. doi: 10.1212/WNL.0000000000002029
11. Guo ZL, Yu SH, Chen X, Zheng P, Hu T, Duan ZH, et al. Suppression of NLRP3 attenuates hemorrhagic transformation after delayed rtPA treatment in thromboembolic stroke rats: involvement of neutrophil recruitment. *Brain Res Bull*. (2018) 137:229–40. doi: 10.1016/j.brainresbull.2017.12.009
12. Stone JA, Willey JZ, Keyrouz S, Butera J, McTaggart RA, Cutting S, et al. Therapies for hemorrhagic transformation in acute ischemic stroke. *Curr Treat Options Neurol*. (2017) 19:15. doi: 10.1007/s11940-017-0438-5
13. Woessner R, Grauer MT, Dieterich HJ, Treib W, Stoll M, Treib J. Influence of long-term volume therapy with hydroxyethyl starch on leukocytes in patients with acute stroke. *Arzneimittelforschung*. (2003) 53:402–6. doi: 10.1055/s-0031-1297127
14. Kidwell CS, Saver JL, Carneado J, Sayre J, Starkman S, Duckwiler G, et al. Predictors of hemorrhagic transformation in patients receiving intra-arterial thrombolysis. *Stroke*. (2002) 33:717–24. doi: 10.1161/hs0302.104110
15. Lee JH, Park KY, Shin JH, Cha JK, Kim HY, Kwon JH, et al. Symptomatic hemorrhagic transformation and its predictors in acute ischemic stroke with atrial fibrillation. *Eur Neurol*. (2010) 64:193–200. doi: 10.1159/000319048
16. Garcia-Culebras A, Duran-Laforet V, Pena-Martinez C, Ballesteros I, Pradillo JM, Diaz-Guzman J, et al. Myeloid cells as therapeutic targets in neuroinflammation after stroke: specific roles of neutrophils and neutrophil-platelet interactions. *J Cereb Blood Flow Metab*. (2018) 38:2150–64. doi: 10.1177/0271678X18795789
17. Tang CM, Wang C, Zhang Y, Xue LJ, Li YY, Ju CY, et al. Recognition, intervention, and monitoring of neutrophils in acute ischemic stroke. *Nano Lett*. (2019) 19:4470–7. doi: 10.1021/acs.nanolett.9b01282
18. Cuartero MI, Ballesteros I, Lizasoain I, Moro MA. Complexity of the cell-cell interactions in the innate immune response after cerebral ischemia. *Brain Res*. (2015) 1623:53–62. doi: 10.1016/j.brainres.2015.04.047
19. McCabe DJH, Harrison P, Mackie IJ, Sidhu PS, Lawrie AS, Watt H, et al. Increased platelet count and leucocyte-platelet complex formation in acute symptomatic compared with asymptomatic severe carotid stenosis. *J Neurol Neurosurg Psychiatry*. (2005) 76:1249–54. doi: 10.1136/jnnp.2004.051003
20. Vital SA, Becker F, Holloway PM, Russell J, Perretti M, Granger DN, et al. Formyl-peptide receptor 2/3/lipoxin A(4) receptor regulates

neutrophil-platelet aggregation and attenuates cerebral inflammation impact for therapy in cardiovascular disease. *Circulation*. (2016) 133:2169–79. doi: 10.1161/CIRCULATIONAHA.115.020633
21. Li G, Sanders JM, Bevard MH, Sun Z, Chumley JW, Galkina EV, et al. CD40 ligand promotes Mac-1 expression, leukocyte recruitment, and neointima formation after vascular injury. *Am J Pathol*. (2008) 172:1141–52. doi: 10.2353/ajpath.2008.070633
22. Rosell A, Cuadrado E, Ortega-Aznar A, Hernandez-Guillamon M, Lo EH, Montaner J. MMP-9-Positive neutrophil infiltration is associated to blood-brain barrier breakdown and basal lamina type IV collagen degradation during hemorrhagic transformation after human ischemic stroke. *Stroke*. (2008) 39:1121–6. doi: 10.1161/STROKEAHA.107.500868
23. Jiao YG, Li GC, Xing YL, Nie DA, Liu XT. Influencing factors of hemorrhagic transformation in non-thrombolysis patients with cerebral infarction. *Clin Neurol Neurosurg*. (2019) 181:68–72. doi: 10.1016/j.clineuro.2019.04.018
24. Jin PP, Li XM, Chen J, Zhang ZR, Hu WW, Chen LY, et al. Platelet-to-neutrophil ratio is a prognostic marker for 90-days outcome in acute ischemic stroke. *J Clin Neurosci*. (2019) 63:110–15. doi: 10.1016/j.jocn.2019.01.028
25. Wei XB, Liu YH, He PC, Yu DQ, Tan N, Zhou YL, et al. The impact of admission neutrophil-to-platelet ratio on in-hospital and long-term mortality in patients with infective endocarditis. *Clin Chem Lab Med*. (2017) 55:899–906. doi: 10.1515/cclm-2016-0527
26. Kim JT, Park MS, Choi KH, Kim BJ, Han MK, Park TH, et al. Clinical outcomes of posterior versus anterior circulation infarction with low National Institutes of Health stroke scale scores. *Stroke*. (2017) 48:55–62. doi: 10.1161/STROKEAHA.116.013432
27. Sulter G, Steen C, De Keyser J. Use of the Barthel Index and Modified Rankin Scale in acute stroke trials. *Stroke*. (1999) 30:1538–41. doi: 10.1161/01.STR.30.8.1538
28. Hacke W, Kaste M, Fieschi C, von Kummer R, Davalos A, Meier D, et al. Randomised double-blind placebo-controlled trial of thrombolytic therapy with intravenous alteplase in acute ischaemic stroke (ECASS II). Second European-Australasian Acute Stroke Study Investigators. *Lancet*. (1998) 352:1245–51. doi: 10.1016/S0140-6736(98)08020-9
29. Rovira A, Grive E, Rovira A, Alvarez-Sabin J. Distribution territories and causative mechanisms of ischemic stroke. *Eur Radiol*. (2005) 15:416–26. doi: 10.1007/s00330-004-2633-5
30. Brott T, Marler JR, Olinger CP, Adams HP Jr, Tomsick T, Barsan WG, et al. Measurements of acute cerebral infarction: lesion size by computed tomography. *Stroke*. (1989) 20:871–5. doi: 10.1161/01.STR.20.7.871
31. Lu GF, He QW, Shen Y, Cao F. Potential biomarkers for predicting hemorrhagic transformation of ischemic stroke. *Int J Neurosci*. (2018) 128:79–89. doi: 10.1080/00207454.2017.1349766
32. Leigh R, Jen SS, Hillis AE, Krakauer JW, Barker PB, Investigators SVI. Pretreatment blood-brain barrier damage and post-treatment intracranial hemorrhage in patients receiving intravenous tissue-type plasminogen activator. *Stroke*. (2014) 45:2030–5. doi: 10.1161/STROKEAHA.114.005249
33. Simpkins AN, Dias C, Leigh R, National Institutes of Health Natural History of Stroke Investigators. Identification of reversible disruption of the human blood-brain barrier following acute ischemia. *Stroke*. (2016) 47:2405–8. doi: 10.1161/STROKEAHA.116.013805
34. Montaner J, Alvarez-Sabin J, Molina CA, Angles A, Abilleira S, Arenillas J, et al. Matrix metalloproteinase expression is related to hemorrhagic transformation after cardioembolic stroke. *Stroke*. (2001) 32:2762–7. doi: 10.1161/hs1201.99512
35. Chen HS, Guan BH, Chen X, Chen XM, Li CM, Qiu JH, et al. Baicalin attenuates blood-brain barrier disruption and hemorrhagic transformation and improves neurological outcome in ischemic stroke rats with delayed t-PA treatment: involvement of ONOO–MMP-9 pathway. *Transl Stroke Res*. (2018) 9:515–29. doi: 10.1007/s12975-017-0598-3
36. Dang QB, Lapergue B, Alexy TD, Diallo D, Moreno JA, Mazighi M, et al. High-density lipoproteins limit neutrophil-induced damage to the blood-brain barrier *in vitro*. *J Cereb Blood Flow Metab*. (2013) 33:575–82. doi: 10.1038/jcbfm.2012.206
37. Bechmann I, Galea I, Perry VH. What is the blood-brain barrier (not)? *Trends Immunol*. (2007) 28:5–11. doi: 10.1016/j.it.2006.11.007
38. Turner RJ, Sharp FR. Implications of MMP9 for blood brain barrier disruption and hemorrhagic transformation following ischemic stroke. *Front Cell Neurosci*. (2016) 10:13. doi: 10.3389/fncel.2016.00056
39. Adeoye O, Walsh K, Woo JG, Haverbusch M, Moomaw CJ, Broderick JP, et al. Peripheral monocyte count is associated with case fatality after intracerebral hemorrhage. *J Stroke Cerebrovasc Dis*. (2014) 23:E107–11. doi: 10.1016/j.jstrokecerebrovasdis.2013.09.006
40. Morotti A, Phuah CL, Anderson CD, Jessel MJ, Schwab K, Ayres AM, et al. Leukocyte count and intracerebral hemorrhage expansion. *Stroke*. (2016) 47:1473–8. doi: 10.1161/STROKEAHA.116.013176
41. Rossaint J, Margraf A, Zarbock A. Role of platelets in leukocyte recruitment and resolution of inflammation. *Front Immunol*. (2018) 9:13. doi: 10.3389/fimmu.2018.02712
42. Vanichakarn P, Blair P, Wu C, Freedman JE, Chakrabarti S. Neutrophil CD40 enhances platelet-mediated inflammation. *Thromb Res*. (2008) 122:346–58. doi: 10.1016/j.thromres.2007.12.019
43. McDonald B, Davis RP, Kim SJ, Tse M, Esmon CT, Kolaczkowska E, et al. Platelets and neutrophil extracellular traps collaborate to promote intravascular coagulation during sepsis in mice. *Blood*. (2017) 129:1357–67. doi: 10.1182/blood-2016-09-741298
44. Maugeri N, Baldini M, Ramirez GA, Rovere-Querini P, Manfredi AA. Platelet-leukocyte deregulated interactions foster sterile inflammation and tissue damage in immune-mediated vessel diseases. *Thromb Res*. (2012) 129:267–73. doi: 10.1016/j.thromres.2011.12.001
45. Bhasym A, Annarapu GK, Saha S, Shrimali N, Gupta S, Seth T, et al. Neutrophils develop rapid proinflammatory response after engulfing Hb-activated platelets under intravascular hemolysis. *Clin Exp Immunol*. (2019) 197:131–40. doi: 10.1111/cei.13310
46. Sreeramkumar V, Adrover JM, Ballesteros I, Cuartero MI, Rossaint J, Bilbao I, et al. Neutrophils scan for activated platelets to initiate inflammation. *Science*. (2014) 346:1234–8. doi: 10.1126/science.1256478
47. Ho-Tin-Noe B, Demers M, Wagner DD. How platelets safeguard vascular integrity. *J Thromb Haemost*. (2011) 9:56–65. doi: 10.1111/j.1538-7836.2011.04317.x
48. Stalker TJ, Welsh JD, Brass LF. Shaping the platelet response to vascular injury. *Curr Opin Hematol*. (2014) 21:410–17. doi: 10.1097/MOH.0000000000000070
49. Jones LD, Jackson JW, Maggirwar SB. Modeling HIV-1 induced neuroinflammation in mice: role of platelets in mediating blood-brain barrier dysfunction. *PloS ONE*. (2016) 11:26. doi: 10.1371/journal.pone.0151702
50. Lee YB, Yoon W, Lee YY, Kim SK, Baek BH, Kim JT, et al. Predictors and impact of hemorrhagic transformations after endovascular thrombectomy in patients with acute large vessel occlusions. *J NeuroInterventional Surg*. (2019) 11:469–73. doi: 10.1136/neurintsurg-2018-014080
51. Molina CA, Alvarez-Sabin J, Montaner J, Abilleira S, Arenillas JF, Coscojuela P, et al. Thrombolysis-related hemorrhagic infarction - a marker of early reperfusion, reduced infarct size, and improved outcome in patients with proximal middle cerebral artery occlusion. *Stroke*. (2002) 33:1551–6. doi: 10.1161/01.STR.0000016323.13456.E5
52. Kablau M, Kreisel SH, Sauer T, Binder J, Szabo K, Hennerici MG, et al. Predictors and early outcome of hemorrhagic transformation after acute ischemic stroke. *Cerebrovasc Dis*. (2011) 32:334–41. doi: 10.1159/000331702
53. De Marchis GM, Kohler A, Renz N, Arnold M, Mono ML, Jung S, et al. Posterior versus anterior circulation strokes: comparison of clinical, radiological and outcome characteristics. *J Neurol Neurosurg Psychiatry*. (2011) 82:33–7. doi: 10.1136/jnnp.2010.211151

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BRIEF RESEARCH REPORT
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Measurement of Platelet Function in an Experimental Stroke Model With Aspirin and Clopidogrel Treatment

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Dual antiplatelet treatment (DAPT) increases the risk of tPA-associated hemorrhagic transformation (HT) in ischemic stroke. To investigate the effects of DAPT in rodents, reliable indicators of platelet function utilizing a minimally invasive procedure are required. We here established a fluorescence-based assay to monitor DAPT efficiency in a mouse model of ischemic stroke with HT. Male C57/BL6 mice were fed with aspirin and clopidogrel (ASA+CPG). Venous blood was collected, stimulated with thrombin, labeled with anti-CD41-FITC and anti-CD62P-PE, and analyzed by flow cytometry. Subsequently, animals were subjected to experimental stroke and tail bleeding tests. HT was quantified using NIH ImageJ software. In ASA+CPG mice, the platelet activation marker CD62P was reduced by 40.6 ± 4.2% ($p < 0.0001$) compared to controls. *In vitro* platelet function correlated inversely with tail bleeding tests ($r = -0.8$, $p = 0.0033$, $n = 12$). Twenty-four hours after drug withdrawal, platelet activation rates in ASA+CPG mice were still reduced by 20.2 ± 4.1% ($p = 0.0026$) compared to controls, while tail bleeding volumes were increased by 4.0 ± 1.4 μl ($p = 0.004$). Conventional tests using light transmission aggregometry require large amounts of blood and thus cannot be used in experimental stroke studies. In contrast, flow cytometry is a highly sensitive method that utilizes small volumes and can easily be incorporated into the experimental stroke workflow. Our test can be used to monitor the inhibitory effects of DAPT in mice. Reduced platelet activation is indicative of an increased risk for tPA-associated cerebral hemorrhage following experimental stroke. The test can be applied to individual animals and implemented flexibly prior and subsequent to experimental stroke.

Keywords: dual antiplatelet therapy, aspirin, clopidogrel, flow cytometry, CD62P, CD41, hemorrhagic transformation, MCAO

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INTRODUCTION

The use of dual antiplatelet treatment (DAPT) in the acute phase of stroke is becoming increasingly common (1, 2). Apart from patients prescribed DAPT for other indications (3), this includes the application of DAPT in patients with transient ischemic attacks and minor strokes, as well as DAPT treatment during acute revascularization procedures (4, 5). By irreversibly impairing platelet function, DAPT increases the risk of hemorrhagic complications after thrombolysis with tPA (6–8).

Current European and American guidelines do not recommend to perform platelet function tests during the acute phase of ischemic stroke (9, 10). Although platelet function testing has become widely accessible with the use of point of care devices, investigations whether patients could potentially benefit are still lacking. On the other hand, rapid testing of the platelet count is a well-established standard in the treatment of stroke. Here, low platelet counts are excluded from tPA treatment due to the significantly increased risk of hemorrhagic transformation (HT) in platelet depleted patients (11). However, the question of whether and how platelet function contributes to tPA-associated HT has not been answered to date.

To address this gap of knowledge, we recently established a model of tPA-associated hemorrhagic transformation (HT) in mice pretreated with Aspirin and Clopidogrel [ASA+CPG, (12)]. While establishing antiplatelets in rodents, the individual hemostatic status of the single mouse becomes relevant. We therefore looked for a simple platelet function test to check for individual drug response.

Conventional platelet function tests like light transmission aggregometry (LTA) require significant volumes of blood (13), which means either collecting a lethal amount of blood from one mouse or collecting and pooling blood from different animals (14). As a result LTA is too invasive to be used as a measure of platelet status at the time of experimental stroke surgery. A simple-to-conduct but unspecific method is the tail bleeding test, which illustrates platelet adhesion *in vivo* and corresponds to the bleeding time test formerly used in patients (15, 16). The principle underlying this test is that platelet function directly affects primary hemostasis after tissue damage, resulting in prolonged bleeding time (time to complete cessation of the bleeding) and increased blood volumes during testing in mice. It can only be done once at the end of an experiment, and precludes the evaluation of drug efficacy in advance and repeated measurements. Accordingly, methods are required that utilize small sample volumes. Flow cytometry is a highly sensitive method requiring only small volumes of blood. Platelet activation assays based on flow cytometry have been introduced (17–19) but at present they are not widely used and have not been tested in the context of experimental stroke studies.

The aim of this study was to demonstrate the feasibility of incorporating flow cytometry-based platelet function testing in a mouse model of DAPT followed by an ischemic stroke and tPA infusion. In addition to monitoring the efficacy of antiplatelet therapy, we also sought to further investigate the impact of platelet function on the development of HT in order to predict bleeding risks and to define inclusion and exclusion criteria for subsequent experiments.

MATERIALS AND METHODS

Animals and Experimental Design

In total 28 male C57/B6 mice (Jackson, Bar Harbor, ME, USA) aged 9–10 weeks with a mean body weight of 26 ± 2g were used in this study. All experiments conformed to an institutionally approved protocol in accordance with the National Institute of Health's guide for the care and use of laboratory animals. We used exclusively male animals to limit variability due to sex differences. The operators performing surgical procedures and the investigators evaluating data were blinded to the treatment groups. At first, we assessed platelet function *in vitro* using our flow cytometry-based assay (as explained below) in mice treated with ASA+CPG and in controls. Mice were then subjected to 2 h MCAO followed by tPA administration. Twenty-four hours later, standard tail bleeding tests were conducted (14, 20), and mice were sacrificed to quantify HT development. A second *in vitro* analysis of platelet function was performed at the end of the experiment in order to demonstrate the feasibility of monitoring treatment effects beyond drug withdrawal (**Figure 1A**).

Antiplatelet Pretreatment

Mice were allocated randomly to a treatment or control group. ASA (Bayer Health Care, Morristown, NJ, USA) and CPG (Dr. Reddy's Laboratories Ltd., Beverley, UK) diluted in drinking water (ASA 0.4 mg/mL, CPG 0.15 mg/mL) were supplied *ad libitum* for 72 h. A water consumption of 15 ml/100 g per 24 h was assumed, providing an estimated daily intake of 60 mg/kg ASA and 22.5 mg/kg CPG per mouse. These dosages were selected based on previous experimental stroke studies (14, 21). Control mice received regular drinking water.

Flow Cytometry Based Platelet Function Test

In vitro platelet function testing was based on the platelet expression of CD41 and CD62P. We used the platelet marker CD41 to distinguish platelets from other events. CD62P is usually located in the membrane of platelet α-granules in the cytoplasm and only translocates to the plasma membrane after platelet activation (22). By detecting the surface co-expression of these antigens following thrombin treatment using a BD LSRII analyzer (Becton-Dickinson, San Jose, CA, USA), we gained an individual platelet activation rate for each single mouse. A high activation rate (indicated by increased CD62P expression) demonstrates sufficient platelet function, whereas a lower activation rate indicates impaired platelet function due to the partial inhibition of platelets, hence also serving as an indicator for an efficient anti-platelet treatment. Briefly, mice were anesthetized with isoflurane (1.25–1.5% in a nitrous oxide/oxygen mixture with spontaneous respiration). Venous blood (10–20 μl) was collected from the left jugular vein and given into sodium citrate (final 0.32%, Sigma) to prevent unintended platelet activation and blood clotting. Five microliters of whole blood were diluted in thirty microliters of PBS containing 0.32% sodium citrate. Fifteen microliters of vehicle or agonist solution were added, where the vehicle was composed of PBS containing 0.32% sodium citrate, and the agonist solution consisted of thrombin (final 2 μ/ml) supplemented with 10 mM GPRP (final 2.5 mM) and 6 mM CaCl2. The samples were then incubated shaking at 37°C for 5 min. Mouse blood was incubated with 1:100 dilution of CD41-FITC and CD62P-PE monoclonal antibodies (BD Biosciences and eBioscience™, respectively) for 15 min at room temperature in the dark. After staining, samples were fixed with 650 μl of fixative solution containing 0.1% formalin, 0.1% dextrose,

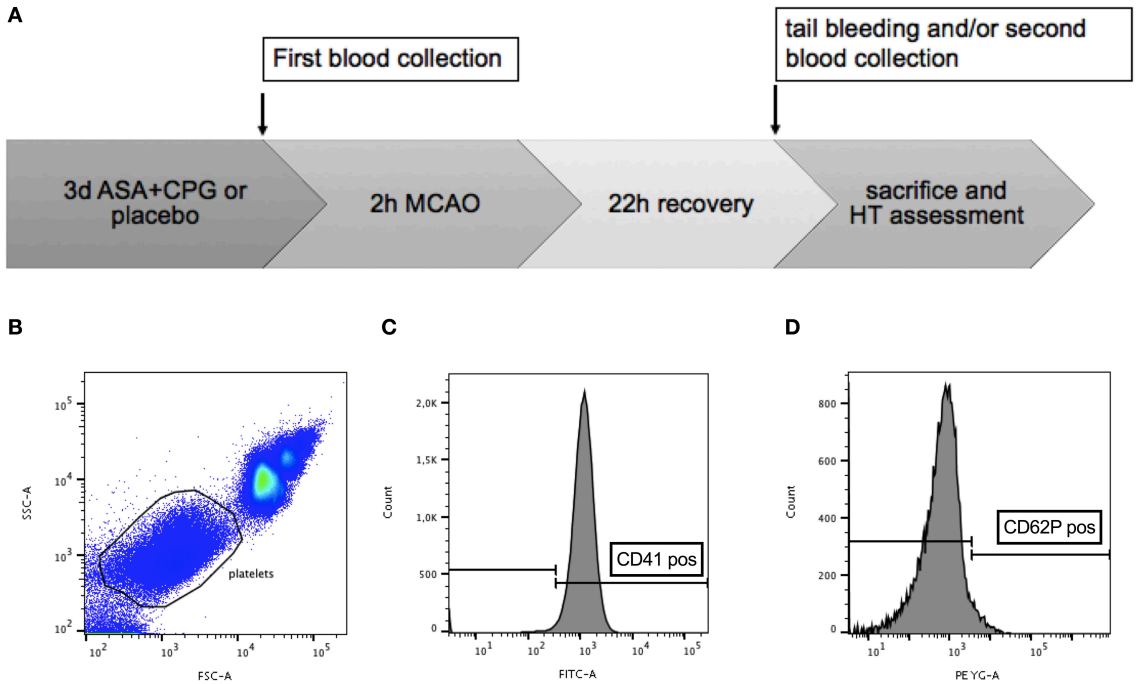

FIGURE 1 | (A) Timeline diagram of the experimental procedures: platelet function in control and ASA+CPG mice was tested *in vitro* using flow cytometry at the end of 3 days pretreatment. Mice were subjected to 2 h MCAO and tPA administration. Twenty-two hours later, tail bleeding tests and a second flow cytometry analysis were performed, followed by sacrifice and histological assessment of hemorrhage. **(B–D)** Gating strategy: Ungated whole blood **(B)**. Gated cells scored high for platelet identification marker FITC-CD41 **(C)** and low for the platelet activation marker PE-CD62P **(D)**, consistent with the properties of resting platelets (Analysis performed with FlowJo).

and 0.2% BSA in PBS. Labeled, diluted and fixed samples were analyzed. Platelets were gated based on their characteristic forward and side scatter properties (**Figure 1B**), as well as for antiplatelet immunoreactivity for CD41 (**Figure 1C**). The third gate was set on a positive staining with monoclonal antibody CD62P-PE (**Figure 1D**). Analyses were performed with BD FACS DIVA software (BD Biosciences) and FloJo (v10).

MCAO

Animals were anesthetized, and a 6–0 silicone-coated monofilament was introduced into the right internal carotid artery until the tip occluded the ostium of the MCA. Regional cerebral blood flow was monitored by laser doppler flowmetry with the use of a probe fixed to the intact skull above the territory of the right MCA. Rectal temperature was maintained between 36.5 and 37°C with a heating pad. After surgery, animals were allowed to recover from anesthesia. Hundred and twenty minutes after MCAO, the filament was withdrawn to initiate reperfusion, and 62.5 µL of tPA (4 mg/ml, final 10 mg/kg BW) were given by intravenous infusion into the right jugular vein over 15 min using a perfusion pump. 0.1 mg/kg BW buprenorphine hydrochloride (Buprenex®, Reckitt Benckiser Healthcare Ltd, UK) were administered at the end of all procedures.

Standard Tail Bleeding

To control for confounders, we first assessed the body temperature: Hypothermic mice were excluded, while normothermic mice were anesthetized and placed on a heating pad in prone position. A distal 5 mm segment of the tail was amputated with a razor blade. Tails were immediately inserted into microtubes containing 1 microliter saline placed in a water bath pre-warmed to 37°C. After 3 min, the tail was removed from the microtube, and hemostatic measures were taken by electro-cauterization using Bovie®. The microtube was vortexed, and ultrasound was applied for 15 s to lyse erythrocyte cell membranes. 3 × 100 µL were transferred to a 96 well plate containing 40 µL of Drabkin's reagent and incubated for 15 min. Absorption rates at 540 nm were determined using a SpectraMax M5 photometer. Bleeding volumes were calculated using a standard curve (14, 20, 23).

HT Determination

For HT measurement in the brain, mice were lethally anesthetized and perfused transcardially with saline. The brains were removed and sectioned into 1 mm thick slices and photographed. HT was assessed as red areas in brain sections, outlined and measured using ImageJ. Hemorrhages were classified according to the ECASS II morphologic definitions (24, 25) adapted to animal models as used in previous publications (12, 26, 27). Therefore, every section was individually scored on a 5 point ordinal scale (1 = no HT; 2 = hemorrhagic infarction type 1; 3 = hemorrhagic infarction type 2; 4 = parenchymal hemorrhage type 1; 5 = parenchymal hemorrhage type 2) and

an overall grade for every brain was determined according to the highest grade occurring among the sections.

Exclusion Criteria

Death within the 22 h recovery period led to exclusion from further assessment. Post-stroke tail bleeding tests were not performed in surviving animals when the general condition was too severe (indicated by a low body temperature (<32°C) and slow breathing rate, for details please see **Supplementary Material**).

Post-stroke Flow Cytometry Analysis of Platelet Function

Post-stroke flow cytometry analysis was performed with blood collected from 5 randomly chosen control mice and 3 ASA+CPG treated mice by cardiac puncture at the time point of sacrifice and conducted as described above.

Statistics and Data Analysis

Data is presented as mean ± SEM. Values were tested for Gaussian distribution with D'Agostino-Pearson omnibus normality test and Kolmogorov test. Statistical significance was determined for two group comparisons with Welch's *t*-test or Mann-Whitney test, and data sets were considered different if $p < 0.05$. Correlations were calculated with Spearman's test. All statistics were performed using Prism 7 graphpad software.

RESULTS

Pre-stroke Platelet Function Testing

In control mice ($n = 12$) platelet pre-activation in unstimulated samples was 3.6 ± 2.0% vs. 2.0 ± 0.7% in unstimulated blood obtained from ASA+CPG treated mice ($n = 15$, $p = 0.365$, **Figure 2A**). *In vitro* stimulation with thrombin platelet activation was 88.0 ± 0.6% in controls compared to 47.5 ± 3.7% in

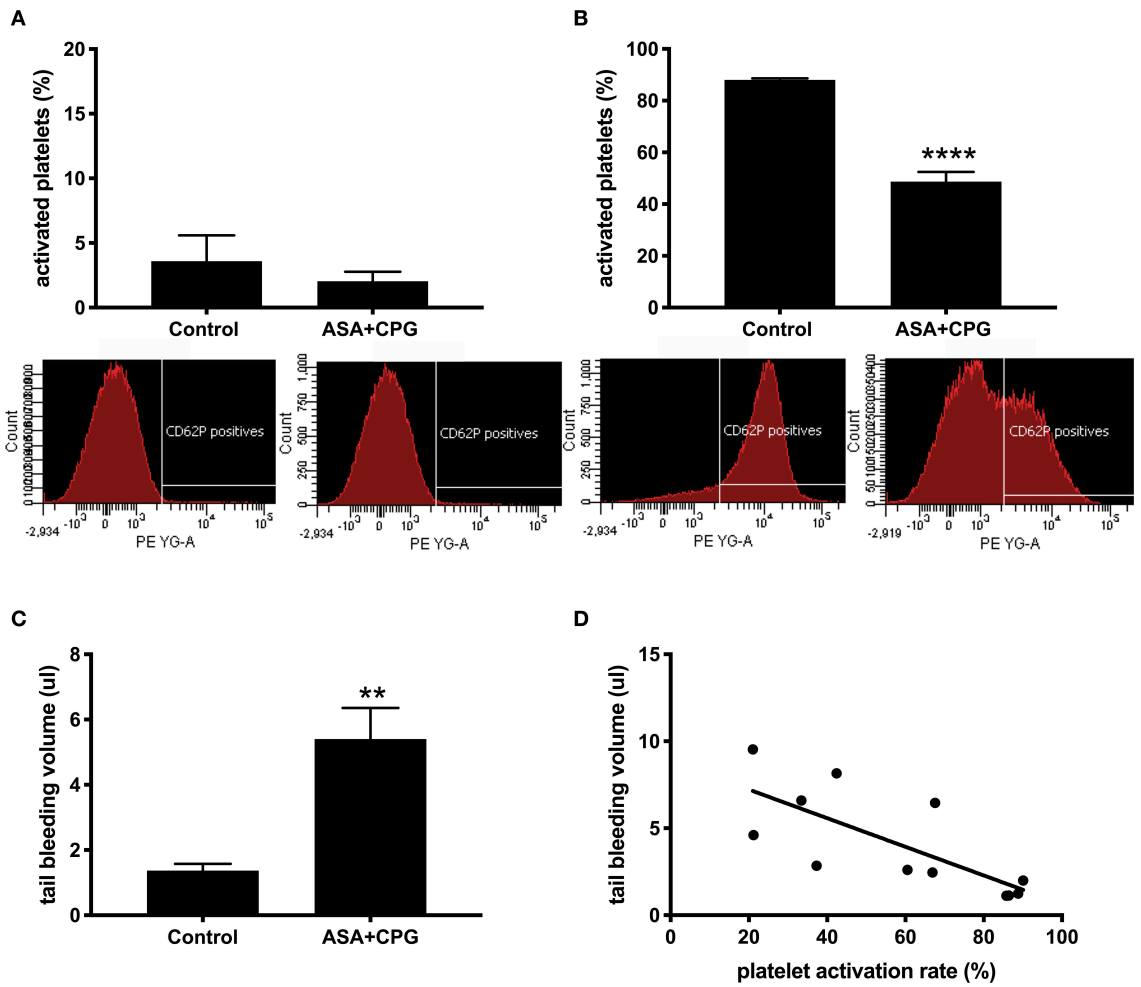

FIGURE 2 | (A) Platelet activation rates in unstimulated samples: Control: 3.6 ± 2.0%, $n = 12$; ASA+CPG: 2.0 ± 0.7%, $n = 15$, $p = 0.3649$ with selected example images of fluorescence intensity for platelet activation marker anti-CD62P-PE in unstimulated blood samples (FACSDiva software). **(B)** Platelet activation rates in thrombin stimulated samples: Controls: 88.0 ± 0.6%, $n = 12$; ASA+CPG: 47.5 ± 3.8%, $n = 15$, $p < 0.0001$, images of anti-CD62P-PE fluorescence intensity after *in vitro* thrombin stimulation. **(C)** Blood volumes in post-stroke tail bleeding testing: Controls: 1.4 ± 0.2 µl/3 min, $n = 4$; ASA+CPG: 5.4 ± 0.9 µl/3 min, $n = 8$, $p = 0.004$. **(D)** Correlation of *in vitro* platelet function with *in vivo* tail bleeding volumes: Linear regression: $R^2 = 0.5518$, y = −0.08175 (95% CI: −0.134 to −0.03)*X+8.846). Correlation: $r = -0.7902$, $p = 0.0033$, $n = 12$. **$p < 0.01$; ****$p < 0.0001$.

ASA+CPG treated mice ($p < 0.0001$, **Figure 2B**), indicating a partial platelet inhibition in these animals. Thus, we sufficiently demonstrated the *in vitro* detection of ASA+CPG drug effects in whole blood collected from mice following 3 days of oral drug administration.

Implementation Into Experimental Stroke

Subsequently, we subjected all mice to 2 h MCAO and tPA infusion, followed by a 22 h recovery period before performing tail bleeding tests (**Figure 1A**). During the recovery period, 4 control mice and 2 ASA+CPG treated mice died. We examined the deceased animals, focusing on intracerebral- or gastrointestinal bleeding or any other obvious hemorrhagic complication. Neither the ASA+CPG mice nor the control mice showed severe hemorrhages, suggesting that mortality was mainly related to the fairly severe ischemia induced by 2 h of MCAO. Tail bleeding volumes measured in 8 ASA+CPG treated mice were on average 5.4 ± 0.9 µl compared to 1.4 ± 0.2 µl measured in 4 control mice ($p = 0.004$, **Figure 2C**), suggesting platelet inhibition was still effective 24 h after cessation of treatment.

In comparing the individual results of pre-stroke platelet function testing with post-stroke tail bleeding volumes (the standard *in vivo* measure of platelet function), we found that low platelet activation rates upon *in vitro* thrombin stimulation correlated significantly with increased blood loss in the tail bleeding test and vice versa ($r = -0.7902$, $p = 0.0033$, $n = 12$; **Figure 2D**), suggesting our method is feasible to depict platelet function in mice.

Investigating the effect of platelet function on HT development, we found that HT did not occur in mice that demonstrated beforehand high activation rates upon *in vitro* stimulation with thrombin (CD62P expression >80%), whereas mice showing reduced platelet activation rates (CD62P expression <80%) were at higher risk for HT development (CD62P expression >80%: 0.9 ± 0.3 mm^2; CD62P expression <80%: 10.8 ± 4.4mm^2, $p = 0.0335$, **Figure 3A**). No hemorrhages were found remote from the infarct. The morphological classification of the HT types showed that 0/7 control mice

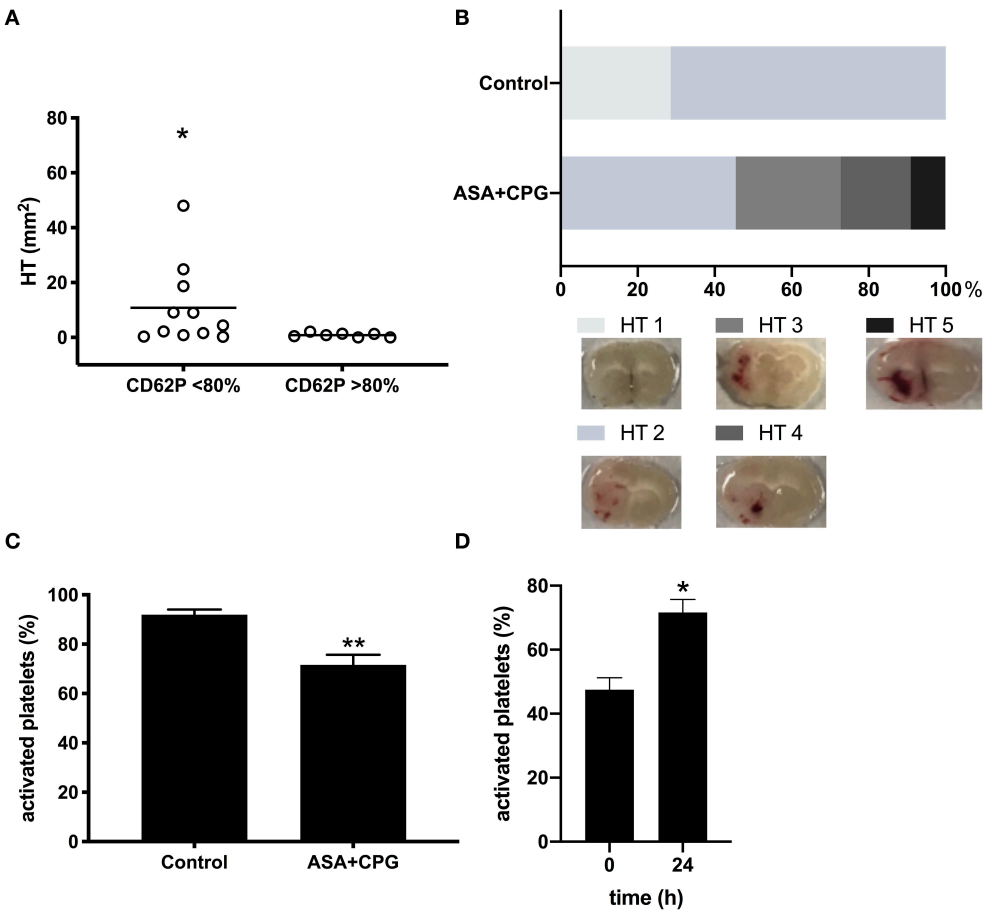

FIGURE 3 | (A) Hemorrhage area measurements in brain sections: Platelet activation rate >80% CD62P expression: 0.9 ± 0.3 mm^2, $n = 7$; Platelet activation rate <80% CD62P expression: 10.8 ± 4.5 mm^2, $n = 11$, $p = 0.0335$. **(B)** Morphological classification of the bleeding types: 0/7 control mice were rated HI-2, compared to 6/11 mice in the ASA+CPG ($p = 0.0125$). **(C)** Platelet activation rates in thrombin stimulated samples (post-stroke): Controls: 91.9 ± 2.1%, $n = 5$; ASA+CPG: 71.6 ± 4.1%, $n = 3$, $p = 0.0026$. **(D)** Platelet activation following thrombin stimulation pre- and post-stroke in ASA+CPG pretreated mice: Platelet activation rates increased significantly by 22.9 ± 8.9% ($p = 0.0204$) within 24 h between the two measurements. *$p < 0.05$; **$p < 0.01$.

were rated HI-2, compared to 6/11 mice in the ASA+CPG ($p = 0.0125$, **Figure 3B**, **Supplementary Table I**).

Post-stroke Platelet Function Testing

In analogy to the post-stroke tail bleeding testing, we performed a second *in vitro* platelet function testing post-stroke in randomly selected mice at the time point of sacrifice. In ASA+CPG pretreated mice, platelet activation upon *in-vitro* thrombin stimulation 24 h after drug withdrawal was 71.6 ± 4.1% compared to 91.9 ± 2.1% in controls (**Figure 3C**, $p = 0.0026$). Although the antiplatelet effect of ASA+CPG pretreatment was still detectable 24 h after stroke and drug withdrawal, platelet function in ASA+CPG pretreated mice already improved significantly compared to the platelet activation rates of the first *in vitro* testing (**Figure 3D** platelet activation rates in ASA+CPG mice pre-stroke 47.5 ± 3.8% vs. 71.6 ± 4.1% post-stroke). We here present the feasibility of repeated sampling within a stroke model using our flow cytometry-based platelet function assay.

DISCUSSION

With this study, we successfully developed a practical and straightforward method to test platelet function *in vitro* using flow cytometry, and we applied this assay to ASA+CPG treated mice in the context of experimental stroke studies in order to monitor antithrombotic treatment efficacy. Furthermore, we demonstrated that our method can be implemented at different time points within the stroke model and that repeated measurements are also feasible. Our method can be used to identify animals that are at a higher risk for developing HT, in order to study the pathophysiology and to explore new therapeutic approaches.

Thus, far, tail bleeding has been the only measure that assesses platelet function in an individual mouse without the need to pool blood from different subjects (which is needed for LTA). However, when it comes to its implementation into stroke models, this approach faces serious limitations. First, tail bleeding testing can only be performed at the end of an experiment before sacrifice due to a high risk of severe and uncontrolled bleeding. Secondly, the tail bleeding test is relatively unspecific (20). It can be affected by secondary hemostasis, blood pressure and body temperature. Another disadvantage is that mice cannot be tested repetitively, precluding dynamic measurements. In contrast, our flow cytometry based technique of platelet function analysis requires only 0.5–1% of the total blood volume of a mouse, allowing for repeated sampling.

We showed that there was no significant difference in platelet activation in unstimulated blood between ASA+CPG treated mice and control mice prior to stroke, which indicates that our samples were handled equally, resulting in acceptable pre-activation rates. However, pre-activation rates in ASA+CPG treated mice were slightly reduced compared to controls, which is in line with the findings of Kassassir et al. (19), who demonstrated that pre-activation rates in mice intravenously treated with cangrelor (another ADP-receptor antagonist like CPG) were reduced compared to untreated controls (19).

As we had hypothesized, ASA+CPG treatment led to significantly reduced platelet activation rates upon *in vitro* stimulation with thrombin. These treatment effects occurred in a comparable range to previously reported results in humans (28), although relatively high treatment dosages (of 60 mg/kg ASA and 22.5 mg/kg CPG) were used. The faster metabolism of mice and different administration protocols (crushing and diluting tablets which are designed for enteric resorption in humans) may explain these comparable effects in spite of a higher absolute dose (19, 22, 29, 30). Ultimately, while there was some variability in the response, we did not identify any non-responders, and all ASA+CPG mice showed reduced platelet reactivity.

Accordingly, we demonstrated that the impaired platelet function after ASA+CPG pre-treatment is strongly correlated with higher tail bleeding volumes. Thus, our method can be used to identify mice with decreased platelet function. Based on this, cohorts with an overall increased risk of HT could be identified. However, HT prediction on an individual animal level remains difficult. Similar to humans, not every pretreated mouse developed HT following thrombolytic treatment, although mice that showed hemorrhages generally had lower activation rates. These findings are in line with previous attempts to better stratify the risk of tPA-associated HT in human patients (7, 31–36), which aimed to define risk scores and predictors for HT. With only modest predictive power, those models failed to identify common key contributors. More research on pre-stroke platelet function and its impact on later HT development and neurological outcome may help to fill this gap of knowledge.

The current literature also revealed that despite the higher risk of tPA-associated HT in patients on DAPT, mortality and neurologic outcomes did not differ from patients without DAPT; thus, the benefit of tPA seemed to outweigh its risk (37–39). In order to get a clearer picture, it may be useful to implement platelet function testing during the early phase of acute ischemic stroke through using flow cytometry-based assays or point of care devices, which have already replaced the previous standard screening methods for platelet dysfunction (40). Testing the platelet function rather than the platelet count could potentially make discussion about platelet threshold superfluous (41).

We also need to address the limitations of our study. In conventional aggregation assays, typically ADP is used to demonstrate CPG effects (32). ADP receptor activation in mice leads to the release of platelet dense granules but not to the release of alpha granules (14). We used CD62P which is only located in alpha granules; thus, ADP stimulation could not be illustrated using anti-CD62P. Another consideration addressing the flow cytometry test relates to our method of quantification. An accepted strategy to quantify platelet aggregation using flow cytometry is the determination of the loss of single platelets due to aggregation upon platelet activation (17, 18, 28, 42, 43). Since we were using two separate samples when comparing unstimulated vs. stimulated blood, we were unable to consistently detect and quantify the loss of single platelets in these samples. A complementary strategy to deal with these limitations for future studies is to use only one sample and repeat measurements before and after platelet stimulation. However, this would require the platelet count to be determined. Which implies another

limitation to our study, since platelet function can be affected by platelet count (44, 45). Also, we have not tested platelet function following long-term treatment yet, in which other hemostatic factors besides platelets may also be affected. In addition, our study was designed as a single-center study at the Neuroprotection Research Laboratory (Massachusetts General Hospital), and needs to be confirmed by replication in other laboratories. A last but major concern is the validation of our flow cytometry assay by using tail bleeding. Since platelet function *in vitro* and *in vivo* may differ (44, 46, 47), it is insufficient to base examinations on either *in vitro* or *in vivo* only. By combining the FACS analysis with tail bleeding, we intended to achieve a complementary insight into the hemostatic status of an individual mouse. As expected, tail bleeding volumes were increased in the pretreated mice 24 h after the ischemic event. At the same time, FACS analysis of blood from the same mice showed reduced platelet activation in the DAPT treated mice, and these results correlated well ($r = -0.7902$, $p = 0.0033$, **Figure 2D**). We also considered cross-validation by comparison with aggregometry, but decided against it because (a) Due to the large volume required for the aggregometer used in the MGH pathology core facility, we would have had to pool the blood of several mice, which practically could not be combined with a stroke model; (b) The procedure used to initiate platelet activation differs significantly from our approach. For example, the core facility uses ADP or arachidonic acid to activate the platelets *in vitro*, while we used thrombin in our study; (c) Measurements with aggregometers are manufacturer and laboratory specific, which implies a restricted comparability between centers. These limitations would have ruled out a quantitative comparison of the two methods. However, the previous study by Lauer et al. demonstrated that the dosages of ASA and CPG used in our study were sufficient to significantly reduce platelet aggregation as measured by aggregometry (14), which is in line with our current results based on FACS measurements.

In summary, we describe a versatile and easy-to-implement tool to investigate the impact of platelet function in experimental stroke. We demonstrated that ASA+CPG treatment resulted in significantly impaired platelet function contributing to an increased risk of HT after experimental stroke and tPA administration. Our test can be used in animal studies to monitor drug effects. To identify and exclude mice with insufficient platelet inhibition from future experiments, it can be implemented at various times within the model and repeated using dynamic measurements. We here offer an experimental model to better investigate and understand the mechanisms underlying the impaired platelet function contributing to HT.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) at Massachusetts General Hospital (MGH).

AUTHOR CONTRIBUTIONS

CF, KL, and FL conceived the idea, designed the model and analyzed the data. FL carried out the experiments and performed the flow cytometry measurements. YZ performed the MCAO surgery. FL processed the experimental data, performed the statistical analysis, drafted the article, and designed the figures. JS aided in interpreting the results. KL and CF supervised the work. CF and KL made critical revisions of the article for important intellectual content. All authors discussed the results and commented on the article.

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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.2020.00085/full#supplementary-material

REFERENCES

1. Johnston SC, Easton JD, Farrant M, Barsan W, Conwit RA, Elm JJ, et al. Clopidogrel and aspirin in acute ischemic stroke and high-risk TIA. *N Engl J Med*. (2018) 379:215–25. doi: 10.1056/NEJMoa1800410
2. Wang Y, Wang Y, Zhao X, Liu L, Wang D, Wang C, et al. Clopidogrel with aspirin in acute minor stroke or transient ischemic attack. *N Engl J Med*. (2013) 369:11–9. doi: 10.1056/NEJMoa1215340
3. Levine GN, Bates ER, Blankenship JC, Bailey SR, Bittl JA, Cercek B, et al. 2011 ACCF/AHA/SCAI guideline for percutaneous coronary intervention. *J Am Coll Cardiol*. (2011) 58:e44–122. doi: 10.1016/j.jacc.2011.08.007
4. Brott TG, Hobson RW, Howard G, Roubin GS, Clark WM, Brooks W, et al. Stenting versus endarterectomy for treatment of carotid-artery stenosis. *N Engl J Med*. (2010) 363:11–23. doi: 10.1056/NEJMx100035
5. Valgimigli M, Bueno H, Byrne RA, Collet JP, Costa F, Jeppsson A, et al. 2017 ESC focused update on dual antiplatelet therapy in coronary artery

disease developed in collaboration with EACTS. *Eur J Cardio-thoracic Surg*. (2018) 53:34–78. doi: 10.1093/eurheartj/ehx419
6. Yang Y, Zhou M, Zhong X, Wang Y, Zhao X, Liu L, et al. Dual versus mono antiplatelet therapy for acute non-cardioembolic ischaemic stroke or transient ischaemic attack : a systematic review and meta-analysis. *Stroke Vasc Neurol*. (2018) 3:107–16. doi: 10.1136/svn-2018-000168
7. Mazya M, Egido JA, Ford GA, Lees KR, Mikulik R, Toni D, et al. Predicting the risk of symptomatic intracerebral hemorrhage in ischemic stroke treated with intravenous alteplase: Safe Implementation of Treatments in Stroke (SITS) symptomatic intracerebral hemorrhage risk score. *Stroke*. (2012) 43:1524–31. doi: 10.1161/STROKEAHA.111.644815
8. Diedler J, Ahmed N, Sykora M, Uyttenboogaart M, Overgaard K, Luijckx GJ, et al. Safety of intravenous thrombolysis for acute ischemic stroke in patients receiving antiplatelet therapy at stroke onset. *Stroke*. (2010) 41:288–94. doi: 10.1161/STROKEAHA.109.559724
9. Powers WJ, Rabinstein AA, Ackerson T, Adeoye OM, Bambakidis NC, Becker K, et al. 2018 guidelines for the early management of patients with acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke*. (2018) 49:46–110. doi: 10.1161/STR.0000000000000163
10. The European Stroke Organisation (ESO) Executive Committee and the ESO Writing Committee. Guidelines for Management of Ischaemic Stroke and Transient Ischaemic Attack 2008. *Cerebrovasc Dis*. (2008) 25:457–507. doi: 10.1159/000131083
11. Gensicke H, Al Sultan AS, Strbian D, Hametner C, Zinkstok SM, Moulin S, et al. Intravenous thrombolysis and platelet count. *Neurology*. (2018) 90:e1–8. doi: 10.1212/WNL.0000000000004982
12. Zheng Y, Lieschke F, Schaefer JH, Wang X, Foerch C, van Leyen K. Dual antiplatelet therapy increases hemorrhagic transformation following thrombolytic treatment in experimental stroke. *Stroke*. (2019) 50:3650–3. doi: 10.1161/STROKEAHA.119.027359
13. Hughes CE. How to perform aggregometry and lumi-aggregometry in mouse platelets. *Platelets*. (2018) 29:638–43. doi: 10.1080/09537104.2018.1478074
14. Lauer A, Schlunk F, Van Cott EM, Steinmetz H, Lo EH, Foerch C. Antiplatelet pretreatment does not increase hematoma volume in experimental intracerebral hemorrhage. *J Cereb Blood Flow Metab*. (2011) 31:1736–42. doi: 10.1038/jcbfm.2011.22
15. Kruse-Jarres R, Singleton TC, Leissinger CA. Identification and basic management of bleeding disorders in adults. *J Am Board Fam Med*. (2014) 27:549–64. doi: 10.3122/jabfm.2014.04.130227
16. Peterson P, Hayes TE, Arkin CF, Bovill EG, Fairweather RB, Rock William AJ, et al. The preoperative bleeding time test lacks clinical benefit: college of American Pathologists' and American Society of Clinical Pathologists' position article. *JAMA Surg*. (1998) 133:134–9. doi: 10.1001/archsurg.133.2.134
17. Armstrong PCJ, Kirkby NS, Chan MV, Finsterbusch M, Hogg N, Nourshargh S, et al. Novel whole blood assay for phenotyping platelet reactivity in mice identifies ICAM-1 as a mediator of platelet-monocyte interaction. *Blood*. (2015) 126:e11–8. doi: 10.1182/blood-2015-01-621656
18. De Cuyper IM, Meinders M, Van De Vijver E, De Korte D, Porcelijn L, De Haas M, et al. A novel fl ow cytometry – based platelet aggregation assay. *Blood*. (2013) 121:70–80. doi: 10.1182/blood-2012-06-437723
19. Kassassir H, Siewiera K, Sychowski R, Watała C. Can the antiplatelet effects of cangrelor be reliably studied in mice under *in vivo* and *in vitro* conditions using flow cytometry? *Pharmacol Rep*. (2013) 65:870–83. doi: 10.1016/S1734-1140(13)71068-5
20. Liu Y, L JN, M DA, Du X-J. Standardizing a simpler, more sensitive and accurate tail bleeding assay in mice. *World J Exp Med*. (2012) 2:30. doi: 10.5493/wjem.v2.i2.30
21. Momi S, Pitchford SC, Alberti PF, Minuz P, Del Soldato P, Gresele P. Nitroaspirin plus clopidogrel versus aspirin plus clopidogrel against platelet thromboembolism and intimal thickening in mice. *Thromb Haemost*. (2005) 93:535–43. doi: 10.1160/TH04-07-0464
22. Tsakiris DA, Scudder L, Hodivala-Dilke K, Hynes RO, Coller BS. Hemostasis in the mouse (Mus musculus): a review. *Thrombosis Haemostasis*. (1999) 81:177–88. doi: 10.1055/s-0037-1614439
23. Saito MS, Lourenço AL, Kang HC, Rodrigues CR, Cabral LM, Castro HC, et al. New approaches in tail-bleeding assay in mice: improving an important method for designing new anti-thrombotic agents. *Int J Exp Pathol*. (2016) 97:285–92. doi: 10.1111/iep.12182
24. Von Kummer R, Broderick JP, Campbell BCV, Demchuk A, Goyal M, Hill MD, et al. The heidelberg bleeding classification: Classification of bleeding events after ischemic stroke and reperfusion therapy. *Stroke*. (2015) 46:2981–6. doi: 10.1161/STROKEAHA.115.010049
25. Yaghi S, Willey JZ, Cucchiara B, Goldstein JN, Gonzales NR, Khatri P, et al. Treatment and outcome of hemorrhagic transformation after intravenous alteplase in acute ischemic stroke: a scientific statement for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke*. (2017) 48:e343–61. doi: 10.1161/STR.0000000000000152
26. García-Yébenes I, Sobrado M, Zarruk JG, Castellanos M, De La Ossa NP, Dávalos A, et al. A mouse model of hemorrhagic transformation by delayed tissue plasminogen activator administration after in situ thromboembolic stroke. *Stroke*. (2011) 42:196–203. doi: 10.1161/STROKEAHA.110.600452
27. Copin J-C, Gasche Y. Effect of the duration of middle cerebral artery occlusion on the risk of hemorrhagic transformation after tissue plasminogen activator injection in rats. *Brain Res*. (2008) 1243:161–6. doi: 10.1016/j.brainres.2008.09.025
28. Van Velzen JF, Laros-Van Gorkom BAP, Pop GAM, Van Heerde WL. Multicolor flow cytometry for evaluation of platelet surface antigens and activation markers. *Thromb Res*. (2012) 130:92–8. doi: 10.1016/j.thromres.2012.02.041
29. Li Y, Landqvist C, Grimm SW. Disposition and metabolism of ticagrelor, a novel P_2Y_{12} receptor antagonist, in mice, rats, and marmosets. *Drug Metab Dispos*. (2011) 39:1555–67. doi: 10.1124/dmd.111.039669
30. Wientjes MG, Levy G. Nonlinear pharmacokinetics of aspirin in rats. *J Pharmacol Exp Ther*. (1988) 245:809–15.
31. Cucchiara B, Tanne D, Levine SR, Demchuk AM, Kasner S. A Risk score to predict intracranial hemorrhage after recombinant tissue plasminogen activator for acute ischemic stroke. *J Stroke Cerebrovasc Dis*. (2008) 17:331–3. doi: 10.1016/j.jstrokecerebrovasdis.2008.03.012
32. Lou M, Safdar A, Mehdiratta M, Kumar S, Schlaug G, Caplan L, et al. The HAT score. *Neurology*. (2008) 71:1417–23. doi: 10.1212/01.wnl.0000330297.58334.dd
33. Strbian D, Engelter S, Michel P, Meretoja A, Sekoranja L, Ahlhelm FJ, et al. Symptomatic intracranial hemorrhage after stroke thrombolysis: the SEDAN Score. *Ann Neurol*. (2012) 71:634–41. doi: 10.1002/ana.23546
34. Saver JL. Hemorrhage after thrombolytic therapy for stroke: the clinically relevant number needed to harm. *Stroke*. (2007) 38:2279–83. doi: 10.1161/STROKEAHA.107.487009
35. Menon BK, Saver JL, Prabhakaran S, Reeves M, Liang L, Olson DM, et al. Risk score for intracranial hemorrhage in patients with acute ischemic stroke treated with intravenous tissue-type plasminogen activator. *Stroke*. (2012) 43:2293–9. doi: 10.1161/STROKEAHA.112.660415
36. Whiteley WN, Bruins SK, Peter F, Peter S, Joanna W. Risk Factors for intracranial hemorrhage in acute ischemic stroke patients treated with recombinant tissue plasminogen activator. *Stroke*. (2012) 43:2904–9. doi: 10.1161/STROKEAHA.112.665331
37. Luo S, Zhuang M, Zeng W, Tao J. Intravenous thrombolysis for acute ischemic stroke in patients receiving antiplatelet therapy: a systematic review and meta-analysis of 19 studies. *J Am Heart Assoc*. (2016) 5:1–14. doi: 10.1161/JAHA.116.003242
38. Xian Y, Federspiel JJ, Grau-Sepulveda M, Hernandez AF, Schwamm LH, Bhatt DL, et al. Risks and benefits associated with prestroke antiplatelet therapy among patients with acute ischemic stroke treated with intravenous tissue plasminogen activator. *JAMA Neurol*. (2016) 73:50–9. doi: 10.1001/jamaneurol.2015.3106
39. Tsivgoulis G, Katsanos AH, Mavridis D, Gdovinova Z, Karlinski M, Macleod MJ, et al. Intravenous thrombolysis for ischemic stroke patients on dual antiplatelets. *Ann Neurol*. (2018) 84:89–97. doi: 10.1002/ana.25269
40. Chen F, Maridakis V, O'Neill EA, Beals C, Radziszewski W, De Lepeleire I, et al. A randomized clinical trial comparing point-of-care platelet function assays and bleeding time in healthy subjects treated with aspirin or clopidogrel. *Platelets*. (2012) 23:249–58. doi: 10.3109/09537104.2011.604806
41. Breuer L, Huttner HB, Kiphuth IC, Ringwald J, Hilz MJ, Schwab S, et al. Waiting for platelet counts causes unsubstantiated delay of thrombolysis therapy. *Eur Neurol*. (2013) 69:317–20. doi: 10.1159/000345702

42. Ramström S, Södergren A, Tynngård N, Lindahl T, Lindahl TL. Platelet function determined by flow cytometry: new perspectives? platelet function determined by flow cytometry -new perspectives? *Semin Thromb Hemost*. (2016) 3:268–81. doi: 10.1055/s-0035-1570082
43. Gremmel T, Koppensteiner R, Panzer S. Comparison of aggregometry with flow cytometry for the assessment of agonists'-induced platelet reactivity in patients on dual antiplatelet therapy. *PLoS ONE*. (2015) 10:e0129666. doi: 10.1371/journal.pone.0129666
44. Vinholt PJ. The role of platelets in bleeding in patients with thrombocytopenia and hematological disease. *Clin Chem Lab Med*. (2019) 57:1808. doi: 10.1515/cclm-2019-0380
45. Krishnegowda M, Rajashekaraiah V. Platelet disorders: an overview. *Blood Coagul Fibrinolysis*. (2015) 26:479–91. doi: 10.1097/01.mbc.0000469521.23628.2d
46. Panzer S, Rieger M, Vormittag R, Eichelberger B, Dunkler D, Pabinger I. Platelet function to estimate the bleeding risk in autoimmune thrombocytopenia. *Eur J Clin Invest*. (2007) 37:814–9. doi: 10.1111/j.1365-2362.2007.01855.x
47. Frelinger AL, Grace RF, Gerrits AJ, Berny-Lang MA, Brown T, Carmichael SL, et al. Platelet function tests, independent of platelet count, are associated with bleeding severity in ITP. *Blood*. (2015) 126:873–9. doi: 10.1182/blood-2015-02-628461

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CASE REPORT
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A Case of Ischemic Stroke With Congenital Protein C Deficiency and Carotid Web Successfully Treated by Anticoagulant and Carotid Stenting

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Objective: A rare case of thromboembolic cerebral infarction due to carotid web in a patient with congenital protein C deficiency is reported.

Case Presentation: A patient in her 40's with left-side hemiparesis was transferred to our hospital under continuous intravenous injection of heparin. Magnetic resonance angiography demonstrated occlusion of the right middle cerebral artery (MCA). Conventional angiography revealed recanalization of the right MCA and a carotid web at the origin of the right internal carotid artery. Ultrasound scan of the carotid artery on the 19th day revealed thrombus formation on the distal portion of the carotid web. We performed carotid artery stenting to prevent thrombus formation by suppressing the carotid web to the vessel wall and by regulating the turbulent flow. The patient had no recurrence of stroke under-anticoagulation with warfarin during the 2-year follow-up period.

Conclusion: To our knowledge, this is the first report in which an immediate thrombus formation on the carotid web was observed in a patient with congenital protein C deficiency. In a case of acute ischemic stroke with carotid web, especially when congenital coagulopathy such as protein C deficiency is suspected, careful follow-up with ultrasound imaging should be performed.

Keywords: protein C deficiency, thrombus formation, carotid web, cerebral infarction, carotid artery stenting

INTRODUCTION

Carotid web is a shelf-like projection in the lumen of the internal carotid artery bulb and is recognized as a cause of stroke. However, the optimal treatment for symptomatic carotid web has not been thoroughly investigated (1). Moreover, congenital protein C deficiency induces thrombogenicity and also increases the risk of arterial thromboembolism, especially in young patients. There is no report on the clinical course and treatment strategy for a case complicated by both carotid web and protein C deficiency. Here, we report a case of thromboembolic cerebral infarction in which protein C deficiency was thought to accelerate thrombus formation on the carotid web. We confirmed thrombus formation with carotid ultrasound.

CASE PRESENTATION

A patient in her 40's with a wake-up stroke resulting in left-side hemiparesis was admitted to a previous hospital. The patient's head CT showed no evidence of cerebral hemorrhage, and ischemic stroke was suspected. The patient was then transferred to our hospital under continuous intravenous injection of low-dose unfractionated heparin (5,000 U intravenously, followed by continuous infusion of 500 U/h) prescribed by the physician of the previous hospital. Her past medical history was unremarkable, and she was under no medications. She did not use tobacco or thrombogenic drugs such as steroids or oral contraceptives. She had a negative family history for stroke. Her pulse was 90 beats per minute, pulse rhythm was regular, and blood pressure was 160/80 mmHg. She did not have any history of atrial fibrillation (AF). Her general physical examination results were normal. Neurological examinations revealed eye deviation to the right, severe inattentiveness on the left side, and left-side hemiparesis with Manual Muscle Testing scores of 1/5 for her left arm and 4/5 for her left leg. Laboratory studies yielded the following results: white blood cell count, 13,200/μl; C-reactive protein, 0.2 mg/dl; LDL-cholesterol, 83 mg/dl; fibrin/fibrinogen degradation products, 0.0 μg/ml; D-dimer, 0.2 μg/ml; prothrombin time-international normalized ratio, 1.32; and activated partial thromboplastin time, 100 s (normal range: 25.0–38.0 s). Protein C activity was 45% (normal range: 64–146%), total protein S antigen was 78% (normal range: 60–150%), and antithrombin activity was 84% (normal range: 80–120%). Tests for lupus anticoagulant, anti-cardiolipin antibodies, and anti-cardiolipin-beta 2-glycoprotein I complex antibody were negative.

Head magnetic resonance imaging (MRI) demonstrated acute cerebral infarction in the right middle cerebral artery (MCA) territory (**Figure 1A**) and magnetic resonance angiography (MRA) demonstrated occlusion of the main trunk of the right MCA (**Figure 1B**).

Because 7 h had passed since her condition had been confirmed normal, we attempted endovascular therapy. However, conventional angiography revealed recanalization of the right MCA and a carotid web at the origin of the right internal carotid artery, which showed approximately 30% stenosis according to the North American Symptomatic Carotid Endarterectomy Trial criteria (**Figure 1C**).

CLINICAL COURSE AFTER ADMISSION

On the second day, carotid duplex Doppler ultrasound confirmed the angiographic diagnosis (**Figures 1E,F**). Ultrasound examination was performed on a Logiq 7 pro (GE healthcare, Illinois, Chicago, USA) with a linear array transducer (10 L probe).

We initiated antiplatelet therapy (clopidogrel 75 mg/day and aspirin 100 mg/day) and anticoagulant therapy for a short duration (argatroban, 60 mg/day for 2 days followed by 20 mg/day for 5 days). Subsequently, we stopped anticoagulant therapy and continued with only antiplatelet therapy. However, the follow-up ultrasound scan of the carotid artery on the 19th day revealed thrombus formation on the distal portion of the

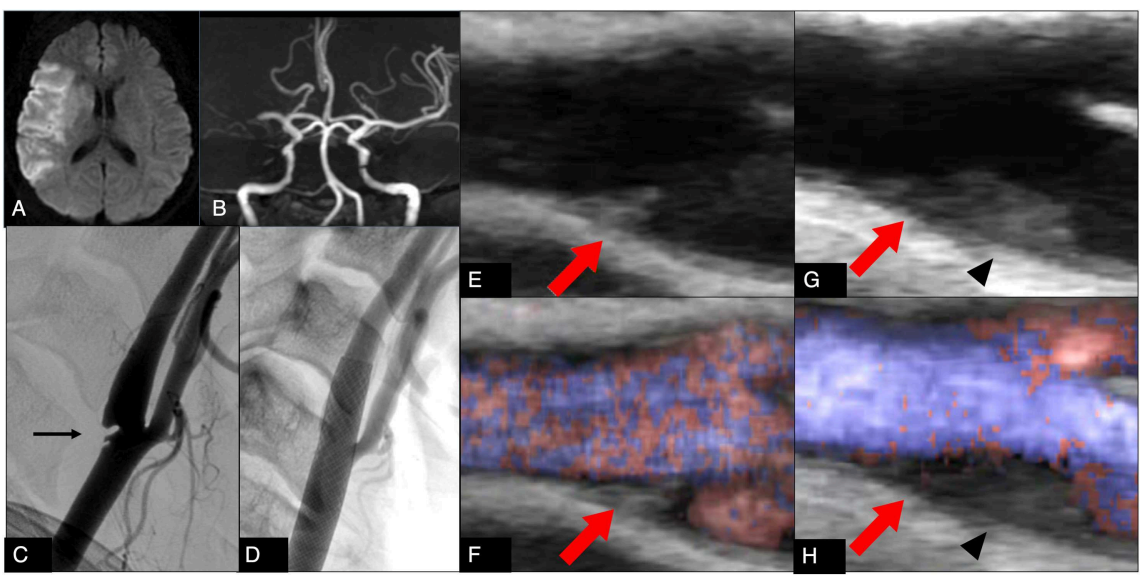

FIGURE 1 | (A) Diffusion-weighted magnetic resonance imaging on admission shows a hyperintense area in the territory of the right middle cerebral artery. **(B)** Magnetic resonance angiography on admission shows right middle cerebral artery occlusion. **(C)** Digital subtraction angiography on admission shows a carotid web (arrow) at the origin of the right internal carotid artery. **(D)** Digital subtraction angiography after carotid artery stenting on the 32nd day shows disappearance of the web protruding into the carotid artery lumen. **(E,F)** Ultrasonography on the 2nd day shows a carotid web (arrow) at the origin of the right internal carotid artery (**E**: brightness mode, **F**: B-flow mode). **(G,H)** Ultrasonography on the 19th day shows a carotid web (arrow) and new thrombus formation on the distal portion of the carotid web (arrowhead) (**G**: brightness mode, **H**: B-flow mode).

carotid web (**Figures 1G,H**). We restarted anticoagulant therapy (continuous infusion of 500 U/h heparin, followed by 4 mg/day warfarin). On the 29th day, follow-up carotid ultrasound scanning showed a decrease of the thrombus on the distal portion of the carotid web. There was no new ischemic lesion on MRI. After obtaining informed consent from the proband, genetic analysis was done. The genetic analysis identified a single-base substitution, c.631G>A; p.Arg211Trp (protein C Tochigi), demonstrating a heterozygosity of the proband for congenital protein C deficiency (2). Neither persistent nor paroxysmal AF was detected by 24-h Holter monitoring. Transesophageal echocardiography showed no embolic source in her heart or aorta. Ultrasonography of the lower extremities showed no deep vein thrombosis. Therefore, we speculated protein C deficiency to have promoted thrombus formation on the web when anticoagulation therapy was stopped, and we initially proposed carotid endarterectomy to eliminate the web, providing a scaffold for thrombus formation. However, because the patient refused surgery, we changed our plan to perform carotid artery stenting (CAS) to prevent thrombus formation by regulating turbulent flow by suppressing the protruding web into the carotid wall.

She underwent CAS on the 32nd day using a closed stent under proximal and distal protection, with successful results and no complications (**Figure 1D**). She was discharged home with dexterity movement disorder in the right hand on the 50th day and had no recurrence of stroke while under anticoagulant therapy with warfarin during the 2-year follow-up period.

We have obtained a written informed consent from the patient. This study was approved by the Kokura Memorial Hospital Human Genome and Gene Analysis Research Ethics Committee.

DISCUSSION

We report a case of thromboembolic cerebral infarction in which protein C deficiency was thought to accelerate thrombus formation on the distal portion of the carotid web. No recurrence of stroke was noted for 2 years after carotid stenting with anticoagulant therapy with warfarin.

Carotid web is a shelf-like intraluminal projection within the internal carotid artery bulb and is referred to as a type of fibromuscular dysplasia (3). It has been reported that the prevalence of carotid web ranges from 9.4 to 37% in cases of cryptogenic stroke of anterior circulation (4, 5). Moreover, carotid web is recognized as a cause of recurring ischemic stroke (6), the etiology of which is thought to be the web projecting into the carotid arterial lumen, thus providing a scaffold for thrombus formation and creating turbulent flow or flow stasis promoting blood coagulation and resulting in thrombus formation (7). This thrombus on the web, when scattered distally by the blood flow, causes thromboembolic stroke. Our case showed an immediate increase in thrombus formation on the web when anticoagulant therapy was interrupted.

Protein C serves as the main anticoagulant factor responsible for the negative feedback in the blood coagulation cascade. Protein C-Tochigi houses one of the main gene mutations (p.Arg211Trp) in the Japanese population and does not function as an anticoagulant factor, thus resulting in protein C deficiency (2). Protein C deficiency induces a hypercoagulable state and mainly causes venous thromboembolism(VTE), including deep vein thrombosis, pulmonary thromboembolism, and mesenteric vein thrombosis (8). It has been reported that, in protein C deficiency, the incidence of VTE is frequent but that of arterial thrombosis is relatively rare (8–10). However, protein C deficiency contributes to the younger onset of arterial thromboembolism (11, 12). Moreover, in recent years, it has been reported that protein C deficiency has a strong association with arterial thromboembolism, especially in the presence of additional vascular risks such as diabetes mellitus (13).

Our case was thought to be associated with an extremely high risk of recurrent stroke because of both carotid web, which is a high-risk lesion related to recurrent stroke, and protein C deficiency, which causes a hypercoagulable state. Here, we confirmed rapidly increasing thrombus formation at the distal portion of the carotid web using carotid ultrasound despite the provision of antiplatelet therapy after discontinuation of anticoagulant therapy. If the additional ultrasonic examination had not been conducted, we would not have restarted anticoagulant or considered surgical therapy, because we would not have noticed the increase in thrombus formation. As a result, there would have been a high possibility of recurrence of cerebral infarction.

The optimal management strategy to prevent ischemic stroke in patients with both carotid web and protein C deficiency remains unknown. As for carotid web, Joux et al. reported that the recurrence rate of ischemic stroke was 0% in patients in whom it had been surgically removed and 30% in patients receiving alternative medical therapy (14). Haussen et al. performed sixteen CAS for symptomatic carotid web and observed no recurrent stroke for the median follow up of 4 months. (6) As in our case, recurrence of stroke has not been observed for 2 years after CAS under anticoagulant therapy with warfarin, and carotid stenting and anticoagulant therapy may be an effective treatment option to prevent ischemic stroke in patients with both carotid web and protein C deficiency. However, careful follow-up is needed because protein C deficiency can cause in-stent thrombus formation after carotid stenting (15). To our knowledge, this is the first report in which thrombus formation on the carotid web following cessation of anticoagulant therapy was confirmed by carotid ultrasonography in a patient with protein C deficiency. In the case of acute ischemic stroke with carotid web, especially when congenital coagulopathy such as protein C deficiency is suspected, careful follow-up with ultrasound imaging should be conducted. A limitation of this report is that only one case was reported, and the follow-up period was relatively short. Long-term follow-up should be performed to confirm the efficacy of our treatment strategy for prevention of lifetime recurrent stroke. Further studies should be accumulated to confirm the optimal treatment for cases with carotid web and congenital coagulopathy.

ETHICS STATEMENT

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

SW, SM, and INak contributed to the conception and design of this paper. SW, SM, INak, AI, TH, and INag contributed to drafting the text and preparing the figures. MM and EM contributed to sequence analysis of the deoxyribonucleic acid sample of this case.

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REFERENCES

1. Kim SJ, Nogueira RG, Haussen DC. Current understanding and gaps in research of carotid webs in ischemic strokes: a review. *JAMA Neurol.* (2018) 76:355–61. doi: 10.1001/jamaneurol.2018.3366
2. Matsuda M, Sugo T, Sakata Y, Murayama H, Mimuro J, Tanabe S, et al. A thrombotic state due to an abnormal protein C. *N Engl J Med*. (1988) 319:1265–8. doi: 10.1056/NEJM198811103191907
3. Sajedi PI, Gonzalez JN, Cronin CA, Kouo T, Steven A, Zhuo J, et al. Carotid bulb webs as a cause of "cryptogenic" ischemic stroke. *Am J Neuroradiol*. (2017) 38:1399–404. doi: 10.3174/ajnr.A5208
4. Coutinho JM, Derkatch S, Potvin AR, Tomlinson G, Casaubon LK, Silver FL, et al. Carotid artery web and ischemic stroke: a case-control study. *Neurology*. (2017) 88:65–69. doi: 10.1212/WNL.0000000000003464
5. Joux J, Boulanger M, Jeannin S, et al. Association between carotid bulb diaphragm and ischemic stroke in young afro-caribbean patients: a population-based case-control study. *Stroke*. (2016) 47:2641–4. doi: 10.1161/STROKEAHA.116.013918
6. Haussen DC, Grossberg JA, Bouslama M, Pradilla G, Belagaje S, Bianchi N, et al. Carotid web (Intimal Fibromuscular Dysplasia) has high stroke recurrence risk and is amenable to stenting. *Stroke*. (2017) 48:3134–7. doi: 10.1161/STROKEAHA.117.019020
7. Choi PM, Singh D, Trivedi A, Qazi E, George D, Wong J, et al. Carotid webs and recurrent ischemic strokes in the Era of CT angiography. *AJNR Am J Neuroradiol*. (2015) 36:2134–9. doi: 10.3174/ajnr.A4431
8. Allaart CF, Poort SR, Rosendaal FR, Reitsma PH, Bertina RM, Briët E, et al. Increased risk of venous thrombosis in carriers of hereditary protein C deficiency defect. *Lancet*. (1993) 341:134–8. doi: 10.1016/0140-6736(93)90003-Y
9. Pabinger I, Kyrle PA, Heistinger M, Eichinger S, Wittmann E, Lechner K, et al. The risk of thromboembolism in asymptomatic patients with protein C and protein S deficiency: a prospective cohort study. *Thromb Haemost.* (1994) 71:441–5. doi: 10.1055/s-0038-1642457
10. De Stefano V, Leone G, Micalizzi P, Teofili L, Falappa PG, Pollari G, et al. Arterial thrombosis as clinical manifestation of congenital protein C deficiency. *Ann Hematol*. (1991) 62:180–3. doi: 10.1007/BF017 03145
11. Sakata T, Kario K, Katayama Y, Matsuyama T, Kato H, Miyata T, et al. Analysis of 45 episodes of arterial occlusive disease in Japanese patients with congenital protein C deficiency. *Thromb Res.* (1999) 94:69–78. doi: 10.1016/S0049-3848(98)00194-7
12. Mahmoodi BK, Brouwer JL, Veeger NJ, van der Meer J. Hereditary deficiency of protein C or protein S confers increased risk of arterial thromboembolic events at a young age: results from a large family cohort study. *Circulation*. (2008) 118:1659–67. doi: 10.1161/CIRCULATIONAHA.108.780759
13. Mahmoodi BK, Veeger NJ, Middeldorp S, Lijfering WM, Brouwer J-LP, Berg JT, et al. Interaction of hereditary thrombophilia and traditional cardiovascular risk factors on the risk of arterial thromboembolism: pooled analysis of four family cohort studies. *Circ Cardiovasc Genet*. (2016) 9:79–85. doi: 10.1161/CIRCGENETICS.115.001211
14. Joux J, Chausson N, Jeannin S, Saint-Vil M, Mejdoubi M, Hennequin JL, et al. Carotid-bulb atypical fibromuscular dysplasia in young Afro-Caribbean patients with stroke. *Stroke*. (2014) 45:3711–3. doi: 10.1161/STROKEAHA.114.007313
15. Iwamoto Y, Kitano T, Matsubara S, Uno M, Yagita Y. In-stent thrombosis after carotid artery stenting in a patient with protein C deficiency. *Neurol Sci.* (2018) 39:2229–30. doi: 10.1007/s10072-018-3544-6

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Hemodynamic Significance of Middle Cerebral Artery Stenosis Associated With the Severity of Ipsilateral White Matter Changes

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Background: Previous studies conflicted in the association between intracranial atherosclerotic stenosis (ICAS) and the severity of white matter changes (WMC).

Aims: We aimed to investigate the relationships between the severity of luminal stenosis and the hemodynamic significance of middle cerebral artery (MCA) stenosis, and the severity of ipsilateral WMC.

Methods: In this cross-sectional study, patients with a recent ischemic stroke or transient ischemic attack and a 50–99% MCA-M1 stenosis in the Chinese Intracranial Atherosclerosis study cohort were analyzed. The post- to pre-stenotic signal intensity ratio (SIR) was obtained in time-of-flight MR angiography (MRA) to represent the hemodynamic significance of MCA-M1 stenosis, with a lower SIR indicating a hemodynamically more severe lesion. The severity of ipsilesional WMC was assessed by an age-related WMC (ARWMC) scale in T2-weighted fluid attenuated inversion recovery MR imaging. The relationships between the degree of MCA-M1 stenosis, SIR, and ipsilesional ARWMC scale were analyzed. The MCA-M1 lesion with a higher percentage of stenosis was chosen for analyses in patients with bilateral MCA-M1 stenoses.

Results: Among 180 subjects (mean age, 64 years), a lower SIR of MCA-M1 stenosis (Spearman correlation coefficient, −0.543; $p < 0.001$), but not the degree of stenosis ($p = 0.93$), was significantly linearly correlated with a higher ipsilateral ARWMC. Multivariate ordinal logistic regression identified older age (OR = 1.037; 95% CI, 1.008–1.066; $p = 0.011$) and lower SIR (OR = 0.010; 95% CI, 0.002–0.058; $p < 0.001$) as independent predictors for more severe ipsilateral WMC.

Conclusion: Patients with hemodynamically more severe ICAS are more likely to have more severe ipsilateral WMC. Longitudinal studies with sequential imaging exams may further reveal the impact of hemodynamic significance of ICAS on the development and progression of WMC.

Keywords: ischemic stroke, intracranial atherosclerosis, magnetic resonance angiography, imaging, white matter changes

INTRODUCTION

White matter changes (WMC) have been linked to cognitive dysfunction, gait impairment and falls, depression, and increased risk of future stroke (1–4), but the pathophysiology underlying development and progression of WMC are not fully elucidated. Previous studies showed conflicting results regarding whether extra- or intra-cranial atherosclerotic disease is associated with WMC, and which is more closely correlated with WMC (5–8).

We had previously proposed an index termed signal intensity ratio (SIR) to quantify the hemodynamic significance of intracranial atherosclerotic stenosis (ICAS), defined as the ratio of distal (post-stenotic) and proximal (pre-stenotic) signal intensities (SIs) obtained in the vessel lumen in time-of-flight magnetic resonance angiography (MRA), which has been recently validated against CT perfusion measures (9–12). In the present study, we aimed to investigate the associations of severity of luminal narrowing and hemodynamic significance of middle cerebral artery (MCA) stenosis by SIR, with the severity of ipsilateral WMC, in ischemic stroke or transient ischemic attack (TIA) patients with MCA stenosis. This may improve our understanding regarding how the presence of ICAS would act on presence or progression of WMC, and may provide therapeutic targets for prevention of the symptoms subsequent to severe WMC.

METHODS

Subjects

This was a cross-sectional study based on the Chinese Intracranial Atherosclerosis (CICAS) study (13). CICAS was a prospective, multicenter, hospital-based, cohort study carried out in 22 hospitals covering a wide geographic area throughout China, enrolling non-cardioembolic ischemic stroke or TIA patients (aged 18–80 years) admitted within 7 days of ictus (13). All patients underwent 1.5- or 3.0-T MR exams, including brain and vascular imaging sequences. Details of the inclusion and exclusion criteria, patient demographics, clinical features, and follow-up information have been described previously (13, 14). The study had been approved by Institutional Review Board at the participating hospitals. Each patient gave written informed consent.

Consecutive patients recruited to the CICAS study, who had 50–99% stenosis [Warfarin-Aspirin Symptomatic Intracranial Disease (WASID) criteria (15)] of the M1 segment of unilateral or bilateral MCA (MCA-M1) in time-of-flight MRA, were screened for the current study. Those with time-of-flight MRA and T2-weighted fluid-attenuated inversion recovery (T2-FLAIR) MR images of adequate quality were enrolled and analyzed in the present study. Patients were excluded if (1) there were no T1/T2-weighted images, time-of-flight MRA or T2-FLAIR images, or the image quality was insufficient for assessment of SIR and WMC; or (2) the MCA lesion was located adjacent to an arterial bifurcation or trifurcation, or a perforator, that was not suitable for the measurement of SIR on MRA. A flow chart of patient screening and selection for the current study is provided in **Figure 1**. Demographics and characteristics of the index ischemic stroke or TIA were collected.

Image Assessment

For each patient, we assessed the percentage of luminal stenosis [the WASID criteria (13, 15)] and the hemodynamic significance (by SIR) of MCA-M1 stenotic lesion in time-of-flight MRA using Phillips DICOM viewer 3.0 (Koninklijke Philips Electronics N.V.), and the severity of ipsilateral WMC by an age-related WMC (ARWMC) scale in axial T1/T2-weighted images and T2-FLAIR images using RadiAnt DICOM Viewer (Medixant). For patients with bilateral MCA-M1 stenoses, the side with a higher degree of stenosis was chosen for image assessment and subsequent analyses.

SIR was obtained in time-of-flight MRA to represent the hemodynamic significance of an MCA-M1 stenosis. The detailed methodology was described previously (10, 16). Briefly, it quantified the relative change in SIs across an ICAS lesion, adjusted by the background SI. Mean SI distal and proximal to MCA-M1 stenosis were measured on the maximum intensity projections (MIPs) showing the highest degree of stenosis of the lesion. In addition, mean background SI was calculated as the mean value of background SIs within the left and right halves of the anterior–posterior direction MIP, in areas adjacent to intracranial internal carotid arteries but free of vessel signals. SIR was then calculated as: SIR = (mean post-stenotic SI – mean background SI)/(mean pre-stenotic SI – mean background SI), with a lower SIR indicating a hemodynamically more severe lesion. The intra-rater (Pearson correlation coefficient, 0.975) and inter-rater reproducibilities (Pearson correlation coefficient, 0.847) of SIR measurement of ICAS lesions have been previously demonstrated (9, 16).

WMC was defined as ill-defined hyperintensities $\geq$5 mm on both T2-weighted and FLAIR imaging and isointensity on T1-weighted imaging (17, 18). The severity of WMCs was scaled in four regions in the supratentorial hemisphere ipsilateral to the index MCA-M1 stenosis, including the frontal area, the parieto-occipital area, the temporal area (scores 0–3 for each region, respectively, indicating no lesion, focal lesions, beginning confluence of lesions, and diffuse lesions), and the basal ganglia area (scores 0–3, respectively, indicating no lesion, one focal lesion, more than one focal lesion, confluent lesions) (17, 18). The ipsilesional ARWMC scale, ranging from 0 to 12, was calculated as a sum of WMC scores in the four areas, with a larger scale indicating more severe WMC. Two authors (HF and XL) independently evaluated unilateral, supratentorial ARWMC in 20 cases, for the assessment of inter-rater reproducibility of the ARWMC scale. The hemisphere for ARWMC assessment in each patient was determined before the assessment; the two investigators conducting ARWMC assessment were blinded to the data of sites and degrees of stenosis in ICAS lesions in each patient, which were centrally measured by CICAS investigators prior to this sub-study. Kappa test showed that the inter-rater reproducibility of ARWMC assessment was 0.79 for frontal lobe, 0.957 for parietal–occipital lobe, 0.773 for temporal lobe, and 1 for basal ganglia, indicating substantial agreement between the two observers.

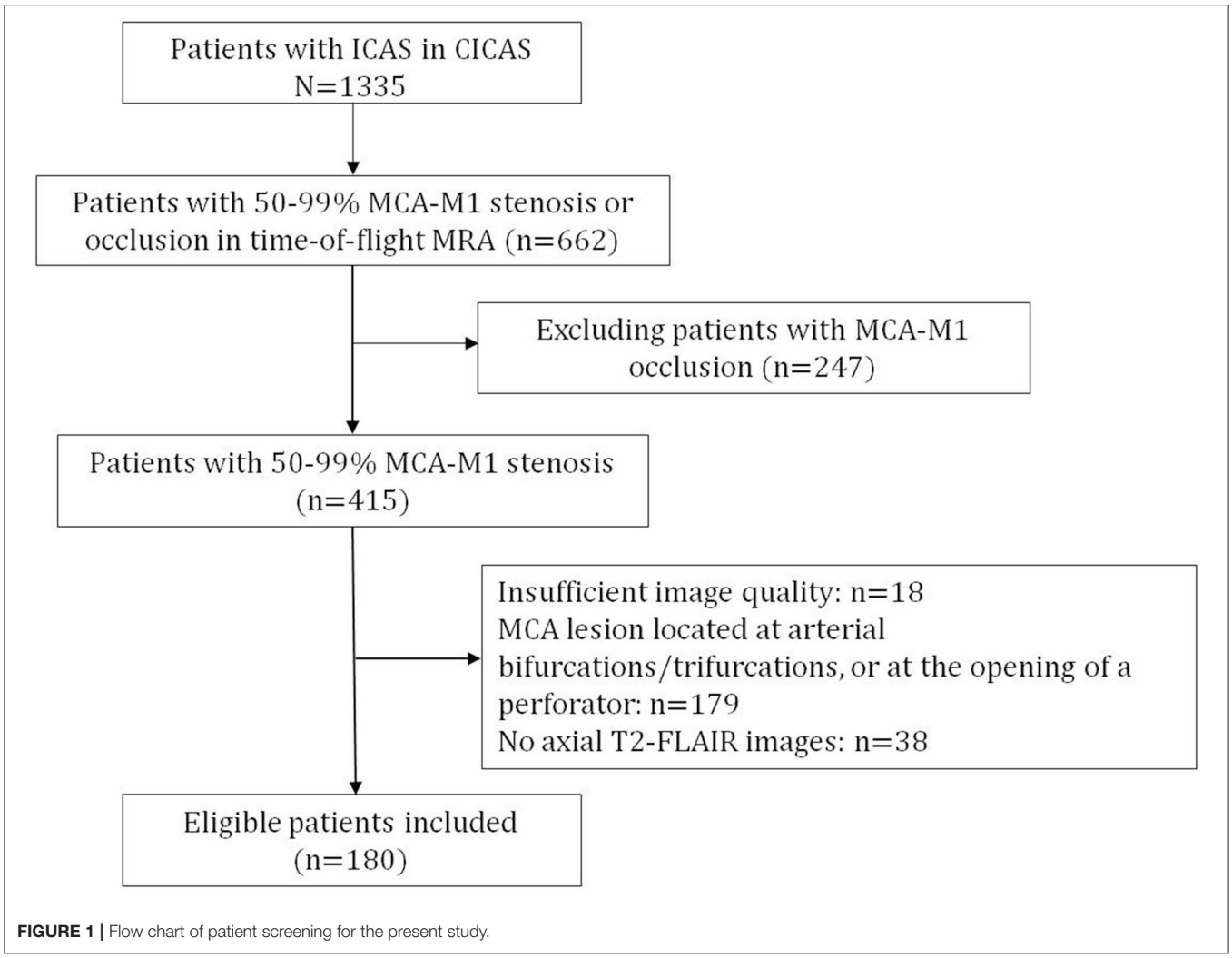

FIGURE 1 | Flow chart of patient screening for the present study.

Statistical Analysis

Inter-rater reproducibilities of unilateral ARWMC scales of the four regions of interest were assessed by the kappa statistic, with ARWMC as an ordinal variable. Chi-square tests were conducted for the correlations of the degree of MCA-M1 stenosis (50–69% vs. 70–99% stenosis) and SIR ($<$ or $\geq$ median) as dichotomized variables, with ipsilateral ARWMC scale as an ordinal variable in its quartiles. Spearman correlation coefficients and scatterplots were used to reveal linear correlations between the degree of MCA-M1 stenosis, SIR, and the ipsilateral ARWMC scale, all as continuous variables.

In addition to the degree of MCA-M1 stenosis and its hemodynamic significance by SIR, the differences in the following factors among patients with different supratentorial ARWMC scales by quartiles were also assessed in univariate analyses, including patient demographics, characteristics of the index stroke or TIA, and vascular risk factors, using chi-square tests for categorical variables and ANOVA for continuous variables. Factors with two-sided $p < 0.1$ in univariate analyses were further analyzed in a multivariate, ordinal logistic regression model, to identify factors independently associated with the ARWMC scale in quartiles. Two-sided $p < 0.05$ were considered statistically significant. All statistical analyses were performed using SAS software version 9.1 (SAS Institute Inc, Cary, NC).

RESULTS

Patient Characteristics

Of the 1,335 patients with ICAS recruited to the CICAS study (13), 415 had 50–99% stenosis [WASID criteria (15)] of unilateral or bilateral MCA-M1(s) in time-of-flight MRA. After excluding 18 patients with insufficient image quality, 179 patients with stenosis located adjacent to an arterial bifurcation or trifurcation, or a perforator, and 38 patients without axial T2-FLAIR images, 180 patients were analyzed in the current study (flow chart shown in **Figure 1**).

Among the 180 patients, 116 were men and 64 were women, with a mean age of 63.7 $\pm$ 11.6 years. Premorbid modified Rankin scale was 0–2 in all patients. The median interval between symptom onset and admission was 2 days [interquartile

TABLE 1 | Univariate comparisons of clinical and imaging characteristics* in patients grouped by ARWMC scales in quartiles.

Variables	Overall ($n = 180$)	Quartile 1 ($n = 19$)	Quartile 2 ($n = 63$)	Quartile 3 ($n = 47$)	Quartile 4 ($n = 51$)	*P*-value
Age	63.7 ± 11.6	55.3 ± 10.7	61.0 ± 12.6	64.7 ± 10.8	69.4 ± 8.2	<0.001
Sex (male)	116 (64.4)	16 (84.2)	40 (63.5)	29 (61.7)	31 (60.8)	0.294
Current smoker	51 (28.3)	8 (42.1)	23 (36.5)	12 (25.5)	8 (15.7)	0.039
History of hypertension	126 (71.2)	13 (72.2)	39 (61.9)	34 (73.9)	40 (80)	0.194
History of diabetes mellitus	55 (31.1)	6 (31.6)	20 (31.8)	16 (36.4)	13 (25.5)	0.721
History of dyslipidemia	34 (23.1)	5 (35.7)	11 (21.2)	9 (22.5)	9 (22)	0.705
History of coronary heart disease	18 (11.0)	1 (6.3)	5 (8.6)	6 (14)	6 (12.8)	0.658
Prior ischemic stroke/TIA	48 (26.7)	2 (10.5)	19 (30.2)	11 (23.4)	16 (31.4)	0.289
NIHSS at admission	4 (2–7)	4 (1–6)	3 (2–7)	4 (1–8)	4 (1–7)	0.95
Premorbid mRS	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–1)	0.059
Interval from onset to admission, days	2 (1–4)	3 (1–6)	2 (1–4)	2 (1–3)	1 (0–4)	0.617
Interval from onset to MRI, days	5 (2–8)	5 (1–8)	4 (2–7)	4 (2–8)	5 (3–8)	0.858
Fasting blood glucose, mmol/L	5.52 (4.7–6.9)	5.8 (4.7–7.9)	5.5 (4.8–6.7)	5.8 (4.7–8)	5.3 (4.6–6.6)	0.625
Total cholesterol, mmol/L	4.82 (4.1–5.5)	5 (4–5.4)	5 (4.2–5.6)	4.9 (4.2–5.6)	4.3 (3.9–5.1)	0.062
Triglycerides, mmol/L	1.5 (1.1–2.2)	2.4 (1.4–3.2)	1.5 (1.1–2.2)	1.4 (1.1–2.4)	1.3 (1–1.8)	0.014
High-density lipoprotein, mmol/L	1.1 (0.9–1.3)	1 (0.9–1.2)	1.1 (0.9–1.3)	1. (0.9–1.3)	1.1 (1–1.3)	0.39
Low-density lipoprotein, mmol/L	2.99 (2.38–3.67)	3.2 (2.4–3.4)	3 (2.5–3.6)	3.1 (2.6–4)	2.6 (2.1–3.4)	0.116
70–99% luminal stenosis of the index ICAS lesion	52 (28.9%)	2 (10.5%)	21 (33.3%)	19 (40.4%)	10 (19.6%)	0.031
SIR < median (0.89)	89 (49.4%)	2 (10.5%)	25 (39.7%)	18 (38.3%)	44 (86.3%)	<0.001

TIA, transient ischemic attack; NIHSS, national institutes of health stroke scale; mRS, modified Rankin scale; MRI, magnetic resonance imaging; ICAS, intracranial atherosclerotic stenosis; SIR, signal intensity ratio.

**Variables are presented in means ± standard deviations, medians (interquartile ranges), or numbers (percentages).*

range (IQR), 1–4]. The median National Institutes of Health Stroke Scale (NIHSS) upon admission was 4 (IQR 2–7). Among the patients, 126 (71.2%) had a history of hypertension, 34 (23.1%) had dyslipidemia, and 55 (31.1%) had diabetes mellitus (**Table 1**). No patient had a history of intracranial hemorrhage or atrial fibrillation.

Characteristics of the Index MCA-M1 Lesions

The median interval between symptom onset and MRI examination was 2 days (IQR 1–4). Sixty-seven (37.2%) patients underwent 1.5-T MR exams and 113 (62.8%) underwent 3.0-T MR exams. Fifty-two (28.9%) and 128 (71.1%) patients had severe (70–99%) and moderate (50–69%) MCA-M1 stenosis, respectively. The mean SIR of all index MCA-M1 lesions was 0.86 ± 0.21, and the median SIR was 0.89 (IQR 0.73–0.97). The median ipsilesional, supratentorial ARWMC scale was 2 (IQR 1–2).

Correlations of Degree of MCA-M1 Stenosis and SIR With Ipsilateral ARWMC

As shown in **Table 1**, the proportions of cases with severe (70–99%) MCA-M1 stenosis ($p = 0.031$) and a SIR <median ($p < 0.001$) were significantly higher in those with higher ARWMC scale in univariate analyses. However, the severity of MCA luminal stenosis was not significantly, linearly related to ipsilesional ARWMC scale (Spearman correlation coefficient, 0.007; $p = 0.93$; **Figure 2**).

The proportions of patients with SIR <median were significantly higher in those with a higher ARWMC scale ($p < 0.001$), which was 86.3% in those with an ARWMC in the highest quartile (**Table 1**). SIR and the ipsilateral ARWMC scale were significantly, linearly, and negatively correlated (Spearman correlation coefficient, −0.543; $p < 0.001$; **Figure 2**), which means that lower SIR (more hemodynamically significant) of MCA-M1 stenosis was associated with more severe ipsilesional WMC. **Figure 3** shows the MRA and FLAIR images of two cases, one with a high SIR and a low ARWMC scale, and the other with a low SIR and a high ARWMC scale.

Independent Predictors of Ipsilesional ARWMC

In addition to the severity of MCA-M1 stenosis and the SIR, other factors differed ($p < 0.1$) between patients with different ARWMC scales by quartiles in univariate analyses, including age ($p < 0.001$), current smoker ($p = 0.039$), premorbid modified Rankin Scale ($p = 0.059$), and total cholesterol ($p = 0.062$) and triglycerides ($p = 0.014$) levels (**Table 1**). These factors were included in multivariate, ordinal logistic regression analysis, which revealed that older age (OR = 1.037, 95% CI 1.008–1.066; $p = 0.011$) and lower SIR (OR = 0.010, 95% CI 0.002–0.058; <0.001), but not the degree of MCA stenosis (OR = 0.725, 95% CI 0.374–1.403; $p = 0.340$), were significantly, independently associated with a higher ipsilateral ARWMC scale (**Table 2**).

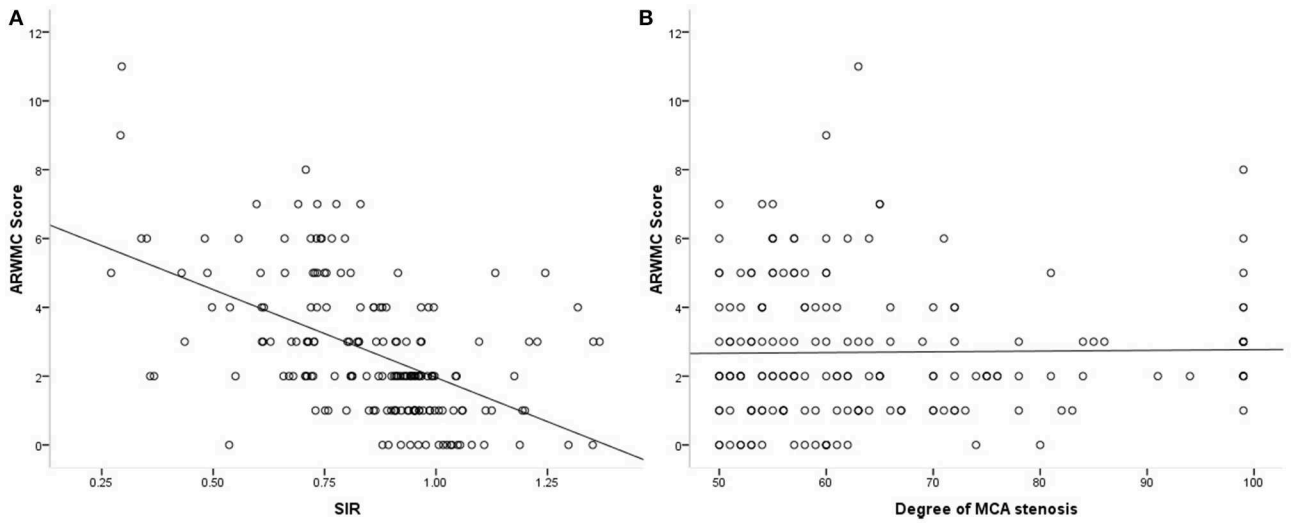

FIGURE 2 | Scatterplots showing the correlations of SIR and degree of MCA stenosis (in percentage) with ipsilateral ARWMC scale.

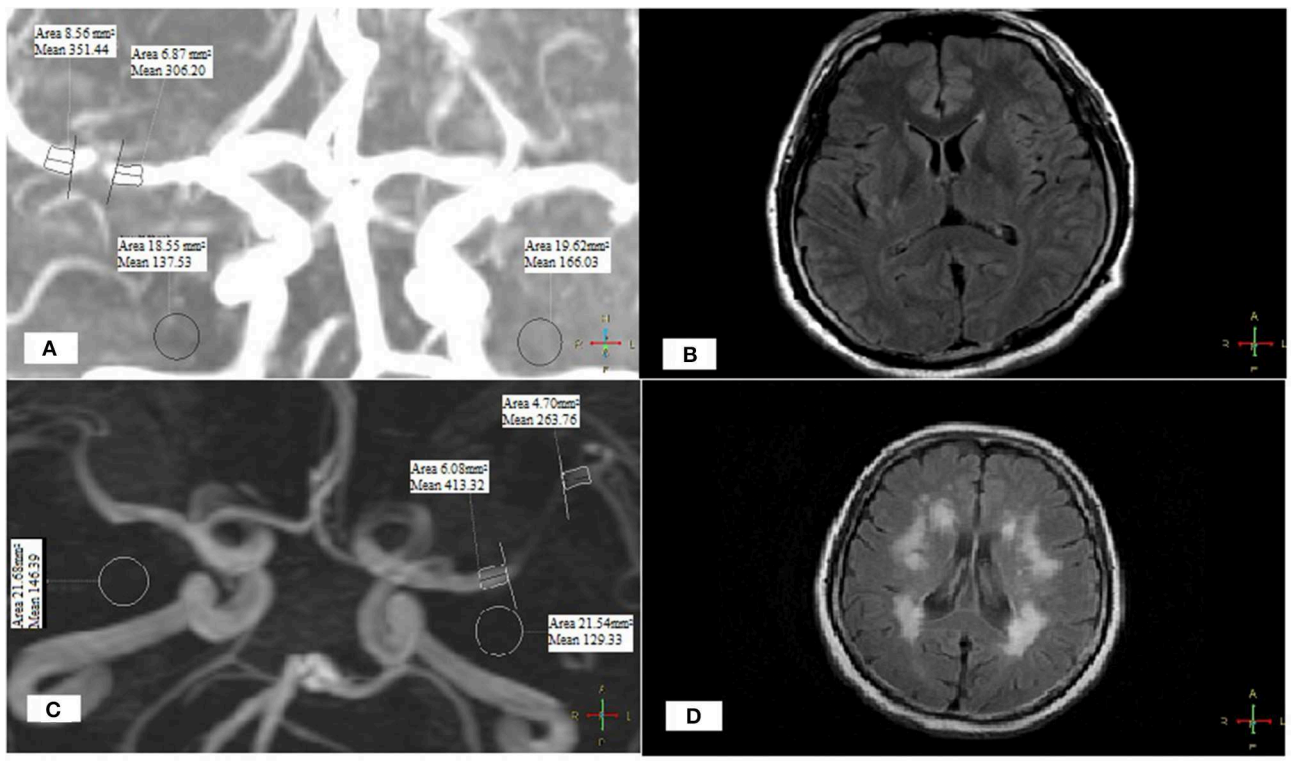

FIGURE 3 | Two cases with different SIR and ARWMC scales.

DISCUSSION

In the present study, we investigated whether the severity of luminal stenosis and the hemodynamic significance of MCA stenosis (as quantified by SIR in time-of-flight MRA) were associated with the severity of ipsilateral WMC (as assessed by the ARWMC scale in MRI). The results showed that SIR was significantly, linearly, and negatively correlated with ipsilateral, supratentorial ARWMC scale, and multivariate analyses further corroborated the association of lower SIR with higher ARWMC

TABLE 2 | Multivariate ordinal logistic regression analysis for independent predictors of the ipsilateral ARWMC scale in quartiles.

Variables	Odds ratio	95% confidence interval	*P*-value
Age	1.037	1.008–1.066	0.011
Current smoker	0.578	0.296–1.128	0.108
Premorbid mRS	1.316	0.738–2.347	0.351
Total cholesterol, mmol/L	0.870	0.689–1.100	0.245
Triglycerides, mmol/L	0.927	0.761–1.130	0.456
Severity of stenosis (70–99%)	0.725	0.374–1.403	0.340
SIR < median (0.89)	0.010	0.002–0.058	<0.001

mRS, modified Rankin scale; SIR, signal intensity ratio.

scale, independent of other confounding factors. The findings indicated that patients with hemodynamically more severe MCA stenosis were more likely to have more severe WMC in the ipsilateral hemisphere. However, the degree of luminal narrowing in MCA stenosis was not significant associated with the severity of ipsilateral WMC in this study. Of note, older age, as an established risk factor of WMC, was also found as an independent predictor of higher ARWMC scale in the current study.

For patients with ICAS, SIR could be easily obtained with the wide application of time-of-flight MRA in clinical practice. Based on the contrast mechanism (flow-related enhancement) of time-of-flight MRA, a lower SIR of an ICAS lesion could, to some extent, reflect downstream hemodynamic impairment caused by the stenotic lesion, which has been correlated with prolonged cerebral perfusion (12), larger acute infarct volume (10), and higher risk of recurrent stroke (11), in patients with a recent ischemic stroke or TIA in previous studies. With previously demonstrated intra- and inter-rater reproducibilities (16), the index SIR could be considered as a simple and effective marker to gauge the hemodynamic significance of ICAS, to be used in relevant research areas. On the other hand, for inter-rater reproducibility of ARWMC assessment in the current study, the kappa test result was 0.79 for frontal lobe, 0.957 for parietal–occipital lobe, 0.773 for temporal lobe, and 1 for basal ganglia, indicating substantial agreement between two observers, which had been demonstrated in previous studies (18). Therefore, this ARWMC assessment scale is operational in clinical practice for assessment of severity of WMC (18).

The roles of extracranial and intracranial large artery atherosclerosis in the development and progression of WMC are uncertain. As mentioned earlier, previous studies showed conflicting results concerning the correlations of presence/extent of WMC with different stages of carotid atherosclerosis (increased intima-media thickness, non-stenotic and stenotic carotid plaques) and intracranial atherosclerosis (mostly intracranial stenosis) (5–8). Some of the studies reported significant correlations while others not. The current study indicated that it might be the hemodynamic impact of ICAS lesions and the possibly subsequent hypoperfusion, but not the degree of luminal stenosis, that governs the severity of ipsilateral WMC. Such inferences are supported by significant correlations between larger white matter lesion volumes and reduced total cerebral blood flow in previous large cohort studies (19–21). Compared with the cortex, the white matter might be more vulnerable to hemodynamic insufficiency, which is supplied by single source subependymal arteries with scarce anastomoses (22–24). This may partly explain the conflicting results in previous studies: the diverse hemodynamic severities of extra- and intra-cranial atherosclerotic lesions in those studies might have interfered with the analyses of their correlations with WMC.

The current findings in the important role of hemodynamic impact of ICAS in determining the severity of WMC may have clinical implications in the management of ICAS patients. As mentioned above, WMC have been related to increased risks of cognitive impairment, dementia, and stroke (1–4). Thus, in this regard, patients with ICAS may benefit from hemodynamic restoring treatment methods, such as angioplasty/stenting, and other treatment methods such as external counter pulsation that can enhance cerebral perfusion (25, 26). However, these inferences need further investigations in future prospective studies.

This study had several limitations. First, patients who underwent 1.5- and 3.0-T MR exams were both included in the present study. The relatively inferior quality of 1.5-T MR images might, to some extent, interfere with the evaluation of SIR and ARWMC, and the severity of MCA luminal stenosis may be overestimated with time-of-flight MRA (27). Second, the influence of isolated or concurrent extracranial stenosis was not considered in the present study, which may be a potential confounder in the correlations between ICAS and WMC. Third, treatment strategies such as medications of the patients receiving may interfere with the associations between SIR and ARWMC, which were not adjusted in the multivariate ordinal logistic regression analyses. Last but not least, the flow-dependent artery visualization mechanism of time-of-flight MRA prohibits accurate calculation of SIR when an ICAS lesion is located adjacent to arterial bifurcations, trifurcations, or perforators, in which case the SIs may alter with or without the presence of ICAS. A considerable number of patients were excluded from the current study due to this reason, which may arouse selection bias.

In conclusion, older age and lower SIR, but not the percentage of luminal stenosis, were independent predictors of higher ipsilateral ARWMC in stroke or TIA patients with MCA-M1 stenosis. In other words, in patients with atherosclerotic MCA stenosis, the hemodynamic impact of the stenotic lesion, rather than the severity of luminal narrowing, may partly determine the severity of ipsilateral WMC. However, it is unclear how the hemodynamic significance of ICAS affects the development and progression of white matter changes based on this cross-sectional study. Further longitudinal studies with sequential imaging exams are warranted.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

All authors participated in the interpretation of study results and in the drafting, critical revision, and approval of the final version of the article. HF and XL was responsible for study design, data analyses, and drafting and revising the article. HF was responsible for acquisition of data, data analyses, and drafting the article. YPu, XZo, BS, YS, TL, CW, XZh, YiW, and YoW were involved in revising the article for important intellectual content. YPa conducted the statistical analysis. LL and YX was responsible for study concept or design, technical, material support, administrative, and supervision.

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REFERENCES

1. Debette S, Markus HS. The clinical importance of white matter hyperintensities on brain magnetic resonance imaging: systematic review and meta-analysis. *BMJ.* (2010) 341:c3666. doi: 10.1136/bmj.c3666
2. de Groot JC, de Leeuw FE, Oudkerk M, van Gijn J, Hofman A, Jolles J, et al. Cerebral white matter lesions and cognitive function: the rotterdam scan study. *Ann Neurol.* (2000) 47:145–51. doi: 10.1002/1531-8249(200002)47:2<145::aid-ana3>3.3.co;2-p
3. Thomas AJ, Kalaria RN, O'Brien JT. Depression and vascular disease: what is the relationship? *J Affect Disord.* (2004) 79:81–95. doi: 10.1016/S0165-0327(02)00349-X
4. Kuller LH, Longstreth WT Jr, Arnold AM, Bernick C, Bryan RN, Beauchamp NJ Jr, et al. White matter hyperintensity on cranial magnetic resonance imaging: a predictor of stroke. *Stroke J Cereb Circul.* (2004) 35:1821–5. doi: 10.1161/01.STR.0000132193.35955.69
5. Bots ML, van Swieten JC, Breteler MM, de Jong PT, van Gijn J, Hofman A, et al. Cerebral white matter lesions and atherosclerosis in the rotterdam study. *Lancet.* (1993) 341:1232–7.
6. Pico F, Dufouil C, Levy C, Besancon V, de Kersaint-Gilly A, Bonithon-Kopp C, et al. Longitudinal study of carotid atherosclerosis and white matter hyperintensities: the EVA-MRI cohort. *Cerebrovasc Dis.* (2002) 14:109–15. doi: 10.1159/000064741
7. Chutinet A, Biffi A, Kanakis A, Fitzpatrick KM, Furie KL, Rost NS. Severity of leukoaraiosis in large vessel atherosclerotic disease. *AJNR Am J Neuroradiol.* (2012) 33:1591–5. doi: 10.3174/ajnr.A3015
8. Park JH, Kwon HM, Lee J, Kim DS, Ovbiagele B. Association of intracranial atherosclerotic stenosis with severity of white matter hyperintensities. *Eur J Neurol.* (2015) 22:44–52.e2-3. doi: 10.1111/ene.12431
9. Leng X, Wong LKS, Soo Y, Leung T, Zou X, Wang Y, et al. Signal intensity ratio as a novel measure of hemodynamic significance for intracranial atherosclerosis. *Int J Stroke.* (2013) 8:E46. doi: 10.1111/ijs.12080
10. Leng X, Wong KS, Soo Y, Leung T, Zou X, Wang Y, et al. Magnetic resonance angiography signal intensity as a marker of hemodynamic impairment in intracranial arterial stenosis. *PLoS ONE.* (2013) 8:e80124. doi: 10.1371/journal.pone.0080124
11. Liebeskind DS, Kosinski AS, Lynn MJ, Scalzo F, Fong AK, Fariborz P, et al. Noninvasive fractional flow on mra predicts stroke risk of intracranial stenosis. *J Neuroimaging.* (2015) 25:87–91. doi: 10.1111/jon.12101
12. Lan L, Leng X, Abrigo J, Fang H, Ip VH, Soo YO, et al. Diminished signal intensities distal to intracranial arterial stenosis on time-of-flight MR angiography might indicate delayed cerebral perfusion. *Cerebrovasc Dis.* (2016) 42:232–9. doi: 10.1159/000445842
13. Wang Y, Zhao X, Liu L, Soo YO, Pu Y, Pan Y, et al. Prevalence and outcomes of symptomatic intracranial large artery stenoses and occlusions in China: the Chinese intracranial atherosclerosis (CICAS) study. *Stroke J Cereb Circul.* (2014) 45:663–9. doi: 10.1161/strokeaha.113.003508
14. Pu Y, Liu L, Wang Y, Zou X, Pan Y, Soo Y, et al. Geographic and sex difference in the distribution of intracranial atherosclerosis in China. *Stroke.* (2013) 44:2109–14. doi: 10.1161/strokeaha.113.001522
15. Samuels OB, Joseph GJ, Lynn MJ, Smith HA, Chimowitz MZ. A standardized method for measuring intracranial1 arterial stenosis. *Am J Neuroradiol.* (2000) 21:643–6.
16. Leng X, Ip H, Soo Y, Leung T, Liu L, Feldmann E. Interobserver reproducibility of signal intensity ratio on magnetic resonance angiography for hemodynamic impact of intracranial atherosclerosis. *J Stroke Cerebrovasc Dis.* (2013) 22:e615– e9. doi: 10.1016/j.jstrokecerebrovasdis.2013.07.036
17. Wahlund LO, Barkhof F, Fazekas F, Bronge L, Augustin M, Sjogren M, et al. A new rating scale for age-related white matter changes applicable to MRI and CT. *Stroke.* (2001) 32:1318–22. doi: 10.1161/01.str.32.6.1318
18. Xiong Y, Yang J, Wong A, Wong CH, Chan SS, Li HH, et al. Operational definitions improve reliability of the age-related white matter changes scale. *Eur J Neurol.* (2011) 18:744–9. doi: 10.1111/j.1468-1331.2010.03272.x
19. Appelman AP, van der Graaf Y, Vincken KL, Tiehuis AM, Witkamp TD, Mali WP, et al. Total cerebral blood flow, white matter lesions and brain atrophy: the SMART-MR study. *J Cereb Blood Flow Metab.* (2008) 28:633–9. doi: 10.1038/sj.jcbfm.9600563
20. Vernooij MW, van der Lugt A, Ikram MA, Wielopolski PA, Vrooman HA, Hofman A, et al. Total cerebral blood flow and total brain perfusion in the general population: the rotterdam scan study. *J Cereb Blood Flow Metab.* (2008) 28:412–9. doi: 10.1038/sj.jcbfm.9600526
21. Appelman AP, Exalto LG, van der Graaf Y, Biessels GJ, Mali WP, Geerlings MI. White matter lesions and brain atrophy: more than shared risk factors? A systematic review. *Cerebrovasc Dis.* (2009) 28:227–42. doi: 10.1159/000226774
22. Moody DM, Bell MA, Challa VR. Features of the cerebral vascular pattern that predict vulnerability to perfusion or oxygenation deficiency: an anatomic study. *AJNR Am J Neuroradiol.* (1990) 11:431–9.
23. Mayer PL, Kier EL. The controversy of the periventricular white matter circulation: a review of the anatomic literature. *AJNR Am J Neuroradiol.* (1991) 12:223–8.
24. Pantoni L. Pathophysiology of age-related cerebral white matter changes. *Cerebrovasc Dis.* (2002) 13:7–10. doi: 10.1159/000049143
25. Lin W, Xiong L, Han J, Leung TW, Soo YO, Chen X, et al. External counterpulsation augments blood pressure and cerebral flow velocities in

ischemic stroke patients with cerebral intracranial large artery occlusive disease. *Stroke.* (2012) 43:3007–11. doi: 10.1161/STROKEAHA.112.659144
26. Xiong L, Lin W, Han J, Chen X, Leung T, Soo Y, et al. Enhancing cerebral perfusion with external counterpulsation after ischaemic stroke: how long does it last? *J Neurol Neurosurg Psychiatry.* (2016) 87:531–6. doi: 10.1136/jnnp-2014-309842
27. Feldmann E, Wilterdink JL, Kosinski A, Lynn M, Chimowitz MI, Sarafin J, et al. The stroke outcomes and neuroimaging of intracranial atherosclerosis (SONIA) trial. *Neurology.* (2007) 68:2099–106. doi: 10.1212/01.wnl.0000261488.05906.c1

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Neoplastic and Non-neoplastic Acute Intracerebral Hemorrhage in CT Brain Scans: Machine Learning-Based Prediction Using Radiomic Image Features

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Background: Early differentiation of neoplastic and non-neoplastic intracerebral hemorrhage (ICH) can be difficult in initial radiological evaluation, especially for extensive ICHs. The aim of this study was to evaluate the potential of a machine learning-based prediction of etiology for acute ICHs based on quantitative radiomic image features extracted from initial non-contrast-enhanced computed tomography (NECT) brain scans.

Methods: The analysis included NECT brain scans from 77 patients with acute ICH ($n = 50$ non-neoplastic, $n = 27$ neoplastic). Radiomic features including shape, histogram, and texture markers were extracted from non-, wavelet-, and log-sigma-filtered images using regions of interest of ICH and perihematomal edema (PHE). Six thousand and ninety quantitative predictors were evaluated utilizing random forest algorithms with five-fold model-external cross-validation. Model stability was assessed through comparative analysis of 10 randomly drawn cross-validation sets. Classifier performance was compared with predictions of two radiologists employing the Matthews correlation coefficient (MCC).

Results: The receiver operating characteristic (ROC) area under the curve (AUC) of the test sets for predicting neoplastic vs. non-neoplastic ICHs was 0.89 [95% CI (0.70; 0.99); $P < 0.001$], and specificities and sensitivities reached >80%. Compared to the radiologists' predictions, the machine learning algorithm yielded equal or superior results for all evaluated metrics. The MCC of the proposed algorithm at its optimal operating point (0.69) was significantly higher than the MCC of the radiologist readers (0.54); $P = 0.01$.

Conclusion: Evaluating quantitative features of acute NECT images in a machine learning algorithm provided high discriminatory power in predicting non-neoplastic vs. neoplastic ICHs. Utilized in the clinical routine, the proposed approach could improve patient care at low risk and costs.

Keywords: intracerebral hemorrhage, neoplastic hemorrhage, radiomics, machine learning, artificial intelligence

INTRODUCTION

While quality and resolution in both computed tomography (CT) and magnetic resonance imaging (MRI) technology has greatly increased in the past decades, the interpretation of images remains largely descriptive, subjective, and non-quantitative (1). With the expansion of computational power and information content in clinical imaging data, novel machine learning-based algorithms increasingly contribute to patient-specific diagnosis and treatment, especially in neuro-oncology (2, 3).

About 10% of intracerebral neoplastic lesions initially present as spontaneous hemorrhagic stroke (4). Acute non-contrast-enhanced computed tomography (NECT) imaging is the preferred screening method when intracerebral hemorrhage (ICH) is suspected; however, follow-up imaging is required for final diagnosis (4, 5). Interpretive challenges emerge from intra-hemorrhage and spatial heterogeneity as well as from the wide variety of different encompassing entities (6). Hence, initial radiological evaluation may be unreliable (4, 7). A recent pooled analysis identified 18 reported cases of glioblastoma-induced hemorrhage that were misdiagnosed as hypertensive ICHs, leading to significant diagnostic delays in two-thirds of the cases (7). Frequently, time-consuming and often negative neurovascular workup is being performed additionally (7). In these cases, extraction of quantitative radiomic image features and evaluation of these data in automated machine learning approaches might offer additional information for discriminating neoplastic and non-neoplastic ICHs. Facilitating early and sensitive detection of neoplastic hemorrhage, such an approach could optimize diagnostic workup, reduce misclassifications and delayed final diagnosis, and hence improve patient care at low risk and cost in the clinical routine.

Radiomic analysis is built on the hypothesis that imaging data reflect the underlying morphology and dynamics of smaller-scale biologic phenomena (8, 9). In this context, two important imaging markers of ICH have been described: firstly, the presence and extent of perihematomal edema (PHE), and secondly, the dynamics of hemorrhage attenuation (4, 10). However, radiomic analysis aims to capture also image information not assessable by human eyes, such as texture metrics or the evaluation of filtered images.

We hypothesized that quantitative radiomic image features extracted from NECT brain scans can be used to differentiate neoplastic and non-neoplastic ICHs. To test and evaluate this hypothesis, we employed a previously published and established radiomics machine learning approach on NECT brain scans of patients presenting with acute ICH of unknown etiology (3, 11). Furthermore, we evaluated the predictive performance of the proposed algorithm in comparison to conventional visual assessments of two radiologist readers.

METHODS

This single-center retrospective study was approved by the ethics committee (Ethik-Kommission der Ärztekammer Hamburg, WF-054/19), and written informed consent was waived according to paragraph 9 section 2 of the Hamburg federal state legislation and paragraph 15 section 1 of the medical association's professional code of conduct in Hamburg. All study protocols and procedures were conducted in accordance with the Declaration of Helsinki. The data that support the findings of this study are available, upon reasonable request from the corresponding author, if in accordance with the institution's data security regulations.

A graphical flow chart of the proposed machine learning-based prediction of the ICH etiology is shown in **Figure 1**, its components are detailed in the following.

Patients

We retrospectively reviewed the database of our center for patients with acute ICH in whom NECT imaging was performed from January 2010 through December 2017. Patients were consecutively included according to inclusion following criteria: (1) acute, non-traumatic single subcortical, or lobar ICH, (2) NECT imaging within 72 h, (3) MRI follow-up imaging confirming cause of acute ICH, and (4) documented time of symptom onset. In cases of suspected vascular malformation, additional digital subtraction angiography (DSA) was performed. Out of 560 systematically reviewed patients, 136 patients met the inclusion criteria. Fifty-nine patients were retrospectively excluded from the study for the following reasons: intraventricular hemorrhage or subarachnoid hemorrhage (SAH)–predominant cases ($n = 43$); multiple hemorrhagic lesions ($n = 9$); cerebral venous thrombosis as cause of ICH ($n = 5$); and aneurysm-associated ICH ($n = 2$). Extracted clinical patient data comprised patient age and patient sex. Seventy-seven patients ($n = 27$ with neoplastic, $n = 50$ with non-neoplastic ICH) remained in the final study population (**Table 1**). Median age of patients with neoplastic ICH was 71 years [inter-quartile range (IQR): 63–75], 40.7% females; median age of patients with non-neoplastic ICH was 72 years (IQR: 54.8–79.0), 56% females. Among the 77 study patients, 11 had primary ICHs ($n = 11$), 12 patients had an underlying vascular malformation or a cavernoma (AVM, $n = 5$; cavernoma, $n = 7$); 6 patients had an underlying amyloid angiopathy ($n = 6$); 21 patients had an unclear but neither neoplastic nor vascular pathology ($n = 21$), 21 patients had underlying brain metastasis ($n = 21$), and 6 patients had primary brain tumors ($n = 6$). All diagnoses were confirmed by follow-up MRI. Study patients were dichotomized for the binary outcome neoplastic vs. non-neoplastic ICH. Age, sex, time interval from symptom onset to NECT, and localization of ICH were not significantly different ($P > 0.05$; **Table 1**).

Image Acquisition

All patients received stroke imaging protocols at admission with NECT performed in equal order on 256 dual slice scanners (Philips iCT 256). NECT brain images were obtained from the vertex to the skull base (120 kV, 280–320 mA, 4.0 mm slice thickness, <0.6 mm in plane resolution). Additional CT angiography (CTA) was partially performed when atypical ICH was suspected. CT perfusion (CTP) was omitted. All NECT data sets were inspected for quality and excluded in case of severe motion artifacts as described in the section above.

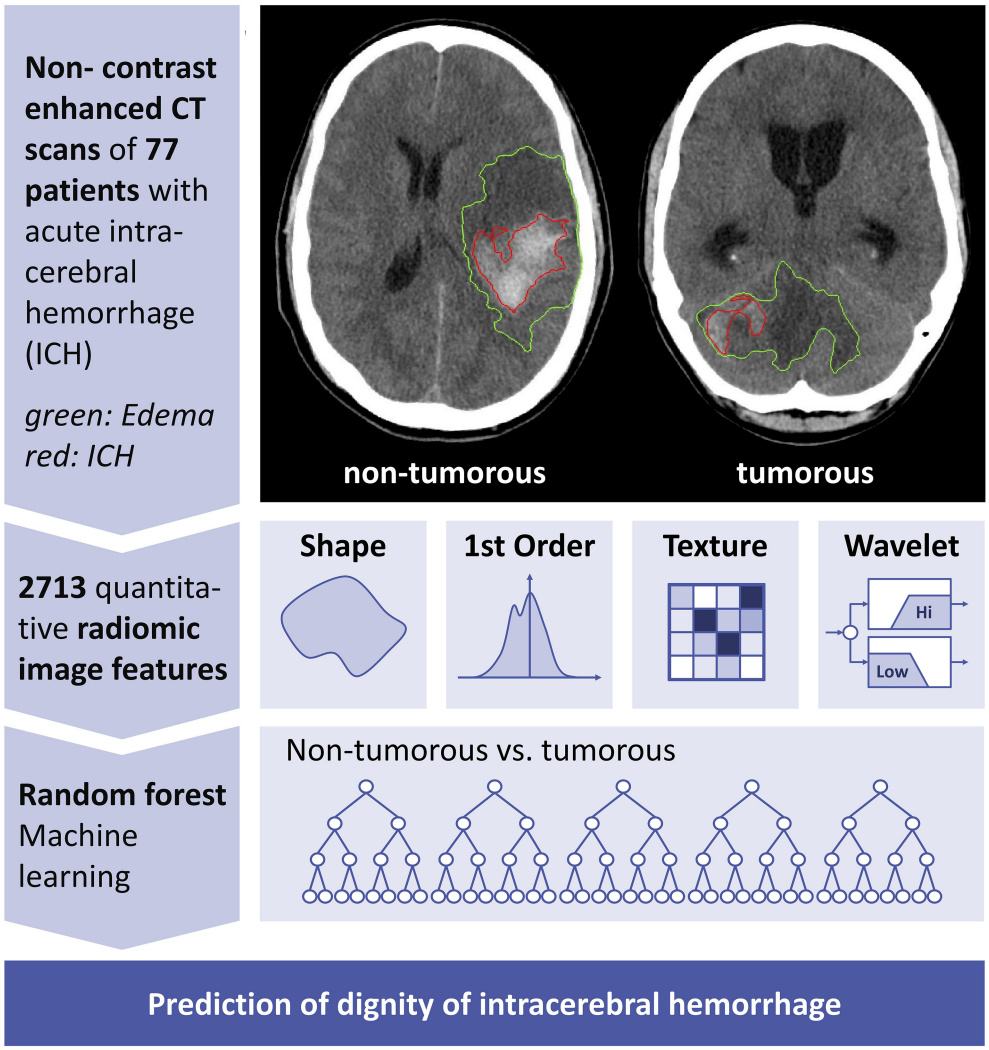

FIGURE 1 | Conceptual overview of proposed neoplastic intracerebral hemorrhage prediction. Conceptual overview of the proposed machine learning approach showing the major processing steps: CT-based image acquisition and segmentation, feature extraction ($n = 2{,}713$), and statistical learning (random forest algorithm). NECT, non-contrast-enhanced computed tomography; ICH, intracerebral hemorrhage; PHE, perihematomal edema.

Segmentation of Intracerebral Hemorrhage and Perihematomal Edema

ICH and PHE were segmented semi-automatically by two MDs (UH: 8 years clinical experience in diagnostic neuroradiology in an academic full-service hospital, research with focus on clinical applications of image processing and predictive modeling; JN: 2 years clinical experience in diagnostic neuroradiology in an academic full-service hospital) on the basis of the original NECT images. Both readers were blinded to all clinical information. Regions of interest (ROIs) were delineated using Analyze 11.0 Software (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN). Consensus ROIs were derived based on overlapping segmentations of both readers.

Machine Learning Approach

Machine learning-based classification was performed using random forest algorithms [Python scikit-learn environment v0.18.1 (12)]. Random forest classifiers were shown to have a comparably low tendency to overfit (13) and allow classification tasks also for data sets with a large number of heterogeneous predictors. Based on stability analysis of the total model out-of-bag error, the number of trees was set to 500, and the number of features per node was set to the square root of the total number of features (13).

Model Validation

Model validation was conducted using five-fold cross-validation with independent training and validation sets in a model-external approach (14). Model stability was examined through comparative analysis of 10 randomly permuted cross-validation sets.

Feature Extraction

Extracted radiomic features were defined according to the PyRadiomics Python package v2.1.0 (11), ROIs were resampled to $1 \times 1 \times 1$ mm isotropic resolution using sitk BSpline

TABLE 1 | Demographic data of study population.

Baseline characteristics	Non-neoplastic ICH ($n = 50$)	Neoplastic ICH ($n = 27$)	*P*-value
Age (years), mean (mean ± SD)	72 (54; 79)	71 (62; 75)	0.70
Sex female, *n* (%)	28 (56.0)	11 (40.7)	0.20
Time onset to imaging (h), median (IQR)	8.25 (2.88; 24)	21.0 (4.0; 54.0)	0.06
Localization supratentorial, *n* (%)	46 (92.0)	23 (85.2)	0.35
Density (HU), mean (mean ± SD)	54.6 (54.8; 79.0)	48.0 (40.00; 54.6)	0.001
Total hemorrhage volume (cm^3), median (IQR)	35.5 (16.7; 72.4)	47.2 (47.2; 108.2)	0.13
ICH volume (cm^3), median (IQR)	15.4 (63.8; 36.0)	13.2 (8.7; 32.1)	0.54
PHE volume (cm^3), median (IQR)	13.6 (7.7; 34.9)	38.2 (11.4; 80.5)	0.007

Continuous variables are represented as mean ± standard deviation (SD) and categorical variables as number (n), and percentages (%). IQR, inter-quartile range; ICH, intracerebral hemorrhage; PHE, perihematomal edema; HU, Hounsfield units.

interpolators. Extracted features comprised 252 first-order features (thereof 18 based on unfiltered images, 144 based on wavelet decompositions, 90 based on log-sigma Laplacian of Gaussian filters), 902 texture features (thereof 68 based on unfiltered images, 544 based on wavelet decompositions, 290 based on log-sigma Laplacian of Gaussian filters), and 14 shape features. In total, 1,218 quantitative image features were extracted from the ICH, PHE, and ICH plus PHE ROIs. Furthermore, feature ratios of ICH/PHE and ICH/(PHE plus ICH) were calculated, resulting in a total of 6,090 extracted quantitative image features.

In brief, shape features were extracted from the hemorrhage and edema ROIs and do not depend on gray level distributions of the image. Shape features include descriptors of the three-dimensional size and shape of the ROI, e.g., volume, surface area, diameter, and sphericity. First-order and texture features were derived from the original images, from wavelet filtered images (high and low passes in three different directions), and from log-sigma-filtered images [log-sigma function at different sizes (1–5, 1 mm increment]. First-order statistics describe the distribution of voxel intensities within the image region defined by the ROI through basic metrics, e.g., mean, median, percentiles, and kurtosis. Texture features quantify the distribution of gray levels in an image with regard to, e.g., the size and position of zones of equal gray levels. The gray level co-occurrence matrix (GLCM) represents the number of times specific combination of gray levels occur in two pixels of an image that are separated by a specific distance. The gray level size zone matrix (GLSZM) quantifies specific gray level zones in an image. The gray level run length matrix (GLRLM) quantifies gray level runs that are defined as the length of consecutive pixels that have the same gray level value. The neighboring gray tone difference matrix (NGTDM) quantifies the difference between a gray value and the average gray value of its neighbors. The gray level dependence matrix (GLDM) quantifies gray level dependencies in an image. A gray level dependency is defined as the number of connected voxels within a specific distance that are dependent on the center voxel.

Feature Selection

Selection of features with the highest predictive value was performed separately for each training data set considering Gini impurity measures (15). Feature sets with outliers greater than six standard deviations (SDs) were excluded from the analysis. For final model training and validation, we employed the 100 most important features of each set.

Radiologist Reading

Two MDs (UH, JN) predicted the dignity of ICHs based on the acute NECT images. For each ICH, the readers rated "neoplastic" or "non-neoplastic." Both readers were blinded to the ground truth, the classifier prediction, and the other reader's prediction.

Statistics

The shown receiver operating characteristic (ROC) curve was calculated based on means of all cross-validation sets. For each set, classifiers were trained and tested on the set's unique training and validation samples employing the 100 most important features of the respective training data. Hence, mean ROC curves can be considered as unbiased estimates of general model classification performance. Statistical significance of the mean area under the curve (AUC) was assumed if $P < 0.05$ for all cross-validation sets. Model prediction instability was derived from the SD of ROC curves. *P*-values were calculated according to Mann–Whitney/Wilcoxon U statistics using the verification R-package v1.42 (16). Confidence intervals (CIs) for sensitivities and specificities were bootstrapped (2,000 replicates) using pROC v1.10 (17) and qwraps2 v0.3.0 R-packages. Statistical significance of differences in specificities was evaluated with McNemar test statistics (DTComPair v1.0.3 R-package). Total classification performance of radiologist readers and the machine learning classifier was compared using the Matthews correlation coefficient (MCC) (18). MCC integrates all fields of the confusion matrix and is generally considered as a favorable metric for unbiased comparisons of binary classifiers (19). Further, MCC evaluates balance ratios of the four confusion matrix categories (true positives, true negatives, false positives, false negatives) and allows comparison of classifiers also for unbalanced data sets (20–22). With *TP*: true positives, *TN*: true negatives, *FP*: false positives, and *FN*: false negatives, MCC is defined as:

$$MCC = \frac{TP\ x\ TN - FP\ x\ FN}{\sqrt{(TP + FP)\ (TP + FN)\ (TN + FP)\ (TN + FN)}}$$

Statistical significances of differences in MCC were calculated using the "psych" v1.8.12 R-package.

RESULTS

Our analysis includes NECT images of 77 patients with acute ICH, thereof 50 with non-neoplastic and 27 with neoplastic cause defined by final diagnosis in follow-up MRI.

Classifier Performance

ROC AUC of the validation sets for predicting the dignity of ICH was 0.89 [95% CI: (0.70; 0.99); SD: 0.013]; all $P < 0.01$. Depending on selected cutoff values, the classifier yielded specificities and sensitivities of >80% (**Figure 2A**). The highest MCC measures of 0.69 were calculated at 70% sensitivity and 95% specificity with a Youden index of 0.65 and accuracy of 86% (**Table 2**).

Feature Importance

The top-100 features with the highest predictive power were mainly derived from ROIs comprising both PHE and ICH segmentations (52% of total predictive power). The lowest predictive value was calculated for ICH segmentations alone (8%) (**Figure 3A**). Regarding feature classes, fist-order histogram-based measures and texture features ranked highest with 52 and 46% of total predictive power, and shape-based features only contributed 2.5%. Filter-based extractions significantly increased predictive power: Wavelet and log-sigma-filtered images contributed 44 and 37%; unfiltered images contributed only 20% to total predictive power (**Figure 3B**). Of the 100 most important feature values, 86 were significantly different for neoplastic and non-neoplastic ICHs ($P < 0.05$). Normalized feature value box plots of the 10 most important predictors demonstrate differences in feature expressions for non-neoplastic and neoplastic ICHs and show typical radiomic signatures of the entities (**Figure 3C**). The most important feature comprises both ROIs, PHE, and ICH, and measures the 10th percentile of a 2 mm log-sigma-filtered image. Features #2 to #5 are first-order density metrics extracted from original and wavelet low-pass filtered (LLL) images.

Radiologist Reading

Reader 1 predicted the dignity of ICHs with a sensitivity of 85% and a specificity of 72%; accuracy was 77%, Youden index was 0.57, and MCC was 0.55. Reader 2 achieved 70% sensitivity at 84% specificity with accuracy of 79%, Youden index of 0.54, and MCC of 0.54 (**Figure 2B**, **Table 2**).

Comparison of Classifier and Radiologist Reader Prediction Performance

Comparative analysis of specificities at the reader's sensitivity set points suggests that classification performance of the machine learning algorithms was equal or superior for all evaluated metrics. Whereas reader 1 achieved classification results equivalent to the proposed algorithm, the metrics of reader 2 were lower, with specificity at −11% (84 vs. 95%, $P = 0.06$) and MCC at −0.15 (0.54 vs. 0.69, $P = 0.08$). When comparing the combined human rating results (reader 1 and reader 2) with the classifier's predictions at its optimal operating point, MCC of the proposed algorithm (0.69) was significantly higher than MCC of the radiologist readers (0.54); $P = 0.01$ (**Table 2**).

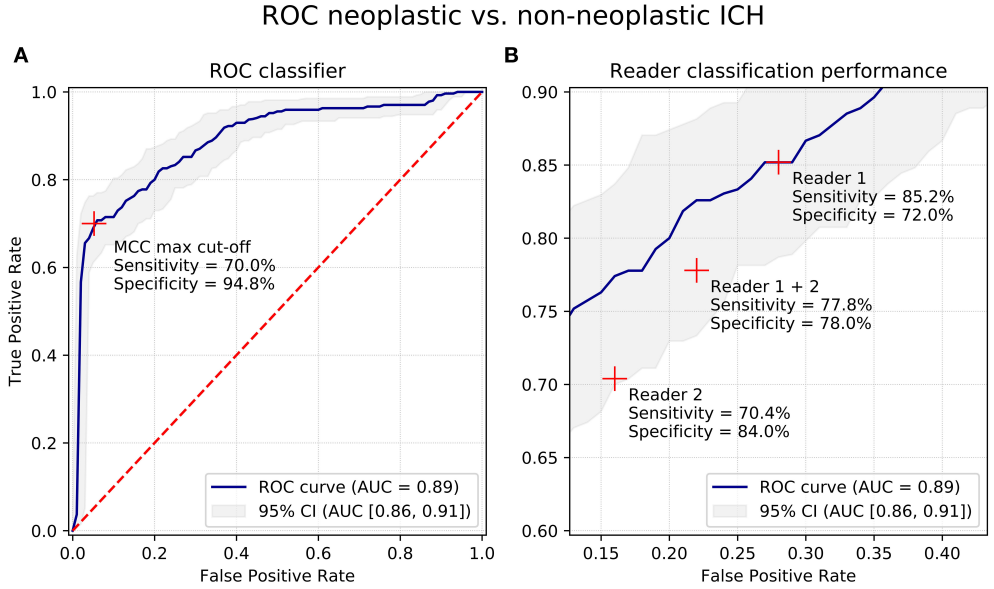

FIGURE 2 | Receiver-Operating-Characteristics curves for differentiation of neoplastic and non-neoplastic ICHs. **(A)** Receiver-Operating-Characteristics (ROC) curves for differentiation of neoplastic and non-neoplastic ICHs of the proposed machine learning classifier based on quantitative radiomic image features. **(B)** Cut-out of panel **(A)** showing classification results of human reader 1 and 2. Blue line shows ROC curve, grey area shows 95% confidence interval (CI). Red crosses show cut-off points/prediction performance. AUC, area under the curve; CI, confidence interval; ROC, Receiver-Operating-Characteristics; ICH, intracerebral hemorrhage; MCC, Matthews correlation coefficient.

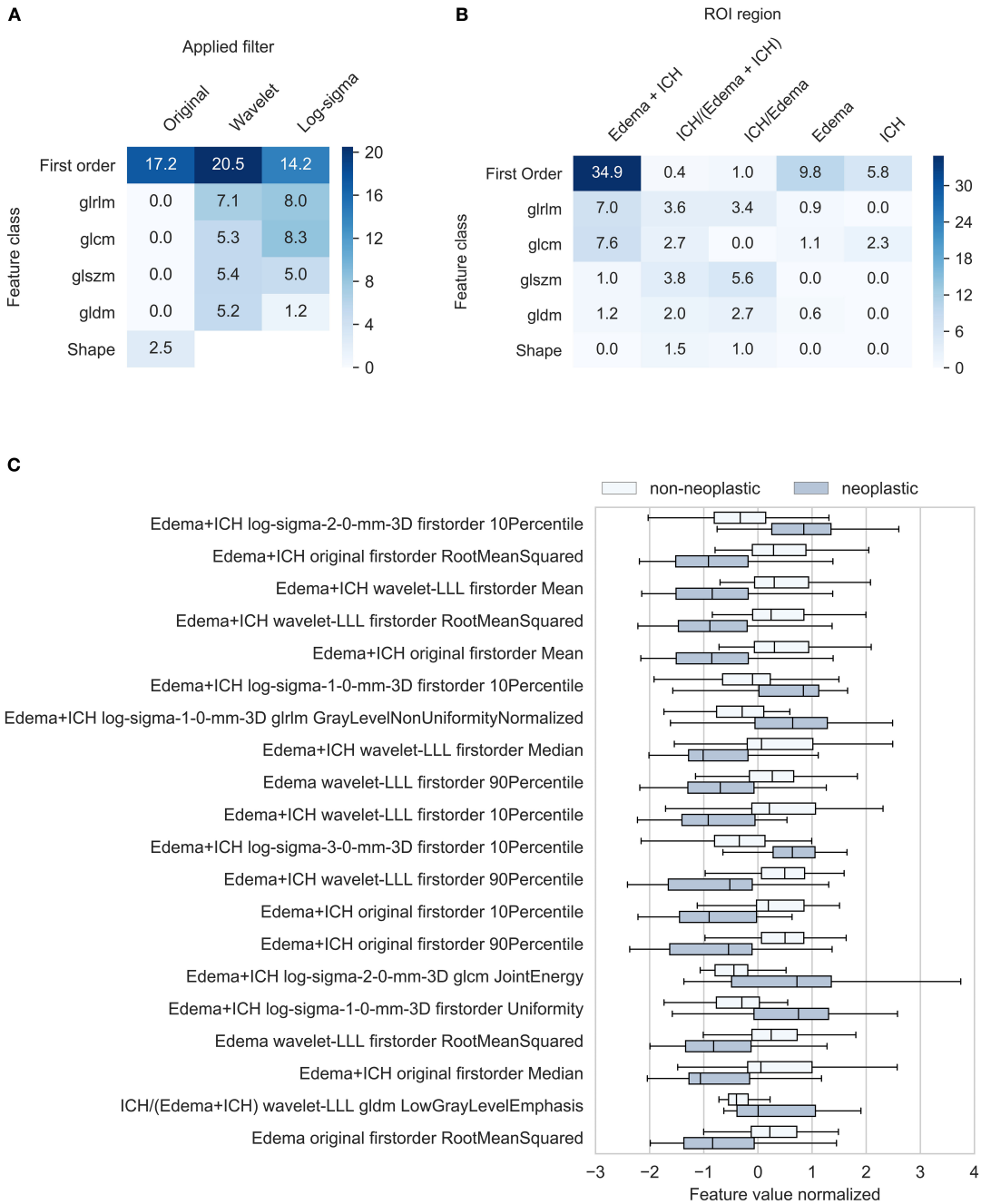

FIGURE 3 | Characterization of most important features. Feature importance contribution of 100 most important features in % **(A)** By applied filter and feature class **(B)** by region and feature class. Texture feature class includes gray level size zone matrix, gray level dependence matrix, gray level run length matrix, and gray level size zone. **(C)** Radiomic feature signatures of neoplastic and non-neoplastic intracerebral hemorrhage. Box-plots show normalized means of the 20 most important image features. All mean feature values significantly different between neoplastic and non-neoplastic ICHs ($P < 0.05$). ROI, region of interest; ICH, intracerebral hemorrhage; PHE, perihematomal edema; gldm, gray level dependence matrix; H, high-pass wavelet decomposition; L, low-pass wavelet decomposition; glnu-norm, gray level non-uniformity normalized; RMS, root mean squared.

DISCUSSION

The main findings of our study are, firstly, that the proposed machine learning approach employing quantitative image features derived from NECT scans provides high discriminatory accuracy in predicting neoplastic ICHs. Secondly, depending on the classifier operating point, the proposed algorithm reaches significantly higher MCC metrics compared to visual ratings.

The proposed classifier yielded an AUC of 0.89 for the prediction of neoplastic ICHs with sensitivities and specificities

TABLE 2 | Classification performance metrics of radiologist readers and machine learning classifier.

Prediction	Cutoff point	Sensitivity (95% CI)	Specificity (95% CI)	Accuracy	Youden index	MCC	
Reader 1	–	85% (70; 96%)	72% (59; 84%)	77%	0.57	0.55	
Reader 2	–	70% (52; 86%)	84% (73; 93%)	79%	0.54	0.54	
Reader 1+2	–	78% (66; 88%)	78% (70; 86%)	78%	0.56	0.54	
Classifier	Sensitivity: 85% (R1)	85% (80; 89%)	73% (65; 81%)	77%	0.68	0.66	
Classifier	Sensitivity: 70% (R2)	70% (64; 75%)	95% (93; 97%)	86%	0.65	0.69	$p = 0.001$
Classifier	Sensitivity: 78% (R1+R2)	78% (72; 83%)	83% (76; 90%)	81%	0.61	0.60	
Classifier	Maximum MCC	70% (64; 75%)	95% (93; 97%)	86%	0.65	0.69	

Classifier metrics are shown at cutoff points according to radiologist readers' sensitivities and at the classifiers' optimal operating point. MCC at the classifier's optimal operating point (0.69) is significantly higher compared to the combined result of readers 1 and 2 (0.54); P = 0.01. MCC, Matthews correlation coefficient; CI, confidence interval.

reaching >80% depending on the cutoff value. Narrow CIs and low SDs of ROC curves suggest high stability of predictive performance. Whereas visual ratings of an 8-years-experienced senior neuroradiologist (reader 1) yielded similar metrics, results of the less experienced reader 2 were inferior, with a −11% loss in specificity ($P = 0.06$). Overall, MCC, a widely accepted metric for comparing binary classifiers, was significantly higher for the machine learning algorithm, with 0.69 vs. 0.54 for visual ratings of readers 1 and 2 ($P = 0.01$) (19). Hence, utilized as a supportive decision tool in clinical practice, the proposed algorithm improved and facilitated initial triaging, diagnostic workup, and precision of final diagnosis in patients presenting with acute ICHs. Also, the utilization of the tool for training and quality control especially for inexperienced residents is an interesting aspect, as MCC was different between the resident and the experienced neuroradiologist.

Although numerous interrelations between quantitative image features and clinical diagnoses have been demonstrated, radiomic analyses are still lacking wide clinical acceptance (2). In particular, the missing link between quantitative metrics, traditional imaging features, and the underlying biology has been a major point of criticism (2). To address these concerns, we evaluated the employed quantitative predictors with respect to their interpretation in visual assessments and established ties to traditional semantic imaging features. It is widely accepted that tumors and metastases are surrounded by an extensive PHE prior to a bleeding event. Preliminary studies underline the CT-based diagnostic importance of this pathophysiological process, as recently published (23). In line with this, Choi et al. (4) have described that a reduced hematoma attenuation in ICH can differentiate neoplastic from non-neoplastic lesions with high diagnostic accuracy. Accordingly, our analysis of the 100 most important features demonstrates that intensity distribution-based predictors (first-order histogram) contribute 51.9% of the cumulated feature importance (**Figure 3A**). Corresponding to classic semantic image readings, our by-region assessment shows that image features extracted from the entire lesion (ICH and PHE) yield the highest contribution (52%) to predictive performance (**Figure 3B**). However, our analysis also proves that the NECT imaging information is much richer: With a 45.6% share in cumulated importance, texture features play a similar important role as classic first-order predictors (**Figure 3A**). Furthermore, differentiation of features by applied filter demonstrates that wavelet and log-sigma-filtered images with a contribution of 44 and 37%, respectively, yield superior importance compared to non-filtered images, with a share of 19% (**Figure 3A**). **Figure 3C** shows box plots of normalized feature values of the 10 most important predictors for neoplastic and non-neoplastic ICHs. The graph demonstrates that the 10th percentile of log-sigma (2 mm) filtered images is the metric with the highest predictive power. This suggests that neoplastic ICHs express significantly sharper density edges compared to non-neoplastic ICHs. Features #2 to #5 are intensity measures extracted from original and from wavelet low-pass filtered images. In line with clinical studies proposing hematoma density as a diagnostic marker for neoplastic ICH on CT (4), these metrics suggest that neoplastic lesions are hypodense compared to non-neoplastic ICHs.

To our knowledge, this is the first study that investigates the use of quantitative radiomic image features extracted from NECT scans to differentiate neoplastic and non-neoplastic ICHs. The proposed method integrates the merits from quantitative radiomic features and machine learning algorithms and relates the employed predictors to traditional radiographic imaging findings. Unlike our study, existing radiomics-based analyses regarding CT imaging have mainly focused so far on prompt ICH diagnosis and automated volume quantification (24, 25).

Our study had general limitations typically associated with quantitative radiomics-based image analysis and classification (3, 8, 26, 27). These limitations include differences in image acquisition techniques, under- or overfitting of machine learning algorithms, and potential misclassifications in the ground truth

definitions. All of these limitations could bias classification and may lead to less generalizable results. Furthermore, we observed study-specific limitations: First, we only included a limited number of patients in a retrospective analysis. An expansion of sample size in a prospective study design would certainly contribute to further improving generalizability of results. Small sample sizes are a general concern for radiomics analysis and are due to the limited availability of standardized multi-center databases. However, results of our model stability analysis suggest sufficient robustness for assessing general feasibility and limitations of the proposed algorithm. Second, the manual definition of ROIs still implies a certain degree of observer-dependence within the machine learning process. To minimize its influence, we employed consensus segmentations from two independent readers and applied a semi-automated delineation segmentation method that was shown to have favorable inter- and intra-observer reliability (10). Noteworthily, variabilities are lower in automatic vs. semi-automatic vs. manual delineation; however, semi-automatic delineation was mandatory in our case (28–31). Further, it was shown that radiomic features are comparably stable with regard to variations in segmentations (30, 32). Third, the underlying NECT images of our analysis were acquired with the same scanner at the same hospital. This might reduce generalizability of results. However, due to standardized and calibrated quantitative imaging parameters and signal intensity processing of CT scanners, we assume neglectable bias on classifier performance in a generalized setting. Lastly, the hematoma density difference between neoplastic and non-neoplastic ICHs can be discussed critically, as symptom onset to imaging time differed by trend between the two categories. As ICH density decreases over time, this might have biased results. However, the difference in onset to imaging times was statistically not significant and in line with current literature (4).

From our results, we conclude that the additional imaging information extracted through texture analysis and filtering as well as the standardized and fully automated machine learning algorithm is the main factor determining the observed high prediction performance and stability. As this information is not assessable by human eyes, the proposed approach can be used as supportive tool to improve the radiologist's diagnostic decision. Through facilitating efficient triage, reducing initial misclassifications, and preventing delayed diagnosis, the proposed algorithm could improve patient care in the daily clinical routine at low risk and costs.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available, upon reasonable request from the corresponding author, if in accordance with the institution's data security regulations.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethik-Kommission der Ärztekammer Hamburg, WF-054/19. The Ethics Committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

JN contributed to the study design, the patient data collection, took a lead in writing the manuscript, conceived, and planned the experiments. HK contributed to the image processing, image analysis, statistical analysis, data analysis, drafting the manuscript, and revising it critically. RK contributed to the acquisition of data, drafting the manuscript, and revising it critically. GB, TF, and CT contributed to the data analysis, drafting the manuscript, and revising it critically. GS contributed to the statistical analysis, data analysis, drafting the manuscript, and revising it critically. JF contributed to the study design, data analysis, drafting the manuscript, and revising it critically. UH contributed to the study design, acquisition of data, image analysis, data analysis, drafting the manuscript, and revising it critically.

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REFERENCES

1. Nawabi J, Kniep H, Kabiri R, Broocks G, Faizy TD, Schoen G, et al. Neoplastic and non-neoplastic acute intracerebral hemorrhage in CT brains brain scans: machine learning based prediction of dignity using radiomic image features. *Clin Neuroradiol.* (2019) 29:5–6. doi: 10.1007/s00062-019-00774-4
2. Zhou M, Scott J, Chaudhury B, Hall L, Goldgof D, Yeom KW, et al. Radiomics in brain tumor: image assessment, quantitative feature descriptors, and machine-learning approaches. *Am J Neuroradiol.* (2018) 39:208–16. doi: 10.3174/ajnr.A5391
3. Kniep HC, Madesta F, Schneider T, Hanning U, Schönfeld MH, Schön G, et al. Radiomics of brain MRI: utility in prediction of metastatic tumor type. *Radiology.* (2018) 180946: 479–87.
4. Choi YS, Rim TH, Ahn SS, Lee S-K. Discrimination of tumorous intracerebral hemorrhage from benign causes using CT densitometry. *Am J Neuroradiol.* (2015) 36:886–92. doi: 10.3174/ajnr.A4233
5. Hemphill JC, Greenberg SM, Anderson CS, Becker K, Bendok BR, Cushman M, et al. Guidelines for the management of spontaneous intracerebral hemorrhage: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke.* (2015) 46:2032–60. doi: 10.1161/STR.0000000000000069
6. Shankar JJS, Sinha N. Diagnosing neoplastic hematoma: role of MR perfusion. *Clin Neuroradiol.* (2018) 29:263–8. doi: 10.1007/s00062-018-0664-6
7. Joseph DM, O'Neill AH, Chandra RV, Lai LT. Glioblastoma presenting as spontaneous intracranial haemorrhage: case report and review of the literature. *J Clin Neurosci.* (2017) 40:1–5. doi: 10.1016/j.jocn.2016.12.046
8. Gillies RJ, Kinahan PE, Hricak H. Radiomics: Images are more than pictures, they are data. *Radiology.* (2016) 278:563–77. doi: 10.1148/radiol.2015151169

9. Kumar V, Gu Y, Basu S, Berglund A, Eschrich SA, Schabath MB, et al. Radiomics: the process and the challenges. *Magn Reson Imaging.* (2012) 30:1234–48. doi: 10.1016/j.mri.2012.06.010
10. Urday S, Beslow LA, Goldstein DW, Vashkevich A, Ayres AM, Battey TWK, et al. Measurement of perihematomal edema in intracerebral hemorrhage. *Stroke.* (2015) 46:1116–9. doi: 10.1161/STROKEAHA.114.007565
11. van Griethuysen JJM, Fedorov A, Parmar C, Hosny A, Aucoin N, Narayan V, et al. Computational radiomics system to decode the radiographic phenotype. *Cancer Res.* (2017) 77:e104–e7. doi: 10.1158/0008-5472.CAN-17-0339
12. Pedregosa F, Varoquaux G, Gramfort A, Michel V, Thirion B, Grisel O, et al. Scikit-learn: machine learning in Python. *J Mach Learn Res.* (2011) 12:2825–30. Available online at: http://jmlr.org/papers/v12/pedregosa11a.html
13. Breiman L. Random forests. *Mach Learn*. (2001) 45:5–32. doi: 10.1023/A:1010933404324
14. Limkin EJ, Sun R, Dercle L, Zacharaki EI, Robert C, Reuzé S, et al. Promises and challenges for the implementation of computational medical imaging (radiomics) in oncology. *Ann Oncol.* (2017) 28:1191–206. doi: 10.1093/annonc/mdx034
15. Louppe G, Wehenkel L, Sutera A GP. Understanding variable importances in forests of randomized trees. *Adv Neural Inf Process Syst*. (2013) 1:431–9.
16. Mason SJ, Graham NE. Areas beneath the relative operating characteristics (ROC) and relative operating levels (ROL) curves: statistical significance and interpretation. *Q J R Meteorol Soc.* (2002) 128:2145–66. doi: 10.1256/003590002320603584
17. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez J-C, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinform*. (2011) 12:77. doi: 10.1186/1471-2105-12-77
18. Matthews BW. Comparison of the predicted and observed secondary structure of T4 phage lysozyme. *Biochim Biophys Acta.* (1975) 405:442–51. doi: 10.1016/0005-2795(75)90109-9
19. Powers DMW. Evaluation: from precision, recall and F-measure to ROC, informedness, markedness and correlation. *J Mach Learn Tech.* (2011) 2:37–63. doi: 10.9735/2229-3981
20. Boughorbel S, Jarray F, El-Anbari M. Optimal classifier for imbalanced data using Matthews Correlation Coefficient metric. *PLoS One.* (2017) 12:e0177678. doi: 10.1371/journal.pone.0177678
21. Chicco D, Jurman G. The advantages of the Matthews correlation coefficient (MCC) over F1 score and accuracy in binary classification evaluation. *BMC Genomics.* (2020) 21:6. doi: 10.1186/s12864-019-6413-7
22. Chicco D. Ten quick tips for machine learning in computational biology. *BioData Min.* (2017) 10:35. doi: 10.1186/s13040-017-0155-3
23. Abstracts. Available online at: https://link.springer.com/content/pdf/10.1007%2Fs00062-018-0719-8.pdf [cited March 13, 2019].
24. Arbabshirani MR, Fornwalt BK, Mongelluzzo GJ, Suever JD, Geise BD, Patel AA, et al. Advanced machine learning in action: identification of intracranial hemorrhage on computed tomography scans of the head with clinical workflow integration. *npj Digit Med.* (2018) 1:9. doi: 10.1038/s41746-017-0015-z
25. Scherer M, Cordes J, Younsi A, Sahin Y-A, Götz M, Möhlenbruch M, et al. Development and validation of an automatic segmentation algorithm for quantification of intracerebral hemorrhage. *Stroke.* (2016) 47:2776–82. doi: 10.1161/STROKEAHA.116.013779
26. Aerts HJWL. The potential of radiomic-based phenotyping in precision medicine. *JAMA Oncol.* (2016) 2:1636–42. doi: 10.1001/jamaoncol.2016.2631
27. Lambin P, Leijenaar RTH, Deist TM, Peerlings J, de Jong EEC, van Timmeren J, et al. Radiomics: the bridge between medical imaging and personalized medicine. *Nat Rev Clin Oncol.* (2017) 14:749–62. doi: 10.1038/nrclinonc.2017.141
28. Zhao B, Tan Y, Tsai W-Y, Qi J, Xie C, Lu L, et al. Reproducibility of radiomics for deciphering tumor phenotype with imaging. *Sci Rep.* (2016) 6:23428. doi: 10.1038/srep23428
29. Balagurunathan Y, Kumar V, Gu Y, Kim J, Wang H, Liu Y, et al. Test-retest reproducibility analysis of lung CT image features. *J Digit Imaging.* (2014) 27:805–23. doi: 10.1007/s10278-014-9716-x
30. Parmar C, Rios Velazquez E, Leijenaar R, Jermoumi M, Carvalho S, Mak RH, et al. Robust radiomics feature quantification using semiautomatic volumetric segmentation. *PLoS One.* (2014) 9:e102107. doi: 10.1371/journal.pone.0102107
31. Rios Velazquez E, Aerts HJWL, Gu Y, Goldgof DB, De Ruysscher D, Dekker A, et al. A semiautomatic CT-based ensemble segmentation of lung tumors: comparison with oncologists' delineations and with the surgical specimen. *Radiother Oncol.* (2012) 105:167–73. doi: 10.1016/j.radonc.2012.09.023
32. Yip SSF, Aerts HJWL. Applications and limitations of radiomics. *Phys Med Biol.* (2016) 61:R150–R66. doi: 10.1088/0031-9155/61/13/R150

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Assessment of Evidence Regarding Minimally Invasive Surgery vs. Conservative Treatment on Intracerebral Hemorrhage: A Trial Sequential Analysis of Randomized Controlled Trials

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Introduction: The recent publication of a trial failed to prove the efficacy of minimally invasive surgery (MIS) in patients with intracerebral hemorrhage. The aim of this study was to answer the question: Do we need more trials to compare MIS vs. conservative treatment in these patients?

Methods: Databases were searched for relevant randomized trials on MIS (endoscopic surgery or stereotactic evacuation) vs. conservative treatment. The primary outcome was significant neurological debilitation or death at the follow-up, and the secondary outcome was death. Both conventional meta-analysis and trial sequential analysis (TSA) were performed.

Results: Twelve trials with 2,049 patients were included. In the conventional meta-analysis, the risk ratios of MIS vs. conservative treatment were 0.82 [95% confidence interval (CI), 0.72–0.94] and 0.74 (95% CI, 0.62–0.88) for the primary and secondary outcomes, respectively. In TSA, the cumulative *z* curve crossed the superiority boundary, which confirmed an 18.8% relative risk reduction of MIS vs. conservative treatment for the primary outcome. It was also highly likely that MIS would reduce mortality by 24.3%. Several sensitivity analyses suggested the robustness of our results, including different prior settings, including only trials with blind outcome assessment, and the assumption of future trials to be futile.

Conclusions: Minimally invasive surgery seems to be more effective than conservative treatment in patients with intracerebral hemorrhage in reducing both morbidity and mortality. Repeating a clinical trial with similar devices, design, and outcomes is unlikely to change the current evidence.

Keywords: endoscope, stereotactic evacuation, thrombolysis, stroke, meta-analysis, mortality

INTRODUCTION

Stroke contributes 5% to all disability-adjusted life-years loss (1) and 10% to all deaths worldwide (2). Hemorrhagic stroke accounts for more disability-adjusted life-years loss than ischemic stroke (1). In theory, surgical evacuation of hemorrhage may improve the patient's outcome, but several randomized trials (3, 4) failed to prove its effectiveness. Morbidity and mortality remained high even in patients treated. With the development of endoscopic and stereotactic evacuation technique, more and more patients were treated with these minimally invasive surgeries (MISs).

The arguments of the benefit by MIS were controversial among several randomized trials; nearly half of the published trials were futile. Several previous systematic reviews and meta-analyses of MIS include retrospective or prospective nonrandomized studies with potential confounding and bias (5–7). The results of the meta-analyses on randomized trials also varied from "nonsuperiority" (8) to "superiority" (9, 10) with different outcome measurements and different control group selections. The recent publication of a phase 3 trial failed to prove the efficacy of MIS, which made the question more suspicious (11).

The question still lies in which treatment is better for patients with intracerebral hemorrhage: MIS or conservative treatment. Do we need more trials to compare MIS vs. conservative treatment in these patients? Trial sequential analysis (TSA) borrows the idea from interim monitoring from a single randomized trial by treating every trial in the meta-analysis as an interim sample (12). Similar to interim monitoring, TSA has rules for early stopping if the result meets superiority boundary or futility boundary. In this review, we aimed to apply TSA on data from randomized trials comparing MIS vs. conservative treatment to answer the questions as mentioned above.

METHODS

Search Strategy and Selection Criteria

This study is reported according to PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (**Supplement Table 1**). We searched randomized controlled trials (RCTs) published up to March 1, 2019, using the combination of stroke ("intracranial hemorrhage," "intracerebral hemorrhage," "cerebral hemorrhage," "brain hemorrhage," "stroke") and surgical modality ("endoscope," "evacuation," "minimally") in PubMed, Web of Science, and China Knowledge Resource Integrated Database (detailed strategy in **Supplement Table 2**). We screened for additional eligible trials in reference lists of retrieved studies and relevant review articles. The exclusion criteria were as follows: studies with brain hemorrhage due to traumatic brain injury, tumor, coagulopathy, or vascular disease; studies with both craniotomy and MIS, but the decision of craniotomy or MIS was made at the discretion of surgeons; nonrandomized studies; and trials in which outcome information was not available.

Type of Interventions

Minimally invasive surgery comprised endoscopic surgery or stereotactic evacuation with or without thrombolysis. Conservative treatment was the best conventional medical treatment.

Outcomes

The primary outcome was the proportion of patients with significant neurological debilitation or death at the postrandomization follow-up. Significant neurological debilitation or death was defined as modified Rankin Score of more than 3 or Glasgow Outcome Scale of <4. As we expected the literature to be heterogeneous in terms of follow-up duration, we adopted the primary outcome time point reported in the original trial. The secondary outcome was the proportion of patients who died at the postrandomization follow-up. Regarding crossover in the included trials, we used the intention-to-treat effect. We also imputed the loss to follow-up data as the worst outcome.

Data Extraction

X.Z. and L.X. independently screened the literature, selected studies, extracted the relevant information, and assessed the risk of bias with the Cochrane risk of bias tool (13). Risk of bias for each item was classified as either low risk, unclear, or high risk. Any controversies were resolved by consensus and arbitration by the entire review team including a senior consultant physician (N.Q.).

Data Synthesis and Statistical Methods

Outcomes were recorded as the proportion in each arm. A conventional meta-analysis was used to pool risk ratios comparing MIS with conservative treatment. We initially used random-effects models to aggregate data and the I^2 tests to examine heterogeneity [more than 50% indicates notable heterogeneity (14)]. When no significant heterogeneity was observed, we changed our models into fixed-effects models. We performed subgroup analysis by different mean ages (<60 or >60 years old), follow-up period (≤1 year), study quality (blind or unblind outcome assessment), publication year (before 2010 or after 2010), study location (Eastern Asia or Western), and surgical modality (endoscopic surgery or stereotactic evacuation).

We conducted a TSA assuming 5% as an acceptable risk of type I error (α). We set several prior to the TSA: (1) effect size: we selected an 18.8% relative risk reduction as *a priori*, which was estimated from the conventional meta-analysis; (2) statistical power: we chose 80%; (3) event proportion in the control arm: we used 67.4% as an estimate from the pooled primary outcome from all the control groups; (4) amount of heterogeneity: 81.9% as the observed diversity across the included trials. For the secondary outcome, we used the same procedures with the prior obtained from the currently available evidence. The TSA combines the information size (cumulated sample size) with trial sequential monitoring boundaries. Whether the conclusion is sufficient was determined based on the following criteria: the evidence is adequate when the cumulative z curve

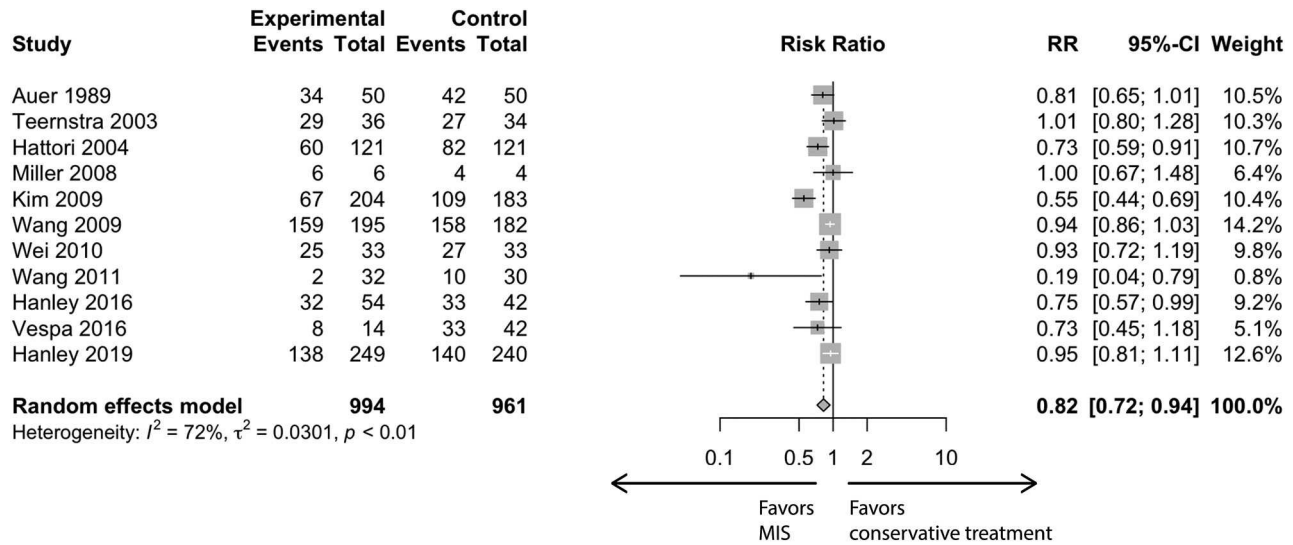

FIGURE 1 | Conventional meta-analysis.

crosses the monitoring boundary, and the evidence is insufficient if the z curve does not intersect any of the boundaries, and the required information size has not been reached (15).

We performed several sensitivity analyses. First, we used more conservative analyses prior, such as a reduced risk reduction (15 and 10%), an increased power (90%), or a decreased event proportion in the control arm (58.0%) according to the most recent trial (11). Second, we repeated the analysis only in trials with high quality (blind outcome assessment). Third, we further assumed the result of the ongoing RCT (NCT02880878) to be futile to discern the impact on the analysis.

Statistical analyses were performed with RStudio version 1.0.143 (Boston, MA) for the conventional meta-analysis and Trial Sequential Analysis software 0.9 (Copenhagen Trials Unit, Copenhagen, Denmark) for the TSA.

RESULTS

Twelve trials (11, 16–26) were included with nine trials in English and three in Chinese. Flowchart of inclusion and exclusion is provided in **Supplement Figure 1**, and baseline characteristics of the included studies are listed in **Supplement Table 3**. There were 2,049 patients included in this study. Nine trials investigated the effect of stereotactic evacuation, and three trials examined the effect of endoscopic surgery. Quality assessment showed none of the trials were blind to patients, but five of the trials applied blinding in outcome assessment. Nearly half of the trials did not provide information on random sequence generation or allocation concealment (**Supplement Figure 2**).

In the conventional meta-analysis, the risk ratios of MIS vs. conservative treatment were 0.82 [95% confidence interval (CI), 0.72–0.94] for the primary outcome (**Figure 1**) and 0.74 (95% CI, 0.62–0.88) for the secondary outcome. In subgroup analysis for the primary outcome, the point estimation kept relatively constant from 0.73 to 0.90 in regard to different subgroups (age, follow-up period, outcome blinding, publication year, study location or surgical modality; **Figure 2**).

Figure 3 summarizes TSA results for the primary outcome. Analysis with the addition of the Vespa trial in 2016 (26) was inconclusive with a significant variation (95% CI, 0.61–1.01). After the addition of the latest Hanley trial (11), the cumulative z curve ($z = 2.93$) crossed the α spending superiority boundary ($z = 2.37$, dashed red line), although the required information size (2,578 patients) was not reached. The result confirmed an 18.8% relative risk reduction of MIS vs. conservative treatment with moderate confidence ($\alpha = 0.05, \beta = 80\%$). For the secondary outcome, it was also highly likely that MIS would reduce mortality by 24.3% with moderate confidence ($\alpha = 0.05, \beta = 80\%$, **Table 1**).

In the sensitivity analysis with different prior (**Table 1**), the result would be inconclusive if the risk reduction were assumed to be 15 or 10%, because the cumulative z curve did not cross the monitoring boundary; neither had the required information size been reached. If we assume higher confidence ($\beta = 90\%$) or a lower event proportion (58.0%) in the control group, both analyses would yield a crossed superiority boundary indicating the efficacy of MIS. For the secondary outcome, the evidence was sufficient to conclude MIS is better even if the effect size decreased to 10%, the power increased to 90%, or the heterogeneity increased to 30%.

In another sensitivity analysis, we included only trials with blind outcome assessment (**Figure 4A**). The cumulative z score crossed the required information size with an adjusted risk ratio of 0.86 (95% CI, 0.73–1.02) using the same prior in the main analysis. We further assumed that the results of the ongoing RCT (NCT02880878: estimated 300 participants) are futile (in this case, both the treatment and control groups would have the

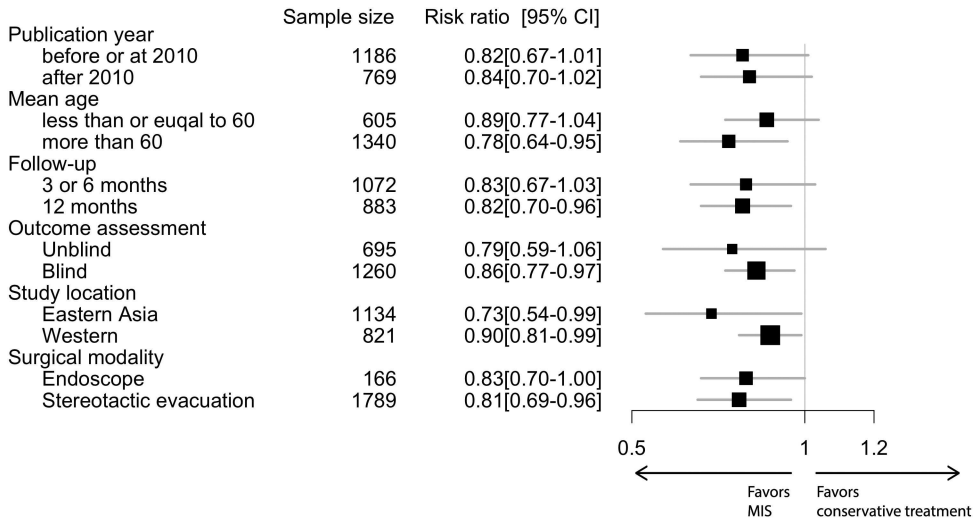

FIGURE 2 | Subgroup analysis in conventional meta-analysis.

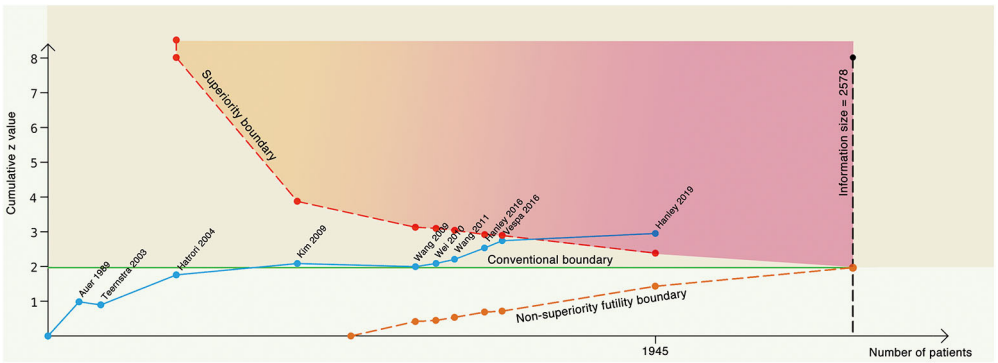

FIGURE 3 | Trial sequential analysis with $\alpha = 5\%$, $\beta = 80\%$ to detect 18.8% relative risk reduction. The blue line represents the cumulative z line, the green line represents the conventional boundary, the red dotted line represents the superiority boundary, the orange dotted line represents the noninferiority futility boundary, and the black dotted line represents the acquired information size.

same outcome incidence: 58%) to inspect the robustness of our results (**Figure 4B**). The cumulative *z* curve still stood above the superiority boundary that suggested our analysis might not be subjective in future trials.

DISCUSSION

Minimally invasive surgery demonstrated improved clinical outcome (the proportion of surviving patients without or with slight neurological debilitation) over conservative treatment in patients with intracerebral hemorrhage. The robust results with several sensitivity analyses indicated a sufficient amount of evidence favoring MIS such that further trials are unlikely to change the conclusion.

Several meta-analyses (5–10) have been published on the similar topic, but we argue these meta-analyses have their limitations compared to our approach, including a mixture control group, involving nonrandomized trials and comprising trials with selection bias (**Table 2**). The CI may shrink when combining many studies with different controls. Nonrandomized studies have the potential risk of selection bias, in which surgeons may select those patients with better outcome probability. Studies with both craniotomy and MIS in which the decision of craniotomy or MIS was made at the discretion of surgeons also introduced selection bias if only the MIS patients were included in the meta-analysis (4, 27). We also argue that our analysis answered the question of which treatment is better, especially in the circumstances that the latest trial (11) was futile, and the question whether we need more trials to compare MIS vs. conservative treatment in patients with intracerebral hemorrhage.

Trial sequential analysis can avoid premature conclusion when meta-analyses based on traditional hypothesis testing would have falsely identified the effect as significant (12, 15). Another advantage of TSA is to estimate the sample size of future trials if the current result is inconclusive. The major limitation of

TABLE 1 | Trial sequential analysis on primary and secondary outcome with different prior.

	Relative risk reduction	Power	Incidence in the control	Heterogeneity (diversity)	Information size	Risk ratio	Boundary	Explanation
(A) TRIAL SEQUENTIAL ANALYSIS ON PRIMARY OUTCOME (PROPORTION OF PATIENTS WITH MODIFIED rANKIN SCORE > 3)								
Conventional meta-analysis with random-effects model						0.82 (0.72–0.94)		
TSA	18.8 (Estimated)	80%	67.4%	81.9% (Estimated)	2578, not reached	0.81 (0.69–0.96)	Superiority crossed	MIS better
	15	80%	67.4%	81.9% (Estimated)	3994, not reached	0.81 (0.66–1.01)	Superiority nearly cross	Inconclusive
	10	80%	67.4%	81.9% (Estimated)	8807, not reached	0.81 (0.65–1.02)	Superiority not crossed	Inconclusive
	18.8 (Estimated)	90%	67.4%	81.9% (Estimated)	3452, not reached	0.81 (0.67–0.99)	Superiority crossed	MIS better
	18.8 (Estimated)	80%	58.0% (Latest study)	81.9% (Estimated)	4885, not reached	0.81 (0.66–1.00)	Superiority crossed	MIS better
(B) TRIAL SEQUENTIAL ANALYSIS ON SECONDARY OUTCOME (MORTALITY)								
Conventional meta-analysis with fix-effects model						0.76 (0.64–0.89)		
TSA	24.3 (Estimated)	80%	25.3%	0.0% (Estimated)	1435, reached	0.76 (0.63–0.90)	Superiority crossed	MIS better
	20	80%	25.3%	0.0% (Estimated)	2157, not reached	0.76 (0.63–0.91)	Superiority crossed	MIS better
	15	80%	25.3%	0.0% (Estimated)	3898, not reached	0.76 (0.61–0.94)	Superiority crossed	MIS better
	10	80%	25.3%	0.0% (Estimated)	8956, not reached	0.76 (0.60–0.96)	Superiority crossed	MIS better
	24.3 (Estimated)	90%	25.3%	0.0% (Estimated)	1921, reached	0.76 (0.62–0.93)	Superiority crossed	MIS better
	24.3 (Estimated)	80%	25.3%	30.0%	2050, not reached	0.76 (0.64–0.90)	Superiority crossed	MIS better

MIS, Minimal invasive surgery; TSA, Trial sequential analysis.

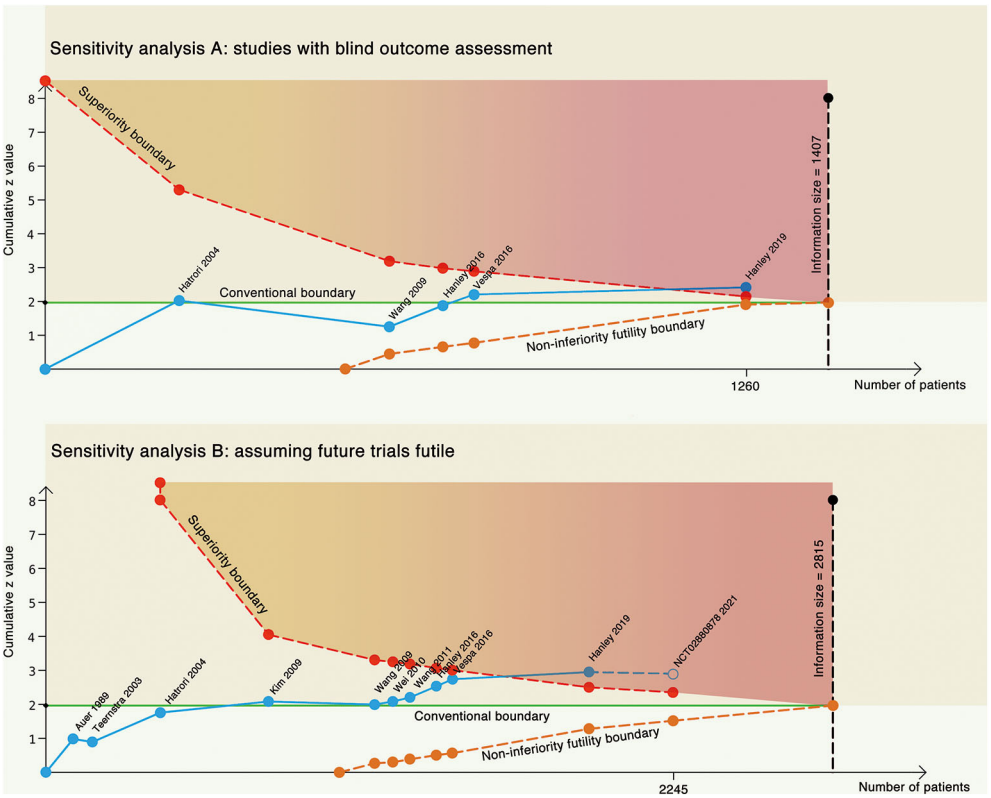

FIGURE 4 | Two sensitivity analyses. **(A)** Trial sequential analysis on only studies with blind outcome assessment. **(B)** Trial sequential analysis on all the studies, assuming future trial futile. The blue line represents the cumulative z line, the green line represents the conventional boundary, the red dotted line represents the superiority boundary, the orange dotted line represents the non-inferiority futility boundary, and the black dotted line represents the acquired information size.

TABLE 2 | Summary of previous published meta-amylases on the similar topic.

References	Treatment	Control	Included studies	Primary outcomes	Limits	Conclusion
Zhou et al. (9)	MIS	Conservative treatment or craniotomy	RCT	Death or dependence	The selection of MIS in Zuccarello Mendelow studies was biased; mixture control	MIS better
Akhigbe et al. (8)	MIS	Conservative treatment	RCT	Mortality	The selection of MIS in Zuccarello study was biased; only include five studies	Inconclusive
Yao et al. (7)	Endoscope	Stereotactic evacuation, conservative treatment or craniotomy	RCT + non-RCT	Mortality	Biased due to non-randomized studies; mixture control	Endoscope better
Xia et al. (6)	MIS	Craniotomy	RCT + non-RCT	Mortality	Biased due to non-randomized studies	MIS better
Tang et al. (5)	MIS	Conservative treatment or craniotomy	RCT + non-RCT	Death or dependence	Only include Eastern Asian patients; biased due to non-randomized studies; mixture control	MIS better
Scaggiante et al. (10)	MIS	Conservative treatment or craniotomy	RCT	Death or dependence	The selection of MIS in Zuccarello studies was biased; mixture control	MIS better

MIS, Minimal invasive surgery; RCT, randomized control trial.

applying TSA lies in that prespecified prior may have a significant impact on the result, which requires many sensitivity analyses to test the robustness.

We also calculated the required sample size of a single trial based on our meta-analysis: 554 patients are required to have an 80% chance of detection, to be significant at the 5% level, a decrease in the primary outcome measure from 67.4% in the control group to 55.9% in the experimental group. None of the current trials reached the required sample size including the latest published one. We also have to acknowledge the population diversity in calculating the sample size. Our subgroup analysis suggested the population might be an effective modifier with a higher effect in the Asian population. Although the mechanism was not clear, sample size calculation in future trials should incorporate the population information.

Because of the nature of comparing a surgical approach vs. a nonsurgical treatment, blinding of the patients was neither possible nor ethical, but several trials applied blinding to the outcome assessment. Our subgroup analysis shows that unblinded assessment had a larger effect size and a wider CI, suggesting the results were biased.

The effect of endoscopic surgery might be different from that of stereotactic evacuation. But several studies found that residue hemorrhage may be a risk factor for unfavorable outcomes in these patients (11, 28). The mean posttreatment day 1 hemorrhage volume reduction was roughly 30% in two stereotactic trials (11, 17). Only 59% of participants achieved the target hemorrhage volume reduction in the Hanley trial (11). In endoscopic trials, the compliance rate could be 95% (26), and the reduction of hemorrhage could be as high as 70% (26), which suggest a potential higher effect. For the per-protocol effect, the benefit of MIS would be larger in practice, considering the crossover rate is higher in the conservative group than in the surgical group (3, 27) as long as surgery was not inferior to conservative treatment.

Our study has several strengths. Our work added further evidence to the existing literature supporting MIS over conservative treatment in patients with intracerebral hemorrhage. Moreover, we answered the question whether we need more trials to save the cost of future unnecessary trials. Second, although the results of TSA were dependent on the prior, we used several sensitivity analyses to show that our results were robust at higher power assumption or within studies with high quality. We also included studies in both English and Chinese as there was geographic variation in the lifetime risk of stroke, with the highest risks in East Asia, Central Europe, and Eastern Europe (29, 30).

The major limitation of our study lies in the mixture of treatments as the differentiation between the two investigated treatments may lead to underpower. Comparative risks and effectiveness of different techniques have never been studied. Even trials using stereotactic evacuation were heterogeneous in applying different modalities. Another limitation of our study was the various definitions of outcome and follow-up time, which should be taken into account when interpreting these data. We were unable to answer the question which subgroup might benefit more from MIS, although we observed that mean age might be an effective modifier, which is consistent with the result in the latest trial (11).

CONCLUSION

Minimally invasive surgery seems to be more effective than conservative treatment in patients with intracerebral hemorrhage in reducing both morbidity and mortality. Repeating a clinical trial with similar devices, design, and outcomes is unlikely to change current evidence. Future trials should target at comparative effectiveness among different approaches.

AUTHOR CONTRIBUTIONS

XZ and LX collected the data. YA did the analysis. XZ and NQ wrote the 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.2020.00426/full#supplementary-material

REFERENCES

1. GBD 2016 Multiple Sclerosis Collaborators. Global, regional, and national burden of multiple sclerosis 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Neurol.* (2019) 18:269–85. doi: 10.1016/S1474-4422(18)30443-5
2. GBD 2016 Causes of Death Collaborators. Global, regional, and national age-sex specific mortality for 264 causes of death, 1980-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet*. (2017) 390:1151–210. doi: 10.1016/S0140-6736(17)32152-9
3. Mendelow AD, Gregson BA, Rowan EN, Murray GD, Gholkar A, Mitchell PM, et al. Early surgery vs. initial conservative treatment in patients with spontaneous supratentorial lobar intracerebral haematomas (STICH II): a randomised trial. *Lancet.* (2013) 382:397–408. doi: 10.1016/S0140-6736(13)60986-1
4. Zuccarello M, Brott T, Derex L, Kothari R, Sauerbeck L, Tew J, et al. Early surgical treatment for supratentorial intracerebral hemorrhage: a randomized feasibility study. *Stroke.* (1999) 30:1833–9. doi: 10.1161/01.STR.30.9.1833
5. Tang Y, Yin F, Fu D, Gao X, Lv Z, Li X. Efficacy and safety of minimal invasive surgery treatment in hypertensive intracerebral hemorrhage: a systematic review and meta-analysis. *BMC Neurol.* (2018) 9:1–11. doi: 10.1186/s12883-018-1138-9
6. Xia Z, Wu X, Li J, Liu Z, Chen F, Zhang L, et al. Minimally invasive surgery is superior to conventional craniotomy in patients with spontaneous supratentorial intracerebral hemorrhage: a

systematic review and meta-analysis. *World Neurosurg.* (2018) 115:266–73. doi: 10.1016/j.wneu.2018.04.181
7. Yao Z, Hu X, You C, He M. Effect and feasibility of endoscopic surgery in spontaneous intracerebral hemorrhage: a systematic review and meta-analysis. *World Neurosurg.* (2018) 113:348–56.e2. doi: 10.1016/j.wneu.2018.02.022
8. Akhigbe T, Okafor U, Sattar T, Rawluk D, Fahey T. Stereotactic-Guided evacuation of spontaneous supratentorial intracerebral hemorrhage: systematic review and meta-analysis. *World Neurosurg.* (2015) 84:451–60. doi: 10.1016/j.wneu.2015.03.051
9. Zhou X, Chen J, Li Q, Ren G, Yao G, Liu M, et al. Minimally invasive surgery for spontaneous supratentorial intracerebral hemorrhage: a meta-analysis of randomized controlled trials. *Stroke.* (2012) 43:2923–30. doi: 10.1161/STROKEAHA.112.667535
10. Scaggiante J, Zhang X, Mocco J, Kellner CP. Minimally invasive surgery for intracerebral hemorrhage. *Stroke.* (2018) 49:2612–20. doi: 10.1161/STROKEAHA.118.020688
11. Hanley DF, Thompson RE, Rosenblum M, Yenokyan G, Lane K, McBee N, et al. Efficacy and safety of minimally invasive surgery with thrombolysis in intracerebral haemorrhage evacuation (MISTIE III): a randomised, controlled, open-label, blinded endpoint phase 3 trial. *Lancet.* (2019) 393:1021–32. doi: 10.1016/S0140-6736(19)30195-3
12. Wetterslev J, Jakobsen JC, Gluud C. Trial Sequential Analysis in systematic reviews with meta-analysis. *BMC Med Res Methodol.* (2017) 17:39. doi: 10.1186/s12874-017-0315-7
13. Higgins JPT, Altman DG, Gøtzsche PC, Peter J, David M, Andrew OD, et al. The Cochrane Collaboration's tool for assessing risk of bias in randomised trials. *BMJ.* (2011) 343:d5928. doi: 10.1136/bmj.d5928
14. Higgins JPT, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. *BMJ.* (2003) 327:557–60. doi: 10.1136/bmj.327.7414.557
15. Thorlund K, Devereaux PJ, Wetterslev J, Guyatt G, Ioannidis JPA, Thabane L, et al. Can trial sequential monitoring boundaries reduce spurious inferences from meta-analyses? *Int J Epidemiol.* (2009) 38:276–86. doi: 10.1093/ije/dyn179
16. Auer LM, Deinsberger W, Niederkorn K, Gell G, Kleinert R, Schneider G, et al. Endoscopic surgery vs. medical treatment for spontaneous intracerebral hematoma: a randomized study. *J Neurosurg.* (1989) 70:530–5. doi: 10.3171/jns.1989.70.4.0530
17. Teernstra OPM, Evers SMAA, Lodder J, Leffers P, Franke CL, Blaauw G, et al. Stereotactic treatment of intracerebral hematoma by means of a plasminogen activator: a multicenter randomized controlled trial (SICHPA). *Stroke.* (2003) 34:968–74. doi: 10.1161/01.STR.0000063367.52044.40
18. Hattori N, Katayama Y, Maya Y, Gatherer A. Impact of stereotactic hematoma evacuation on activities of daily living during the chronic period following spontaneous putaminal hemorrhage: a randomized study. *J Neurosurg.* (2004) 101:417–20. doi: 10.3171/jns.2004.101.3.0417
19. Miller CM, Vespa P, Saver JL, Kidwell CS, Carmichael ST, Alger J, et al. Image-guided endoscopic evacuation of spontaneous intracerebral hemorrhage. *Surg Neurol.* (2008) 69:441–6–discussion: 446. doi: 10.1016/j.surneu.2007.12.016
20. Kim YZ, Kim KH. Even in patients with a small he morrhagic volume, stereotactic-guided evacuation of spontaneous intracerebral hemorrhage improves functional outcome. *J Korean Neurosurg Soc.* (2009) 46:109–15. doi: 10.3340/jkns.2009.46.2.109
21. Luo JB, Peng B, Quan W, Cao ZK, Xiao GC, Lu JP, et al. Therapeutic effects of aspiration with a directional soft tube and conservative treatment on mild hemorrhage in the basal ganglion. *J First Military Med Univ.* (2008) 28:1352–3.
22. Wang W-Z, Jiang B, Liu H-M, Li D, Lu C-Z, Zhao Y-D, et al. Minimally invasive craniopuncture therapy vs. conservative treatment for spontaneous intracerebral hemorrhage: results from a randomized clinical trial in China. *Int J Stroke.* (2009) 4:11–6. doi: 10.1111/j.1747-4949.2009.00239.x
23. Wei PB, You C, Chen H, Zhang GB, He J, Yang M. Three treatments for moderate hypertensive intracerebral hemorrhage:a comparative therapeusis. *Chin J Cerebrovasc Dis.* (2010) 7:519–22. doi: 10.3969/j.issn.1672-5921.2010.10.004
24. Wang XY, Yang SY, Huang Y, Sun M, Zhao L, Zhuo J, et al. Effects of craniopuncture and drainage of intracerebral hemorrhage on brain edema and neurological outcome. *Chin J Contemp Neurol Neurosurg.* (2011) 11:230–5. doi: 10.3969/j.issn.1672-6731.2011.02.021
25. Hanley DF, Thompson RE, Muschelli J, Rosenblum M, McBee N, Lane K, et al. Safety and efficacy of minimally invasive surgery plus alteplase in intracerebral haemorrhage evacuation (MISTIE): a randomised, controlled, open-label, phase 2 trial. *Lancet Neurol.* (2016) 15:1228–37. doi: 10.1016/S1474-4422(16)30234-4
26. Vespa P, Hanley D, Betz J, Hoffer A, Engh J, Carter R, et al. ICES (Intraoperative stereotactic computed tomography-guided endoscopic surgery) for brain hemorrhage: a multicenter randomized controlled trial. *Stroke.* (2016) 47:2749–55. doi: 10.1161/STROKEAHA.116.013837
27. Mendelow AD, Gregson BA, Fernandes HM, Murray GD, Teasdale GM, Hope DT, et al. Early surgery vs. initial conservative treatment in patients with spontaneous supratentorial intracerebral haematomas in the International Surgical Trial in Intracerebral Haemorrhage (STICH): a randomised trial. *Lancet.* (2005) 365:387–97. doi: 10.1016/S0140-6736(05)70233-6
28. Zuo Y, Cheng G, Gao D-K, Zhang X, Zhen H-N, Zhang W, et al. Gross-total hematoma removal of hypertensive basal ganglia hemorrhages: a long-term follow-up. *J Neurol Sci.* (2009) 287:100–4. doi: 10.1016/j.jns.2009.08.046
29. GBD 2016 Lifetime Risk of Stroke Collaborators, Feigin VL, Nguyen G, Cercy K, Johnson CO, Alam T, et al. Global, Regional, and Country-Specific Lifetime Risks of Stroke, 1990 and 2016. *N Engl J Med.* (2018) 379:2429–37. doi: 10.1056/NEJMoa1804492
30. Holst LB, Petersen MW, Haase N, Perner A, Wetterslev J. Restrictive vs. liberal transfusion strategy for red blood cell transfusion: systematic review of randomised trials with meta-analysis and trial sequential analysis. *BMJ.* (2015) 350:h1354–4. doi: 10.1136/bmj.h1354

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Hemorrhagic Transformation in Ischemic Moyamoya Disease: Clinical Characteristics, Radiological Features, and Outcomes

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Objective: Hemorrhagic transformation (HT) in ischemic moyamoya disease (MMD), reasonably defined as hemorrhage events in patients with ischemic onset manifestation, leads to a poor outcome. This study aims to reveal factors associated with HT in patients with ischemic onset manifestation and to assess the outcome of these patients.

Methods: A total of 683 surgically managed patients with onset ischemic manifestation were included. The clinical variables of the HT and non-HT groups were compared, and risk factors were analyzed using logistic regression analysis. Recurrent stroke events (including hemorrhagic and ischemic) during the follow-up were documented. The cumulative incidence rate of stroke events was generated *via* Kaplan–Meier survival analysis. Outcomes were compared between HT and non-HT groups using propensity score analysis to account for between-group differences in baseline characteristics.

Results: Of 683 patients surgically treated in the overall cohort, 29 (4.3%) were classified as cases of HT. The majority manifestation of these patients was transient ischemic attack. Multivariate analysis showed that the normal cerebral perfusion according to the CT perfusion was identified as factors associated with HT [odds ratio (OR) 13.464, 95% CI 3.529–51.363, $P < 0.001$]. Patients who occurred HT had a worse outcome than patients without HT.

Conclusions: HT in adult ischemic MMD is a rare phenomenon, but it is strongly associated with increased disability rates and mortality. The normal cerebral perfusion is a possible risk factor associated with HT in adult ischemic MMD. Recognition of HT in adult ischemic MMD may contribute to an improved outcome.

Keywords: hemorrhage, hemorrhagic transformation, moyamoya disease, outcome, stroke

INTRODUCTION

Moyamoya disease (MMD) is a chronic occlusive-stenosis disorder at the terminal portion of the internal carotid artery, leading to intracranial ischemia and hemorrhage (1). The exact underlying mechanism between ischemic MMD and hemorrhagic MMD may be different and poorly understood. Although intracranial hemorrhage was less common than ischemic attack, it does contribute to the poor outcome in patients with MMD (2). Unlike most ischemic MMD patients who suffer from ischemic events, some patients with ischemic onset manifestation also developed intracranial hemorrhage, which we define as hemorrhagic transformation (HT). However, the clinical characteristics, radiological features, and outcomes of the ischemic MMD patients with HT remain unclear. At present, it is challenging to distinguish whether the patients will develop HT. As intracranial hemorrhage is hardly observed in pediatric MMD patients, in this study, we aim to explore the difference of clinical and radiological features between adult ischemic MMD patients with HT and without HT. Moreover, we studied the association of clinical outcomes and HT in adult ischemic MMD patients.

MATERIALS AND METHODS

Patients and Materials

The participants included in this study were from a multicenter cohort of Chinese MMD patients who had been treated between 2009 and 2018 (**Figure 1**). All patient's data were retrospectively reviewed, including clinical records and radiological data. The diagnosis of MMD was confirmed with digital subtraction angiography (DSA) and/or MR angiography based on the criteria of the Research Committee on Spontaneous Occlusion of the Circle of Willis (2012) (3). Pediatric and patients with hemorrhagic symptoms as the initial presentation and moyamoya syndrome caused by other systemic diseases were excluded.

Baseline clinical characteristics and imaging data were reviewed, such as age, sex, onset manifestation, past medical history, neurological status, and imaging findings. The onset manifestations were divided as follows: cerebral infarction, transient ischemic attack (TIA), headache, epilepsy, and asymptomatic. Suzuki stage was recorded as previously described (4). CT perfusion (CTP) was initiated 4 s after injection of a bolus of 40 ml of iobitridol (350 mg/ml, Xenetix; Guerbet, Aulnay-sous-Bois, France) at a rate of 5 ml/s into the antecubital vein (with a 20-gauge intravenous cannula) using a power injector. The acquisition parameters were as follows: 80 kVp, 150 mA, 0.28 s/rotation, 25 s CT data acquisition time, 5-mm section thickness, field matrix 512 × 512, and 40 images per section. The scan layers that contained basal ganglia were chosen in all patients. Cerebral perfusion assessment was performed by the new CTP staging system (pre-infarction staging system) proposed by our previous study (5). Neurological status was recorded using the modified Rankin Scale (mRS) score.

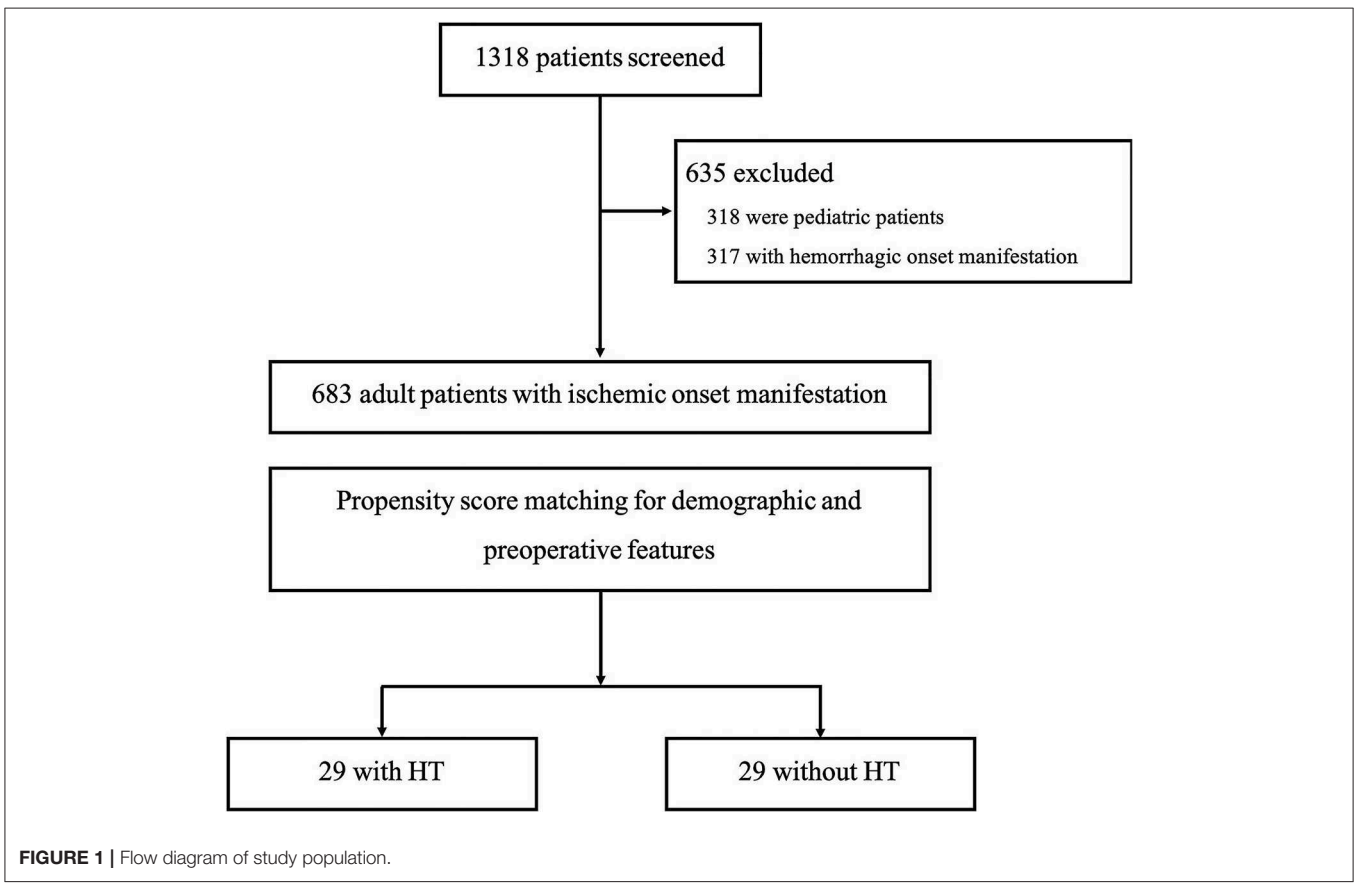

FIGURE 1 | Flow diagram of study population.

Surgical Modalities

Our principles of the surgical strategies were as follows. Revascularization surgery was performed on the symptomatic and hemodynamically affected hemisphere. Patients usually reviewed 3–6 months after the first surgery. If the patient's symptoms were significantly relieved after the first surgery and the patient did not have symptoms that could be attributed to the contralateral hemisphere, operative treatment of the contralateral hemisphere was not considered. Otherwise, surgery on the contralateral hemisphere was performed. The surgical procedures of MMD could be divided into two categories in our population: indirect bypass and combined bypass (6).

Follow-Up

Patients were followed up by clinical visit or telephone interviews at 3 and 6 months after surgery and annually thereafter. Doctors performing follow-up assessments were blinded to baseline information. Clinical outcomes including recurrent intracranial hemorrhage and ischemic stroke and neurological status were collected during follow-ups.

Statistical Analysis

All analyses were conducted using IBM SPSS statistical software (version 26.0). The categorical variables are presented as counts (with percentages); continuous variables are presented as the means ± standard deviations. The chi-square test was used to compare categorical variables. The Mann–Whitney *U*-test was performed for ordinal variables. Logistic regression and proportional odds regression were used to generate odds ratios (ORs) and 95% confidence intervals (CIs). A logistic regression model was built to identify predictors of HT in ischemic MMD patients.

Propensity score matching was used to reduce imbalances in the baseline characteristics between patients with or without HT. The propensity scores for the development of HT in ischemic MMD were estimated with a logistic multivariate regression model containing demographic characteristics (age and sex), pre-operative angiopathy (Suzuki stage, posterior circulation involvement, and bilateral lesions), and clinical status (mRS score on admission, CTP stage, and previous revascularization surgery). Using the nearest-neighbor method without replacement for propensity score matching, pairs of patients were matched with a match tolerance of 0.02 and a ratio of 1:1. In the matched pairs, the outcomes of interest were stroke events (including intracranial hemorrhage and ischemic stroke), neurological function deterioration, disability-free rate, and mRS scores during follow-up. Cumulative risk of hemorrhage was estimated by the Kaplan–Meier product-limit method. A P value < 0.05 was considered to be statistically significant.

RESULTS

Patient Characteristics

A total of 683 cases were included in this study (**Figure 1**). Baseline presentation and characteristics of the patient cohort are presented in **Table 1**. Of them, 29 patients developed HT. The occurrence of HT in adult ischemic MMD patients was 4.2%. The mean age of the patients with HT was 39.07 ± 10.39 years (range, 19∼59 years). The female-to-male ratio was 1:1. The most common onset manifestation was TIA (18/29, 62.1%). Twenty-one patients (21/29, 72.4%) developed the HT before the revascularization surgery, 11 of them (TIA in 10 cases and infarction in 1 case) suffered an intracranial hemorrhage within the 3 months after the initial ischemic symptom. And eight patients (8/29, 28.6%) developed the HT after the revascularization surgery (mean time, 34.8 ± 19.8

TABLE 1 | Baseline characteristics.

Characteristic	Total ($n = 683$)	Hemorrhagic transformation		p-value
		Present ($n = 29$)	Absent ($n = 654$)	
Mean age, y	38.99 ± 9.92	39.07 ± 10.39	38.99 ± 9.91	0.967
Sex male/female	312/371	17/12	295/359	0.153
Onset manifestation				0.168
TIA	289 (42.3)	18 (62.1)	271 (41.4)	
Infarction	325 (47.6)	11 (37.9)	314 (48.0)	
Headache	49 (7.2)	0 (0.0)	49 (7.5)	
Epilepsy	12 (1.8)	0 (0.0)	12 (1.8)	
Asymptomatic	8 (1.2)	0 (0.0)	8 (1.2)	
mRS on admission				0.567
0	38 (5.6)	0 (0.0)	38 (5.8)	
1	452 (66.2)	19 (65.5)	433 (66.2)	
2	145 (21.2)	8 (27.6)	137 (20.9)	
3	36 (5.3)	2 (6.9)	34 (5.2)	
4	12 (1.8)	0 (0.0)	12 (1.8)	
Medical history				
Hypertension	238 (34.8)	8 (27.6)	230 (35.2)	0.402
Diabetes	79 (11.6)	3 (10.3)	76 (11.6)	0.833
Hyperlipemia	43 (6.3)	4 (13.8)	39 (6.0)	0.089
Smoking	94 (13.8)	5 (17.2)	89 (13.6)	0.578
Suzuki stage*				0.760
I	5 (0.9)	0 (0.0)	5 (0.9)	
II	31 (5.4)	2 (6.9)	29 (5.3)	
III	168 (29.2)	8 (27.6)	160 (29.3)	
IV	205 (35.7)	8 (27.6)	197 (36.1)	
V	122 (21.2)	7 (24.1)	115 (21.1)	
VI	44 (7.7)	4 (13.8)	40 (7.3)	
Posterior involvement*	177 (30.8)	13 (44.8)	164 (30.0)	0.093
Bilateral lesions	607 (88.9)	27 (93.1)	580 (88.7)	0.459
CTP stage				<0.001
Normal	15 (2.3)	5 (17.2)	10 (1.6)	
I	28 (4.3)	0 (0.0)	28 (4.5)	
II	189 (29.2)	6 (20.7)	183 (29.6)	
III	202 (31.2)	11 (37.9)	191 (30.9)	
IV	213 (32.9)	7 (24.1)	206 (33.3)	

Values are numbers of cases (%) unless otherwise indicated. Mean values are presented with SDs.

**DSA was available in 575 patients.*

†CT perfusion was available in 647 patients.

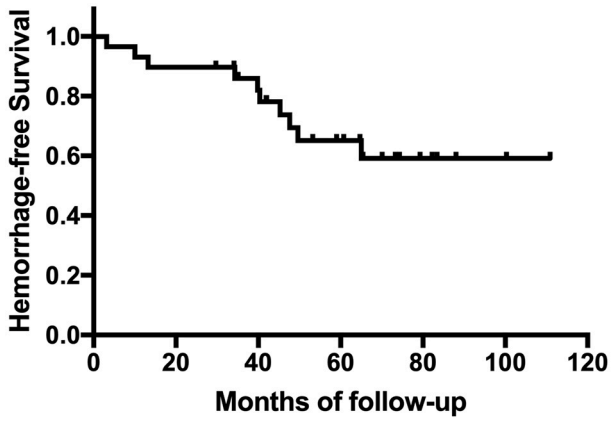

FIGURE 2 | Kaplan–Meier curve for cumulative rates of hemorrhage for patients with hemorrhagic transformation (HT).

months; range, 3∼65 months). Intraventricular hemorrhage (IVH) and intracerebral hemorrhage (ICH) were the most frequent presentations observed on CT scans, accounting for 41.4% (12/29) and 37.9% (11/29), respectively. Besides, four patients were demonstrated ICH + IVH and two patients were demonstrated subarachnoid hemorrhage (SAH). The Suzuki stage of patients mostly were stage III and stage IV according to the Suzuki classification. All patients with HT underwent CTP. The measure of CTP was performed within 3 days after admission, and all patients were not in the acute stage of cerebral hemorrhage. Of them, five patients (5/29, 17.2%) with normal perfusion, zero patients (0.0%) in stage I, six patients (6/29, 20.7%) in stage II, 11 patients (11/29, 37.9%) in stage III, and seven patients (7/29, 24.1%) in stage IV. Kaplan–Meier curves for intracranial hemorrhage-free survival of patients with HT are shown in **Figure 2**.

Risk Factors of Hemorrhagic Transformation

Univariable and multivariable ORs for the risk factors of HT are shown in **Table 2**. Univariate analysis showed that patients with normal cerebral perfusion status were more likely to develop HT. The multivariate analysis demonstrated that the normal cerebral perfusion status remains significantly associated with an increased risk of HT. Age, sex, hypertension, diabetes, hyperlipemia, smoking, TIA manifestation, Suzuki stage, posterior involvement, bilateral lesions, and previous revascularization surgery history were not associated with any increased risk of HT in the analysis ($P > 0.05$).

Hemorrhagic Transformation and Long-Term Clinical Outcomes

Within the overall propensity score-matched cohort of 58 patients, the two groups were compared with each other to verify that no significant differences were present in baseline characteristics between these two groups after the propensity score matching (**Table 3**). Outcomes of patients in the propensity score-matched cases are shown in **Table 4**. During the follow-up period (mean time, 71.9 ± 29.0 months; range, 12∼116 months), 15 stroke events (including 10 intracranial hemorrhages and five ischemic strokes) were observed, two patients experienced both intracranial hemorrhage and ischemic stroke. One of them experienced multiple hemorrhage events, which contributed to a poor outcome. There was a significant difference in the mRS score between these two groups at follow-up ($P = 0.047$; **Figure 3**). Forty-six patients (46/58, 79.3%) recovered without disability at the final follow-up. More patients in the HT group were disabled or dead (mRS score 3∼6) than in the non-HT group (7/29, 24.1 vs. 3/29, 10.3%, $P = 0.086$). In comparison to the mRS score on admission, the mRS score at the last follow-up showed improvement of 1–2 points in 35 cases (35/58, 60.3%), remained unchanged in 15 cases (15/58, 25.9%), and eight patients (8/58, 13.8%) had worse neurological functions. Follow-up mRS scores showed deterioration (compared to the scores on admission) in seven patients (7/29, 24.1%) in the HT group but only one patient (1/29, 3.4%) in the non-HT group ($P = 0.05$; **Figure 3**). However, it should also be noted that five patients (5/29, 17.2%) in the non-HT group had poor neurological functions on admission (mRS score 3∼4), in contrast to one patient (1/29, 3.4%) in the HT group (**Figure 3**).

TABLE 2 | Logistic regression analysis for hemorrhagic transformation in adult ischemic MMD.

Characteristic	Univariate analyses			Multivariate analyses		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Mean age, y	1.001	0.964–1.039	0.967	1.008	0.963–1.506	0.720
Male	1.724	0.810–3.668	0.157	1.878	0.785–4.494	0.157
Hypertension	0.702	0.306–1.611	0.404	0.530	0.200–1.408	0.203
Diabetes	0.878	0.259–2.969	0.834	1.010	0.243–4.195	0.989
Hyperlipemia	2.523	0.837–7.610	0.100	3.437	0.880–13.417	0.076
Smoking	1.323	0.492–3.556	0.580	0.854	0.259–2.817	0.796
TIA manifestation	2.313	1.075–4.975	0.032	2.150	0.936–4.939	0.071
Suzuki stage	1.170	0.820–1.670	0.387	1.111	0.743–1.663	0.608
Posterior involvement	1.893	0.890–4.024	0.097	1.902	0.830–4.361	0.129
Bilateral lesions	1.722	0.401–7.391	0.464	1.406	0.285–6.935	0.676
CTP stage						
Normal	REF	REF	REF	REF	REF	REF
I	<0.001	–	0.998	<0.001	–	0.998
II	0.066	0.017–0.252	<0.001	0.057	0.012–0.261	<0.001
III	0.115	0.034–0.395	0.001	0.132	0.031–0.558	0.006
IV	0.068	0.018–0.252	<0.001	0.057	0.013–0.254	<0.001
Previous revascularization	1.512	0.681–3.356	0.309	1.406	0.572–3.460	0.458

DISCUSSION

There were mainly two typical manifestations in MMD patients: intracranial hemorrhage, which was commonly observed in adults, and ischemic stroke, which was mostly observed in

TABLE 3 | Baseline characters of patients in the propensity score–matched cases.

Characteristic	Total ($n = 58$)	Hemorrhagic transformation		*P*-value
		Present ($n = 29$)	Absent ($n = 29$)	
Mean age, y	38.88 ± 10.16	39.07 ± 10.39	38.69 ± 10.10	0.888
Sex male/female	38/20	17/12	21/8	0.269
Onset manifestation				0.188
TIA	31 (53.4)	18 (62.1)	13 (44.8)	
Infarction	27 (46.6)	11 (37.9)	16 (55.2)	
mRS on admission				0.335
1	38 (65.5)	19 (65.5)	18 (62.1)	
2	14 (24.1)	8 (27.6)	6 (20.7)	
3	4 (6.9)	2 (6.9)	3 (10.3)	
4	2 (3.4)	0 (0.0)	2 (6.9)	
Medical history				
Hypertension	16 (27.6)	8 (27.6)	8 (27.6)	1.000
Diabetes	7 (12.1)	3 (10.3)	4(13.8)	0.687
Hyperlipemia	8 (13.8)	4 (13.8)	4 (13.8)	1.000
Smoking	9 (15.5)	5 (17.2)	4 (13.8)	0.717
Suzuki stage				0.729
I	0 (0.0)	0 (0.0)	0 (0.0)	
II	3 (5.2)	2 (6.9)	1 (3.4)	
III	13 (22.4)	8 (27.6)	5 (17.2)	
IV	20 (34.5)	8 (27.6)	12 (41.4)	
V	15 (25.9)	7 (24.1)	8 (27.6)	
VI	7 (12.1)	4 (13.8)	3 (10.3)	
Posterior involvement	21 (36.2)	13 (44.8)	8 (27.6)	0.172
Bilateral lesions	53 (91.4)	27 (93.1)	26 (89.7)	0.640
CTP stage				0.220
Normal	7 (12.1)	5 (17.2)	2 (6.9)	
I	0 (0.0)	0 (0.0)	0 (0.0)	
II	19 (32.8)	6 (20.7)	13 (44.8)	
III	19 (32.8)	11 (37.9)	8 (27.6)	
IV	13 (24.1)	7 (24.1)	6 (20.7)	

pediatrics. Ischemic manifestations were more common than hemorrhagic manifestations (7, 8). MMD manifested as ischemia or hemorrhage at the initial attack may differ greatly in many aspects such as pathological features, disease progression, and prognosis (1, 9). The exact cause for hemorrhage in MMD has not been fully elucidated. Previous studies have revealed that the rupture of microaneurysms in the collateral vessels at the base of the skull was related to the hemorrhagic presentation in patients with MMD (10). The most common adverse event which contributed to a poor outcome in patients manifested as ischemia was recurrent ischemic stroke, while rebleeding plays an important role in patients with hemorrhagic manifestation. Recently, a long follow-up study reported that the average annual ischemic stroke incidence in patients with hemorrhagic manifestation was only 0.3% (11). However, there were few reports about intracranial hemorrhage in patients manifested as ischemia, considering that intracranial hemorrhage contributed to higher mortality than ischemic events in MMD patients (12, 13). This large cohort study extends our knowledge of the clinical characteristics, radiological features, and long-term outcome of adult ischemic patients with HT.

In this study, we found that the incidence of HT in adult ischemic MMD patients was 4.2%. Previous studies reported that HT occurs in as many as 10–40% of patients with ischemic stroke (14). The use of alteplase and the brain–blood barrier disruption might contribute to HT (15, 16). However, in our cohort, 11 patients developed HT within 3 months after the initial ischemic manifestation, while 18 patients experienced ischemic strokes or TIAs for more than 2 years. The underlying mechanism of HT in patients with MMD might differ from those with acute ischemic stroke. In addition, TIA was the most common symptom before intracranial hemorrhage in this cohort (18/29, 62.1%), and univariate analysis confirmed the significant association between TIA manifestation and incidence of HT (OR 2.313, 95% CI 1.075–4.975, $P = 0.032$). However, multivariate analysis showed no significance. It should also be noted that eight of them developed HT after the revascularization surgery. We suggest that adult ischemic MMD patients take 100 mg of aspirin (The Bayer Company) daily for antiplatelet therapy. Although our previous study showed that aspirin might not increase the risk of hemorrhages in adult ischemic MMD patients (17), whether aspirin is safe for patients with the potential of HT remains uncertain. The outcome of patients who takes aspirin may be worse when developing an intracranial hemorrhage than those who do not. In this study, we observed that patients who occurred HT after the revascularization surgery have a worse neurological function at the last follow-up than patients occurred HT before the revascularization surgery (mRS score 2.75 ± 2.32 vs. 1.05 ± 1.47, $P = 0.025$). Moreover, among 29 patients occurred HT, eight of these occurred HT after the surgery (six direct revascularization vs. two indirect revascularization). Patients with direct revascularization have a higher percentage of HT. The vessels of MMD patients might be abnormal vasodilation due to the chronic hypoperfusion. The immediate increase of the blood flow after the direct revascularization might increase the risk of hemorrhage.

The determination of the HT risk is essential to provide information for appropriate management for adult ischemic MMD. At present, various factors were found to be associated with intracranial hemorrhage in MMD. Kikuta et al. (18) reported the presence of multiple microbleeds on magnetic resonance imaging to be an independent predictor of intracranial hemorrhage. Liu et al. (19) found that the involvement of the posterior circulation was associated with the hemorrhage in MMD. Meanwhile, other factors such as smoking and hypertension have already been considered as risk factors for hemorrhagic stroke (20–22). However, in this study, univariable analysis revealed that posterior circulation involvement, smoking, and hypertension were not significant predictors of HT.

Furthermore, the results of CTP demonstrated that patients with HT had a higher proportion of normal cerebral perfusion than patients without HT (5/29, 17.2 vs. 10/654, 1.6%, *P*

TABLE 4 | Outcomes of patients in the propensity score–matched cases.

Outcomes	Total ($n = 58$)	HT Group ($n = 29$)	non-HT Group ($n = 29$)	*P*-value	OR (95% CI)	Adjusted *P*-value
mRS score at discharge				0.321†	0.752 (0.198–2.861)‡	0.676
1	42 (72.4)	22 (75.9)	20 (69.0)			
2	10 (17.2)	6 (20.7)	4 (13.8)			
3	4 (6.9)	1 (3.4)	3 (10.3)			
4	2 (3.4)	0 (0.0)	2 (6.9)			
Stroke event during follow-up	13 (22.4)	10 (34.5)	3 (10.3)	0.028*	4.561(1.103–18.859)	
Hemorrhage during follow-up	10 (17.2)	10 (34.5)	0 (0.0)	0.001*	1.526 (1.172–1.988)	
Infarction during follow-up	5 (10.3)	2 (6.9)	3 (8.6)	0.640*	0.642 (0.099–4.159)	
mRS score at follow-up				0.476†	1.693 (1.007–2.846)	0.047
0	22 (37.9)	10 (34.5)	12 (41.4)			
1	24 (41.4)	11 (37.9)	13 (44.8)			
2	2 (3.4)	1 (3.4)	1 (3.4)			
3	5 (8.6)	3 (10.3)	2 (6.9)			
4	1 (1.7)	0 (0.0)	1 (3.4)			
5	2 (3.4)	2 (6.9)	0 (0.0)			
6	2 (3.4)	2 (6.9)	0 (0.0)			
Disability at follow-up (mRS > 2)	10 (17.2)	7 (24.1)	3 (10.3)	0.164*	4.355 (0.812–23.367)	0.086
Neurological function deterioration	8 (13.8)	7 (24.1)	1 (3.4)	0.022*	8.696 (0.986–76.722)	0.050

Values are numbers of cases (%) unless otherwise indicated. Mean values are presented with SDs.

* *McNemar test.*

† *Marginal homogeneity test.*

‡ *Adjusted for mRS score on admission.*

< 0.001). Interestingly, only 2.3% of patients in this cohort had normal cerebral perfusion, yet nearly more than half of them occurred HT. Multivariate analysis confirmed that normal cerebral perfusion status was significantly associated with HT (OR 13.464, 95% CI 3.529–51.363, $P < 0.001$). It should be noted that the patients were mostly in stage III and stage IV according to the Suzuki classification. In order to maintain a normal perfusion status, these patients are usually accompanied with good collateral circulation compensations even some of which were regarded as dangerous collateral vessels and closely related to hemorrhagic stroke recurrence in adult hemorrhagic MMD, while the rupture of these vasodilated abnormal collateral vessels might contribute to intracranial hemorrhage (23). In this study, most patients presented with IVH and ICH (41.4 and 37.9%, respectively), only two patients presented with SAH, which represent the typical presentation of the ruptured collateral vessels in MMD. Therefore, it might provide some support for our hypothesis.

In propensity score-matched cases, we have shown that the mRS score at the last follow-up was significantly different between patients with HT and without HT ($P = 0.047$). The severe disability and mortality of patients with HT were higher than that of patients without HT, while it did not have a significant difference ($P = 0.086$). We also observed that more patients observed with neurological function deterioration were in the HT group ($P = 0.05$), but it might be because some patients developed HT during the follow-up period which caused neurological function deterioration. As aforementioned, we suggest adult ischemic MMD patients take aspirin for antiplatelet therapy. Therefore, we advise that the time period of taking aspirin should be more accurate in these patients to decrease the incidence and mortality of hemorrhage events.

There are study limitations that need to be addressed for accurate interpretation of our data. In this study, only adult ischemic patients treated surgically were included, and this introduced selection bias as patients with less severe MMD disease might have been treated conservatively and were excluded; therefore, the conclusion drawn may not be generalizable to all patients with MMD. Although cerebral CT or magnetic resonance imaging scans have been performed at the point of new symptom manifestations during follow-up, DSA was not routinely performed, and the data are, therefore, not available in some of the patients; thus, limited radiographic evidence of disease progression must be noted. In addition, similar to all retrospective studies, this is a multicenter study over a time span of ≈10 years, and not all patients were followed up on a regular basis. This may render the results prone to potential attrition biases.

CONCLUSIONS

In summary, HT in adult ischemic MMD is a rare phenomenon. Our data support that normal cerebral perfusion is a possible risk factor associated with HT in adult ischemic MMD. HT was strongly associated with increased disability rates and

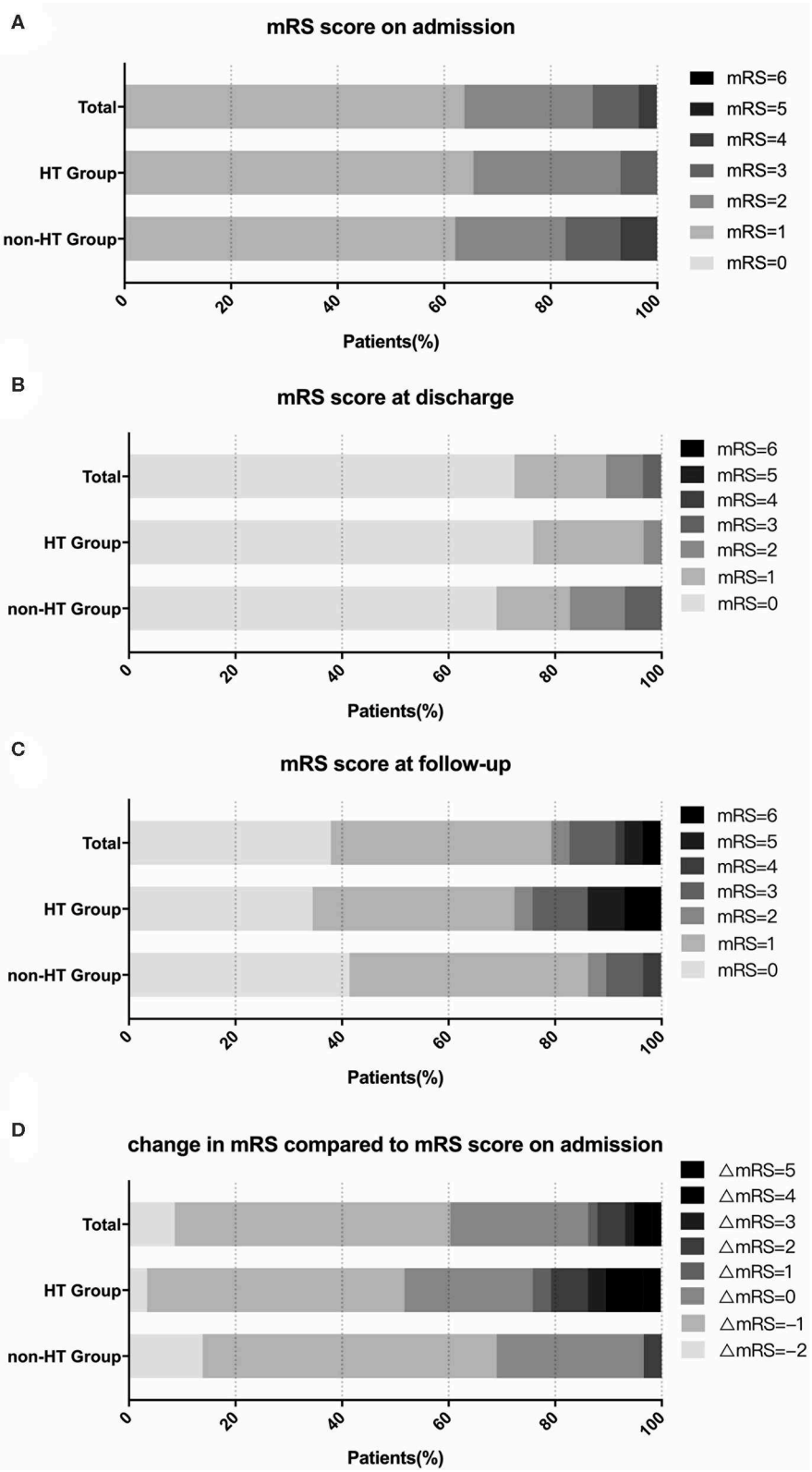

FIGURE 3 | Change in modified Rankin Scale (mRS) scores in the propensity score-matched cohort. The graphs show the proportions of patients stratified by mRS score. There was no statistically significant difference in mRS scores on admission **(A)**, at discharge **(B)**, and at follow-up **(C)** between the hemorrhagic transformation (HT) and non-HT groups. Compared to their neurological status on admission, the mRS score at follow-up showed a deterioration in 20.7% of patients in the HT group but 0% of the non-HT group **(D)**.

mortality. The concept of HT in adult ischemic MMD may contribute to further improvement in the outcome of MMD as a result of appropriate antiplatelet management arising from HT risk stratification.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB of Beijing Tiantan Hospital, Capital Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JL designed the study, drafted the manuscript, performed data collection and data analysis, contributed to the discussion, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. ZL performed data collection and contributed to the discussion. XC and YZ contributed to the discussion and edited the manuscript. GS performed data collection. JZ provided approval for publication of the content. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Scott RM, Smith ER. Moyamoya disease and moyamoya syndrome. *N Engl J Med*. (2009) 360:1226–37. doi: 10.1056/NEJMra0804622
2. Miyamoto S, Yoshimoto T, Hashimoto N, Okada Y, Tsuji I, Tominaga T, et al. Effects of extracranial-intracranial bypass for patients with hemorrhagic moyamoya disease: results of the Japan Adult Moyamoya Trial. *Stroke*. (2014) 45:1415–21. doi: 10.1161/STROKEAHA.113.004386
3. Research Committee on the P, Treatment of Spontaneous Occlusion of the Circle of W, Health Labour Sciences Research Grant for Research on Measures for Infractable D. Guidelines for diagnosis and treatment of moyamoya disease (spontaneous occlusion of the circle of Willis). *Neurol Med Chir*. (2012) 52:245–66. doi: 10.2176/nmc.52.245
4. Suzuki J, Takaku A. Cerebrovascular "moyamoya" disease. disease showing abnormal net-like vessels in base of brain. *Arch Neurol*. (1969) 20:288–99. doi: 10.1001/archneur.1969.00480090076012
5. Yin H, Liu X, Zhang D, Zhang Y, Wang R, Zhao M, et al. A novel staging system to evaluate cerebral hypoperfusion in patients with moyamoya disease. *Stroke*. (2018) 49:2837–43. doi: 10.1161/STROKEAHA.118.022628
6. Acker G, Fekonja L, Vajkoczy P. Surgical management of moyamoya disease. *Stroke*. (2018) 49:476–82. doi: 10.1161/STROKEAHA.117.018563
7. Liu XJ, Zhang D, Wang S, Zhao YL, Teo M, Wang R, et al. Clinical features and long-term outcomes of moyamoya disease: a single-center experience with 528 cases in China. *J Neurosurg*. (2015) 122:392–9. doi: 10.3171/2014.10.JNS132369
8. Duan L, Bao XY, Yang WZ, Shi WC, Li DS, Zhang ZS, et al. Moyamoya disease in China: its clinical features and outcomes. *Stroke*. (2012) 43:56–60. doi: 10.1161/STROKEAHA.111.621300
9. Kuroda S, Houkin K. Moyamoya disease: current concepts and future perspectives. *Lancet Neurol*. (2008) 7:1056–66. doi: 10.1016/S1474-4422(08)70240-0
10. Liu X, Zhang D, Shuo W, Zhao Y, Wang R, Zhao J. Long term outcome after conservative and surgical treatment of haemorrhagic moyamoya disease. *J Neurol Neurosurg Psychiatry*. (2013) 84:258–65. doi: 10.1136/jnnp-2012-302236
11. Kang S, Liu X, Zhang D, Wang R, Zhang Y, Zhang Q, et al. Natural course of moyamoya disease in patients with prior hemorrhagic stroke. *Stroke*. (2019) 50:1060–6. doi: 10.1161/STROKEAHA.118.022771
12. Morioka M, Hamada J, Todaka T, Yano S, Kai Y, Ushio Y. High-risk age for rebleeding in patients with hemorrhagic moyamoya disease: long-term follow-up study. *Neurosurgery*. (2003) 52:1049–54. doi: 10.1227/01.NEU.0000058223.73857.F4
13. Zhao M, Deng X, Zhang D, Wang S, Zhang Y, Wang R, et al. Risk factors for and outcomes of postoperative complications in adult patients with moyamoya disease. *J Neurosurg*. (2018) 130:337–673. doi: 10.3171/2017.10.JNS171749
14. Jickling GC, Liu D, Stamova B, Ander BP, Zhan X, Lu A, et al. Hemorrhagic transformation after ischemic stroke in animals and humans. *J Cereb Blood Flow Metab*. (2014) 34:185–99. doi: 10.1038/jcbfm.2013.203
15. Yaghi S, Willey JZ, Cucchiara B, Goldstein JN, Gonzales NR, Khatri P, et al. Treatment and outcome of hemorrhagic transformation after intravenous alteplase in acute ischemic stroke: a scientific statement for healthcare professionals from the American heart Association/American Stroke association. *Stroke*. (2017) 48:e343–e61. doi: 10.1161/STR.0000000000000152
16. Villringer K, Sanz Cuesta BE, Ostwaldt AC, Grittner U, Brunecker P, Khalil AA, et al. DCE-MRI blood-brain barrier assessment in acute ischemic stroke. *Neurology*. (2017) 88:433–40. doi: 10.1212/WNL.0000000000003566
17. Zhao Y, Zhang Q, Zhang D, Zhao Y. Effect of aspirin in postoperative management of adult ischemic moyamoya disease. *World Neurosurg*. (2017) 105:728–31. doi: 10.1016/j.wneu.2017.06.057
18. Kikuta K, Takagi Y, Nozaki K, Sawamoto N, Fukuyama H, Hashimoto N. The presence of multiple microbleeds as a predictor of subsequent cerebral hemorrhage in patients with moyamoya disease. *Neurosurgery*. (2008) 62:104–11. doi: 10.1227/01.NEU.0000311067.41239.E6
19. Liu P, Liu AH, Han C, Chen C, Lv XL, Li DS, et al. Difference in angiographic characteristics between hemorrhagic and nonhemorrhagic hemispheres associated with hemorrhage risk of moyamoya disease in adults: a self-controlled study. *World Neurosurg*. (2016) 95:348–56. doi: 10.1016/j.wneu.2016.08.033

20. Nordahl H, Osler M, Frederiksen BL, Andersen I, Prescott E, Overvad K, et al. Combined effects of socioeconomic position, smoking, and hypertension on risk of ischemic and hemorrhagic stroke. *Stroke*. (2014) 45:2582–7. doi: 10.1161/STROKEAHA.114.005252
21. Qureshi AI, Palesch YY, Barsan WG, Hanley DF, Hsu CY, Martin RL, et al. Intensive blood-pressure lowering in patients with acute cerebral hemorrhage. *N Engl J Med*. (2016) 375:1033–43. doi: 10.1056/NEJMoa1603460
22. Lattanzi S, Silvestrini M. Optimal achieved blood pressure in acute intracerebral hemorrhage: INTERACT2. *Neurology*. (2015) 85:557–8. doi: 10.1212/01.wnl.0000470918.40985.d0
23. Kim KM, Kim JE, Cho WS, Kang HS, Son YJ, Han MH, et al. Natural history and risk factor of recurrent hemorrhage in hemorrhagic adult moyamoya disease. *Neurosurgery*. (2017) 81:289–96. doi: 10.1093/neuros/nyw179

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Reversal Treatment in Oral Anticoagulant-Related Intracerebral Hemorrhage—An Observational Study Based on the Swedish Stroke Register

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Introduction: Intracerebral hemorrhage (ICH) is the most serious adverse effect of oral anticoagulant (OAC) treatment. The effect of OAC reversal therapy on outcome is uncertain. We compared 90-day survival and functional outcome in patients with OAC-ICH who received OAC reversal therapy with those who did not.

Methods: Data from The Swedish Stroke Register (Riksstroke) for all registered cases of OAC-ICH during 2017 (572 patients) were used to obtain information on reversal ($n = 369$) and non-reversal ($n = 203$) treatment receiving patients. Univariate and multivariate Cox regression analysis stratified for level of consciousness (LOC) on admission, and adjustment for relevant baseline variables, was used to compare 90-day Hazard Ratios (HR) for mortality.

Results: Sixty-five percent of patients received reversal treatment. These patients were younger, more often pre-stroke independent and alert at presentation. Withholding reversal treatment was associated with an increased death rate ($HR = 1.47$; 95% CI: 1.08–2.01) in a Cox regression model stratified for LOC and adjusted for baseline imbalances. Additional factors associated with an increased 90-day death rate were male sex ($HR = 1.42$; 95% CI: 1.06–1.92), age ($HR = 1.05$; 95% CI: 1.02–1.07), and intraventricular hemorrhage ($HR = 2.41$; CI: 1.77–3.29).

Conclusion: In this large observational study 35% of patients with OAC-ICH did not receive reversal treatment. Patients receiving OAC-reversal treatment had an improved 90-day mortality outcome compared to those not receiving treatment. Mortality was strongly related to LOC. Further, and larger, studies are required to determine which patient groups may benefit from reversal therapy and in whom non-reversal is adequate.

Keywords: patient outcome, acute stroke, intracranial hemorrhage, mortality, survival, anticoagulants, prognosis, hemostatic techniques

INTRODUCTION

Intracerebral hemorrhage (ICH) is the most serious complication of oral anticoagulant therapy (OAC) with an approximate yearly incidence of 0.1–0.6% (1, 2). The increased use of OAC for the prevention of ischemic stroke in patients with non-valvular atrial fibrillation has led to an increased number of OAC-related ICH (3, 4). Prognosis after OAC-ICH is poor, with reported mortality rates reaching 45%, and functional dependency rates of 75% by 3 months, regardless of OAC type (5–8). However, recent data show that 30-day mortality rates following OAC-ICH may be lower in patients with non-vitamin K oral anticoagulant (NOAC)—related ICH compared to vitamin K antagonist (VKA)—related ICH (9). Factors predicting poor outcome and high early case fatality in OAC-ICH include hematoma expansion, large hematoma volumes, and intraventricular hemorrhage (10–14). Early administration of hemostatic therapy can inhibit hematoma expansion, yet the benefit of this measure on patient outcome is uncertain (15, 16).

The 2014 European Stroke Organization (ESO) guidelines on management of spontaneous intracerebral hemorrhage provided no recommendation regarding hemostatic treatment following OAC-ICH (17). However, reversal therapy for OAC-ICH is recommended in the ESO guidelines specifically on this topic published 2019 (18), although these recommendations are based on weak quality evidence. Vitamin K and four-factor prothrombin complex concentrate (PCC) are recommended for reversal of VKA activity (19, 20). Direct antidotes are recommended for the reversal of NOAC, and PCC is recommended in the absence of a specific antidote (20, 21).

Our study aims to delineate mortality and functional outcome at 90 days post-OAC-ICH by comparing individuals receiving OAC-reversal therapy to individuals not receiving reversal therapy in a large nationwide stroke cohort representing recent clinical practice in Sweden.

METHODS

Database and Study Population

All patients >18 years who presented with ICH (ICD.10 I61.9) during ongoing anticoagulant treatment, and who were registered in The Swedish Stroke Register (Riksstroke) between January 1, 2017 and December 31, 2017 were included. Patients were anticoagulated prior to ICH with either VKA, Apixaban, Dabigatran, or Rivaroxaban.

Riksstroke is a nationwide hospital-based stroke quality register, with a coverage of >90% of hospital admitted stroke cases in Sweden (22). Patients were registered in Riksstroke during their hospital stay. Three-month follow-up data were attained from Riksstroke's follow-up questionnaire, which was distributed by mail, with two postal reminders to non-respondents. The follow-up questionnaire was either completed by the patient alone, with caregiver assistance, or by caregiver alone. Data on indications for anticoagulation therapy other than atrial fibrillation, reasons for withstanding reversal treatment, and laboratory values regarding NOAC serum concentrations were not available. All-cause mortality status was obtained from the Swedish Causes of Death Register, with a coverage of >98% (23). Patients who died within the 90 days were considered followed up.

TABLE 1 | Baseline characteristics of 572 patients with OAC-ICH comparing patients that received reversal treatment compared to patients who did not receive reversal treatment.

Variables	Reversal (*n* = 369) *n* (%)	Non-reversal (*n* = 203) *n* (%)	*p*-value
Demographics			
Mean age	79.0 (±9.2)*	81.4 (±8.9)*	0.003
Sex (male)	205 (55.6)	104 (51.2)	0.32
Pre-stroke dependent	116 (32.7)	94 (46.3)	< 0.001
Vascular risk factors			
Hypertension	296 (80.2)	167 (82.3)	0.79
Atrial fibrillation	319 (86.4)	182 (89.7)	0.18
Diabetes	80 (21.7)	36 (17.8)	0.27
Previous stroke	98 (26.6)	73 (36.0)	0.02
Previous TIA	42 (11.4)	21 (10.3)	0.84
Clinical characteristics			
Symptom onset to hospital arrival (time)			0.49
0–3 h	141 (38.2)	88 (43.3)	
3–6 h	110 (29.8)	50 (24.6)	
>6 h	88 (23.8)	46 (22.7)	
Admitted to stroke unit or ICU	364 (90.1)	164 (80.8)	0.001
Length of hospital stay (median days)	11	5	< 0.001
Level of consciousness at hospital admission			< 0.001
Alert	238 (65.0)	92 (45.5)	
Drowsy	95 (26.0)	48 (23.8)	
Comatose	33 (9.0)	62 (30.7)	
Hemorrhage location			
Supratentorial	311 (85.2)	183 (91.0)	0.05
Intraventricular hemorrhage	*140/311 (45.8)*	*85/183 (47.5)*	0.71
Neurosurgery	*9/311 (2.9)*	*2/183 (1.1)*	0.19
Infratentorial	54 (14.8)	18 (9.0)	0.05
Intraventricular hemorrhage	*14/54 (26.9)*	*5/18 (27.8)*	0.94
Neurosurgery	*7/54 (13.0)*	*0/18 (0.0)*	0.11
Anticoagulant			
NOAC	118 (32.0)	117 (57.6)	
Apixaban	*78 (66.1)*	*83 (70.9)*	0.64
Rivaroxaban	*31 (26.3)*	*28 (23.9)*	
Dabigatran	*9 (7.6)*	*6 (5.1)*	
VKA	251 (68.0)	86 (42.4)	
INR <1.7	*16 (6.4)*	*10 (11.6)*	0.17
INR 1.7–3	*149 (59.4)*	*43 (50.0)*	
INR >3	*86 (34.3)*	*33 (38.4)*	

**Standard deviation of the mean. OAC, oral anticoagulant; ICH, intracerebral hemorrhage; VKA, Vitamin K antagonist; NOAC, non-vitamin K oral anticoagulant; INR, international normalized ratio; ICU, intensive care unit; TIA, transitory ischemic attack.*

Proportion of missing data varied between 0 and 1.0% for all variables except for VKA reversal type (2.3%), intraventricular hemorrhage (2.5%), pre-stroke dependency (4.5%), NOAC reversal type (11%), and time interval between symptom onset to hospital arrival (8.6%). The characteristics seen in italics comparing Supra vs infratentorial under hemorrhage location.

TABLE 2 | Cox regression analysis stratified for level of consciousness showing Hazard Ratios (HR) for 90-day mortality in 572 patients with OAC-ICH.

Variable	HR	95% CI		*P*-value
		Lower	Upper	
Crude model (non-stratified)				
No OAC-reversal	1.92	1.48	2.49	< 0.001
Adjusted model (stratified)*				
No OAC-reversal	1.47	1.08	2.01	0.02
Male sex	1.42	1.06	1.92	0.02
Age	1.05	1.02	1.07	< 0.001
Diabetes	1.03	0.70	1.50	0.89
Hypertension	0.80	0.57	1.14	0.22
Atrial fibrillation	0.74	0.48	1.14	0.17
Pre-stroke dependency	1.02	0.74	1.40	0.92
Intraventricular hemorrhage	2.41	1.77	3.29	< 0.001
Neurosurgery not performed	2.13	0.91	5.02	0.08
Infratentorial hemorrhage	1.47	0.96	2.24	0.08

Simple analysis is displayed as a crude model. OAC, oral anticoagulant; ICH, intracerebral hemorrhage; CI, confidence interval.
**Stratified for level of consciousness (alert, drowsy, and comatose).*

Outcome Variables

Baseline characteristics included: age, sex, vascular risk factors, history of previous stroke, or transitory ischemic attack (TIA), and pre-stroke dependency. Stroke care characteristics included: time interval between symptom onset to hospital arrival, hemorrhage location (supra- or infratentorial), presence of intraventricular hemorrhage, international normalized ratio (INR), neurosurgery, hemostatic drug, stroke unit/ICU admission, and median days of hospital admittance. Level of consciousness (LOC) at admission based on the reaction level scale (RLS-85) was used as a proxy for stroke severity. The RLS-85 is an eighth grade single line scale commonly used in Sweden to assess LOC, and correlates with the Glasgow Coma Scale (24). Riksstroke presents RLS-85 as three main categories: alert, drowsy, and comatose. Primary outcome variables were mortality and functional outcome at 90 days post-OAC-ICH.

At 3 months post-OAC-ICH, we used a modified Rankin Scale (mRS) score (translated using a previously validated translation algorithm) to assess patient-reported outcome variables on dressing and toileting (independent or assistance required), living conditions (living independently, living independently with homecare, residing at an assisted living facility or in need of in-patient care), mobility (fully mobile both indoors and outdoors, mobile but only indoors or fully dependent on assistance for mobility), and dependency on next of kin for support (fully dependent on relatives, partially dependent, not dependent) (25). The final mRS score was categorized as mRS 0–2 (independent), mRS 3–5 (dependent), or mRS 6 (deceased).

Statistical Methods

Statistical analysis was performed using IBM SPSS Statistics version 24. Patient baseline characteristics were grouped according to those receiving OAC-reversal treatment vs. non-OAC reversed patients and are presented as simple proportions, medians and means. Independent samples *t*-tests and Mann-Whitney tests were used to analyze continuous variables. A Chi-squared test determined differences between categorical variables. Survival curves were constructed using the Kaplan-Meier method. In order to determine whether death rates were affected by OAC-reversal treatment, we conducted two Cox regression analyses; one simple and one adjusted for age, sex, diabetes, hypertension, atrial fibrillation, pre-stroke dependency, anticoagulant reversal, hemorrhage location (supratentorial vs. infratentorial), intraventricular hemorrhage, and neurosurgery. Due to the indication of non-proportionality in mortality rates corresponding to different LOC categories, a stratified model was applied to the multivariate analysis using LOC category as the stratification variable.

Simple and stratified multivariable linear regressions were also performed comparing mortality with and without reversal treatment for patients with VKA-ICH and NOAC-ICH, separately. Presenting INR level (<1.7, 1.7–3, >3) was included as a covariate in VKA-ICH multivariate analysis. A $p < 0.05$ was considered statistically significant.

Ethical Considerations

This study was approved by the local Research Ethics Committee in Lund, Sweden (dnr 2017/529). Anonymized data were used. Individual consent was not required for this study as patients were informed of the handling of their data for possible future research purposes on entry into the quality register. This article aligns with STROBE criteria for observational studies (26).

RESULTS

The study included 572 patients presenting with acute OAC-ICH during 2017. Of these, 369 patients were receiving OAC-reversal treatment (118 NOAC, 251 VKA) and 203 patients were not receiving OAC-reversal treatment (117 NOAC, 86 VKA).

Patient Characteristics

Baseline patient characteristics and missing data are displayed in **Table 1**. Patients receiving reversal treatment were younger, more often pre-stroke independent and more likely treated with VKA than NOAC. Level of consciousness at hospital admission differed between groups: 65.0% presented as alert, 26.0% drowsy, and 9.0% comatose in the reversal treatment group, compared to 45.5, 23.8, and 30.7%, respectively, in the non-reversal treatment group ($p < 0.001$). Time from symptom onset to hospital admission was similar in patients receiving reversal vs. non-reversal treatment, with most patients arriving within 3 h of symptom onset ($p = 0.49$). Irrespective of treatment group, drowsy and comatose patients were more frequently admitted within 3 h of symptom onset compared to alert individuals ($p = 0.02$). Patients receiving reversal treatment were more likely cared for in a stroke care unit or ICU setting ($p = 0.001$). Patient characteristics of those lost to follow-up are displayed in **Supplemental Table 1**.

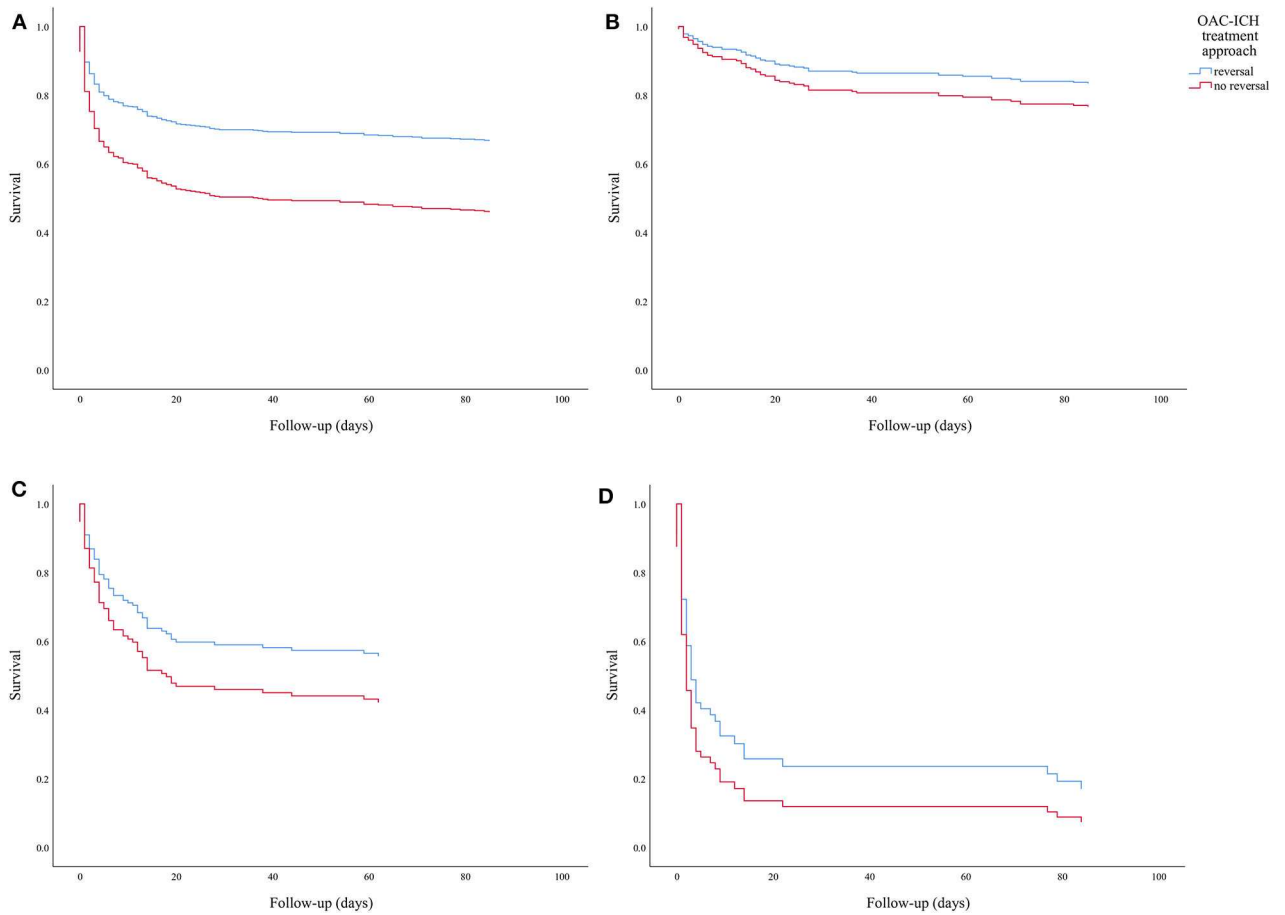

FIGURE 1 | (A) Simple analysis showing Kaplan-Meier survival curve of 90-day cumulative survival following OAC-ICH comparing patients with reversal treatment vs. non-OAC reversal patients ($n = 572$). **(B–D)** Survival curves based on multivariate analysis stratified for level of consciousness, comparing 90-day cumulative survival following OAC-ICH in patients receiving reversal treatment vs. non-OAC reversed patients. State of consciousness according to graph is as follows: **(B)** alert ($n = 316$), **(C)** drowsy ($n = 128$), **(D)** comatose ($n = 78$).

Reversal Treatment

In patients exhibiting VKA-ICH, 85.7% of reversal treatment cases were treated with PCC and Vitamin K, while 11.6% received PCC alone. In NOAC-ICH patients receiving reversal treatment, 82.2% were treated with PCC, 5.9% received Idarucizumab, and one patient received both PCC and Idarucizumab. Data on type of hemostatic agent were missing in 2.3% of VKA-ICH and 11% of NOAC-ICH cases.

Cumulative Mortality

All 572 patients were included in the mortality analysis. All-cause mortality in patients receiving reversal treatment was 19.2% at 7 days, 30.1% at 30 days, and 33.6% at 90 days. The corresponding numbers for non-reversal treatment patients were 41.9, 48.8, and 52.7%, respectively. Mortality differed significantly between patients receiving OAC-reversal and non-reversal treatment at all endpoints when the simple Cox analysis was applied. At 90 days, Hazard ratio (HR) for death using the simple analysis was 1.92 (95% CI: 1.48–2.49; **Table 2**).

All-cause mortality at 90 days differed according to presenting LOC, irrespective of reversal treatment, and was as follows: alert 18.4%, drowsy 53.1%, and comatose 89.0%. **Figures 1A–D** display the Kaplan-Meier survival curves. Multivariate Cox regression analysis stratified for LOC confirmed an increased risk of death in patients not receiving reversal treatment ($HR = 1.47$; 95% CI: 1.08–2.01; **Table 2**).

In the VKA-ICH subgroup, HR for death in patients not receiving OAC-reversal treatment was 1.49 (95% CI: 0.94–2.37; **Table 3A**). Patients presenting with INR 1.7–3 had a death rate of 1.96 (95% CI: 0.69–5.54), and those presenting with INR values >3 had a death rate of 2.16 (95% CI: 0.75–6.25). In NOAC-ICH patients not receiving reversal treatment, HR for death was 1.41 (95% CI: 0.88–2.24; **Table 3B** and **Figure 2**). Ninety-day mortality did not differ between VKA vs. NOAC-ICH (NOAC $HR = 0.95$; 95% CI: 0.70–1.28; **Supplemental Table 2**).

Functional Outcome

Ninety-day functional outcome follow-up rate was 86%. In crude analysis regarding 90-day functional outcome, a favorable

TABLE 3A | Cox regression analysis stratified for level of consciousness showing Hazard Ratios (HR) for 90-day mortality in 337 patients with VKA-ICH.

Variable	HR	95% CI		*P*-value
		Lower	Upper	
Crude model (non-stratified)				
No VKA reversal	2.20	1.54	3.13	< 0.001
Adjusted model (stratified)*				
No VKA reversal	1.49	0.94	2.37	0.09
Male sex	1.42	0.93	2.16	0.10
Age	1.06	1.03	1.09	< 0.001
Diabetes	1.68	1.01	2.79	0.05
Hypertension	0.68	0.44	1.06	0.09
Atrial fibrillation	0.53	0.28	1.01	0.05
Pre-stroke dependency	0.79	0.50	1.25	0.31
INR				
<1.7	1			
1.7–3	1.96	0.69	5.54	0.21
>3	2.16	0.75	6.25	0.15
Intraventricular hemorrhage	2.23	1.45	3.43	< 0.001
Neurosurgery not performed	2.38	0.80	7.12	0.12
Infratentorial hemorrhage	1.37	0.78	2.39	0.28

Simple analysis is displayed as a crude model. ICH, intracerebral hemorrhage; CI, confidence interval; VKA, vitamin K antagonist; INR, international normalized ratio.
**Stratified for level of consciousness (alert, drowsy, and comatose).*

TABLE 3B | Cox regression analysis stratified for level of consciousness showing Hazard Ratios (HR) for 90-day mortality in 235 patients with NOAC-ICH.

Variable	HR	95% CI		*P*-value
		Lower	Upper	
Crude model (non-stratified)				
No NOAC reversal	1.72	1.15	2.57	0.008
Adjusted model (stratified)*				
No NOAC reversal	1.41	0.88	2.24	0.15
Male sex	1.53	0.98	2.39	0.06
Age	1.03	1.00	1.07	0.04
Diabetes	0.57	0.32	1.05	0.07
Hypertension	1.17	0.65	2.13	0.60
Atrial fibrillation	0.94	0.49	1.79	0.84
Pre-stroke dependency	1.22	0.76	1.95	0.42
Intraventricular hemorrhage	2.82	1.75	4.53	< 0.001
Neurosurgery not performed	1.63	0.38	7.03	0.52
Infratentorial hemorrhage	1.69	0.83	3.45	0.15

Simple analysis is displayed as a crude model. ICH, intracerebral hemorrhage; CI, confidence interval; NOAC, non-vitamin K antagonist oral anticoagulant.
**Stratified for level of consciousness (alert, drowsy, and comatose).*

outcome was more often observed in patients receiving reversal treatment (n = 349; mRS 0–2: 15.4%, mRS 3: 10.3%, mRS 4: 14.9%, mRS 5: 12.2%, mRS 6: 33.6%) compared to those not receiving treatment (n = 174; mRS 0–2: 8.9%, mRS 3: 7.4%, mRS 4: 7.4%, mRS 5: 9.4%, mRS 6: 52.7%) (**Figure 3**). When analyzing crude functional outcome separately based on LOC category at admission (alert, drowsy, or comatose), a trend toward a more favorable outcome was seen in individuals receiving OAC-reversal treatment compared to no treatment (**Supplemental Figures 1A–C**). The proportion of patients with a favorable outcome was more distinct in patients with VKA-ICH who had received reversal treatment compared to no treatment, although this trend was not as clear regarding NOAC-ICH patients (**Figure 4**). Pre-stroke independent patients (n = 336) were more likely to remain independent at 90 days if reversal treatment was given, compared to no treatment (**Supplemental Figure 2A**). However, this trend was not as clear in pre-stroke dependent individuals in analysis of crude data (n = 210) (**Supplemental Figure 2B**).

The 90-day self-reported follow-up questionnaire was completed independently in 21.1% of cases, 39.6% required assistance, and 31.1% were completed by a caregiver alone. Patients lost to follow-up were alive and included 50 individuals receiving reversal treatment (13.6%) and 29 receiving no reversal treatment (14.3%).

DISCUSSION

In this large observational study on reversal treatment following OAC-ICH, we found that 35% of OAC-ICH patients did not receive reversal treatment. Our study shows improved survival and functional outcome in patients receiving reversal treatment compared to those who did not. Importantly, improved survival remained significant after adjusting for imbalances in relevant baseline data (HR = 0.68) and was thus not restricted to younger patients and several other predictors of good outcome in ICH.

Our findings regarding 90-day mortality are consistent with results from other studies (15, 27, 28). However, some studies have shown contradicting results, including a recent systematic review of 21 studies that did not show improved survival following the use of reversal agents in VKA-ICH (29). The sample size of each study included in this systematic review was small and confounded by limited data interpretation. Non-significant findings have also been observed in two multicenter observational studies concerning NOAC-ICH (10, 30). It is possible that these studies were underpowered or confounded by residual center effects.

Level of consciousness was strongly related to prognosis. This is not surprising, since LOC may be regarded as a proxy for stroke severity and hematoma volume (31). In our study, 90-day all-cause mortality in comatose patients was 82% in OAC-reversal patients and 95% in non-reversal patients, with low functional independence achieved in only 1.6–3% of cases. Comatose patients presented most often within 3 h of symptom onset, yet they represented only 9% of patients in the reversal treatment group. Early arrival within 3 h of symptom onset did thus not in itself determine treatment approach. In comparison, alert and drowsy patients received reversal treatment to a greater extent compared to comatose patients, suggesting a preference toward treating milder OAC-ICH, a practice that is not in line with recently published ESO guideline recommendations which promote treatment regardless of hemorrhagic stroke severity (18). Additional factors predictive of death included male sex, age, and intraventricular hemorrhage. Patients withheld

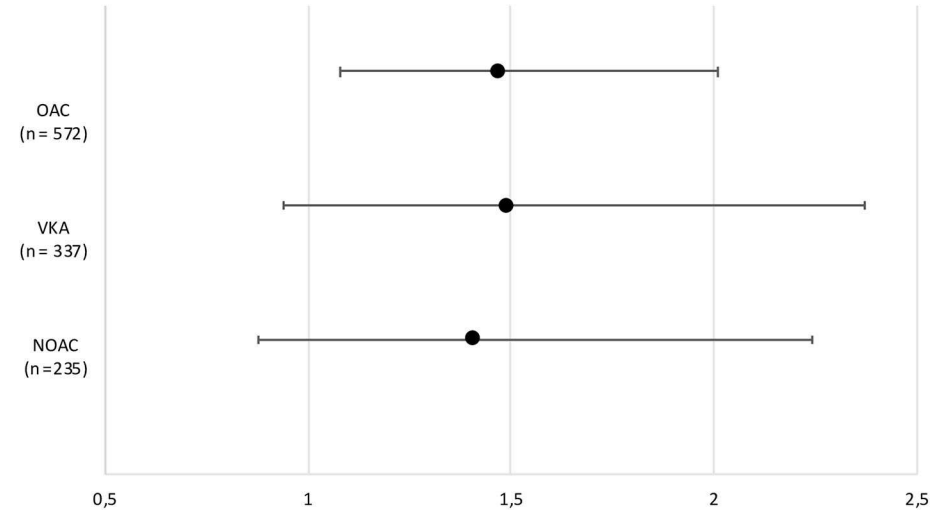

FIGURE 2 | Forest plot demonstrating hazard ratios and confidence intervals for 90-day mortality in patients who did not receive reversal treatment following OAC related ICH, and in subgroup analyses regarding VKA-ICH and NOAC-ICH separately. ICH, intracerebral hemorrhage; VKA, vitamin K antagonist; NOAC, non-vitamin K antagonist oral anticoagulant.

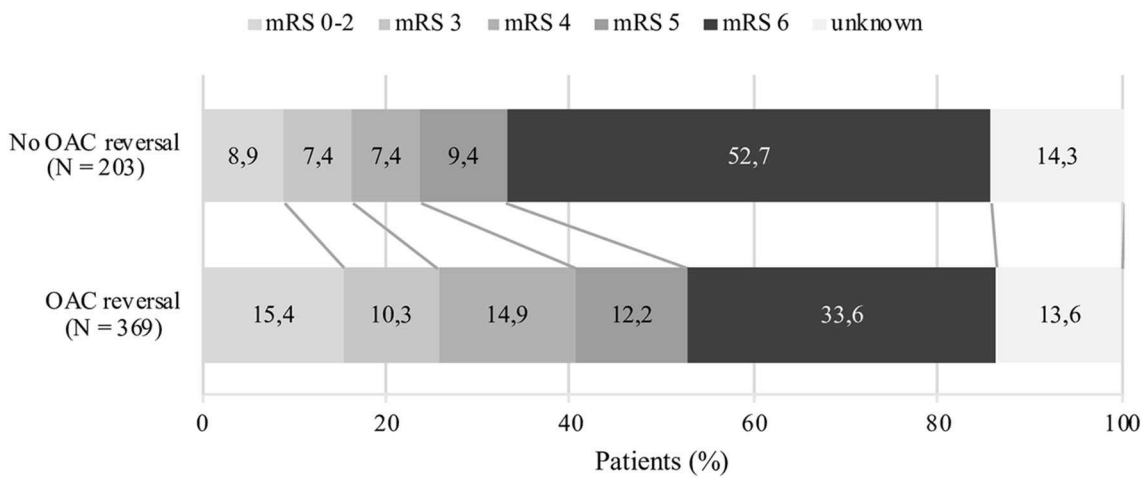

FIGURE 3 | Crude data comparing 90-day functional outcome following oral anticoagulant related intracerebral hemorrhage. Figure includes patients lost to follow-up. VKA, vitamin K antagonist; NOAC, non-vitamin K antagonist oral anticoagulant.

reversal treatment were more often older, comatose, pre-stroke dependent, and taking NOAC. Clinical treatment decisions like withdrawal of care may have been based on individual physician perceptions on appropriateness of reversal therapy, thereby presuming that older and comatose patients did not receive reversal treatment to the same extent as patients with a seemingly better prognosis. Furthermore, the questionable efficacy of PCC in NOAC-ICH patients may have deterred physicians from using this intervention, thus accounting for the large number of NOAC-ICH patients who did not receive reversal treatment. Only few patients were treated with direct antidote Idarucizumab, and Andexanet alfa was not available during the inclusion period.

Patients receiving OAC reversal therapy had improved 90-day survival but had a greater proportion of dependency compared to non-reversed patients (mRS 3–5: 37.4 vs. 24.2%), which may be explained by the increased number of deaths in the non-reversed group (**Figure 3**). In a separate crude functional outcome analysis, functional outcome following reversal of NOAC did not show as clear of a trend toward a more favorable outcome as observed in patients who received VKA reversal. NOAC reversal may have been less effective due to the unavailability of specific antidotes for factor Xa inhibitors during the study period. Considering that the majority of patients in our study were on VKA treatment, reversal treatment may have been more effective in this group. Since imbalances in baseline

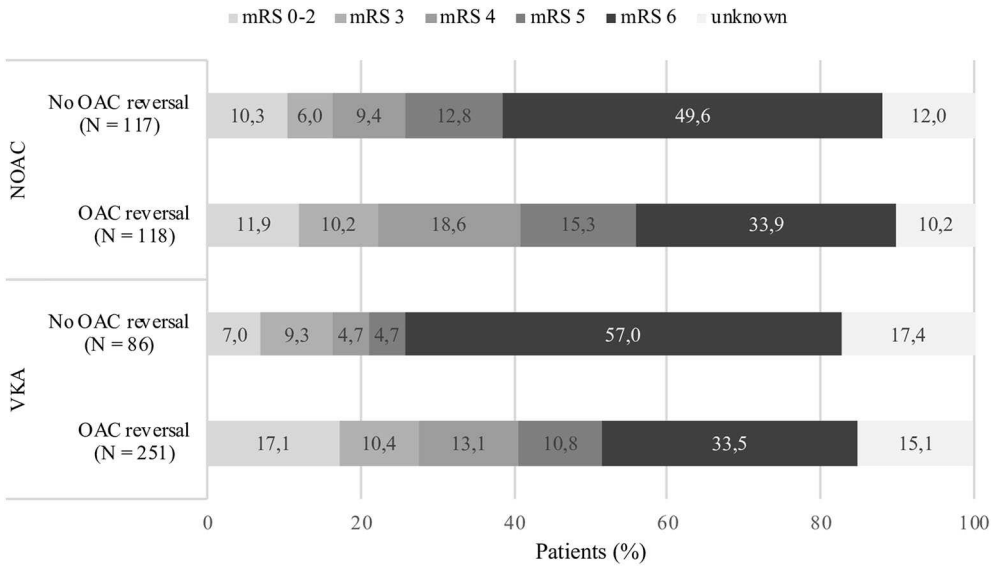

FIGURE 4 | Crude data comparing 90-day functional outcome following oral anticoagulant related intracerebral hemorrhage. Figure includes patients lost to follow-up. ICH, intracerebral hemorrhage; VKA, vitamin K antagonist; NOAC, non-vitamin K antagonist oral anticoagulant.

data, specifically baseline mRS, were not adjusted for in 90-day functional outcome analyses and confounding by indication was unable to be accounted for, these results should be interpreted with caution.

Strengths and Limitations

Riksstroke is a national database with >90% coverage, identifying the vast majority of OAC-related ICH cases in Sweden during 2017. The risk of selection bias is therefore estimated to be low. Secondly, the validity of this study is improved by the complete coverage of mortality status and few patients lost to 3-month follow-up (14%). Our data reflect recent clinical practice in use of reversal therapies in Sweden.

Several limitations of our study should be recognized. (A) The study was observational and retrospective. (B) There were imbalances in baseline data between the OAC reversal and non-reversal groups, suggesting the presence of confounding by indication (i.e., treatment decisions may have been based on individual physician perception of appropriateness of reversal therapy). Despite adjusting for all relevant confounders available in Riksstroke, the difference in survival may be influenced by residual confounding. The presumed efficacy of PCC for NOAC-ICH may be a result of residual confounding since previous studies have shown PCC to be ineffective in improving survival outcome in this patient group, hence the introduction of specific antidotes (10, 30). In line with this argument, our subgroup analysis of NOAC-ICH patients only showed a non-significant survival trend favoring patients who received reversal treatment compared to those who did not. We were unable to determine the effect on outcome related to the use of direct antidotes in NOAC-ICH patients since only few patients with Dabigatran related ICH had received Idarucizumab during our inclusion period. In addition, the direct antidote for factor Xa inhibitors, Andexanet alfa, was unavailable during the inclusion period. (C) The lack of radiologic data regarding hemorrhage volumes and hematoma expansion may account for discrepancies observed between our results and the results from aforementioned studies. However, LOC was exploited as a proxy for hemorrhage severity (31). (D) Data on clinical treatment decisions like withdrawal of care were not available. (E) Data on the dosage and timing of administration of the hemostatic agent were not available. (F) Data on acute blood pressure lowering were not available, an intervention that has shown to improve outcome (32). (G) Whereas 90-day functional outcome data are self-reported, a validation study has shown good agreement with objective data collection (25). However, only one third of patients could estimate their functional status independently, reflecting poor prognosis following OAC-ICH. Fourteen percent of individuals who did not answer the follow-up questionnaire were more often pre-stroke dependent and required lengthier hospital stays, suggesting that functional outcome rates may still be overestimated.

Hence, our results be should be interpreted with caution, and further studies are needed to more precisely determine the effects of reversal therapies in oral anticoagulant-related ICH.

CONCLUSION

In this large national study reflecting recent (2017) clinical practice in treatment of OAC-related ICH in Sweden, 35% of OAC-ICH patients did not receive reversal therapy. Current ESO guidelines (2019) recommend the use of anticoagulant reversal treatment in OAC-ICH (18). Patients either on NOAC, or comatose at admission were less likely to receive OAC-reversal treatment. Overall, we found that patients

receiving anticoagulant reversal treatment had superior 90-day survival compared to patients not receiving reversal treatment. Reversal treatment may be inappropriately withheld in a large proportion of patients. On the other hand, favorable prognosis (mRS 0–2) was observed in only 15% of OAC-ICH patients receiving reversal treatment, emphasizing the need for further studies on reversal therapies in specific groups of patients with stratification for known risk factors such as LOC, timing of treatment and ICH volume.

DATA AVAILABILITY STATEMENT

Requests to access the dataset supporting the conclusions of this article may be sent to Riksstroke after obtaining the appropriate ethics approval.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethical approval for this study was obtained from the local Research Ethics Committee in Lund, Sweden (dnr 2017/529). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

TA-H was involved in data analysis, researched literature, and wrote the first draft of the manuscript. TA-H and TU were involved in gaining ethical approval. TA-H, TU, MP, BN, and JP reviewed and edited the manuscript and approved the definitive version of the manuscript. TA-H, TU, BN, and JP conceived the study. 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.2020.00760/full#supplementary-material

REFERENCES

1. Hankey GJ, Stevens SR, Piccini JP, Lokhnygina Y, Mahaffey KW, Halperin JL, et al. Intracranial hemorrhage among patients with atrial fibrillation anticoagulated with warfarin or rivaroxaban: the rivaroxaban once daily, oral, direct factor Xa inhibition compared with vitamin K antagonism for prevention of stroke and embolism trial in atrial fibrillation. *Stroke.* (2014) 45:1304–12. doi: 10.1161/STROKEAHA.113.004506
2. Hart RG, Diener HC, Yang S, Connolly SJ, Wallentin L, Reilly PA, et al. Intracranial hemorrhage in atrial fibrillation patients during anticoagulation with warfarin or dabigatran: the re-ly trial. *Stroke.* (2012) 43:1511–17. doi: 10.1161/STROKEAHA.112.650614
3. Steiner T, Kohrmann M, Schellinger PD, Tsivgoulis G. Non-vitamin K oral anticoagulants associated bleeding and its antidotes. *J Stroke.* (2018) 20:292–301. doi: 10.5853/jos.2018.02250
4. Riksstroke. *Stroke och tia - riksstrokes årsrapport 2018*. Malmö (2020).
5. Apostolaki-Hansson T, Ullberg T, Norrving B, Petersson J. Prognosis for intracerebral hemorrhage during ongoing oral anticoagulant treatment. *Acta Neurol Scand*. (2019) 139:415–21. doi: 10.1111/ane.13068
6. Wilson D, Seiffge DJ, Traenka C, Basir G, Purrucker JC, Rizos T, et al. Outcome of intracerebral hemorrhage associated with different oral anticoagulants. *Neurology.* (2017) 88:1693–700. doi: 10.1212/WNL.0000000000003886
7. Boulouis G, Morotti A, Pasi M, Goldstein JN, Gurol ME, Charidimou A. Outcome of intracerebral haemorrhage related to non-vitamin K antagonists oral anticoagulants versus vitamin K antagonists: a comprehensive systematic review and meta-analysis. *J Neurol Neurosurg Psychiatry.* (2018) 89:263–70. doi: 10.1136/jnnp-2017-316631
8. Tsivgoulis G, Wilson D, Katsanos AH, Sargento-Freitas J, Marques-Matos C, Azevedo E, et al. Neuroimaging and clinical outcomes of oral anticoagulant associated ich. *Ann Neurol.* (2018) 84:694–704. doi: 10.1002/ana.25342
9. Tsivgoulis G, Katsanos AH, Seiffge DJ, Paciaroni M, Wilson D, Koga M, et al. Fatal intracranial haemorrhage occurring after oral anticoagulant treatment initiation for secondary stroke prevention in patients with atrial fibrillation. *Eur J Neurol.* (2020) 27:1612–17. doi: 10.1111/ene.14280
10. Purrucker JC, Haas K, Rizos T, Khan S, Wolf M, Hennerici MG, et al. Early clinical and radiological course, management, and outcome of intracerebral hemorrhage related to new oral anticoagulants. *JAMA Neurol.* (2016) 73:169–77. doi: 10.1001/jamaneurol.2015.3682
11. Roquer J, Vivanco-Hidalgo RM, Capellades J, Ois A, Cuadrado-Godia E, Giralt-Steinhauer E, et al. Ultra-early hematoma growth in antithrombotic pretreated patients with intracerebral hemorrhage. *Eur J Neurol.* (2018) 25:83–89. doi: 10.1111/ene.13458
12. Melmed KR, Lyden P, Gellada N, Moheet A. Intracerebral hemorrhagic expansion occurs in patients using non-vitamin K antagonist oral anticoagulants comparable with patients using warfarin. *J Stroke Cerebrovasc Dis.* (2017) 26:1874–82. doi: 10.1016/j.jstrokecerebrovasdis.2017.04.025
13. Yang NR, Kim SJ, Seo EK. Spontaneous intracerebral hemorrhage with antiplatelets/anticoagulants/none: a comparison analysis. *Acta Neurochirurgica.* (2014) 156:1319–25. doi: 10.1007/s00701-014-2080-2
14. Inohara T, Xian Y, Liang L, Matsouaka RA, Saver JL, Smith EE, et al. Association of intracerebral hemorrhage among patients taking non-vitamin K antagonist vs vitamin K antagonist oral anticoagulants with in-hospital mortality. *JAMA.* (2018) 319:463–73. doi: 10.1001/jama.2017.21917
15. Kuwashiro T, Yasaka M, Itabashi R, Nakagaki H, Miyashita F, Naritomi H, et al. Effect of prothrombin complex concentrate on hematoma enlargement and clinical outcome in patients with anticoagulant-associated intracerebral hemorrhage. *Cerebrovasc Dis.* (2011) 31:170–76. doi: 10.1159/000321766
16. Huttner Hagen B, Schellinger Peter D, Hartmann M, Köhrmann M, Juettler E, Wikner J, et al. Hematoma growth and outcome in treated neurocritical care patients with intracerebral hemorrhage related to oral anticoagulant therapy. *Stroke.* (2006) 37:1465–70. doi: 10.1161/01.STR.0000221786.81354.d6

17. Steiner T, Al-Shahi Salman R, Beer R, Christensen H, Cordonnier C, Csiba L, et al. European Stroke Organisation (ESO) guidelines for the management of spontaneous intracerebral hemorrhage. *Int J Stroke.* (2014) 9:840–55. doi: 10.1111/ijs.12309
18. Christensen H, Cordonnier C, Kõrv J, Lal A, Ovesen C, Purrucker JC, et al. European stroke organisation guideline on reversal of oral anticoagulants in acute intracerebral haemorrhage. *Eur Stroke J.* (2019) 4:294–306. doi: 10.1177/2396987319849763
19. Steiner T, Poli S, Griebe M, Husing J, Hajda J, Freiberger A, et al. Fresh frozen plasma versus prothrombin complex concentrate in patients with intracranial haemorrhage related to vitamin K antagonists (INCH): a randomised trial. *Lancet Neurol.* (2016) 15:566–73. doi: 10.1016/S1474-4422(16)00110-1
20. Läkemedelsverket. *Antikoagulantiabehandling vid förmaksflimmer – behandlingsrekommendation*. (Malmö): Information från läkemedelsverket (2017).
21. Kuramatsu JB, Sembill JA, Huttner HB. Reversal of oral anticoagulation in patients with acute intracerebral hemorrhage. *Crit Care.* (2019) 23:206 doi: 10.1186/s13054-019-2492-8
22. Asplund K, Hulter Asberg K, Norrving B, Stegmayr B, Terent A, Wester PO, et al. Riks-stroke - a swedish national quality register for stroke care. *Cerebrovasc Dis.* (2003) 15(Suppl. 1):5–7. doi: 10.1159/000068203
23. Socialstyrelsen. *Bortfall och kvalitet på dödsorsaksregistret*. Malmö (2020).
24. Starmark JE, Stalhammar D, Holmgren E, Rosander B. A comparison of the glasgow coma scale and the reaction level scale (RLS85). *J Neurosurg.* (1988) 69:699–706. doi: 10.3171/jns.1988.69.5.0699
25. Eriksson M, Appelros P, Norrving B, Terent A, Stegmayr B. Assessment of functional outcome in a national quality register for acute stroke: can simple self-reported items be transformed into the modified rankin scale? *Stroke.* (2007) 38:1384–6. doi: 10.1161/01.STR.0000260102.97954.9c
26. Cuschieri S. The strobe guidelines. *Saudi J Anaesth.* (2019) 13:S31–S34. doi: 10.4103/sja.SJA_543_18
27. Hanger HC, Geddes JAA, Wilkinson TJ, Lee M, Baker AE. Warfarin-related intracerebral haemorrhage: better outcomes when reversal includes prothrombin complex concentrates. *Intern Med J.* (2013) 43:308–16. doi: 10.1111/imj.12034
28. Huhtakangas J, Tetri S, Juvela S, Saloheimo P, Bode MK, Karttunen V, et al. Improved survival of patients with warfarin-associated intracerebral haemorrhage: a retrospective longitudinal population-based study. *Int J Stroke.* (2015) 10:876–81. doi: 10.1111/j.1747-4949.2012.00926.x
29. Ko D, Razouki Z, Otis J, Marulanda-Londoño E, Hylek EM. Anticoagulation reversal in vitamin K antagonist–associated intracerebral hemorrhage: a systematic review. *J Thromb Thrombol.* (2018) 46:227–37. doi: 10.1007/s11239-018-1667-5
30. Gerner ST, Kuramatsu JB, Sembill JA, Sprugel MI, Endres M, Haeusler KG, et al. Association of prothrombin complex concentrate administration and hematoma enlargement in non-vitamin K antagonist oral anticoagulant-related intracerebral hemorrhage. *Ann Neurol.* (2018) 83:186–96. doi: 10.1002/ana.25134
31. Portenoy RK, Lipton RB, Berger AR, Lesser ML, Lantos G. Intracerebral haemorrhage: a model for the prediction of outcome. *J Neurol Neurosurg Psychiatry.* (1987) 50:976–9. doi: 10.1136/jnnp.50.8.976
32. Moullaali TJ, Wang X, Martin RH, Shipes VB, Robinson TG, Chalmers J, et al. Blood pressure control and clinical outcomes in acute intracerebral haemorrhage: a preplanned pooled analysis of individual participant data. *Lancet Neurol.* (2019) 18:857–64. doi: 10.1016/S1474-4422(19)30196-6

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CASE REPORT
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Lupus Antibody Mimicking Reduced Plasmatic Coagulation in a Patient With Atrial Fibrillation and Ischemic Stroke

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Background: Lupus anticoagulant (LA) owns procoagulant properties *in vivo* and prolongs phospholipid-dependent clotting times *in vitro*. The prolonged *in vitro* clotting time can be misinterpreted as a bleeding disorder. In some cases, it is necessary to differentiate LA-associated *in vitro* changes from *in vivo* coagulation factor deficiency. In this case, we used different laboratory testing in a patient with ischemic stroke and reduced prothrombin time (PT) to identify an *in-vitro* effect of LA excluding an *in-vivo* bleeding disorder.

Methods: The activity of various coagulation factors was evaluated both with recombinant thromboplastin Innovin (Siemens Healthcare) and reagent tissue extracted thromboplastin Thromborel® (Siemens Healthcare). Moreover, a 1:1 plasma mixing test with standard plasma was performed. In order to exclude the interaction of tromboplastin and LA thromboplastin, an independent global coagulation test, thromboelastography, was used. Diluted-Russel-Viper-Venom (dRVVT) assay was applied to detect the presence of LA detection.

Results: The activity of several coagulation factors measured with recombinant thromboplastin Innovin (Siemens Healthcare) showed a reduced activity of the following coagulation factors: Factor V (20.9%), Factor VII (23.8%), Factor X (19.7%) and international normalized ratio (INR) of 2.33. Re-assessment of the factor's activity with another reagent tissue extracted thromboplastin Thromborel® (Siemens Healthcare) showed a normalization of INR and factor's activity in comparison to thromboplastin reagent Innovin®: Factor V (77%), Factor VII (45.4%), Factor X (64.2%), and INR of 1.28. A plasma mixing study with 1:1 standard plasma revealed reduced (<50%) normalization of INR as well as coagulation factor's activity confirming a LA-inhibitor in the patient plasma. Diagnostic LA testing was also performed with dRVVT assay showing a significantly prolonged (112.8 s) test time. Thromboelastography revealed no abnormalities.

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Conclusions: Different thromboplastin reagents and plasma mixing tests as well as thromboplastin independent coagulation tests may be helpful to differentiate LA and *in vitro* changes from *in vivo* factor deficiency in patients with LA.

Keywords: lupus anticoagulant, coagulation factor deficiency, thromboplastin, ischemic stroke, anticoagulation

INTRODUCTION

Lupus anticoagulant (LA) is an antiphospholipid antibody that binds phospholipids and proteins associated with the cell membrane (1). Phospholipids are essential *in vivo* because of their biological role in cell membranes and are used *in vitro* to accelerate coagulation. LA have procoagulant properties *in vivo*, but since they bind phospholipids in the testing assay, they prolong phospholipid-dependent clotting times *in vitro* (2). The presence of LA and arterial and/or venous thrombosis and/or pregnancy morbidity is one of the characteristics of antiphospholipid syndrome (3). The diagnosis and management of this disease can be challenging, as increased *in vitro* clotting time can be misinterpreted and may delay anticoagulation.

CASE PRESENTATION

A 77-year-old female patient presented to the emergency department with an acute onset of aphasia, dysphagia and right-sided hemiplegia. The patient had a history of hypertension as well as cutaneous lupus erythematosus without any bleeding events. Prior medication was only verapamil (Ca-channel blocker). She did not take any other medication during the last few weeks. The neurological investigation revealed global aphasia, right-sided homonymous hemianopsia, right-sided hemiplegia and hemihypoalgesia. As an acute stroke was suspected, the patient underwent multinodal cranial CT scanning within the first 15 min of arrival in the emergency department to obtain the necessary information needed for further acute treatment.

There were no signs of an acute intracerebral bleeding but a proximal occlusion of the M1 segment of the left middle cerebral artery (MCA) was detected as well as corresponding perfusion deficit in the territory of the left MCA. As the presentation was within 30 min symptom onset, intravenous thrombolysis with a recombinant tissue plasminogen activator, in combination with a mechanical thrombectomy, where a complete recanalization of the MCA could be achieved, was immediately performed. New onset atrial fibrillation, which was detected in the emergency room, was suspected as possible cause of the stroke. The routine blood coagulation tests sampled at admission showed normal platelet count (171/nl) and reduced plasmatic coagulation. International normalized ratio (INR) 2.33 (Reference Range (RR) 0.9–1.15), activated partial thromboplastin time (aPTT) of 30.4 s (RR 15–30 S) (Citrate blood, using reagent Innovin® and Actin FS® on SYSMEX 2100i Siemens Health Care Diagnostics, Marburg, Germany). As no coagulation disorder was known and the test results were completed within 30 min of arrival in the emergency, the thrombolysis therapy was performed.

Abbreviations: aPTT, activated partial thromboplastin time; APS, antiphospholipid syndrome; dRVVT, diluted-Russel-Viper-Venom; INR, international normalized ratio; LA, lupus anticoagulant; MCA, middle cerebral artery.

CLINICAL COURSE AFTER ADMISSION

The patient was referred to the neurological intensive care unit. A subsequent up cranial CT scanning revealed full demarcation of a large frontoparietal infarction in the territory of the left MCA and no evidence of bleeding despite *in-vitro* reduced plasmatic coagulation (**Figure 1**).

Because of the repeated reduced plasmatic coagulation test, haemostaseology specialists were involved. Further laboratory investigation and the analysis of the activity of separate coagulation factors revealed a reduced activity of coagulation Factor V (20.9%), Factor VII (23.8%), Factor IX (66%), Factor X (19.7%), Factor XI (53%), Factor XII (26%), and Factor XIII (65%) (Citrate blood, using reagents Innovin®/ActinFS® and SYSMEX 2100i Siemens Health Care Diagnostics, Marburg, Germany. FXIII activity was measured by ammonia released chromogenic assay (Berichrom® F XIII, Siemens Sysmex System CS 5100). Von Willebrand Factor antigen (VWF Ag®, Siemens System BCS®XP) and platelet agglutination caused by ristocetin cofactor assay (INNOVANCE® VWF, Siemens System BCS®XP), fibrinogen (Dade®, Siemens Sysmex System CS 5100), and Factor VIIIc (ActinFS®, Siemens Sysmex System CS 5100) as well as platelet function tests (Platelet Function Assay (PFA-100) und Platelet Aggregation by Born) were normal. The global *in vitro* coagulation test – thromboelastography showed normal test results. The LA test showed pathological results: the diluted-Russel-Viper-Venom (dRVVT) assay was significantly prolonged (112.8 S). The antiphospholipid antibodies were slightly increased in the screening test, however the further IgM and IgG Testing showed results in the RR (**Table 1**). 1:1 Plasma mixing study with standard plasma revealed a normalization of INR as well as of coagulation factors activity (**Table 2**) as effect of inhibitory activity of patient plasma. Re-assessment of the factor's activity with another reagent Thromborel® (Siemens Healthcare) showed a normalization of INR and factor's activity in comparison to the previously used thromboplastin reagent Innovin® (**Table 2**). The results of the test showed the presence of an *in vitro* coagulation factor activity inhibitor having different sensitivity to tissue extracted and recombinant thromboplastin. Furthermore, the thromboplastin independent coagulation test thromboelastography revealed no relevant coagulation disorders (*r*-Time 14.6 min [9–27 Min], *k*-Time 3.2 Min [2–9 min], max. amplitude 60 mm [44–64 mm]). As a haemorrhagic disorder was no longer suspected, the patient was anticoagulated with enoxaparin. During further hospital course, endoscopic gastrostomy was performed because

TABLE 1 | Initial laboratory parameter.

	Result	Reference range		Result	Reference range
Factor II	101%	70–140%	PFA-EPI	117 Sec	8–150 Sec
Factor V	20%	70–120%	Lupus anticoagulant LA1	112.8 Sec	0–44 Sec
Factor VII	23%	70–140%	Lupus anticoagulant LA 2	42.0 Sec	28–32 Sec
Factor VIIIc	188%	70–200%	LA1/LA2 ratio	2.69	0-1,2
Factor IX	66%	70–140%	Lupus sensitive PTT	55.6 Sec	25–34 Sec
Factor X	19%	70–140%	Platelets	167 10E9/L	165-387E9/L
Factor XI	53%	70–140%	Anti-B2-glycoprotein screening	15 U/ml	10 U/ml
Factor XII	20%	70–140%	Anti-B2-glycoprotein IgG	2.50 U/ml	0–8 U/ml
Factor XIII	65%	70–140%	Anti-B2-glycoprotein IgM	3.90 U/ml	0–8 U/ml
INR	2.33	0.9–1.15	Anti-cardiolipin screening	16.30 U/ml	0–10 U/ml
aPTT	30.4	15–30 Sec	Anti-cardiolipin IgG	1.20 U/ml	0–7 U/ml
vWF-Antigen	>200%	70–150%	Anti-cardiolipin IgM	4.50 U/ml	0–10 U/ml
Ristocetin-Cofactor	>150%	70–150%	Anti-Annexin V IgM/IgG	<8 U/ml	0–10 U/ml

aPTT, activated partial thromboplastin time; INR, international normalized ratio; PFA-EPI, platelet function assay with epinephrine membrane; vWF, von-Willebrand-Factor.

TABLE 2 | Advanced testing of coagulation factor activity.

	Innovin®/ ActinFS® for FXII	Thromborel®	50% Patient plasma, 50% standard plasma	RR
INR	2.33	1.28	1.86	0.9–1.15
Factor V	20.9%	77%	33.6%	70–120%
Factor VII	23.8%	45.4%	33.6%	70–140%
Factor X	19.7%	64.2%	37.4%	70–140%
Factor XII	20.9%	–	46.2%	70–140%

INR, international normalized ratio.

of dysphagia without any bleeding complication and the patient was discharged to a neurological rehabilitation center.

DISCUSSION

Both, atrial fibrillation and antiphospholipid syndrome (APS) are associated with cerebrovascular events (4, 5). The state of the art treatment of an acute stroke is thrombolytic therapy with the thrombolytic drug – tissue plasminogen activator alteplase (6). However, impaired coagulation can be a contraindicated for fibrinolytic therapy. Thus, INR values above 1,7 are associated with increased risk of intracranial bleeding. Therefore, thrombolytic therapy is contraindicated in these cases (7). In addition hemorrhagic disorders and a deficiency of coagulation factors, lupus antibodies impair global coagulation tests (8). In the present case, the patient did benefit from thrombolytic therapy and further secondary prevention of thromboembolic events due to APS or atrial fibrillation. However, medical therapy in this case was challenging: on the one hand, there was the need of fibrinolytic therapy and anticoagulation as secondary prevention of thromboembolic events and, on the other hand, *in vitro* signs of hemorrhagic coagulation disorder due to impaired INR and reduced activity of separate coagulation factors. The fact that there were no signs of impaired liver function and no history of bleeding or any hemorrhagic disorders, gave suspicion that the laboratory results do not depict *in-vivo* hemostasis. Furthermore, thrombolytic therapy did not cause any bleeding complications, which could be typical for a case of severe coagulation factor deficiency. Therefore, indirect methods, such as a plasma mixing test and testing with other less lupus sensitive reagents were used.

LUPUS ANTICOAGULANT

LA is one of the most mysterious antibodies in patients with an antiphospholipid syndrome. On the one hand, an antiphospholipid antibody may cause a phospholipid-dependent prolongation of the clotting time *in vitro* and, on the other hand, it is associated with an increased risk of thrombosis (9). This ambivalence is based on the feature of building complexes which slow down coagulation reactions *in vitro* by forming stable complexes on coagulation active phospholipids (8). Laboratory diagnostic tests for the LA are heterogeneous and include phospholipid-dependent clotting assays, plasma mixing studies, and demonstration of the phospholipid-dependency of the inhibitory activity. Due to the heterogeneity of antibodies, reagents and the variability of the analyser, the general algorithms of LA detection are missing (3).

LA is usually detected with clotting assays based on aPTT and dRVVT. Both can be performed at low and high phospholipid concentration, or on 1:1 mixtures of tested sample and a normal plasma pool (10). Some prothrombin assays have demonstrated to be sensitive to the effects of LA, leading to a prolonged PT and consequently false INR values. Thromboplastin reagents are widely used as clotting activator *in vitro* and they contain either recombinant tissue factor or tissue factor extracted from the brain and placental sources. Recombinant thromboplastins have been discussed to be more sensitive to variation in the presence of LA. Accordingly, tissue extracted thromboplastins are more suitable for measurements (11). The reason for such correlations is not known. Possible explanation is an interaction of LA either with recombinant replicated tissue factor or a different phospholipid

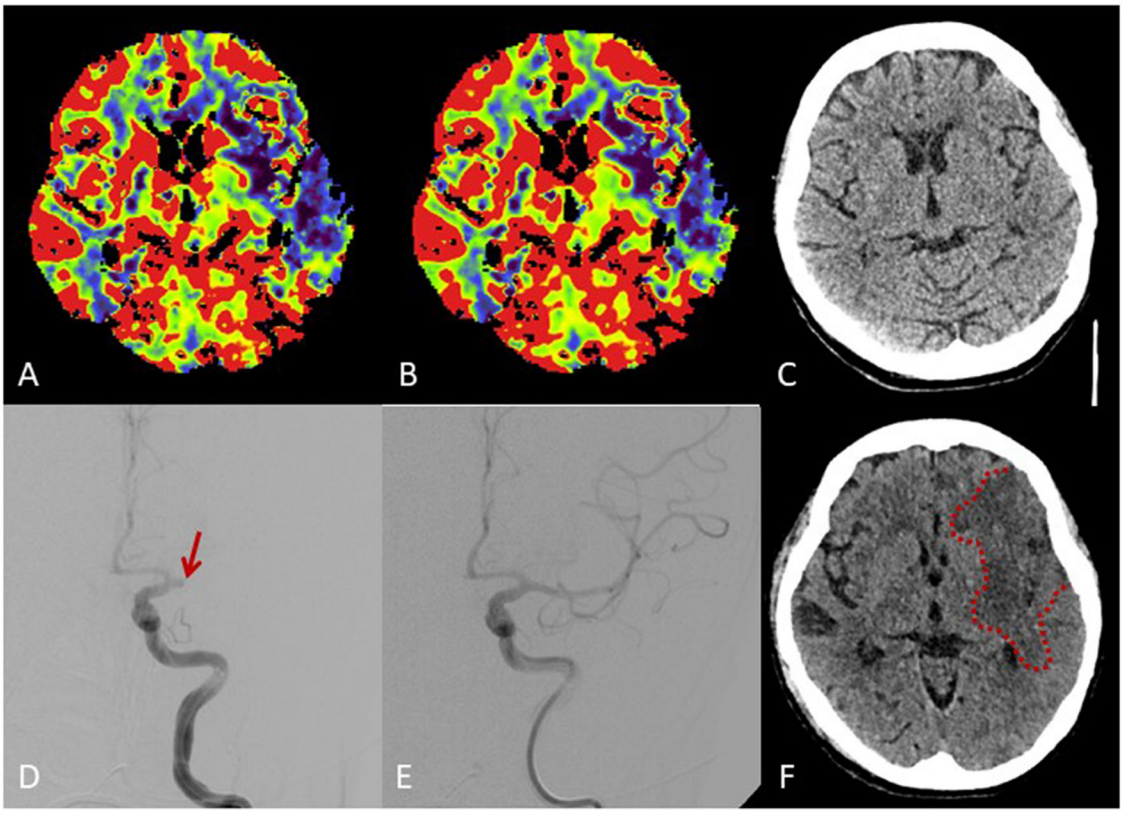

FIGURE 1 | Brain imaging. Multimodal cranial CT imaging at admission shows reduced perfusion in cerebral blood flow maps **(A)** und cerebral blood volume maps **(B)** in the territory of the middle cerebral artery (MCA) without early ischemic changes on non-contrast-CT **(C)**. Angiography **(D)** shows proximal occlusion of the MCA (arrow) with complete recanalization after intravenous thrombolysis and thrombectomy **(E)**. Follow-up CT shows a large frontoparietal MCA infarction despite complete recanalization **(F)**.

composition (12). In summary, the fact of different results using different reagents could be a good evidence of the existence of LA in the present case. Both, plasma mixing test and the use of different reagents were helpful to choose the anticoagulation strategy for this patient. Furthermore, thromboplastin independent coagulation tests, such as thromboelastography, should be used to rule out a severe bleeding coagulation disorder.

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

Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

AH, TB, MB, and UH contributed to the conception and design of the paper. VH and MK contributed to preparing laboratory data. MN and AA contributed to developing the idea of the study and revising the article critically for important intellectual content. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Molhoek JE, de Groot PG, Urbanus RT. The lupus anticoagulant paradox. *Semin Thromb Hemost.* (2018) 44:445–52. doi: 10.1055/s-0037-1606190
2. Linnemann B. Antiphospholipid syndrome - an update. *Vasa.* (2018) 47:451–64. doi: 10.1024/0301-1526/a000723
3. Moore GW. Recent guidelines and recommendations for laboratory detection of lupus anticoagulants. *Semin Thromb Hemost.* (2014) 40:163–71. doi: 10.1055/s-0033-1364185
4. Hanly JG, Li Q, Su L, Urowitz MB, Gordon C, Bae SC, et al. Cerebrovascular events in systemic lupus erythematosus: results from an international inception cohort study. *Arthritis Care Res.* (2018) 70:1478–87. doi: 10.1136/lupus-2017-000215.439

5. Wolf PA, Dawber TR, Thomas HE Jr, Kannel WB. Epidemiologic assessment of chronic atrial fibrillation and risk of stroke: the Framingham study. *Neurology*. (1978) 28:973–7. doi: 10.1212/WNL.28.10.973
6. Powers WJ, Rabinstein AA, Ackerson T, Adeoye OM, Bambakidis NC, Becker K, et al. 2018 guidelines for the early management of patients with acute ischemic stroke: a guideline for healthcare professionals from the American heart association/American stroke association. *Stroke*. (2018) 49:e46–110. doi: 10.1161/STR.0000000000000163
7. Mazya MV, Lees KR, Markus R, Roine RO, Seet RC, Wahlgren N, et al. Safety of intravenous thrombolysis for ischemic stroke in patients treated with warfarin. *Ann Neurol.* (2013) 74:266–74. doi: 10.1002/ana.23924
8. Kershaw G. Performance of Activated Partial Thromboplastin Time (APTT): determining reagent sensitivity to factor deficiencies, heparin, and lupus anticoagulants. *Methods Mol Biol.* (2017) 1646:75–83. doi: 10.1007/978-1-4939-7196-1_5
9. Devreese KM. Antiphospholipid antibodies: evaluation of the thrombotic risk. *Thromb Res.* (2012) 130(Suppl. 1):S37–40. doi: 10.1016/j.thromres.2012.08.270
10. Amiral J, Peyrafitte M, Dunois C, Vissac AM, Seghatchian J. Anti-phospholipid syndrome: Current opinion on mechanisms involved, laboratory characterization and diagnostic aspects. *Transfus Apher Sci.* (2017) 56:612–25. doi: 10.1016/j.transci.2017.07.014
11. Isert M, Miesbach W, Schuttfort G, Weil Y, Tirneci V, Kasper A, et al. Monitoring anticoagulant therapy with vitamin K antagonists in patients with antiphospholipid syndrome. *Ann Hematol.* (2015) 94:1291–9. doi: 10.1007/s00277-015-2374-3
12. Tripodi A, Chantarangkul V, Clerici M, Negri B, Galli M, Mannucci PM. Laboratory control of oral anticoagulant treatment by the INR system in patients with the antiphospholipid syndrome and lupus anticoagulant. Results of a collaborative study involving nine commercial thromboplastins. *Br J Haematol.* (2001) 115:672–8. doi: 10.1046/j.1365-2141.2001.03178.x

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Liver Fibrosis Is Associated With Hemorrhagic Transformation in Patients With Acute Ischemic Stroke

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Background: Hemorrhagic transformation (HT) is a frequent, often asymptomatic event that occurs after acute ischemic stroke (AIS). Liver fibrosis, usually subclinical, is common and crucial in the development of liver disease. We aimed to investigate the association between liver fibrosis and HT in patients with AIS.

Methods: We performed a single-center and retrospective study. A total of 185 consecutive participants with HT and 199 age- and sex-matched stroke patients without HT were enrolled in this study. We calculated one validated fibrosis index—Fibrosis-4 (FIB-4) score—to assess the extent of liver fibrosis. HT was detected by routine CT or MRI and was radiologically classified as hemorrhagic infarction type 1 or 2 or parenchymal hematoma type 1 or 2. HT was also classified into asymptomatic or symptomatic. We used logistic regression models adjusted for previously established risk factors to assess the risks for HT.

Results: The median FIB-4 score was significantly higher among patients who developed HT than among those without HT, whereas standard hepatic assays were largely normal. Patients were assigned to groups of high FIB-4 score and low FIB-4 score based on the optimal cutoff value. Compared with the subjects in the low-FIB-4-score group, incidence of HT for the high-FIB-4-score group was significantly higher. After adjustment for potential confounders, the patients with high FIB-4 score had 3.461-fold risk of HT in AIS compared to the patients with low FIB-4 score [odds ratio, 3.461 (95% CI, 1.404–8.531)].

Conclusion: Liver fibrosis, measured by FIB-4 score, was independently associated with the risk of HT in AIS patients.

Keywords: liver fibrosis, acute ischemic stroke, hemorrhagic transformation, risk factors, liver disease

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INTRODUCTION

Liver diseases could increase the risk of cardiovascular disease and lead to worse hospital discharge disposition and higher in-hospital mortality after stroke (1–3). Non-alcoholic fatty liver disease (NAFLD) is the most common cause of liver dysfunction, affecting about 25% of the adult population globally, which spans pathologies ranging from simple fatty liver (steatosis) to necroinflammatory non-alcoholic steatohepatitis (NASH), including scarring (fibrosis) and

the formation of nodules surrounded by fibrotic bands (cirrhosis) (4, 5). A previous study has demonstrated that liver cirrhosis is associated with an increased risk of stroke, particularly hemorrhagic stroke (6). In addition, a recent study showed liver fibrosis, not simple steatosis, is a strong predictor of long-term mortality in the ischemic stroke population (7). Although these studies highlight a possible relevance between advanced liver diseases and poor stroke outcomes, it is still unsure if these findings can also apply to subclinical liver disease.

Liver fibrosis, a histological precursor to cirrhosis, is an often clinically (4) chronic liver disease and is preceded and promoted by an inflammatory process in conjunction with the accumulation of extracellular matrix in the liver (8). Studies previously have indicated an unexpectedly high prevalence of liver fibrosis in up to 9% individuals without known liver disease (9, 10). Furthermore, the presence and severity of liver fibrosis can predict cardiovascular mortality in patients with chronic liver disease as well as the risk of ischemic stroke (11, 12) and are associated with the outcomes after primary intracerebral hemorrhage (ICH) according to previous studies (13).

Hemorrhagic transformation (HT) is a common and severe complication that patients may develop in acute ischemic stroke (AIS) (14–16). And it is a major cause of early mortality and disability, which is potentially linked with clinical deterioration and poor outcomes (17–19). Previously identified risk factors for HT in ischemic stroke patients include increasing age (20), higher systolic blood pressure (SBP) (21), atrial fibrillation (22), thrombolysis (23), and symptom severity (24). In fact, several studies have found that liver fibrosis is associated with cerebral microbleeds and admission hematoma volume, hematoma expansion, and mortality after ICH (13, 25). Although subclinical liver fibrosis or steatosis may not be rare in patients with stroke (26), data are lacking regarding the association between liver fibrosis and HT for patients with AIS. We, therefore, investigated the association between subclinical liver disease, defined using the liver fibrosis score, and HT using a population of patients without overt liver disease. In the present study, we hypothesized that liver fibrosis may be associated with HT in patients with AIS.

MATERIALS AND METHODS

Subjects

We performed a retrospective study using data from the HT database, which collected data on patients admitted to the First Affiliated Hospital of Wenzhou Medical University. All sampled subjects were objectively diagnosed with HT consecutively between October 2011 and March 2019. Approved by the Institutional Review Board and Research Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University, this study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki. No informed consent was required as this study was retrospective and all included data were anonymous.

Patients were sampled if they (1) were 18–90 years old; (2) were hospitalized within 7 days from the onset of stroke; and (3) were identified as AIS after admission by computed tomography (CT) or magnetic resonance imaging (MRI). Subjects were excluded when one of the following conditions was met: (1) a diagnosis of hemorrhagic stroke or transient ischemic attack (TIA); (2) alcohol use; (3) current use of hepatotoxic medications; (4) severe renal disease or known overt liver disease, such as cirrhosis and chronic viral hepatitis B; (5) unavailability of a repeated CT/MRI scan; (6) intravenous thrombolytic therapy received by the patient; or (7) incomplete laboratory data. Ultimately, a total of 185 consecutive participants meeting the requirements were enrolled in this study. According to the same inclusion and exclusion criteria, we included another 199 AIS patients with gender and age matching yet without HT from the stroke unit of our institution.

Data Collection

A complete survey of all patients was performed using a review of the medical records upon admission. Demographic and clinical data [age, gender, marital status (married or single), and body mass index (BMI)] were documented at baseline. The following stroke risk factors were also identified: hypertension, diabetes mellitus, dyslipidemia, atrial fibrillation, previous history of stroke, current cigarette smoking, and alcohol consumption. Laboratory tests [including white cell count, platelet count, fibrinogen, creatinine, glucose levels (fast blood glucose), HbA1c, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, HDL cholesterol, and LDL cholesterol] and blood pressure measurements were conducted within 24 h after admission. In addition, information on treatment for acute stroke before HT using anticoagulant, antiplatelet, and lipid-lowering agents was collected for all patients. All patients were investigated to clarify the stroke subtype according to the TOAST criteria (27). The size of each infarction area was classified as follows: less than one-half of a lobe was defined as small, and more than one-half of a lobe was defined as large (16, 28, 29). Stroke severity was assessed at admission by experienced neurologists using the National Institutes of Health Stroke Scale (NIHSS) (30). Ranging from 0 to 42, the score on the NIHSS quantifies the extent of neurological deficits.

All patients' platelet count and liver chemistries (AST and ALT) were measured at our hospital's clinical laboratory using routine laboratory methods. All measurements were performed by laboratory personnel blinded to the study samples, baseline characteristics, and outcomes.

Assessment of Liver Fibrosis

We assessed the extent of liver fibrosis by calculating the non-invasive liver fibrosis score for each subject at the time of admission: the Fibrosis-4 (FIB-4) score. The index is calculated from laboratory data and demographic variables (13, 31, 32) using the following formula: $\left[\left(age\left(years\right)\times AST\left(\frac{U}{L}\right)\right)\div\left(platelet\ count\left(\frac{10^9}{L}\right)\times\sqrt{ALT(\frac{U}{L})}\right)\right]$. Data on liver imaging are not available in the study. However, this index has been tested to be a non-invasive parameter of liver fibrosis with high diagnostic accuracy in patients with NAFLD (33). The patients were then categorized into two groups according to the optimal cutoff value of the FIB-4 score for

further comparisons. The FIB-4 score quantifies the extent of liver fibrosis.

Definition and Classification of HT

HT was defined as hemorrhage inside the infarct region or parenchyma outside the infarct territory on a follow-up CT scan or MRI (34) which included diffusion-weighted imaging (DWI) and T2-weighted gradient echo, and all patients in this study were detected to have HT within 24 h and 7 days (±2) of the stroke onset. Furthermore, with the aim of diagnosing HT promptly, an imaging examination was performed whenever the patient's clinical condition appeared to worsen. All imaging studies were reviewed retrospectively by consensus of two experienced neurologists blinded to the clinical data, and the presence of HT and its subtypes was confirmed according to the results of CT/MRI tests.

HT can be further categorized radiologically and symptomatically on the basis of the recommendations of the European Cooperative Acute Stroke Study (35). The different types of HT after AIS were divided into hemorrhagic infarction (HI) types 1 and 2 (HI-1 and HI-2) and parenchymal hematoma (PH) types 1 and 2 (PH-1 and PH-2) (35, 36). HT was then further classified as symptomatic or asymptomatic according to whether the neurological deterioration was present. The diagnosis of symptomatic HT (sHT) required an increase of more than 4 points on the NIHSS score, which indicated clinical deterioration; the remaining HT not meeting the requirement was considered asymptomatic HT (asHT) (37).

Statistical Analysis

Standard statistical methods were used for descriptive statistics. Categorical variables were presented as frequencies and continuous variables as mean ± standard deviation or median (quartiles), as appropriate. Depending on the normality of distribution, the Student t test or the Mann–Whitney test was used for continuous variables, as appropriate; the Pearson χ^2 test or Fisher exact test was used for categorical variables. The receiver operating characteristic (ROC) curve was applied to determine an optimal cutoff value for the FIB-4 score according to the Youden index. Statistical comparisons among the different degrees of HT were conducted using the Kruskal–Wallis test or one-way analysis of variance (ANOVA) with liver fibrosis index as the variable. Known confounding factors and main baseline variables associated with HT identified in the univariate analysis were selected to be covariates. We used multivariate-adjusted binary logistic regression to identify whether the liver fibrosis index might be an independent predictor of HT, HI, and PH. A two-tailed P-value of <0.05 was considered to be statistically significant for all tests. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS 19.0 for Windows, SPSS. Inc., Chicago, IL, USA) and GraphPad Prism, version 8.0.2.

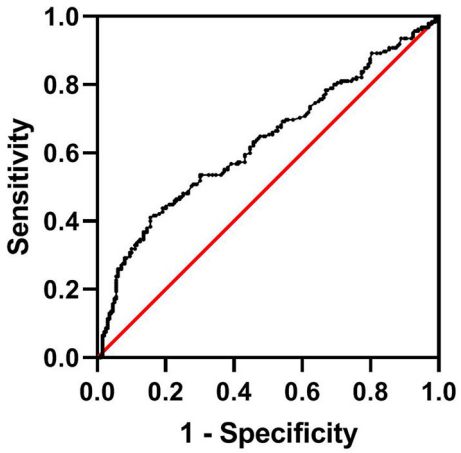

FIGURE 1 | Determination of the cutoff value for the FIB-4 score in AIS patients undergoing HT by ROC analysis. AIS, acute ischemic stroke; HT, hemorrhagic transformation; FIB-4, Fibrosis-4 score; ROC, receiver operating characteristic.

RESULTS

Characteristics of the Study Population

Of the total 287 AIS patients who suffered HT, a total of 102 patients were excluded from the study. These included 4 patients who had known overt liver disease, 93 patients with alcohol use, 3 patients with hepatotoxic medication use, and 2 patients with missing laboratory data. Another 199 age- and sex-matched AIS inpatients without HT from the stroke center at our institution were also enrolled. Therefore, a total of 185 consecutive participants with HT and 199 age- and sex-matched stroke patients without HT were enrolled in the final study. In this study, the median age of patients was 73 years (range 25–96). There were 221 males (57.6%) and 163 females (42.4%). Among the patients with HT, the majority (96.2%) had asHT, while only seven patients (3.8%) were classified as having sHT. According to the imaging features, HI-1 occurred in 31 (17.7%) patients, HI-2 in 63 (36.0%), PH-1 in 40 (22.9%), and PH-2 in 41 (23.4%). The optimal cutoff value for the FIB-4 score determined by the ROC curve was 2.68 (area under the ROC curve: 0.631, 95% CI: 0.575–0.687, $P < 0.001$) with a sensitivity of 41.1% and specificity of 84.4% (**Figure 1**). The patients were assigned into two groups based on a high (≥2.68) or low (<2.68) FIB-4 score.

The demographic, clinical, and laboratory characteristics of the patients with and without HT are presented in **Table 1**. As expected, in both groups, patients had generally normal standard liver chemistry examination indices; 7.0% patients without HT and 16.2% with HT had an AST >40 IU/L, while 8.5% patients without HT and 9.2% with HT had an ALT >40 IU/L (**Table 1**). In this study sample, patients with a history of atrial fibrillation, a large infarction area, and anticoagulation therapy were more likely to undergo HT, while those with a history of antiplatelet or lipid-lowering therapies were less likely to undergo HT. In comparison to patients without HT, those in the HT group had

TABLE 1 | Baseline characteristics of AIS patients with and without HT.

Variables	Non-HT ($n = 199$)	HT ($n = 185$)	*P*
PATIENT CHARACTERISTICS			
Age (years)	73.0 (17.0)	73.0 (17.0)	0.891
Male, *n* (%)	115 (57.8%)	106 (57.3%)	0.922
BMI (kg/m^2)	23.0 (4.0)	23.0 (3.4)	0.898
Married, *n* (%)	183 (92.4%)	173 (93.5%)	0.677
History of stroke, *n* (%)	21 (10.7%)	29 (15.7%)	0.146
History of atrial fibrillation, *n* (%)	20 (10.1%)	79 (42.7%)	0.000*
History of hypertension, *n* (%)	145 (73.6%)	121 (65.4%)	0.082
History of diabetes, *n* (%)	59 (29.9%)	44 (23.8%)	0.175
History of dyslipidemia, *n* (%)	14 (7.1%)	12 (6.5%)	0.821
Current smoking, *n* (%)	48 (24.1%)	30 (16.2%)	0.054
BIOCHEMISTRY AND VITAL SIGNS ON ADMISSION			
Baseline SBP (mmHg)	158.0 ± 22.7	148.1 ± 24.2	0.000*
Baseline DBP (mmHg)	79.5 (18.0)	81.0 (17.0)	0.343
White cell count ($\times 10^9$/L)	6.4 (2.0)	8.3 (3.6)	0.000*
Platelets ($\times 10^9$/L)	208.0 (58.0)	191.0 (83.0)	0.006
Fibrinogen (g/L)	3.4 (1.2)	3.9 (1.7)	0.000*
Creatinine (μmol/L)	71.5 (28.3)	65.0 (24.0)	0.007
Glucose levels (mmol/L)	5.3 (1.8)	5.7 (2.1)	0.029
HbA1c (%)	6.0 (1.6)	6.0 (1.3)	0.186
AST (units/L)	21.0 (8.0)	26.0 (12.0)	0.000
AST > 40 units/L	14 (7.0%)	30 (16.2%)	0.005
ALT (units/L)	17.0 (11.0)	17.0 (13.0)	0.416
ALT > 40 units/L	17 (8.5%)	17 (9.2%)	0.824
Total cholesterol (mmol/L)	1.9 (2.4)	4.4 (1.6)	0.000*
HDL cholesterol (mg/dl)	1.0 (0.3)	1.1 (0.4)	0.113
LDL cholesterol (mmol/L)	2.6 (1.4)	2.6 (1.3)	0.685
Large size of the infarction area, *n* (%)	5 (2.5%)	64 (34.6%)	0.000*
NIHSS on admission, median (IQR)	3.0 (4.0)	10.0 (9.0)	0.000*
FIB-4	1.92 (1.10)	2.36 (1.77)	**0.000***
Stroke mechanisms			0.000*
Atherosclerotic, *n* (%)	152 (84.9%)	113 (63.1%)	
Cardioembolic, *n* (%)	14 (7.8%)	65 (36.3%)	
Lacunar, *n* (%)	2 (1.1%)	0 (0.0%)	
Other causes, *n* (%)	11 (6.1%)	1 (0.6%)	
INITIAL TREATMENT IN HOSPITAL			
Antiplatelets, *n* (%)	180 (90.5%)	104 (56.2%)	0.000*
Anticoagulants, *n* (%)	22 (11.1%)	55 (29.7%)	0.000*
Lipid-lowering agents, *n* (%)	183 (95.3%)	157 (85.8%)	0.002

**P < 0.001. AIS, acute ischemic stroke; HT, hemorrhagic transformation; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; AST, aspartate aminotransferase; ALT, alanine aminotransferase; NIHSS, National Institutes of Health Stroke Scale; FIB-4, Fibrosis-4 score. The bold values were the P values of our object of study.*

higher baseline white cell count, fibrinogen, glucose levels, AST levels, total cholesterol, and initial NIHSS scores.

The median FIB-4 score was 2.07 (interquartile, 1.30). The baseline demography and disease characteristics of the patients stratified by the FIB-4 score (<2.68 vs. ≥2.68) are shown in **Table 2**. Compared to patients in the low-FIB-4-score group, those in the high-FIB-4-score group were older and had higher creatinine, total cholesterol, and NIHSS scores and were more likely to undergo atrial fibrillation, a large size of infarction, and anticoagulant treatments. Moreover, the patients in the high-FIB-4-score group tended to have lower platelets and were less likely to undergo diabetes mellitus,

TABLE 2 | Baseline characteristics of patients with AIS, stratified by FIB-4 scores of <2.68 and ≥2.68.

Variables	FIB-4 score < 2.68 (*n* = 277)	FIB-4 score ≥ 2.68 (*n* = 107)	*P*
PATIENT CHARACTERISTICS			
Age (years)	70.0 (16.0)	79.0 (8.0)	0.000*
Male, *n* (%)	159 (57.4%)	62 (57.9%)	0.923
BMI (kg/m^2)	23.4 ± 3.1	21.3 ± 2.4	0.006
History of atrial fibrillation, *n* (%)	54 (19.6%)	45 (42.1%)	0.000*
History of hypertension, *n* (%)	192 (69.8%)	74 (69.2%)	0.900
History of diabetes mellitus, *n* (%)	87 (31.6%)	16 (15.0%)	0.001
History of dyslipidemia, *n* (%)	20 (7.3%)	6 (5.6%)	0.556
Current smoking, *n* (%)	68 (24.5%)	10 (9.3%)	0.001
BIOCHEMISTRY AND VITAL SIGNS ON ADMISSION			
Baseline SBP (mmHg)	152.8 ± 25.0	154.1 ± 20.8	0.621
White cell count (×10^9/L)	7.0 (2.8)	7.5 (3.7)	0.266
Platelets (×10^9/L)	215.0 (65.0)	162.0 (66.0)	0.000*
Fibrinogen (g/L)	3.6 (1.3)	3.7 (1.6)	0.315
Creatinine (μmol/L)	68.0 (27.8)	72.0 (28.0)	0.083
Glucose levels (mmol/L)	5.5 (2.2)	5.6 (1.7)	0.935
AST (units/L)	21.0 (9.5)	29.0 (18.0)	0.000*
AST > 40 units/L	14 (5.1%)	30 (28.0%)	0.000*
ALT (units/L)	17.0 (11.0)	16.0 (12.0)	0.268
ALT > 40 units/L	22 (7.9%)	12 (11.2%)	0.311
Total cholesterol (mmol/L)	3.2 (3.2)	4.0 (1.6)	0.001
HDL cholesterol (mg/dl)	1.0 (0.3)	1.1 (0.3)	0.024
LDL cholesterol (mmol/L)	2.6 (1.4)	2.5 (1.2)	0.099
Large size of the infarction area, *n* (%)	35 (12.7%)	34 (31.8%)	0.000*
NIHSS on admission, median (IQR)	4.0 (6.0)	10.0 (10.0)	0.000*
HT, *n* (%)	109 (39.4%)	76 (71.0%)	**0.000***
Stroke mechanisms			0.000*
Atherosclerotic, *n* (%)	201 (78.8%)	64 (62.1%)	
Cardioembolic, *n* (%)	41 (16.1%)	38 (36.9%)	
Lacunar, *n* (%)	1 (0.4%)	1 (1.0%)	
Other causes, *n* (%)	12 (4.7%)	0(0.0%)	
INITIAL TREATMENT IN HOSPITAL			
Antiplatelets, *n* (%)	219 (79.1%)	65 (60.7%)	0.000*
Anticoagulants, *n* (%)	41 (14.8%)	36 (33.6%)	0.000*
Lipid-lowering agents, *n* (%)	254 (93.7%)	86 (82.7%)	0.001

*$P < 0.001$.

AIS, acute ischemic stroke; HT, hemorrhagic transformation; BMI, body mass index; SBP, systolic blood pressure; AST, aspartate aminotransferase; ALT, alanine aminotransferase; NIHSS, National Institutes of Health Stroke Scale; FIB-4, Fibrosis-4 score. The bold values were the P values of our object of study.

antiplatelet, or lipid-lowering therapies in comparison to those in the low-FIB-4-score group. Baseline characteristics of the patients according to the subcategorized groups of HT are shown in **Table 3**.

Fibrosis Indices and HT

Baseline FIB-4 scores were significantly higher in patients with HT than in those without HT (1.92 ± 1.10 vs. 2.36 ± 1.77, $P < 0.001$; **Table 1**). As for the radiological status of HT, baseline FIB-4 scores were significantly different among subjects in three groups ($H = 18.924$, $P < 0.001$ by Kruskal–Wallis test; **Table 3**, **Figure 2**). Additionally, **Figure 2** shows that the FIB-4 scores were significantly higher in patients with HI or PH when compared to those without HT after the Bonferroni modification [2.29 (1.60–3.29) vs. 1.92 (1.35–2.45), $P = 0.002$; 2.36 (1.64–3.40) vs. 1.92 (1.35–2.45), $P < 0.001$, respectively]. However, there is no significant difference in FIB-4 scores between patients with HI and PH. The proportions of subjects

TABLE 3 | Baseline characteristics of the patients according to the subcategorized groups of HT.

Variables	Non-HT ($n = 199$)	HI ($n = 94$)	PH ($n = 81$)	*P*
PATIENT CHARACTERISTICS				
Age (years)	73.0 (17.0)	73.0 (16.0)	71.0 (22.0)	0.501
Male, *n* (%)	115 (57.8%)	53.0 (56.4%)	46 (56.8%)	0.971
BMI (kg/m^2)	23.0 (4.0)	22.8 (3.7)	23.8 (4.7)	0.904
History of atrial fibrillation, *n* (%)	20 (10.1%)	34 (36.2%)	43 (53.1%)	0.000*
History of hypertension, *n* (%)	145 (73.6%)	65 (69.1%)	49 (60.5%)	0.096
History of diabetes mellitus, *n* (%)	59 (29.9%)	22 (23.4%)	19 (23.5%)	0.367
History of dyslipidemia, *n* (%)	14 (7.1%)	9 (9.6%)	3 (3.8%)	0.324
Current smoking, *n* (%)	48 (24.1%)	15 (16.0%)	13 (16.0%)	0.150
BIOCHEMISTRY AND VITAL SIGNS ON ADMISSION				
Baseline SBP (mmHg)	158.0 ± 22.7	149.7 ± 25.3	145.0 ± 22.6	0.000*
White cell count ($\times 10^9$/L)	6.4 (2.0)	7.6 (3.8)	8.6 (3.3)	0.000*
Platelets ($\times 10^9$/L)	208.0 (58.0)	196.0 (65.8)	183.0 (98.0)	0.016
Fibrinogen (g/L)	3.4 (1.2)	3.9 (1.5)	3.9 (2.0)	0.000*
Creatinine (μmol/L)	71.5 (28.3)	64.0 (22.5)	69.0 (23.0)	0.007
Glucose levels (mmol/L)	5.3 (1.8)	5.7 (2.1)	5.8 (2.1)	0.026
AST (units/L)	21.0 (8.0)	26.5 (11.3)	24.0 (12.5)	0.000*
AST > 40 units/L	14 (7.0%)	16 (17.0%)	11 (13.6%)	0.027
ALT (units/L)	17.0 (11.0)	17.5 (13.3)	16.0 (12.5)	0.283
ALT > 40 units/L	17 (8.5%)	7 (7.4%)	8 (9.9%)	0.849
FIB-4	1.92 (1.10)	2.29 (1.70)	2.36 (1.76)	**0.000***
Total cholesterol (mmol/L)	1.9 (2.4)	4.3 (2.0)	4.5 (1.3)	0.000*
HDL cholesterol (mg/dl)	1.0 (0.3)	1.1 (0.3)	1.1 (0.4)	0.073
LDL cholesterol (mmol/L)	2.6 (1.4)	2.5 (1.4)	2.7 (1.2)	0.793
Large size of the infarction area, *n* (%)	5 (2.5%)	33 (35.1%)	30 (37.0%)	0.000*
NIHSS on admission, median (IQR)	3.0 (4.0)	8.0 (9.0)	12.0 (9.0)	0.000*
Stroke mechanisms				0.000*
Atherosclerotic, *n* (%)	152 (84.9%)	68 (76.4%)	38 (47.5%)	
Cardioembolic, *n* (%)	14 (7.8%)	21 (23.6%)	41 (51.2%)	
Lacunar, *n* (%)	2 (1.1%)	0 (0.0%)	0 (0.0%)	
Other causes, *n* (%)	11 (6.1%)	0 (0.0%)	1 (1.3%)	
INITIAL TREATMENT IN HOSPITAL				
Antiplatelets, *n* (%)	180 (90.5%)	54 (57.4%)	44 (54.3%)	0.000*
Anticoagulants, *n* (%)	22 (11.1%)	25 (26.6%)	28 (34.6%)	0.000*
Lipid-lowering agents, *n* (%)	183 (95.3%)	80 (87.0%)	67 (82.7%)	0.002

*$P < 0.001$. *HT, hemorrhagic transformation; HI, hemorrhagic infarct; PH, parenchymal hematoma; BMI, body mass index; SBP, systolic blood pressure; AST, aspartate aminotransferase; ALT, alanine aminotransferase; NIHSS, National Institutes of Health Stroke Scale; FIB-4, Fibrosis-4 score. The bold values were the P values of our object of study.*

diagnosed with HT were higher in the high-FIB-4-score group than in the low-FIB-4-score group (39.4 vs. 71.0%, chi-square test $P < 0.001$; **Figure 3**).

In univariate analyses, patients in the high-FIB-4-score group were associated with an increased risk for HT with an odds ratio (OR) (95% CI = 2.333–6.120, $P < 0.001$) of 3.779 without adjustment and showed significant associations with a high risk of HI (OR = 4.295, 95% CI = 2.459–7.499, $P < 0.001$) as well as PH (OR = 3.188, 95% CI = 1.764–5.761, $P < 0.001$; **Figure 4**). Risk for HT was also linked with atrial fibrillation, elevated baseline NIHSS score, total cholesterol, and lack of antiplatelet use.

A distinction was made between the two adjusted multivariable models, and the covariates for each model were the same for each dependent variable (**Figure 4**). Compared with the patients in the low-FIB-4-score group, those in the high-FIB-4-score group had an OR (95% CI) of 4.720 (2.795, 7.970) for HT, 4.664 (2.567, 8.473) for HI, and 4.486 (2.312, 8.706) for PH after adjusting for age and gender (all $P < 0.001$; **Figure 4**). After further adjusting for factors already identified

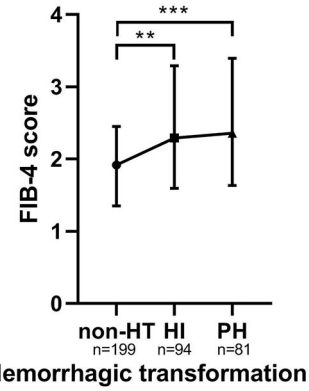

FIGURE 2 | The FIB-4 score in the subcategorized groups of HT. Each data point and error bar corresponds to the median and interquartile range of the FIB-4 score by the subcategorized groups of HT. HT, hemorrhagic transformation; HI, hemorrhagic infarct; PH, parenchymal hematoma. $^{**}P < 0.01$; $^{***}P < 0.001$.

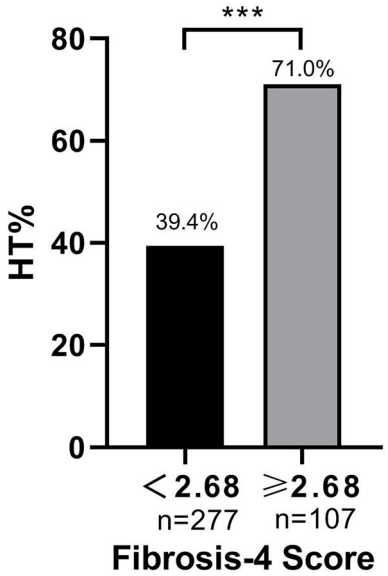

FIGURE 3 | The incidence of HT at different FIB-4 score. $^{***}P < 0.001$.

as risk factors for HT and those potential factors detected in the univariate analysis (Model 2: adjusting for age, gender, SBP, baseline NIHSS score, hypertension, atrial fibrillation, diabetes, dyslipidemia, smoking status, stroke mechanism, size of the infarction area, baseline white cell count, fibrinogen, total cholesterol, and anticoagulant, antiplatelet, and lipid-lowering therapies), the risk of HT (OR 3.461 [95% CI, 1.404–8.531]) and HI (OR 4.387 [95% CI, 1.542–12.480]) remained significant ($P = 0.007$ and $P = 0.006$, respectively). However, the risk of PH was not significantly higher in the high-FIB-4-score group (OR 3.088 [95% CI, 0.757–12.605], $P = 0.116$).

DISCUSSION

In this study, the liver fibrosis index was independently associated with an increased risk for HT, even after adjustment for potential and known confounders. To our knowledge, this is the first study to explore and analyze the relationship between liver fibrosis and HT in patients with AIS. It is noteworthy that these associations were found in a study population in which standard liver enzyme levels were commonly normal.

The liver dysfunction has been found to contribute to hematoma expansion in spontaneous ICH (38–41) and HT in AIS (42). In addition, the HAS-BLED score, which includes liver dysfunction as a fundamental item, is widely used for evaluating hemorrhagic risk (43). One such study found that derangements in individual liver enzymes, such as increased serum alkaline phosphatase, were associated with high-risk sHT in AIS patients (44). However, these studies were mostly based on individual, non-specific hepatic enzyme tests in populations with heavy alcohol use. In contrast, our study, in accordance with prior studies, suggested a novel association between a relatively validated liver fibrosis index and HT in patients with AIS. Several studies have identified that standard hepatic chemistries are generally normal in patients with chronic liver disease. Moreover, the proportion of individuals with imaging evidence of significant liver fibrosis whose levels of transaminase are normal is nearly three quarters (9, 45). Interestingly, a recent retrospective cohort study showed the associations between liver fibrosis indices and admission hematoma volume, hematoma enlargement, and 3-month mortality despite largely normal standard hepatic chemistries among patients with ICH (13). These findings inspired us to consider the presence of subclinical liver fibrosis and raised the possibility that liver fibrosis is associated with HT in AIS patients without clinically overt liver disease.

Recently, some reports have considered either patients without known overt liver disease as well as alcohol use or isolated derangements in individual hepatic enzyme levels when analyzing the association between liver disease and outcomes of stroke. Tan et al. found that in univariable analysis, subclinical abnormalities in individual liver enzymes were linked with poor prognosis in ICH, but these associations were not significant after adjusting for confounding factors (46). In another study, associations were found between liver cirrhosis and higher in-hospital mortality in ICH among patients with overt liver disease caused by virus or alcohol (47). Additionally, with regard to alcohol use or clinically overt liver disease, recent studies have suggested that liver fibrosis was independently associated with the risk of incident of cardiovascular events and prognosis of stroke (3, 5, 48). Based upon these data, our study demonstrated an association between the liver fibrosis score and HT among AIS patients with largely normal standard liver chemistries. sHT is an important clinical outcome of AIS patients, increasing the worse prognosis together with asHT (49). According to a recent study, about 8% of patients will develop sHT and subsequent worsening of the outcomes after thrombolysis therapies (50). However, due to the limited number

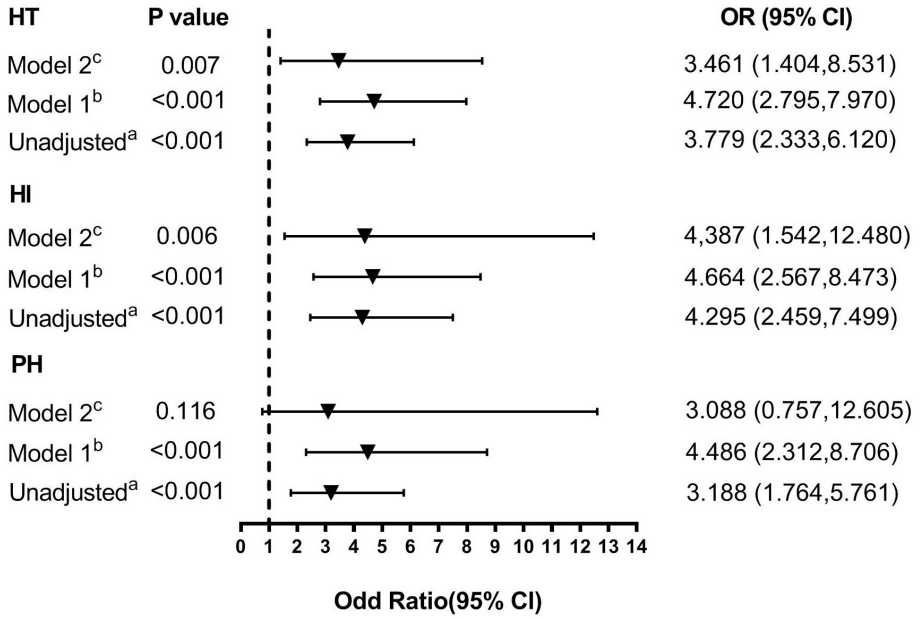

FIGURE 4 | Multivariate adjusted odds ratios for the association between FIB-4 score and the subcategorized groups of HT (including HT, HI, and PH, respectively). OR, odds radio; CI, confidence level; HI, hemorrhagic infarct; HT: hemorrhagic transformation; PH, parenchymal hematoma. **(A)** Reference OR (1.000) is an FIB-4 score <2.68. **(B)** Model 1: adjusted for age and sex. **(C)** Model 2: adjusted for covariates from Model 1 and further adjusted for vascular risk factors (history of hypertension, atrial fibrillation, diabetes, dyslipidemia, and current smoking) and systolic blood pressure, stroke mechanism, size of the infarction area, baseline NIHSS score, baseline white cell counts, fibrinogen, total cholesterol, and anticoagulant, antiplatelet, and lipid-lowering therapies.

of patients with sHT in our study, we did not perform this analysis on the association between liver fibrosis and sHT. More studies need to be conducted to further analyze the relationship between liver fibrosis and sHT. In the process of research, we have hypothesized that the liver fibrosis index was a predictor of HT severity. It was previously shown that PH represented poor clinical outcomes in stroke patients. However, there was no statistically significant difference between HI and PH in this study. We hope that more research can be designed to further confirm the results.

Although the mechanisms underlying the association between liver fibrosis and HT remain obscure, several explanations may account for the observed association. First, it has been demonstrated that liver fibrosis increases endothelial dysfunction (51, 52). Second, inflammation is typically present in all liver disease stages and associated with the development of liver fibrosis (53). An inflammatory response resulting in the increasing release of plasma biomarkers of inflammation can aggravate endothelial dysfunction (8, 54). Third, according to previous study, oxidative stress represents a shared pathophysiological disorder between liver disease and cardiovascular risk factors (55). Studies have suggested that oxidative stress could act as a potential trigger of HT by undermining the integrity of both basal lamina and endothelial tight junctions in the blood–brain barrier (56). Furthermore, liver fibrosis may contribute to HT through the mechanism of subclinical coagulopathy (57). Progression of liver fibrosis reduces the production of thrombopoietin by hepatocytes and, hence, reduced platelet production (58). Finally, fibrogenesis can be intensified by interfering with the fibrolytic activity of the TIMP-1/MMP system, which is closely related to HT in patients with AIS (59, 60). Though unconfirmed, the expression of the MMP family in liver fibrosis may play a vital role in HT.

There are limitations in our study. First, patients with thrombolytic therapy were excluded in this study, and a subgroup analysis of patients with and without thrombolytic therapy was not performed. Therefore, we expect further investigation to eliminate the effect of thrombolytic therapy on the results in this study. Second, we could not establish causality as this study is a retrospective, single-center study. Further prospective, multicenter studies are still needed to confirm the results. Third, we purposefully excluded the patients who consumed alcohol because the liver fibrosis index was proven to be accurate in patients with NAFLD. Fourth, the infarct size was taken as a categorical variable in the analysis, and it is better to calculate the infarct size using the Alberta Stroke Program Early CT Score (ASPECTS) system by trained radiologists. Moreover, we did not analyze the relationship between liver fibrosis and sHT due to the limited sample size of this study and because the majority of patients with HT were asymptomatic. Finally, we did not have liver imaging or liver biopsy data to ascertain the presence of liver fibrosis in our study population though two simple, non-invasive, and validated biomarkers were used to assess the extent of liver fibrosis.

CONCLUSION

In conclusion, our study demonstrated that liver fibrosis was associated with HT among patients with AIS despite largely normal standard liver chemistries.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: Research data are not shared. Requests to access these datasets should be directed to Jin-Cai He, hjc@wmu.edu.cn.

ETHICS STATEMENT

The studies including human participants were reviewed and approved by the Institutional Review Board and Research Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. Written informed consent was not required as the study was a retrospective study.

AUTHOR CONTRIBUTIONS

C-XY and J-CH designed the study. Y-TR and Y-YZ interpreted data. C-XY wrote the manuscript. H-RC, Q-QC, and Y-BC prepared the figures. W-LH and Y-TR did the statistical analyses. G-QH, Q-QC, and Y-YZ screened and extracted the data. J-CH supervised the study. All authors have made an intellectual contribution to the manuscript and approved the submission. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Targher G, Bertolini L, Poli F, Rodella S, Scala L, Tessari R, et al. Nonalcoholic fatty liver disease and risk of future cardiovascular events among type 2 diabetic patients. *Diabetes*. (2005) 54:3541–6. doi: 10.2337/diabetes.54.12.3541
2. Parikh NS, Merkler AE, Schneider Y, Navi BB, Kamel H. Discharge disposition after stroke in patients with liver disease. *Stroke*. (2017) 48:476–8. doi: 10.1161/STROKEAHA.116.016016
3. Baratta F, Pastori D, Angelico F, Balla A, Paganini AM, Cocomello N, et al. Nonalcoholic fatty liver disease and fibrosis associated with increased risk of cardiovascular events in a prospective study. *Clin Gastroenterol Hepatol Off Clin Pract J Am Gastroenterol Assoc*. (2019). doi: 10.1016/j.cgh.2019.12.026. [Epub ahead of print].
4. Paul S, Davis AM. Diagnosis and management of nonalcoholic fatty liver disease. *JAMA*. (2018) 320:2474. doi: 10.1001/jama.2018.17365
5. Walker AP. Ischaemic stroke and liver fibrosis. *Atherosclerosis*. (2017) 260:153–5. doi: 10.1016/j.atherosclerosis.2017.03.028
6. Parikh NS, Navi BB, Schneider Y, Jesudian A, Kamel H. Association between cirrhosis and stroke in a nationally representative cohort. *JAMA Neurol*. (2017) 74:927–32. doi: 10.1001/jamaneurol.2017.0923
7. Baik M, Kim SU, Kang S, Park HJ, Nam HS, Heo JH, et al. Liver fibrosis, not steatosis, associates with long-term outcomes in ischaemic stroke patients. *Cerebrovasc Dis*. (2019) 47:32–9. doi: 10.1159/000497069
8. Bataller R, Brenner DA. Liver fibrosis. *J Clin Invest*. (2005) 115:209–18. doi: 10.1172/JCI24282
9. Caballería L, Pera G, Arteaga I, Rodríguez L, Alumà A, Morillas RM, et al. High prevalence of liver fibrosis among european adults with unknown liver disease: a population-based study. *Clin Gastroenterol Hepatol Off Clin Pract J Am Gastroenterol Assoc*. (2018) 16:1138–45.e5. doi: 10.1016/j.cgh.2017.12.048
10. You SC, Kim KJ, Kim SU, Kim BK, Park JY, Kim DY, et al. Factors associated with significant liver fibrosis assessed using transient elastography in general population. *World J Gastroenterol*. (2015) 21:1158–66. doi: 10.3748/wjg.v21.i4.1158
11. Kim SU, Song D, Heo JH, Yoo J, Kim BK, Park JY, et al. Liver fibrosis assessed with transient elastography is an independent risk factor for ischemic stroke. *Atherosclerosis*. (2017) 260:156–62. doi: 10.1016/j.atherosclerosis.2017.02.005
12. Kim D, Kim WR, Kim HJ, Therneau TM. Association between noninvasive fibrosis markers and mortality among adults with nonalcoholic fatty liver disease in the United States. *Hepatology*. (2013) 57:1357–65. doi: 10.1002/hep.26156
13. Parikh NS, Kamel H, Navi BB, Iadecola C, Merkler AE, Jesudian A, et al. Liver fibrosis indices and outcomes after primary intracerebral hemorrhage. *Stroke*. (2020) 51:830–7. doi: 10.1161/STROKEAHA.119.028161
14. Yaghi S, Willey JZ, Cucchiara B, Goldstein JN, Gonzales NR, Khatri P, et al. Treatment and outcome of hemorrhagic transformation after intravenous alteplase in acute ischemic stroke: a scientific statement for healthcare professionals from the american heart association/american stroke association. *Stroke*. (2017) 48:e343–61. doi: 10.1161/STR.0000000000000152
15. Jickling GC, Liu D, Stamova B, Ander BP, Zhan X, Lu A, et al. Hemorrhagic transformation after ischemic stroke in animals and humans. *J Cereb blood flow Metab Off J Int Soc Cereb Blood Flow Metab*. (2014) 34:185–99. doi: 10.1038/jcbfm.2013.203
16. Álvarez-Sabín J, Maisterra O, Santamarina E, Kase CS. Factors influencing haemorrhagic transformation in ischaemic stroke. *Lancet Neurol*. (2013) 12:689–705. doi: 10.1016/S1474-4422(13)70055-3
17. Zhu F, Labreuche J, Haussen DC, Piotin M, Steglich-Arnholm H, Taschner C, et al. Hemorrhagic Transformation After Thrombectomy for Tandem Occlusions. *Stroke*. (2019) 50:516–9. doi: 10.1161/STROKEAHA.118.023689
18. Paciaroni M, Agnelli G, Corea F, Ageno W, Alberti A, Lanari A, et al. Early hemorrhagic transformation of brain infarction: rate, predictive factors, and influence on clinical outcome: results of a prospective multicenter study. *Stroke*. (2008) 39:2249–56. doi: 10.1161/STROKEAHA.107.510321
19. Fagan SC, Lapchak PA, Liebeskind DS, Ishrat T, Ergul A. Recommendations for preclinical research in hemorrhagic transformation. *Transl Stroke Res*. (2013) 4:322–7. doi: 10.1007/s12975-012-0222-5
20. Hacke W, Donnan G, Fieschi C, Kaste M, von Kummer R, Broderick JP, et al. Association of outcome with early stroke treatment: pooled analysis of ATLANTIS, ECASS, and NINDS rt-PA stroke trials. *Lancet*. (2004) 363:768–74. doi: 10.1016/S0140-6736(04)15692-4
21. Castellanos M, Leira R, Serena J, Pumar JM, Lizasoain I, Castillo J, et al. Plasma metalloproteinase-9 concentration predicts hemorrhagic transformation in acute ischemic stroke. *Stroke*. (2003) 34:40–6. doi: 10.1161/01.STR.0000046764.57344.31
22. Burton TM, Luby M, Nadareishvili Z, Benson RT, Lynch JK, Latour LL, et al. Effects of increasing IV tPA-treated stroke mimic rates

at CT-based centers on clinical outcomes. *Neurology*. (2017) 89:343–8. doi: 10.1212/WNL.0000000000004149
23. Larrue V, von Kummer R, del Zoppo G, Bluhmki E. Hemorrhagic transformation in acute ischemic stroke. Potential contributing factors in the European cooperative acute stroke study. *Stroke*. (1997) 28:957–60. doi: 10.1161/01.STR.28.5.957
24. Lansberg MG, Albers GW, Wijman CAC. Symptomatic intracerebral hemorrhage following thrombolytic therapy for acute ischemic stroke: a review of the risk factors. *Cerebrovasc Dis*. (2007) 24:1–10. doi: 10.1159/000103110
25. Kim YD, Song D, Heo JH, Kim SU, Kim BK, Park JY, et a. Relationship between cerebral microbleeds and liver stiffness determined by transient elastography. *PLoS ONE*. (2015) 10:e0139227. doi: 10.1371/journal.pone.0139227
26. Moshayedi H, Ahrabi R, Mardani A, Sadigetegad S, Farhudi M. Association between non-alcoholic fatty liver disease and ischemic stroke. *Iran J Neurol*. (2014) 13:144–148.
27. Adams HPJ, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, et al. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. *Stroke*. (1993) 24:35–41. doi: 10.1161/01.STR.24.1.35
28. Brott T, Adams HPJ, Olinger CP, Marler JR, Barsan WG, Biller J, et al. Measurements of acute cerebral infarction: a clinical examination scale. *Stroke*. (1989) 20:864–70. doi: 10.1161/01.STR.20.7.864
29. Selim M, Fink JN, Kumar S, Caplan LR, Horkan C, Chen Y, et al. Predictors of hemorrhagic transformation after intravenous recombinant tissue plasminogen activator: prognostic value of the initial apparent diffusion coefficient and diffusion-weighted lesion volume. *Stroke*. (2002) 33:2047–52. doi: 10.1161/01.STR.0000023577.65990.4E
30. Goldstein LB, Samsa GP. Reliability of the national institutes of health stroke scale. Extension to non-neurologists in the context of a clinical trial. *Stroke*. (1997) 28:307–10. doi: 10.1161/01.STR.28.2.307
31. Yong SH, Leem AY, Kim YS, Park MS, Chang J, Kim SU, et al. Hepatic fibrosis assessed using fibrosis-4 index is predictive of all-cause mortality in patients with chronic obstructive pulmonary disease. *Int J Chron Obstruct Pulmon Dis*. (2020) 15:831–9. doi: 10.2147/COPD.S242863
32. Peleg N, Issachar A, Sneh-Arbib O, Shlomai A. AST to platelet ratio index and fibrosis 4 calculator scores for non-invasive assessment of hepatic fibrosis in patients with non-alcoholic fatty liver disease. *Dig liver Dis Off J Ital Soc Gastroenterol Ital Assoc Study Liver*. (2017) 49:1133–8. doi: 10.1016/j.dld.2017.05.002
33. Xiao G, Zhu S, Xiao X, Yan L, Yang J, Wu G. Comparison of laboratory tests, ultrasound, or magnetic resonance elastography to detect fibrosis in patients with nonalcoholic fatty liver disease: A meta-analysis. *Hepatology*. (2017) 66:1486–501. doi: 10.1002/hep.29302
34. Ott BR, Zamani A, Kleefield J, Funkenstein HH. The clinical spectrum of hemorrhagic infarction. *Stroke*. (1986) 17:630–7. doi: 10.1161/01.STR.17.4.630
35. Hacke W, Kaste M, Fieschi C, von Kummer R, Davalos A, Meier D, et al. Randomised double-blind placebo-controlled trial of thrombolytic therapy with intravenous alteplase in acute ischaemic stroke. (ECASS II). Second European-Australasian Acute Stroke Study Investigators. *Lancet*. (1998) 352:1245–51. doi: 10.1016/S0140-6736(98)08020-9
36. Larrue V, von Kummer RR, Müller A, Bluhmki E. Risk factors for severe hemorrhagic transformation in ischemic stroke patients treated with recombinant tissue plasminogen activator: a secondary analysis of the European-Australasian Acute Stroke Study. (ECASS II). *Stroke*. (2001) 32:438–41. doi: 10.1161/01.STR.32.2.438
37. Libman RB, Kwiatkowski T, El-Zammar ZMK, Levine SR. Is asymptomatic hemorrhagic transformation really innocuous? *Neurology*. (2012) 78:1703. doi: 10.1212/WNL.0b013e3182598e5b
38. Fujii Y, Tanaka R, Takeuchi S, Koike T, Minakawa T, Sasaki O. Hematoma enlargement in spontaneous intracerebral hemorrhage. *J Neurosurg*. (1994) 80:51–7. doi: 10.3171/jns.1994.80.1.0051
39. Fujii Y, Takeuchi S, Sasaki O, Minakawa T, Tanaka R. Multivariate analysis of predictors of hematoma enlargement in spontaneous intracerebral hemorrhage. *Stroke*. (1998) 29:1160–6. doi: 10.1161/01.STR.29.6.1160
40. Kazui S, Minematsu K, Yamamoto H, Sawada T, Yamaguchi T. Predisposing factors to enlargement of spontaneous intracerebral hematoma. *Stroke*. (1997) 28:2370–5. doi: 10.1161/01.STR.28.12.2370
41. Niizuma H, Suzuki J, Yonemitsu T, Otsuki T. Spontaneous intracerebral hemorrhage and liver dysfunction. *Stroke*. (1988) 19:852–56. doi: 10.1161/01.STR.19.7.852
42. Tan G, Lei C, Hao Z, Chen Y, Yuan R, Liu M. Liver function may play an uneven role in haemorrhagic transformation for stroke subtypes after acute ischaemic stroke. *Eur J Neurol*. (2016) 23:597–604. doi: 10.1111/ene.12904
43. Pisters R, Lane DA, Nieuwlaat R, de Vos CB, Crijns HJGM, Lip GYH. A novel user-friendly score. (HAS-BLED) to assess 1-year risk of major bleeding in patients with atrial fibrillation: the Euro Heart Survey. *Chest*. (2010) 138:1093–100. doi: 10.1378/chest.10-0134
44. Liu J, Wang D, Li J, Xiong Y, Liu B, Wei C, et al. Increased serum alkaline phosphatase as a predictor of symptomatic hemorrhagic transformation in ischemic stroke patients with atrial fibrillation and/or rheumatic heart disease. *J stroke Cerebrovasc Dis Off J Natl Stroke Assoc*. (2016) 25:2448–52. doi: 10.1016/j.jstrokecerebrovasdis.2016.06.017
45. Calvaruso V, Craxì A. Implication of normal liver enzymes in liver disease. *J Viral Hepat*. (2009) 16:529–36. doi: 10.1111/j.1365-2893.2009.01150.x
46. Tan G, Hao Z, Lei C, Chen Y, Yuan R, Xu M, et al. Subclinical change of liver function could also provide a clue on prognosis for patients with spontaneous intracerebral hemorrhage. *Neurol Sci Off J Ital Neurol Soc Ital Soc Clin Neurophysiol*. (2016) 37:1693–700. doi: 10.1007/s10072-016-2656-0
47. Hoya K, Tanaka Y, Uchida T, Takano I, Nagaishi M, Kowata K, et al. Intracerebral hemorrhage in patients with chronic liver disease. *Neurol Med Chir*. (2012) 52:181–5. doi: 10.2176/nmc.52.181
48. Papagianni M, Tziomalos K. Non-alcoholic fatty liver disease: an emerging predictor of stroke risk, severity and outcome. *Eur J Neurol*. (2018) 25:610–11. doi: 10.1111/ene.13584
49. Park JH, Ko Y, Kim W-J, Jang MS, Yang MH, Han M-K, et al. Is asymptomatic hemorrhagic transformation really innocuous? *Neurology*. (2012) 78:421–6. doi: 10.1212/WNL.0b013e318245d22c
50. Castellanos M, van Eendenburg C, Gubern C, Kádár E, Huguet G, Puig J, et al. Low levels of caveolin-1 predict symptomatic bleeding after thrombolytic therapy in patients with acute ischemic stroke. *Stroke*. (2018) 49:1525–7. doi: 10.1161/STROKEAHA.118.020683
51. Targher G, Bertolini L, Scala L, Zoppini G, Zenari L, Falezza G. Non-alcoholic hepatic steatosis and its relation to increased plasma biomarkers of inflammation and endothelial dysfunction in non-diabetic men. Role of visceral adipose tissue. *Diabet Med*. (2005) 22:1354–8. doi: 10.1111/j.1464-5491.2005.01646.x
52. Jagavelu K, Routray C, Shergill U, O'Hara SP, Faubion W, Shah VH. Endothelial cell toll-like receptor 4 regulates fibrosis-associated angiogenesis in the liver. *Hepatology*. (2010) 52:590–601. doi: 10.1002/hep.23739
53. Seki E, Schwabe RF. Hepatic inflammation and fibrosis: functional links and key pathways. *Hepatology*. (2015) 61:1066–79. doi: 10.1002/hep.27332
54. Lee HJ, Lee CH, Kim S, Hwang SY, Hong HC, Choi HY,et al. Association between vascular inflammation and non-alcoholic fatty liver disease: analysis by (18)F-fluorodeoxyglucose positron emission tomography. *Metabolism*. (2017) 67:72–9. doi: 10.1016/j.metabol.2016.11.004
55. Polimeni L, Del Ben M, Baratta F, Perri L, Albanese F, Pastori D, et al. Oxidative stress: new insights on the association of non-alcoholic fatty liver disease and atherosclerosis. *World J Hepatol*. (2015) 7:1325–36. doi: 10.4254%2Fwjh.v7.i10.1325
56. Khatri R, McKinney AM, Swenson B, Janardhan V. Blood-brain barrier, reperfusion injury, and hemorrhagic transformation in acute ischemic stroke. *Neurology*. (2012) 79:S52–7. doi: 10.1212/WNL.0b013e3182697e70
57. Tripodi A, Mannucci PM. The coagulopathy of chronic liver disease. *N Engl J Med*. (2011) 365:147–56. doi: 10.1056/NEJMra1011170
58. Adinolfi LE, Giordano MG, Andreana A, Tripodi MF, Utili R, Cesaro G, et al. Hepatic fibrosis plays a central role in the pathogenesis of thrombocytopenia in patients with chronic viral hepatitis. *Br J Haematol*. (2001) 113:590–5. doi: 10.1046/j.1365-2141.2001.02824.x

59. Wang W, Li M, Chen Q, Wang J. Hemorrhagic transformation after tissue plasminogen activator reperfusion therapy for ischemic stroke: mechanisms, models, and biomarkers. *Mol Neurobiol*. (2015) 52:1572–9. doi: 10.1007/s12035-014-8952-x
60. Borkham-Kamphorst E, Alexi P, Tihaa L, Haas U, Weiskirchen R. Platelet-derived growth factor-D modulates extracellular matrix homeostasis and remodeling through TIMP-1 induction and attenuation of MMP-2 and MMP-9 gelatinase activities. *Biochem Biophys Res Commun*. (2015) 457:307–13. doi: 10.1016/j.bbrc.2014.12.106

Conflict of Interest: The 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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Plasmin Generation Potential and Recanalization in Acute Ischaemic Stroke; an Observational Cohort Study of Stroke Biobank Samples

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Rationale: More than half of patients who receive thrombolysis for acute ischaemic stroke fail to recanalize. Elucidating biological factors which predict recanalization could identify therapeutic targets for increasing thrombolysis success.

Hypothesis: We hypothesize that individual patient plasmin potential, as measured by *in vitro* response to recombinant tissue-type plasminogen activator (rt-PA), is a biomarker of rt-PA response, and that patients with greater plasmin response are more likely to recanalize early.

Methods: This study will use historical samples from the Barcelona Stroke Thrombolysis Biobank, comprised of 350 pre-thrombolysis plasma samples from ischaemic stroke patients who received serial transcranial-Doppler (TCD) measurements before and after thrombolysis. The plasmin potential of each patient will be measured using the level of plasmin-antiplasmin complex (PAP) generated after *in-vitro* addition of rt-PA. Levels of antiplasmin, plasminogen, t-PA activity, and PAI-1 activity will also be determined. Association between plasmin potential variables and time to recanalization [assessed on

serial TCD using the thrombolysis in brain ischemia (TIBI) score] will be assessed using Cox proportional hazards models, adjusted for potential confounders.

Outcomes: The primary outcome will be time to recanalization detected by TCD (defined as TIBI $\geq$4). Secondary outcomes will be recanalization within 6-h and recanalization and/or haemorrhagic transformation at 24-h. This analysis will utilize an expanded cohort including ∼120 patients from the Targeting Optimal Thrombolysis Outcomes (TOTO) study.

Discussion: If association between proteolytic response to rt-PA and recanalization is confirmed, future clinical treatment may customize thrombolytic therapy to maximize outcomes and minimize adverse effects for individual patients.

Keywords: acute stroke therapy, fibrinolysis, rtPA, thrombolysis, plasmin, stroke, recanalization

INTRODUCTION

Despite the increasing use of mechanical thrombectomy (MT), thrombolysis with recombinant tissue-type plasminogen activator (rt-PA) remains a cornerstone of acute ischaemic stroke treatment. Upon administration, rt-PA cleaves endogenous plasminogen (a free-floating but inactive protein) into its active form plasminogen; using fibrin as a cofactor so that the majority of plasmin generation occurs at the sight of a fibrinous clot. Plasmin is rapidly bound by α2-antiplasmin to form the inactive plasmin-antiplasmin (PAP) complex but is a potent fibrinolytic nonetheless (1). However, more than 60% of patients who receive rt-PA fail to recanalize (2) and this failure of recanalization has been associated with poor patient outcomes, even compared to patients who did not receive any reperfusion therapy (3). While great strides have been made with regard to selecting patients who are likely to have a favorable clinical outcome after thrombolysis (4–6), the reasons some patients fail to recanalize remain largely unknown. Previous studies have examined baseline levels of individual fibrinolysis markers in acute ischemic stroke patients (7) but this approach is limited as the potency of thrombolysis depends on the net capacity of a patient's plasma to generate plasmin from plasminogen in response to rt-PA and this may not necessarily correlate with baseline levels of individual fibrinolysis markers. We aim to examine association between net plasmin generation in the presence of rt-PA and clinical outcomes, particularly time to recanalization.

METHODS AND ANALYSIS

Sample Collection

Blood samples were collected via venepuncture into sodium-citrate anticoagulated vacuum-tubes during routine pre-thrombolysis work-up. Extracted plasma was stored at −80°C until analyzed.

Inclusion and Exclusion Criteria

Samples from patients who received thrombolysis and underwent transcranial doppler (TCD) before thrombolysis, and at least once within 6 h after thrombolysis will be included in the primary analysis. In addition, samples from patients who received thrombolysis and underwent angiography, either computed tomography (CT) or magnetic resonance imaging (MRI), before and 24-h post-thrombolysis will be included in secondary analyses.

Plasmin Assays (Lab Work)

Pre-thrombolysis plasma samples will be analyzed for both baseline fibrinolytic markers and *ex-vivo* plasmin generating capacity using different modalities.

All samples will be tested for baseline total plasminogen, α2-antiplasmin, plasminogen activator inhibitor-1 (PAI-1) levels, and baseline t-PA activity using specific enzyme-linked immunosorbent assays (ELISA) from Molecular Innovations, USA.

Plasmin-antiplasmin (PAP) complexes will be measured as a surrogate for rt-PA inducible plasmin activity generated after addition of rt-PA. To achieve this, PAP complexes will be measured at baseline and also after the *ex-vivo* addition of 50 nM rt-PA ("Inducible PAP") in the presence and absence of a soluble fibrin co-factor, Cyanogen bromide activated Fibrinogen (CNBr-Fib, 0.25 mg/ml) Both ELISA kit and CNBr-Fib used for the PAP analysis are from DRG International (USA).

Inducible PAP levels will be measured following stimulation of the plasma samples with vehicle (for baseline levels), addition of CNBr-Fib alone, rt-PA alone, and both rt-PA + CNBr-Fib for 10 min at 37°C and the reaction stopped by combining with aprotinin (20 μM), a serine protease inhibitor which competitively inhibits plasmin. The generated PAP complexes will then be quantified using a commercial ELISA (DRG International, USA). Treatment of samples with cofactor alone is to determine whether plasma is able to generate a significant amount of inducible PAP on its own, due to elevated levels of endogenous t-PA that may occur during acute ischaemic stroke.

Furthermore, the rate of plasmin generation within each sample will also be evaluated with an amidolytic assay using S2251, a chromogenic substrate specific for plasmin (8). The production of plasmin within the sample hydrolyses S-2251, liberating a pNA chromogenic group detected on a plate reader at an absorbance wavelength of 405 nm.

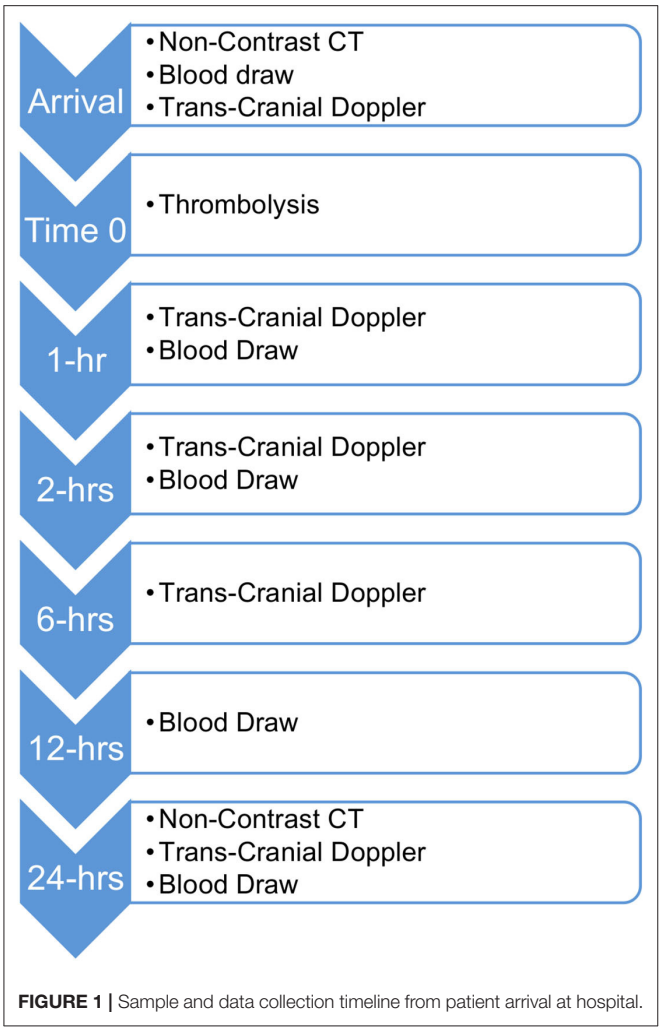

FIGURE 1 | Sample and data collection timeline from patient arrival at hospital.

PAP complex levels will also be determined in plasma samples obtained at various time points post-rt-PA thrombolysis (1, 2, 12, and 24 h). Hence the degree of rt-PA inducible PAP complexes determined in the pre-thrombolysis samples will then be correlated to the actual degree of PAP complexes formed *in vivo* after rt-PA administration. This will also provide relevant information as to the temporal nature of the response to rt-PA treatment.

Imaging/Recanalization Assessment

Patients in the primary analysis received non-contrast computed tomography (NCCT) to rule out hemorrhage and TCD pre-thrombolysis and at 1, 2, 6, and 24-h post-thrombolysis (see **Figure 1**). The initial occlusion and subsequent recanalization were assessed using the TIBI score. For the purposes of this analysis a TIBI score ≥4 will be considered as recanalization.

Statistical Analysis

The primary analysis will use Cox proportional hazards regression to estimate the relationship between plasmin potential variables and time to recanalization, defined by a thrombolysis in brain ischemia (TIBI) score of 4 or 5 (9).

The key predictive variables will be the fibrinolytic potential, as measured by the *in vitro* response to thrombolytic agent. This response will be measured in terms of the amount of PAP formed, the ratio of formed PAP to baseline PAP or the rate of plasmin activity. The Kaplan-Meier survival function for various levels of the key predictors will be visualized and a Cox Proportional-Hazard regression will be used to estimate the hazard ratio with 95% confidence intervals for the effect of each key predictor on time to recanalization after adjusting for potential confounders. Potential confounders include blood glucose level, initial occlusion size (as measured by baseline TIBI), age, sex, prior medications, and chronic illness. The proportional hazards assumption will be tested using standard statistical tests and residual plots. Predictive accuracy will be quantified using c-statistics.

Secondary analyses will use logistic regressions to examine the correlations between fibrinolytic markers and recanalization within 6 h and outcome at 24-h post-thrombolysis, recanalization and haemorrhagic transformation of cerebral infarcts detected on follow-up CT and disability at 3 months as measured by the modified Rankin scale.

TABLE 1 | Number of patients with Trans-Cranial Doppler at each time point in the Barcelona Biobank.

Timepoint	*N*
0	350
1	332
2	300
6	220
24	194

Available Sample Size

The Barcelona Stroke Thrombolysis Biobank consists of samples from a total 350 patients who underwent TCD before thrombolysis and NCCT before and 24-h after thrombolysis, 332 of whom underwent TCD at least once after thrombolysis (see **Table 1**). The TOTO (10) sample is forecast to contain samples from ∼250 patients by the time analysis is conducted, ∼120 of these patients will have vessel occlusions visible on CTA or MRA.

The sample size available for the primary recanalization analysis will consist of the 332 patients from the Barcelona Stroke Thrombolysis Biobank who underwent at least 1 post-thrombolysis TCD (11). Initially 50% of these samples will be analyzed in a pilot study. If a larger sample is required, this pilot study will allow for formal power calculations to determine the final sample size. The sample size for the logistic regression analysis of recanalization at 24-h will be ∼450 including Barcelona Stroke Thrombolysis Biobank samples and those from the TOTO study with clear vessel occlusions. Analysis of haemorrhagic transformation will include all 600 samples (350 from the Barcelona Stroke Thrombolysis Biobank and 250 from TOTO).

DISCUSSION

Individual variation in fibrinolytic potential remain poorly understood, especially with regard to its effects on clinical outcomes after thrombolysis. The Barcelona Stroke Thrombolysis Biobank provides an exceptional sample in which to examine correlations between fibrinolytic markers and recanalization as the use of serial TCD assessments allows direct assessment of early recanalization, and being collected before mechanical thrombectomy was common practice, the biobank consists entirely of patients who received thrombolysis only. Where subsequent biobank studies such as TOTO have collected much more detailed baseline data in the form of multi-modal CT, such examinations cannot be repeated as often as TCD so these studies lack the timing data available from the Barcelona Stroke Thrombolysis Biobank. In addition, the Barcelona Stroke Thrombolysis Biobank recruited patients between 2003 and 2009, before the widespread use of mechanical thrombectomy and was therefore purely focussed on thrombolysis. Subsequent biobanks (including TOTO) have been forced to either recruit patients who undergo mechanical thrombectomy, or recruit fewer patients with large-vessel occlusions.

If individual fibrinolytic potential does correlate with time to recanalization, future studies could focus on reducing variation in fibrinolytic potential, for example by supplementing plasminogen in patients before thrombolysis.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Barcelona Stroke Biobank received ethical approval from Vall d'Hebron Hospital ethics committee [Registration No. PT(HG)89/2003]. The TOTO Biobank Study received ethical approval from the Hunter New-England Health Human Research Ethics Committee (Registration No. 14/10/15/4.02). The study combining samples from these two biobanks was approved by the Monash University Human Research Ethics Committee (Registration No. 21640) with appropriate material transfer agreements between the University of Newcastle and Monash University, and between VHIR and Monash University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RM conceived and oversaw the development of the study. TL and EH wrote the statistical analysis plan. DD, JC, HH, SH, and BN developed the laboratory protocols. TL, EH, RM, CL, CG-E, NS, PG, ABi, MP, GC, JMM, LL, TK, JA, SK, MH-B, BW, and RM all oversee the TOTO biobank and contribute to the design of studies arising from it. JM, ABu, and IC contributed to the management of and access to the Barcelona Stroke Biobank and the design of the study. TL drafted the manuscript. All authors critically reviewed and edited the manuscript and have approved the final draft.

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REFERENCES

1. Abdul S, Leebeek FW, Rijken DC, Uitte de Willige S. Natural heterogeneity of α2-antiplasmin: functional and clinical consequences. *Blood.* (2016) 127:538–45. doi: 10.1182/blood-2015-09-670117
2. Bhatia R, Hill MD, Shobha N, Menon B, Bal S, Kochar P, et al. Low rates of acute recanalization with intravenous recombinant tissue plasminogen activator in ischemic stroke: real-world experience and a call for action. *Stroke.* (2010) 41:2254–8. doi: 10.1161/strokeaha.110.592535
3. Iglesias-Rey R, Rodríguez-Yáñez M, Rodríguez-Castro E, Pumar JM, Arias S, Santamaría M, et al. Worse outcome in stroke patients treated with rt-PA without early reperfusion: associated factors. *Transl Stroke Res.* (2018) 9:347–55. doi: 10.1007/s12975-017-0584-9
4. Saarinen JT, Rusanen H, Sillanpää N. Collateral score complements clot location in predicting the outcome of intravenous thrombolysis. *Am J Neuroradiol.* (2014) 35:1892–6. doi: 10.3174/ajnr.A3983
5. Bivard A, Levi C, Krishnamurthy V, McElduff P, Miteff F, Spratt NJ, et al. Perfusion computed tomography to assist decision making for stroke thrombolysis. *Brain.* (2015) 138:1919–31. doi: 10.1093/brain/awv071
6. Bivard A, Spratt N, Miteff F, Levi C, Parsons MW. Tissue is more important than time in stroke patients being assessed for thrombolysis. *Front Neurol.* (2018) 9:41. doi: 10.3389/fneur.2018.00041
7. Martí-Fàbregas J, Borrell M, Cocho D, Belvís R, Castellanos M, Montaner J, et al. Hemostatic markers of recanalization in patients with ischemic stroke treated with rt-PA. *Neurology.* (2005) 65:366–70. doi: 10.1212/01.wnl.0000171704.50395.ba
8. Niego B, Horvath A, Coughlin PB, Pugsley MK, Medcalf RL. Desmoteplase-mediated plasminogen activation and clot lysis are inhibited by the lysine analogue tranexamic acid. *Blood Coagulat Fibrinol.* (2008) 19:322–4. doi: 10.1097/MBC.0b013e3282f54568
9. Demchuk AM, Burgin WS, Christou I, Felberg RA, Barber PA, Hill MD, et al. Thrombolysis in brain ischemia (TIBI) transcranial doppler flow grades predict clinical severity, early recovery, and mortality in patients treated with intravenous tissue plasminogen activator. *Stroke.* (2001) 32:89–93. doi: 10.1161/01.STR.32.1.89
10. Holliday E, Lillicrap T, Kleinig T, Choi PMC, Maguire J, Bivard A, et al. Developing a multivariable prediction model for functional outcome after reperfusion therapy for acute ischaemic stroke: study protocol for the Targeting Optimal Thrombolysis Outcomes (TOTO) multicentre cohort study. *BMJ Open.* (2020) 10: e038180. doi: 10.1136/bmjopen-2020-038180
11. Bustamante A, Ning M, García-Berrocoso T, Penalba A, Boada C, Simats A, et al. Usefulness of ADAMTS13 to predict response to recanalization therapies in acute ischemic stroke. *Neurology.* (2018) 90:e995–1004. doi: 10.1212/wnl.0000000000005162

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Machine Learning Analysis of the Cerebrovascular Thrombi Proteome in Human Ischemic Stroke: An Exploratory Study

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Objective: Mechanical retrieval of thrombotic material from acute ischemic stroke patients provides a unique entry point for translational research investigations. Here, we resolved the proteomes of cardioembolic and atherothrombotic cerebrovascular human thrombi and applied an artificial intelligence routine to examine protein signatures between the two selected groups.

Methods: We specifically used $n = 32$ cardioembolic and $n = 28$ atherothrombotic diagnosed thrombi from patients suffering from acute stroke and treated by mechanical thrombectomy. Thrombi proteins were successfully separated by gel-electrophoresis. For each thrombi, peptide samples were analyzed by nano-flow liquid chromatography coupled to tandem mass spectrometry (nano-LC-MS/MS) to obtain specific proteomes. Relative protein quantification was performed using a label-free LFQ algorithm and all dataset were analyzed using a support-vector-machine (SVM) learning method. Data are available via ProteomeXchange with identifier PXD020398. Clinical data were also analyzed using SVM, alone or in combination with the proteomes.

Results: A total of 2,455 proteins were identified by nano-LC-MS/MS in the samples analyzed, with 438 proteins constantly detected in all samples. SVM analysis of LFQ proteomic data delivered combinations of three proteins achieving a maximum of 88.3% for correct classification of the cardioembolic and atherothrombotic samples in our cohort. The coagulation factor XIII appeared in all of the SVM protein trios, associating with cardioembolic thrombi. A combined SVM analysis of the LFQ proteome and clinical data did not deliver a better discriminatory score as compared to the proteome only.

Conclusion: Our results advance the portrayal of the human cerebrovascular thrombi proteome. The exploratory SVM analysis outlined sets of proteins for a proof-of-principle characterization of our cohort cardioembolic and atherothrombotic samples. The integrated analysis proposed herein could be further developed

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and retested on a larger patients population to better understand stroke origin and the associated cerebrovascular pathophysiology.

Keywords: stroke, thrombus, cerebrovascular, mechanical thrombectomy, proteome, support vector machine learning, neuroradiology

INTRODUCTION

Stroke is a major public health burden and the second most common cause of death worldwide (1–3). Currently, the incomplete molecular understanding of stroke pathophysiology negatively impacts patients' management, follow-up, and secondary prevention (3, 4). A recent consensus indicates that examinations of patients' intracranial thrombi could help unveil novel disease mechanisms (5). Studying the intracranial thrombi composition could advance our knowledge of the molecular mechanisms of local cerebrovascular cell damage in this disease setting (6–9).

Mechanical thrombectomy (MT) is a standard of care for patients presenting with acute ischemic stroke (AIS) due to large vessel occlusion (LVO) (10). MT allows the retrieval of cerebral thrombi from brain arteries, enabling subsequent samples storage and analysis. A few studies have analyzed the histological composition of intracranial thrombi (11, 12), describing architecture or reporting the presence of fibrin and leucocytes (13). However, an in depth characterization of the thrombi molecular components is currently lacking (11).

Here, we performed a quantitative proteomic analysis of intracranial thrombi retrieved using MT from a cohort of $n = 32$ cardioembolic and $n = 28$ atherothrombotic diagnosed AIS patients. We resolved the thrombi proteomes for our cohort samples and next applied a support-vector machine (SVM) learning approach to estimate whether specific sets of proteins, alone or in combination with available clinical data, could help differentiate the cardioembolic from atherothrombotic origin in our selected population.

MATERIALS AND METHODS

Inclusion Criteria

Patients with suspected ischemic stroke secondary to an LVO were prospectively recruited at a high-volume, comprehensive stroke center in France. Patients were required to present imaging evidence of occlusion of the internal carotid artery (ICA, cervical or intracranial part), the M1 or M2 branches of the middle cerebral artery (MCA), the basilar artery, or a tandem atheromatous occlusion defined by the occlusion of both cervical carotid artery and intracranial artery (carotid artery or MCA). Use of intravenous thrombolysis (IVT) treatment was allowed and administrated according to current guidelines (10). Stroke cause was defined by a stroke neurologist blinded to the proteomics analysis, according to the TOAST (Trial of ORG 10172 in Acute Stroke Treatment) (14) classification, after an exhaustive in-hospital workup (15) including at least computed tomography and magnetic resonance imaging, duplex sonography of the cervical arteries, blood coagulation tests, long-term electrocardiography, and transthoracic or transesophageal echocardiography. Stroke etiology was defined as "atherothrombotic tandem" when CT angiography and MR angiography demonstrated >50% stenosis or occlusion of the cervical carotid artery with associated intracranial ICA or MCA occlusion ipsilateral to the symptomatic hemisphere, in addition to exclusion of potential sources of cardiac embolism. Stroke etiology was defined as "cardioembolic" when at least one cardiac source for an embolus was identified after a complete cardiological work-up including Holter monitoring and echocardiography, in the absence of any stenosis of ipsilateral large extracranial arteries or atherosclerosis, excluding atrial fibrillation with non-cardioembolic strokes.

Exclusion criteria for the present study were: (1) failure of thrombus retrieval (failure of catheterization, patients with spontaneous reperfusion at the beginning of the procedure), (2) patients non-suitable for MT with a pre-stroke modified Rankin Scale (mRS) score of >3; (3) patients with non-atheromatous or non-cardioembolic tandem occlusions (intimal dysplasia/web, dissection), (4) patients having had MT but with a thromboembolic material unsuitable for proteomic analyses (mainly due to insufficient material amounts retrieved), (5) patients with no clear etiology or "undefined etiology" (defined as at least two possible etiologies found after a complete clinical, laboratory, and imaging work-up).

The study was approved by the local ethics committee, with the patients providing written informed consent in acute phase whenever possible. Otherwise, the consent form was signed by the patient's relatives.

Patient Characteristics

Patient demographics, vascular risk factors, imaging data, vital signs before treatment, severity of ischemic stroke, and clinical outcomes were collected with a structured questionnaire. Age, sex, cardiovascular risk factors (hypertension, dyslipidemia, diabetes mellitus, and smoking habits), time of symptom onset, National Institutes of Health Stroke Scale (NIHSS) at baseline, use of IVT, and its time from symptom onset were collected (see **Table 1**). The Alberta Stroke Program Early CT Score (ASPECT) on diffusion-weighted magnetic resonance or CT imaging was assessed by a neuroradiologist.

Endovascular Procedure

All patients were treated in a dedicated neuroangiography suite under general anesthesia or conscious sedation, after evaluation by the anesthesiology team.

Most of the procedures were performed using the Trevo® device (Stryker, Kalamazoo, Michigan) or the Solitaire FR™ device (Medtronic, Dublin, Ireland) via the femoral artery

TABLE 1 | Patients data, Treatment Characteristics, Complications, and Outcomes according to stroke etiology.

Characteristics	Cardioembolic ($n = 32$)	Atherothrombotic ($n = 28$)	*P*-Values
Age, years	79.5 (72.0–85.5)	67.5 (57.5–77.5)	0.005
Men	18/32 (56.3)	20/28 (71.4)	0.22
Medical history			
Hypertension	16/32 (50.0)	20/28 (71.4)	0.091
Diabetes	6/32 (18.8)	3/28 (10.7)	0.48
Hypercholesterolemia	10/32 (31.3)	8/28 (28.6)	0.82
Current smoking	6/32 (18.8)	10/28 (35.7)	0.14
Alcohol abuse	3/32 (9.4)	4/28 (14.3)	0.70
Coronary artery disease/Myocardial Infarction	5/32 (15.6)	3/28 (10.7)	0.71
Previous stroke or TIA	8/32 (25.0)	2/28 (7.1)	0.088
Cardiac failure	6/32 (18.8)	0/28 (0.0)	0.047
Coronary stent	1/32 (3.1)	1/28 (3.6)	NA
Coronary bypass	1/32 (3.1)	1/28 (3.6)	NA
Carotid endarterectomy	0/32 (0.0)	0/28 (0.0)	NA
Atrial fibrillation	20/32 (62.5)	1/28 (3.6)	<0.001
Previous antithrombotic medications	20/32 (62.5)	11/28 (39.3)	0.073
Aspirin	10/32 (31.3)	8/28 (28.6)	0.82
Clopidogrel	1/32 (3.1)	2/28 (14.3)	NA
Vitamin K Antagonist	7/32 (21.9)	0/28 (0.0)	0.0110
New oral anticoagulant	3/32 (9.4)	0/28 (0.0)	NA
Current stroke event			
Systolic blood pressure, mmHg	136 (111–151)	152 (139–170)	0.006
Diastolic blood pressure, mmHg	80 (65–90)	90 (76–96)	0.033
Heart rate	76 (64–90)	80 (70–96)	0.51
Weight, kg	71 (60–81)	79 (64–88)	0.18
Body Mass Index	24.4 (23.0–27.7)	26.0 (23.5–30.2)	0.23
Glycemia, mmol/L	6.8 (6.2–7.5)	6.8 (5.7–8.1)	0.77
NIHSS score	19 (10–24)	19 (14–23)	0.85
Pre-stroke mRS≥1	6/32 (18.8)	2/28 (7.1)	0.26
ASPECTS	8 (6–10)	8 (7–9)	0.84
Site of occlusion			
MCA (M1 or M2)	18/32 (56.3)	2/28 (7.1)	<0.001
Intracranial ICA	11/32 (34.4)	1/28 (3.6)	
Tandem	1/32 (3.1)	24/28 (85.7)	
Basilar artery	1/32 (3.1)	0/28 (0.0)	
Cervical ICA	1/32 (3.1)	1/28 (3.6)	
Complete blood count			
Hemoglobin	13.5 (12.4–14.9)	13.8 (12.8–14.7)	0.65
Platelets	203 (177–237)	250 (212–301)	0.011
White cells	8.9 (6.6–12.0)	10.3 (8.5–11.6)	0.15
Treatment characteristics			
Intravenous rt-PA	13/32 (40.6)	15/28 (53.6)	0.32
General anesthesia	9/32 (28.1)	15/28 (53.6)	0.45
Onset to groin puncture time, min	173 (147–327)	289 (184–693)	0.034
Onset to Intravenous rt-PA, min	130 (110–180)	130 (105–180)	0.58
Total number of passes	1 (1–2)	1 (1–2)	0.55
Reperfusion success	29/32 (90.6)	28/28 (100.0)	NA
Groin puncture to reperfusion, min	40 (31–59)	72 (50–97)	0.002
Adverse events			
Per-procedural complication	1/20 (5.0)	3/20 (15.0)	NA

(Continued)

TABLE 1 | Continued

Characteristics	Cardioembolic ($n = 32$)	Atherothrombotic ($n = 28$)	*P*-Values
Any ICH	12/32 (37.5)	11/28 (39.3)	0.89
PH or symptomatic ICH	0/32 (0.0)	0/32 (0.0)	NA
Functional outcome			
Favorable outcome (mRS 0–2)	10/32 (31)	14/28 (50)	0.14

Values expressed as no/total no. (%), or median (interquartile range).
ASPECTS, Alberta stroke program early computed tomography score; CT, computed tomography; ICA, internal carotid artery; ICH, Intracranial hemorrhage; MCA, middle cerebral artery; mRS, modified Rankin scale; NIHSS, national institutes of health stroke scale; PH, parenchyma hematoma; rt-PA, recombinant tissue plasminogen activator; TIA, transient ischemic attack.

approach. A balloon catheter was positioned in the ICA to allow flow arrest during thrombus retrieval. The stent retriever was delivered through a microcatheter and deployed across the thrombus. A distal aspiration during the stent retrieval was performed, according to the SAVE technique (16). A control angiogram was obtained to assess recanalization and reperfusion. This sequence was repeated until mTICI 2b or mTICI 2C/3 flow (defined as successful reperfusion) was established (17). The "retrograde approach" (also known as the distal-to-proximal or intracranial-first approach), aiming to recanalize the distal and symptomatic intracranial occlusion before addressing the cervical carotid lesion, was generally chosen for tandem occlusions. The interventional neuroradiologist used another thrombectomy device in the case of reperfusion failure (mTICI <2b) with the first stent retriever. Reperfusion results were reported by using the mTICI score (18). Peri-procedural complications [embolization in a new territory (defined as an angiographic occlusion in a previously unaffected vascular territory observed on the angiogram after clot removal), arterial dissection or perforation, vasospasm, and subarachnoid hemorrhage] were recorded.

Follow-Up and Outcome

All patients underwent cross-sectional imaging (computed tomography or magnetic resonance imaging) within a range of 18–24 h after the procedure. Intracranial hemorrhage was classified according to the ECASS (European Cooperative Acute Stroke Study) criteria (19). Symptomatic intracranial hemorrhage was defined as any intracerebral hemorrhage with an increase of at least four NIHSS points within 24 h, or resulting in death. The mRS at 90 days was assessed by trained research nurses unaware of the study group assignments, during face-to-face interviews, or via telephone conversations with the patients, their relatives, or their general practitioners.

Collection and Processing of Intracranial Thrombi

In the angiography room, after retrieval (**Figure 1E**), thrombi were immediately frozen at −80°C in a dedicated transportable azote freezer (Voyager, Air Liquide). In the laboratory, samples were prepared for mass spectrometry analysis. After initial mashing in a glass potter at 4°C in RIPA buffer, thrombi were further dissolved using an ultrasonic liquid processor (10 applications of 1 second each at 4°C; Vibra-cell VCX130PB, VWR) and then centrifuged (Eppendorf 5427R) at 1,200 RPM for 10 min at 4°C. Protein concentration was assessed by a bicinchoninic acid (BCA) assay. Protein extracts (20 μg) were separated by SDS-PAGE using a short (2 cm) migration. Single pieces of gel including separated proteins except hemoglobin were excised for each sample and proteins were digested in-gel using Trypsin (Trypsin Gold, Promega), as previous described (20).

Mass Spectrometry

The resulting peptide samples were analyzed online using Q-Exactive HF mass spectrometer coupled with an Ultimate 3000 RSLC (Thermo Fisher Scientific) fitted with a stainless-steel emitter (Thermo Fisher Scientific). Desalting and pre-concentration of samples were performed online on a Pepmap® pre-column (0.3 mm × 10 mm, Thermo Fisher Scientific). A gradient consisting of 2–40% of buffer B in 123 min, then 90% of buffer B during 5 min (A: 0.1% formic acid in water; B: 0.1% formic acid 80% ACN) at 300 nL/min was used to elute peptides from the capillary reverse-phase column (0.075 mm × 500 mm, Pepmap® C18, Thermo Fisher Scientific). Spectra were acquired with Xcalibur software (v4.1 Thermo Fisher Scientific). MS/MS analyses were performed in a data-dependent mode with standard settings. MS data analysis was performed using the MaxQuant software with default settings (v1.5.5.1) (21). All MS/MS spectra were searched using the Andromeda search engine (22) against the UniProtKB Reference proteome UP000005640 database for Homo sapiens (release 2019_03, https://www.uniprot.org/) and the contaminant database in MaxQuant. Default search parameters were applied. Oxidation (Met) and Acetylation (N-term) were used as variable modifications and Carbamidomethyl (Cys) was used as fixed modification. FDR was set to 1% for peptides and proteins. A representative protein ID in each protein group was automatically selected using an in-house bioinformatics tool (Leading v3.2). First, proteins with the most numerous identified peptides were isolated in a "match group" (proteins from the "Protein IDs" column with the maximum number of "peptides counts"). For the match groups where more than one protein ID were present after filtering, the best annotated protein in UniProtKB [reviewed entries rather than automatic ones, highest evidence for protein existence, most annotated protein according to the number of Gene Ontology Annotations (UniProtKB-GOA, made on 20190416)]

was defined as the "leading" protein. Graphical representation and statistical analysis of MS/MS data were performed using Perseus (v1.6.1.1). Label-free quantification (MaxQuant LFQ) was used to highlight proteins differentially expressed between samples (23).

The mass spectrometry proteomics data have been uploaded to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD020398 (24).

Data Analysis

Descriptive Analysis

Data in **Table 1** are presented as median (range) for quantitative variables, and percentage (count) for categorical variables. Baseline and treatment characteristics, complications and outcomes were compared according to stroke etiology using Chi-Square or Fisher's exact tests for categorical variables and the Mann-Whitney *U*-test for quantitative variables. No statistical comparisons were done for categorical variables with frequency <5. Statistical testing was done at the 2-tailed α level of 0.05. Data were analyzed using the SAS package, release 9.4 (SAS Institute, Cary, NC).

A support-vector machine (SVM) approach was implemented using MATLAB (r2018a, MathWorks, Natick, MA, USA). The SVM algorithm analyzes and learns from the dataset (**Supplementary Table 2**) to identify the hyperplanes for the best segregation of data according to a known discriminatory characteristic (25). In our work, the relatively small sample size prevents from achieving a correct validation step and SVM was used as a statistical tool to examine whether hyperplanes exist splitting our two groups. Here, we specifically tested whether samples segregation is attainable using combinations of up to 3 proteins (trios) from those commonly detected in all samples. Each possible combinations of three proteins from the data set in **Supplementary Table 2** was tested (n = 13,908,836), the corresponding X/Y/Z hyperplanes were defined by the SVM (see **Figure 3A**), and the percentage of correct sample classification was obtained. The protein combinations achieving the best discriminatory score for our populations were retained. SVM analysis was also performed using clinical data in **Table 1**.

RESULTS

Clinical Data, Peripheral Blood and Thrombi Characteristics

Baseline clinical data, treatment characteristics, early complications and outcomes are provided in **Table 1**. In the selected population, subjects suffering from atherothrombotic stroke were younger (67.5 vs. 79.5 years old, $p = 0.005$), presented no cardiac failure (0 vs. 18.8%, $p = 0.047$), no significant atrial fibrillation (3.6 vs. 62.5%, $p < 0.001$), and displayed higher systolic and diastolic blood pressure at admission (152 and 90 mmHg vs. 136 and 80 mmHg, $p = 0.006$ and 0.033). M1 occlusions were more frequent in the cardioembolic group (56.3 vs. 7.1%, $p < 0.001$). Groin puncture to reperfusion time was longer in the atherothrombotic group, which included 85.7% of tandem occlusions (72 vs. 40 min., $p = 0.002$). Complete blood count at admission indicated that platelet levels were higher in the atherothrombotic group (250×10^9/L vs. 203×10^9/L, $p = 0.011$; **Table 1**). Weight of the retrieved thrombi was 31.2 mg for the cardioembolic group (range 5.8–206.2 mg) and 36 mg for the atherothrombotic group (range 3.2–136.2; $p = 0.85$). Total protein concentrations were 11.20 μg/μl (5.3–22.1) and 11.1 μg/μl (4–26.5; $p = 0.82$) for the cardioembolic and atherothrombotic groups, respectively.

Analysis of the Intracranial Human Thrombi Proteome

All thrombus samples were individually processed by SDS-page chromatography and the hemoglobin band excised (**Figures 1 A–F**). Mass spectrometry analysis identified a total of 2,455 proteins in the samples analyzed. The complete list of all proteins detected in each sample is provided in **Supplementary Table 1**. A total of 438 proteins were commonly present in all the samples analyzed (**Supplementary Table 2**). Analysis of ClueGO annotations of the thrombi proteome, according to UniProtKB or EBI GOA databases, showed protein clusters for key biological pathways including metabolic processes, cytokines production, and cell proliferation, activation, or motility (**Figure 2A**). Indicating an inflammatory track are proteins associated with leukocytes activation, migration, and cell adhesion (**Figure 2B**; *high definition zoom-in*). This dataset constitutes the largest human thrombus proteome available and a shared library for the investigation of the molecular mechanisms of thrombus formation and ischemic stroke pathophysiology.

Exploring the Use of Support-Vector-Machine Learning to Analyse the Thrombi Proteome

The proteomic LFQ data obtained from our samples cohort were analyzed using a SVM routine to mathematically examine potential signatures existing between the cardioembolic and atherothrombotic proteomes. The SVM algorithm does not handle missing data across samples and the analysis was performed using the proteins commonly detected in all thrombi (438 proteins; **Supplementary Table 2**). In our SVM study we specifically aimed at identifying small set of discriminatory elements, here up to 3 proteins (see Methods). As a result, proteins trios were found by SVM providing a 88.3% accuracy of correct classification of our two sample groups. Proteins and their biological functions are detailed in **Table 2**. Factor XIII, which catalyzes the last step of the coagulation cascade by crosslinking fibrin fibers, was present in all combinations. **Figure 3A** shows an illustration of the SVM hyperplane classification for the cardioembolic and atherothrombotic samples according to the protein trio Eukaryotic translation initiation factor 2 subunit 3, Ras GTPase-activating-like protein IQGAP2, and Coagulation factor XIII. Using this specific setting, four and three patients were misclassified (light green squares in **Figure 3A**) as cardioembolic and atherothrombotic, respectively. In univariate analysis (Wilcoxon test), the coagulation Factor XIII, the Eukaryotic translation initiation factor 2 subunit 3, and the Myosin light chain kinase levels were significantly

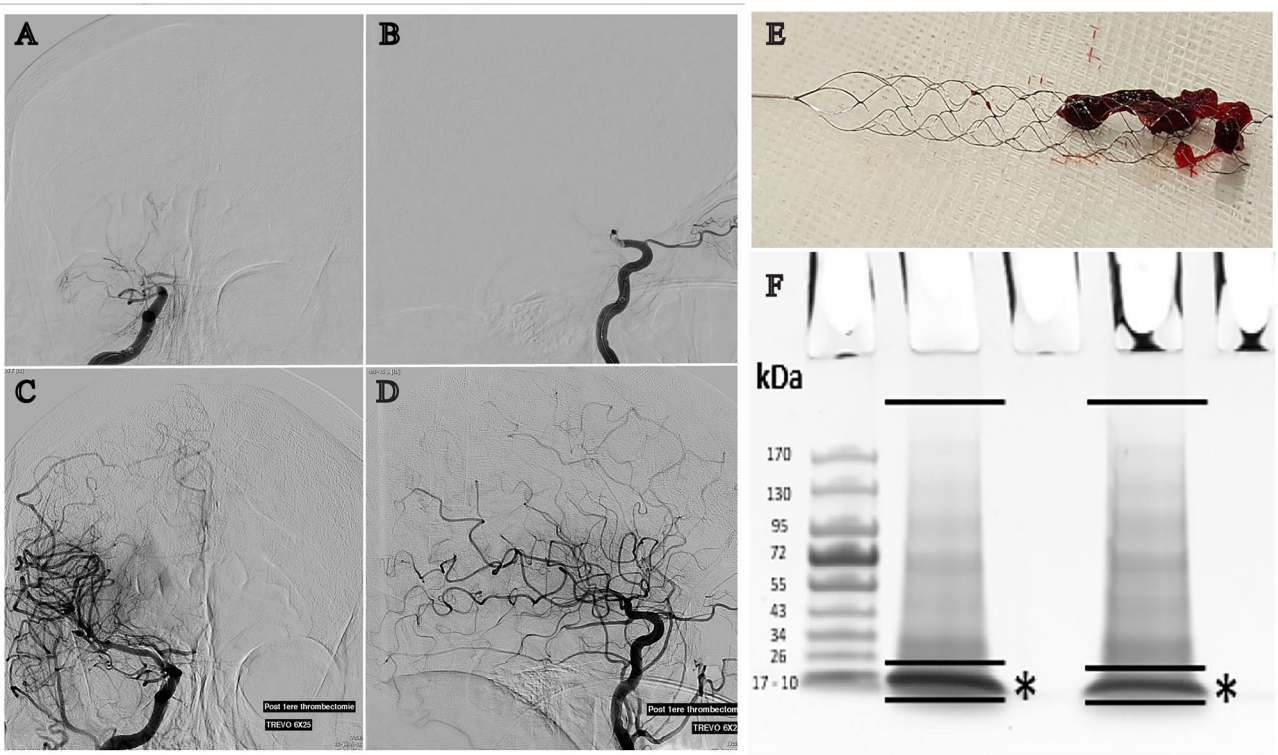

FIGURE 1 | Cerebral angiography showing a right MCA occlusion before (**A**: anteroposterior projection, **B**: lateral projection) and after recanalization (**C**: anteroposterior projection, **D**: lateral projection) achieved by mechanical thrombectomy. **(E)** Clot engaged by the Trevo® stent-retriever. **(F)** Illustration of thrombi protein separation on 4–15% polyacrylamide gels and Hemoglobin depletion (*) prior to in-gel protein digestion by trypsin.

different between the cardioembolic and atherothrombotic groups, with respective *p*-values of 0.002, 0.04, and 0.01 (see **Table 2**). These results have a dual value, suggesting potential molecular differences between cardioembolic and atherothrombotic thrombi while supporting the notion of protein biomarkers to understand clot origin.

Integrating SVM Analyses of Clinical Data and Thrombi Proteome

In an attempt to identify additional SVM differentiation factors, we performed an analysis using patients clinical data (**Table 1**; age, sex, history of cardiac failure or atrial fibrillation, previous antithrombotic medication, glycemia, weight and BMI, thrombus weight and global protein concentration, hemoglobin, leucocytes, and platelet rate). SVM identified history of cardiac failure and atrial fibrillation as variables differentiating the two population with an 81.36% accuracy. This result is obvious considering our study design and because history of cardiac failure was used as one of the criteria to diagnose etiology at enrollement (see Methods). Cardiac failure and atrial fibrillation are two known risk factors linked to cardioembolic stroke (3). Interestingly, when atrial fibrillation was excluded from the SVM analysis, patient age and thrombus protein concentration provided a differentiation level of 74.58% within our sample cohorts. The latter results indicate thrombus total protein concentration as a new SVM analytical variable. Addition of a third variable did not improve the SVM score (*not shown*). We do acknowledge that combining the protein trio 1 (see **Table 2**), history of cardiac failure, and protein concentration we obtained a SVM score of 96.6%.

Testing Proteome Using LFQ Statistics

The selected SVM method tests all combinations of three inter-dependent proteins, obtaining solutions for data clusterization that are not executable using LFQ and standard statistics (26). Thus, a Student's *T*-test (Perseus algorithms) analysis on the proteins (log2 transformed) detected in all samples did not deliver significant difference between the studied cardioembolic and atherothrombotic populations. Furthermore, we applied a conventional method where proteomes (**Supplementary Table 1**) are filtered to include proteins with at least 50% of valid LFQ values. By using this approach, Student's *t*-test identified four proteins (PHB, SLC25A11, ATP5A1, and APOE; see **Table 3**) that display an abundance in cardioembolic as compared to atherothrombotic thrombi (volcano plot in **Figure 3B**). However, LFQ *T*-test difference was low (x-axis = −1.2; red dots in **Figure 3B**) with the crucial caveat that, because of method design, these proteins were undetectable in an elevated number of

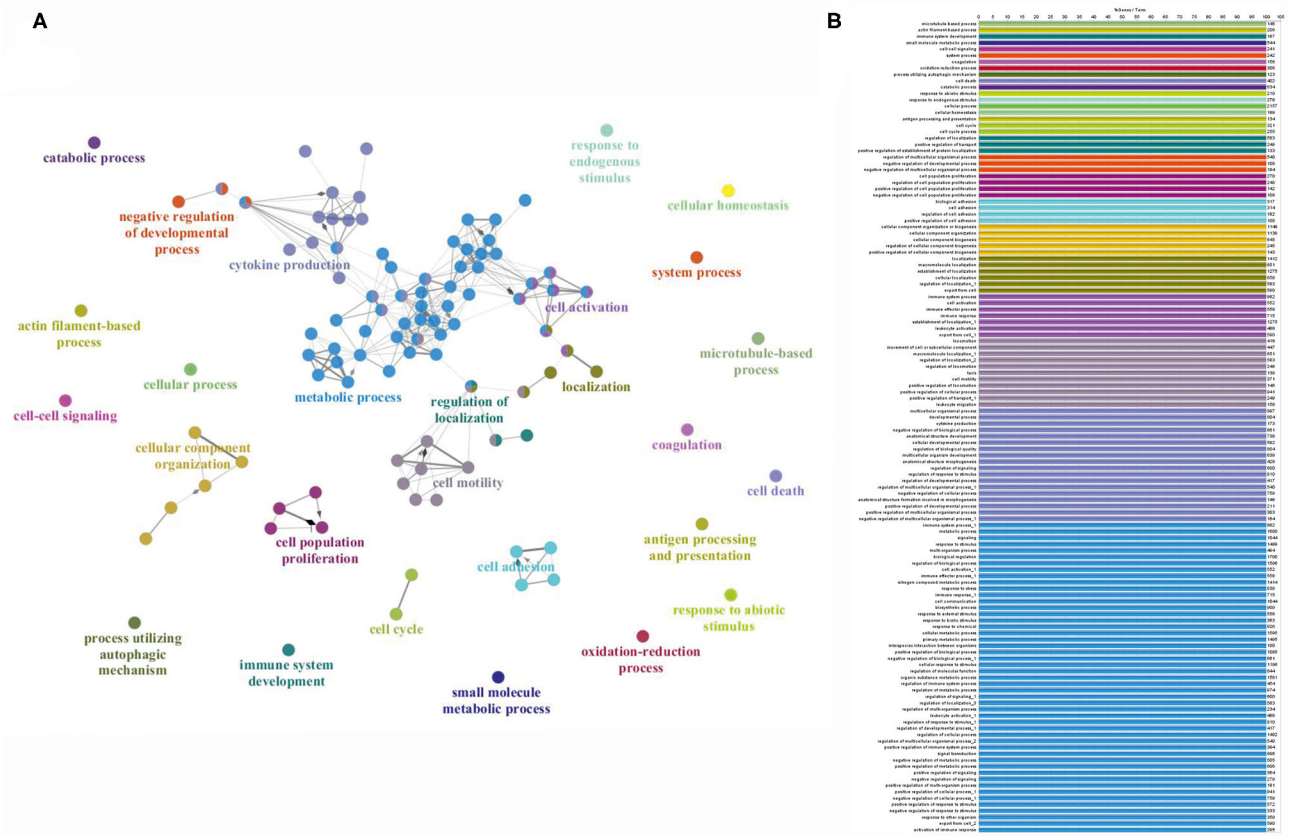

FIGURE 2 | (A) Graphic representation of the proteome fingerprint and protein clustering according to cellular functions. **(B)** List of specific cellular processes color-coded to panel A (high resolution zoom-in).

samples (**Supplementary Table 1**), therefore impeding group discrimination. These results support the relevance and the efficiency of SVM to analyze the proteome thombi dataset in our experimental settings.

DISCUSSION

Our study advances the knowledge of the human cerebrovascular thombi composition by delivering the largest proteome dataset available to date. We focused on the protemic analysis of cardioembolic and atherothrombotic thrombi and we applied a support-vector machine learning routine in an exploratory, proof-of-concept, attempt to identify protein candidates segragating the two selected populations. Our research supports the general notion that direct analysis of the thrombi material could unveil, in the future, new disease players and candidate biomarkers potentially aiding stroke diagnosis. The SVM method used herein was set to identify combinations of protein trios (**Table 2**) in the intracranial thrombi, and it allowed for an 88.3% correct classification of our selected cardioembolic and atherothrombotic populations (**Table 1**). We here underscore that histological, cellular (e.g., red blood cells, platelets, white blood cells), and molecular (omics) analyses should be all integrated to obtain a complete and multi-level depiction of the thrombi structure and biology.

Understanding the composition of the human clot was previously attempted in two studies, although limited in sample size or lacking SVM analysis (12, 27) A first proteomic investigation correlated 2 inflammation-associated proteins (integrin alpha-M and mitochondrial superoxide dismutase) to high blood LDL (27). Mitochondrial superoxide dismutase was previously associated to unstable carotid plaques (28). These proteins were detected in our study, although without significant differences between cardioembolic and atherothrombotic thrombi. A second study analyzed 4 thrombi, with ∼1,600 proteins identified (12). An earlier investigation, focused on human coronary thrombi in patients with ST-segment elevation in acute myocardial infarction, identified 708 proteins. The implication of platelet activation during the formation of thrombus causing acute coronary syndrome was suggested (29).

Combining Mass-Spectrometry With SVM Analysis: Initial Feasibility and Proposed Applicability to Human Ischemic Stroke

An innovative aspect of the presented study is the methodological combination of large-scale proteomic tools and machine learning

TABLE 2 | SVM protein trios allowing 88.3% accuracy of correct classification of cardioembolic and atherothrombotic thrombi.

Trio combinations	Proteins	Protein function (40)	Univariate *p*-value (bilateral Wilcoxon rank sum test)
1	• Coagulation factor XIII	Catalyzes the last step of the coagulation cascade by crosslinking fibrin fibers	0.0022
	• Eukaryotic translation initiation factor 2 subunit 3	Involved in the early steps of protein synthesis	0.0439
	• Ras GTPase-activating-like protein IQGAP2	Binds to activated CDC42 and RAC1. Associates with calmodulin	0.1326
2	• Coagulation factor XIII	Catalyzes the last step of the coagulation cascade by crosslinking fibrin fibers	0.0022
	• F-actin-capping protein	Binds to the fast-growing ends of actin filaments in a Ca^{2+} independent manner	0.1752
	• Myosin light chain kinase	Calcium/calmodulin-dependent kinase implicated in smooth muscle contraction via phosphorylation of myosin light chains	0.0142
3	• Coagulation factor XIII	Catalyzes the last step of the coagulation cascade by crosslinking fibrin fibers	0.0022
	• Septin-7	Filament-forming cytoskeletal GTPase. Required for normal organization of the actin cytoskeleton	0.1897
	• Gamma-adducin	Membrane-cytoskeleton-associated protein that promotes the assembly of the spectrin-actin network.	0.0589

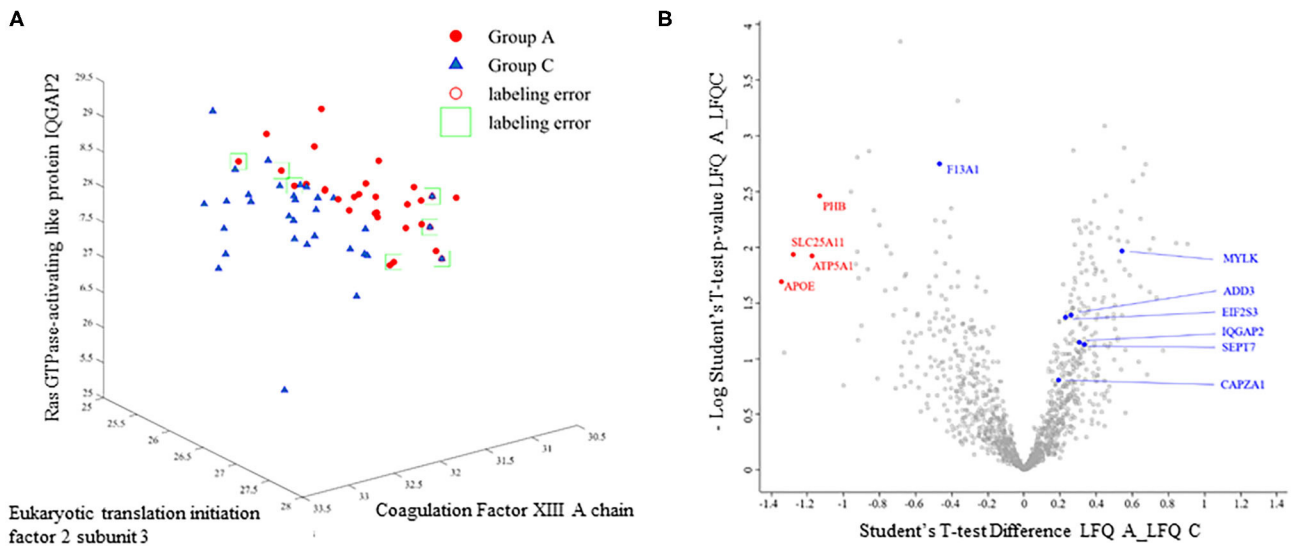

FIGURE 3 | (A) Example of SVM plot and classification for the combination of Eukaryotic translation initiation factor 2 subunit 3, Ras GTPase-activating-like protein IQGAP2, and coagulation Factor XIII. Groups A: atherothrombotic; Group C: cardioembolic. Light green squares and red circles indicate labeling errors for misclassified patients. **(B)** Volcano plot showing proteins significantly ($p < 0.05$) enriched using LFQ values log2 transformed (red dots; see **Table 3** for protein details). Student's *t*-test is performed by using Perseus algorithms. Blue dots indicate the SVM-identified proteins (see **Table 2** for details).

models or algorithms to define and potentially categorize the thrombi proteomes (3). In our patients' cohort, the fibrin stabilizing or coagulation Factor XIII (FXIII) was identified by SVM as one potential differentiating element between the cardioembolic and atherothrombotic thrombi analyzed (**Table 2**). FXIII is a key enzyme in the coagulation cascade that allows the cross-linking of fibrin chains with subsequent increase of mechanical clot strength and resistance to fibrinolysis (30). FXIII was also reported in embolized thrombi from the cardiac left atrial appendage in atrial fibrillation patients (31).

Interestingly, it has been recently shown that FXIII levels are higher after myocardial injury and that FXIII harbors an important role in cardiac healing and remodeling (32). Moreover, valine-to-leucine (V34L) single-nucleotide polymorphism (SNP), which is associated with higher levels of FXIIIa, appears to be associated with a lower risk of pathological thrombosis in

TABLE 3 | LFQ (log2 transformed) of single proteins enriched in cardioembolic as compared to atherothrombotic thrombi.

Gene	Uniprot Identification (40)	Protein name	Main known protein function (40)	Subcellular Location	Student's *T*-test Difference Log2 LFQ intensity Atheroma_Log2 LFQ intensity Cardioembolic	-log *P*-value
SLC25A11	Q02978	Mitochondrial 2-oxoglutarate/malate carrier protein	Catalyzes the transport of 2-oxoglutarate at mitochondrial membrane	Mitochondrion	−1.278498	1.94
APOE	P02649	Apolipoprotein E	Lipid transport between organs via the plasma and interstitial fluids	Extracellular	−1.34310437	1.69
PHB	P35232	Prohibitin	Inhibits DNA synthesis	Mitochondrion	−1.1333107	2.46
ATP5A1	P25705	ATP synthase subunit alpha	Produces ATP from ADP	Mitochondrion, plasma membrane	−1.1749357	1.93

ischemic heart disease (33, 34). Importantly, atrial fibrillation or atrial cardiopathies that share a common mechanism of thrombus formation in the left atrial appendage should be identified as soon as possible after stroke occurrence to initiate anticoagulation therapy (35). Our SVM learning analysis also identified proteins involved in the cellular cytoskeleton assembly (**Table 2**), namely the myosin light chain kinase and F-actin-capping protein. In general, the large scale proteomic analysis of human clots here executed discloses pathways and molecular players of clot-endothelium interplay and local inflammation related to cerebrovascular damage (**Figure 2**). The latter is important because cerebrovascular breakdown contributes to the development of central nervous system disease (6–8, 36), in this case potentially enabling post-stroke sequelae.

Study Limitations and Prospectives

To further explore the utility of the protein candidates here discovered (**Table 2**) a validation step using an independent, and larger sample population will be necessary to define reproducibility and accuracy parameters (e.g., sensitivity, specificity, positive ad negative predictive values). Our SVM analysis, due to a relatively small sample size, only allowed accuracy estimation. A compelling question is whether our integrated proteomic-SVM method could be next used to examine specific signatures in case of cryptogenic stroke. We are aware that the proteins here identified may be not helpful in a population of cryptogenic stroke that includes etiologies other that the two studied here. We are aware that an efficient transition from SVM proteome analysis to clinical laboratory tools (e.g., Elisa) could be challenging and time consuming. (12, 27). The latter will be possible only when definitive molecular candidate(s) will be confirmed in larger populations with results replicated across stroke centers. Nevertheless, our study provide a proof of principle model that could be further developed and applied. Our proteome results (**Supplementary Tables 1**, **2**) are shared and available to be re-analyzed using more advanced or alternative SVM methods.

We here recognize that the cohort used in the present study is heterogeneous in respect to age and blood platelet levels. Although blood platelet levels have been associated to stroke outcome (37), it is unknown whether a correlation with stroke etiology exists. One study showed that high platelet content of intracranial thrombi associates with large artery atherosclerosis. However, the authors did not study the correlation between blood platelet content and stroke cause (38). Another possible limitation of our approach concerns the retrieved material that may not represent the entire thrombus, although the analyses presented here were performed on the largest portion of clots retrieved at one pass of the thrombectomy device. IVT may also alter the samples, although this effect is likely to be limited due to the short time between IVT and thrombus extraction and processing. Finally, pre-stroke antithrombotic therapy may alter thrombus proteome composition (39).

Conclusions

In summary, quantitative proteomics and SVM analysis can be feasibly combined to examine the variation of intracranial human thrombi proteomes. If further developed and tested on larger cohorts, these methods have the potential to discover precise and novel pathophysiological players and biomarkers, with the ideal goal of aiding cerebrovascular stroke diagnosis and secondary prevention.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD020398.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité de Protection des Personnes ≪Sud-Méditerranée IV≫, Centre Hospitalier Universitaire de Montpellier, hôpital Saint-Eloi, 34295 Montpellier Cedex 5. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CD, JD, PM, VC, and NM: conception and design of the study, analysis of data, and drafting of the manuscript. EZ, MD, FB, and JL: acquisition and analysis of data, drafting of the manuscript, and figures. PH-L, GG, ID, CR, FC, and AB: acquisition of data. CD and VC: emergency surgery interventions, samples collection and patients' approval. 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.2020.575376/full#supplementary-material

Supplementary Table 1 | Complete list of proteins and mass-spectrometry data for each sample.

Supplementary Table 2 | List of proteins commonly present in all samples.

REFERENCES

1. Amarenco P, Bogousslavsky J, Caplan LR, Donnan GA, Hennerici MG. Classification of stroke subtypes. *Cerebrovasc Dis Basel Switz.* (2009) 27:493–501. doi: 10.1159/000210432
2. Ornello R, Degan D, Tiseo C, Carmine CD, Perciballi L, Pistoia F, et al. Distribution and temporal trends from 1993 to 2015 of ischemic stroke subtypes: a systematic review and meta-analysis. *Stroke.* (2018) 49:814–9. doi: 10.1161/STROKEAHA.117.020031
3. Yaghi S, Bernstein RA, Passman R, Okin PM, Furie KL. Cryptogenic stroke: research and practice. *Circ Res.* (2017) 120:527–40. doi: 10.1161/CIRCRESAHA.116.308447
4. Jickling GC, Sharp FR. Biomarker panels in ischemic stroke. *Stroke J Cereb Circ.* (2015) 46:915–20. doi: 10.1161/STROKEAHA.114.005604
5. De Meyer SF, Andersson T, Baxter B, Bendszus M, Brouwer P, Brinjikji W, et al. Analyses of thrombi in acute ischemic stroke: a consensus statement on current knowledge and future directions. *Int J Stroke.* (2017) 12:606–14. doi: 10.1177/1747493017709671
6. Sweeney MD, Zhao Z, Montagne A, Nelson AR, Zlokovic BV. Blood-brain barrier: from physiology to disease and back. *Physiol Rev.* (2019) 99:21–78. doi: 10.1152/physrev.00050.2017
7. Librizzi L, de Cutis M, Janigro D, Runtz L, de Bock F, Barbier EL, et al. Cerebrovascular heterogeneity and neuronal excitability. *Neurosci Lett.* (2018) 667:75–83. doi: 10.1016/j.neulet.2017.01.013
8. Giannoni P, Badaut J, Dargazanli C, De Maudave AF, Klement W, Costalat V, et al. The pericyte-glia interface at the blood-brain barrier. *Clin Sci Lond Engl 1979.* (2018) 132:361–74. doi: 10.1042/CS20171634
9. Nation DA, Sweeney MD, Montagne A, Sagare AP, D'Orazio LM, Pachicano M, et al. Blood-brain barrier breakdown is an early biomarker of human cognitive dysfunction. *Nat Med.* (2019) 25:270–6. doi: 10.1038/s41591-018-0297-y
10. Furie KL, Jayaraman MV. 2018 guidelines for the early management of patients with acute ischemic stroke. *Stroke.* (2018) 49:509–10. doi: 10.1161/STROKEAHA.118.020176
11. Brinjikji W, Duffy S, Burrows A, Hacke W, Liebeskind D, Majoie CBLM, et al. Correlation of imaging and histopathology of thrombi in acute ischemic stroke with etiology and outcome: a systematic review. *J NeuroInterventional Surg.* (2017) 9:529–34. doi: 10.1136/neurintsurg-2016-012391
12. Muñoz R, Santamaría E, Rubio I, Ausín K, Ostolaza A, Labarga A, et al. Mass spectrometry-based proteomic profiling of thrombotic material obtained by endovascular thrombectomy in patients with ischemic stroke. *Int J Mol Sci.* (2018) 19:498. doi: 10.3390/ijms19020498
13. Dargazanli C, Rigau V, Omer E. High CD3+ cells in intracranial thrombi represent a biomarker of atherothrombotic stroke. *PLoS ONE.* (2016) 11:e0154945. doi: 10.1371/journal.pone.0154945
14. Adams HP, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, et al. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. trial of org 10172 in acute stroke treatment. *Stroke.* (1993) 24:35–41. doi: 10.1161/01.STR.24.1.35
15. McMahon NE, Bangee M, Benedetto V, Bray EP, Georgiou RF, Gibson JME, et al. Etiologic Workup in Cases of Cryptogenic Stroke. *Stroke.* (2020) 51:1419–27. doi: 10.1161/STROKEAHA.119.027123
16. Maus V, Behme D, Kabbasch C, Borggrefe J, Tsogkas I, Nikoubashman O, et al. Maximizing first-pass complete reperfusion with save. *Clin Neuroradiol.* (2018) 28:327–38. doi: 10.1007/s00062-017-0566-z
17. Dargazanli C, Fahed R, Blanc R, Gory B, Labreuche J, Duhamel A, et al. Modified thrombolysis in cerebral infarction 2c/thrombolysis in cerebral infarction 3 reperfusion should be the aim of mechanical thrombectomy: insights from the aster trial (contact aspiration versus stent retriever for successful revascularization). *Stroke.* (2018) 49:1189–96. doi: 10.1161/STROKEAHA.118.020700
18. Fugate JE, Klunder AM, Kallmes DF. What is meant by "tici"? *Am J Neuroradiol.* (2013) 34:1792–7. doi: 10.3174/ajnr.A3496
19. Hacke W, Kaste M, Fieschi C, Toni D, Lesaffre E, von Kummer R, et al. Intravenous thrombolysis with recombinant tissue plasminogen activator for acute hemispheric stroke. The European cooperative acute stroke study (ECASS). *JAMA.* (1995) 274:1017–25. doi: 10.1001/jama.1995.03530130023023
20. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc.* (2006) 1:2856–60. doi: 10.1038/nprot.2006.468
21. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol.* (2008) 26:1367–72. doi: 10.1038/nbt.1511
22. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M. Andromeda: a peptide search engine integrated into the MaxQuant environment. *J Proteome Res.* (2011) 10:1794–805. doi: 10.1021/pr101065j
23. Tyanova S, Temu T, Sinitcyn P, Carlson A, Hein MY, Geiger T, et al. the perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods.* (2016) 13:731–40. doi: 10.1038/nmeth.3901
24. Perez-Riverol Y, Csordas A, Bai J, Bernal-Llinares M, Hewapathirana S, Kundu DJ, et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res.* (2019) 47:D442–50. doi: 10.1093/nar/gky1106
25. Gholami R, Fakhari N. Support vector machine: principles, parameters, applications. In: Samui P, Sekhar S, Balas VE, editors. *Handbook of Neural Computation*. Academic Press (2017). p. 515–35. doi: 10.1016/B978-0-12-811318-9.00027-2
26. Tyanova S, Albrechtsen R, Kronqvist P, Cox J, Mann M, Geiger T. Proteomic maps of breast cancer subtypes. *Nat Commun.* (2016) 7:10259. doi: 10.1038/ncomms10259

27. Rao NM, Capri J, Cohn W, Abdaljaleel M, Restrepo L, Gornbein JA, et al. Peptide composition of stroke causing emboli correlate with serum markers of atherosclerosis and inflammation. *Front Neurol.* (2017) 8:427. doi: 10.3389/fneur.2017.00427
28. Lepedda AJ, Cigliano A, Cherchi GM, Spirito R, Maggioni M, Carta F, et al. A proteomic approach to differentiate histologically classified stable and unstable plaques from human carotid arteries. *Atherosclerosis.* (2009) 203:112–8. doi: 10.1016/j.atherosclerosis.2008.07.001
29. Alonso-Orgaz S, Moreno-Luna R, López JA, Gil-Dones F, Padial LR, Moreu J, et al. Proteomic characterization of human coronary thrombus in patients with ST-segment elevation acute myocardial infarction. *J Proteomics.* (2014) 109:368–81. doi: 10.1016/j.jprot.2014.07.016
30. Muszbek L, Yee VC, Hevessy Z. Blood coagulation factor XIII: structure and function. *Thromb Res.* (1999) 94:271–305. doi: 10.1016/S0049-3848(99)00023-7
31. Gosk-Bierska I, McBane RD, Wu Y, Mruk J, Tafur A, McLeod T, et al. Platelet factor XIII gene expression and embolic propensity in atrial fibrillation. *Thromb Haemost.* (2011) 106:75–82. doi: 10.1160/TH10-11-0765
32. Frey A, Gassenmaier T, Hofmann U, Schmitt D, Fette G, Marx A, et al. Coagulation factor XIII activity predicts left ventricular remodelling after acute myocardial infarction. *ESC Heart Fail.* 7:2354–64. doi: 10.1002/ehf2.12774
33. Bagoly Z, Koncz Z, Hársfalvi J, Muszbek L. Factor XIII, clot structure, thrombosis. *Thromb Res.* (2012) 129:382–7. doi: 10.1016/j.thromres.2011.11.040
34. Wartiovaara U, Mikkola H, Szôke G, Haramura G, Kárpáti L, Balogh I, et al. Effect of Val34Leu polymorphism on the activation of the coagulation factor XIII-A. *Thromb Haemost.* (2000) 84:595–600. doi: 10.1055/s-0037-1614073
35. Kamel H, Okin PM, Elkind MSV, Iadecola C. Atrial fibrillation and mechanisms of stroke: time for a new model. *Stroke.* (2016) 47:895–900. doi: 10.1161/STROKEAHA.115.012004
36. Klement W, Blaquiere M, Zub E, deBock F, Boux F, Barbier E, et al. A pericyte-glia scarring develops at the leaky capillaries in the hippocampus during seizure activity. *Epilepsia.* (2019) 60:1399–411. doi: 10.1111/epi.16019
37. Yang M, Pan Y, Li Z, Yan H, Zhao X, Liu L, et al. Platelet count predicts adverse clinical outcomes after ischemic stroke or TIA: subgroup analysis of CNSR II. *Front Neurol.* (2019) 10:370. doi: 10.3389/fneur.2019.00370
38. Fitzgerald S, Dai D, Wang S, Douglas A, Kadirvel R, Layton KF, et al. Platelet-rich emboli in cerebral large vessel occlusion are associated with a large artery atherosclerosis source. *Stroke.* (2019) 50:1907–10. doi: 10.1161/STROKEAHA.118.024543
39. Marcone S, Dervin F, Fitzgerald DJ. Proteomic signatures of antiplatelet drugs: new approaches to exploring drug effects. *J Thromb Haemost.* (2015) 13:S323–31. doi: 10.1111/jth.12943
40. UniProt Consortium T. UniProt: the universal protein knowledgebase. *Nucleic Acids Res.* (2018) 46:2699. doi: 10.1093/nar/gky092

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Tissue-Type Plasminogen Activator and Tenecteplase-Mediated Increase in Blood Brain Barrier Permeability Involves Cell Intrinsic Complement

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Background: Tissue-type plasminogen activator (t-PA) has been the mainstay of therapeutic thrombolysis for patients with acute ischaemic stroke (AIS). However, t-PA can cause devastating intracerebral hemorrhage. t-PA can also influence the CNS in part by modulation of BBB permeability. Complement activation also occurs after AIS and has also been reported to increase BBB permeability. The complement components, C3 and C5, can also be activated by t-PA via plasmin formation and cell intrinsic complement may be involved in this process. Tenecteplase (TNK-tPA) is a t-PA variant with a longer plasma half-life, yet the ability of TNK-tPA to modulate the BBB and complement is less clear.

Aim: To evaluate the effect of C5 and C5a-receptor 1 (C5aR1) inhibitors on t-PA- and TNK-tPA-mediated opening of the BBB.

Methods: We used an *in vitro* model of the BBB where human brain endothelial cells and human astrocytes were co-cultured on the opposite sides of a porous membrane assembled in transwell inserts. The luminal (endothelial) compartment was stimulated with t-PA or TNK-tPA together with plasminogen, in the presence of PMX205 (a non-competitive C5aR1 antagonist), Avacopan (a competitive C5aR1 antagonist) or Eculizumab (a humanized monoclonal inhibitor of human C5). BBB permeability was assessed 5 and 24 h later. Immunofluorescence was also used to detect changes in C5 and C5aR1 expression in endothelial cells and astrocytes.

Results: PMX205, but not Avacopan or Eculizumab, blocked t-PA-mediated increase in BBB permeability at both the 5 and 24 h time points. PMX205 also blocked TNK-tPA-mediated increase in BBB permeability. Immunofluorescence analysis revealed intracellular staining of C5 in both cell types. C5aR1 expression was also detected on the cell surfaces and also located intracellularly in both cell types.

Conclusion: t-PA and TNK-tPA-mediated increase in BBB permeability involves C5aR1 receptor activation from cell-derived C5a. Selective inhibitors of C5aR1 may have therapeutic potential in AIS.

Keywords: fibrinolysis, plasminogen activation, complement, blood brain barrier, tissue-type plasminogen activator, tenecteplase

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INTRODUCTION

The advent of recombinant tissue-type plasminogen activator (t-PA) revolutionized the management of acute ischaemic stroke more than two decades ago, providing a new therapeutic intervention for a devastating neurological disorder (1). However, tPA thrombolysis remains ineffective in achieving successful recanalization in 60% of the stringently selected patients (2). Furthermore, the risk of symptomatic intracerebral hemorrhage, the most feared and devastating complication of tPA thrombolysis, occurs in ∼6.8% of cases (3). Despite the poor efficacy and risks, t-PA still remains the only approved pharmacological treatment available in patients with acute ischaemic stroke. While there have been no successful attempts to improve the efficacy of t-PA thrombolysis, there are also no treatments available to reduce the risk of t-PA induced ICH. Even in the current era of thrombectomy, tPA is still generally delivered in conjunction with mechanical clot retrieval. The need to minimize tPA's adverse effects while still harnessing its therapeutic potential remains a clinical and scientific priority.

Challenging tPA is tenecteplase (TNK-tPA), a t-PA variant developed over 25 years ago to circumvent the short (5 min) plasma half-life of t-PA. Only 6 amino acids are altered in TNK-tPA (hence is 99% identical to tPA), but these critical substitutions extended the plasma half-life of TNK-tPA to ∼30 min. It is evident that any attempts to improve the efficacy and safety of t-PA should also include parallel studies on TNK-tPA in the event that TNK-tPA becomes the front line thrombolytic in the future.

The plasminogen activating system is traditionally known for its role in fibrinolysis. However, both tPA and plasmin have numerous non-fibrinolytic properties. In the CNS, plasmin and tPA are important in modulating NMDA receptors and synaptic plasticity, thus contributing to elements of learning and behavior (4, 5). tPA also has the ability to directly increase permeability of the blood brain barrier (BBB), which is considered the precursor to the development of intracerebral hemorrhage (6). Several plasmin-dependent and -independent mechanisms contributing to these permeability changes have been described (7–9). Intuitively, studies are exploring targeted inhibition of these pathways, aimed at reducing tPA's unwanted effects on the BBB while still maintaining its desired plasmin dependent fibrinolytic capacity.

Many signaling pathways are triggered either directly by t-PA or indirectly via plasmin generation that modulate permeability properties of brain microvascular endothelial cells or astrocytes that in turn impact on BBB integrity (6). Among these include the complement system that has received much attention in CNS disorders in recent years. The complement system, a crucial line of host innate immune defense, comprises a cascade of molecules which when activated, leads to cleavage of central components C3 and C5 to C3a and C5a, which then bind to their receptors (C3aR and C5aR1) and induce immune modulation. Furthermore, emerging literature recognizes the importance of intracellular complement components or the "complosome" in cell signaling repertoire (10). Whether this pathway is also involved in the tPA and plasmin mediated complement activation within astrocytes and other neuronal cell populations requires further investigation.

Complement's role in neuroinflammation, neurodegenerative disease and CNS injury is well-recognized (11–13). Plasmin's ability to directly activate complement proteins independent of the classical central C3 convertase, introduces a new paradigm to the understanding of complement activation (14). Recent studies have shown that tPA promotes C3 cleavage both *in vitro* and in the ischaemic mouse brain through a plasmin-mediated pathway (15). These authors also showed that the C3a-receptor is strongly expressed on the endothelium of ischaemic brain and that exogenous C3a dramatically enhanced brain endothelial cell permeability. tPA therapy was shown to exacerbate brain oedema and hemorrhage in stroke and these effects were ameliorated by treatment with a C3a receptor inhibitor (15). These findings established that intravenous tPA can upregulate complement activation in ischaemic brain tissue and that complement inhibition can protect against the adverse outcomes of tPA-mediated thrombolysis in stroke (15).

In contrast, the role of C5 and C5a in tPA-mediated neuroinflammation and stroke has not been explored. In relation to BBB integrity, Mahajan et al. demonstrated that C5a regulates BBB integrity during neuroinflammation, where it affects both endothelial and astroglial cells in a human *in vitro* model of systemic lupus erythematosus (SLE) (12). Furthermore, Eculizumab, a humanized monoclonal anti-C5 antibody approved for the treatment of the rare paroxysmal nocturnal haemoglobinuria (PNH) and atypical haemolytic uraemic syndrome (aHUS) was found to reduce the number of neurological episodes in neuromyelitis optica (NMO); a demyelinating disease characterized by BBB disruption and inflammation/degeneration of the optic nerve and spinal cord (16). Small molecule C5aR1 inhibitors (PMX205 and PMX53) have had some promising results in animal models of spinal cord injury (17), Alzheimer's disease (18), Amyotrophic lateral sclerosis (19, 20) and Huntington's disease (21, 22). C5aR1 deficient mice have also been reported to be protected in ischaemic stroke models, in a manner linked to neuronally derived (cell intrinsic) C5a (23). Avacopan, a clinically advanced small molecule C5aR1 antagonist, may have disease ameliorating and steroid sparing effects in ANCA associated vasculitis (24).

While tPA can increase BBB permeability and trigger the infiltration of immune cells into the CNS, complement activation may occur concomitantly with immune cell infiltration and has been linked to BBB permeability in other CNS injury models. Since plasmin is known to possess C3 and C5 convertase activity, we hypothesized that plasmin-driven complement activation is central to the capacity of the complement system to increase BBB permeability and inhibition of this pathway may offer a novel means to attenuate this process and abrogate the deleterious effects of thrombolytic therapy on the BBB and elsewhere in the brain.

Curiously, despite the fact that TNK-tPA has been available for over 25 years, it is still unclear whether or to what extent it shares any of these emerging non-canonical properties with tPA, particularly on the BBB and complement activation at the cellular level. Previous studies have reported that TNK-tPA

can also increase BBB permeability in *in vitro* models but was ∼50% as effective as tPA on a molar basis (25). TNK-tPA can also bind to NMDA subunits but whether it also influences neurological parameters to the same extent as tPA is unknown. In this study, we investigated the role of complement activation during t-PA and TNK-tPA-mediated opening of the BBB using an established *in vitro* model (26) using C5 and C5a-receptor 1 (C5aR1) inhibitors.

MATERIALS AND METHODS

Reagents

Human t-PA (rt-PA; Actilyse®) was purchased from Boehringer Ingelheim GmbH (Rhein, Germany) and dialysed against 0.4 M HEPES at pH 7.4 to remove the original vehicle component from the compound. Tenecteplase was obtained from expired hospital stocks and kindly provided by Prof Christopher Levi (Hunter Valley Hospital, Newcastle, NSW, Australia). Human Glu-plasminogen was purchased from Enzyme research laboratories (South Bend, IN, USA). Fluorescein isothiocyanate- conjugated bovine serum albumin (FITC-BSA) was obtained from Sigma Aldrich (St Louis, MO, USA). Rat Collagen-I was purchased from Cultrex (Minneapolis, MN, USA).

PMX205 was synthesized and purified as previously described (27). Avacopan was purchased from MedKoo Biosciences, Morrisville, USA. Eculizumab remaining from therapeutic use was kindly donated by the Pharmacy department at Monash Health, Victoria, Australia.

Institutional Ethics Approval

Normal human plasma used for the amidolytic assays was approved by the Local Institutional Human Research Ethics Committee, approval number 2017-9712-13995.

Cell Culture

Primary human brain microvascular endothelial cells (BECs; ACBRI 376, Cell Systems, Kirkland, WA, USA) and human transformed SVG astrocytes were cultured to form an *in vitro* human BBB as described below. Endothelial Cell Growth Medium MV2 with Supplement Mix (Promocell, Heidelberg, Germany) was used as the BECs culturing growth medium. Minimum Essential Medium (MEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 20% heat inactivated fetal calf serum, 1% L-Glutamine and 0.5% Penicillin/Streptomycin was used to culture the SVG astrocytes. BECs and SVGs were cultured for 3–4 days with a media change on day 3 and passage splitting on day 3 or 4.

Human monocytic leukaemic cells (THP-1) were cultured in RPMI supplemented with 10% heat inactivated fetal calf serum, serum, 1% L-Glutamine and 0.5% Penicillin/Streptomycin.

In vitro Human Blood Brain Barrier Model

An *in vitro* model of the BBB was utilized in line with our pre-existing protocol developed within our laboratory (25, 26) (**Figure 1A**). This involves co-culture of the BECs and SVGs on opposing surfaces of a porous, collagen-I-coated membrane using Transwell® inserts (polyester membrane, 6.5 mm diameter, 0.4 μm pore size, Costar, Corning, NY, USA).

Firstly, 4×10^4 SVGs were allowed to adhere onto the underside of an inverted Transwell® insert over 3–4 h. Subsequently, 2×10^4 BECs were cultured on the collagen-coated inner surface of the porous membrane on the same insert. This co-culture system was maintained in BEC growth medium over 3 days in a humidified 37°C incubator at 5% CO_2 and 21% O_2.

Experimentation on the BBB construct was undertaken after 3 days of incubation. Both luminal and abluminal chambers were washed once with serum free BEC medium not containing the Supplement Mix for 60 min. Thereafter, the luminal medium was replaced again with serum free BEC medium containing a cocktail of tPA (25 nM) or TNK-tPA (25 or 50 nM), plasminogen (100 nM) and the varied drugs of investigation at the specified concentrations. The investigational drugs, PMX205 (a cyclic peptide non-competitive C5aR1 antagonist) was reconstituted in sterile milliQ water and used at a final concentration of 10, 50, or 100 μM; Avacopan (a small molecule competitive C5aR1 antagonist) was reconstituted in 100% dimethyl sulfoxide (DMSO) and used at 2 μM; and Eculizumab, a humanized monoclonal inhibitor of human C5, was supplied as a 10 mg/ml aqueous solution and used at a final concentration of 200 μg/ml (1.35 μM). These reagents were added to both endothelial (luminal) and astrocytic (abluminal) compartments simultaneously. tPA, TNK-tPA and plasminogen were only added to the luminal/endothelial compartments. We chose doses of PMX205 and Avacopan based on the known pharmacological properties of these compounds in human cells (28, 29). Eculizumab was used at a 1:50 final dilution of the 10 mg/ml stock solution (200 μg/ml) and approximates the concentration used clinically for the treatment of patients with PNH (30). In experiments where PMX205 was evaluated on single cell cultures of BECs or SVG astrocytes, it was added to the luminal compartment only.

Incubation durations were either 5 or 24 h. Changes in the BBB permeability were evaluated by adding fluorescent FITC-conjugated BSA 0.385% (w/v) to the luminal chamber 1 h prior to the end point (i.e., at 4 or 23 h post treatment) and sampling the abluminal chamber 1 h later to quantitate the passage of the tracer through the semipermeable BBB over this 1 h period. Fluorescence was measured using a micro-plate reader (FLUOstar Optima, BMG labtech, Australia) at an emission wavelength of 485 nm and absorbance wavelength of 520 nm and gain adjusted to 1,500 nm. Data is presented as a fold-change in the passage of FITC-BSA relative to vehicle treated control wells.

It is important to note that endothelial tight junction proteins like ZO-1 are integral components of blood brain barrier integrity. Using immunofluorescence, we have previously reported the presence of ZO-1 along BEC-BEC boundaries (31). Furthermore, a breakdown of tight-junction proteins in BECs by t-PA and plasminogen and their preservation by a ROCK-2 selective inhibitor (KD025) became apparent, fully correlating with the permeability data. In addition, we confirmed that the ability of t-PA to modulate permeability was not due to any toxic effects on cell viability but rather due to direct changes on cell signaling events. These results indicate the involvement of tight

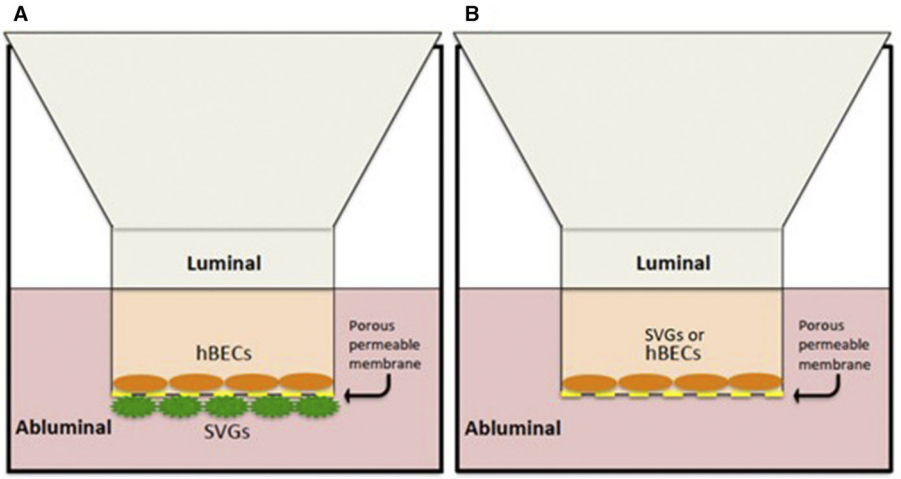

FIGURE 1 | *In vitro* human BBB models. **(A)** Co-culture contact system with hBECs cultured on the luminal side of a porous membrane (0.4 μm pore size) and SVGs on the abluminal surface. **(B)** A non-contact monolayer of hBECs or SVGs cultured only on the luminal compartment.

junction proteins in our experimental system further supporting their utility as a model for the BBB.

A "non-contact" monolayer *in vitro* system was also utilized to isolate and identify any individual contribution from each cell type (BECs or SVGs) to the BBB changes seen (**Figure 1B**). This involved a simplified version of the co-culture system where only the luminal chamber of the inserts was cultured with BECs or SVGs and no cells were cultured on the abluminal surface of the porous membrane. The stimulation "cocktail" was added only to the luminal compartment and evaluation of changes to the cell layer was performed in the same manner as described for the co-culture system.

Amidolytic Chromogenic Assay for Plasmin Generation

Plasmin activity/activation was evaluated using an amidolytic assay with S-2251 (Chromogenix S-2251, Diapharma, Louisville, USA) a chromogenic substrate specific for plasmin. This was performed to exclude any confounding anti-plasmin properties of the complement inhibitors under investigation. Aprotinin (Sigma-Aldrich, Missouri, USA), a serine protease inhibitor that competitively inhibits plasmin was used as a positive control (32). This assay has previously been optimized by our laboratory to evaluate the plasmin generation rate in plasma (33). t-PA (30 nM) in the presence of a cofactor (cyanogen bromide activated (CNBr) fibrinogen at 0.25 mg/mL) and S2251 (1 mM) "spiked" with complement inhibitors PMX205 (10, 100 μM) and Avacopan (2 μM) was incubated with healthy donor control plasma in a clear 96-well plate and absorbance read every cycle (20 s cycle) at 405 nm for 250 cycles using a plate reader pre-set at 37°C. Recordings of the optical densities taken every 20 s was collated and a sigmoidal curve generated. The curves exhibit an initial lag phase, followed by an exponential growth phase reflecting plasmin generation in the sample and a final plateau phase indicating exhaustion of the S2251 substrate.

Phase Contrast Microscopy and Immunofluorescence Confocal Imaging

BECs and SVGs were cultured to confluency over 3–4 days in 12-well plates (for phase contrast imaging) or in μ-slides 8-wells (Ibidi, Munic, Germany) for immunofluorescence. For phase contrast microscopy, wells were coated with gelatine for BECs and treated with serum free medium containing t-PA (25 nM) and plasminogen (100 nM) with or without PMX205, Avacopan or Eculizumab at the same concentrations used in the *in vitro* system. Stimulation times were ∼15–20 h in duration. Following aspiration of the stimulation media, the cells were gently washed with PBS then fixed for 30 min with ice-cold 4% paraformaldehyde (PFA). Phase contrast images were captured using a Nikon Eclipse TS100 inverted microscope.

For immunofluorescence imaging, cells cultured in the μ-slides were treated with serum free medium alone or containing t-PA (25 nM) and plasminogen (100 nM) for a period of 15–20 h. Following aspiration of the stimulation media the cells were fixed with ice-cold 4% PFA for 10 min. Thereafter, the cells were blocked for 10 min with TBS containing 10% heat inactivated horse serum. Following 3 × 5 min washes with TBS, the primary antibody was added and incubated overnight at 4°C with gentle agitation. The primary antibodies used include mouse anti-human C5aR (CD88), clone W17/1 (HycultBiotech, Netherlands) diluted 1:50 and sheep anti-human C5 (Bio-Rad, California, USA) diluted 1:100. Secondary antibodies used include Alexa Fluor 568-conjugated donkey anti-mouse and Alexa Fluor 488-conjugated chicken anti-mouse (Invitrogen, California, USA) incubated for 3 h at 4°C with gentle agitation, diluted 1:1,000. DyLight488-conjugated donkey anti sheep/goat IgG (Bio-Rad, California, USA) was used as the paired secondary to the anti-human C5 primary antibody, diluted 1:100 as per the manufacturer's recommendations. All primary and secondary antibodies were diluted in TBS containing 4% heat inactivated horse serum. Hoechst was used as a nuclear counterstain

(Invitrogen, California, USA) at a final concentration of 5 μg/mL in TBS, incubated for 30 min at 4°C with gentle agitation. Immunofluorescence images were captured on a Nikon A1r confocal inverted microscope using a X40 water objective and fluorescence DAPI, FITC/Alexa488 and Alexa 568. Nikon NIS-Elements software was used to directly acquire the images and further analysis was completed using FIJI (Image J) software.

THP-1 cells were used as a positive control for C5aR and C5 immunofluorescence staining. These non-adherent cells were cultured and adhered to a glass slide using a cytospin centrifuge (1,000 rpm, low acceleration, 5 min). Two-hundred microliter of cell stock at a concentration of 100,000 cells/mL was used for each slide. The cells were fixed and stained alongside the μ-slides containing BECs and SVGs as per the protocol described above.

Statistical Analysis

Each *in vitro* BBB experiment was performed in triplicate and at least four independent experiments were performed. Statistical analysis was performed using GraphPad Prism 8.0 software. Comparisons of experimental data sets were performed by ordinary one-way ANOVA with the application of the Dunnett's multiple comparisons test. *P*-values under 0.05 were considered significant.

RESULTS

Non-competitive C5aR1 Antagonist, PMX205, Reduces t-PA-Mediated Increase in BBB Permeability

To determine whether the t-PA/plasmin-mediated increase in BBB permeability could be inhibited by targeting the complement system, we evaluated the capacity of three different inhibitors of the complement pathway: PMX205 (cyclic peptide non-competitive C5aR1 antagonist), Avacopan (a small molecule competitive C5aR1 antagonist) and Eculizumab (a humanized monoclonal inhibitor of human C5) to attenuate the actions of tPA/plasmin in our established *in vitro* BBB model.

PMX205, significantly reduced t-PA+plasminogen (plg)-mediated increase in BBB permeability at both 5 h (**Figure 2A**) and 24 h (**Figure 2B**) after treatment. This effect of PMX205 was dose-dependent (**Figure 2C**). Indeed, the increase in BBB permeability was reduced by 36% at 5 h ($p < 0.05$) and 34% at 24 h ($p < 0.05$) using a 10 μM concentration. This increased to an 80% reduction in permeability with the higher 50 μM dose ($p < 0.0001$) and 97% using 100 μM of PMX205 ($P < 0.0001$) both at the 5 h time point.

Interestingly, unlike PMX205, Avacopan, had no effect on BBB permeability at the 5 h time point (**Figure 2A**), but showed a non-significant ($P = 0.1$) 29% reduction in this increased permeability at 24 h (**Figure 2B**). Eculizumab had no significant effect at either time point (**Figures 2A,B**).

We have previously reported that t-PA promotes marked morphological changes of both BECS and astrocytes by altering contractility of the cytoskeletal structure of the cells via the Rho-kinase (ROCK) pathway (25). As shown in **Figure 2D**, the capacity of t-PA+plg to alter cell morphology and contractility of both cell types was blocked by PMX205 in a dose-dependent manner as revealed by phase contrast microscopy.

BECs Contribute the Majority of the BBB Permeability Changes to C5aR1 Inhibition With PMX205 Compared to Astrocytes

We next investigated whether this complement inhibitory effect on the BBB was mediated by one particular cellular compartment more than the other. A single cell monolayer BBB model was used to stimulate BECs and SVGs individually with the same stimulation protocol used in the contact (co-culture) system. Both BECs and SVGs, when cultured alone, showed a significant increase in cell permeability following treatment with tPA and plasminogen (**Figures 3A,B**), but this was more marked in BECs (2.13 ± 0.48-fold increase) than astrocytes (1.72 ± 0.32-fold increase), suggesting that BECs are more sensitive to tPA/plasmin. Interestingly, PMX205 reduced the capacity of tPA to increase BEC permeability, with the 100 μM dose significantly reducing permeability by 66% ($p < 0.005$; **Figure 3A**). In contrast, SVGs did not show a significant reduction in permeability fold change in the presence of C5aR1 inhibition (**Figure 3B**), even using the higher dose of 100 μM, although there was a trend toward a benefit.

Small Molecule C5aR1 Inhibitors Do Not Exhibit Any Antiplasmin Properties

The *in vitro* results of the small molecule C5aR1 inhibitors, in particular PMX205, in ameliorating the t-PA and plasminogen-mediated permeability changes to the BBB raised the question of whether off-target direct anti-plasmin effects of these agents could be contributing to the observed reductions in BBB permeability. To address this question, we utilized the S2251 amidolytic assay that is specific for plasmin, which allowed us to evaluate effects of these complement inhibitors on plasmin activity. Normal healthy human control plasma was incubated with t-PA and S2251 in the presence and absence of PMX205 or Avacopan (see Methods). Results indicated there is minimal to absent anti-plasmin effects of PMX205 at 10 or 100 μM concentrations. Similarly, Avacopan at 2 μM did not show any anti-plasmin activity (**Figure 4**). Aprotinin (2 μM), a competitive inhibitor of plasmin was used as a positive control and showed almost complete plasmin inhibition. Furthermore, Tranexamic acid, a lysine analog, which impedes plasminogen and plasmin binding to their active sites significantly slowed down the plasmin generation curve.

Immunofluorescence Imaging Reveals C5 and C5aR Expression in BECs and SVGs

The functional *in vitro* changes we observed in the absence of any serum-derived complement, prompted us to investigate the distribution of complement C5a receptor 1 and its ligand, C5, in BECs and SVGs, and their changes following t-PA and plasminogen treatment of these cells. Confocal immunofluorescence images of BECs and SVGs were obtained

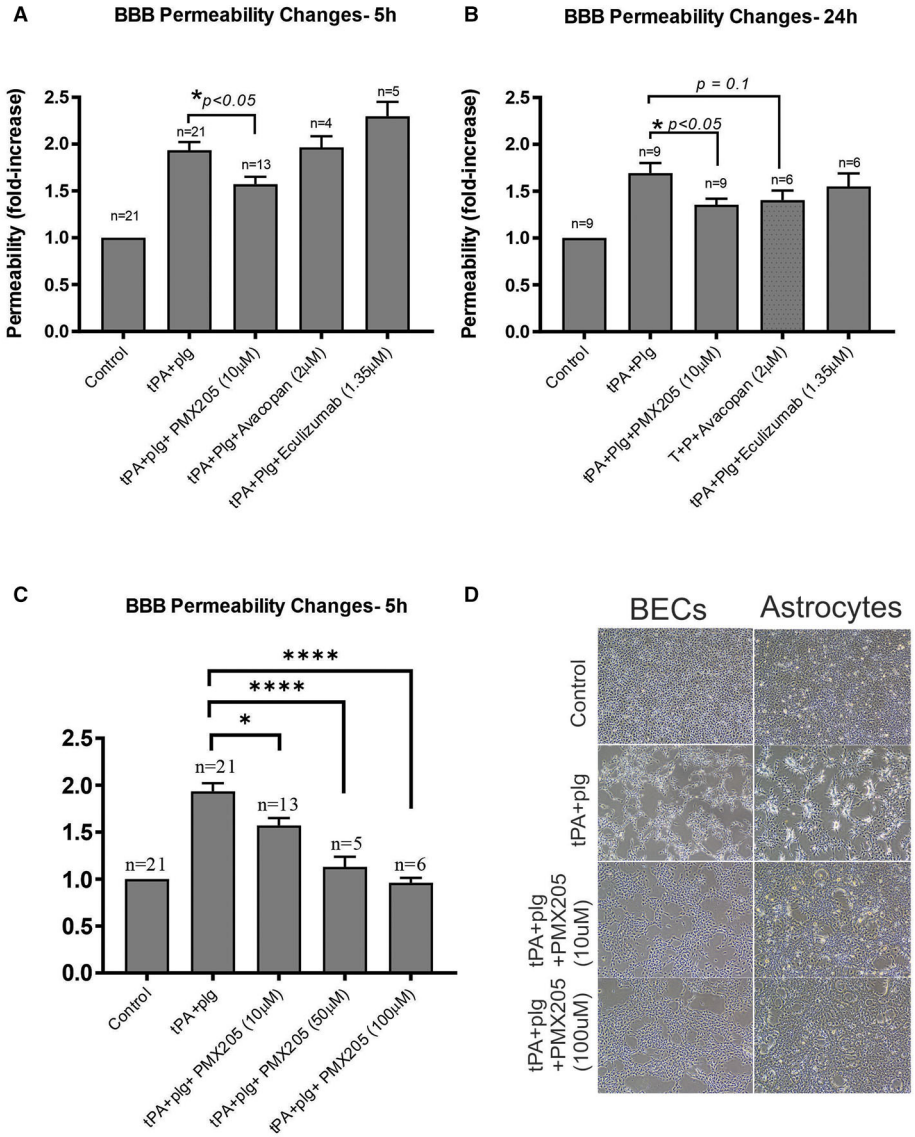

FIGURE 2 | The small molecule C5aR1 inhibitor, PMX205, reduces the t-PA and Plg-mediated increase in BBB permeability at 5 and 24 h. **(A)** Permeability changes in the *in vitro* human BBB 5 h post stimulation with control (serum-free medium) or with t-PA (25 nM) and human Plg (100 nM), with or without PMX205, Avacopan or Eculizumab (10, 2, or 1.35 μM, respectively, added to both luminal and abluminal chambers) **(B)** Permeability changes at 24 h post-stimulation with control (serum-free medium) or with t-PA (25 nM) and human Plg (100 nM), with or without PMX205, Avacopan or Eculizumab at same concentration and protocol as per 5 h experiment. **(C)** Permeability changes in the *in vitro* BBB 5 h post-stimulation with control (serum-free medium) or with t-PA (25 nM) and human Plg (100 nM), with or without PMX205 at increasing concentrations (10, 50, 100 μM, added to both luminal and abluminal chambers). **(D)** Phase-contrast microscopy images (x 4 magnification) of BECs and SVG human astrocytes demonstrating blockade of morphological changes by PMX205 at ∼20 h of treatment compared with control (serum-free medium), or t-PA (25 nM) and Plg (100 nM), in the presence or absence of PMX205 (10 or 100 μM). Data is presented relative to serum-free medium control. Bars represent mean ± SEM. *$p < 0.05$, ****$p < 0.0005$ compared to t-PA+Plg by one-way ANOVA with Dunnett's multiple comparison test.

following co-staining for the C5aR1 (CD88) and C5. Human monocytic leukaemic cells (THP-1) were used as a positive control for C5aR1 and C5 (not shown) as these malignant cells are known to contain high levels of complement components (34).

The vehicle treated BECs exhibited a prominence of C5 signal intracellularly in the cytoplasm with an increased intensity closer to the perinuclear areas. A scattered low intensity signal for C5aR1 was evident predominantly in the cell periphery, with some distribution intracellularly in the cell cytoplasm. Vehicle treated SVGs also showed a similar pattern of distribution of the C5aR1 and C5 at baseline (**Figures 5A,B**). Following treatment with t-PA and plasminogen for approximately 20 h, the BECs exhibited a persistence in C5 signal which was again concentrated predominantly intracellularly, however this time there was distribution throughout the entire cell cytoplasm. The

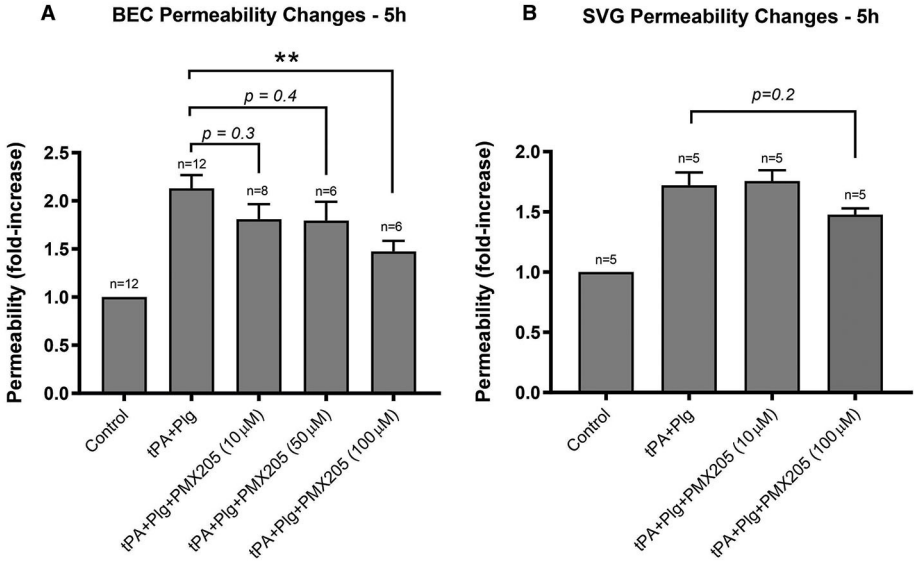

FIGURE 3 | BECs contributed the majority of the BBB permeability changes to C5aR1 inhibition with PMX205 compared to SVG human astrocytes. **(A)** Permeability changes in BEC monolayers alone 5 h post-stimulation with control (serum-free medium) or with t-PA (25 nM) and human plasminogen (plg; 100 nM), with or without PMX205 (10, 50, or 100 μM added only to the luminal chamber) **(B)** Monolayer integrity changes in SVG astrocytes cultured alone 5 h post-stimulation with control (serum-free medium) or with t-PA (25 nM) and human Plg (100 nM), with or without PMX205 (10 or 100 μM added only to the luminal chamber). Data is presented relative to serum-free medium control. Bars represent mean ± SEM. $^{**}p < 0.005$, compared to t-PA+Plg by one-way ANOVA with Dunnett's multiple comparison test.

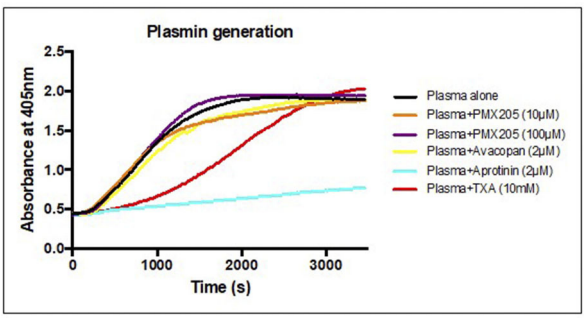

FIGURE 4 | C5aR1 inhibitors have no direct plasmin inhibitory effect. S2251 plasmin generation curve of plasma from healthy subjects in the presence or absence of PMX205 (10, 100 μM), Avacopan (2 μM), Aprotinin (2 μM) or TXA (10 mM). Cleavage of the plasmin-specific chromogenic substrate S2251 was measured by the absorbance of 405 nm.

C5aR1 signal was also present following t-PA and plasminogen treatment, especially in the cell periphery and in some areas, delineating the cell outline quite clearly (**Figure 5**) suggestive of cell membrane expression. In addition to undergoing prominent cytoskeletal changes, the SVGs also showed prominent C5 and C5aR1 expression following t-PA and plasminogen treatment (**Figure 5B**). These immunofluorescence image findings indicate there is C5 and C5aR1 expression in untreated fixed BECs and SVGs and this signal persists following t-PA and plasminogen treatment. Although there appears to be a qualitative prominence of C5 and C5aR1 signal in the t-PA and plasminogen treated cells, quantitative studies, such as qPCR or flow cytometry, are needed to confirm any true upregulation of C5 and C5aR1 in this context.

Delayed Administration of PMX205 Still Ameliorates the t-PA and Plasminogen Mediated Increase in BBB Permeability *in vitro*

Given the results of PMX205 reducing t-PA and Plg-mediated increase in BBB permeability *in vitro* and the cellular prominence of C5aR1 and its ligand, C5, on immunofluorescence imaging, we were keen to understand whether PMX205 could reduce this increase in permeability at delayed timepoints following t-PA and Plg treatment of the BBB. We found the effects of PMX205 were still evident when drug was added up to 3 h after t-PA and Plg treatment (**Figure 6**). Interestingly, there did not appear to be a significant difference in outcome between early treatment with PMX205 (prior to t-PA and Plg) and the delayed administration of PMX205 post-t-PA and Plg.

Tenecteplase Mediated Increase in BBB Permeability Is Also Inhibited by PMX205

TNK treatment of the *in vitro* system resulted in a consistent increase in the BBB permeability both at 25 and 50 nM concentrations, although this was less potent than t-PA which supports previous reports (25). Nonetheless, TNK-tPA-mediated increase in BBB permeability was effectively inhibited by PMX205, although more prominently at the higher dosage of 50 μM. PMX205 treatment at 50 μM resulted in a 59% reduction in BBB permeability induced by TNK at 25 nM ($p < 0.05$) and a

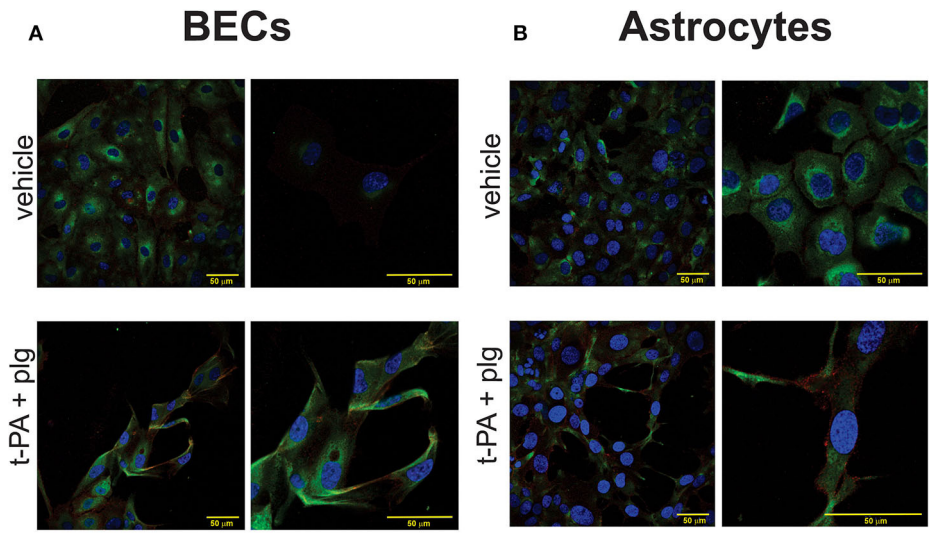

FIGURE 5 | C5 and C5aR1 are expressed in BECs and SVG astrocytes at baseline and following treatment with t-PA and plasminogen. Representative confocal immunofluorescence images of C5 (green), C5aR1 (red) and nuclei (Hoechst; blue) of BECs **(A)** and SVG astrocytes **(B)**, demonstrating a prominence of C5 and C5aR1 in vehicle-treated cells and following treatment with t-PA (25 nM) and plg (100 nM) for ∼20 h. Magnification scale bars are indicated in each panel.

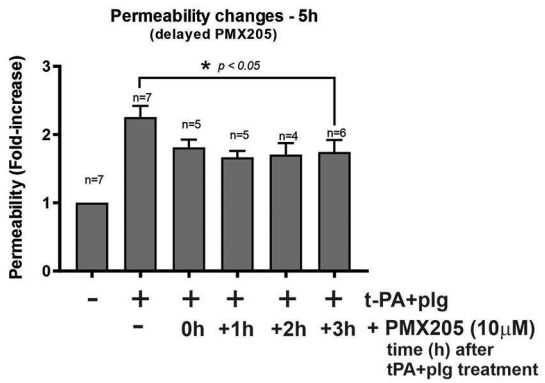

FIGURE 6 | Delayed administration of PMX205 ameliorates the t-PA and plasminogen (plg)-mediated permeability increase of the *in vitro* BBB. Permeability changes in the *in vitro* human BBB 5 h post-stimulation with control (serum-free medium) or with t-PA (25 nM) and human plg (100 nM), with or without PMX205 10 μM added either 1 h prior to t-PA+Plg, or 1, 2, or 3 h after t-PA+Plg. Bars = SEM.

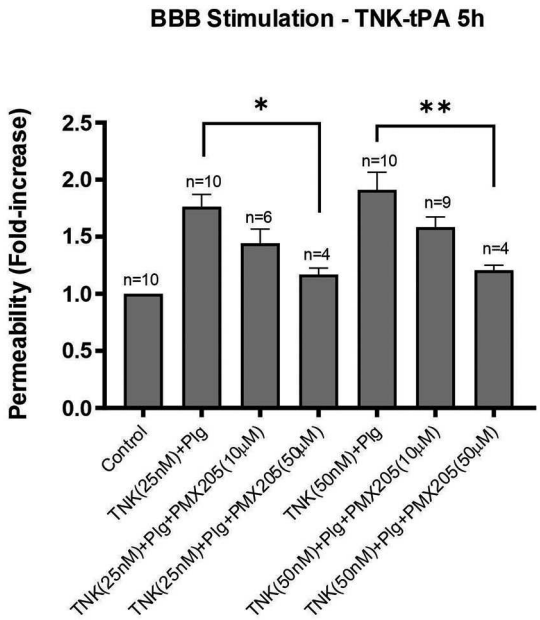

FIGURE 7 | TNK-tPA mediated increase in BBB permeability is blocked by PMX205. Permeability changes in the *in vitro* human BBB 5 h post-stimulation with control (serum-free medium) or with TNK (25 or 50 nM) and human plasminogen (plg; 100 nM), with or without PMX205 (10 or 50 μM). PMX205 was added to both the luminal and abluminal chambers. Bars represent mean ± SEM. *$p < 0.05$, **$p < 0.005$, compared to TNK+Plg one-way ANOVA with Dunnett's multiple comparison test.

more impressive 70% reduction in BBB permeability induced by TNK at 50 nM ($p < 0.005$) (**Figure 7**).

DISCUSSION

The blood-brain barrier and the complement system are both critical for brain defense and homeostasis. Loss of BBB integrity and unregulated complement activation can have deleterious consequences in the CNS (35). This is most notable in ischaemic stroke where BBB breakdown (6) and complement activation (36) occur hand-in-hand. Although well known for its role in innate immunity, the defensive function of complement can actually exacerbate immune and inflammatory conditions (37) and complement inhibition has been reported to be protective in models of ischaemic stroke (38–40). Adding to this is

the observation that complement activation can itself increase BBB permeability (12) further indicating that both processes are intertwined.

In this study, we have evaluated the role of the complement components C5a/C5aR1 during t-PA-mediated opening of the BBB using an *in vitro* model. This model had been previously validated as a relevant *in vitro* BBB model with the expression of the tight junction protein ZO-1 expressed between BEC-BEC boundaries. Our investigation into the role of complement was undertaken not only because the complement pathway is known to modulate the BBB (12), but that the key complement components C3 and C5 can be directly activated by t-PA via plasmin generation (41, 42). Hence, we took the view that the activation of these key complement components in patients with acute ischaemic stroke may be further facilitated by t-PA, providing an additional means by which t-PA can increase BBB permeability and intracellular bleeding. Hence, the role of plasmin-mediated complement activation in this landscape introduces an intriguing avenue contributing to the inflammatory and cellular changes seen within the BBB following t-PA exposure. This could potentially be a key event during t-PA thrombolysis when t-PA levels are transiently increased many hundred-fold leading to excessive plasmin-mediated cleavage of C5 and C3 that could not only exacerbate inflammation, but also increase BBB permeability and intracerebral bleeding.

The effects of tPA/plasmin-mediated C3 cleavage and its impact on the ischaemic brain have previously been explored (15). However, since the role of C5/C5a is yet to be investigated in this particular context, we set out to determine the extent to which C5/C5a participated in the ability of t-PA to increase BBB permeability. To address this, we compared the inhibitory capacity of three distinct C5 and C5aR1 blocking agents using an *in vitro* BBB model previously used in our laboratory, to study BBB permeability changes following t-PA and plasminogen treatment (25, 31). Tenecteplase (TNK-tPA) is closely related to t-PA and under development as a thrombolytic for AIS. However, there is little information available as to the extent to which TNK-tPA can modulate the BBB and whether TNK-tPA can also trigger complement activation. Hence, we were interested to compare and contrast t-PA with TNK-tPA in this study.

We showed that t-PA- and TNK-tPA-mediated increase in BBB permeability was inhibited using the small molecule non-competitive C5aR1 antagonist, PMX205. However, neither Avapocan (competitive C5aR1 antagonist) nor Eculizumab (monoclonal human C5 inhibitor) produced any significant inhibitory activity against t-PA mediated increase in BBB. We have previously shown that brain endothelial cells and astrocytes undergo significant cytoskeletal and morphological changes following exposure to tPA and plasminogen, a feature that is dependent in part, on activation of the Rho-kinase pathway (25). Since PMX205 also blocked the morphological effect of t-PA, this suggests that activation of C5 by t-PA/plasmin occurs upstream of the pathways controlling cytoskeletal changes in the cells forming the BBB, although this requires further investigation. Previous studies have reported on the specificity of PMX205 for C5aR1 and this inhibitor does not inhibit closely related receptors (i.e., C5ar2, C3aR, FPR1), and is inactive in C5aR1$^{-/-}$ mice (43), suggesting the effects observed in our study were truly C5aR1-dependent. We also excluded the possibility that PMX205 was not acting directly on plasmin activity since addition of PMX205 had no effect on the amidolytic activity of plasmin.

We observed that BECs appeared to be the most sensitive to tPA and plasminogen-mediated cytoskeletal changes, reflected in the robust increase in monolayer permeability. Moreover, the blocking capacity of PMX205 seen in the BBB model, appeared to predominantly target BECs, as PMX205 failed to block that ability of t-PA to increase BBB permeability of the SVG astrocytes. Since both cell types displayed increased cell permeability in response to t-PA + plasminogen treatment, yet only the increase in BEC permeability was blocked by PMX205, indicates an important cell-type specific response to this inhibitor. On the other hand, PMX205 did partially reduce the ability of t-PA to promote morphological changes to the astrocytes. This may reflect differences in the level of C5a generated in BECs and astrocytes, but this remains to be determined.

In keeping with the response of the cells to PMX205, we observed cellular expression of C5aR1 in both BECs and SVGs at baseline and following t-PA and plasminogen exposure, as revealed by immunofluorescence. Prominent cytoplasmic staining was seen for both C5aR1 and C5 (especially C5) as well as along the cell periphery, at baseline and following t-PA exposure. It remains to be determined whether treatment of either cell type with t-PA+plasminogen further increases expression of these complement components as quantitative experiments are yet to be performed. Nonetheless, the intracellular presence of C5 and to some extent, C5aR1, is in contrast to the classical thinking that C5aR1 is solely a G-protein coupled transmembrane receptor. Indeed, our findings are more in keeping with recent literature suggesting that many of the complement components are expressed intracellularly within immune and non-immune cells, contributing to the entity coined the "complosome" which has important roles in cellular regulation and signaling (10, 44). Intracellular complement protein stores of C3 and C5 can be cleaved intracellularly or on the cell surface and also undergo binding to their respective intracellular receptors, C3aR and C5aR1 (44). We hypothesize that similar processes are playing a role in non-immune cells, such as astrocytes and endothelial cells used in our studies.

We were surprised that of the three complement inhibitors evaluated, only PMX205 proved to be effective at blocking BBB permeability. The prominent intracellular location of C5 probably explains why Eculizumab, a ∼180 kD antibody molecule was ineffective at blocking the ability of t-PA to increase BBB permeability due to its inability to gain intracellular access. This result also suggests that cells are generating C5a intracellularly (i.e., not secreting C5 to be cleaved extracellularly) in response to t-PA treatment, which is either binding to intracellular or cell surface expressed C5aR1. While Avacopan, a small molecule (580 Da) had no demonstrable blocking effect on the BBB, we did observe that Avacopan did partially block the t-PA induced morphological changes in both BECs and in SVG astrocytes (data not shown), suggesting there was partial activity. Despite Avacopan being much more potent than PMX205 in

other classical immune cell-based assays (45), it may be that higher doses of this compound were needed to observe full functional activity in our model system (and indeed perhaps to achieve intracellular C5aR1 inhibition).

PMX205 also blocked the ability of TNK-tPA to increase BBB permeability, most likely reflective of TNK-tPA-mediated plasmin generation that in turn activates C5. TNK-tPA was less effective than t-PA at increasing permeability overall which is consistent with our previous study (25). Nonetheless, patients treated with TNK-tPA are still at risk of developing symptomatic intracranial hemorrhage so the ability of PMX205 to inhibit this capacity of TNK-tPA also has potential therapeutic implications if TNK-tPA is to become the preferred thrombolytic for AIS.

Another key finding of our study was that delayed addition of PMX205 still effectively prevented t-PA-induced increases in BBB permeability when added up to 3 h after t-PA treatment. In fact, later addition of PMX205 seemed to be slightly more effective compared to the earlier time points, perhaps reflecting PMX205 activity on the upregulated C5aR1 at this timepoint. We have previously reported that the ability of tPA to increase BBB permeability could also be blocked by delayed addition of the plasmin inhibitor, aprotinin (46) over a similar time frame, supporting the notion that the process is reversible. This finding has significant positive clinical implications. If this holds true, delayed complement inhibition could serve as an adjunctive therapy following thrombolysis in acute ischaemic stroke or as part of supportive care in traumatic brain injury to limit the unwanted BBB effects of excess t-PA in these time critical contexts.

In conclusion, our data demonstrate that inhibition of complement C5a-C5aR1 interaction reduces the ability of both t-PA or TNK-tPA to increase BBB permeability, and may offer a novel means to improve the safety profile of thrombolytic therapy for patients with acute ischaemic 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 Monash University Human Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RM and CK: project concept and writing of the manuscript. CK, HH, and ZL: experimentation. CK, RM, and TW: data analysis. TW, ZM, and BN: intellectual input and project concept. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. National Institute of Neurological Diseases and Stroke rt-PA Stroke Study Group. Tissue plasminogen activator for acute ischemic stroke. *N Engl J Med.* (1995) 333:1581–7. doi: 10.1056/NEJM199512143332401
2. Bhatia R, Hill MD, Shobha N, Menon B, Bal S, Kochar P, et al. Low rates of acute recanalization with intravenous recombinant tissue plasminogen activator in ischemic stroke: real-world experience and a call for action. *Stroke.* (2010) 41:2254–8. doi: 10.1161/STROKEAHA.110.592535
3. Emberson J, Lees KR, Lyden P, Blackwell L, Albers G, Bluhmki E, et al. Effect of treatment delay, age, and stroke severity on the effects of intravenous thrombolysis with alteplase for acute ischaemic stroke: a meta-analysis of individual patient data from randomised trials. *Lancet.* (2014) 384:1929–35. doi: 10.1016/S0140-6736(14)60584-5
4. Samson AL, Medcalf RL. Tissue-type plasminogen activator: a multifaceted modulator of neurotransmission and synaptic plasticity. *Neuron.* (2006) 50:673–8. doi: 10.1016/j.neuron.2006.04.013
5. Samson AL, Nevin ST, Croucher D, Niego B, Daniel PB, Weiss TW, et al. Tissue-type plasminogen activator requires a co-receptor to enhance NMDA receptor function. *J Neurochem.* (2008) 107:1091–101. doi: 10.1111/j.1471-4159.2008.05687.x
6. Niego B, Medcalf RL. Plasmin-dependent modulation of the blood-brain barrier: a major consideration during tPA-induced thrombolysis? *J Cereb Blood Flow Metab.* (2014) 34:1283–96. doi: 10.1038/jcbfm.2014.99
7. Su EJ, Fredriksson L, Geyer M, Folestad E, Cale J, Andrae J, et al. Activation of PDGF-CC by tissue plasminogen activator impairs blood-brain barrier integrity during ischemic stroke. *Nat Med.* (2008) 14:731–7. doi: 10.1038/nm1787
8. Suzuki Y, Nagai N, Yamakawa K, Muranaka Y, Hokamura K, Umemura K. Recombinant tissue-type plasminogen activator transiently enhances blood-brain barrier permeability during cerebral ischemia through vascular endothelial growth factor-mediated endothelial endocytosis in mice. *J Cereb Blood Flow Metab.* (2015) 35:2021–31. doi: 10.1038/jcbfm.2015.167
9. Medcalf RL. Fibrinolysis: from blood to the brain. *J Thromb Haemost.* (2017) 15:2089–98. doi: 10.1111/jth.13849
10. Arbore G, Kemper C, Kolev M. Intracellular complement - the complosome - in immune cell regulation. *Mol Immunol.* (2017) 89:2–9. doi: 10.1016/j.molimm.2017.05.012
11. Roselli F, Karasu E, Volpe C, Huber-Lang M. Medusa's head: the complement system in traumatic brain and spinal cord injury. *J Neurotrauma.* (2018) 35:226–40. doi: 10.1089/neu.2017.5168

12. Mahajan SD, Parikh NU, Woodruff TM, Jarvis JN, Lopez M, Hennon T, et al. C5a alters blood-brain barrier integrity in a human in vitro model of systemic lupus erythematosus. *Immunology*. (2015) 146:130–43. doi: 10.1111/imm.12489
13. Lee JD, Coulthard LG, Woodruff TM. Complement dysregulation in the central nervous system during development and disease. *Semin Immunol.* (2019) 45:101340. doi: 10.1016/j.smim.2019.101340
14. Amara U, Flierl MA, Rittirsch D, Klos A, Chen H, Acker B, et al. Molecular intercommunication between the complement and coagulation systems. *J Immunol.* (2010) 185:5628–36. doi: 10.4049/jimmunol.0903678
15. Zhao XJ, Larkin TM, Lauver MA, Ahmad S, Ducruet AF. Tissue plasminogen activator mediates deleterious complement cascade activation in stroke. *PLoS ONE.* (2017) 12:e0180822. doi: 10.1371/journal.pone.0180822
16. Pittock SJ, Lennon VA, McKeon A, Mandrekar J, Weinshenker BG, Lucchinetti CF, et al. Eculizumab in AQP4-IgG-positive relapsing neuromyelitis optica spectrum disorders: an open-label pilot study. *Lancet Neurol.* (2013) 12:554–62. doi: 10.1016/S1474-4422(13)70076-0
17. Li L, Xiong ZY, Qian ZM, Zhao TZ, Feng H, Hu S, et al. Complement C5a is detrimental to histological and functional locomotor recovery after spinal cord injury in mice. *Neurobiol Dis.* (2014) 66:74–82. doi: 10.1016/j.nbd.2014.02.008
18. Fonseca MI, Ager RR, Chu SH, Yazan O, Sanderson SD, LaFerla FM, et al. Treatment with a C5aR antagonist decreases pathology and enhances behavioral performance in murine models of Alzheimer's disease. *J Immunol.* (2009) 183:1375–83. doi: 10.4049/jimmunol.0901005
19. Woodruff TM, Costantini KJ, Crane JW, Atkin JD, Monk PN, Taylor SM, et al. The complement factor C5a contributes to pathology in a rat model of amyotrophic lateral sclerosis. *J Immunol.* (2008) 181:8727–34. doi: 10.4049/jimmunol.181.12.8727
20. Lee JD, Kumar V, Fung JN, Ruitenberg MJ, Noakes PG, Woodruff TM. Pharmacological inhibition of complement C5a-C5a1 receptor signalling ameliorates disease pathology in the hSOD1(G93A) mouse model of amyotrophic lateral sclerosis. *Br J Pharmacol.* (2017) 174:689–99. doi: 10.1111/bph.13730
21. Carpanini SM, Torvell M, Morgan BP. Therapeutic inhibition of the complement system in diseases of the central nervous system. *Front Immunol.* (2019) 10:362. doi: 10.3389/fimmu.2019.00362
22. Woodruff TM, Crane JW, Proctor LM, Buller KM, Shek AB, de Vos K, et al. Therapeutic activity of C5a receptor antagonists in a rat model of neurodegeneration. *FASEB J.* (2006) 20:1407–17. doi: 10.1096/fj.05-5814com
23. Pavlovski D, Thundyil J, Monk PN, Wetsel RA, Taylor SM, Woodruff TM. Generation of complement component C5a by ischemic neurons promotes neuronal apoptosis. *FASEB J.* (2012) 26:3680–90. doi: 10.1096/fj.11-202382
24. Merkel PA, Jayne DR, Wang C, Hillson J, Bekker P. Evaluation of the safety and efficacy of avacopan, a C5a receptor inhibitor, in patients with antineutrophil cytoplasmic antibody-associated vasculitis treated concomitantly with rituximab or cyclophosphamide/azathioprine: protocol for a randomized, double-blind, active-controlled, phase 3 trial. *JMIR Res Protoc.* (2020) 9:e16664. doi: 10.2196/16664
25. Niego B, Freeman R, Puschmann TB, Turnley AM, Medcalf RL. t-PA-specific modulation of a human blood-brain barrier model involves plasmin-mediated activation of the Rho kinase pathway in astrocytes. *Blood.* (2012) 119:4752–61. doi: 10.1182/blood-2011-07-369512
26. Niego B, Medcalf RL. Improved method for the preparation of a human cell-based, contact model of the blood-brain barrier. *J Vis Exp*. (2013) 81:e50934. doi: 10.3791/50934
27. Kumar V, Lee JD, Clark RJ, Noakes PG, Taylor SM, Woodruff TM. Preclinical pharmacokinetics of complement C5a receptor antagonists PMX53 and PMX205 in mice. *ACS Omega.* (2020) 5:2345–54. doi: 10.1021/acsomega.9b03735
28. Bekker P, Dairaghi D, Seitz L, Leleti M, Wang Y, Ertl L, et al. Characterization of Pharmacologic And Pharmacokinetic Properties of CCX168, a potent and selective orally administered complement 5a receptor inhibitor, based on preclinical evaluation and randomized phase 1 clinical study. *PLoS ONE.* (2016) 11:e0164646. doi: 10.1371/journal.pone.0164646
29. Woodruff TM, Pollitt S, Proctor LM, Stocks SZ, Manthey HD, Williams HM, et al. Increased potency of a novel complement factor 5a receptor antagonist in a rat model of inflammatory bowel disease. *J Pharmacol Exp Ther.* (2005) 314:811–7. doi: 10.1124/jpet.105.086835
30. Hillmen P, Young NS, Schubert J, Brodsky RA, Socie G, Muus P, et al. The complement inhibitor eculizumab in paroxysmal nocturnal hemoglobinuria. *N Engl J Med.* (2006) 355:1233–43. doi: 10.1056/NEJMoa061648
31. Niego B, Lee N, Larsson P, De Silva TM, Au AE, McCutcheon F, et al. Selective inhibition of brain endothelial Rho-kinase-2 provides optimal protection of an in vitro blood-brain barrier from tissue-type plasminogen activator and plasmin. *PLoS ONE.* (2017) 12:e0177332. doi: 10.1371/journal.pone.0177332
32. Davis R, Whittington R. Aprotinin. A review of its pharmacology and therapeutic efficacy in reducing blood loss associated with cardiac surgery. *Drugs.* (1995) 49:954–83. doi: 10.2165/00003495-199549060-00008
33. Niego B, Horvath A, Coughlin PB, Pugsley MK, Medcalf RL. Desmoteplase-mediated plasminogen activation and clot lysis are inhibited by the lysine analogue tranexamic acid. *Blood Coagul Fibrinol.* (2008) 19:322–4. doi: 10.1097/MBC.0b013e3282f54568
34. Li R, Coulthard LG, Wu MC, Taylor SM, Woodruff TM. C5L2: a controversial receptor of complement anaphylatoxin, C5a. *FASEB J.* (2013) 27:855–64. doi: 10.1096/fj.12-220509
35. Alexander JJ. Blood-brain barrier (BBB) and the complement landscape. *Mol Immunol.* (2018) 102:26–31. doi: 10.1016/j.molimm.2018.06.267
36. Clarke AR, Christophe BR, Khahera A, Sim JL, Connolly ES Jr. Therapeutic modulation of the complement cascade in stroke. *Front Immunol.* (2019) 10:1723. doi: 10.3389/fimmu.2019.01723
37. Ma Y, Liu Y, Zhang Z, Yang GY. Significance of complement system in ischemic stroke: a comprehensive review. *Aging Dis.* (2019) 10:429–62. doi: 10.14336/AD.2019.0119
38. Arumugam TV, Woodruff TM, Lathia JD, Selvaraj PK, Mattson MP, Taylor SM. Neuroprotection in stroke by complement inhibition and immunoglobulin therapy. *Neuroscience.* (2009) 158:1074–89. doi: 10.1016/j.neuroscience.2008.07.015
39. Alawieh A, Andersen M, Adkins DL, Tomlinson S. Acute complement inhibition potentiates neurorehabilitation and enhances tPA-mediated neuroprotection. *J Neurosci.* (2018) 38:6527–45. doi: 10.1523/JNEUROSCI.0111-18.2018
40. Alawieh A, Langley EF, Tomlinson S. Targeted complement inhibition salvages stressed neurons and inhibits neuroinflammation after stroke in mice. *Sci Transl Med.* (2018) 10:eaao6459. doi: 10.1126/scitranslmed.aao6459
41. Foley JH. Plasmin(ogen) at the nexus of fibrinolysis, inflammation, and complement. *Semin Thromb Hemost.* (2017) 43:135–42. doi: 10.1055/s-0036-1592302
42. Foley JH, Walton BL, Aleman MM, O'Byrne AM, Lei V, Harrasser M, et al. Complement activation in arterial and venous thrombosis is mediated by plasmin. *EBioMedicine.* (2016) 5:175–82. doi: 10.1016/j.ebiom.2016.02.011
43. Jain U, Woodruff TM, Stadnyk AW. The C5a receptor antagonist PMX205 ameliorates experimentally induced colitis associated with increased IL-4 and IL-10. *Br J Pharmacol.* (2013) 168:488–501. doi: 10.1111/j.1476-5381.2012.02183.x
44. West EE, Afzali B, Kemper C. Unexpected roles for intracellular complement in the regulation of Th1 responses. *Adv Immunol.* (2018) 138:35–70. doi: 10.1016/bs.ai.2018.02.001
45. March DR, Proctor LM, Stoermer MJ, Sbaglia R, Abbenante G, Reid RC, et al. Potent cyclic antagonists of the complement C5a receptor on human polymorphonuclear leukocytes. Relationships between structures and activity. *Mol Pharmacol.* (2004) 65:868–79. doi: 10.1124/mol.65.4.868
46. Freeman R, Croucher DR, Pedersen LO, Medcalf RL. t-PA, but not desmoteplase, induces plasmin-dependent opening of a blood-brain barrier model under normoxic and ischaemic conditions. *Brain Res.* (2014) 1565:63–73. doi: 10.1016/j.brainres.2014.03.027

Conflict of Interest: TW has previously consulted to Alsonex Pty Ltd., who are commercially developing PMX205, and Alexion Pharmaceuticals Inc., who developed eculizumab. He holds no stocks, shares or other commercial interest in either company.

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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CASE REPORT
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Cyclosporine-A-Induced Intracranial Thrombotic Complications: Systematic Review and Cases Report

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This study reported two cases of intracranial thrombotic events of aplastic anemia (AA) under therapy with cyclosporine-A (CsA) and reviewed both drug-induced cerebral venous thrombosis (CVT) and CsA-related thrombotic events systematically. We searched PubMed Central (PMC) and EMBASE up to Sep 2019 for publications on drug-induced CVT and Cs-A-induced thrombotic events. Medical subject headings and Emtree headings were used with the following keywords: "cyclosporine-A" and "cerebral venous thrombosis OR cerebral vein thrombosis" and "stroke OR Brain Ischemia OR Brain Infarction OR cerebral infarction OR intracerebral hemorrhage OR intracranial hemorrhage." We found that CsA might be a significant risk factor in inducing not only CVT but also cerebral arterial thrombosis in patients with AA.

Keywords: cyclosporine-A, cerebral venous sinus thrombosis, cerebral arterial infarction, case report, systematic review

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BACKGROUND

Cyclosporine-A (CsA) is widely used as an immunosuppressive agent in organ transplantation (1–3), ulcerative colitis (UC) (4–6), and aplastic anemia (AA) (7). Most commonly, the high incidences of thromboembolic complications in the renal vascular system were found in patients with CsA use after kidney transplantation (8, 9), which might be due to acute and chronic nephrotoxicity of CsA. However, thrombotic complications in other organs secondary to CsA use are not fully analyzed in the clinical settings (10). In particular, cases of CsA-induced intracranial thrombotic complications in patients with AA were rather rare (7). Herein, we presented two cases of AA with CsA-related intracranial thrombotic events, involved in cerebral venous sinuses and cerebral arteries, respectively. Besides, we conducted a systematic literature review of CsA-related thrombotic events to give more clinical references to physicians in this field.

Moreover, it is well-known that oral contraceptive (OCP) use is regarded as the iatrogenic risk factor inducing cerebral venous thrombosis (CVT). However, there is by far no review on if any other medications that could also cause CVT. Therefore, inspired by our case of CsA-induced CVT, we further comprehensively reviewed drug-induced CVT.

CASE PRESENTATION

Case 1

A 15-year-old female with a 4-year history of AA with treatment of CsA (50 mg, bid) complained of an intermittently severe headache on her left frontoparietal areas for 8 months. Her headache

could initially attenuate after intravenous injection of mannitol (125 ml, q8h) for 7 days. However, her headache was recurrent and even became aggressively severe with nausea and projectile vomiting 20 days ago, which could no longer be relieved by the former treatment of mannitol. Physical examination revealed a body temperature of 36.4°C, blood pressure of 105/85 mmHg, heart rate of 78/min, and respiratory rate of 20/min. No abnormal finding was found in the neurological examination. Fundoscopy showed stage V papilledema measured by the Frisén scale (**Supplementary Figure 1**).

Her complete blood cell (CBC) test indicated moderate normocytic normochromic anemia and a decreased platelet level due to her primary disease. The serum iron test was normal, which further excluded the differential diagnosis of iron deficiency anemia. Baseline levels of inflammatory biomarkers, including C-reactive protein (CRP) (37.2 mg/L, normal 1.0–8.0 mg/L), high-sensitivity CRP (hs-CRP) (25.75 mg/L, normal 0.0–3.0 mg/L), and interleukin 6 (IL-6) (19.6 pg/ml, normal 0.0010–7.0 pg/ml) were all above the upper normal limits (**Supplementary Table 1**), which suggested acute inflammatory reaction secondary to the primary disease. An increased level of D-dimer (2.47 μg/ml, normal range 0.01–0.5 μg/ml) and fibrinogen (4.21 g/L, normal range 2.0–4.0 g/L) remained over the upper limit of the normal range for several days after admission, suggesting the formation of thrombosis at acute stage (**Supplementary Table 1**). Serum neuron-specific enolase (NSE) level at admission was 51.52 ng/ml (normal range 0.0–17.0 ng/ml). The elevated NSE was related to damage to both neurons and the blood–brain barrier (BBB). Investigation for vasculitis [antinuclear antibody (ANA), antineutrophil cytoplasmic antibody (ANCA), and antiphospholipid antibody (APLA)] was negative. The cerebrospinal fluid (CSF) profile revealed a slightly increased white blood cell (WBC) count (2×10^6/L), and lumbar puncture opening pressure (LPOP) was over 330 mm H_2O. Contrast-enhanced magnetic resonance venography (CE-MRV) (**Figure 1**) and high-resolution MRI with black-blood thrombus image (MRBTI) of the brain (**Figure 2**) demonstrated subacute thrombosis in the superior sagittal sinus (SSS), straight sinus, right transverse sinus (TS), right sigmoid sinus (SS), and proximal part of right internal jugular vein (IJV). Moreover, no parenchymal lesion was found in MRBTI. The confirmed diagnosis of subacute CVT in multiple sites was made based on imaging findings, with involvement in cerebral

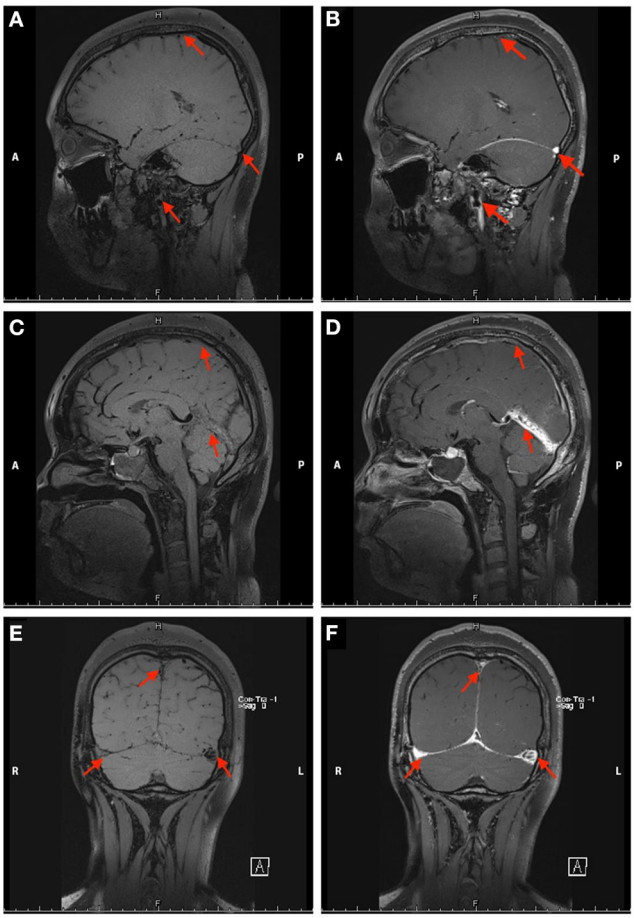

FIGURE 2 | Non-contrast enhanced **(A,C,E)** and contrast-enhanced **(B,D,F)** black-blood thrombus images of the head in Case 1. The red arrow indicates the focal stenosis of the internal jugular vein and cerebral vein sinus.

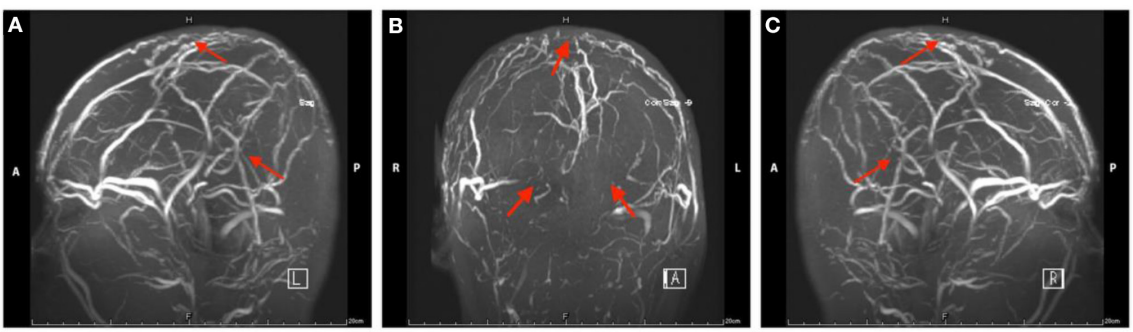

FIGURE 1 | Magnetic resonance venography images of head in Case 1. The red arrow indicates the focal stenosis of cerebral venous sinus and venous collateral circulation.

venous sinuses and IJV. The CVT-induced cerebrospinal venous insufficiency could cause disturbance of CSF circulation, further leading to intracranial hypertension and related symptoms, such as severe headaches and projectile vomiting. However, the etiology of CVT development was hard to be explained in this case due to lacking common risk factors like other female CVT patients, such as obesity, pregnancy, or long-term OCP use. Moreover, no positive result was found in the workup of thrombophilia, including protein S (PS), protein C (PC), antithrombin-III (AT-III), Factor VII/VIII deficiency, or Factor V Leiden mutation. Then, we closely monitored her blood cell counts on an everyday basis. Her hypercoagulable state induced by moderate anemia secondary to AA and probable adverse effect of CsA on damaging venous vessel walls raised our attention.

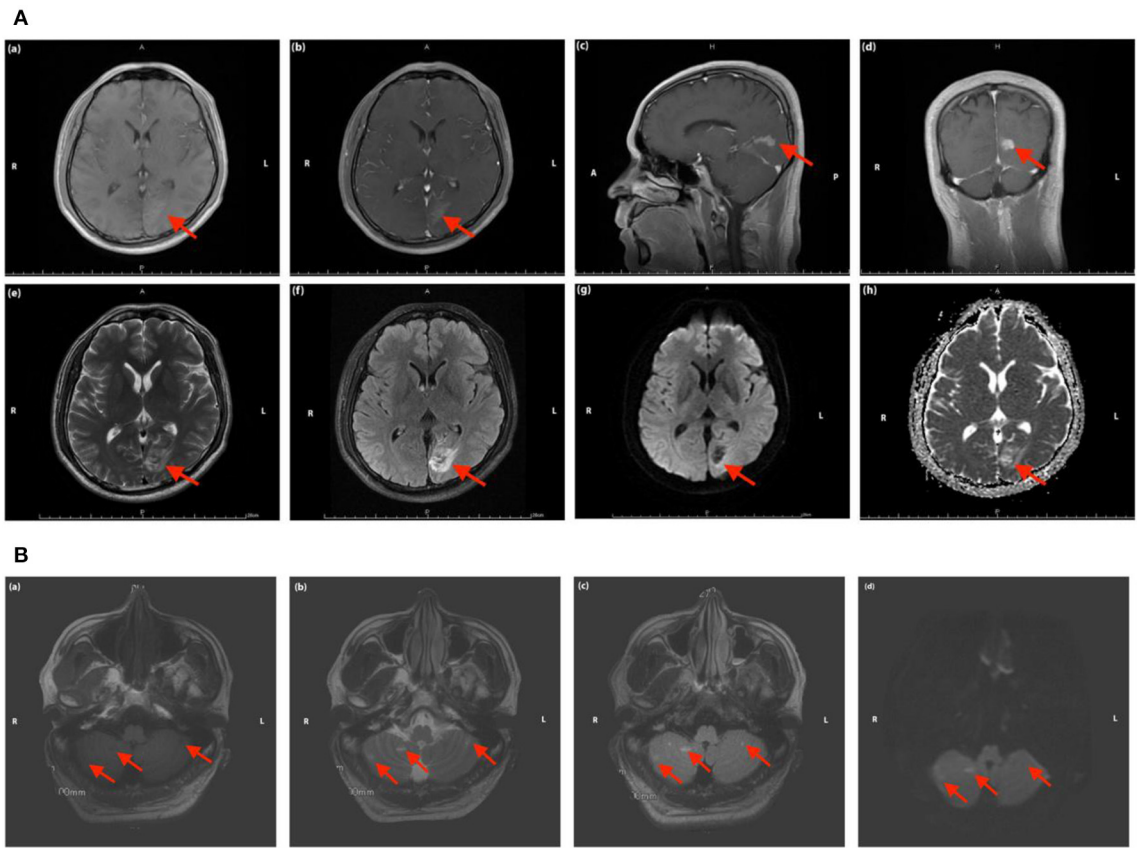

FIGURE 3 | (A) Magnetic resonance images of the head in Case 2. The red arrow indicates the focal ischemic infarction in left occipital lobe [**(a)** T1 sequence; **(b–d)** T1 sequence with contrast-enhancing; **(e)** T2 sequence; **(f)** T2 FLAIR sequence; **(g)** DWI sequence; **(h)** ADC sequence]. **(B)** Magnetic resonance images of the head in Case 2. The red arrow indicates the focal ischemic infarction in cerebellum [**(a)** T1 sequence; **(b)** T2 sequence; **(c)** T2 FLAIR sequence; **(d)** DWI sequence].

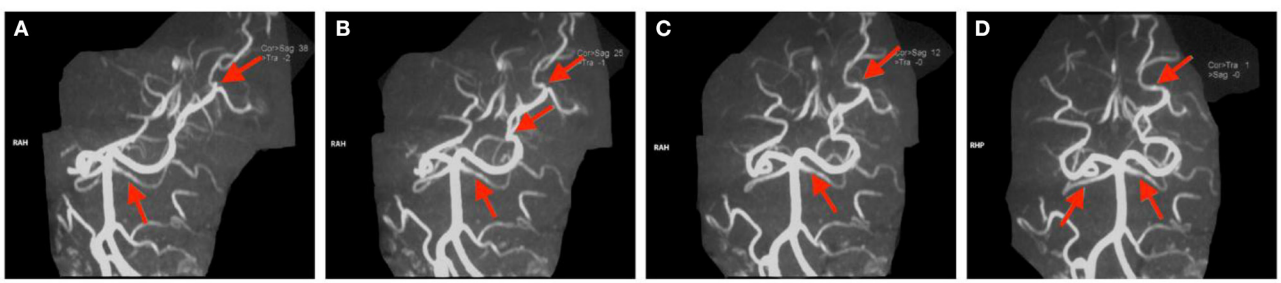

FIGURE 4 | (A–D) Magnetic resonance arthrography images of the head in Case 2. The red arrow indicates partial filling defects.

The procoagulant effect of the two factors might potentiate the formation of CVT.

Intravenous injection of mannitol (125 mL, q8h) was continued after the admission. Subcutaneous injection enoxaparin sodium (0.6 ml, qd) was started when the diagnosis of CVT was confirmed and usage of CsA was suspended after consultation with the department of hematology. The usage of enoxaparin sodium was then bridged to rivaroxaban (20 mg, qd) when she was discharged. Outpatient follow-up after 6 months of standard anticoagulation was evaluated by the Patients' Global Impression of Change (PGIC) scale. The patient reported a definite improvement of her symptoms (PGIC score = 6) and was transferred to the department of hematology to further treat AA.

Case 2

A 34-year-old male with a 1-year treatment of CsA (50 mg, bid) for AA presented with right homonymous hemianopia for 20 days, accompanied by dizziness and right-hand numbness. There was no history of nausea and vomiting, motor or sensory symptoms in the limbs, facial bulbar symptoms, sphincter incontinence, and loss of consciousness or seizures. He denied a family history of blood clotting disorders. Physical examination showed his body temperature was 36.9°C, blood pressure was 130/84 mmHg, heart rate was 72 beats/min, and respiratory rate was 18 beats/min. Neurological examination revealed no positive findings.

Peripheral blood test demonstrated mild normocytic normochromic anemia (hemoglobin, 110 g/L, normal range 120–160 g/L; hematocrit 34.8%, normal range 38.0–50.8%). The evaluation of thrombophilia showed increased levels of fibrinogen (4.11 g/L, normal range 2.0–4.0 g/L), D-dimer (1.4 μg/ml, normal range 2.0–4.0 g/L), AT-III (134%, normal range 80.0–120.0%), and protein C (181%, normal range 65.0–140.0%). All the results of serological tests, including aPL, ANA, ANCA, and complements C3 and C4, were negative. Workups of proinflammatory biomarkers, such as CRP, hs-CRP, and IL-6, were all negative. LPOP was 200 mmH_2O, and a slightly elevated level of protein (57 mg/dl, normal range 15.0–45.0 mg/dl) and WBC count in CSF was found (5×10^6/L). Serum NSE was more than two times higher than the normal upper limit (36.59 ng/ml, normal range 0.0–17.0 ng/ml). MRI indicated cerebral infarction in the left occipital lobe (**Figure 3A**) and both sides of the cerebellum (**Figure 3B**). Magnetic resonance angiography (MRA) showed focal stenosis in the distal branches of the left posterior cerebral artery (PCA) and a partial filling defect in both sides of the superior cerebellar arteries (**Figure 4**). CE-MRV excluded the possibility of CVT (**Figure 5**). As this patient has not been identified to have any vascular risk factors, such as diabetes mellitus (DM), hypertension, hyperlipidemia, obesity or smoking history, family history of small vessel disease, or state of hypercoagulability, and the evidence of systemic autoimmune diseases was also negative, we assumed that the cerebral atrial infarction was caused by emboli from cardiac source or thrombosis *in situ* secondary to certain unknown injuries. Then, to further evaluate the potential cause of stroke, transesophageal echocardiography (TEE) was conducted, with negative findings of atrial septal abnormalities [patent foramen ovale (PFO), atrial septal defect (ASD), or atrial septal aneurysm (ASA)]. Based on the patient's medical history of using CsA, the direct or indirect adverse effect of CsA may contribute

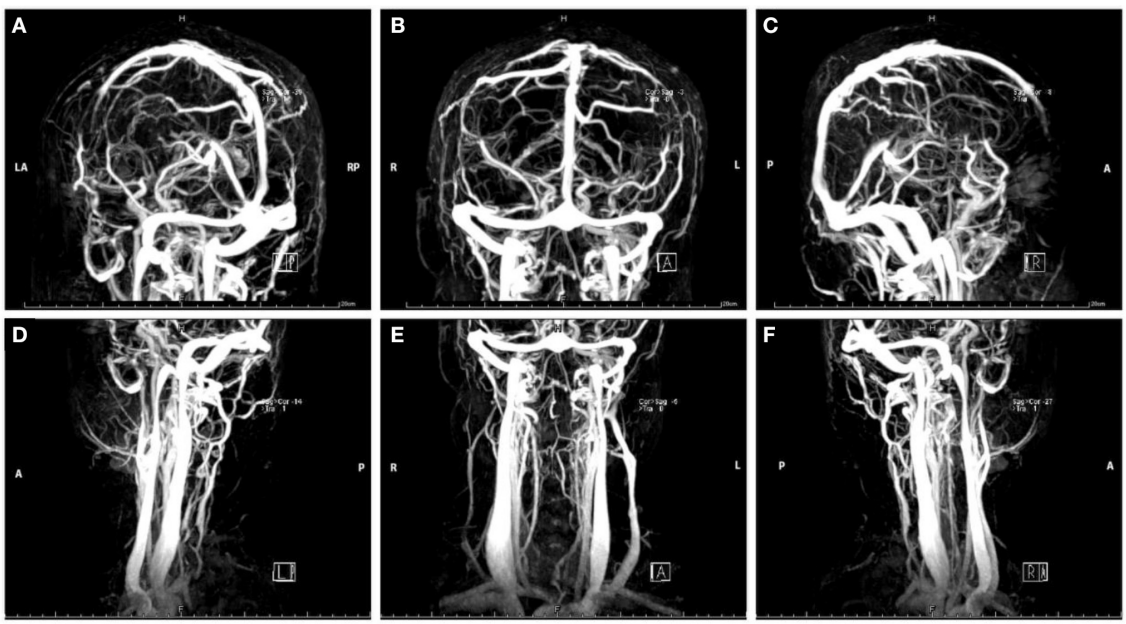

FIGURE 5 | Magnetic resonance venography images of the head **(A–C)** and neck **(D–F)** in Case 2.

to the damage in arterial vessel walls, which further initiated the formation of thrombosis *in situ*. The usage of CsA was withdrawn after consultation with the department of hematology due to his relatively well-controlled condition of AA. Aspirin (100 mg, qd) was prescribed at discharge.

MRI follow-up at 6 months post-stroke showed no new-onset parenchymal lesions, and his symptoms were partially relieved and evaluated by PGIC scale (PGIC score = 6).

Literature Review

We searched PubMed Central (PMC) and EMBASE up to Sep 2019 for publications on CsA-induced thrombotic events and drug-induced CVT. We used Medical subject headings and Emtree headings combining with the following keywords: "cyclosporine-A" and "cerebral venous thrombosis OR cerebral vein thrombosis" and "stroke OR Brain Ischemia OR Brain Infarction OR cerebral infarction OR intracerebral hemorrhage OR intracranial hemorrhage." We also screened reference lists of included articles for additional relevant studies. Intracranial thrombotic events had to be diagnosed by MRI, conventional angiography, computed tomography (CT) angiography, or at surgery or autopsy. Articles written in languages other than English were only selected if they had an English abstract with sufficient data.

We identified 322 publications related to drug-induced cerebral venous sinus thrombosis (CVST), of which 109 were selected for full-length review (**Figure 6**). Among these, 79 articles with a total of 706 patients were included based

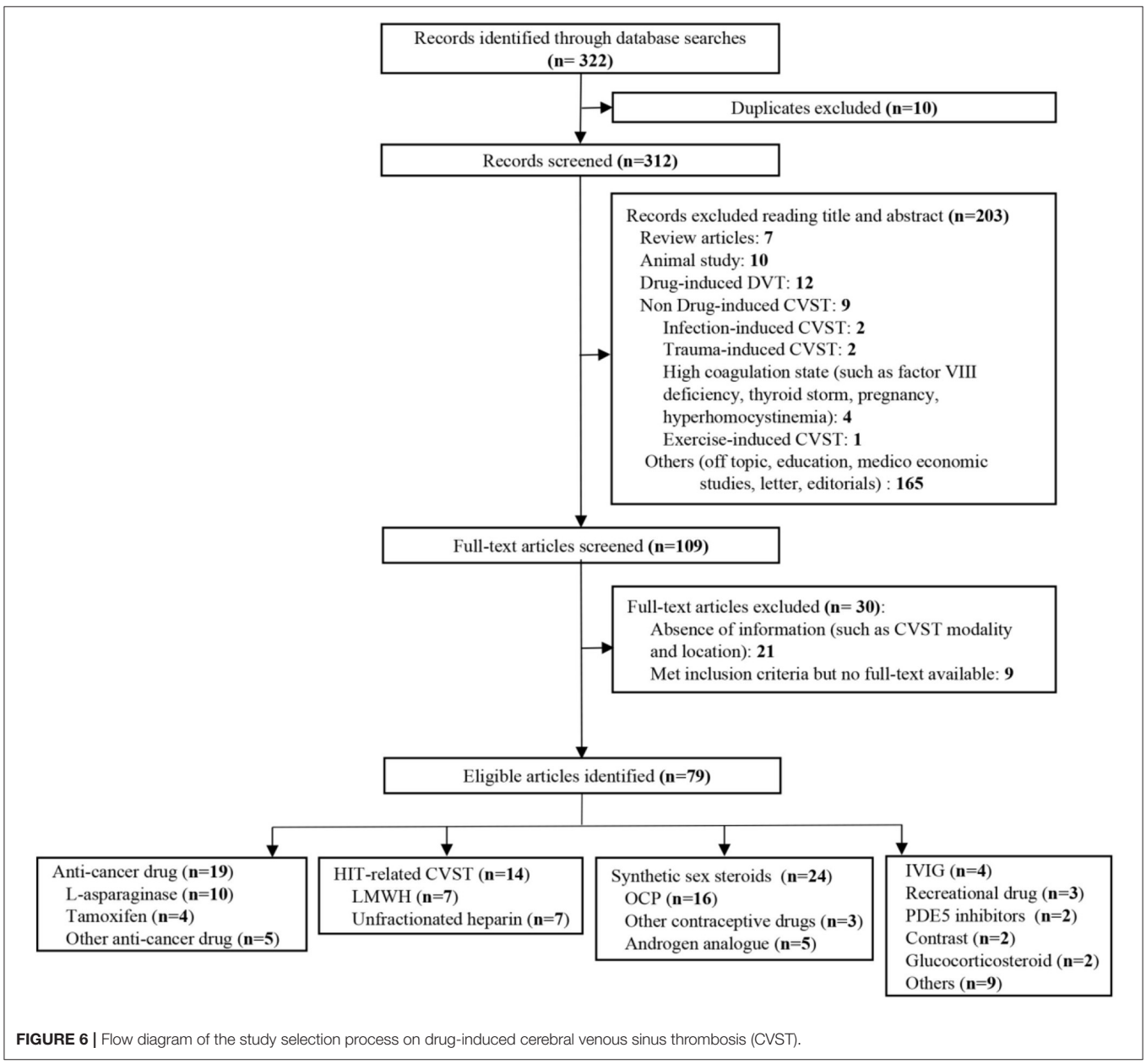

FIGURE 6 | Flow diagram of the study selection process on drug-induced cerebral venous sinus thrombosis (CVST).

TABLE 1 | Drug induced cerebral venous thrombosis.

Drug	Age/gender	Primary disease	Symptoms	CVST		References	Country	Article type	Study size
				Modality	Location				
Contrast									
Iopamidol	36/F	Myelography	NR	DSA	SSS	(11)	France	Case report	1
	24/M	Recurrent left sciatica (Myelography)	Headache (severe), nausea and vomiting	DSA	SSS, RLS	(12)	France	Case report	1
Recreational drug									
MDMA type synthetic drugs									
Ecstasy	22/F	None	Headache, nausea, visual disturbance (photophobia, visual fortification spectra), expressive dysphasia, and right hemisensory loss.	DSA	TS	(13)	UK	Case report	1
Speed	19/F	None	Uncontrollable aggressiveness	MRV	LTS	(14)	Spain	Case report	1
Cocaine	30/M	None	Headache (Occipital) and vomiting	MRV	SSS, TS	(15)	UK	Case report	1
Phosphodiesterase-5 (PDE5) inhibitors									
Tadalafil	45/M	None	Headache (severe, posterior, sudden-onset, 3-day), and seizure (generalized tonic-clonic)	MRI	RCoVT	(16)	Japan	Case report	1
Sildenafil	57/M	Two episodes of venous thrombosis (DVT; hemorrhoid plexus thrombosis)	Headache (occipital, 2-week) and visual disturbance (blurry vision)	MRV	SSS, RSS, RTS	(17)	Italy	Case report	1
IVIG	16/F	ITP	Headache (severe), neck rigidity, and vomiting	MRI	SSS	(18)	USA	Case report	1
	11/M	ITP	Headache (severe, transient frontotemporal)	CTV	SSS	(19)	Canada	Case report	1
	11/M	Humoral immunodeficiency (Bruton's disease)	Expressive aphasia, right upper extremity heaviness	CTV	SSS	(20)	Lebanon	Case report	1
	13/F	ITP	Headache	MRI	LIJV	(21)	USA	Case report	1
Others									
Dulaglutide	52/F	DM-2	Headache (3-day) and visual disturbance (blurry vision)	MRV	RTS	(22)	India	Case report	1
Romiplostim	45/F	ITP	Headache (severe, occipital)	MRV	SSS, TS	(23)	Taiwan	Case report	1
Epoetin alfa	37/F	End stage renal disease	Headache (progressive, several-day)	MRV	SSS, SS	(24)	USA	Case report	1
Dietary supplements	63/M	well-controlled hypertension	Seizure (generalized tonic-clonic)	DSA	SS, vein of Galen	(25)	USA	Case report	1
Lithum	30/F	Bipolar disorder	Headache (progressive), confusion, visual disturbance (blurry vision), and left hemiparesis.	DSA	SSS, LSS, LTS, straight sinus	(26)	USA	Case report	1
Finasteride	35/M (case 1) 41/M (case 2)	Male-pattern hair loss	Headache and seizures (case 1); Headache (case 2)	CTV	SSS	(27)	Japan	Case report	2
Combination tacrolimus/sirolimus	67/M	Renal transplantation	Seizure (generalized) and right hemiparesis	MRI	TS	(28)	Australia	Case report	1
Clozapine	30/F	Chronic paranoid schizophrenia	Vomiting, irritability, fatigability, poor personal care (5-day)	MRV	SSS, ISS, RTS, RIJV	(29)	USA	Case report	1
Levetiracetam	6.5/M	None	Headache and vomiting (2-day), then seizures (generalized)	MRI	LTS, LSS	(30)	UK	Case report	1

(Continued)

TABLE 1 | Continued

Drug	Age/gender	Primary disease	Symptoms	CVST		References	Country	Article type	Study size
				Modality	Location				
Synthetic sex steroids									
Oral contraception									
Phytoestrogens	52/F	None	Headache (diffuse and continuous, 2-month)	MRV	LLS, LSS	(31)	Portugal	Case report	1
Third-generation CHCs (containing desogestrel or gestoden)	16–49/F	CVT[h]	Seizure ($n = 52$)	CTV/MRV	CVT: dural sinuses, DCVT, CoVT, IJV	(32)	USA	Retro	57
Ethinylestradiol/ levonorgestrel	21/F	None	Headache (severe, 4-day)	CTV	SSS, CoVT, RTS, RSS	(33)	USA	Case report	1
NR	18/F (case 1) 23/F (case 2)	Protein C resistance (case 1); Anti-thrombin III deficiency (case 2)	Headache, nausea, vomiting and visual disturbance (photophobia) (case 1); Headache, vomiting, altered sensorium (case 2)	MRI	SSS, TS, LSS (case 1) SSS (case 2)	(34)	India	Case report	2
NR	18/F (case 1) 18/F (case 2)	None (case 1); ADHD and bipolar disorder (case 2)	Headache (intermittent right-sided) and visual disturbance (blurry vision) (3-day) (case 1); Headache (intermittent right-sided) and visual disturbance (double vision) (6-week) (case 2)	MRV	LTS, LSS (case 1); RTS, IJV (case 2)	(35)	USA	Case report	2
NR	27/F (case 1) 23/F (case 2)	None	Headache (retroauricular), vomiting, drowsiness, fever (several days) (case 1); Headache, vomiting, increasing drowsiness and extra-pyramidal movements (1 day) (case 2).	DSA (case 1) MRI (case 2)	LTS, SS (case 1); SSS, SS (case 2)	(36)	Italy	Case report	2
Ethinylestradiol/ desogestrel	24/F	None	Headache (severe, 1 week), vomiting.	DSA	SSS, RLS, RSS, vein of Galen	(37)	Czech Republic	Case report	1
Noracyclin[a] (case 1) Ovulen[b] (case 2)	50/F (case 1) 41/F (case 2)	None (case 1); Thrombosis of left common carotid (case 2)	Fluctuated conscious status, aphasia, right arm weakness, and several epileptic seizures (case 1); Right hand numbness and rapid-onset unconsciousness (case 2)	Necropsy	SSS, CoVT (case 1, 2);	(38)	Switzerland	Case report	2
Lyndiol 2, 5 (case 1) Metrulen-M (case 2) Anovlar (case 3) Gynovlar (case 4) Nuvacon (case 5)	24/F (case 1) 49/F (case 2) 30/F (case 3) 23/F (case 4) 29/F (case 5)	RIJVS (case 1) Diabetes (case 2) Right pulmonary embolus (case 3) Thrombosis of choroid plexus (case 4) Marfan's syndrome; thrombosis of iliac vein (case 5)	Headache, vomiting and drowsiness (5-day) (case 1); Headache, vomiting, seizure (generalized, left-sided) (case 2); Deeply comatose (case 3); Diarrhea, headache (severe) and further unconsciousness (case 4); Abdominal pain, headache (severe, 1-day), and vomiting (5-day) (case 5).	Necropsy	SSS, RTS, SS, CoVT, IJV (case 1); SSS, RTS, LTS, CoVT (case 2, 3, 5); All sinuses thrombosis, CoVT (case 4)	(39)	NR	Case report	5
Norethynodrel and mestranol	35/F	Eclampsia during pregnancy; obesity	Headache (severe, persistent, temporal) (4-day), vomiting, diarrhea, seizure, urinary incontinence, upper limb weakness (left-sided), and visual disturbance (photophobia)	Necropsy	SSS, LTS, CoVT	(40)	NR	Case report	1
Enovid (case 1) Ortho novum (case 2)	29/F (case 1, 2)	Multiple arterial thrombi; thrombosis of left opththalmic vein; Marfan's syndrome (case 2)	NR	Necropsy	All sinuses thrombosis, CoVT (case 1); SSS (case 2)	(41)	NR	Case report	2

(Continued)

TABLE 1 | Continued

Drug	Age/gender	Primary disease	Symptoms	CVST		References	Country	Article type	Study size
				Modality	Location				
Combined oral contraceptives (COCs), progestin-only contraceptives or cyproterone acetate.	15–49/F	Former thromboembolic event (including PE, CVT, ischemic stroke, or MI)	NR	NR	CVT	(42)	France	Retro	452
Yasmin 28[c]	18/F	None	Headache (Throbbing, frontal and occipital, 1-month)	MRV	RTS, RSS, IJV, CoVT	(43)	USA	Case report	1
NR	22/F	None	Severe headache (1 week)	CT/MRV/DSA	LTS, LSS	(44)	China	Case report	1
Cyproterone/gestodene	50/F	Heterozygous factor II polymorphism	NR	NR	CVST	(45)	Italy	Retro	1/28
NR	23–45/F	Prothrombin mutation G20210A ($n = 2$)	Headache ($n = 11$), vomiting ($n = 2$), aphasia ($n = 1$)	MRA ($n = 10$) DSA ($n = 7$)	Straight sinus ($n = 15$) TS ($n = 7$)	(46)	Spain	Retro	15
Other contraceptive drugs									
Norethisterone enanthate injection	23/F	None	Headaches (progressive), vomiting (repeated) and syncope (2–3 min)	MRV	SSS, RTS, RSS	(47)	USA	Case report	1
Vaginal contraceptive ring[d]	28/F	None	Headache	CT	LSS, TS	(48)	USA	Case report	1
	33/F	None	Seizures (multiple tonic-clonic), headaches	CT	LTS, LSS, LIJV	(49)	Canada	Case report	1
Androgen analog									
Oxymetholone	40/F	AA	NR	MRI	SSS, LTS	(50)	South Korea	Case report	1
Nandrolone decaonoate	22/M	None	Headaches and vomiting (repeated) (3-day)	MRV	SSS, TS	(51)	Iran	Case report	1
Fluoxymesterone	52/F (case 1) 39/F (case 2)	Hypoplastic anemia	Headaches (severe), seizures, aphasia and hemiplegia, coma (case 1) Headaches (severe) and seizure (focal) (case 2)	DSA	SSS (case 1) SSS, CoVT (case 2)	(52)	USA	Case report	3
Methenolone-enanthate	26/F (case 3)		Headaches, visual disturbance (blurred vision), and hemiparesis (right-sided) (case 3)		SSS, CoVT (case 3)				
Danazol[e]	40/M	AA	Headache (acute onset) and altered sensorium	CT	CoVT	(53)	India	Case report	1
	19/M	IHA	Headache and visual disturbance (transient obscurations of vision)	DSA	SSS, CoVT, straight sinus	(54)	USA	Case report	1
Steroid	32/F	Relapsing-remitting multiple sclerosis	Numbness and weakness (both legs)	MRV	LTS, LSS	(55)	Turkey	Case report	1
	31/M	IHA	Headaches, anorexia, general malaise	DSA	SSS, CoVT, straight sinus	(52)	Japan	Case report	1
HIT-related CVST									
LMWH	60/F	Bilateral extensive varicose veins in legs	Right focal seizures with secondary generalization followed by headache, slurred speech, and altered sensorium	MRI/CTV	LTS, LSS	(56)	India	Case report	1

(Continued)

TABLE 1 | Continued

Drug	Age/gender	Primary disease	Symptoms	CVST		References	Country	Article type	Study size
				Modality	Location				
	52/F	Kyphoplasty and posterior spinal fusion	Acute onset altered mental status with significant agitation and non-sensical speech.	MRV	LTS, LSS, LIJV	(57)	USA	Case report	1
	55/F	Partial gastrectomy	NR	MRV	SSS, SS, LIJV	(58)	Germany	Case report	1
	57/F	Antiphospholipid syndrome and possible systemic lupus erythematosus	Fever, altered mental status, and aphasia (expressive and sensory)	MRV	SSS, LSS, LIJV	(59)	Greece	Case report	1
	69/F	Knee replacement for osteoarthritis	Seizures (right arm focal type)	MRV	SSS, CoVT	(60)	USA	Case report	1
	72/M	Left knee joint surgery	Comatose	Necropsy	SSS, SS, CoVT	(61)	Germany	Case report	1
	38 ± 28	NR	NR	MRV	CVT	(62)	Germany	Retro	3/120
Unfractionated heparin	61/F	Retinal transient ischemic attack; DVT of the leg	Headache (progressive) and aphasia	MRI	LLS	(63)	France	Case report	1
	18/M	Extensive UC	Headache (severe), nausea and vomiting	MRI	RTS, confluence area	(64)	Sweden	Case report	1
	45/F	Cystic pituitary adenoma.	Aphasia and visual disturbances	MRI	LTS, LSS	(65)	USA	Case report	1
	67/F	NR	NR	CTV	SSS	(66)	Germany	Case report	1
	63/F	Polycythemia vera	Seizures (right-sided focal type)	Contrast-enhanced CT	SSS	(67)	USA	Case report	1
	36/F	PNH	Headache, nausea, then developed dysphasia and right hemiparesis	MRV	LTS, LSS	(68)	Japan	Case report	1
	67/F	Antiphospholipid syndrome	Headache (transient), vertigo, tinnitus and right hemifacial par-aesthesia with propagation down to the ipsilateral arm.	MRV	RTS, RSS, IJV	(69)	Switzerland	Case report	1
Anti-cancer drugs									
Tamoxifen	40/F	Breast cancer	Headache and hemiparesis (left-sided) (10-day)	MRI	SSS, RLS, RIJV	(70)	Turkey	Case report	1
	30/F	Breast cancer	Headache (acute-onset) and hemiparesis (left-sided)	DSA/MRI	SSS	(71)	South Korea	Case report	1
	46/F	Breast cancer	Headache (severe) and nausea (subacute onset, 2-week)	MRI/CT	SSS, straight sinus	(72)	South Korea	Case report	1
	47/F	Breast cancer	Headache (Severe), seizure (generalized tonic-clonic)	MRV	SSS, CoVT	(73)	USA	Case report	1
L-asparaginase	15/M	ALL	Acute severe headache and recurrent vomiting	MRV	SSS, TS, straight sinus	(74)	Germany	Case report	1
	10/M (case 1) 13/F (case 2)	ALL (case 1) Acute mixed phenotypic leukemia (case 2)	Headache, vomiting, seizures and loss of consciousness (case 1) Headache and focal seizure (case 2)	MRV	SSS (case 1 & 2)	(75)	India	Case report	2

(Continued)

TABLE 1 | Continued

Drug	Age/gender	Primary disease	Symptoms	CVST		References	Country	Article type	Study size
				Modality	Location				
	5–16	ALL ($n = 8$) Non-Hodgkin lymphoma ($n = 1$)	Headaches (chronic, daily), and seizures (partial-complex)[g]	MRV	LTS, LSS, LIJV (case 1) CVST (case 2) SSS (case 3) TS (case 4)	(76)	USA	Retro	9/200
	2.3/M (case 1) 3.5/F (case 2)	ALL (case 1 & 2)	Seizure (left focal seizure evolving into generalized tonic-colonic seizure and subsequently status epilepticus) (case 1); Seizure (left focal seizure evolving into status epilepticus) (case 2)	MRV (case 1 & 2)	SSS (case 1 & 2)	(77)	India	Case report	2
	(1–17)/(38/10, M/F)	ALL	Headache ($n = 14$), a decreased level or loss of consciousness ($n = 15$), visual impairment ($n = 3$), focal or generalized seizures ($n = 18$), photophobia ($n = 1$), vomiting ($n = 8$), irritability ($n = 3$), hemiparesis ($n = 5$), ataxia ($n = 2$), speech impairment ($n = 6$), and cranial nerve palsy ($n = 1$).	CT ($n = 38$), MRV ($n = 27$)	CoVT ($n = 3$), CVST ($n = 26$), CVST combined with CoVT ($n = 4$)	(78)	Italy	Retro	33/48
	5.6 (1.0–17.0)/(38/33, M/F)	ALL	NR	MRI	CVT	(79)	Austria	Pro	3/71
	9/M	ALL	Headache (Acute-onset, severe) and then seizures (left-sided focal type) and right arm sensory disturbance.	MRI	SSS	(80)	Saudi Arabia	Case report	1
	32 (15–59)/(144/96, M/F)	ALL or lymphoblastic lymphoma	NR	NR	CVT	(81)	France	Retro	5/214
	NA	ALL	NR	NR	CVT	(82)	Italy	Meta-analysis	26/1,752
	16/M	ALL	Headache, vomiting, and multiple episodes of seizures	Contrast enhanced CT	CoVT	(83)	India	Case report	1
L-asparaginase or Tamoxifen[f]	44.5 (10-71)/(16/4, M/F)	Hematologic malignancies ($n = 9$); Solid tumor ($n = 11$)	Headache ($n = 8$), seizure ($n = 6$), nausea/vomiting ($n = 5$), hemiparesis/aphasia ($n = 4$), altered metal status/coma ($n = 3$), dizziness ($n = 3$), visual disturbance ($n = 2$), gait disturbance ($n = 1$), incidental finding ($n = 1$), not available ($n = 1$)	MRV	SSS ($n = 13$), TS ($n = 8$), SS ($n = 5$), IJV ($n = 4$), straight sinus ($n = 1$)	(84)	USA	Retro	20
Cisplatin and BEP	16/F	Immature teratoma	Hemiparesis (left-sided)	MRI	SSS	(85)	Tunisia	Case report	1
Thalidomide	74/F	Multiple myeloma	Headache (right-sided, frontal), confusion and speech difficulty (acute-onset)	MRI	LTS, straight sinus, CoVT, LIJV	(86)	USA	Case report	1

(Continued)

TABLE 1 | Continued

Drug	Age/gender	Primary disease	Symptoms	CVST		References	Country	Article type	Study size
				Modality	Location				
Methotrexate	12/M	ALL	Hemiparesis (right-sided), aphasia, altered mental status, persistent seizure activity, and progressive neurological deterioration	MRI	LTS, LSS	(87)	USA	Case report	1
ATRA	22/F	APML	Visual disturbance (blurred vision on the right eye)	MRV	SSS, RSS, TS, IJV	(88)	Malaysia	Case report	1

R, right; L, left; SS, sigmoid sinus; SSS, superior sagittal sinus; TS, transverse sinus; LS, lateral sinus; CVT, cerebral venous thrombosis; CVST, cerebral venous sinus thrombosis; CoVT, cortical vein thrombosis; IJVS, internal jugular vein stenosis; DVT, deep vein thrombosis; ALL, acute lymphoblastic leukemia; APML, acute promyelocytic leaukaemia; HIT, Heparin-induced thrombocytopenia; AA, Aplastic anemia; DM, diabetes mellites; PNH, Paroxysmal nocturnal hemoglobinuria; UC, ulcerative colitis; MI, myocardial infarction; ADHD, attention deficit hyperactivity disorder; IHA, immune hemolytic anemia; ITP, immune thrombocytopenia; PE, pulmonary emboli; MRI, magnetic resonance imaging; CTV, CT venography; MRV, magnetic resonance venography; DSA, digital subtraction angiography; CHCs, combined hormonal contraceptives; ATRA, All-trans retinoic acid; BEP, bleomycin and etoposide; IVIG, Intravenous immunoglobulins; Retro, retrospective; Pro, prospective; NR, not reported.

[a]Noracyclin = Lynestrenol/mestranol.

[b]Ovulen = Ethinodiol diacetate/mestranol.

[c]Yasmin 28 = drospirenone.

[d]Vaginal contraceptive ring (NuvaRing): etonogestrel and ethinyl-estradiol per day.

[e]Danazol is an attenuated androgen derived from ethisterone (17 α-ethinyltestosterone).

[f]This study enrolled patients with hematologic malignancies or solid tumors.

[g]All patients survived, with 4 experiencing complications possibly related to CVST. In this article, report these four patients in detail.

[h]Female patients with diagnosed cerebral venous thrombosis (CVT) of the dural sinuses, involvement of the deep venous system (DCVT), cortical venous thrombosis, or with thrombosis of the jugular system.

on our inclusion criteria. However, nine articles within the inclusion criteria were not collected due to no access to full texts despite that we searched for several times and tried to contact corresponding authors by e-mail. Herein, we listed these nine references in **Supplementary Materials**. Most of the eligible studies were case reports or case series (n = 68) and retrospective studies (n = 9), and only one meta-analysis and one prospective study were found (**Table 1**) (11–89). Western countries reported 95% of the cases, followed by eastern countries (4%), while only one case was from African countries. The mean age of patients was 33.8 ± 17.9 years, and 68.5% of patients were female. There were 94 pediatric cases (94/706, 13.3%). The most common symptoms were seizures (48.6%), headaches (38.1%), nausea/vomiting (19.5%), altered mental status (drowsiness, confusion, syncope, or coma) (17.6%), motor/sensory disorder (12.9%), visual disturbance (9.0%), and aphasia/dysphasia (7.6%). The least common symptoms were personality/behavior change (aggressiveness, n = 1; irritability, n = 4; poor personal care, n = 1) (2.9%) and ataxia (2.4%). Only few cases reported symptoms like general malaise/fatigability (n = 2), fever (n = 2), diarrhea (n = 2), and urinary incontinence (n = 1). CVT was confirmed by CE-MRV (n = 55) and MRI (n = 18). Although digital subtraction angiography (DSA) was considered the gold standard, only 13 cases conducted DSA to make the defined diagnosis. Besides, CT (n = 5), CT venography (CTV) (n = 5) and autopsy (n = 5) were also mentioned as method to detect CVT. Among all sinuses, SSS (n = 123) was most likely involved in drug-induced CVT, followed by the TS (n = 119), SS (n = 97), and straight sinus (n = 80). Thrombosis was usually formed bilaterally in the TS (n = 26), while it was less common in the left TS (LTS) (n = 23) and the right TS (RTS) (n = 14). However, the left SS (LSS) more potentially formed thrombosis (n = 18) than the right SS (RSS) (n = 9); 60.3% of cases had multiple sinus thromboses (105/174). CVST combined with cortical vein thrombosis (CoVT) and isolated CoVT were reported in 102 cases and 6 cases, respectively. Drug-induced deep cerebral vein thrombosis was only found in a vein of Galen, combined with CVST (n = 2). Furthermore, CVST was also found to coexist with jugular system thrombosis (n = 70), while isolated jugular system thrombosis was very rare (n = 2). Nineteen articles indicated contraceptive drug-induced CVT, and 14 studies reported heparin-induced thrombocytopenia (HIT) that resulted in CVT. L-Asparaginase was widely used in patients with acute lymphoblastic leukemia (ALL), while 10 publications demonstrated the close relationship between CVT and L-asparaginase. Furthermore, CsA use was also a risk factor for CVT (n = 7).

We further searched articles related to CsA-induced thrombotic events to explore if CsA would bring extensive damage to different kinds of blood vessels. One hundred forty articles were identified, and full texts of 67 articles were screened (**Figure 7**). Only studies with sufficient information and a clear description of the relationship between CsA and thrombosis were finally included (n = 29). CsA was more likely associated with venous thrombotic events (n = 16), followed by capillary thrombotic events (n = 9) and arterial thrombotic events (n = 8). CVT was the most common thrombosis in CsA-induced

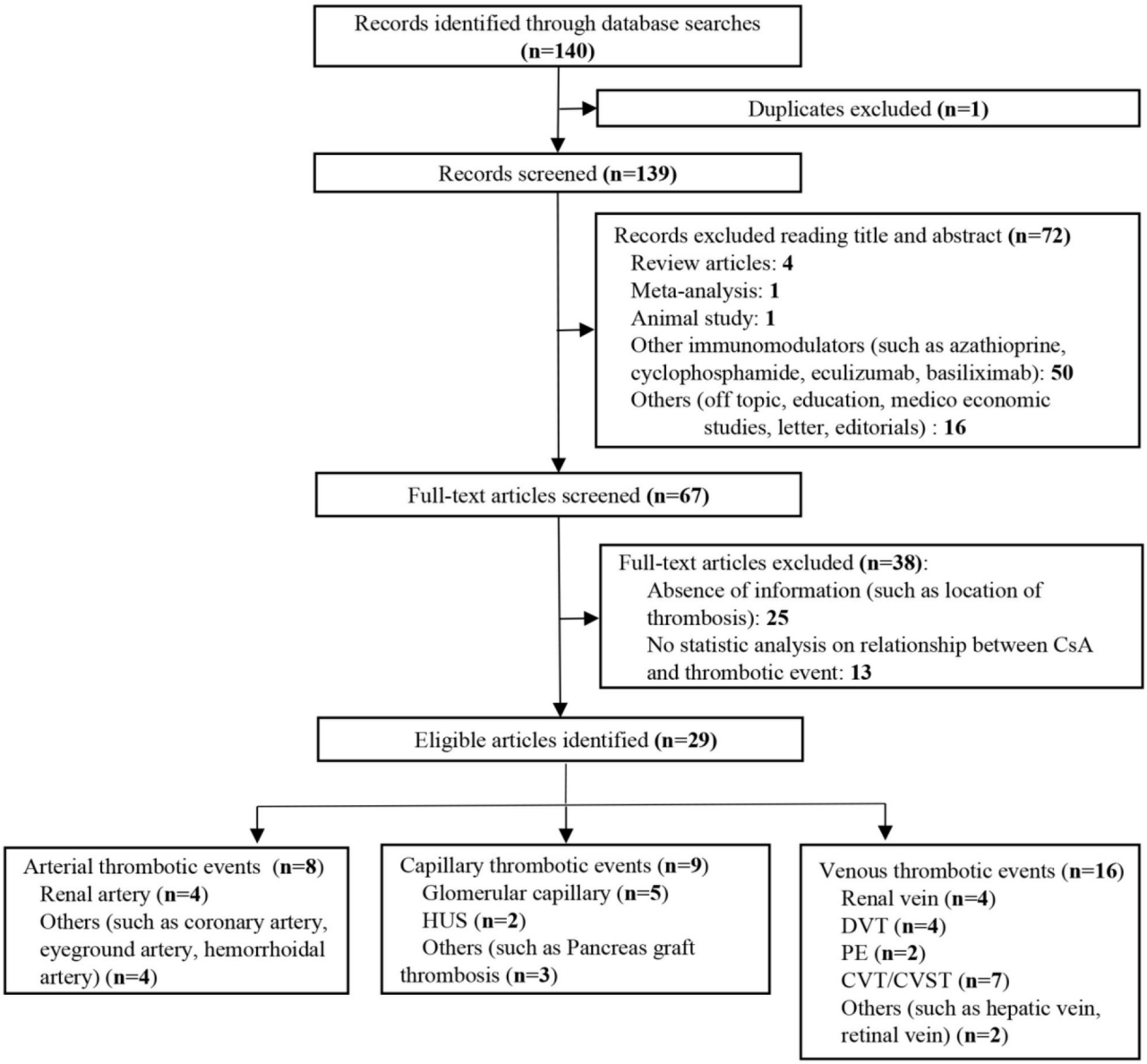

FIGURE 7 | Flow diagram of the study selection process on cyclosporine-A (CsA)-induced thrombosis.

thrombotic events (**Table 2**) (1–9, 90–109). Thrombosis in the renal vessel system was more likely formed due to CsA use in renal transplantation ($n = 13$).

Statistical Analysis

Quantitative variables with a normal distribution were specified as mean ± standard deviation. Analyses were performed with Stata software (version 15.0 SE, Stata Corp, LP, Texas, USA).

DISCUSSION

This was the first systematic review on drug-induced CVT and CsA-related thrombosis based on the clinical cases. CVT is a rare subtype of stroke, accounting for <1% of all strokes (110). Severe CVT can be fatal. Common etiologies of CVT are postpartum period, infection, and coagulopathies (111). However, drug-induced CVT should not be neglected, as this kind of CVT could be reversible and preventable if we avoid certain drugs when treating primary diseases, for instance, the two cases presented in this study. In line with CVT of other etiologies, the most common symptoms in drug-induced CVT were seizures (48.6%) and headaches (38.1%). Furthermore, women or young people were mainly involved. Both CE-MRV and black-blood thrombus image (BBTI) are useful imaging tools to make a definitive diagnosis.

TABLE 2 | Cyclosporine-A induced thrombosis.

Age/gender	Thrombosis location	Primary disease	References	Country	Study size	Article type
NR	DVT ($n = 25$), PE ($n = 4$), DVT with PE ($n = 11$)	Renal transplantation	(90)	UK	40/480	Retro
41 ± 12	DVT	Renal transplantation	(91)	Switzerland	9/97	Pro
52/M (case 1) 26/M (case 2) 32/M (case 3) 61/M (case 4) 11/M (case 5) 54/M (case 6) 45/F (case 7)	Glomerular capillary; Renal afferent artery	Renal transplantation (cadaver-donor)	(92)	UK	7	Case report
NA	Cyclosporine-associated arteriopathy (acute tubular necrosis; acute vasculitis; glomerular ischemia; interstitial intimate; Intima proliferation; venous thrombosis)	Renal transplantation (cadaver-donor)	(93)	USA	16/200	Retro
36.7 ± 1.3/(49/41, M/F)	PE ($n = 10$), Renal vein ($n = 1$), DVT ($n = 3$), Hemorrhoidal artery ($n = 3$)	Renal transplantation (cadaver-donor)	(8)	Belgium	13/90	Retro
35.9 ± 13.8	DVT	Renal transplantation (cadaver-donor, living-donor)	(94)	Sweden	9/97	Pro
53/F (case 1) 33/M (case 2) 48/F (case 3)	HUS, Glomerular capillaries, Renal artery	Renal transplantation	(95)	Canada	3	Case report
33/F	Glomerular capillaries	Renal transplantation (cadaver-donor)	(96)	UK	1	Case report
NA	Glomerular capillaries (Platelet microthrombi)	Renal transplantation	(97)	UK	12/32	Retro
NA	Renal artery	Renal transplantation (cadaver-donor, living-donor)	(98)	China	1/14	Retro
6 (case 1) 6 (case 2) 17 (case 3) 48 (case 4) 53 (case 5) 51 (case 6)	Renal vein	Renal transplantation (cadaver-donor, living-donor)	(99)	UK	6/791	Retro
NA	HUS, Glomerular capillaries	Behcet's disease	(100)	France	2	Case report
NA	CVST	SSINS	(101)	UK	1/53	Retro
45.1 ± 12.3	Renal vein	Renal transplantation (cadaver-donor)	(102)	UK	16	Pro
NA	Eyeground artery	Recurrent nephrotic syndrome	(103)	Japan	1	Case report
NA	CVT	Renal transplantation	(104)	Spain	1	Case report
25.0 ± 26.4	HUS	Renal transplantation	(105)	USA	10/672	Retro
NA	Hepatic vein	Inflammatory bowel disease and latent thrombocythemia	(106)	France	1	Case report
19/M	CVST	Severe active UC	(5)	Japan	1	Case report
48/F	CVST (LTS, LSS), LIJVS	Chronic UC	(4)	USA	1	Case report
NA	Thrombotic microangiopathy	SPK transplantation	(107)	Belgium	1/102	Retro
(18–55)	Pancreas graft thrombosis ($n = 10$), Kidney graft thrombosis ($n = 1$)	SPK transplantation	(9)	Belgium	11/102	Pro
31 ± 11	Graft thrombosis (combined arterial and venous thrombotic occlusion, $n = 5$; arterial occlusion, $n = 3$, venous occlusion, $n = 1$)	SPK transplantation	(3)	Austria	9/67	Retro

(Continued)

TABLE 2 | Continued

Age/gender	Thrombosis location	Primary disease	References	Country	Study size	Article type
30/F	Central retinal vein	Renal transplantation (cadaver-donor)	(108)	Croatia	1	Case report
56.7 ± 10.1	Coronary artery	Heart transplantation	(2)	Canada	18/129	Retro
25/M	CVST (SSS, TS)	Renal transplantation (living-donor)	(1)	Sri Lanka	1	Case report
23/F	CVT	Neuro-Behcet's disease	(109)	Brazil	3/40	Retro
18–64/(28/33, F/M)	Venous thrombosis	Acute steroid-refractory or dependent UC	(6)	Finland	1/61	Pro
44/F	CVST	AA	(7)	China	1	Case report

R, right; L, left; CVT, cerebral vein thrombosis; CVST, cerebral vein sinus thrombosis; CoVT, cortical vein thrombosis; SSS, superior sagittal sinus; TS, transverse sinus; DVT, deep vein thrombosis; PE, Pulmonary emboli; SPK transplantation, Simultaneous pancreas-kidney transplantation; HUS, Hemolytic Uremic Syndrome; AA, Aplastic anemia; SSINS, steroid sensitive idiopathic nephrotic syndrome; UC, ulcerative colitis; IBD, inflammatory bowel disease; Retro, retrospective; Pro, prospective; NR, not reported.

It would be worth noticing that CsA can induce not only CVT but also cerebral arterial thrombosis, as in Case 2 of this report. Interestingly, drug-induced CVT is more likely involved in multiple sinuses, cortical veins, or IJV, such as Case 1 in this paper. It is well-known that OCP can promote CVT in women, whereas CsA-related CVT should also raise our concern.

Cyclosporine thrombogenicity manifested mostly with CVT. However, the underlying mechanism is still controversial. Several adverse effects of CsA had been reported in patients: Firstly, CsA enhanced secretion of von Willebrand factor (VWF), a classic platelet agonist, from endothelial cells (112). Then, platelet aggregation was increased due to a higher level of VWF in circulation (113). Thirdly, CsA-induced endothelial cell dysfunction by suppressing nitric oxide production and initiating intrinsic coagulation pathway (10, 114). Further, CsA was associated with increased D-dimer and fibrinogen levels, which were observed in our patients after the onset of the thrombotic event, which was consistent with other studies (4, 8, 115). However, some animal and clinical studies showed that CsA therapy was not related to thrombosis in renal transplant and even provided strong protection from both reperfusion injury (97) and congestive heart failure (116) or improved recovery after treatment of coronary thrombosis with angioplasty (117).

Moreover, apart from the thrombogenic effect of CsA, patients with AA frequently presented with decreased levels of WBC, RBC, or platelet. Anemia secondary to AA could also be associated with both CVT (118) and arterial ischemic stroke (AIS) (119). More importantly, anemia was correlated with stroke severity and poor clinical outcomes in AIS patients (120, 121). Thus, a well-controlled condition of AA is vital to prevent cerebral thrombotic events. Besides, a stronger association between anemia and CVT in men than in women (118), which reminded us that the potential confounders, such as age and gender, should also be taken into consideration when treating AA patients with thrombotic complications.

Although we cannot prove the clear relationship between the potential adverse effect of CsA, anemia secondary to AA, and intracranial thrombotic events in these two cases due to the rarity of similar cases, CsA-induced intracranial thrombosis in AA patients was firstly reported. This observation may at least warrant caution of monitoring thrombotic events during CsA treatment in patients with AA. Therefore, we suggested that future studies could shed more light on the mechanism of the prothrombotic effects of Cs-A in the treatment of AA patients. Additionally, the systematic literature review on CsA-related thrombotic events and drug-induced CVT would give more clinical references to physicians in this field, especially when treating patients with unknown reasons for stroke.

SUMMARY TABLE

What Is Known About This Topic?

- A possible association may exist between cyclosporine-A use and thrombotic events in patients with aplastic anemia.
- Currently, there is a lack of information on comprehensive review on drug-induced cerebral venous thrombosis and cyclosporine-A-related thrombotic events.

What Does This Paper Add?

- This real-world study provides two cases with aplastic anemia that developed intracerebral thrombotic events due to cyclosporine-A use.
- Articles on cyclosporine-A-related thrombotic events were reviewed. CsA-induced thrombosis may involve the arteries, veins, and capillaries. Damage to the renal vascular system was most commonly reported due to the acute and chronic nephrotoxicity of CsA.
- Studies on drug-induced cerebral venous thrombosis were selected, of which we summarized features of clinical characteristics and neuroimaging findings.

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 Xuanwu Hospital, Beijing, China. 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

RM drafted and revised the manuscript and provided the study concept and design. S-YS drafted and revised the manuscript, provided the study concept and design, and carried out collection, assembly, and interpretation of the data. RM, S-YS, Y-CD, Z-AW, and X-MJ wrote the manuscript and gave final approval of the manuscript. Y-CD intensively edited the revised version and contributed to the critical 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.2020.563037/full#supplementary-material

Supplementary Figure 1 | Funduscopic imaging of Case 1.

Supplementary Table 1 | Follow up of abnormal examination in Case 1.

REFERENCES

1. Rajapakse S, Gnanajothy R, Lokunarangoda N, Lanerolle R. A kidney transplant patient on cyclosporine therapy presenting with dural venous sinus thrombosis: a case report. *Cases J*. (2009) 2:9139. doi: 10.1186/1757-1626-2-9139
2. White M, Ross H, Haddad H, LeBlanc MH, Racine N, Pflugfelder P, et al. Subclinical inflammation and prothrombotic state in heart transplant recipients: impact of cyclosporin microemulsion vs. tacrolimus. *Transplantation*. (2006) 82:763–70. doi: 10.1097/01.tp.0000232286.22319.e0
3. Steurer W, Malaise J, Mark W, Koenigsrainer A, Margreiter R. Spectrum of surgical complications after simultaneous pancreas-kidney transplantation in a prospectively randomized study of two immunosuppressive protocols. *Nephrol Dial Transplant*. (2005) 20:ii54–61. doi: 10.1093/ndt/gfh1083
4. Al-Shekhlee A, Oghlakian G, Katirji B. A case of cyclosporine-induced dural sinus thrombosis. *J Thromb Haemost*. (2005) 3:1327–8. doi: 10.1111/j.1538-7836.2005.01387.x
5. Murata S, Ishikawa N, Oshikawa S, Yamaga J, Ootsuka M, Date H, et al. Cerebral sinus thrombosis associated with severe active ulcerative colitis. *Intern Med*. (2004) 43:400–3. doi: 10.2169/internalmedicine.43.400
6. Nieminen U, Turunen U, Arkkila P, Sipponen T, Af Björkesten CG, Färkkilä MA. Cyclosporin A in acute steroid-refractory or dependent ulcerative colitis: a prospective study on long term outcome. *U Eur Gastroenterol J*. (2014) 2:A535. doi: 10.1177/2050640614548980
7. Gao F, Zhang J, Wang F, Xin X, Sha D. Cyclosporin A-related cerebral venous sinus thrombosis: a case report. *Medicine*. (2018) 97:e11642. doi: 10.1097/MD.0000000000011642
8. Vanrenterghem Y, Lerut T, Roels L. Thromboembolic complications and haemostatic changes in cyclosporin-treated cadaveric kidney allograft recipients. *Lancet*. (1985) 1:999–1002. doi: 10.1016/S0140-6736(85)91610-1
9. Saudek F, Malaise J, Bouček P, Adamec M. Efficacy and safety of tacrolimus compared with cyclosporin microemulsion in primary spk transplantation: 3-year results of the euro-spk 001 trial. *Nephrol Dial Transplant*. (2005) 20:ii3–10. doi: 10.1093/ndt/gfh1076
10. Lopez E, Rosado JA, Redondo PC. Immunophilins and thrombotic disorders. *Curr Med Chem*. (2011) 18:5414–23. doi: 10.2174/092986711798194405
11. Glowinski J, Breuillard P, Delafolie A, Redondo A. thrombosis of the superior longitudinal sinus after sacculoradiculography with iopamidol. *Rev Rhum Mal Osteoartic*. (1986) 53:183.
12. Brugeilles H, Pénisson-Besnier I, Pasco A, Oillic P, Lejeune P, Mercier P. Cerebral venous thrombosis after myelography with iopamidol. *Neuroradiology*. (1996) 38:534–6. doi: 10.1007/BF00626091
13. Rothwell PM, Grant R. Cerebral venous sinus thrombosis induced by 'ecstasy'. *J Neurol Neurosurg Psychiatry*. (1993) 56:1035. doi: 10.1136/jnnp.56.9.1035
14. Méndez-Sánchez F, Guisado JA, Palacios R, Teva I. Intracranial sinus thrombosis secondary to the consumption of inhaled speed. *Actas Esp Psiquiatr*. (2011) 39:404–7.
15. Burns H, Rich P, Al-Memar AY. An unpleasant hit from cocaine: a case of cocaine-induced cerebral venous sinus thrombosis. *J Neurol Neurosurg Psychiatry*. (2012) 83:A1. doi: 10.1136/jnnp-2012-304200a.3
16. Numata K, Shimoda K, Shibata Y, Shioya A, Tokuda Y. The development of cerebral venous thrombosis after tadalafil ingestion in a patient with antiphospholipid syndrome. *Intern Med*. (2017) 56:1235–7. doi: 10.2169/internalmedicine.56.7864
17. Rufa A, Cerase A, Monti L, Dotti MT, Giorgio A, Sicurelli F, et al. Recurrent venous thrombosis including cerebral venous sinus thrombosis in a patient taking sildenafil for erectile dysfunction. *J Neurol Sci*. (2007) 260:293–5. doi: 10.1016/j.jns.2007.05.011
18. Benadiba J, Robitaille N, Lambert G, Itaj NK, Pastore Y. Intravenous immunoglobulin-associated thrombosis: is it such a rare event? Report of a pediatric case and of the quebec hemovigilance system. *Transfusion*. (2015) 55:571–5. doi: 10.1111/trf.12897
19. Al-Riyami AZ, Lee J, Connolly M, Shereck E. Cerebral sinus thrombosis following iv immunoglobulin therapy of immune thrombocytopenia purpura. *Pediatr Blood Cancer*. (2011) 57:157–9. doi: 10.1002/pbc.22968
20. Barada W, Muwakkit S, Hourani R, Bitar M, Mikati M. Cerebral sinus thrombosis in a patient with humoral immunodeficiency on intravenous immunoglobulin therapy: a case report. *Neuropediatrics*. (2008) 39:131–3. doi: 10.1055/s-2008-1077088
21. Iroh Tam PY, Richardson M, Grewal S. Fatal case of bilateral internal jugular vein thrombosis following ivig infusion in an adolescent girl treated for itp. *Am J Hematol*. (2008) 83:323–5. doi: 10.1002/ajh.21107
22. Rajput R, Pathak V, Yadav PK, Mishra S. Dulaglutide-induced cerebral venous thrombosis in a patient with type 2 diabetes mellitus. *BMJ Case Rep*. (2018) 2018:bcr2018226346. doi: 10.1136/bcr-2018-226346
23. Ho P, Khan S, Crompton D, Hayes L. Extensive cerebral venous sinus thrombosis after romiplostim treatment for immune thrombocytopenia (itp) despite severe thrombocytopenia. *Intern Med J*. (2015) 45:682–3. doi: 10.1111/imj.12765
24. Finelli PF, Carlcy MD. Cerebral venous thrombosis associated with epoetin alfa therapy. *Arch Neurol*. (2000) 57:260–2. doi: 10.1001/archneur.57.2.260
25. Newey CR, Sarwal A, Tepper D. Iatrogenic venous thrombosis secondary to supplemental medicine toxicity. *J complement*

integrat Med. (2013) 10:129–34. doi: 10.1515/jcim-2012-0003
26. Wasay M, Bakshi R, Kojan S, Bobustuc G, Dubey N. Superior sagittal sinus thrombosis due to lithium: local urokinase thrombolysis treatment. *Neurology*. (2000) 54:532–3. doi: 10.1212/WNL.54.2.532
27. Tsuji Y, Nakayama T, Bono K, Kitamura M, Imafuku I. Two cases of stroke associated with the use of finasteride, an approved drug for male-pattern hair loss in japan. *Clin Neurol*. (2014) 54:423–8. doi: 10.5692/clinicalneurol.54.423
28. Graves A, Kulkarni H. Cerebral sinus venous thrombosis temporally associated with combination tacrolimus/sirolimus immunosuppression. *Nephrology*. (2013) 18:75–6. doi: 10.1111/nep.12121
29. Srinivasaraju R, Reddy YC, Pal PK, Math SB. Clozapine-associated cerebral venous thrombosis. *J Clin Psychopharmacol*. (2010) 30:335–6. doi: 10.1097/JCP.0b013e3181deb88a
30. Mohamed BP, Prabhakar P. Thrombocytopenia as an adverse effect of levetiracetam therapy in a child. *Neuropediatrics*. (2009) 40:243–4. doi: 10.1055/s-0030-1247524
31. Guimaraes J, Azevedo E. Phytoestrogens as a risk factor for cerebral sinus thrombosis. *Cerebrovasc Dis*. (2005) 20:137–8. doi: 10.1159/000086805
32. Roethlisberger M, Gut L, Zumofen DW, Fisch U, Boss O, Maldaner N, et al. Cerebral venous thrombosis requiring invasive treatment for elevated intracranial pressure in women with combined hormonal contraceptive intake: risk factors, anatomical distribution, and clinical presentation. *Neurosurg focus*. (2018) 45:E12. doi: 10.3171/2018.4.FOCUS1891
33. Wharton HC. A 21-year-old white woman diagnosed with cerebral venous sinus thrombosis related to oral contraceptive and factor v leiden. *Adv Emerg Nurs J*. (2012) 34:10–5. doi: 10.1097/TME.0b013e318243552c
34. Wankhade V, Patil S, Joshi G, Kadhe N, Pawar S, Maulik N. Oc pills induced cerebral venous sinus thrombosis: a case series. *Indian J Pharmacol*. (2013) 45:S162.
35. Tan JJ, Hassoun A, Elmalem VI. Cerebral venous sinus thrombosis with ophthalmic manifestations in 18-year-olds on oral contraceptives. *Clin Pediatr*. (2014) 53:826–30. doi: 10.1177/0009922814533405
36. Rosi R, Stanca A, Monfregola MR, Malandrini A, Fabrizi GM, Galluzzi P, et al. Aggressive treatment of severe acute cerebral venous thrombosis associated with oral contraceptives in young women. *Clin Intensive Care*. (1995) 6:36–9. doi: 10.3109/tcic.6.1.36.39
37. Prochazka V, Rajner J, Prochazka M, Dvorak J, Cizek V. Oral contraceptive induced cerebral venous thrombosis treated by local catheter directed thrombolysis. *Interv Neuroradiol*. (2004) 10:321–8. doi: 10.1177/159101990401000406
38. Poltera AA. The pathology of intracranial venous thrombosis in oral contraception. *J pathol*. (1972) 106:209–19. doi: 10.1002/path.1711060402
39. Atkinson EA, Fairburn B, Heathfield KW. Intracranial venous thrombosis as complication of oral contraception. *Lancet*. (1970) 1:914–8. doi: 10.1016/S0140-6736(70)91046-9
40. Buchanan DS, Brazinsky JH. Dural sinus and cerebral venous thrombosis. incidence in young women receiving oral contraceptives. *Arch neurol*. (1970) 22:440–4. doi: 10.1001/archneur.1970.00480230058006
41. Walsh FB, Clark DB, Thompson RS, Nicholson DH. Oral contraceptives and neuro-ophthalmologic interest. *Arch Ophthalmol*. (1965) 74:628–40. doi: 10.1001/archopht.1965.00970040630009
42. Petitpain N, Gourbil M, Grandvuillemin A, Beyens MN, Massy N, Gras V, et al. Hormonal contraception or cyproterone acetate and thromboembolic events: a study in 30 french public hospitals. *Fundam Clin Pharmacol*. (2014) 28:104–5. doi: 10.1111/fcp.12066
43. Perez MA, Glaser JS, Schatz NJ. "Idiopathic" intracranial hypertension caused by venous sinus thrombosis associated with contraceptive usage. *Optometry*. (2010) 81:351–8. doi: 10.1016/j.optm.2010.01.010
44. Huang Q, Chai X, Xiao C, Cao X. A case report of oral contraceptive misuse induced cerebral venous sinus thrombosis and dural arteriovenous fistula. *Medicine*. (2019) 98:e16440. doi: 10.1097/MD.0000000000016440
45. Girolami A, Spiezia L, Girolami B, Zocca N, Luzzatto G. Effect of age on oral contraceptive-induced venous thrombosis. *Clin Appl Thromb/Hemost*. (2004) 10:259–63. doi: 10.1177/107602960401000308
46. Galarza M, Gazzeri R. Cerebral venous sinus thrombosis associated with oral contraceptives: the case for neurosurgery. *Neurosurg Focus*. (2009) 27:E5. doi: 10.3171/2009.8.FOCUS09158
47. Bahall M, Santlal M. Norethisterone enanthate-induced cerebral venous sinus thrombosis (cvst). *BMJ Case Rep*. (2017) 2017:bcr2017222418. doi: 10.1136/bcr-2017-222418
48. Kolacki C, Rocco V. The combined vaginal contraceptive ring, nuvaring, and cerebral venous sinus thrombosis: a case report and review of the literature. *J Emerg Med*. (2012) 42:413–6. doi: 10.1016/j.jemermed.2011.06.011
49. Dunne C, Malyuk D, Firoz T. Cerebral venous sinus thrombosis in a woman using the etonogestrel-ethinyl estradiol vaginal contraceptive ring: a case report. *J Obstet Gynaecol Can*. (2010) 32:270–3. doi: 10.1016/S1701-2163(16)34454-1
50. Chu K, Kang DW, Kim DE, Roh JK. Cerebral venous thrombosis associated with tentorial subdural hematoma during oxymetholone therapy. *J Neurol Sci*. (2001) 185:27–30. doi: 10.1016/S0022-510X(01)00448-8
51. Sahraian MA, Mottamedi M, Azimi AR, Moghimi B. Androgen-induced cerebral venous sinus thrombosis in a young body builder: case report. *BMC Neurol*. (2004) 4:22. doi: 10.1186/1471-2377-4-22
52. Shiozawa Z, Ueda R, Mano T, Tsugane R, Kageyama N. Superior sagittal sinus thrombosis associated with evans' syndrome of haemolytic anaemia. *J Neurol*. (1985) 232:280–2. doi: 10.1007/BF00313866
53. Sudheer Kumar G, Roopesh Kumar VR, Gopalakrishnan MS, Shankar Ganesh CV, Venkatesh MS. Danazol-induced life-threatening cerebral venous thrombosis in a patient with aplastic anemia. *Neurol India*. (2011) 59:762–4. doi: 10.4103/0028-3886.86557
54. Hamed LM, Glaser JS, Schatz NJ, Perez TH. Pseudotumor cerebri induced by danazol. *Am J Ophthalmol*. (1989) 107:105–10. doi: 10.1016/0002-9394(89)90206-7
55. Gazioglu S, Solmaz D, Boz C. Cerebral venous thrombosis after high dose steroid in multiple sclerosis: a case report. *Hippokratia*. (2013) 17:88–90.
56. Shah SD, Shah C, Vora R. Heparin-induced thrombocytopenia and cerebral venous thrombosis after low-molecular weight heparin. *Neurol India*. (2010) 58:669–70. doi: 10.4103/0028-3886.68688
57. Gleichgerrcht E, Lim MY, Turan TN. Cerebral venous sinus thrombosis due to low-molecular-weight heparin-induced thrombocytopenia. *Neurologist*. (2017) 22:241–4. doi: 10.1097/NRL.0000000000000146
58. Beland B, Busse H, Loick HM, Ostermann H, Van Aken H. Phlegmasia cerulea dolens, cerebral venous thrombosis, and fatal pulmonary embolism due to heparin-induced thrombocytopenic thrombosis syndrome. *Anesth Analg*. (1997) 85:1272–4. doi: 10.1097/00000539-199712000-00016
59. Stavropoulos I, Liverezas A, Papageorgiou E, Tsiara S. A rare case of heparin-induced thrombocytopenia and cerebral venous sinus thrombosis with antiphospholipid syndrome and possible systemic lupus erythematosus. *Aktualnosci Neurologiczne*. (2017) 17:121–5. doi: 10.15557/AN.2017.0013
60. Fesler MJ, Creer MH, Richart JM, Edgell R, Havlioglu N, Norfleet G, et al. Heparin-induced thrombocytopenia and cerebral venous sinus thrombosis: case report and literature review. *Neurocritic Care*. (2011) 15:161–5. doi: 10.1007/s12028-009-9320-y
61. Meyer P, Couzi G, Bavle J, Blanc P, Gibelin P, Camous JP, et al. Disseminated coronary thrombosis and pentosane polysulfate-induced thrombocytopenia. *Arch Mal Coeur Vaiss*. (1988) 81:913–9.
62. Pohl C, Harbrecht U, Greinacher A, Theuerkauf I, Biniek R, Hanfland P, et al. Neurologic complications in immune-mediated heparin-induced thrombocytopenia. *Neurology*. (2000) 54:1240–5. doi: 10.1212/WNL.54.6.1240
63. Richard S, Perrin J, Lavandier K, Lacour JC, Ducrocq X. Cerebral venous thrombosis due to essential thrombocythemia and worsened by heparin-induced thrombocytopenia and thrombosis. *Platelets*. (2011) 22:157–9. doi: 10.3109/09537104.2010.527399
64. Thorsteinsson GS, Magnusson M, Hallberg LM, Wahlgren NG, Lindgren F, Malmborg P, et al. Cerebral venous thrombosis and heparin-induced thrombocytopenia in an 18-year old male with severe ulcerative colitis. World *J Gastroenterol*. (2008) 14:4576–9. doi: 10.3748/wjg.14.4576
65. Refaai MA, Warkentin TE, Axelson M, Matevosyan K, Sarode R. Delayed-onset heparin-induced thrombocytopenia, venous thromboembolism, and cerebral venous thrombosis: a consequence of heparin "flushes". *Thromb Haemost*. (2007) 98:1139–40. doi: 10.1160/TH07-06-0423

66. Merz S, Fehr R, Gülke C. Sinus vein thrombosis. a rare complication of heparin-induced thrombocytopenia type ii. *Anaesthesist*. (2004) 53:551–4. doi: 10.1007/s00101-004-0687-z
67. Kyritsis AP, Williams EC, Schutta HS. Cerebral venous thrombosis due to heparin-induced thrombocytopenia. *Stroke*. (1990) 21:1503–5. doi: 10.1161/01.STR.21.10.1503
68. Ishihara-Kawase K, Ohtsuki T, Sugihara S, Tanaka H, Nakamura T, Kimura A, et al. Cerebral sinus thrombosis and heparin-induced thrombocytopenia in a patient with paroxysmal nocturnal hemoglobinuria. *Intern Med*. (2010) 49:941–3. doi: 10.2169/internalmedicine.49.3053
69. Hsieh J, Kuzmanovic I, Vargas MI, Momjian-Mayor I. Cerebral venous thrombosis due to cryptogenic organising pneumopathy with antiphospholipid syndrome worsened by heparin-induced thrombocytopenia. *BMJ Case Rep*. (2013) 2013:bcr2013009500. doi: 10.1136/bcr-2013-009500
70. Akdal G, Donmez B, Cakmakci H, Yener GG. A case with cerebral thrombosis receiving tamoxifen treatment. *Eur J Neurol*. (2001) 8:723–4. doi: 10.1046/j.1468-1331.2001.00297.x
71. Hwang SK. A case of dural arteriovenous fistula of superior sagittal sinus after tamoxifen treatment for breast cancer. *J Korean Neurosurg Soc*. (2015) 57:204–7. doi: 10.3340/jkns.2015.57.3.204
72. Kim J, Huh C, Kim D, Jung C, Lee K, Kim H. Isolated cortical venous thrombosis as a mimic for cortical subarachnoid hemorrhage. *World Neurosurg*. (2016) 89:727.e5–7. doi: 10.1016/j.wneu.2016.01.009
73. Phuong L, Shimanovsky A. Superior sagittal sinus thrombosis related to the use of tamoxifen: a case report and review of literature. *Conn med*. (2016) 80:487–9.
74. Dietel V, Bührdel P, Hirsch W, Körholz D, Kiess W. Cerebral sinus occlusion in a boy presenting with asparaginase-induced hypertriglyceridemia. *Klin Padiatr*. (2007) 219:95–6. doi: 10.1055/s-2007-921455
75. Wani NA, Kosar T, Pala NA, Qureshi UA. Sagittal sinus thrombosis due to l-asparaginase. *J Pediatr Neurosci*. (2010) 5:32–5. doi: 10.4103/1817-1745.66683
76. Ross CS, Brown TM, Kotagal S, Rodriguez V. Cerebral venous sinus thrombosis in pediatric cancer patients: long-term neurological outcomes. *J Pediatr Hematol/Oncol*. (2013) 35:299–302. doi: 10.1097/MPH.0b013e31827e8dbd
77. Siddaiahgari SR, Makadia D, Lingappa L. Peg asparginase induced superior sagittal sinus thrombosis with status epilepticus pediatric in acute lymphoblastic leukemia (all): a report of 2 cases from India. *J Pharmacol Toxicol*. (2014) 9:129–33. doi: 10.3923/jpt.2014.129.133
78. Santoro N, Colombini A, Silvestri D, Grassi M, Giordano P, Parasole R, et al. Screening for coagulopathy and identification of children with acute lymphoblastic leukemia at a higher risk of symptomatic venous thrombosis: an aieop experience. *J Pediatr Hematol Oncol*. (2013) 35:348–55. doi: 10.1097/MPH.0b013e31828dc614
79. Meister B, Kropshofer G, Klein-Franke A, Strasak AM, Hager J, Streif W. Comparison of low-molecular-weight heparin and antithrombin versus antithrombin alone for the prevention of symptomatic venous thromboembolism in children with acute lymphoblastic leukemia. *Pediatr Blood Cancer*. (2008) 50:298–303. doi: 10.1002/pbc.21222
80. Alsaid Y, Gulab S, Bayoumi M, Baeesa S. Cerebral sinus venous thrombosis due to asparaginase therapy. *Case Rep Hematol*. (2013) 2013:841057. doi: 10.1155/2013/841057
81. Hunault-Berger M, Chevallier P, Delain M, Bulabois CE, Bologna S, Bernard M, et al. Changes in antithrombin and fibrinogen levels during induction chemotherapy with l-asparaginase in adult patients with acute lymphoblastic leukemia or lymphoblastic lymphoma. Use of supportive coagulation therapy and clinical outcome: the capelal study. *Haematologica*. (2008) 93:1488–94. doi: 10.3324/haematol.12948
82. Caruso V, Iacoviello L, Di Castelnuovo A, Storti S, Mariani G, de Gaetano G, et al. Thrombotic complications in childhood acute lymphoblastic leukemia: a meta-analysis of 17 prospective studies comprising 1752 pediatric patients. *Blood*. (2006) 108:2216–22. doi: 10.1182/blood-2006-04-015511
83. Dubashi B, Jain A. L-asparginase induced cortical venous thrombosis in a patient with acute leukemia. *J Pharmacol Pharmacotherapeut*. (2012) 3:194–5. doi: 10.4103/0976-500X.95531
84. Raizer JJ, DeAngelis LM. Cerebral sinus thrombosis diagnosed by mri and mr venography in cancer patients. *Neurology*. (2000) 54:1222–6. doi: 10.1212/WNL.54.6.1222
85. Kridis WB, Khanfir A, Kammoun F, Mahfoudh KB, Triki C, Frikha M. A very rare cerebral complication of chemotherapy in a young girl: a difficult diagnosis. *Curr Drug Saf*. (2015) 10:257–60. doi: 10.2174/1574886310666150518112823
86. Lenz RA, Saver J. Venous sinus thrombosis in a patient taking thalidomide. *Cerebrovasc Dis*. (2004) 18:175–7. doi: 10.1159/000079739
87. Mahadeo KM, Dhall G, Panigrahy A, Lastra C, Ettinger LJ. Subacute methotrexate neurotoxicity and cerebral venous sinus thrombosis in a 12-year old with acute lymphoblastic leukemia and methylenetetrahydrofolate reductase (mthfr) c677t polymorphism: homocysteine-mediated methotrexate neurotoxicity *via* direct endothelial injury. *Pediatr Hematol Oncol*. (2010) 27:46–52. doi: 10.3109/08880010903341904
88. Lee KR, Subrayan V, Win MM, Fadhilah Mohamad N, Patel D. Atra-induced cerebral sinus thrombosis. *J Thromb Thrombolysis*. (2014) 38:87–9. doi: 10.1007/s11239-013-0988-7
89. Shiozawa Z, Yamada H, Mabuchi C, Hotta T, Saito M, Sobue I, et al. Superior sagittal sinus thrombosis associated with androgen therapy for hypoplastic anemia. *Ann Neurol*. (1982) 12:578–80. doi: 10.1002/ana.410120613
90. Allen RD, Michie CA, Morris PJ, Chapman JR. Venous thrombosis and cyclosporin. *Lancet*. (1985) 2:1004. doi: 10.1016/S0140-6736(85)90543-4
91. Bergentz SE, Bergqvist D, Bornmyr S. Venous thrombosis and cyclosporin. *Lancet*. (1985) 2:101–2. doi: 10.1016/S0140-6736(85)90205-3
92. Neild GH, Reuben R, Hartley RB, Cameron JS. Glomerular thrombi in renal allografts associated with cyclosporin treatment. *J Clin Pathol*. (1985) 38:253–8. doi: 10.1136/jcp.38.3.253
93. Sommer BG, Innes JT, Whitehurst RM, Sharma HM, Ferguson RM. Cyclosporine-associated renal arteriopathy resulting in loss of allograft function. *Am J Surg*. (1985) 149:756–64. doi: 10.1016/S0002-9610(85)80181-1
94. Brunkwall J, Bergqvist D, Bergentz SE, Bornmyr S, Husberg B. Postoperative deep venous thrombosis after renal transplantation. Effects of cyclosporine. *Transplantation*. (1987) 43:647–9. doi: 10.1097/00007890-198705000-00008
95. Giroux L, Smeesters C, Corman J, Paquin F, Allaire G, St-Louis G, et al. Hemolytic uremic syndrome in renal allografted patients treated with cyclosporin. *Can J Physiol Pharmacol*. (1987) 65:1125–31. doi: 10.1139/y87-177
96. Muirhead N, Hollomby DJ, Keown PA. Acute glomerular thrombosis with csa treatment. *Ren Fail*. (1987) 10:135–9. doi: 10.3109/08860228709047648
97. Dunnill MS, Gatter KC, Mason DY, Morris PJ. Immunosuppression and thrombosis in renal transplantation: an immunohistological study. *Histopathology*. (1990) 16:79–82. doi: 10.1111/j.1365-2559.1990.tb01065.x
98. Fang GX, Chan PCK, Cheng IKP, Li MK, Wong KK, Chan MK. Haematological changes after renal transplantation: differences between cyclosporin-A and azathioprine therapy. *Int Urol Nephrol*. (1990) 22:181–7. doi: 10.1007/BF02549838
99. Richardson AJ, Higgins RM, Jaskowski AJ, Murie JA, Dunnill MS, Ting A, et al. Spontaneous rupture of renal allografts: the importance of renal vein thromobosis in the cyclosporin era. *Br J Surg*. (1990) 77:558–60. doi: 10.1002/bjs.1800770530
100. Beaufils H, De Groc F, Gubler MC, Wechsler B, Le Hoang P, Baumelou A, et al. Hemolytic uremic syndrome in patients with behcet's disease treated with cyclosporin A: report of 2 cases. *Clin Nephrol*. (1990) 34:157–62.
101. Neuhaus TJ, Fay J, Dillon MJ, Trompeter RS, Barratt TM. Alternative treatment to corticosteroids in steroid sensitive idiopathic nephrotic syndrome. *Arch Dis Child*. (1994) 71:522–6. doi: 10.1136/adc.71.6.522
102. Schleibner S, Krauss M, Wagner E, Erhard J, Christiaans M, Van Hooff J, et al. Fk 506 versus cyclosporin in the prevention of renal allograft rejection - european pilot study: six week results. *Transplant Int*. (1995) 8:86–90. doi: 10.1111/j.1432-2277.1995.tb01481.x

103. Ito S, Hosaka M, Beppu M, Nomura T, Uchida J. Case report of a recurrent nephrotic syndrome patient with sudden onset of blindness during treatment with cyclosporin A. *Jpn J Nephrol*. (1998) 40:27–31.
104. Guerrero AL, Arcaya J, Cacho J, Seisdedos L. Thrombosis of intracranial venous veins in a patient with kidney transplant and toxic serum levels of cyclosporin. *Med Clin*. (1999) 112:238–9.
105. Langer RM, Van Buren CT, Katz SM, Kahan BD. De novo hemolytic uremic syndrome after kidney transplantation in patients treated with cyclosporine-sirolimus combination. *Transplantation*. (2002) 73:756–60. doi: 10.1097/00007890-200203150-00017
106. Decaens T, Maitre S, Marfaing A, Naveau S, Chaput JC, Mathurin P. Inflammatory bowel disease and latent thrombocythemia: a novel cause of hepatic vein thrombosis. *Gastroenterol Clin Biol*. (2004) 28:394–7. doi: 10.1016/S0399-8320(04)94941-X
107. Kuypers DRJ, Malaise J, Claes K, Evenepoel P, Maes B, Coosemans W, et al. Secondary effects of immunosuppressive drugs after simultaneous pancreas-kidney transplantation. *Nephrol Dial Transplant*. (2005) 20:ii33–9. doi: 10.1093/ndt/gfh1080
108. Simic P, Gasparovic V, Skegro M, Stern-Padovan R. Cholelithiasis and thrombosis of the central retinal vein in a renal transplant recipient treated with cyclosporin. *Clin Drug Investig*. (2006) 26:361–5. doi: 10.2165/00044011-200626060-00008
109. Dutra LA, Goncalves CR, Pedroso JL, Braga-Neto P, Gabbai AA, Barsottini OGP, et al. Neuro-behets disease in brazil: higher incidence in females and atypical manifestations. *Arthritis Rheum*. (2011) 63.
110. Einhaupl K, Stam J, Bousser MG, De Bruijn SF, Ferro JM, Martinelli I, et al. EFNS guideline on the treatment of cerebral venous and sinus thrombosis in adult patients. *Eur J Neurol*. (2010) 17:1229–35. doi: 10.1111/j.1468-1331.2010.03011.x
111. Meng R, Ji X, Wang X, Ding Y. The etiologies of new cases of cerebral venous sinus thrombosis reported in the past year. *Intractable Rare Dis Res*. (2012) 1:23–6. doi: 10.5582/irdr.2012.v1.1.23
112. Nolasco LH, Gushiken FC, Turner NA, Khatlani TS, Pradhan S, Dong JF, et al. Protein phosphatase 2b inhibition promotes the secretion of von willebrand factor from endothelial cells. *J Thromb Haemost*. (2009) 7:1009–18. doi: 10.1111/j.1538-7836.2009.03355.x
113. Fishman SJ, Wylonis LJ, Glickman JD, Cook JJ, Warsaw DS, Fisher CA, et al. Cyclosporin A augments human platelet sensitivity to aggregating agents by increasing fibrinogen receptor availability. *J Surg Res*. (1991) 51:93–8. doi: 10.1016/0022-4804(91)90076-X
114. Dusting GJ. Nitric oxide in cardiovascular disorders. *J Vasc Res*. (1995) 32:143–61. doi: 10.1159/000159089
115. Ueda D, Suzuki K, Malyszko J, Pietraszek MH, Takada Y, Takada A, et al. Fibrinolysis and serotonin under cyclosporine a treatment in renal transplant recipients. *Thromb Res*. (1994) 76:97–102. doi: 10.1016/0049-3848(94)90211-9
116. Piot C, Croisille P, Staat P, Thibault H, Rioufol G, Mewton N, et al. Effect of cyclosporine on reperfusion injury in acute myocardial infarction. *N Engl J Med*. (2008) 359:473–81. doi: 10.1056/NEJMoa071142
117. Halestrap AP, Pasdois P. The role of the mitochondrial permeability transition pore in heart disease. *Biochim Biophys Acta Bioenerg*. (2009) 1787:1402–15. doi: 10.1016/j.bbabio.2008.12.017
118. Coutinho JM, Zuurbier SM, Gaartman AE, Dikstaal AA, Stam J, Middeldorp S, et al. Association between anemia and cerebral venous thrombosis: case-control study. *Stroke*. (2015) 46:2735–40. doi: 10.1161/STROKEAHA.115.009843
119. Chang YL, Hung SH, Ling W, Lin HC, Li HC, Chung SD. Association between ischemic stroke and iron-deficiency anemia: a population-based study. *PLoS ONE*. (2013) 8:e82952. doi: 10.1371/journal.pone.0082952
120. Khan MF, Shamael I, Zaman Q, Mahmood A, Siddiqui M. Association of anemia with stroke severity in acute ischemic stroke patients. *Cureus*. (2018) 10:e2870 doi: 10.7759/cureus.2870
121. Tanne D, Molshatzki N, Merzeliak O, Tsabari R, Toashi M, Schwammenthal Y. Anemia status, hemoglobin concentration and outcome after acute stroke: a cohort study. *BMC Neurol*. (2010) 10:22. doi: 10.1186/1471-2377-10-22

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Inside the Thrombus: Association of Hemostatic Parameters With Outcomes in Large Vessel Stroke Patients

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Background: Actual clinical management of ischemic stroke (IS) is based on restoring cerebral blood flow using tissue plasminogen activator (tPA) and/or endovascular treatment (EVT). Mechanical thrombectomy has permitted the analysis of thrombus structural and cellular classic components. Nevertheless, histological assessment of hemostatic parameters such as thrombin-activatable fibrinolysis inhibitor (TAFI) and matrix metalloproteinase 10 (MMP-10) remains unknown, although their presence could determine thrombus stability and its response to thrombolytic treatment, improving patient's outcome.

Methods: We collected thrombi ($n = 45$) from large vessel occlusion (LVO) stroke patients ($n = 53$) and performed a histological analysis of different hemostatic parameters [TAFI, MMP-10, von Willebrand factor (VWF), and fibrin] and cellular components (erythrocytes, leukocytes, macrophages, lymphocytes, and platelets). Additionally, we evaluated the association of these parameters with plasma levels of MMP-10, TAFI and VWF activity and recorded clinical variables.

Results: In this study, we report for the first time the presence of MMP-10 and TAFI in all thrombi collected from LVO patients. Both proteins were localized in regions of inflammatory cells, surrounded by erythrocyte and platelet-rich areas, and their content was significantly associated ($r = 0.41$, $p < 0.01$). Thrombus TAFI was lower in patients who died during the first 3 months after stroke onset [odds ratio (OR) (95%CI); 0.59 (0.36–0.98), $p = 0.043$]. Likewise, we observed that thrombus MMP-10 was inversely correlated with the amount of VWF ($r = -0.30$, $p < 0.05$). Besides, VWF was associated with the presence of leukocytes ($r = 0.37$, $p < 0.05$), platelets ($r = 0.32$, $p < 0.05$), and 3 months mortality [OR (95%CI); 4.5 (1.2–17.1), $p = 0.029$]. Finally, plasma levels of TAFI correlated with circulating and thrombus platelets, while plasma MMP-10 was associated with cardiovascular risk factors and functional dependence at 3 months.

Conclusions: The present study suggests that the composition and distribution of thrombus hemostatic components might have clinical impact by influencing the response to pharmacological and mechanical therapies as well as guiding the development of new therapeutic strategies.

Keywords: ischemic stroke (IS), thrombus, thrombin activatable fibrinolysis inhibitor (TAFI), matrix-metalloproteinase 10 (MMP-10), hemostasis

INTRODUCTION

Stroke is the primary neurovascular disease, being the second cause of death and disability worldwide (5.5 million deaths each year and 176.4 million stroke-related disabled people) with almost 14 million new cases around the world every year (1). Stroke severely hampers the normal daily activities of survivors affecting health and social-care resources (2). Moreover, in 2047, the number of stroke events is expected to increase in almost 40,000 incident strokes and 2.58 million prevalent cases in Europe, in part as a consequence of the aging of the population (3).

Ischemic stroke (IS) accounts for the majority of strokes and in caused by the presence of a thrombus or an embolus in brain vessels. The current goal for the management of IS is based on the restoration of the cerebral blood flow achieved by the use of the thrombolytic drug, tissue plasminogen activator (tPA), and/or endovascular treatment (EVT) to remove thrombi (4). The successful introduction of endovascular thrombectomy procedures within the last decade has allowed thrombus retrieval and its detailed analysis. The study of thrombi is crucial to understand diagnosis, treatment, and secondary prevention of acute IS and to design safe and efficient thrombolytic strategies to improve recanalization and prognosis of IS patients.

Several studies of IS thrombi have focused on their structural and cellular components (5). Among them, platelets and von Willebrand factor (VWF) are important factors in thrombus formation and have previously been shown as key components of acute IS thrombo-emboli (6). Erythrocite dominancy in thrombi has been associated with arterial thrombi from noncardiac source, whereas fibrin/platelet dominancy has been described as related to cardiac thrombi (7–9). Leukocytes are often present in thrombus and seem to be more dominant in cardiac thrombi (7, 8). However, when T cells were analyzed separately by CD3+ immunostaining, the number of T cells was significantly higher in atherothrombotic thrombi than in thrombi from patients with cardioembolic or other causes stroke (10).

In search of new pharmacological alternatives for patients that do not benefit from current therapies, preclinical studies are exploring the potential of new thrombolytic compounds in different models of IS. Specific inhibitors of antifibrinolytic proteins are under development as the diabody against plasminogen activator inhibitor-1 (PAI-1) and thrombin-activatable fibrinolysis inhibitor (TAFI) (11). This simultaneous inhibition of TAFI and PAI-1 showed increased profibrinolytic effects without adverse bleeding (12). Moreover, already approved drugs, as the mucolytic drug N-acetylcisteine, by dissolving the disulfide bonds of large VWF multimers, have been proven to accelerate thrombus dissolution and prevent rethrombosis in rodent models of IS resistant to tPA (13). In line with these results, a disintegrin and metalloproteinase with a thrombospondin type 1 motif member 13 (ADAMTS13), which cleaves VWF, dissolves the t-PA-resistant thrombi. Consequently it reduces cerebral infarct sizes showing a potent thrombolytic activity in experimental models of stroke (14). Finally, matrix metalloproteinases (MMPs) could also play a role in thrombolysis, since the fibrinolytic and the MMPs systems cooperate in thrombus dissolution by acting on fibrin(ogen) directly or by collaborating with plasmin. Precisely, plasmin is able to cleave and activate several MMPs (MMP-1, MMP-3, and MMP-9) that can take part in the dissolution of the fibrin clot directly or interacting with other elements of the fibrinolytic system (15, 16). Specifically, our group has shown the fibrinolytic role of MMP-10 by preventing the activation of TAFI (17). We have reported that the administration of MMP-10 is as efficient as tPA reducing infarct size and demonstrated that a combination of MMP-10 with tPA achieves further reduction in brain damage by blocking tPA-induced neuronal excitotoxicity in IS experimental models (18).

The histological location of TAFI and MMP-10 in stroke thrombi still remains unknown, and their presence could determine thrombus stability and the response to thrombolytic therapy. In this study, we therefore collected thrombi retrieved from large vessel occlusion (LVO) stroke patients and subjected them to histological assessment of different hemostatic parameters with a specific focus on TAFI and MMP-10. Furthermore, we investigated their association with clinical outcomes.

MATERIALS AND METHODS

Study Population

A total of 53 serial acute LVO IS patients admitted to the Complejo Hospitalario de Navarra Stroke Unit who underwent EVT between November 2015 and November 2017 were recruited. Adequate and correctly processed histological material was available only from 45 patients. Depending on the degree of fragmentation, it was either collected in one piece or in multiple pieces. All collected material from the same patient was processed together as one. The decision to perform EVT, associated or not with intravenous tPA, was made according to guidelines at the time of patient admission as the standard of care for acute IS (19). Endovascular procedure was performed

using a stent-retriever [pRESET (Phenox, Germany); Catch (Balt, France); Tigertriever and Comaneci (Rapid Medical, Israel)] or an aspiration device (Penumbra, Penumbra, USA) according to interventionalist's criteria.

Clinical Information

Demographics (age, sex) and other baseline characteristics of the patients, including previous cardiovascular disease, vascular risk factors, systolic, and diastolic blood pressure (SBP and DBP, respectively) at admission, serum glucose, stroke severity assessed by the National Institutes of Health Stroke Scale (NIHSS), previous use of antithrombotic agents (antiplatelet agents and anticoagulants), and treatment with tPA, were recorded. Main vascular risk factors documented were the following: type-2 diabetes mellitus (use of antidiabetic drugs, a casual plasma glucose >200 mg/dl, or fasting blood sugar ≥126 mg/dl or HbA1c ≥6.5%), hypertension (patients taking antihypertensive drugs or with blood pressure >140/90 mmHg on repeated measurements), hypercholesterolemia [patients receiving lipid-lowering agents or with triglycerides ≥200 mg/dl, an overnight fasting cholesterol level ≥240 mg/dl, or low-density lipoprotein (LDL) cholesterol ≥160 mg/dl], and current cigarette smoking. Based on the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification (20), etiological subtypes of ischemic stroke were assesed. C-reactive protein (CRP) and plasma creatinine were measured with autoanalyzers (Architect i2000SR, USA, and Cobas C311, Roche, Germany, respectively).

Alberta Stroke Program Early CT Score (ASPECTS) was collected by two independent radiologists regarding a CT scan obtained at admission for all patients. A second CT scan was done at 24–48 h in all patients to identify hemorragic transformation and evaluate infarct area. A 1.5 MRI scan was obtained within 1 week of stroke onset if not contraindicated (when MRI was contraindicated, delayed CT scan was elective) to confirm IS. The recanalization after thrombectomy was evaluated by angiography during endovascular procedure using the modified treatment in cerebral infarction (mTICI) score.

Histological Analysis

Retrieved thrombi ($n = 45$) were immersed in a saline solution and fixed for 24 h in formalin (PanReac AppliChem, Spain). Later, samples were embedded in paraffin by a tissue automatic processor (Tissue-Tek VIP, Sakura, Japan), and 3-μm sections of clot material were cut with a rotatory microtome (HM-340E, Microm, Germany). Serial slides from each thrombus were stained with Martius Scarlet Blue staining (Atom, UK), hematoxylin and eosin (PanReac AppliChem, Spain), and platelets glycoprotein Ib (CD42b, 42C01, Invitrogen, USA) to visualize their general internal organization (**Figure 1**). H&E allows identification of platelet/fibrin aggregates (pink), red blood cells (RBCs, red), and nucleated cells (dark blue), whereas the presence of fibrin (dark pink/red), red blood cells (RBCs, yellow), and collagen (blue) was demonstrated selectively by Martius Scarlet Blue (MSB) staining. Moreover, specific antibodies against VWF (A0082, Dako), T lymphocytes (anti-CD3, A0452, Dako), leukocytes (antiCD45, NCL-LCA-RP, Leica Biosystems), macrophages (anti-CD68, M0814, Dako), TAFI (AP17235PU, Origene), and MMP-10 (OAAF01865, Aviva) were also assayed.

Deparaffined and hydrated slides were incubated with citrated antigen retrieval solution (pH 6.10, Dako) at 95°C for 20 min or with Tris–ethylenediaminetetraacetic acid (EDTA) pH 9 for CD3 immunostaining (Master Diagnostica). Then, endogenous peroxidases were blocked with 5% hydrogen peroxide for 20 min at room temperature (RT) in the dark. Slides were then washed in Tris saline buffer (TBS, pH 7.36, 25 mM Tris). Sections were blocked using normal goat serum (Dako) for 1 h at RT. Sections were then incubated overnight at 4°C, with the primary antibodies. After washing, slides were incubated with the required secondary antibodies using the anti-rabbit or anti-mouse Dako Envision System-HRP (Dako) for 30 min and developed with diaminobenzidine (DAB, Dako) followed by counterstaining with Harris' hematoxylin. Slides were then mounted with distyrene plasticizer and xylene mixture (DPX, VWR Chemicals).

Double immunofluorescence was performed to localize TAFI and MMP-10 with specific cell types in thrombi tissue. Briefly, slides were incubated with a mix of primary antibodies overnight at 4°C. After washing, slides were incubated with the corresponding secondary antibodies for 30 min, using a goat anti-rabbit Alexa fluor 488 antibody (Invitrogen) or a biotinylated goat anti-mouse antibody (Dako) that was amplified with the Cy3 NEL 704 kit (PerkinElmer). Finally, slides were mounted with VECTASHIELD® Antifade Mounting Medium on DAPI (Novus Biological). Double immunofluorescence for TAFI and MMP-10 was performed with the rabbit anti-TAFI antibody described above and a monoclonal anti-MMP-10 (MAB9101, R&D systems).

Immunostained slides were subsequently scanned (Aperio ImageScope, Leica ByoSistems, Germany and Vectra Polaris, Perkin Elmer, USA) and quantified with ImageJ software (21). The percentage of positively stained area in total tissue area is presented as representative of thrombi content for TAFI, MMP-10, VWF, fibrin, RBC, and platelets (CD42b), whereas the positive cell number per square millimeter is given for nucleated cells (leukocytes, lymphocytes, and macrophages).

Outcome Measures

Individual scores in the modified Rankin Scale (mRS) at 90 days, established by face-to-face interview with a stroke specialized neurologist, were the main clinical outcome. Other clinical outcomes included were as follows: (a) 3-month all-cause mortality; (b) 3-month functional independence (FI), categorized as 90-day mRS <3; (c) successful recanalization, defined as mTICI 2b or 3; and (d) hemorrhagic transformation after ischemic stroke according to the European Cooperative Acute Stroke Study III (ECASS III) classification (22), including hemorrhagic infarcts (HI type 1 or 2), parenchymal hematomas (PH type 1 or 2) and remote hematomas or subarachnoid hemorrhages.

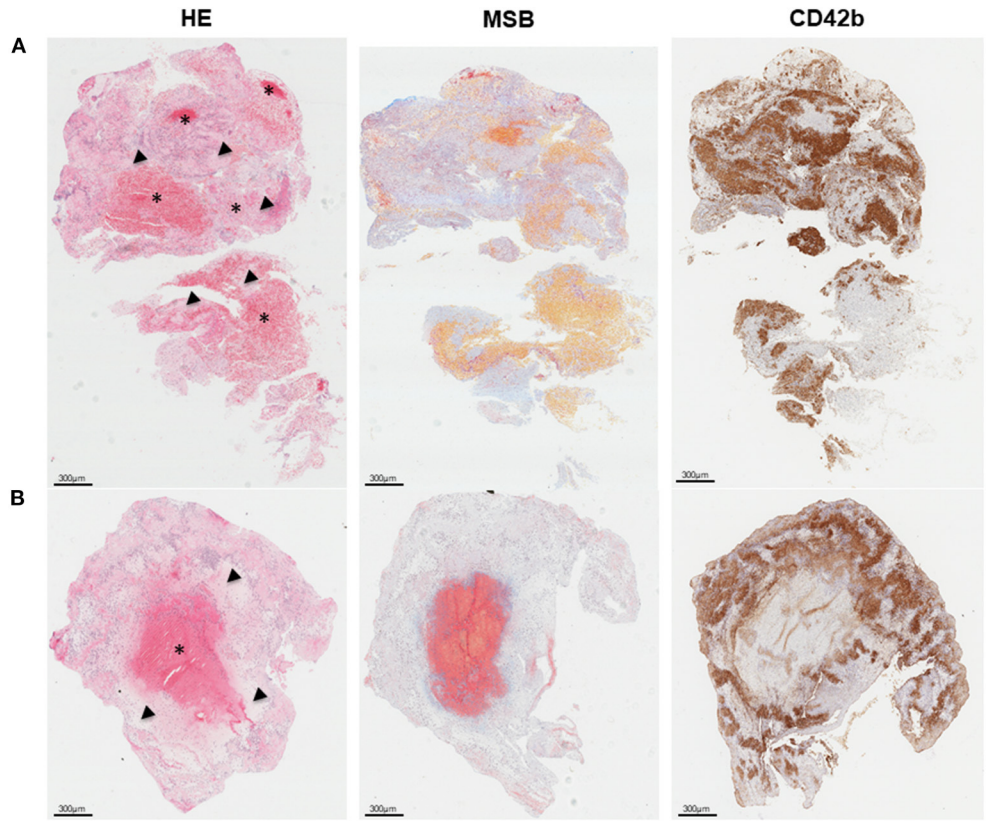

FIGURE 1 | Overall thrombus composition and organization. **(A)** Red blood cell (RCB)-rich (asterisk) and platelet-rich areas (arrow head) are patchly distributed within thrombi. **(B)** Some thrombi present an RBC-rich core surrounded by platelet-rich material. From left to right, representative histological images of consecutive thrombi sections stained with hematoxylin and eosin (H&E), allowing identification of fibrin/platelet aggregates (pink), RBC (red), and nucleated cells (dark blue); Martius Scarlet Blue (MSB) showing the presence of fibrin (dark pink/red), RBC (yellow), and collagen (blue); and CD42b (brown) for platelets. Scale = 300 µm.

Plasma Levels of VWF, MMP-10, and TAFI

Within the following 24 h after admission, venous blood samples were drawn from all patients and centrifuged at 1200 × *g* for 15 min within 2 h of collection and subsequently stored at −80°C for further analysis. VWF activity (Innovance VWFAc, Siemens, Spain), MMP-10 levels (R&D Systems, USA), and TAFI activity (TAFIa, STA STACHROM TAFI, Stago, France) were measured with an automated ELISA analyzer TRITURUS (Grifols, Spain) in citrated plasma samples after being thawed on ice and thoroughly vortexed. The detection limit of the assays was 2.2%, 15.1 pg/ml, and 5% for VWFAc, MMP-10, and TAFIa, respectively. All experiments were performed and analyzed in a blinded manner.

Statistical Analysis

Normality of distributions was assessed graphically and with the Shapiro–Wilk test. Non-normally distributed variables were presented as median with interquartile range (IQR), while continuous variables with normal distributions were presented as mean with standard deviation (SD). Logarithmic transformation was applied for continuous variables with skewed distributions. An unpaired *t*-test or the Wilcoxon rank-sum test was applied to compare continuous variables between groups depending on their distribution. The chi-square test or, in the case of small-expected frequencies, Fisher's exact test were performed to compare binary categorical variables distribution between groups. Correlation between continuous variables was evaluated by pairwise Spearman correlation test. Association between MMP-10 and TAFI thrombi content was assessed by linear regression analysis. Based on TOAST criteria, stroke subtype classification was assessed, and dichotomized etiological groups were created. Three groups of stroke severity by NIHSS score were categorized [(0–7), (7–14), and (>14)], and analysis of variance and trend analysis were performed.

Selected multivariate binary logistic regression models were performed to evaluate associations between thrombi histological parameters and circulating measurements with clinical outcomes. Results were expressed as odds ratios (ORs) with 95% confidence intervals (95% CIs).

Statistical significance was considered for all analyses if $p < 0.05$. STATA software (version 16, StataCorp LLC, Texas, USA) was the statistic software for this study.

RESULTS

Patients Clinical Characteristics

Fifty-three patients were finally included in the study. Clinical characteristics of the patients are shown in **Table 1**.

TABLE 1 | Patients clinical characteristics.

Variable	*n* = 53
Age, years†	74.6 (61.9–78.2)
Female, *n* (%)‡	25 (47.2)
Hypertension, *n* (%)‡	31 (58.5)
Type-2 Diabetes, *n* (%)‡	10 (18.9)
Dyslipidemia, *n* (%)‡	31 (58.5)
Antiplatelet therapy, *n* (%)‡	12 (22.6)
Anticoagulant therapy, *n* (%)‡	18 (34.0)
SBP at admission, mmHg*	143.5 (23.7)
DBP at admission, mmHg*	82.0 (15.1)
Serum glucose at admission, mg/dL†	115 (98–140)
Neutrophil count at admission, $\times 10^9$/L†	5.7 (4.3–7.7)
Lymphocyte count at admission, $\times 10^9$/L†	1.7 (1.3–2.6)
Baseline NIHSS score†	18 (15–21)
ASPECTS, points†	10 (8–10)
Baseline mRS score, *n* (%)‡	
mRS 0	34 (64.2)
mRS 1	10 (18.9)
mRS 2	9 (17.0)
Etiologic subtype by TOAST, *n* (%)	
Atherothrombotic‡	8 (15.1)
Cardioembolic‡	33 (62.3)
Undetermined‡	10 (18.9)
Others‡	2 (3.8)
Intravenous thrombolysis, *n* (%)‡	38 (71.7)
Recanalization (TICI 2B-3), *n* (%)‡	45 (84.9)
Hemorrhagic transformation, *n* (%)‡	19 (35.9)
3-month mortality, *n* (%)‡	13 (25)
3-month functional independence, *n* (%)‡	24 (46.2)

SBP, systolic blood pressure; DBP, diastolic blood pressure; NIHSS, National Institute of Health Stroke Scale; ASPECTS, Alberta Stroke Program Early CT Score; mRS, modified Rankin Scale; TOAST, Trial of Org 10172 in Acute Stroke Treatment; TICI, Treatment in Cerebral Infarction.

**Continuous variables with normal distributions are presented as mean (SD).*

†*Continuous non-normally distributed variables are presented as median (IQR 25–75).*

‡*Categorical variables are presented as n (%).*

Revascularization after EVT was achieved in 84.9% of patients treated. Stent-retriever devices were deployed in 71.2% of patients, and aspiration techniques alone were performed in the remaining 28.9% of patients. A high percentage of patients (71.7%) was treated also with intravenous tPA as IS standard of care when they did not have contraindication to tPA treatment. Median time from stroke onset to EVT treatment was 190 min (IQR, 150–240) with an onset-to-needle time for those with intravenous fibrinolysis of 86 min (IQR, 65–130). No differences in onset-to-femoral puncture time was observed in those patients who received tPA vs. those without tPA treatment [median, (IQR): 190 (155–265) vs 220 (140–232), $p = 0.53$]. Stroke severity was severe with a median NIHSS score of 18 points (IQR, 15–21). ICH incidence was 35.9% (18.9% PH), and mortality rate was 25%.

Histological Characteristics of Thrombi

Only 45 IS thrombi properly retrieved after thrombectomy were analyzed. According to usual description of thrombi microscopic distribution (23, 24), two different patterns are interspersed within the analyzed thrombi (**Figure 1A**): on the one hand, the RBC-rich areas, composed of packed RBC within a meshwork of fibrin and little or no nucleated cells; on the other hand, the platelet-rich areas with fibrin staining through the platelets region. From the 45 analyzed thrombi, this pattern could be identified in 23 with a wide heterogeneity in quantity and distribution of those regions. Some thrombi, however, mainly consisted of a RBC-rich core that was surrounded by a platelet-rich matrix (18/45) (**Figure 1B**).

As shown in **Figure 2**, leukocytes were mainly found at the interface between RBC- and platelet-rich areas but also within platelet-rich zones. Moreover, VWF staining was localized scattered in platelet-rich areas and through fibrin-positive regions.

To assess the relative contribution of each thrombus element, we quantified the stained area of RBC and fibrin (MSB), platelets (CD42b), and VWF. In addition, number of leukocytes (CD45), macrophages (CD68), and T lymphocytes (CD3) were assessed for all thrombi (**Table 2**). Overall, the median amount of RBC-rich material was 12.8% (IQR, 9.3–29.1), similar to fibrin [16.2% (5.4–41)], platelets [15.0% (4.6–26.7)], and VWF content [11.0% (6.3–17.4)]. A median of 308.9 leukocytes/mm^2 (IQR, 216.3–513.3), 32.9 lymphocytes/mm^2 (19.5–63.9), and 98.7 macrophages/mm^2 (44.9–264.2) were observed within the thrombus.

As shown in **Table 3**, analysis of cell types and proteins content in stroke thrombi showed a positive correlation between fibrin and RBC ($r = 0.38$, $p < 0.05$), as well as with platelets ($r = 0.36$, $p < 0.05$). Meanwhile, VWF correlated with platelets ($r = 0.32$, $p < 0.05$) and leukocytes ($r = 0.37$, $p < 0.05$). After linear regression multivariate analysis of thrombi components, only the association between fibrin and platelets [B = −0.07 (−0.11–−0.03), $p = 0.002$], VWF and leukocytes [B = 6.46 (2.33–10.58), $p = 0.003$], and VWF and platelets [B = 0.23 (0.01–0.45), p =0.042] remained significant after adjusting for age and sex.

Interestingly, MMP-10 and TAFI proteins were present in all thrombi [median (IQR): 2.9% (0.15-8.1) MMP-10 and 2.1% (0.9–3.8) TAFI] related to leukocyte distribution and primarily found at the interface between RBC and platelet-rich areas (**Table 2** and **Figure 2**). As shown in **Figure 3**, MMP-10 colocalized with CD68 and with some CD45- and CD42b-positive cells, while TAFI signal was observed in some leukocytes and in platelets. Double immunostaining for TAFI and MMP-10 confirmed the colocalization of both proteins in thrombi (**Figure 4**).

Finally, MMP-10 staining positively correlated with TAFI ($r = 0.41$, $p < 0.01$) and negatively with VWF ($r = -0.30$, $p < 0.05$), while no association was found between thrombus TAFI and their cellular components or other analyzed proteins, except for MMP-10 (**Table 3**). This association between thrombus TAFI and MMP10 remained significant after adjusting for age and sex [B = 0.88 (0.55–1.20), $p < 0.001$].

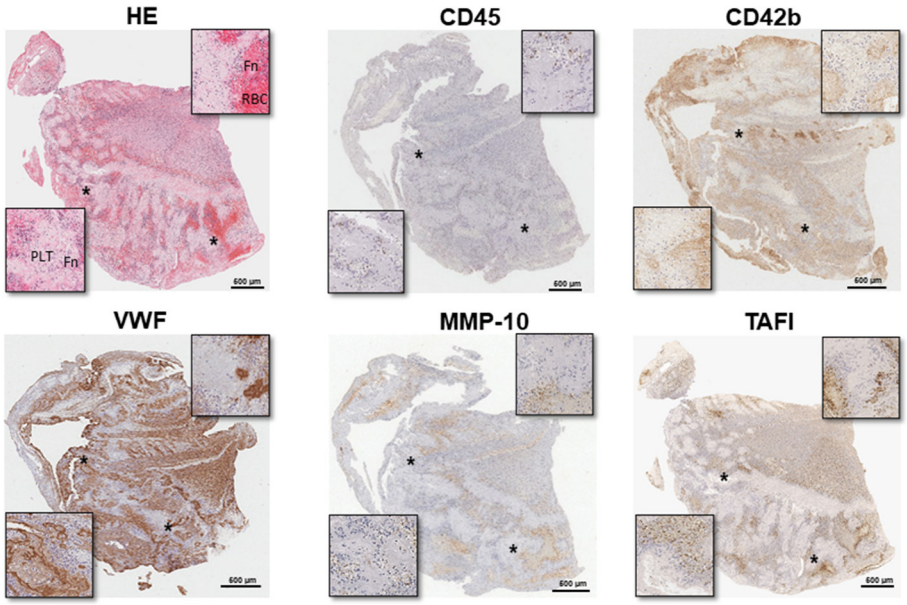

FIGURE 2 | Location and distribution of different cellular and hemostatic parameters in retrieved stroke thrombi. Representative immunohistological images of consecutive thrombi sections stained with hematoxylin & eosin (H&E), allowing the identification of fibrin/platelet aggregates (pink), red blood cell (RCB) (red), and nucleated cells (dark blue); leukocytes (CD45), von Willebrand Factor (VWF), platelets (CD42b), matrix metalloproteinase-10 (MMP-10), and thrombin-activatable fibrinolysis inhibitor (TAFI), stained in brown. Magnification images of selected areas (*). Scale = 300 μm. Fn, fibrin; PLT, platelets; RBC, red blood cells.

TABLE 2 | Quantification of hemostatic parameters in retrieved stroke thrombi.

Thrombi content	% of stained Area
RBC	12.8 (9.3–29.1)
Fibrin	16.2 (5.4–41)
Platelets (CD42b+)	15.0 (4.6–26.7)
VWF	11.0 (6.3–17.4)
TAFI	2.1 (0.9–3.8)
MMP-10	2.9 (0.15–8.1)
Thrombi content	**No. of cells/mm^2**
Leukocytes (CD45+)	308.9 (216.3–513.3)
Macrophages (CD68+)	98.7 (44.9–264.1)
T Lymphocytes (CD3+)	32.9 (19.5–63.9)

Data are presented as median (IQR 25–75).
RCB, red blood cells; VWF, von Willebrand Factor; TAFI, thrombin-activatable fibrinolysis inhibitor; MMP-10, matrix metalloproteinase-10.

TABLE 3 | Correlations between cell types and proteins content in stroke thrombi.

	Fibrin	VWF	TAFI	MMP-10
RBC (MSB)	$r = 0.38$*	$r = 0.15$	$r = -0.22$	$r = -0.14$
Platelets (CD42b+)	$r = -0.36$*	$r = 0.32$*	$r = -0.01$	$r = -0.012$
Leukocytes (CD45+)	$r = -0.09$	$r = 0.37$*	$r = -0.18$	$r = -0.14$
Macrophages (CD68+)	$r = -0.18$	$r = -0.05$	$r = -0.05$	$r = -0.03$
T Lymphocytes (CD3+)	$r = -0.25$	$r = -0.10$	$r = -0.02$	$r = -0.02$
Fibrin (MSB)				
VWF	$r = -0.22$			
TAFI	$r = -0.24$	$r = -0.16$		
MMP-10	$r = -0.13$	$r = -0.30$*	$r = 0.41$**	

RCB, red blood cells; platelets; VWF, von Willebrand Factor; TAFI, thrombin-activatable fibrinolysis inhibitor; MMP-10, matrix metalloproteinase-10; MSB, martius scarlet blue.
*$p < 0.05$; **$p < 0.01$.*

Association of Thrombi Components With Clinical Outcomes

We further analyzed the association of thrombi components with clinical data. We found that the pharmacological intervention with tPA was associated with higher thrombi platelets content in univariate [median (IQR): 17.0% (10.1–29.4) vs 5.4% (1.5–14.8), $p < 0.01$, **Figure 5A**], and multivariate analysis [OR 1.23 (1.03–1.48), $p < 0.05$] after adjustment for confounding factors (baseline mRS).

None of the analyzed thrombus components were associated with complete recanalization after endovascular procedure, but the number of patients without recanalization was small ($n = 8$). Nevertheless, higher frequency of recanalization after the first pass of the device was associated to reduced macrophage content in thrombi [48.9 macrophages/mm^2 (29.0–173.0) 1st pass vs. 189.2 macrophages/mm^2 (74.3–305.6) more than first pass, $p = 0.04$] and remained associated after multivariate analysis by age and sex [OR (95% CI): 0.44 (0.20–0.97), $p < 0.05$]. Other studied components (platelets, leukocytes, T lymphocytes, fibrin, RBCs, VWF, TAFI, or MMP10) were not associated with recanalization or device passes.

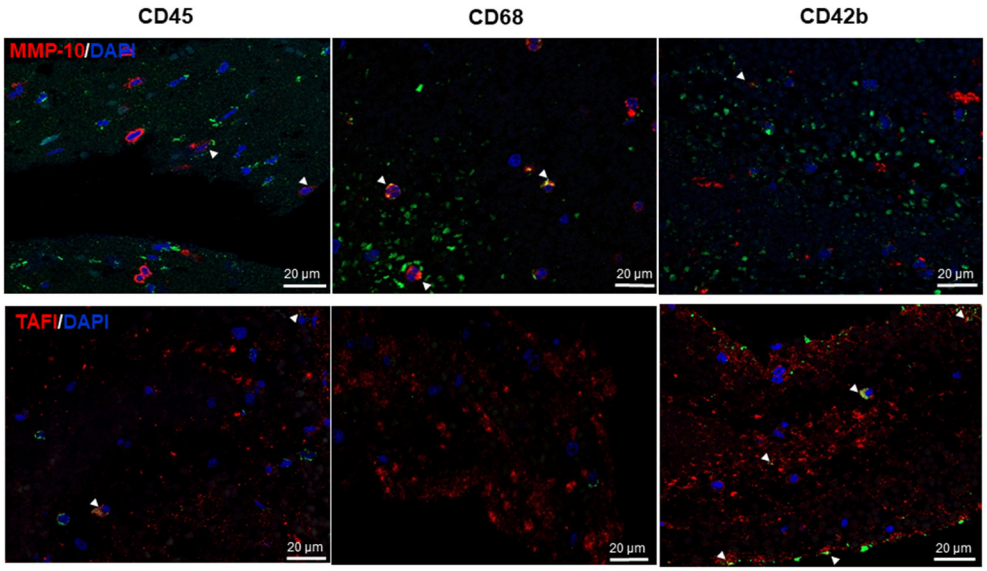

FIGURE 3 | Matrix metalloproteinase-10 (MMP-10) and thrombin-activatable fibrinolysis inhibitor (TAFI) colocalize with inflammatory cells and platelets in thrombi. Double immunofluorescence for MMP-10 (top, red) and TAFI (bottom, red) and leukocytes (CD45, left), macrophages CD68 (middle), and platelets CD42b (right, green); cell nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Arrow heads point to double positive cells for MMP-10 (upper panels) and TAFI (lower panels) and the specified antigens (yellow). Scale = 20 µm.

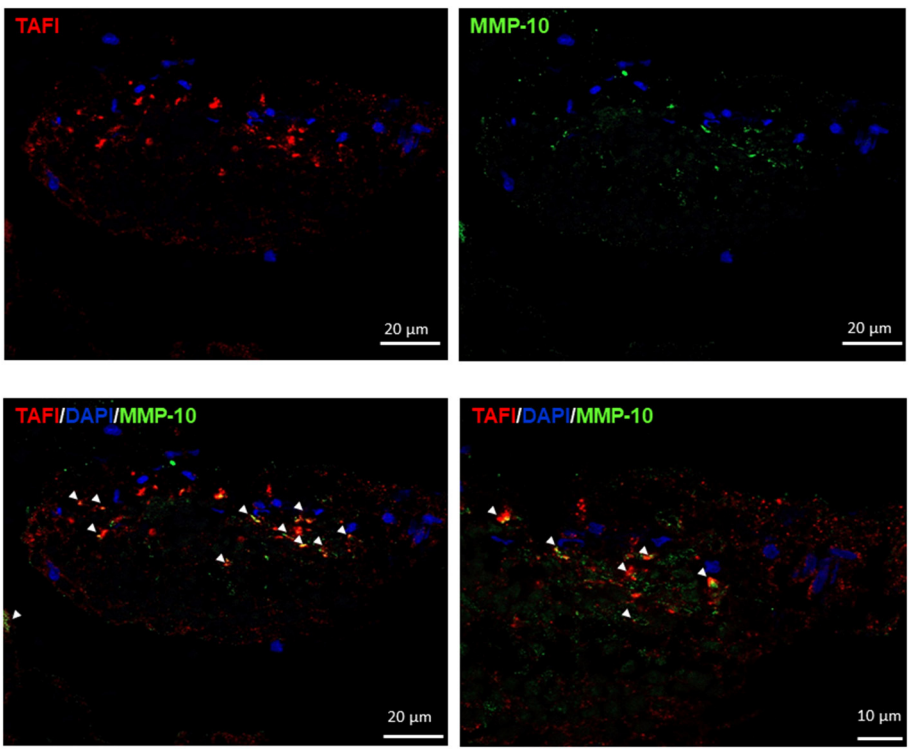

FIGURE 4 | Matrix metalloproteinase-10 (MMP-10) and thrombin-activatable fibrinolysis inhibitor (TAFI) colocalization in thrombi. Immunofluorescence for TAFI (red), MMP-10 (green), and 4′,6-diamidino-2-phenylindole (DAPI) (blue). Arrow heads point to double positive cells for MMP-10 and TAFI. Scale = 20 and 10 µm.

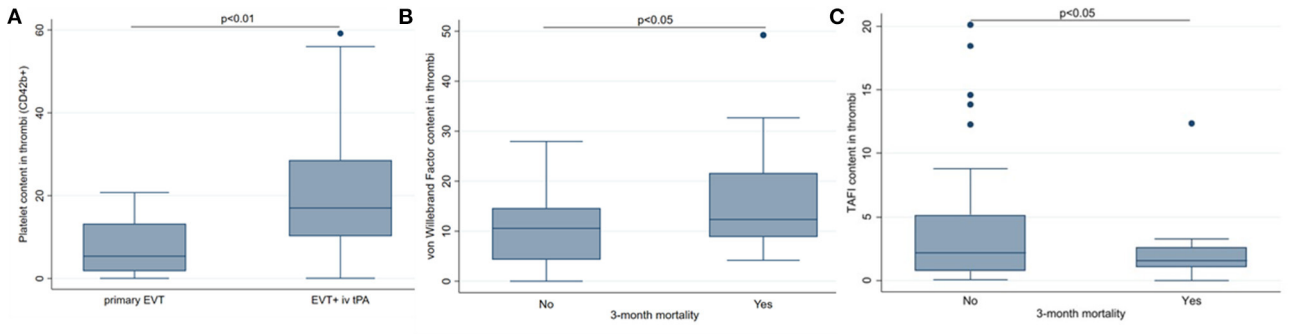

FIGURE 5 | Association of thrombi components with clinical outcomes. **(A)** tissue plasminogen activator (tPA) treatment was associated with higher platelets (CD42b) content. **(B)** Patients who died within 3 months had higher von Willebrand Factor. **(C)** Patients who died within 3 months had lower thrombin-activatable fibrinolysis inhibitor (TAFI) content thrombi. EVT, endovascular treatment. Median (%) and interquartile range (IQR) 25–75, $p < 0.05$ and $p < 0.01$ using Chi-square or Fisher's exact test.

No significant association between functional independence (FI) 3 months after stroke and content in thrombus for any of the studied thrombi components was observed (data not shown). Regarding 3-month mortality, patients who died within 3 months had higher VWF staining in thrombi [12.3% (8.9–21.7) vs. 10.6% (4.3–14.6), $p < 0.05$, **Figure 5B**]. Multivariate analysis adjusting for confounding factors (age and SBP) showed that thrombus VWF remained statistically significantly associated with mortality [OR (95% CI): 4.5 (1.2–17.1), $p = 0.029$]. In contrast, the amount of TAFI in thrombi was associated with lower mortality [1.5% (1.0–2.6) vs. 2.2% (0.7–5.1), **Figure 5C**] even after adjustment for age, glucose, and stroke severity [OR (95% CI): 0.59 (0.36–0.98), $p = 0.043$].

Moreover, a higher presence of leukocytes in thrombus was observed in those patients who died 3 months after stroke [444.7 (273.7–634.8) vs. 294.8 (191.8–439.0) leukocytes/mm^2, $p < 0.05$], and it remained significant after adjustment by stroke severity and age [OR (95% CI): 4.98 (1.01-24.58), $p < 0.05$]. No association of 3-month mortality with other components of thrombus was observed.

Stroke etiological subtypes according to TOAST criteria and hemorrhagic transformation were also assessed in our cohort, and the associations with thrombi components were evaluated, but no association was found.

Association of Circulating Hemostatic Parameters and Clinical Outcomes

When evaluating circulating levels of VWFAc, MMP-10, and TAFIa, no correlation with their thrombus content was found (**Table 4**) and only an association between VWFAc and thrombus lymphocytes was observed ($r = 0.44$, $p < 0.01$). Higher levels of circulating VWFAc were found in patients treated with tPA [60.3% (40.4–140.2) vs. 41.1% (25.3–50.2), $p < 0.03$] and in patients with worse clinical stroke severity by NIHSS score (ANOVA linear trend $p < 0.001$) with median values of NIHSS 0–7 [20% (15.6–35.3)], NIHSS 7–14 [48% (40.4–53.7)], and NIHSS >14 [57.5% (36.8–126.1)].

As previously described, greater plasma levels of MMP-10 were associated with a decline in the glomerular filtration rate ($r = -0.35$, $p < 0.05$), increased C-reactive protein (CRP, $r = 0.37$, $p < 0.05$), and smoking habit [996 pg/ml [472–1150) vs. 359 pg/ml (264–620), $p < 0.01$]. Furthermore, we observed higher plasma levels of MMP-10 in patients with functional dependence [476 pg/ml (336–716) vs. 307 pg/ml (250–620), $p = 0.026$], which remained significant after adjustment for stroke severity and glucose [OR 5.13 (1.42–18.50); $p = 0.013$].

Finally, circulating TAFIa was associated with circulating platelets ($r = 31$, $p < 0.05$), and a trend between blood and thrombi platelets ($r = 0.31$, $p = 0.061$) was also observed (**Table 4**).

Neither of the studied circulating parameters (VWFAc, MMP10, and TAFIa) were associated with mortality after ischemic stroke in our cohort nor with stroke TOAST subtypes (data not shown).

DISCUSSION

In this study, we demonstrate the presence of MMP-10 and TAFI in all thrombi retrieved from LVO stroke patients at the interface between RBC and platelet-rich areas, matching leukocytes. Thrombus MMP-10 and TAFI content correlate independently of confounding factors, the local TAFI expression being significantly lower in patients who died within 3 months after stroke onset. Additionally, we show that thrombus MMP-10 inversely correlates with VWF content, which is also associated with 3-month mortality. Interestingly, the presence of platelets in the thrombus is associated with thrombolysis treatment as well as with thrombus VWF. Finally, plasma TAFI activity is associated with blood and thrombus platelets, whereas plasma MMP-10 is related to cardiovascular risk factors and 3-month functional dependence. Taken together, *in situ* analysis of different hemostatic and proteolytic parameters has prognostic implications in IS patients. These findings will help to understand thrombus stability and the response to IS therapies, leading to the development of individualized treatment strategies

TABLE 4 | Correlations between elements in stroke thrombi and circulating parameters.

Plasma / *Thrombi*	VWF	TAFIa	MMP10	Platelets	Neutrophils	Lymphocytes
RBC (MSB)	$r = 0.17$	$r = 0.12$	$r = -0.10$	$r = -0.16$	$r = -0.09$	$r = 0.05$
Platelets (CD42b+)	$r = 0.10$	$r = -0.07$	$r = 0.13$	$r = 0.31$	$r = 0.09$	$r = 0.30$
Leukocytes (CD45+)	$r = -0.01$	$r = 0.04$	$r = 0.20$	$r = -0.21$	$r = -0.15$	$r = 0.13$
Macrophages (CD68+)	$r = 0.05$	$r = -0.05$	$r = -0.03$	$r = 0.20$	$r = 0.06$	$r = -0.04$
T Lymphocytes (CD3+)	$r = 0.44$**	$r = 0.15$	$r = 0.23$	$r = -0.06$	$r = -0.09$	$r = 0.05$
Fibrin (MSB)	$r = -0.11$	$r = -0.00$	$r = -0.05$	$r = -0.14$	$r = -0.04$	$r = -0.23$
VWF	$r = 0.19$	$r = -0.07$	$r = -0.12$	$r = 0.02$	$r = -0.15$	$r = 0.24$
TAFI	$r = 0.01$	$r = -0.03$	$r = -0.09$	$r = -0.08$	$r = -0.02$	$r = 0.28$
MMP−10	$r = -0.10$	$r = -0.21$	$r = -0.10$	$r = -0.12$	$r = 0.29$	$r = -0.23$

RCB, red blood cells; platelets; VWF, von Willebrand Factor; TAFI, thrombin-activatable fibrinolysis inhibitor; MMP-10, matrix metalloproteinase-10; MSB, martius scarlet blue.
***$p < 0.01$.*

based on clot composition, which ultimately will improve patient outcome.

TAFI is a metallocarboxypeptidase activated by thrombin/thrombomodulin and plasmin that removes C-terminal lysine residues from partially degraded fibrin, preventing t-PA-plasminogen activation and inhibiting fibrinolysis. Previous reports showed the role of TAFI in the stabilization of newly formed fibrin clots (25). It was proposed that thrombin-induced activation of TAFI render newly formed fibrin clots more resistant to plasmin degradation (26). *In vivo* evidence for the role of TAFI in fibrinolysis was obtained in experimental venous and arterial thrombosis models using TAFI inhibitors (27–30). Decreased TAFI activity in rodent models of transient middle cerebral artery occlusion treated with TAFI inhibitor resulted in signs of lower microvascular thrombosis such as reduced fibrin deposition, regardless of infarct volume (29, 30). However, data from TAFI knockout mice indicated that TAFI deficiency did not have a significant impact on the rate of thrombus formation in arterial and venous thrombosis models (26, 31). Beyond fibrinolysis, TAFI also plays a role in inflammatory conditions, processing C-terminal arginine or lysine from bradykinin, complement factors C5a and C3a, etc., leading to a reduced inflammatory/immune response (32). In this regard, our group reported that TAFI deficiency increased brain damage and circulating microvesicles in IS model under thrombolysis, suggesting a higher inflammatory status in these mice (33). In line with these data, this study reports a significant association of thrombus TAFI with lower mortality, suggesting that TAFI could be implicated in IS at various levels, linking coagulation/fibrinolysis and the inflammatory/immune systems.

Furthermore, we previously demonstrated that MMP-10 cleaves TAFI, preventing its activation and enhancing tPA-induced fibrinolysis *in vitro* and in experimental models of thrombosis (17). In this study, we first identified TAFI and MMP-10 in human thrombi sections. Both proteins were localized in the same areas associated with leukocytes, and their stainings even colocalized in specific points of the thrombus surface, suggesting that the processing of TAFI by MMP-10 could be operational locally due to their proximity. Moreover, the strong correlation between both reinforces that their coexpression, at the surface of the thrombus, might favor TAFI inactivation by MMP-10 promoting thrombus lysis.

Interestingly, an inverse linear correlation was also observed between MMP-10 and VWF content, the latter previously associated with platelet-rich clots, dense fibrin structures, and poor revascularization outcome (6, 34). Our data suggest that higher expression of MMP-10 in thrombi might be associated with more effective fibrin lysis, lower VWF-fibrin structures, and better recanalization-related outcome. Moreover, we have also demonstrated an association between higher thrombus content of VWF and leukocytes with 3-month mortality in multivariate analysis. VWF is a large, multimeric glycoprotein that is crucial for normal hemostasis due to its role in the stable platelet plug formation at sites of vascular injury. Not surprisingly, different studies have identified VWF as an important constituent of stroke thrombi with a direct impact on thrombolysis (5, 6).

Next, we studied VWFAc, TAFIa, and MMP-10 in blood and their expression in thrombi. No significant correlation was found between circulating levels of studied proteins and their thrombus content, suggesting a different role of VWF, TAFI, and MMP-10 in circulation and locally, where they might be involved in thrombus formation and/or on cell-dependent thrombolysis. For instance, systemic VWFAc was associated with thrombus lymphocytes. The important role of immune cells on stroke progression is well established, likewise immune cells interact with molecules involved in platelet signaling, such as VWF, contributing to thrombus formation (35).

Furthermore, plasma TAFIa was correlated with platelets in the blood and thrombus. An association between higher plasma TAFI levels and the occurrence of IS was reported in a number of clinical studies (32–34, 36–38). It has been described that TAFI secreted upon platelet activation (39) might contribute to its variations in plasma. Our results support these data demonstrating a correlation between plasma TAFI activity and circulating platelets and locally showing their colocalization in thrombi. Even if TAFI and platelet content in thrombectomies did not correlate, their association within thrombi might suggest a role of locally secreted platelet-derived TAFI in the systemic crosstalk between coagulation and fibrinolysis, protecting thrombus against lysis.

In addition, higher circulating VWFAc were found in patients treated with tPA and in those with greater stroke severity, supporting previous studies showing that increased VWF levels were associated with elevated baseline stroke severity (by the NIHSS score) (40, 41). Moreover, elevated VWF antigen concentrations immediately after and 24 h postthrombolysis have also been associated to poor functional outcomes 3 months after ischemia (41), and tPA has been shown as potentially implicated with brain microvascular endothelial injury during postischemia in experimental models (42). Thus, it could be hypothesized that the increased levels of VWFAc after thrombolysis could be due to increased VWF antigen following endothelial damage caused by the thrombolytic agent.

Moreover, patients treated with tPA who underwent thrombectomy presented higher platelet fraction in thrombi. This fact has not been previously described but has been suggested in some studies (43). A paradoxical platelet activation has been reported secondary to fibrinolysis (44) as responsible for delayed thrombosis in some patients with tPA-resistant thrombi causing reocclusion and rethrombosis (45). Other additional mechanisms have been implicated in a higher platelets content in stroke thrombi of patients treated with tPA. An outer shell composed of platelets, extracellular DNA, and tight cross-linking of fibrin that confers resistance to fibrinolysis has been described in acute IS thrombi (46) and could support the higher platelet percentage found in thrombus of tPA-treated patients.

On the other hand, thrombus composition has been shown to be related to interventional times and efficacy of mechanical thrombectomy treatment for LVO stroke (9, 47). In this line, in our cohort, a higher macrophage presence in the thrombus was associated with lower frequencies of recanalization with the first pass of the device. There are previous data reporting that fibrin-organized thrombi need longer recanalization times (47) or a higher number of maneuvers during mechanical thrombectomy (9); thus, further studies are needed to analyze more deeply this association.

Finally, in this study, we observed an association of plasma MMP-10 levels with cardiovascular risk factors and 3-month functional dependence. In line with these results, we had previously reported that higher serum MMP-10 levels were associated with inflammatory markers and the presence of atherosclerotic plaques in asymptomatic subjects (48). Moreover, in IS patients, serum proMMP-10 concentration was independently associated with higher infarct volume, severe brain edema, neurological deterioration, and poor functional outcome at 3 months (49). Altogether, this study confirms that plasma MMP-10 might play a key role in cardiovascular diseases and therefore could be a potential biomarker for LVO stroke patients.

There are some limitations to this report that are worth considering. First, the modest sample size and the retrospective analysis of prospectively collected data are important methodological shortcomings. Second, only thrombi from those LVO patients in whom the thrombus could be partially or totally retrieved were available for study, whereas not recovered clots or those clots dissolved after tPA treatment could not be studied, and this impedes evaluation of tPA susceptibility and thrombectomy resistance. Third, the observational study design and the use of correlations to evaluate the association between variables do not allow to establish causal relationship and is only a rough approach to probably complex interrelationships between components in thrombi.

CONCLUSION

Histological structure of thrombi is crucial to better understand their pathogenesis, properties, and clinical management in IS. The present findings suggest that the histological composition and distribution of different thrombi hemostatic components have prognostic implications, and it would most likely determine the clinical impact of pharmacological and mechanical strategies in order to guide personalized therapies for stroke 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 committee of the Navarra Government (84/2018). The patients or their legally authorized representative provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JM-E participated in the experimental work, analysis of data, and edited and reviewed the manuscript. MN-O participated in the design of the project, experimental work and wrote, reviewed, and edited the manuscript. RM participated in the design of the project, samples collection, and reviewed the manuscript. GZ, RL, MM, JO-A, and JAP participated in the design of the project and reviewed the manuscript. CR and JO-A were in charge of the whole project design, supervised the work, and wrote, edited, and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Virani SS, Alonso A, Benjamin EJ, Bittencourt MS, Callaway CW, Carson AP, et al. Heart disease and stroke statistics-2020 update: a report from the american heart association. *Circulation.* (2020) 141:e139–e596. doi: 10.1161/CIR.0000000000000746
2. Luengo-Fernandez R, Violato M, Candio P, Leal J. Economic burden of stroke across Europe: a population-based cost analysis. *Eur Stroke J.* (2020) 5:17–25. doi: 10.1177/2396987319883160
3. Wafa HA, Wolfe CDA, Emmett E, Roth GA, Johnson CO, Wang Y. Burden of stroke in Europe: thirty-Year projections of incidence, prevalence, deaths, and disability-Adjusted life years. *Stroke.* (2020) 51:2418–27. doi: 10.1161/STROKEAHA.120.029606
4. Powers WJ, Rabinstein AA, Ackerson T, Adeoye OM, Bambakidis NC, Becker K, et al. Guidelines for the early management of patients with acute ischemic stroke: 2019 update to the 2018 guidelines for the early management of acute ischemic stroke a guideline for healthcare professionals from the American Heart Association/American stroke a. *Stroke.* (2019) 50:344–418. doi: 10.1161/STR.0000000000000211
5. De Meyer SF, Andersson T, Baxter B, Bendszus M, Brouwer P, Brinjikji W, et al. Analyses of thrombi in acute ischemic stroke: a consensus statement on current knowledge and future directions. *Int J Stroke.* (2017) 12:606–14. doi: 10.1177/1747493017709671
6. Douglas A, Fitzgerald S, Mereuta OM, Rossi R, O'Leary S, Pandit A, et al. Platelet-rich emboli are associated with von Willebrand factor levels and have poorer revascularization outcomes. *J Neurointerv Surg.* (2019) 12:557–62. doi: 10.1136/neurintsurg-2019-015410
7. Boeckh-Behrens T, Kleine JF, Zimmer C, Neff F, Scheipl F, Pelisek J, et al. Thrombus histology suggests cardioembolic cause in cryptogenic stroke. *Stroke.* (2016) 47:1864–71. doi: 10.1161/STROKEAHA.116.013105
8. Sporns PB, Hanning U, Schwindt W, Velasco A, Minnerup J, Zoubi T, et al. Ischemic stroke: what does the histological composition tell us about the origin of the thrombus? *Stroke.* (2017) 48:2206–10. doi: 10.1161/STROKEAHA.117.016590
9. Maekawa K, Shibata M, Nakajima H, Mizutani A, Kitano Y, Seguchi M, et al. Erythrocyte-rich thrombus is associated with reduced number of maneuvers and procedure time in patients with acute ischemic stroke undergoing mechanical thrombectomy. *Cerebrovasc Dis Extra.* (2018) 8:39–49. doi: 10.1159/000486042
10. Dargazanli C, Rigau V, Eker O, Bareiro CR, Machi P, Gascou G, et al. High cD3+ cells in intracranial thrombi represent a biomarker of atherothrombotic stroke. *PLoS ONE.* (2016) 11:e154945. doi: 10.1371/journal.pone.0154945
11. Develter J, Booth NA, Declerck PJ, Gils A. Bispecific targeting of thrombin activatable fibrinolysis inhibitor and plasminogen activator inhibitor-1 by a heterodimer diabody. *J Thromb Haemost.* (2008) 6:1884–91. doi: 10.1111/j.1538-7836.2008.03137.x
12. Wyseure T, Rubio M, Denorme F, De Lizarrondo SM, Peeters M, Gils A, et al. Innovative thrombolytic strategy using a heterodimer diabody against tAFI and pAI-1 in mouse models of thrombosis and stroke. *Blood.* (2015) 125:1325–32. doi: 10.1182/blood-2014-07-588319
13. Martinez de Lizarrondo S, Gakuba C, Herbig BA, Repessé Y, Ali C, Denis C V, et al. Potent thrombolytic effect of n-Acetylcysteine on arterial thrombi. *Circulation.* (2017) 136:646–60. doi: 10.1161/CIRCULATIONAHA.117.027290
14. Denorme F, Langhauser F, Desender L, Vandenbulcke A, Rottensteiner H, Plaimauer B, et al. ADAMTS13-mediated thrombolysis of t-PA-resistant occlusions in ischemic stroke in mice. *Blood.* (2016) 127:2337–45. doi: 10.1182/blood-2015-08-662650
15. Lijnen HR. Matrix metalloproteinases and cellular fibrinolytic activity. *Biochemistry.* (2002) 67:92–8. doi: 10.1023/A:1013908332232
16. Mühl D, Ghosh S, Uzuelli JA, Lantos J, Tanus-Santos JE. Increases in circulating matrix metalloproteinase-9 levels following fibrinolysis for acute pulmonary embolism. *Thromb Res.* (2010) 125:549–53. doi: 10.1016/j.thromres.2010.02.015
17. Orbe J, Barrenetxe J, Rodriguez JA, Vivien D, Orset C, Parks WC, et al. Matrix metalloproteinase-10 effectively reduces infarct size in experimental stroke by enhancing fibrinolysis via a thrombin-activatable fibrinolysis inhibitor-mediated mechanism. *Circulation.* (2011) 124:2909–19. doi: 10.1161/CIRCULATIONAHA.111.047100
18. Roncal C, Martinez de Lizarrondo S, Salicio A, Chevilley A, Rodriguez JA, Rosell A, et al. New thrombolytic strategy providing neuroprotection in experimental ischemic stroke: mMP10 alone or in combination with tissue-type plasminogen activator. *Cardiovasc Res.* (2017) 113:1219–29. doi: 10.1093/cvr/cvx069
19. Powers WJ, Derdeyn CP, Biller J, Coffey CS, Hoh BL, Jauch EC, et al. 2015 American Heart Association/American Stroke Association Focused Update of the 2013. Guidelines for the Early Management of Patients With Acute Ischemic Stroke Regarding Endovascular Treatment: A Guideline for Healthcare Professionals From the American Heart Association/American Stroke Association. *Stroke.* (2015) 46:3020–35. doi: 10.1161/STR.0000000000000074
20. Love BB, Bendixen BH. Classification of subtype of acute ischemic stroke definitions for use in a multicenter clinical trial. *Stroke.* (1993) 24:35–41. doi: 10.1161/01.STR.24.1.35
21. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods.* (2012) 9:676–82. doi: 10.1038/nmeth.2019
22. Hacke W, Kaste M, Fieschi C, Toni D, Lesaffre E, Von Kummer R, et al. Intravenous thrombolysis with recombinant tissue plasminogen activator for acute hemispheric stroke: the Europe an cooperative acute stroke study (ECASS). *JAMA.* (1995) 274:1017–25. doi: 10.1001/jama.274.13.1017
23. Staessens S, Denorme F, François O, Desender L, Dewaele T, Vanacker P, et al. Structural analysis of ischemic stroke thrombi: histological indications for therapy resistance. *Haematologica.* (2020) 105:498–507. doi: 10.3324/haematol.2019.219881
24. Staessens S, Fitzgerald S, Andersson T, Clarençon F, Denorme F, Gounis MJ, et al. Histological stroke clot analysis after thrombectomy: technical aspects and recommendations. *Int J Stroke.* (2020) 15:467–76. doi: 10.1177/1747493019884527
25. Broze GJJ, Higuchi DA. Coagulation-dependent inhibition of fibrinolysis: role of carboxypeptidase-U and the premature lysis of clots from hemophilic plasma. *Blood.* (1996) 88:3815–23. doi: 10.1182/blood.V88.10.3815.bloodjournal88103815
26. Nagashima M, Yin Z-F, Zhao L, White K, Zhu Y, Lasky N, et al. Thrombin-activatable fibrinolysis inhibitor (TAFI) deficiency is compatible with murine life. *J Clin Invest.* (2002) 109:101–10. doi: 10.1172/JCI0212119
27. Minnema MC, Friederich PW, Levi M, Von Dem Borne PAK, Mosnier LO, Meijers JCM, et al. Enhancement of rabbit jugular vein thrombolysis by neutralization of factor XI. *In vivo* evidence for a role of factor XI as an anti-fibrinolytic factor. *J Clin Invest.* (1998) 101:10–4. doi: 10.1172/JCI781
28. Vercauteren E, Emmerechts J, Peeters M, Hoylaerts MF, Declerck PJ, Gils A. Evaluation of the profibrinolytic properties of an anti-TAFI monoclonal antibody in a mouse thromboembolism model. *Blood.* (2011) 117:4615–22. doi: 10.1182/blood-2010-08-303677
29. Mertens JC, Boisseau W, Leenaerts D, Di Meglio L, Loyau S, Lambeir AM, et al. Selective inhibition of carboxypeptidase U may reduce microvascular thrombosis in rat experimental stroke. *J Thromb Haemost.* (2020) 18:3325–35. doi: 10.1111/jth.15071
30. Denorme F, Wyseure T, Peeters M, Vandeputte N, Gils A, Deckmyn H, et al. Inhibition of thrombin-Activatable fibrinolysis inhibitor and plasminogen activator inhibitor-1 reduces ischemic brain damage in mice. *Stroke.* (2016) 47:2419–22. doi: 10.1161/STROKEAHA.116.014091
31. Kraft P, Schwarz T, Meijers JCM, Stoll G, Kleinschnitz C. Thrombin-activatable fibrinolysis inhibitor (TAFI) deficient mice are susceptible to intracerebral thrombosis and ischemic stroke. *PLoS ONE.* (2010) 5:e11658. doi: 10.1371/journal.pone.0011658
32. Plug T, Meijers JCM. Structure-function relationships in thrombin-activatable fibrinolysis inhibitor. *J Thromb Haemost.* (2016) 14:633–44. doi: 10.1111/jth.13261
33. Orbe J, Alexandru N, Roncal C, Belzunce M, Bibiot P, Rodriguez JA, et al. Lack of tAFI increases brain damage and microparticle generation after thrombolytic therapy in ischemic stroke. *Thromb Res.* (2015) 136:445–50. doi: 10.1016/j.thromres.2015.06.010
34. Leebeek FWG, Van Goor MPJ, Guimaraes AHC, Brouwers GJ, De Maat MPM, Dippel DWJ, et al. High functional levels of thrombin-activatable fibrinolysis

inhibitor are associated with an increased risk of first ischemic stroke. *J Thromb Haemost.* (2005) 3:2211–8. doi: 10.1111/j.1538-7836.2005.01484.x
35. Nieswandt B, Kleinschnitz C, Stoll G. Ischaemic stroke: a thrombo-inflammatory disease? *J Physiol.* (2011) 589:4115–23. doi: 10.1113/jphysiol.2011.212886
36. Santamaría A, Oliver A, Borrell M, Mateo J, Belvis R, Martí-Fábregas J, et al. Risk of ischemic stroke associated with functional thrombin-activatable fibrinolysis inhibitor plasma levels. *Stroke.* (2003) 34:2387–91. doi: 10.1161/01.STR.0000088642.07691.15
37. Montaner J, Ribó M, Monasterio J, Molina CA, Alvarez-Sabín J. Thrombin-activable fibrinolysis inhibitor levels in the acute phase of ischemic stroke. *Stroke.* (2003) 34:1038–40. doi: 10.1161/01.STR.0000063139.06585.45
38. Rooth E, Wallen H, Antovic A, Von Arbin M, Kaponides G, Wahlgren N, et al. Thrombin activatable fibrinolysis inhibitor and its relationship to fibrinolysis and inflammation during the acute and convalescent phase of ischemic stroke. *Blood Coagul Fibrinolysis.* (2007) 18:365–70. doi: 10.1097/MBC.0b013e3281139c34
39. Mosnier LO, Buijtenhuijs P, Marx PF, Meijers JCM, Bouma BN. Identification of thrombin activatable fibrinolysis inhibitor (TAFI) in human platelets. *Blood.* (2003) 101:4844–6. doi: 10.1182/blood-2002-09-2944
40. Prochazka V, Jonszta T, Czerny D, Krajca J, Roubec M, Macak J, et al. The role of von willebrand factor, aDAMTS13, and cerebral artery thrombus composition in patient outcome following mechanical thrombectomy for acute ischemic stroke. *Med Sci Monit.* (2018) 24:3929–45. doi: 10.12659/MSM.908441
41. Tóth NK, Székely EG, Czuriga-Kovács KR, Sarkady F, Nagy O, Lánczi LI, et al. Elevated factor VIII and von Willebrand factor levels predict unfavorable outcome in stroke patients treated with intravenous thrombolysis. *Front Neurol.* (2018) 8:721. doi: 10.3389/fneur.2017.00721
42. Gong P, Li M, Zou C, Tian Q, Xu Z. Tissue plasminogen activator causes brain microvascular endothelial cell injury after oxygen glucose deprivation by inhibiting sonic hedgehog signaling. *Neurochem Res.* (2019) 44:441–9. doi: 10.1007/s11064-018-2697-2
43. Heo JH, Nam HS, Kim YD, Choi JK, Kim BM, Kim DJ, et al. Pathophysiologic and therapeutic perspectives based on thrombus histology in stroke. *J Stroke.* (2020) 22:64–75. doi: 10.5853/jos.2019.03440
44. Coller BS. Platelets and thrombolytic therapy. *N Engl J Med.* (1990) 322:33–42. doi: 10.1056/NEJM199001043220107
45. Heo JH, Lee KY, Kim SH, Kim DI. Immediate reocclusion following a successful thrombolysis in acute stroke: a pilot study. *Neurology.* (2003) 60:1684–7. doi: 10.1212/01.WNL.0000063323.23493.98
46. Di Meglio L, Desilles JP, Ollivier V, Nomenjanahary MS, Di Meglio S, Deschildre C, et al. Acute ischemic stroke thrombi have an outer shell that impairs fibrinolysis. *Neurology.* (2019) 93:e1686–e98. doi: 10.1212/WNL.0000000000008395
47. Sporns PB, Hanning U, Schwindt W, Velasco A, Buerke B, Cnyrim C, et al. Ischemic stroke: histological thrombus composition and pre-Interventional cT attenuation are associated with intervention time and rate of secondary embolism. *Cerebrovasc Dis.* (2017) 44:344–50. doi: 10.1159/000481578
48. Orbe J, Montero I, Rodríguez JA, Beloqui O, Roncal C, Páramo JA. Independent association of matrix metalloproteinase-10, cardiovascular risk factors and subclinical atherosclerosis. *J Thromb Haemost.* (2007) 5:91–7. doi: 10.1111/j.1538-7836.2006.02276.x
49. Rodríguez JA, Sobrino T, Orbe J, Purroy A, Martínez-Vila E, Castillo J, et al. proMetalloproteinase-10 is associated with brain damage and clinical outcome in acute ischemic stroke. *J Thromb Haemost.* (2013) 11:1464–73. doi: 10.1111/jth.12312

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Prognostic Value of Various Hemostasis Parameters and Neurophysiological Examinations in Spontaneous Intracerebral Hemorrhage: The IRONHEART Study Protocol

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Rationale: Stroke is one of the leading causes of death in all developed countries. In Hungary, more than 10,000 patients die annually due to cerebrovascular diseases according to the WHO Mortality Database. Of these patients, 10–15 % suffer non-traumatic intracerebral hemorrhage (ICH). ICH results in a higher rate of mortality as compared to ischemic stroke and outcomes are difficult to predict. In the IRONHEART study, we aim to test various hemostasis parameters and clinical neurophysiological examinations in evaluating outcome in ICH.

Methods: In this prospective, observational study, we plan to enroll consecutive patients with non-traumatic spontaneous ICH admitted to a single Stroke Center (Department of Neurology, University of Debrecen, Hungary). The protocol of the IRONHEART study includes the investigation of detailed clinical, laboratory investigations, and various neurophysiological examinations. Stroke severity is quantified based on the National Institutes of Health Stroke Scale (NIHSS) on admission and day 7, 14, and 90 after the onset of stroke. Cranial CT is performed on admission, day 14, and 90 to estimate the ICH volume. Modified Rankin Scale (mRS) is used for evaluating the long-term outcome (90 days post-event). Blood is drawn immediately on admission for specific hemostasis tests. Digital and quantitative EEG techniques and motor evoked potential (MEP) are performed to evaluate the prognosis of cerebral hemorrhage on admission (within 24–48 h), immediately before discharge (∼10–14 days later), and 3 months after the event.

Outcomes: The following outcomes are investigated: primary outcomes: mortality by day 14 and day 90, secondary long-term outcome at 90 days post-event where mRS 0–2 is defined as favorable long-term outcome.

Discussion: If associations between outcomes and the investigated parameters (hemostasis and neurophysiological examinations) are confirmed, results might aid prognosis assessment in this subtype of stroke with particularly high mortality. Improving clinical grading systems on ICH severity and outcomes by including the investigated parameters could help to better guide the management of these patients in the future.

Keywords: intracerebral hemorrhage, outcome, clot lysis, motor evoked potential, quantitative electroencephalography

INTRODUCTION

Stroke is one of the leading causes of death in all developed countries (1). In Hungary, more than 10,000 patients die annually due to cerebrovascular diseases according to the WHO Mortality Database (2). Of these patients, 10–15 % suffer non-traumatic intracerebral hemorrhage (ICH), which has the highest case fatality among all strokes (3). Age over 80 years, infratentorial origin, intraventricular hemorrhage, hemorrhage volume $\geq$30 cm^3, Glasgow Coma Scale score under 5 are particularly poor prognostic factors (4). Although its mortality is higher than that of ischemic stroke, no similar therapeutic options are available. This might be explained by the fact that its etiology often remains unknown and outcomes in ICH are difficult to predict. In the IRONHEART study, detailed clinical, laboratory investigations, and the results of various neurophysiological examinations will be correlated with ICH stroke severity and clinical outcomes.

Despite the significant mortality of ICH and the considerable health care burden related to treatment and rehabilitation of ICH patients, management strategies have not improved significantly in the past decade. This is at least partly due to an existing knowledge gap of the pathophysiology of the intracerebral bleeding and mechanisms driving hematoma progression. In the IRONHEART study, we hypothesize that by learning more about the levels of hemostasis factors and the fibrinolytic potential in ICH patients and their relation to clinical outcomes we might better understand the underlying pathomechanism that could be the key for improved therapeutic approaches in the future. Neurophysiological examinations might equally have considerable potentials as prognostic tools in predicting changes in the extent of bleeding and brain edema. Here we hypothesize that identification of the above parameters employing neurophysiological examinations could be important because they could have a significant impact on the outcome of brain hemorrhage and might provide an early tool for the personalization of ICH treatment.

The main goals of the hemostasis substudy are the following: (1) to examine the levels of certain coagulation and fibrinolysis markers in ICH patients and correlate results with outcomes and cerebral hematoma volume (2) to introduce a new, modified global assay of clot formation and fibrinolysis that includes the effect of NETs and to test its potential clinical utility in ICH patients.

The main goals of the neurophysiological substudy are the following: (1) to find out whether quantitative EEG results correlate with the improvement or deterioration of cerebral functions by comparing data with clinical parameters (NIHSS score, modified Rankin Scale) and cranial CT findings (estimated hematoma and edema volume). (2) Early detection of epileptiform discharges or non-convulsive seizures to select patients who may benefit from early-initiated antiseizure drug treatment. Parallel with the quantitative EEG we will perform TMS examinations to evaluate the prognosis and the probable effectiveness of further rehabilitation. The main purpose is to look for a correlation between intracerebral blood volume, magnetic evoked potential, and functional outcome.

METHODS AND ANALYSIS

Patients

In this prospective, observational study, we plan to enroll consecutive patients with ICH admitted to a single Stroke Center (Department of Neurology, University of Debrecen, Hungary). Inclusion criteria: patients over 18 years with acute non-traumatic, spontaneous ICH verified with cranial CT, not meeting exclusion criteria. Exclusion criteria: the presence of cerebral aneurysm, arteriovenous malformation, malignancy, subdural and epidural hemorrhage, severe hepatic- or renal insufficiency, hemorrhagic diathesis. A detailed flowchart of examinations is depicted in **Figure 1**. ICH will be diagnosed with complex physical examination and non-contrast computerized tomography (NCCT) scan on arrival. If necessary, contrast-enhanced CT or CT-angiography is performed on admission to rule out secondary causes of ICH (e.g., aneurysm, cerebral tumors). Follow-up NCCT scans will be performed 14 $\pm$ 2 days and 3 months $\pm$ 7 days after the stroke onset. CT images will be analyzed simultaneously by 3 independent radiologists, and a detailed list of radiological data, such as location and volume of bleeding, extent of perihematomal edema, degree of midline shift, involvement of pyramidal tract, and presence of intraventricular or subarachnoidal component will be recorded (5). The volume of the IVH will be not measured. Manual CT volumetry will be performed by tracing the focal hyperdense (blood) the and the perifocal hypodense area surrounding the hemorrhage (edema) on each slice. Hypodensity attributable to microangiopathy will be omitted as far as possible by comparison with the contralateral hemisphere. Total lesion volume will be calculated by multiplying the traced area (ROI, cm^2) with the slice thickness and adding up the results (6–8). All tracings will be performed by one radiologist.

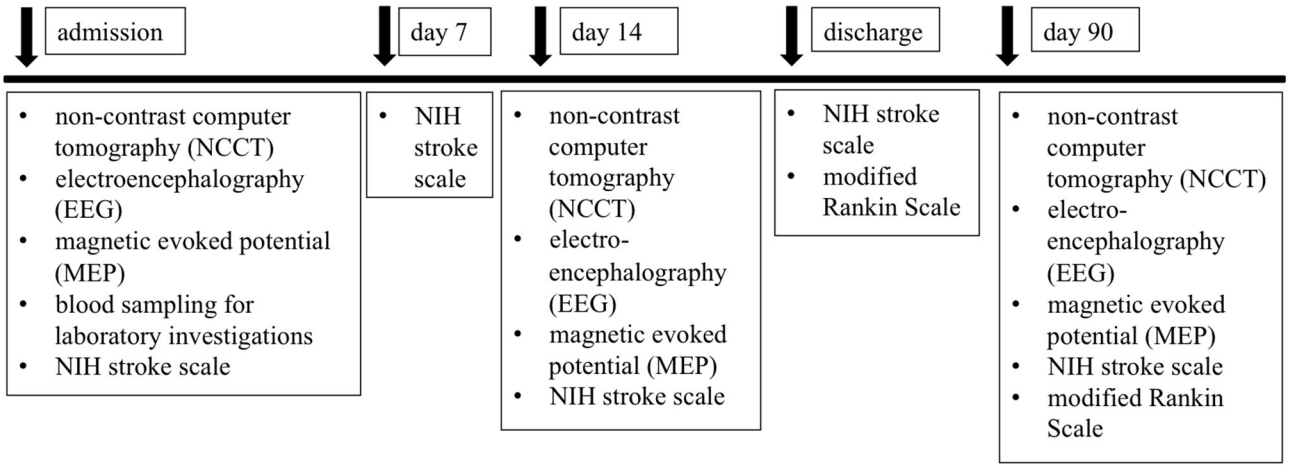

FIGURE 1 | Flow chart of examinations planned during the study.

The time of symptom onset, baseline characteristics (age, sex, BMI, cerebrovascular risk factors, history of cerebrovascular and cardiovascular diseases, previous medications) will be recorded on admission. National Institutes of Health Stroke Scale (NIHSS) will be used to quantify stroke severity on admission and day 7 after the onset of hemorrhage stroke (9). Modified Rankin Scale (mRS) will be used for evaluating long-term outcomes (90 days post-event) (10) performed by neurologists of the study including physical examination. Mortality by day 14 and day 90 will be assessed. All patients who die during the hospital stay will be autopsied and the cause of death will be assessed and determined by a pathologist. When autopsy is performed amyloid angiopathy and microbleeding will be examined. Cerebral microbleeds may help to predict the prognosis of ICH (11).

Outcomes

The following primary outcomes are defined: 1/ Mortality by day 14 and day 90 2/Long-term outcome at 90 days post-event: mRS 0–2 is defined as favorable long-term outcome.

Informed Consent

The study design was developed by the guiding principles of the Declaration of Helsinki and was approved by the Institutional Ethics Committee of the University of Debrecen and the Ethics Committee of the National Medical Research Council. All patients or their relatives are required to provide written informed consent to be enrolled in the study. A general consent form encompasses the consent for blood sampling, imaging studies, electrophysiological examinations, and autopsy also. All patient data will be treated anonymously.

Blood Sampling and Laboratory Measurements

Peripheral venous blood samples will be taken from all enrolled patients on admission (within 1 h of admittance). Routine laboratory tests (ions, glucose level, renal and liver function tests, high-sensitivity C-reactive protein measurement, complete blood count) will be carried out immediately after blood drawing by standard laboratory methods (Roche Diagnostics, Mannheim, Germany, and Sysmex Europe GmbH, Hamburg, Germany). For the examination of hemostasis tests, blood samples will be collected to vacutainer tubes containing 0.109 M sodium citrate (Becton Dickinson, Franklin Lane, NJ) and plasma samples will be processed immediately (centrifugation twice at 1,500 g, room temperature, 15 min). Screening tests of coagulation (prothrombin time, activated partial thromboplastin time, and thrombin time) will be carried out immediately using standard methods (BCS coagulometer, Siemens Healthcare Diagnostic Products, Marburg, Germany) Plasma samples will be labeled with a code and stored at −80 C until further analysis of specific hemostasis measurements. All measurements will be carried out by investigators blinded to clinical data. Quantitative D-dimer levels will be measured by a particle-enhanced, immuno-turbidimetric assay (Innovance D-dimer) on a BCS coagulometer according to the manufacturer's instructions (Siemens Healthcare Diagnostic Products, Marburg, Germany). The levels of von Willebrand factor (VWF) antigen, chromogenic factor VIII (FVIII) activity, α2-plasmin inhibitor (α2-PI) activity, and plasminogen activity will be measured by commercially available methods (Siemens Healthcare Diagnostic Products, Marburg, Germany). Fibrinogen levels will be analyzed according to the method of Clauss. Plasma levels of factor FXIII (FXIII) activity will be determined by ammonia release assay using a commercially available reagent kit (REA-chrom FXIII kit, Reanalker, Budapest, Hungary). PAI-1 activity and antigen levels will be measured using commercially available ELISA tests (Technozym PAI-1 Actibind ELISA and Technozym PAI-1 Antigen ELISA assays, respectively), according to the manufacturer's instructions. Thrombin generation assay will be performed using the Thrombinoscope CAT (Calibrated Automated Thrombogram, Maastricht, The Netherlands) assay according to the manufacturer's instructions (Diagnostica Stago, Asnières, France).

Neurophysiological Examinations

As a part of this study, we will perform digital and quantitative EEG and MEP examinations on all enrolled participants. Patients will be examined on 3 occasions with the above neurophysiological examinations within 24–48 h after admission, at 14 days ± 2 days and 3 months ± 7 days after the event. The EEG and MEP will be analyzed by clinical neurophysiologists participating in the study (KF and IF). EEGs will be recorded in the EEG laboratory of the Department of Neurology according to the International Federation of Clinical Neurophysiology guidelines using Micromed BrainQuick Plus System with surface Ag/AgCl electrodes. Each recording session will last for 40 min. There are no exclusion criteria for digital and quantitative EEG. Monophasic, single-pulse stimulation is used by Magstim 200 magnetic stimulator. Central motor conduction time and amplitudes will be recorded and analyzed. Patients would be selected after strict consideration of contraindications (e.g., metallic implantations, pacemaker are excluding factors) as described in safety regulations of MEP examination protocol. Recording electrodes will be placed over the abductor digiti minimi muscle of the upper limb, and on the anterior tibial muscle of the lower limb. The investigation is suitable to show the severity of corticospinal tract damage which may correlate with the prognosis and outcome of rehabilitation even in a hemiplegic patient (12).

Sample Size Calculations and Statistical Analysis

Sample size calculations were performed using the power/sample size calculator software of the Department of Biostatistics, Vanderbilt University. Based on our calculations using the Casagrande, Pike and Smith's method, assuming a 15% true difference between the compared groups based on primary and secondary outcomes, an estimated sample size of 87 patients was determined by setting the value of α to 0.05 and the statistical power to 0.80 (in case of at least 30% anticipated incidence of outcome event, at least 25 patients in each outcome group should be enrolled plus 6% additional subjects for attrition). Patient groups will be dichotomized according to mortality by day 14 and day 90, and to groups according to favorable (mRS 0–2) or unfavorable outcomes (mRS 3–6). According to previous records, this sample size can be reached in our center in a period of ∼4 years. For the statistical evaluation of the results, the Statistical Package for Social Sciences (SPSS, Version 26.0, Chicago, IL), and GraphPad Prism 8.0 (GraphPad Prism Inc., La Jolla, CA) software will be used. The normality of data will be studied using the Shapiro-Wilk test. Student's *t*-test or Mann–Whitney *U*-test will be performed for independent two-group analyses. In the case of paired data, paired *t*-test or Wilcoxon signed-rank test will be applied. ANOVA with Bonferroni *post-hoc* test or Kruskal–Wallis analysis with Dunn's *post-hoc* test will be used for multiple comparisons. Pearson's or Spearman's correlation will be used to determine the strength of association between continuous variables. Differences between categorical variables will be assessed by χ^2 test or by Fisher's exact where appropriate. In the case of hemostasis variables, optimal tests and threshold values will be chosen using receiver operating characteristic (ROC) curves, built by plotting sensitivity vs. 1-specificity, calculating the area under the curve (AUC), and defining threshold values based on Youden's J statistics. Test characteristics of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) will be calculated using contingency tables and χ^2 test or Fisher's exact at statistically optimal threshold values. The Kaplan-Meier method will be applied to plot survival vs. non-survival of patients, based on previously calculated optimal test parameter cut-offs. Survival curves will be compared using the log-rank test.

DISCUSSION

Non-traumatic, spontaneous ICH is one of the most disabling forms of stroke, with high mortality and limited therapeutic choices. To make treatment decisions and being able to determine a prognosis, it is important to know which factors predict outcome. Most studies in this field investigate clinical and radiographic predictors of ICH outcome, e.g., age, premorbid functional state, initial GCS, blood pressure, hematoma location, and volume (13, 14). Non-invasive neuromonitoring techniques, such as quantitative EEG and evoked potentials, are increasingly being used to monitor brain damage in traumatic brain injury and subarachnoidal hemorrhage to evaluate functional recovery (15, 16), but only a few studies deal with primary ICHs. As this field of stroke research is relatively understudied, new data is warranted by studying relatively large prospective patient cohorts to understand the pathophysiology and the progression of the disease.

Few studies showed that coagulation disorders and impaired hemostasis increase the risk of ICH (17, 18) but the impact of hemostasis and fibrinolytic abnormalities on the outcome of non-traumatic ICH has not been thoroughly evaluated. Besides a handful of studies indicating that increased admission D-dimer levels are associated with ICH mortality (19–21), the role of hemostasis or fibrinolytic system alterations potentially occurring in ICH has remained mostly unexplored in predicting outcomes. Gaining knowledge on hemostasis factors that potentially drive the enlargement or the dissolution of the ICH might be crucial for future therapeutic approaches. As a first step, the underlying pathomechanism leading to poor outcomes must be understood in patients, and adequate diagnostic tools are substantial for this. It is important to learn about the levels of certain individual hemostasis and fibrinolytic factors in ICH patients as they might play a role in the development and dissolution of the hematoma. However, a global assay of coagulation and fibrinolysis might prove to be more useful in the acute clinical setting as compared to laborious individual tests. The clot lysis assay (CLA) is a global assay of clot formation and fibrinolysis that provides valuable information about the fibrinolytic potential of the plasma. This measurement is performed using plasma samples therefore the cellular components of hemostasis are not investigated by this test. In the past decade, it has been reported that neutrophil extracellular

trap (NET) components are important modulating factors of hemostasis: NET components intercalate to fibrin and alter clot formation and fibrinolysis (22, 23). The prothrombotic and antifibrinolytic effects of NETs may play a role in the clinical outcome of ICH. To our knowledge, our study is the first that plans to examine whether a modified CLA that incorporates the effects of NETs might be a prognostic factor in patients with non-traumatic ICH.

Neurophysiological examinations might also have tremendous potentials in predicting ICH outcomes, however, the prognostic value of EEG and MEP in ICH outcomes has not been extensively studied. Changes in the extent of bleeding, moreover, the onset and progression of brain edema can be monitored by using digital and quantitative EEG techniques (24). During early management, identification of these parameters is important because it could have a significant impact on the outcome of brain hemorrhage. In the early stages, localized polymorphic delta wave activity appears ipsilaterally in patients with ICH without a shift of midline structures, regardless of the location of hematoma. In patients with larger hematomas of 30 mL or more, causing a shift of the midline structures, delta wave activity appears over both hemispheres, while superposition of faster frequencies on slow waves indicates the development of brain edema (25). The incidence of seizures after spontaneous ICH reportedly ranges from 2.8 to 18.7% (26). Provoking factors are related to hemorrhage volume, hemorrhage location within the cerebrum, cortical involvement, and the severity of neurological deficits (12). Seizures can be non-convulsive, which remain undiscovered without an EEG examination. The incidence of non-convulsive seizures has been reported in 8–20% of ICU patients, and in case it progresses to status epilepticus, the mortality could be as high as 70% (27). Epileptiform discharges detected by EEG or early seizures are associated with deterioration of mental functions and should be treated with antiepileptic drugs (28). There is a strong relationship between the pathophysiology of ICH and the development of epilepsy. Therefore, early antiepileptic treatment has a positive effect on glutaminergic synaptic transmission, neuronal cell apoptosis, and permeability of the blood-brain barrier through decreasing inflammatory processes (29). Damage to the corticospinal tract can be examined with motor evoked potentials (MEP) elicited by transcranial magnetic stimulation (TMS). Based on the literature, the absence of MEP in the acute stage indicates poor recovery of muscle strength, while the presence of MEP in a completely hemiplegic patient predicted some recovery of motor function (12). The suppression of amplitude was more accurate than the prolongation of latency in predicting functional recovery. MEP monitoring of patients with hypertensive ICH in the acute stage can predict the outcome of motor function (30–32).

The IRONHEART study is a single-centered observational study, with the disadvantage of somewhat limited sample size, but with the clear advantage of uniform patient management, uniform neurophysiological examination, blood sample handling, and testing, which are crucial for the evaluation of results. In multicentric studies, specific investigations, including neurophysiological and hemostasis tests are difficult to harmonize due to the lack of standardized methods and due to the various sensitivity and specificity of the tests used. In the IRONHEART study, a complex evaluation of ICH progression will be carried out, and associations with specific hemostasis test results, neurophysiological data, and imaging data will be investigated. If associations between outcomes and the investigated parameters are confirmed, results might aid prognosis assessment in this subtype of stroke, with particularly high mortality. In our study, patients who die during the hospital stay will be autopsied, which will provide the possibility to differentiate between hemorrhagic stroke-related death and non-stroke related mortality as an outcome. Gaining knowledge on factors that potentially drive the enlargement or the dissolution of the ICH might be crucial for future therapeutic approaches. In the IRONHEART study, ICH size and its subsequent resolution or enlargement will be followed in every patient, providing potentially useful data in this respect. In the future, improving clinical grading systems on ICH outcomes by including the investigated parameters of the IRONHEART study might help to better guide the management of these patients. The MEP examinations will show us the proper time to evaluate the prognosis and will show the best biological marker and the appropriate parameter. The findings of the study will help clinicians estimate long-term outcome by using functional examinations such as EEG and MEP, instead of using imaging techniques only. Clinical neurophysiological examinations and CT findings together help planning the rehabilitation program tailored to the patients' status. The early epileptiform activity could predict early and delayed epileptic seizures, and presumably, early treatment might lead to more favorable outcome of the stroke.

ETHICS STATEMENT

The study design was built by the guiding principles of the Declaration of Helsinki and was approved by the Institutional Ethics Committee of the University of Debrecen and the Ethics Committee of the National Medical Research Council (Registration Number: 16343-5/2017/EÜIG). All patients or their relatives will provide written informed consent to be enrolled in the study.

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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REFERENCES

1. Feigin VL. Stroke in developing countries: can the epidemic be stopped and outcomes improved? *Lancet Neurol.* (2007) 6:94–7. doi: 10.1016/S1474-4422(07)70007-8
2. Hartley A, Marshall DC, Salciccioli JD, Sikkel MB, Maruthappu M, Shalhoub J. Trends in mortality from ischemic heart disease and cerebrovascular disease in Europe: 1980 to 2009. *Circulation.* (2016) 133:1916–26. doi: 10.1161/CIRCULATIONAHA.115.018931
3. Feigin VL, Lawes CM, Bennett DA, Barker-Collo SL, Parag V. Worldwide stroke incidence and early case fatality reported in 56 population-based studies: a systematic review. *Lancet Neurol.* (2009) 8:355–69. doi: 10.1016/S1474-4422(09)70025-0
4. Hemphill JC, 3rd, Bonovich DC, Besmertis L, Manley GT, Johnston SC. The ICH score: a simple, reliable grading scale for intracerebral hemorrhage. *Stroke.* (2001) 32:891–7. doi: 10.1161/01.STR.32.4.891
5. Kothari RU, Brott T, Broderick JP, Barsan WG, Sauerbeck LR, Zuccarello M, et al. The ABCs of measuring intracerebral hemorrhage volumes. *Stroke.* (1996) 27:1304–5. doi: 10.1161/01.STR.27.8.1304
6. Al-Mufti F, Thabet AM, Singh T, El-Ghanem M, Amuluru K, Gandhi CD. Clinical and radiographic predictors of intracerebral hemorrhage outcome. *Interv Neurol.* (2018) 7:118–36. doi: 10.1159/000484571
7. Vinciguerra L, Bosel J. Noninvasive neuromonitoring: current utility in subarachnoid hemorrhage, traumatic brain injury, and stroke. *Neurocrit Care.* (2017) 27:122–40. doi: 10.1007/s12028-016-0361-8
8. Volbers B, Staykov D, Wagner I, Dorfler A, Saake M, Schwab S, et al. Semi-automatic volumetric assessment of perihemorrhagic edema with computed tomography. *Eur J Neurol.* (2011) 18:1323–8. doi: 10.1111/j.1468-1331.2011.03395.x
9. Brott T, Adams HP, Jr, Olinger CP, Marler JR, Barsan WG, et al. Measurements of acute cerebral infarction: a clinical examination scale. *Stroke.* (1989) 20:864–70. doi: 10.1161/01.STR.20.7.864
10. van Swieten JC, Koudstaal PJ, Visser MC, Schouten HJ, van Gijn J. Interobserver agreement for the assessment of handicap in stroke patients. *Stroke.* (1988) 19:604–7. doi: 10.1161/01.STR.19.5.604
11. Charidimou A, Imaizumi T, Moulin S, Biffi A, Samarasekera N, Yakushiji Y, et al. Brain hemorrhage recurrence, small vessel disease type, and cerebral microbleeds: A meta-analysis. *Neurology.* (2017) 89:820–9. doi: 10.1212/WNL.0000000000004259
12. Zhao Y, Li X, Zhang K, Tong T, Cui R. The Progress of Epilepsy after Stroke. *Curr Neuropharmacol.* (2018) 16:71–8. doi: 10.2174/1570159X15666170613083253
13. Hendricks HT, van Limbeek J, Geurts AC, Zwarts MJ. Motor recovery after stroke: a systematic review of the literature. *Arch Phys Med Rehabil.* (2002) 83:1629–37. doi: 10.1053/apmr.2002.35473
14. Shah SD, Kalita J, Misra UK, Mandal SK, Srivastava M. Prognostic predictors of thalamic hemorrhage. *J Clin Neurosci.* (2005) 12:559–61. doi: 10.1016/j.jocn.2004.08.010
15. Matsubara S, Sato S, Kodama T, Egawa S, Nakamoto H, Toyoda K, et al. Nonconvulsive status epilepticus in acute intracerebral hemorrhage. *Stroke.* (2018) 49:1759–61. doi: 10.1161/STROKEAHA.118.021414
16. Biffi A, Rattani A, Anderson CD, Ayres AM, Gurol EM, Greenberg SM, et al. Delayed seizures after intracerebral haemorrhage. *Brain.* (2016) 139:2694–705. doi: 10.1093/brain/aww199
17. Aguilar MI, Freeman WD. Spontaneous intracerebral hemorrhage. *Semin Neurol.* (2010) 30:555–64. doi: 10.1055/s-0030-1268865
18. van Asch CJ, Luitse MJ, Rinkel GJ, van der Tweel I, Algra A, Klijn CJ. Incidence, case fatality, and functional outcome of intracerebral haemorrhage over time, according to age, sex, and ethnic origin: a systematic review and meta-analysis. *Lancet Neurol.* (2010) 9:167–76. doi: 10.1016/S1474-4422(09)70340-0
19. Chiu CC, Li YN, Lin LJ, Hsiao CT, Hsiao KY, Chen IC. Serum D-dimer as a predictor of mortality in patients with acute spontaneous intracerebral hemorrhage. *J Clin Neurosci.* (2012) 19:810–3. doi: 10.1016/j.jocn.2011.08.032
20. Hu X, Fang Y, Ye F, Lin S, Li H, You C, et al. Effects of plasma D-dimer levels on early mortality and long-term functional outcome after spontaneous intracerebral hemorrhage. *J Clin Neurosci.* (2014) 21:1364–7. doi: 10.1016/j.jocn.2013.11.030
21. Delgado P, Alvarez-Sabin J, Abilleira S, Santamarina E, Purroy F, Arenillas JF, et al. Plasma d-dimer predicts poor outcome after acute intracerebral hemorrhage. *Neurology.* (2006) 67:94–8. doi: 10.1212/01.wnl.0000223349.97278.e0
22. Varju I, Kolev K. Networks that stop the flow: A fresh look at fibrin and neutrophil extracellular traps. *Thromb Res.* (2019) 182:1–11. doi: 10.1016/j.thromres.2019.08.003
23. Longstaff C, Varju I, Sotonyi P, Szabo L, Krumrey M, Hoell A, et al. Mechanical stability and fibrinolytic resistance of clots containing fibrin, DNA, and histones. *J Biol Chem.* (2013) 288:6946–56. doi: 10.1074/jbc.M112.404301
24. Wang F, Zhang XY, Hu FY, Li FL, Tian YC. Quantitative electroencephalography analysis for improved assessment of consciousness in cerebral hemorrhage and ischemic stroke patients. *IEEE Access.* (2019) 7:63674–85. doi: 10.1109/ACCESS.2019.2916165
25. Hirose G, Saeki M, Kosoegawa H, Takado M, Yamamoto T, Tada A. Delta waves in the EEGs of patients with intracerebral hemorrhage. *Arch Neurol.* (1981) 38:170–5. doi: 10.1001/archneur.1981.00510030064009
26. Woo KM, Yang SY, Cho KT. Seizures after spontaneous intracerebral hemorrhage. *J Korean Neurosurg Soc.* (2012) 52:312–9. doi: 10.3340/jkns.2012.52.4.312
27. Laccheo I, Sonmezturk H, Bhatt AB, Tomycz L, Shi Y, Ringel M, et al. Non-convulsive status epilepticus and non-convulsive seizures in neurological ICU patients. *Neurocrit Care.* (2015) 22:202–11. doi: 10.1007/s12028-014-0070-0
28. Hemphill JC, 3rd, Greenberg SM, Anderson CS, Becker K, Bendok BR, Cushman M, et al. Guidelines for the management of spontaneous intracerebral hemorrhage: a guideline for healthcare professionals from the American heart association/American stroke association. *Stroke.* (2015) 46:2032–60. doi: 10.1161/STR.0000000000000069
29. Doria JW, Forgacs PB. Incidence, implications, and management of seizures following ischemic and hemorrhagic stroke. *Curr Neurol Neurosci Rep.* (2019) 19:37. doi: 10.1007/s11910-019-0957-4
30. Cortes M, Black-Schaffer RM, Edwards DJ. Transcranial magnetic stimulation as an investigative tool for motor dysfunction and recovery in stroke: an overview for neurorehabilitation clinicians. *Neuromodulation.* (2012) 15:316–25. doi: 10.1111/j.1525-1403.2012.00459.x
31. Nagao S, Kawai N. Prediction of motor function by magnetic brain stimulation in patients with intracerebral hematoma. *Neurol Med Chir.* (1992) 32:268–74. doi: 10.2176/nmc.32.268
32. Tsai SY, Tchen PH, Chen JD. The relation between motor evoked potential and clinical motor status in stroke patients. *Electromyogr Clin Neurophysiol.* (1992) 32:615–20.

Conflict of Interest: The 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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A Modified *in vitro* Clot Lysis Assay Predicts Outcomes in Non-traumatic Intracerebral Hemorrhage Stroke Patients—The IRONHEART Study

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Background: Non-traumatic intracerebral hemorrhage (ICH) accounts for 10–15% of all strokes and results in a higher rate of mortality as compared to ischemic strokes. In the IRONHEART study, we aimed to find out whether a modified *in vitro* clot lysis assay method, that includes the effect of neutrophil extracellular traps (NETs) might predict ICH outcomes.

Patients and Methods: In this prospective, observational study, 89 consecutive non-traumatic ICH patients were enrolled. Exclusion criteria included aneurysm rupture, cancer, liver- or kidney failure or hemorrhagic diathesis. On admission, detailed clinical and laboratory investigations were performed. ICH volume was estimated based on CT performed on admission, day 14 and 90. A conventional *in vitro* clot lysis assay (CLA) and a modified CLA (mCLA) including cell-free-DNA and histones were performed from stored platelet-free plasma taken on admission. Clot formation and lysis in case of both assays were defined using the following variables calculated from the turbidimetric curves: maximum absorbance, time to maximum absorbance, clot lysis times (CLT) and area under the curve (CLA AUC). Long-term ICH outcomes were defined 90 days post-event by the modified Rankin Scale (mRS). All patients or relatives provided written informed consent.

Results: Patients with more severe stroke (NIHSS>10) presented significantly shorter clot lysis times of the mCLA in the presence of DNA and histone as compared to patients with milder stroke [10%CLT: NIHSS 0–10: median 31.5 (IQR: 21.0–40.0) min vs. NIHSS>10: 24 (18–31.0) min, $p = 0.032$]. Shorter clot lysis times of the mCLA showed significant association with non-survival by day 14 and with unfavorable long-term outcomes [mRS 0–1: 36.0 (22.5.0–51.0) min; mRS 2–5: 23.5 (18.0–36.0) min and mRS 6: 22.5 (18.0–30.5) min, $p = 0.027$]. Estimated ICH volume showed significant negative correlation with mCLA parameters, including 10%CLT ($r = -0.3050$, $p = 0.009$). ROC analysis proved good diagnostic performance of mCLA for predicting poor long-term outcomes [AUC: 0.73 (0.57–0.89)]. In a Kaplan-Meier survival analysis, those patients

who presented with an mCLA 10%CLT result of >38.5 min on admission showed significantly better survival as compared to those with shorter clot lysis results ($p = 0.010$).

Conclusion: Parameters of mCLA correlate with ICH bleeding volume and might be useful to predict ICH outcomes.

Keywords: hemorrhagic stroke, clot lysis, outcome, intracerebral hemorrhage, neutrophil extracellular traps

INTRODUCTION

Stroke is a leading cause of death and disability in all developed countries. Non-traumatic intracerebral hemorrhage (ICH) constitutes ∼10–15% of acute strokes and has a much higher risk of morbidity and mortality than ischemic strokes or subarachnoid hemorrhage (1, 2). Despite advances in acute stroke care, pharmacological treatment in ICH is still limited, and it remains the most devastating cerebral vascular disease with a mortality of up to 30–50% (1). Coagulation disorders and impaired hemostasis have been shown to increase the risk of ICH (3, 4). However, studies on associations between hemostasis or fibrinolysis abnormalities and the outcome of ICH remains limited. Besides a handful of studies indicating that increased admission D-dimer levels predict mortality (5–7), the impact of the fibrinolytic system on the outcome of acute non-traumatic ICH has not been fully investigated.

The clot lysis assay (CLA) is a global assay of the fibrinolytic system reflecting the overall plasma fibrinolytic potential (8). This test has been used to study clot formation and fibrinolysis in a wide spectrum of pathologies, including acute ischemic stroke (8–13). Although the CLA is feasible and has a potential for clinical use to examine fibrin clot properties and fibrinolysis, the assay is not free of analytical challenges and protocols used in different laboratories may vary significantly. Moreover, the assay is performed using plasma and therefore potential cellular contributors of hemostasis and fibrinolysis are not incorporated in the test. Recent data identified neutrophil extracellular trap (NET) components, released from activated neutrophils as important modulators of fibrinolysis (14–16). NETs are networks of DNA and histones, decorated with granular neutrophil proteins (e.g., elastase), originally described as a first line defense mechanism of the innate immune system (17). It has been shown that NETs intercalate to fibrin and create a dense network that is resistant to fibrinolysis (14–16). NETs have been implicated as important constituents of venous and arterial thrombi, and the prothrombotic and antifibrinolytic effects of NETs have been in the focus of wide range of research in recent years (14, 18–25).

The aim of this study was to evaluate whether a modified CLA (mCLA) incorporating the effect of NETs might predict outcomes in a cohort of patients with non-traumatic, spontaneous ICH.

MATERIALS AND METHODS

Patients

In this prospective observational study, consecutive patients with non-traumatic intracerebral hemorrhage stroke (ICH) were enrolled in a single stroke center (Department of Neurology, University of Debrecen, Hungary). Patient enrollment started in June 2017 and finished in September 2020. Inclusion criteria were: patients over 18 years of age with acute non-traumatic intracerebral hemorrhage, verified with non-contrast computerized tomography (NCCT) scan. Exclusion criteria included the presence of cerebral aneurysm, AV malformation, epidural hemorrhage, subdural hemorrhage, malignancy, severe hepatic- and renal insufficiency, hemorrhagic diathesis and SARS-CoV-2 infection at hospital admission or during follow-up. The presence of ICH was diagnosed by complex neurological examination based on clinical symptoms, brain imaging using NCCT scan. Follow-up NCCT scans were performed 14 days and 3 months after the event. CT images were analyzed simultaneously by 3 independent investigators and a comprehensive list of radiographic features and estimated ICH volume was recorded (26). For each patient, the time of symptom onset, demographic and clinical characteristics (age, sex, BMI, previous medications, history of cerebrovascular and cardiovascular diseases, cerebrovascular risk factors including smoking) were registered on admission. Stroke severity was determined by the National Institutes of Health Stroke Scale (NIHSS) on admission and on day 7 (27). Risk stratification of each patient was performed using the ICH score (based on GCS score, age, infratentorial origin, intraventricular hemorrhage and ICH volume) (28). Patients were followed and long-term functional outcomes were determined at 3 months after the stroke event using the modified Rankin Scale (mRS) (29). As from March 2020, all patients were investigated about potential acquiration and symptoms of SARS-CoV-2 infection on admission and during follow-up. In case of a suspected infection, the diagnosis was confirmed by a routine method of reverse transcriptase polymerase chain reaction testing of RNA extracted from nasopharyngeal/oropharyngeal swabs.

The following outcomes were investigated: 1/Mortality by day 14 and day 90. 2/Long-term outcome at 90 days post-event: mRS 0–1 was defined as favorable long-term outcome (30).

Informed Consent

The study design was in accordance with the guiding principles of the Declaration of Helsinki and was approved by the Institutional Ethics Committee of the University of Debrecen and the Ethics Committee of the National Medical Research Council. All patients or their relatives provided written informed consent.

Blood Sampling and Laboratory Measurements

Peripheral venous blood samples were taken from all patients on admission. Routine laboratory tests (ions, glucose level,

TABLE 1 | Baseline characteristics of enrolled patients, imaging data and outcomes.

Number of patients, *n*	**89**
Age, y, mean ± SD	68 (±11.6)
Male sex, *n* (%)	57 (64.0)
Stroke severity on admission, NIHSS, median (IQR)	14 (8–20)
Stroke severity on discharge, NIHSS, median (IQR)	19 (8–43)
ICH score, median (IQR)	1 (1–3)
Glasgow coma scale, median (IQR)	13 (9–14)
Cerebrovascular risk factors, *n* (%)	
Arterial hypertension	86 (96.6)
Atrial fibrillation	11 (12.4)
Diabetes mellitus	39 (43.8)
Hyperlipidemia	48 (53.9)
Active smoker	15 (16.9)
BMI, kg/m^2, median (IQR)	27.0 (24.1–31.9)
Laboratory measurements on admission, median (IQR)	
INR	0.97 (0.93–1.05)
APTT, s	27.7 (25.4–31.2)
WBC, G/L	8.6 (6.7–11.5)
Platelet count, G/L	226 (170–265)
Serum glucose, mmol/L	7.6 (6.0–10.4)
hsCRP, mg/L	2.7 (1.2–6.5)
Creatinine, μmol/L	69.0 (61.0–84.5)
Fibrinogen, g/L	3.8 (3.1–4.4)
Plasminogen activity (%)	110 (100–122)
α2- plasmin inhibitor activity (%)	107 (98–113)
Factor XIII activity (%)	166 (139–176)
Imaging data, *n* (%)	
Presence of hydrocephalus on admission	
No	59 (66.3)
External hydrocephalus	2 (2.2)
Internal hydrocephalus	21 (23.6)
Both	7 (7.9)
Hemisphere localization of ICH on admission	
Left hemisphere	44 (49.4)
Right hemisphere	41 (46.1)
Bilateral hemisphere	4 (4.5)
Presence of intraventricular hemorrhage on admission	
No	43 (48.3)
Subarachnoideal	11 (12.4)
Lateral ventricule	8 (9.0)
III. ventricule	1 (1.1)
IV. ventricule	1 (1.1)
Combined	25 (28.1)
Infratentorial origin	
Yes	4 (4.5)
No	85 (95.5)
Estimated volume of hemorrhage, cm^3, median (IQR)	
On admission	20.0 (3.7–48.0)
Day 14	10.0 (2.8–27.0)
Day 90	0 (0.0–2.4)
Outcomes, *n* (%)	
Mortality by day 14	26 (29.0)
Long-term outcome (mRS, day 90)	
Favorable (mRS 0–1)	15 (16.9)
Unfavorable (mRS 2–5)	32 (36.0)
Death (mRS 6)	39 (43.8)
Undetermined	3 (3.3)

Data are means ± SD or medians (interquartile ranges). APTT, activated partial thromboplastin time; BMI, body mass index; hsCRP, high sensitivity C-reactive protein measurement; ICH, intracerebral hemorrhage, INR, international normalized ratio; IQR, interquartile range; i.v., intravenous; mRS, modified Rankin Scale; NIHSS, National Institutes of Health Stroke Scale; WBC, white blood cell.

TABLE 2 | Clot lysis assay (CLA) and modified CLA (mCLA)* parameters in the total cohort.

	CLA	**mCLA**	***p***
Max. absorbance (OD)	1.41 (1.30–1.59)	1.42 (1.32–1.60)	0.001
Time to max. absorbance (min)	10.5 (9.0–14.0)	11.5 (8.0–15.0)	0.906
10%CLT (min)	23.5 (15.5–33.0)	25.5 (18.5–35.0)	0.023
50%CLT (min)	34.5 (24.5–44.0)	35.5 (28.0–49.5)	0.012
90%CLT (min)	76.0 (66.0–87.0)	75.0 (68.0–87.0)	0.254
CLA AUC (OD*min)	24.2 (18.4–28.3)	25.2 (19.6–30.0)	0.004

**mCLA is performed in the presence of cell-free DNA and histones. Data are medians (interquartile ranges); cfDNA, cell-free DNA; CLA; clot lysis assay, 10%CLT, 10% clot lysis time; 50%CLT, 50% clot lysis time; 90%CLT, 90% clot lysis time; CLA AUC, clot lysis assay area under the curve.*

renal and liver function tests, high-sensitivity C-reactive protein measurement, complete blood count) were carried out immediately by standard laboratory methods (Roche Diagnostics, Mannheim, Germany and Sysmex Europe GmbH, Hamburg, Germany). For the examination of hemostasis tests, blood samples were collected to vacutainer tubes containing 0.109 M sodium citrate (Becton Dickinson, Franklin Lane, NJ) and were processed immediately (centrifugation twice at 1,500 g, room temperature, 15 min). Screening tests of coagulation (prothrombin time, activated partial thromboplastin time, and thrombin time) were performed immediately on a BCS coagulometer using routine methods (Siemens Healthcare Diagnostic Products, Marburg, Germany). For the execution of *in vitro* clot lysis assays (CLA and mCLA) and other specific hemostasis tests, aliquots of citrated plasma were labeled with a unique code and stored at −80°C until analysis. Specific hemostasis tests including CLA and mCLA were performed from stored plasma aliquots by investigators blinded to patient identification and clinical data. Fibrinogen levels were measured according to the method of Clauss on a BCS coagulometer (Siemens Healthcare Diagnostic Products, Marburg, Germany). Plasminogen and α2-plasmin inhibitor (α2-PI) activities were measured by commercially available methods on a BCS coagulometer. Plasma levels of FXIII activity were determined by

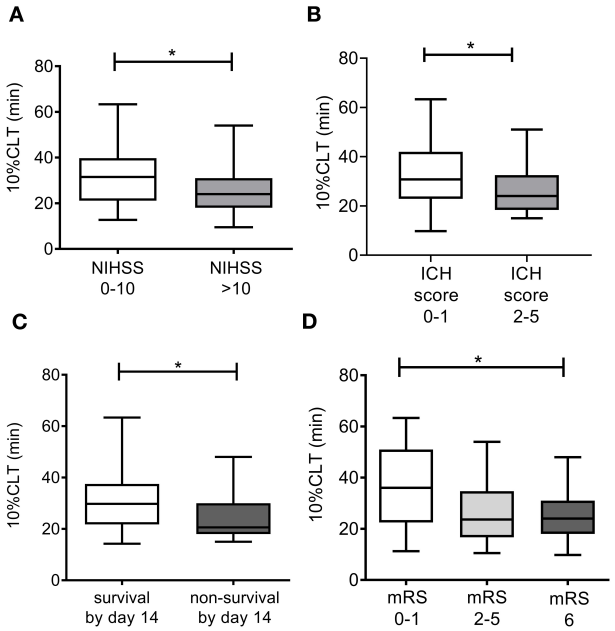

FIGURE 1 | Associations of the 10%CLT parameter of the modified clot lysis assay (mCLA) with stroke severity on admission **(A)**, ICH score calculated on admission **(B)**, survival by day 14 **(C)**, and the modified Rankin Scale (mRS) on day 90 **(D)**. mCLA is performed in the presence of cell-free DNA and histones. Box and whisker plots indicate median, interquartile range, and total range. 10% CLT: 10% clot lysis time, ICH, intracerebral hemorrhage; NIHSS, National Institutes of Health Stroke Scale; mRS, modified Rankin Scale. *$p < 0.05$.

ammonia release assay using a commercially available reagent kit (REA-chrom FXIII kit, Reanalker, Budapest, Hungary).

In vitro CLA and mCLA Measurements

Recombinant t-PA-driven lysis of tissue factor-induced plasma clots was studied in 96-well microtiter plates by monitoring changes in turbidity. Assay conditions in our study were based on previously described methods, with some modifications (8, 13, 31–33). Two assay conditions were used, and plasma samples were run in quadruplicates in both assay conditions. All concentrations provided refer to final concentrations in the 100 μL final well volume. Plasma samples were thawed in a water bath at 37°C. In the first assay condition (CLA), citrated plasma was mixed with 1,000-fold diluted human tissue factor (Innovin, Siemens, Marburg, Germany) and 100 ng/ml rt-PA (Alteplase, Boehringer Ingelheim, Ingelheim, Germany) in HEPES buffer (10 mM HEPES, 150 mM NaCl, 0.05% Tween 20, pH:7.4). In order to mimick the effect of NETs, in the second assay condition (mCLA) 150 μg/ml pure and cell-free DNA (cfDNA) (calf thymus DNA, Sigma-Aldrich, Darmstadt, Germany) and 50 μg/ml calf thymus histone (TIII S, Calbiochem, La Jolla, CA, USA) were also added to the sample solutions. Optimal concentrations of cfDNA and histones were tested in preliminary experiments based on literature where the combined effect of histones (50 μg/ml) and various concentrations of cfDNA (50–250 μg/ml) were studied on fibrinolysis kinetics in purified experimental conditions (16). Dilution of plasma samples with buffer was 1.2-fold in case of both assay conditions. Clot formation in both conditions was initiated by automated sample pipetting of HEPES buffer, containing 21 mM $CaCl_2$, to each sample well. Optical density was measured at 340 nm, 37°C every minute for 300 min in a TECAN Infinite m200 microplate

TABLE 3 | Baseline clinical data and modified clot lysis assay (mCLA)* parameters according to mortality by day 14.

	Survival by day 14 ($n = 63$)	Non-survival by day 14 ($n = 26$)	p
Age, y, mean ± SD	67 ± 12	71 ± 10	0.082
Male sex, *n* (%)	29 (46)	10 (39)	0.474
Stroke severity on admission, NIHSS, median (IQR)	11 (5–17)	19 (16–23)	<0.0001
Cerebrovascular risk factors, *n* (%)			
Arterial hypertension	59 (94)	26 (100)	0.552
Atrial fibrillation	7 (11)	3 (12)	0.999
Diabetes mellitus	24 (38)	14 (54)	0.191
Hyperlipidemia	34 (54)	14 (54)	0.932
Active smoker	14 (22)	2 (8)	0.032
BMI, kg/m^2, median (IQR)	27.1 (23.3–32.4)	26.9 (25.4–30.6)	0.810
Laboratory measurements on admission, median (IQR)			
INR	0.96 (0.93–1.00)	1.02 (0.96–1.68)	0.009
APTT, s	27.7 (25.4–30.1)	28.3 (25.1–36.5)	0.430
WBC, G/L	8.6 (6.5-11.2)	9.3 (6.9-12.0)	0.538
Platelet count, G/L	238 (172–283)	203 (167–242)	0.049
Serum glucose, mmol/L	7.3 (5.8–9.7)	8.2 (6.5–11.4)	0.094
hsCRP, mg/L	3.2 (1.2–7.1)	2.4 (1.3–3.8)	0.373
Creatinine, μmol/L	69 (60-82)	76 (62-95)	0.326
Fibrinogen, mg/ml	4.0 (3.2–4.6)	3.4 (2.9–4.2)	0.086
Plasminogen activity (%)	112 (97-123)	107 (103-114)	0.212
α2- plasmin inhibitor activity (%)	107 (99–113)	105 (94–114)	0.422
Factor XIII activity (%)	167 (147–175)	161.5 (132–177)	0.414
mCLA parameters			
Maximal absorbance (OD)	1.48 ± 0.25	1.41 ± 0.21	0.374
Time to maximal absorbance (min)	11.5 (8.0–15.0)	10.0 (8.0–14.0)	0.724
10%CLT (min)	30.0 (22.0–37.5)	20.5 (18.0–30.0)	0.037
50%CLT (min)	39.0 (28.0–51.0)	34.0 (27.5–45.5)	0.491
90%CLT (min)	76.5 (69.0–87.0)	74.0 (61.0–82.5)	0.179
CLA AUC (OD*min)	25.6 (19.8–31.5)	24.1 (20.3–28.5)	0.459

**mCLA is performed in the presence of cell-free DNA and histones. Data are means ± SD or medians (interquartile ranges). 10%CLT, 10% clot lysis time; 50%CLT, 50% clot lysis time; 90%CLT, 90% clot lysis time; CLA, clot lysis assay; CLA AUC, clot lysis assay area under the curve; APTT, activated partial thromboplastin time; BMI, body mass index; hsCRP, high sensitivity C-reactive protein measurement; INR, international normalized ratio.*

TABLE 4 | Baseline clinical data and modified clot lysis assay (mCLA)* parameters according to long-term functional outcomes at 90 days post-event.

	mRS 0–1 ($n = 15$)	mRS 2–5 ($n = 32$)	mRS 6 ($n = 39$)	p
Age, y, mean ± SD	64 ± 12	67 ± 13	71 ± 10	0.054
Male sex, *n* (%)	10 (67)	21 (66.0)	28 (72)	0.920
Stroke severity on admission, NIHSS, median (IQR)	5 (4–6)	13 (10–17)	19 (15–23)	<0.0001
				<0.0001[§]
				<0.001[#]
				0.015[†]
Cerebrovascular risk factors, *n* (%)				
Arterial hypertension	13 (87)	32 (100)	39 (100)	0.060
Atrial fibrillation	0 (0)	5 (16)	5 (13)	0.283
Diabetes mellitus	6 (40)	12 (38)	20 (51)	0.541
Hyperlipidemia	11 (73)	14 (44)	23 (59)	0.095
Active smoker	5 (33)	6 (19)	4 (10)	0.109
BMI, kg/m^2, median (IQR)	23.6 (21.5–26.0)	30.2 (25.0–33.2)	27.1 (25.4–31.6)	0.012
				0.037[§]
				0.011[#]
Laboratory measurements on admission, median (IQR)				
INR	0.96 (0.94–0.99)	0.95 (0.91–0.99)	1.01 (0.94–1.21)	0.029
				0.033[†]
APTT, s	27.6 (25.7–29.6)	28.0 (24.8–30.4)	27.9 (25.4–33.3)	0.822
WBC, G/L	6.9 (6.5–9.4)	8.8 (7.6–12.3)	8.8 (6.7–11.4)	0.315
Platelet count, G/L	250 (158–273)	253 (209–288)	200 (168–243)	0.032
				0.027[#]
Serum glucose, mmol/L	7.4 (5.5–11.2)	7.3 (6.0–9.8)	7.7 (6.3–11.2)	0.563
hsCRP, mg/L	1.5 (0.7–4.1)	4.2 (1.3–8.1)	2.7 (1.2–7.0)	0.193
Creatinine, μmol/L	70 (54–88)	68 (55–83)	72 (64–88)	0.478
Fibrinogen, mg/ml	4.0 (3.2–4.3)	3.8 (3.3–4.6)	3.5 (3.0–4.5)	0.339
Plasminogen activity (%)	111 (93–123)	113 (100–132)	107 (103–117)	0.456
α2-plasmin inhibitor activity (%)	103 (99–109)	112 (103–115)	105 (96–114)	0.135
Factor XIII activity (%)	160 (138–175)	174 (160–180)	165 (141–178)	0.071
mCLA parameters				
Maximal absorbance (OD)	1.37 (1.3–1.6)	1.50 (1.4–1.6)	1.42 (1.3–1.6)	0.454
Time to maximal absorbance (min)	15.0 (12.0–16.5)	10.5 (8.0–14.0)	9.5 (7.5–14.5)	0.039
				0.047[§]
10%CLT (min)	36.0 (22.5–51.0)	23.5 (17.8–36.0)	22.5 (18.0–30.5)	0.027
				0.032[§]
50%CLT (min)	48.0 (42.0–63.0)	32.0 (28.0–49.5)	34.5 (27.0–45.0)	0.041
				0.043[§]
90%CLT (min)	81.0 (68.0–90.0)	75.0 (69.0–85.5)	75.0 (68.0–87.0)	0.490
CLA AUC (OD*min)	29.4 (23.7–34.5)	24.6 (18.8–29.7)	24.4 (19.5–29.3)	0.149

**mCLA is performed in the presence of cell-free DNA and histones. Data are means ± SD or medians (interquartile ranges). 10%CLT, 10% clot lysis time; 50%CLT, 50% clot lysis time; 90%CLT, 90% clot lysis time; APTT, activated partial thromboplastin time; BMI, body mass index; hsCRP, high sensitivity C-reactive protein measurement; CLA, clot lysis time; CLA AUC, clot lysis assay area under the curve; INR, international normalized ratio; mRS, modified Rankin Scale.*

[§]mRS 0–1 vs. mRS 6 (ANOVA, Bonferroni post-hoc-test or Kruskal-Wallis, Dunn's post-hoc-test).

[†]mRS 2–5 vs. mRS 6 (ANOVA, Bonferroni post-hoc-test).

[#]mRS 0–1 vs. mRS 2-5 (ANOVA, Bonferroni post-hoc-test or Kruskal-Wallis, Dunn's post-hoc-test).

reader (TECAN Trading AG, Männedorf, Switzerland). Curves were analyzed using the Shiny app software tool (34). The following parameters were calculated from the turbidimetric curves in case of both assay conditions: maximum absorbance, time to maximum absorbance, various points of clot lysis time (CLT): 10% clot lysis time (10%CLT), 50%CLT, 90%CLT and area under the curve (CLA AUC). Clot lysis times were defined as the time from the 10, 50, or 90% point, from clear to maximum turbidity, to the 10, 50, or 90% point, respectively, in the transition from maximum turbidity to the final baseline turbidity (10%CLT, 50%CLT, and 90%CLT parameters, respectively). Analytical precision of both assay conditions was evaluated

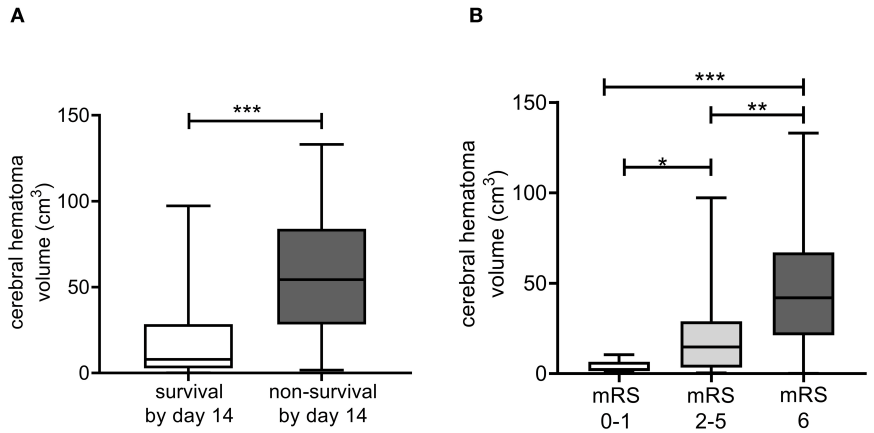

FIGURE 2 | Associations of the estimated cerebral hematoma volume as calculated from on admission CT scans with patient survival by day 14 **(A)**, and the modified Rankin Scale (mRS) on day 90 **(B)**. Box and whisker plots indicate median, interquartile range, and total range. mRS, modified Rankin Scale. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

according to the guidelines of Clinical and Laboratory Standards Institutes (CLSI document EP05-A3) (35, 36). Precision was tested using healthy control plasmas, each run in quadruplicate, for 20 days. Coefficients of variation (CVs) of the within-run and total (within-laboratory) precision assessments were 8.6 and 8.9%, respectively. Precision results were essentially similar in both assay conditions. Representative CLA and mCLA curves and reference parameters of healthy individuals as compared to patients are provided as **Supplementary Figure 1** and **Supplementary Table 1**.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, Version 26.0, Chicago, IL), and GraphPad Prism 8.0 (GraphPad Prism Inc., La Jolla, CA). Normality of data was studied using the Shapiro-Wilk-test. Student's *t*-test or Mann–Whitney *U*-test was performed for independent two-group analyses. In case of paired data, paired *t*-test or Wilcoxon signed-rank-test was applied. ANOVA with Bonferroni *post-hoc*-test or Kruskal–Wallis analysis with Dunn's *post-hoc*-test was used for multiple comparisons. Spearman's correlation coefficient was used to determine the strength of correlation between continuous variables. Differences between categorical variables were assessed by χ^2-test or by Fisher's exact where appropriate. Receiver operating characteristic (ROC) curves were built by plotting sensitivity vs. 1-specificity and calculating the area under the curve (AUC). Optimal threshold values were calculated based on Youden's J statistics. Test characteristics of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated using contingency tables and χ^2-test or Fisher's exact at statistically optimal threshold values. The Kaplan-Meier method was applied to plot survival vs. non-survival of patients, based on the calculated optimal test parameter cut-off. Survival curves were compared using the log-rank test. Binary backward logistic regression models were used to determine independent predictors of mortality and long-term functional outcome. Adjustments of the models were based on the results of preliminary statistical analyses of baseline characteristics between groups (Student's *t*-test or Mann–Whitney *U*-test, χ^2-test or Fisher's exact), literature data, and methodological principles (dichotomized variables when possible). Results of the logistic regression analysis were expressed as odds ratio (OR) and 95% confidence interval (CI). A *p*-value of <0.05 was considered statistically significant.

RESULTS

In the IRONHEART study, 89 patients with non-traumatic, spontaneous ICH were enrolled. One patient was excluded from the study due to SARS-CoV-2 infection on admission. One patient acquired SARS-CoV-2 infection on day 25 after the event, thus long-term follow-up results were excluded in this case. The assumed cause of ICH was hypertension in all patients, as based on the exclusion criteria, other causes, including cerebral aneurysm, AV malformation, malignancy, severe liver insufficiency, hemorrhagic diathesis, amyloidosis or vasculitis were excluded. Baseline characteristics of patients, imaging data and outcomes are shown in **Table 1**. The mean age of the cohort was 68 ($\pm$ 11.6) years, 64% of patients were men. Median NIHSS on admission was 14 (IQR: 8–20), median ICH score was 1 (IQR: 1–3). The most frequent cerebrovascular risk factor was hypertension (96.6%). Screening tests of coagulation and fibrinogen levels did not indicate a hemorrhagic defect in any of the patients. The median volume of hemorrhage was 20.0 (IQR: 3.7–48.0) cm^3 on admission and 46 (51.7%) of patients had intraventricular hemorrhage extension. Mortality was 29.0% within the first 14 days after event and 43.8% by day 90.

Clot Lysis Results

As expected, clot lysis parameters (max. absorbance, 10%CLT, 50% CLT, and AUC) became significantly prolonged in the total cohort when cfDNA and histones were added to the sample

solutions (**Table 2**). Patients with ICH showed significantly shorter clot lysis times as compared to a healthy reference group (**Supplementary Table 1**), indicating faster fibrinolysis, that was independent of the addition of cfDNA and histones. However, stroke severity and outcomes showed no association with the conventional CLA in the absence of DNA and histones (**Supplementary Tables 2–4**). Stroke severity and outcomes showed no association with the difference obtained between mCLA and CLA parameters (data not shown). On the contrary, patients with more severe stroke (NIHSS > 10) showed significantly shorter clot lysis (10%CLT) in the modified test as compared to patients with milder stroke (NIHSS 0–10) (**Figure 1A**). Similarly, significantly shorter clot lysis was observed using the mCLA in patients with higher ICH score (2–5) as compared to those with ICH 0–1 (**Figure 1B**). Key proteins of the fibrinolytic system (plasminogen, α2-PI, and FXIII activity) showed significant correlation with the maximal absorbance parameter of the conventional and mCLA, moreover, plasminogen activity showed significant correlation with most parameters of both assays (**Supplementary Table 5**). However, stroke severity (data not shown) and outcomes showed no association with any of the tested coagulation or fibrinolysis protein activity levels (**Tables 3**, **4**).

Mortality by day 14 was associated with significantly shorter 10%CLT of the mCLA (**Table 3** and **Figure 1C**). The median 10%CLT was 9.5 min shorter in those patients who died by day 14 as compared to those who survived ($p = 0.037$). Besides CLA parameters, admission NIHSS, INR, smoking and platelet count showed association with mortality by day 14. Similarly to short-term outcomes by day 14, results of long-term functional outcomes showed significant association with parameters of the modified assay (**Table 4** and **Figure 1D**). Those patients, who died or had unfavorable outcomes (mRS ≥ 2) by the end of the 3rd month, demonstrated significantly shorter mCLA parameters on admission as compared to those with good functional outcomes. Besides mCLA parameters, admission NIHSS, BMI, INR and platelet count were associated with outcomes by day 90. In addition, estimated hemorrhage volume on admission showed strong association with day 14 and day 90 mortality (**Figure 2**). Notably, mCLA parameters correlated significantly with estimated intracerebral hemorrhage volume (**Figure 3**). mCLA parameters indicating faster clot formation and lysis (shorter 10%CLT and time to maximal absorbance parameters, lower CLA AUC) showed significant association with larger hemorrhage volumes.

ROC analysis was performed for all outcomes to investigate the diagnostic performance of mCLA parameters (**Figure 4**). The best AUC of ROC was 0.73 (95%CI: 0.57–0.89) for the parameter 10%CLT for predicting mRS 0–1 as outcome (**Figure 4B**). Based on the optimal threshold value as defined by the Youden index (32.25 min), best sensitivity and specificity was provided by the 10%CLT parameter (77.0 and 67.7%, respectively, **Figure 4B**). When performing ROC analysis for mortality by day 14 and day 90, similar optimal threshold values were defined (10%CLT cut-off: >38.5 min for 90 day survival, curves not shown). In a Kaplan-Meier survival analysis, those patients who presented with a 10%CLT result of >38.5 min on admission showed significantly better survival as compared to those with shorter clot lysis results ($p = 0.010$; **Figure 5**).

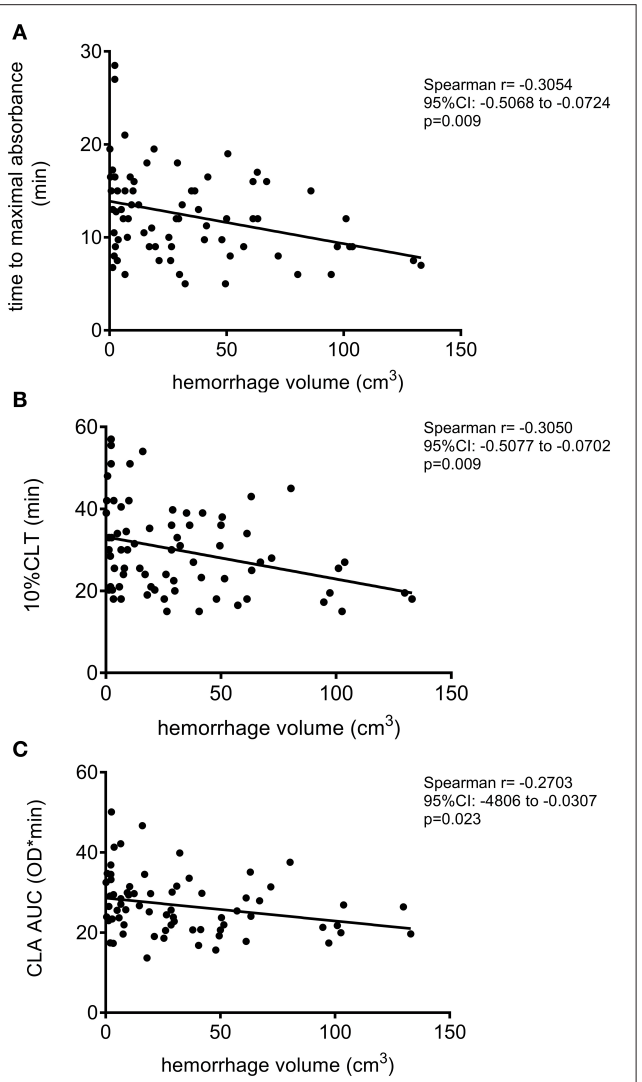

FIGURE 3 | Correlation between the modified clot lysis assay (mCLA) parameters and estimated intracerebral hematoma volume of the ICH patients. mCLA is performed in the presence of cell-free DNA and histones. Correlation between estimated intracerebral hemorrhage volume and time to maximal absorbance parameter **(A)**, 10% clot lysis time (10%CLT) **(B)**, and clot lysis assay area under the curve (CLA AUC) **(C)** are depicted.

Using binary backward logistic regression models (including age, sex, NIHSS on admission, hypertension, INR, platelet count, smoking status, cerebral hemorrhage volume, 10%CLT and BMI), 10%CLT of the mCLA did not prove to be an independent predictor of mortality by 14 days and 90 days post-event (**Table 5**). On the other hand, a binary backward logistic regression model (including age, sex, NIHSS on admission, INR, BMI, platelet count, 10%CLT, hemorrhage volume, hypertension) revealed that a shorter 10%CLT of the mCLA (<32.25 min) is a significant, independent predictor of unfavorable long-term functional outcome (mRS ≥ 2) (OR: 6.14, 95%CI: 1.11–34.02, $p = 0.038$) (**Table 5**).

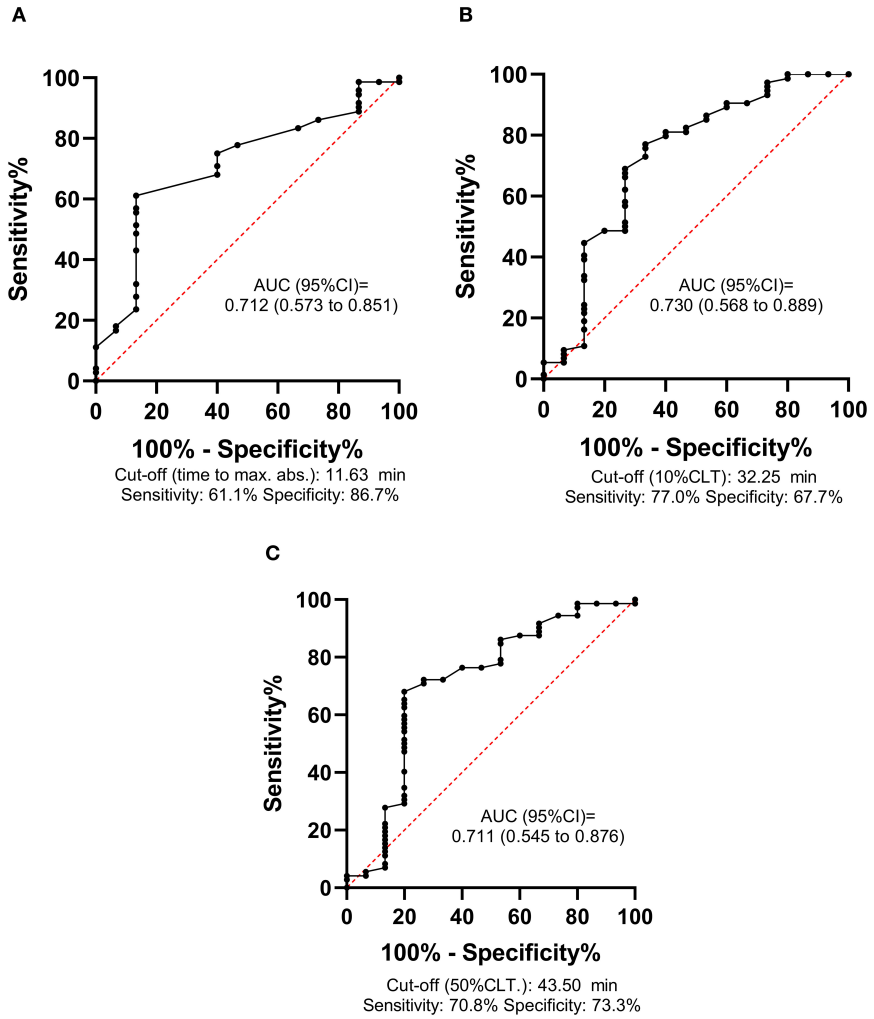

FIGURE 4 | Receiver operator characteristic (ROC) curves of the modified clot lysis assay (mCLA) parameters for predicting long-term functional outcomes (mRS 0–1 vs. 2–6) of intracerebral hemorrhage stroke patients. mCLA is performed in the presence of cell-free DNA and histones. ROC curve and descriptive statistics including best cut-off value as determined by the Youden index are depicted for time to maximal absorbance parameter **(A)**, 10% clot lysis time (10%CLT) **(B)**, 50% clot lysis time (50%CLT) **(C)**.

DISCUSSION

To our knowledge, the current study shows for the first time that a CLA might be a promising tool to predict the outcome of intracerebral hemorrhagic stroke. Despite the clear benefit of diagnostic tests with acceptable predictive value regarding outcomes in acute ICH stroke patients, surprisingly few studies are available on this topic. Here we show that mCLA parameters of patients on admission correlate with the estimated size of hematoma on admission, which is an important predictor of outcomes. Shorter clot formation and lysis times, indicating faster break-down of the newly formed clot were associated with larger hematoma volume, more severe stroke, and worse outcomes in this cohort. These results are in line with previous studies revealing that an elevated D-dimer, indicating a more extensive break-down of clots, is associated with adverse outcomes in patients with ICH (5–7).

Our results clearly show a potential effect of the distortion of fibrinolytic balance on the evolution of the intracerebral hematoma. Fibrinogen levels were within the normal range in this cohort, suggesting that fibrinolytic factors rather than fibrinogen itself might be involved in this process. On the other hand, FXIII activity, plasminogen and α2-PI activity did not show an association with outcomes, suggesting the presence of other fibrinolytic alterations. Gaining knowledge on the factors that drive the enlargement or the dissolution of the bleeding are potentially important when designing future pharmacological therapies. As a first step, it is crucial to understand the underlying pathomechanism leading to poor outcomes in patients, and adequate diagnostic tools are a pre-requisite of such approaches.

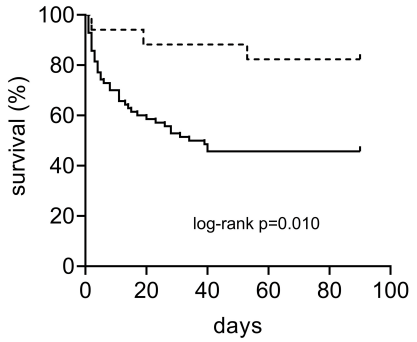

FIGURE 5 | Kaplan-Meier survival curves of patients with spontaneous intracerebral hemorrhage according to the result of the modified clot lysis assay on admission (10%CLT below and above the limit of 38.5 min).

TABLE 5 | Independent predictors of outcomes in the studied cohort.

	OR	95%CI	*p*
Mortality by day 14[§]			
NIHSS on admission	1.20	1.09–1.32	0.0001
INR	3.54	1.06–11.85	0.041
Mortality by day 90 (mRS 0–5 vs. mRS 6)[#]			
NIHSS on admission	1.17	1.07–1.28	0.001
INR	2.02	0.71–5.75	0.190
mCLA 10%CLT <38.5 min	3.69	0.84–16.15	0.083
Unfavorable long-term outcome (mRS 0–1 vs. mRS 2–6)[†]			
NIHSS on admission	1.57	1.21–2.05	0.001
mCLA 10%CLT <32.25 min	6.14	1.11–34.02	0.038

Last step of backward multiple regression analysis is provided.

[§]Backward multiple regression model included age, sex, NIHSS on admission, hypertension, INR, platelet count, smoking status, hemorrhage volume, mCLA 10%CLT (threshold: <30.25 min).

[#]Backward multiple regression model included age, sex, NIHSS on admission, hypertension, BMI, INR, platelet count, hemorrhage volume, mCLA 10%CLT (threshold: <38.5 min).

[†]Backward multiple regression model included age, sex, NIHSS on admission, hypertension, BMI, INR, platelet count, hemorrhage volume, mCLA 10%CLT (threshold: <32.25 min).

95%CI, 95% confidence interval; 10%CLT, 10% clot lysis time; INR, international normalized ratio; mCLA, modified clot lysis assay performed in the presence of cell-free DNA and histones; NIHSS, National Institutes of Health Stroke Scale; OR, odds ratio.

The CLA is a method that has been shown to be potentially useful to predict outcomes in a wide range of pathologies where the fibrinolytic balance has been tilted (14, 18–25). The assay has a list of benefits and limitations, and most importantly, optimal conditions of the test have not been clearly defined, as yet. An undisputable advantage of the assay is being a global test of clot formation and lysis, thus saving efforts to determine the levels of individual factors of fibrinolysis using laborous and time-consuming methods. On the other hand, despite attempts to standardize the test, analytical challenges remain to be elaborated. In our study, we aimed to improve the diagnostic performance of the assay by incorporating the effect of cellular components that are known to influence fibrinolysis. In our assay conditions of mCLA, optimal concentrations of cfDNA and histones were adapted from previous *in vitro* studies using purified fibrinogen and various concentrations of cfDNA and histones, testing their combined effect on fibrinolysis (16). It must be emphasized, that our primary goal was to find assay conditions where fibrinolysis kinetics are optimally influenced by the addition of cfDNA and histones. The mechanisms behind the observed effect of cfDNA and histones resulting in prolongation of clot lysis are complex (14, 37). Among others, the addition of cfDNA promotes the formation of densely packed networks of thick fibers less susceptible to plasmin digestion, while the addition of histones competitively inhibits plasmin and delays fibrinolysis. Assay conditions of the mCLA represent an increased pool of DNA and histones, likely to be present during *in vivo* clot formation, as published previously (16, 24). The source of cfDNA and histones in the intracerebral compartment could also originate from tissue death (38) and might have an important modulatory effect on coagulation and fibrinolysis. Using such assay conditions in this study, the diagnostic performance of the assay was found to be particularly good to predict unfavorable long-term outcomes. In a binary logistic regression model, a shorter 10%CLT of the mCLA (<32.25 min) proved to be an independent predictor of unfavorable long-term outcomes (mRS $\geq$ 2).

The CLA is a test with the potential to represent *in vivo* global hemostasis and fibrinolysis upon a few modifications. In future studies, assay conditions might be further improved and standardized, allowing direct comparison between laboratories. The ultimate goal in the development and standardization of the CLA and related global fibrinolytic assays will be to generate standardized assay conditions that lead to highly sensitive and specific tests that aid clinical decision making, while allowing interlaboratory comparison of larger datasets. Clinical studies testing the utility of various fibrinolysis tests in predicting the outcome of thrombotic events are scarcely found in the literature as yet. Our study, similarly to few previous studies testing fibrinolysis in patient cohorts, is a hypothesis-generating study to obtain insights whether fibrinolytic abnormalities are associated with poor outcomes in ICH patients (39–41). Further long-term follow up studies are warranted to verify our results and optimize assay conditions.

CONCLUSIONS

Parameters of the mCLA correlate well with ICH bleeding volume and could suggest unfavorable outcomes in spontaneous, non-traumatic ICH. Future studies including large cohorts of patients with ICH are warranted to further study the relevance of fibrinolysis alterations in the evolution of intracerebral hematoma and patient outcomes. Further modifications of the test might allow better diagnostic performance and easier implementation, which might be necessary for its potential clinical utilization in the future.

LIMITATIONS

Results of the present study should be interpreted in the context of its limitations and strengths. The sample size is limited,

however, as compared to other published prospective studies including consecutive patients with non-traumatic, spontaneous ICH, involving the measurement of hemostasis biomarkers from admission samples, it is among the largest studies as yet. Nevertheless, results presented here must be confirmed and validated by larger studies. The study was single-centered, which contributed to the limited sample size, but it had the advantages of uniform sample handling and patient care, and the major benefit that few patients were lost to follow-up. During follow-up only one patient acquired SARS-CoV-2 infection post-event, and in this case long-term follow-up results were excluded.

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 Ethics Committee of the University of Debrecen and the Ethics Committee of the National Medical Research Council. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RO-K collected clinical samples, performed experiments, and analyzed and interpreted data. TÁ, IF, KF, MH, and JT collected clinical data and analyzed and interpreted data. FS collected clinical samples and performed experiments. LC designed the research and analyzed and interpreted the data. ZB analyzed and interpreted the data and wrote the manuscript. All authors have 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.2021.613441/full#supplementary-material

Representative CLA curves and reference parameters of healthy controls as compared to patients are provided as **Supplementary Figure 1** and **Supplementary Table 1**. Associations between parameters of the conventional CLA and stroke severity or outcomes are shown in **Supplementary Tables 2–4**. Correlation between fibrinolysis parameters and clot lysis assay parameters in the absence (CLA) or presence of cfDNA and histones (mCLA) are provided in **Supplementary Table 5**.

REFERENCES

1. Pinho J, Costa AS, Araujo JM, Amorim JM, Ferreira C. Intracerebral hemorrhage outcome: a comprehensive update. *J Neurol Sci*. (2019) 398:54–66. doi: 10.1016/j.jns.2019.01.013
2. Qureshi AI, Tuhrim S, Broderick JP, Batjer HH, Hondo H, Hanley DF. Spontaneous intracerebral hemorrhage. N *Engl J Med*. (2001) 344:1450–60. doi: 10.1056/NEJM200105103441907
3. Zakai NA, Olson NC, Judd SE, Kleindorfer DO, Kissela BM, Howard G, et al. Haemostasis biomarkers and risk of intracerebral haemorrhage in the reasons for geographic and racial differences in stroke study. *Thromb Haemost*. (2017) 117:1808–15. doi: 10.1160/TH17-03-0189
4. Quinones-Hinojosa A, Gulati M, Singh V, Lawton MT. Spontaneous intracerebral hemorrhage due to coagulation disorders. *Neurosurg Focus*. (2003) 15:E3. doi: 10.3171/foc.2003.15.4.3
5. Chiu CC, Li YN, Lin LJ, Hsiao CT, Hsiao KY, Chen IC. Serum D-dimer as a predictor of mortality in patients with acute spontaneous intracerebral hemorrhage. *J Clin Neurosci*. (2012) 19:810–3. doi: 10.1016/j.jocn.2011.08.032
6. Hu X, Fang Y, Ye F, Lin S, Li H, You C, et al. Effects of plasma D-dimer levels on early mortality and long-term functional outcome after spontaneous intracerebral hemorrhage. *J Clin Neurosci*. (2014) 21:1364–7. doi: 10.1016/j.jocn.2013.11.030
7. Delgado P, Alvarez-Sabin J, Abilleira S, Santamarina E, Purroy F, Arenillas JF, et al. Plasma d-dimer predicts poor outcome after acute intracerebral hemorrhage. *Neurology*. (2006) 67:94–8. doi: 10.1212/01.wnl.0000223349.97278.e0
8. Lisman T, de Groot PG, Meijers JC, Rosendaal FR. Reduced plasma fibrinolytic potential is a risk factor for venous thrombosis. *Blood*. (2005) 105:1102–5. doi: 10.1182/blood-2004-08-3253
9. Siudut J, Natorska J, Wypasek E, Wiewiorka L, Ostrowska-Kaim E, Wisniowska-Smialek S, et al. Impaired fibrinolysis in patients with isolated aortic stenosis is associated with enhanced oxidative stress. *J Clin Med*. (2020) 9:2002. doi: 10.3390/jcm9062002
10. Gitto S, Romanelli RG, Cellai AP, Lami D, Vizzutti F, Abbate R, et al. Altered clot formation and lysis are associated with increased fibrinolytic activity in ascites in patients with advanced cirrhosis. *Intern Emerg Med*. (2020) 16:339–47. doi: 10.1007/s11739-020-02375-3
11. Pitkanen HH, Karki M, Niinikoski H, Tanner L, Nanto-Salonen K, Pikta M, et al. Abnormal coagulation and enhanced fibrinolysis due to lysinuric protein intolerance associates with bleeds and renal impairment. *Haemophilia*. (2018) 24:e312–21. doi: 10.1111/hae.13543
12. Bembenek JP, Niewada M, Siudut J, Plens K, Czlonkowska A, Undas A. Fibrin clot characteristics in acute ischaemic stroke patients treated with thrombolysis: the impact on clinical outcome. *Thromb Haemost*. (2017) 117:1440–7. doi: 10.1160/TH16-12-0954
13. Cieslik J, Mrozinska S, Broniatowska E, Undas A. Altered plasma clot properties increase the risk of recurrent deep vein thrombosis: a cohort study. *Blood*. (2018) 131:797–807. doi: 10.1182/blood-2017-07-798306
14. Varju I, Kolev K. Networks that stop the flow: a fresh look at fibrin and neutrophil extracellular traps. *Thromb Res*. (2019) 182:1–11. doi: 10.1016/j.thromres.2019.08.003
15. Varju I, Longstaff C, Szabo L, Farkas AZ, Varga-Szabo VJ, Tanka-Salamon A, et al. DNA, histones and neutrophil extracellular traps exert anti-fibrinolytic effects in a plasma environment. *Thromb Haemost*. (2015) 113:1289–98. doi: 10.1160/TH14-08-0669
16. Longstaff C, Varju I, Sotonyi P, Szabo L, Krumrey M, Hoell A, et al. Mechanical stability and fibrinolytic resistance of clots containing fibrin, DNA, and histones. *J Biol Chem*. (2013) 288:6946–56. doi: 10.1074/jbc.M112.404301

17. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science*. (2004) 303:1532–5. doi: 10.1126/science.1092385
18. Laridan E, Denorme F, Desender L, Francois O, Andersson T, Deckmyn H, et al. Neutrophil extracellular traps in ischemic stroke thrombi. *Ann Neurol*. (2017) 82:223–32. doi: 10.1002/ana.24993
19. Ducroux C, Di Meglio L, Loyau S, Delbosc S, Boisseau W, Deschildre C, et al. Thrombus neutrophil extracellular traps content impair tPA-induced thrombolysis in acute ischemic stroke. *Stroke*. (2018) 49:754–7. doi: 10.1161/STROKEAHA.117.019896
20. Hisada Y, Grover SP, Maqsood A, Houston R, Ay C, Noubouossie DF, et al. Neutrophils and neutrophil extracellular traps enhance venous thrombosis in mice bearing human pancreatic tumors. *Haematologica*. (2020) 105:218–25. doi: 10.3324/haematol.2019.217083
21. Fuchs TA, Brill A, Wagner DD. Neutrophil extracellular trap (NET) impact on deep vein thrombosis. *Arterioscler Thromb Vasc Biol*. (2012) 32:1777–83. doi: 10.1161/ATVBAHA.111.242859
22. Thalin C, Hisada Y, Lundstrom S, Mackman N, Wallen H. Neutrophil extracellular traps: villains and targets in arterial, venous, and cancer-associated thrombosis. *Arterioscler Thromb Vasc Biol*. (2019) 39:1724–38. doi: 10.1161/ATVBAHA.119.312463
23. Valles J, Lago A, Santos MT, Latorre AM, Tembl JI, Salom JB, et al. Neutrophil extracellular traps are increased in patients with acute ischemic stroke: prognostic significance. *Thromb Haemost*. (2017) 117:1919–29. doi: 10.1160/TH17-02-0130
24. Fuchs TA, Brill A, Duerschmied D, Schatzberg D, Monestier M, Myers DD, Jr., et al. Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci USA*. (2010) 107:15880–5. doi: 10.1073/pnas.1005743107
25. Geddings JE, Mackman N. New players in haemostasis and thrombosis. *Thromb Haemost*. (2014) 111:570–4. doi: 10.1160/TH13-10-0812
26. Kothari RU, Brott T, Broderick JP, Barsan WG, Sauerbeck LR, Zuccarello M, et al. The ABCs of measuring intracerebral hemorrhage volumes. *Stroke*. (1996) 27:1304–5. doi: 10.1161/01.STR.27.8.1304
27. Brott T, Adams HP Jr., Olinger CP, Marler JR, Barsan WG, Biller J, et al. Measurements of acute cerebral infarction: a clinical examination scale. *Stroke*. (1989) 20:864–70. doi: 10.1161/01.STR.20.7.864
28. Hemphill JC III, Bonovich DC, Besmertis L, Manley GT, Johnston SC. The ICH score: a simple, reliable grading scale for intracerebral hemorrhage. *Stroke*. (2001) 32:891–7. doi: 10.1161/01.STR.32.4.891
29. van Swieten JC, Koudstaal PJ, Visser MC, Schouten HJ, van Gijn J. Interobserver agreement for the assessment of handicap in stroke patients. *Stroke*. (1988) 19:604–7. doi: 10.1161/01.STR.19.5.604
30. Banks JL, Marotta CA. Outcomes validity and reliability of the modified Rankin scale: implications for stroke clinical trials: a literature review and synthesis. *Stroke*. (2007) 38:1091–6. doi: 10.1161/01.STR.0000258355.23810.c6
31. Talens S, Malfliet JJ, Rudez G, Spronk HM, Janssen NA, Meijer P, et al. Biological variation in tPA-induced plasma clot lysis time. *Thromb Haemost*. (2012) 108:640–6. doi: 10.1160/TH12-02-0124
32. Pieters M, Philippou H, Undas A, de Lange Z, Rijken DC, Mutch NJ, Subcommittee on factor X, fibrinogen, the subcommittee on F. An international study on the feasibility of a standardized combined plasma clot turbidity and lysis assay: communication from the SSC of the ISTH. *J Thromb Haemost*. (2018) 16:1007–12. doi: 10.1111/jth.14002
33. Posch F, Hofer S, Thaler J, Hell L, Konigsbrugge O, Grilz E, et al. *Ex vivo* properties of plasma clot formation and lysis in patients with cancer at risk for venous thromboembolism, arterial thrombosis, and death. *Transl Res*. (2020) 215:41–56. doi: 10.1016/j.trsl.2019.08.009
34. Longstaff C, Subcommittee on Fibrinolysis. Development of Shiny app tools to simplify and standardize the analysis of hemostasis assay data: communication from the SSC of the ISTH. *J Thromb Haemost*. (2017) 15:1044–6. doi: 10.1111/jth.13656
35. Chesher D. Evaluating assay precision. *Clin Biochem Rev*. (2008) 29(Suppl. 1):S23–6.
36. Clinical and Laboratory Standards Institute. *Evaluation of Precision of Quantitative Measurement Procedures; Approved Guideline-Third Edition. CLSI Document EP05-A3*. Wayne, PA: Clinical and Laboratory Standards Institute (2014).
37. Locke M, Longstaff C. Extracellular histones inhibit fibrinolysis through noncovalent and covalent interactions with fibrin. *Thromb Haemost*. (2020) 121:464–76. doi: 10.1055/s-0040-1718760
38. Rodrigues Filho EM, Simon D, Ikuta N, Klovan C, Dannebrock FA, Oliveira de Oliveira C, et al. Elevated cell-free plasma DNA level as an independent predictor of mortality in patients with severe traumatic brain injury. *J Neurotrauma*. (2014) 31:1639–46. doi: 10.1089/neu.2013.3178
39. Fraczek P, Krzysztofik M, Stanisz A, Undas A. Clinical outcomes and plasma clot permeability and lysability in patients with venous thromboembolism on rivaroxaban: a cohort study. *Pol Arch Intern Med*. (2019) 129:377–85. doi: 10.20452/pamw.14824
40. Undas A. Altered fibrin clot properties and fibrinolysis in patients with atrial fibrillation: practical implications. *Europace*. (2020) 22:185–94. doi: 10.1093/europace/euz271
41. Kleinegris MF, Konings J, Daemen JW, Henskens Y, de Laat B, Spronk HMH, et al. Increased clot formation in the absence of increased thrombin generation in patients with peripheral arterial disease: a case-control study. *Front Cardiovasc Med*. (2017) 4:23. doi: 10.3389/fcvm.2017.00023

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Sex Differences in Risk Factors for Transient Ischemic Attack in a Chinese Population

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Introduction: This study aimed to collect and evaluate basic information of a stroke screening population in eastern China and to compare distribution differences in risk factors between males and females in a transient ischemic attack (TIA) population.

Methods: A standardization of the risk factors for stroke was performed according to an implementation plan of stroke in a high-risk population screening and intervention project in Shandong Province. Of the 231,289 residents, 8,603 patients with a previous TIA were identified and risk factors in this cohort were analyzed for sex differences.

Results: In our initial cohort of 231,289 residents, we found 3,390 men and 5,213 women with TIA, accounting for a prevalence of 3.1 and 4.2%, respectively. Risk factors for TIA were hypertension, atrial fibrillation, diabetes, smoking, lack of exercise, overweight, and family history of stroke. In our TIA cohort, we found that the prevalence of smoking was significantly higher in men (41.3%) compared with that found in women (4.2%). Further, hypertension (58.8 vs. 55.5%) and family history of stroke (22.3 vs. 20.0%) were more prevalent in men compared with women, whereas atrial fibrillation (AF) (14.7 vs. 16.4%), diabetes (11.1 vs. 13.2%), lack of exercise (27.2 vs. 28.0%), and overweight (29.5 vs. 35.7%) were less prevalent.

Conclusions: In our TIA cohort from eastern China, we found significant sex differences for the risk factors of hypertension, atrial fibrillation, smoking, diabetes, and overweight.

Keywords: distributional differences, Eastern China, transient ischemic attack population, risk factors, stroke, sex differences

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INTRODUCTION

Stroke is a severe disease of global significance with a high disability rate and high incidence (1). In the past, transient ischemic attack (TIA) was defined as any focal ischemic event with symptoms lasting 24 h. Stroke 2009 mated that a revision of TIA definitions to"a transient episode of neurological dysfunction caused by focal brain, spinal cord, or retinal ischemia without acute infarction" (2). As TIA is a significant predictor of subsequent ischemic stroke (3), it has been hypothesized that there may be sex differences in the incidence of TIA over time. Further, because TIA constitutes a major risk factor for stroke, patients with TIA are an important group for secondary intervention. The fact that diabetes mellitus, atrial fibrillation, and smoking are more common in individuals with TIA compared with the general population suggests that these factors are risk factors for TIA (4). In addition, as a risk factor and precursor of cerebral infarction, TIA is of high clinical value. Therefore, understanding the risk factors of TIA in the context of stroke risk

will enable timely and effective implementation of preventive countermeasures, thereby reducing the probability that a TIA event leads to infarction. There are also substantial sex differences in age-adjusted stroke incidence and stroke prevalence as well as in the prevalence and risk of various cardiometabolic factors, including hypertension, atrial fibrillation, diabetes mellitus, and smoking (5). An improved understanding of these differences is needed to ensure that stroke prevention strategies are effective for both women and men.

It has been reported that sex differences contribute to differences in the prevalence and/or control of TIA risk factors (5). One study reported that ischemic stroke incidence has declined over time for men but not women. In males, they found that incidence decreased from 153 per 100,000 (95% confidence interval [CI] 139–167) in 1993 and 1994 to 117 per 100,000 (95% CI 107–128) in 2010 ($P < 0.05$ for trend test), with no significant change observed among females for the same period [107 vs. 102 per 100,000 (95% CI 97–116 and 94–111, respectively; $P > 0.05$)] (6). The recently reported decreases in TIA are likely a result of improved control of risk factors, including hypertension, diabetes, dyslipidemia, and smoking (7). Because of known sex differences in risk factor profiles, a better understanding of temporal patterns in the prevalence of TIA risk factors by sex is necessary to reduce TIA incidence and mortality in both sexes.

The purpose of this study was to determine whether there were sex differences in the distribution of risk factors among a population of individuals with TIA from eastern China and provide further recommendations for stroke management and prevention in high-risk populations.

SUBJECTS AND METHODS

Ethics Statement

The study was conducted according to the guidelines of the Helsinki Declaration. Ethical approval was obtained prior to the start of the study from the Ethics Committee of Shandong Provincial Hospital affiliated to Shandong University, China. Written informed consent was obtained from all participants.

Study Population

The study population and proportion of the population to screen were determined according to specific criteria. First, provinces in China were selected based on accessibility and feasibility for long-term follow up of high-risk groups. Second, the proportion of the target population for screening in each region was determined based on the Sixth National Population Census of the urban and rural resident population for all persons >40 years of age. Proportional screening was undertaken according to the age distribution and sex ratio of the Sixth National Population Census of the population in each province. Further, based on the Census definition of a permanent resident population, we classified the population in a city divided into districts and the entire population in a city not divided into districts as urban residents, and the rest of the population as rural residents. Lastly, cluster sampling was used in 18 urban regions and 18 rural regions of Shandong province as a representative province of eastern China, characterized by a large population and rapid economic development.

The number of individuals screened within each region was no fewer than 6,000. We identified 231,289 (108,230 men and 123,059 women) permanent residents over 40 years old (date of birth between January 1, 1937 and December 31, 1971) in our screened regions who were eligible to participate in our study. Residents living for ≥6 months in an area were also included in our study, but recent migrants between urban and rural areas were not considered for inclusion. We selected 8,603 participants (3,390 men and 5,213 women) who had accurate data on a diagnosis of transient ischemic attack (TIA) in a medical institution above the township level. The prevalence of TIA was 3.1% in men and 4.2% in women.

Data Collection

All data were collected using a nationally agreed questionnaire. The survey questionnaire included: (1) basic information about the respondent(s), including age, gender, BMI, exercise status, community, education level and smoking history; (2) incidence or family history of hypertension, atrial fibrillation, diabetes grade, and stroke; (3) whether TIA was diagnosed by a medical institution above the township level, and whether there are symptoms of transient neurological impairment at the time of onset, including transient dizziness, visual rotation, limb weakness or numbness, language disorders, and coughing while drinking water. Duration, number of TIA, and time of the last TIA were also recorded. If the respondent could accurately provide a basis for the diagnosis of TIA in a medical institution above the township level, we diagnosed the patient as suffering from TIA.

Assessment Criteria

This study was conducted to evaluate the use of symptom duration of 24 h or less as a segmentation point for the diagnosis of TIA. Previously in 2009, the American heart association and the American stroke association (ASA/AHA) had published new guidelines for TIA diagnosis. However, community epidemiology studies routinely use the histologic standard for diagnosis, and given the results of previous epidemiological studies on international comparability of data, we chose to adopt symptom duration of 24 h or less for the diagnosis of TIA. Patients with a previous TIA episode, irrespective of whether it was the first episode or a recurrence, were included in the diagnosis and patients with a simultaneous stroke episode were also categorized as TIA, regardless of whether the stroke occurred before or after the TIA.

RF1: hypertension, defined as a history of high blood pressure (≥140/90 mmHg) reported by the participant or the current use of antihypertensives. RF2: Atrial Fibrillation (AF) reported by the participant or indicated by electrocardiogram. RF3: diabetes mellitus defined by previous diagnosis, treatment with insulin/oral hypoglycemic medications, or a fasting plasma glucose level ≥126 mg/dL or glycosylated hemoglobin ≥6.5%. RF4: smoking defined by either the current or former practice of smoking. RF5: lack of physical exercise defined as physical exercise <3 times a week and each <30 min in duration

(industrial and agricultural labor was considered as physical exercise). RF6: overweight, defined as a body mass index ≥25 kg/m2. RF7: self-reported family history of stroke.

Statistical Analysis

Descriptive characteristics of study subjects according to differences in sex or residence are reported as percentages for categorical variables and mean ± standard deviation for continuous variables. Student's *t*-test was performed to assess differences in age, whereas Chi-square test was used to compare frequencies of education level and TIA risk assessments between men and women. Binary logistic regression modeling was used to estimate associations between sex and hypertension, diabetes mellitus, atrial fibrillation, smoking, lack of exercise, overweight, and family history of stroke after adjusting for age and degree of education. Results are expressed as multivariable-adjusted odds ratios (ORs) and 95% CI. A two-sided *P*-value < 0.05 was considered statistically significant. All data analyses were performed using SPSS 25.0.

RESULTS

Descriptive characteristics of our study population are shown in **Table 1**. Our analysis cohort consisted of 8,603 individuals with TIA from Shandong province in eastern China, of which 3,390 (39.4%) were male and 5,213 (60.6%) were female with an average age of 61.9 ± 9.9 and 60.75 ± 9.9 years, respectively. We found that the mean age difference of TIA occurrence and education level between males and females were significantly different ($P < 0.001$), although there was no evidence of a sex difference in urban-rural distribution ($P = 0.063$). The age of TIA occurrence and education level in men were higher compared with those of women in our cohort.

Next, the distribution of different TIA risk factors were analyzed between men and women (**Tables 2**, **3**). As shown in **Table 2**, we found that hypertension was the most common risk factor, with a higher prevalence in men (58.8%) compared with women (55.5%). Further, smoking was the risk factor with the greatest difference between sexes, in which 41.3% of men smoked compared with 4.2% of women. With the exception of exercise deficiency ($P = 0.385$), we also found evidence of increased rates of other TIA risk factors namely, hypertension and family history of stroke (58.8 and 22.3%, respectively) in men compared with women (55.5 and 20.0%, respectively). In contrast, the incidence of atrial fibrillation, diabetes, and overweight was higher in women (16.4, 13.2, and 35.7%, respectively) compared with that found in men (14.7, 11.1, and 29.5%, respectively). Using multivariate analysis (**Table 3**), we found significant differences between men and women in the distribution of four TIA risk factors specifically, hypertension (OR = 0.865, 95% CI 0.791–0.984), atrial fibrillation (OR = 1.230, 95% CI 1.087–1.392), diabetes (OR = 0.786, 95% CI 0.684–0.901), and overweight (OR = 0.754, 95% CI 0.686–0.830). In addition, among the variables included in logistic regression, we found that hypertension and smoking were more common in men.

We also studied gender differences in the prevalence of risk factors in the non-TIA population (**Table 4**) and compared it with the TIA population (**Table 5**). In addition to smoking (32.2 vs. 2.2%), hypertension (19.7 vs. 22.5%), atrial fibrillation (2.5 vs. 3.6%), diabetes (4.0 vs. 5.3%), reduced physical activity (13.6 vs. 16.7%), and prevalence of overweight (20.5 vs. 27.5%), a family history of stroke (6.8 vs. 7.4%) was more common in women than in men. Further, analysis of combined data provided in **Tables 4**, **5** showed that the prevalence of risk factors, in both men and women, was higher in the TIA group than in the non-TIA group. However, family history of stroke and hypertension were more common in men than women in the TIA group, whereas a greater number of women were present in the TIA group.

DISCUSSION

After adjusting for multiple cofactors, we found significant differences in the distribution of a number of risk factors for TIA namely, hypertension, atrial fibrillation, smoking, overweight, and diabetes, between men and women. In the current study, the mean age of males was 61.9 years compared to 60.7 years in women. The prevalence of TIA in men and women was 3.1 and 4.2%, respectively, with a higher prevalence in women than in men. Concurrently, previous studies have also reported that the lower incidence of stroke in women disappeared with increasing age (8). Women over 80 have higher mortality and poorer functional outcomes compared to age-matched men after stroke. Previous studies have reported that women have a longer life expectancy than men, and that women are 4–6 years older than men at the onset of stroke, which may account for their higher disability rate and poorer prognosis (9, 10). TIA prevalence rates vary, depending on the age distribution of the study population. Other studies have found that a prevalence of TIA in men of 2.7% for 65 to 69 years of age and 3.6% for 75 to 79 years of age. For women, TIA prevalence was 1.6% for 65 to 69 years of age and 4.1% for 75 to 79 years of age (11). Similarly, a population-based study in Sweden found stroke incidence to be lower among women than men at ages 55–64 years; however, this association was reversed among individuals aged 75–85 years as incidence was higher among women than men (12). Other studies have reported contrasting results, with higher stroke risk seen in men compared to women, which then persisted after midlife or diminished, but never reversed with age (13). Our findings from the current study differ from those previously reported, which may be accounted for by population or ethnicity differences. In contrast to these earlier studies that tested other populations, our study investigated a Chinese cohort. In studies of gender differences in pre-hospital and hospitalization delays, women arrived both later than (14) and earlier than men (15). One study found that 40% of women arrived within 3 h of onset compared with 47% of men (14). However, there was no conclusive evidence on clinically important differences in pre-hospital delay between women and men.

Although stroke mortality and case fatality have declined in eastern China, improvements in standard of living has resulted in poorer control of risk factors, such as hypertension, diabetes mellitus, obesity, and smoking. In the current study,

TABLE 1 | Descriptive characteristics of the study cohort.

Risk factors	Male	Female	t/x^2	*P**
Age	61.9 ± 9.90	60.75 ± 9.93	5.226	0.000
Education level			243.729	0.000
≤Primary school	1,866 (55.0%)	3,722 (71.4%)		
Middle school	1,043 (30.8%)	1,040 (20.0)		
High school	360 (10.6%)	350 (6.7%)		
University	118 (3.5%)	100 (1.9%)		
Master's degree	3 (0.1%)	1 (0%)		
Group			3.448	0.063
Urban	1,660 (49.0%)	2,446 (46.9%)		
Rural	1,730 (31.0%)	2,767 (53.1%)		

Values are mean (SD) or percentages.
**Student's t-test was used for comparison of mean values and x^2 test was used for comparison of proportions.*

TABLE 2 | Risk factors for transient ischemic attack (TIA) in men and women.

Risk factors	Male	Female	t/x^2	*P*
Hypertension	1,993 (58.8%)	2,893 (55.5%)	9.087	0.003
Atrial fibrillation	498 (14.7%)	856 (16.4%)	4.637	0.031
Smoking	1,400 (41.3%)	220 (4.2%)	847.593	0.000
Diabetes	373 (11.1%)	686 (13.2%)	8.358	0.004
Sport lack	972 (27.2%)	1,462 (28.0%)	0.755	0.385
Overweight	1,001 (29.5%)	1,863 (35.7%)	35.666	0.000
Family history of stroke	757 (22.3%)	1044 (20.0%)	6.5855	0.01

TABLE 3 | Associations between risk factors for transient ischemic attack and sex in our study cohort.

Risk factors	*P*	OR	95%CI
Hypertension	0.002	0.865	0.791–0.984
Atrial fibrillation	0.001	1.230	1.087–1.392
Smoking	0.000	17.064	14.614–19.924
Diabetes	0.001	0.786	0.685–0.901
Overweight	0.000	0.754	0.686–0.830
Family history of stroke	0.079	1.103	0.989–1.231

TABLE 4 | Risk factors for non-TIA in men and women.

Risk factors	Male	Female
Hypertension	20,683 (19.7%)	26,491 (22.5%)
Atrial Fibrillation	2,610 (2.5%)	4,270 (3.6%)
Smoking	33,757 (32.2%)	2,627 (2.2%)
Diabetes	4,225 (4.0%)	6,261 (5.3%)
Sport lack	14,287 (13.6%)	19,626 (16.7%)
Overweight	21,449 (20.5%)	32,420 (27.5%)
Family history of stroke	7,173 (6.8%)	8,750 (7.4%)

hypertension were found to be the most common risk factor in the TIA population. The incidence of hypertension was 58.8% in males and 55.5% in females and there was a significant difference between males and females in the distribution of Chinese Population (OR = 0.8650, 95% CI 0.791–0.984). In the non-TIA population, the incidence of hypertension was 19.7% in men and 22.5% in women. Hypertension is the most common modifiable stroke risk factor known to differ in prevalence, rate of control, and degree of associated stroke risk between women and men (16). Studies have shown that blood pressure is higher in men than women of similar ages (17); however, the import of sex differences in the prevalence of hypertension may be more complicated. A different study reported that prevalence is lower in women compared with men <60 years of age, but higher in women after that time point (18). Further, women in older age groups are less likely to control their hypertension compared with men (19). Whereas, data from UK found that women were less likely to be discharged on dual antiplatelet therapy, angiotensin-converting enzyme inhibitors, or statins (20). We hypothesized that women with low adherence to medication may be at greater risk for TIA in the presence of hypertension. A recent Chinese study found evidence of a strong association between hypertension and stroke risk in older women (OR = 6.73, 95% CI 2.14–21.15) compared with older men (OR = 3.18, 95% CI 1.65–6.14) (21). First, biological, genetic, or hormonal mechanisms linking hypertension to vascular dysfunction and

TABLE 5 | Risk factors in the non-TIA population compared with the TIA population.

	TIA		Non-TIA	
Prevalence rates	**Male**	**Female**	**Male**	**Female**
Hypertension	58.8%	55.5%	19.7%	22.5%
Atrial Fibrillation	14.7%	16.4%	2.5%	3.6%
Smoking	41.3%	4.2%	32.2%	2.2%
Diabetes	11.1%	13.2%	4.0%	5.3%
Sport lack	27.2%	28.0%	13.6%	16.7%
Overweight	29.5%	35.7%	20.5%	27.5%
Family history of stroke	22.3%	20.0%	6.8%	7.4%

disease may differ between the sexes (22). It is also possible that there are sex differences in hypertension treatment and adherence, which leads to increased stroke risk for women.

In our study, we also found that smoking (OR = 17.064, 95% CI 14.614–19.924) was more frequent in men. Among non-TIA subjects, men were more likely to smoke than women (32.2 vs. 2.2%) Whereas atrial fibrillation (OR = 1.230, 95% CI 1.087-1.392), diabetes (OR = 0.786, 95% CI 0.685–0.901), and overweight (OR = 0.754, 95% CI 0.686–0.830) were more frequent in women. These findings are consistent with those of previous studies that reported unhealthy lifestyle habits, such as smoking, drinking, and eating a diet high in salt and fat, were more pronounced in men (23). Impaired endogenous fibrinolysis and reduced blood flow in the brain attributable to vasoconstriction by smoking are also associated with lacunar stroke development (24). Studies have shown that continuous constriction of blood vessels can lead to high blood pressure, and that smokers with high blood pressure have a 5-fold greater relative risk of stroke compared to smokers with normal blood pressure. This risk increases to 20-fold higher in smokers with high blood pressure (25). Generally, published evidence supports a similar association between hypertension and stroke risk for men and women, and a stronger association with diabetes and atrial fibrillation for women (26). Atrial fibrillation is more prevalent among elderly people, and female stroke patients are older at the time of their first TIA compared to men (27). Women with atrial fibrillation have nearly double the risk of stroke than men with the same risk factor (28). Use of warfarin anticoagulation for thrombo-embolic prophylaxis in patients with atrial fibrillation has been shown to be less common in women than in men (29). Several studies have also shown that women are generally at a higher risk for atrial fibrillation-related cardioembolic stroke (30). The underlying reason for the increased risk for stroke in women with atrial fibrillation is not fully known (31), although a study from the Swedish Stroke Register has shown that female patients with atrial fibrillation receive oral anticoagulant therapy less often than men (32). The higher prevalence of embolic strokes among women may to a large part explain their higher stroke severity.

Although both diabetes and metabolic syndrome (the clustering of obesity, abdominal obesity, dyslipidaemia, hypertension, and high plasma glucose) are recognized to increase the risk of ischaemic stroke in men and women (17). However, the study indicated that both risk factors, namely diabetes and metabolic syndrome affect women more (33). In our study, the prevalence of diabetes in the TIA group was 11.15% in men and 13.2% in women, while in the non-TIA group it was only 4.0% in men and 5.3% in women. The prevalence of overweight in men and women in the TIA group was 29.5 and 35.7%, respectively, and these values were 20.5 and 27.5%, respectively, in the non-TIA group. In both groups, compared to men, women showed a greater prevalence of diabetes and overweight population-based study in Denmark found that type 2 diabetes doubled the risk of stroke in men across all age groups, whereas in women the effect of diabetes on stroke risk was significantly higher (risk ratio [RR] 2.5–6.5) (34). A recent study found that metabolic syndrome doubled the risk of ischaemic stroke in women but had no effect in men (35). The increase in TIA can be associated with a rise of obesity and other metabolic-associated diseases as a result of significant lifestyle changes in the last several decades; a phenomenon that warrants our continued attention. Moreover, the association between TIA and body mass index (BMI) is well-established (36). A study from the United Kingdom found that a higher BMI is associated with increased risk of ischemic stroke in women (37). Our study found that, compared to men, a greater number of women reported physical inactivity, in both TIA and non-TIA groups. The protective effect of physical activity may be partly mediated by its role in reducing blood pressure and controlling other risk factors, such as diabetes and excess body weight (38). We propose that policy strategies to prevent TIA should include educating people on the importance of a healthy balanced lifestyle, especially among women.

In addition to these risk factors, other aspects such as hormonal status, pregnancy, and migraine headaches put women at greater risk than men. The results of this meta-analysis indicate that people with migraine are at an increased risk of ischemic stroke, and that this increased risk is only apparent in those who have migraines with aura and not in those with migraines without an aura (39). Additionally, these results suggest an ∼2-fold higher risk of migraine among women compared to men, and factors that further increased their risk of ischemic stroke were age <45 years, smoking, and use of oral contraceptives. Among people with migraine, the risk of TIA appeared to be higher than their risk of ischemic stroke. Pre-menopausal estrogen in women may have a protective effect on the heart and cerebrovascular systems, but understandably, the risk of microemboli caused by migraines, birth control pills, menopause, and other risk factors is higher in women than in men (40). It is now well-recognized that estradiol plays a vital role in the ischemic brain. Animal models of experimental stroke have shown that estrogen (17-βestradiol) is a robust neuroprotective agent in both males and females (41). This may be one reason why the incidence of TIA in pre-menopausal women is lower than in men. But Among healthy post-menopausal women in the Women's Health Initiative (WHI) study, exogenous estrogen increased the risk of stroke (42). Some risk factors are specific to women of reproductive age. A recent meta-analysis concluded that oral

contraceptives increased the risk of ischemic stroke by nearly 3-fold (RR 2.75,95%CI 2.24–3.38) (43). Pregnancy can lead to hemostatic changes, including increased levels of clotting factors, decreased levels of anticoagulants, and fibrinolytic activity, all of which can increase risk of thrombosis. However, the overall incidence of pregnancy-related strokes was low (44).

Regarding TIA risk factors as a whole, the underlying causes that lead to the observed sex-specific differences may be accounted for by several points. First, men are more likely to be affected by bad habits, which may lead to an earlier onset of hypertension compared to women. Second, underlying biological factors such as hormonal mechanisms or genetics may confer a higher susceptibility to diabetes or atrial fibrillation in women. Furthermore, in our cohort we found that women have a lower education level than men, which may contribute to a lack of public knowledge regarding TIA as well as poor drug compliance for the management of risk factors (45).

Major strengths of the current study are the large representative sample from eastern China, the identification of all relevant TIA risk factors, and a thorough statistical analysis in our investigation to determine whether there was evidence of sex differences in TIA risk factors in our cohort. However, there are several limitations that should be considered in the context of our findings. First, the scope of our study was restricted by the age of our participants. Individuals $\geq$40 but $<$80 years of age were included in our cohort, thereby preventing a more thorough analysis of age-related changes in the adult population. Second, there is a possible selection bias of the screening population because of geographic location, society, and structure of the questionnaire survey. Third, binary logistic regression modeling was implemented to analyze risk factors independently, without considering the possibility of interactions between them. In addition, we are unable to conduct further in-depth analyses of certain risk factors.

Nevertheless, despite the aforementioned limitations, our study significantly contributes to a better understanding of TIA populations in eastern China by identifying and characterizing sex differences in TIA risk factors. Our findings demonstrate that there are significant sex differences in most of the risk factors examined, with increased rates of atrial fibrillation, diabetes, and overweight found in women and increased prevalence of hypertension and smoking in men. A better understanding of the sex differences and underlying temporal patterns in the prevalence of stroke risk factors are necessary to develop strategies to reduce stroke incidence and mortality in both sexes, such as greater promotion of good living habits in men and increased awareness of stroke among women. An improved understanding of such differences are needed to ensure that TIA prevention strategies are effective for both women and men.

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 Shandong Provincial Hospital affiliated to Shandong First Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WW: investigation, data curation, writing-reviewing, and editing. PS: investigation, conceptualization, methodology. FH: data curation and writing-original draft preparation. CQ: writing-reviewing, editing, and supervision. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REFERENCES

1. Li J, Wang Y. Blood biomarkers in minor stroke and transient ischemic attack. *Neurosci Bull.* (2016) 32:463–8. doi: 10.1007/s12264-016-0038-5
2. Easton JD, Saver JL, Albers GW, Alberts MJ, Chaturvedi S, Feldmann E, et al. Definition and evaluation of transient ischemic attack: a scientific statement for healthcare professionals from the American heart association/American Stroke association stroke council; council on cardiovascular surgery and anesthesia; council on cardiovascular radiology and intervention; council on cardiovascular nursing; and the interdisciplinary council on peripheral vascular disease. The American academy of neurology affirms the value of this statement as an educational tool for neurologists. *Stroke*. (2009) 40:2276–93. doi: 10.1161/STROKEAHA.108192218
3. Kleindorfer D, Panagos P, Pancioli A, Khoury J, Kissela B, Woo D, et al. Incidence and short-term prognosis of transient ischemic attack in a population-based study. *Stroke.* (2005) 36:720–3. doi: 10.1161/01.STR.0000158917.59233b7
4. Ström JO, Tavosian A, Appelros P. Cardiovascular risk factors and TIA characteristics in 19,872 Swedish TIA patients. *Acta Neurologica Scandinavica.* (2016) 134:427–33. doi: 10.1111/ane12560
5. Madsen TE, Howard VJ, Jiménez M, Rexrode KM, Acelajado MC, Kleindorfer D, et al. Impact of conventional stroke risk factors on stroke in women: an update. *Stroke.* (2018) 49:536–42. doi: 10.1161/STROKEAHA.117018418
6. Madsen TE, Khoury J, Alwell K, Moomaw CJ, Rademacher E, Flaherty ML, et al. Sex-specific stroke incidence over time in the greater cincinnati/Northern Kentucky stroke study. *Neurology.* (2017) 89:990–6. doi: 10.1212/WNL0000000000004325
7. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, et al. Heart disease and stroke statistics—2014 update: a report from the American Heart Association. *Circulation*. (2014) 129:e28–292.
8. Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, et al. heart disease and stroke statistics—2011 update: a report from the American heart association. *Circulation*. (2011) 123:e18–209. doi: 10.1161/CIR0b013e3182009701

9. Baghshomali S, Bushnell C. Reducing stroke in women with risk factor management: blood pressure and cholesterol. *Womens Health.* (2014) 10:535–44. doi: 10.2217/WHE.1447
10. Kes VB, Jurašić MJ, Zavoreo I, Lisak M, Jelec V, Matovina LZ. Age and gender differences in acute stroke hospital patients. *Acta Clin Croatica.* (2016) 55:69–78. doi: 10.20471/acc.2016.55.0111
11. Price TR, Psaty B, O'Leary D, Burke G, Gardin J. Assessment of cerebrovascular disease in the Cardiovascular Health Study. *Ann Epidemiol.* (1993) 3:504–7. doi: 10.1016/1047-2797(93)90105-D
12. Löfmark U, Hammarström A. Evidence for age-dependent education-related differences in men and women with first-ever stroke. Results from a community-based incidence study in northern Sweden. *Neuroepidemiology.* (2007) 28:135–41. doi: 10.1159/000102141
13. Vega T, Zurriaga O, Ramos JM, Gil M, Alamo R, Lozano JE, et al. Stroke in Spain: epidemiologic incidence and patterns; a health sentinel network study. *J Stroke Cerebrovasc Dis.* (2009) 18:11–6. doi: 10.1016/j.jstrokecerebrovasdis.2008.06010
14. Menon SC, Pandey DK, Morgenstern LB. Critical factors determining access to acute stroke care. *Neurology.* (1998) 51:427–32. doi: 10.1212/WNL.51.2427
15. Lacy CR, Suh DC, Bueno M, Kostis JB. Delay in presentation and evaluation for acute stroke: stroke time registry for outcomes knowledge and epidemiology (S.T.R.O.K.E.). *Stroke.* (2001) 32:63–9. doi: 10.1161/01.STR.32.163
16. Madsen TE, Howard G, Kleindorfer DO, Furie KL, Oparil S, Manson JE, et al. Sex differences in hypertension and stroke risk in the regards study: a longitudinal cohort study. *Hypertension (Dallas, Tex.: 1979).* (2019) 74:749–55. doi: 10.1161/HYPERTENSIONAHA.11912729
17. Goldstein LB, Adams R, Alberts MJ, Appel LJ, Brass LM, Bushnell CD, et al. Primary prevention of ischemic stroke: a guideline from the American heart association/American stroke association stroke council: cosponsored by the atherosclerotic peripheral vascular disease interdisciplinary working group; cardiovascular nursing council; clinical cardiology council; nutrition, physical activity, and metabolism council; and the quality of care and outcomes research interdisciplinary working group. *Circulation.* (2006) 113:e873–923. doi: 10.1161/01.STR.0000223048.70103F1
18. Yoon SS, Carroll MD, Fryar CD. Hypertension prevalence and control among adults: United States, 2011-2014. *NCHS Data Brief.* (2015) 220:1–8.
19. Daugherty SL, Masoudi FA, Ellis JL, Ho PM, Schmittdiel JA, Tavel HM, et al. Age-dependent gender differences in hypertension management. *J Hypertens.* (2011) 29:1005–11. doi: 10.1097/HJH0b013e3283449512
20. McInnes C, McAlpine C, Walters M. Effect of gender on stroke management in Glasgow. *Age Age.* (2008) 37:220–2. doi: 10.1093/ageing/afm153
21. Lai YJ, Chen HC, Chou P. Gender difference in the interaction effects of diabetes and hypertension on stroke among the elderly in the Shih-Pai study, Taiwan. *PLoS ONE.* (2015) 10:e0136634. doi: 10.1371/journal.pone0136634
22. Doumas M, Papademetriou V, Faselis C, Kokkinos P. Gender differences in hypertension: myths and reality. *Curr Hypertens Rep.* (2013) 15:321–30. doi: 10.1007/s11906-013-0359-y
23. Deijle IA, Van Schaik SM, Van Wegen EE, Weinstein HC, Kwakkel G, Van den Berg-Vos RM. Lifestyle interventions to prevent cardiovascular events after stroke and transient ischemic attack: systematic review and meta-analysis. *Stroke.* (2017) 48:174–9. doi: 10.1161/STROKEAHA.116013794
24. Jackson C, Sudlow C. Are lacunar strokes really different? A systematic review of differences in risk factor profiles between lacunar and nonlacunar infarcts. *Stroke.* (2005) 36:891–901. doi: 10.1161/01.STR.0000157949.3498630
25. Aldoori MI, Rahman SH. Smoking and stroke: a causative role. Heavy smokers with hypertension benefit most from stopping. *BMJ.* (1998) 317:962–3. doi: 10.1136/bmj.317.7164962
26. Appelman Y, van Rijn BB, Ten Haaf ME, Boersma E, Peters SA. Sex differences in cardiovascular risk factors and disease prevention. *Atherosclerosis.* (2015) 241:211–8. doi: 10.1016/j.atherosclerosis.2015.01027
27. Roquer J, Campello AR, Gomis M. Sex differences in first-ever acute stroke. *Stroke.* (2003) 34:1581–5. doi: 10.1161/01.STR.0000078562.82918F6
28. Wang TJ, Massaro JM, Levy D, Vasan RS, Wolf PA, D'Agostino RB, et al. A risk score for predicting stroke or death in individuals with new-onset atrial fibrillation in the community: the Framingham Heart Study. *JAMA.* (2003) 290:1049–56. doi: 10.1001/jama.290.81049
29. Go AS, Hylek EM, Chang Y, Phillips KA, Henault LE, Capra AM, et al. Anticoagulation therapy for stroke prevention in atrial fibrillation: how well do randomized trials translate into clinical practice? *JAMA.* (2003) 290:2685–92. doi: 10.1001/jama.290.20.2685
30. Fang MC, Singer DE, Chang Y, Hylek EM, Henault LE, Jensvold NG, et al. Gender differences in the risk of ischemic stroke and peripheral embolism in atrial fibrillation: the AnTicoagulation and Risk factors In Atrial fibrillation (ATRIA) study. *Circulation.* (2005) 112:1687–91. doi: 10.1161/CIRCULATIONAHA.105.553438
31. Lip GY, Watson T, Shantsila E. Anticoagulation for stroke prevention in atrial fibrillation: is gender important?. *Eur Heart J.* (2006) 27:1893–4. doi: 10.1093/eurheartj/ehl140
32. Glader EL, Stegmayr B, Norrving B, Terént A, Hulter-Asberg K, Wester PO, et al. Sex differences in management and outcome after stroke: a Swedish national perspective. *Stroke.* (2003) 34:1970–5. doi: 10.1161/01.STR.0000083534.81284C5
33. Galassi A, Reynolds K, He J. Metabolic syndrome and risk of cardiovascular disease: a meta-analysis. *Am J Med.* (2006) 119:812–9. doi: 10.1016/j.amjmed.2006.02031
34. Almdal T, Scharling H, Jensen JS, Vestergaard H. The independent effect of type 2 diabetes mellitus on ischemic heart disease, stroke, and death: a population-based study of 13,000 men and women with 20 years of follow-up. *Arch Intern. Med.* (2004) 164:1422–6. doi: 10.1001/archinte.164.131422
35. Boden-Albala B, Sacco RL, Lee HS, Grahame-Clarke C, Rundek T, Elkind MV, et al. Metabolic syndrome and ischemic stroke risk: Northern Manhattan study. *Stroke.* (2008) 39:30–5. doi: 10.1161/STROKEAHA.107496588
36. Goldstein LB, Bushnell CD, Adams RJ, Appel LJ, Braun LT, Chaturvedi S, et al. Guidelines for the primary prevention of stroke: a guideline for healthcare professionals from the American heart association/American stroke association. *Stroke.* (2011) 42:517–84. doi: 10.1161/STR0b013e3181fcb238
37. Kroll ME, Green J, Beral V, Sudlow CL, Brown A, Kirichek O, et al. Adiposity and ischemic and hemorrhagic stroke: prospective study in women and meta-analysis. *Neurology.* (2016) 87:1473–81. doi: 10.1212/WNL0000000000003171
38. Zhang FL, Guo ZN, Wu YH, Liu HY, Luo Y, Sun MS, et al. Prevalence of stroke and associated risk factors: a population based cross sectional study from northeast China. *BMJ Open.* (2017) 7:e015758. doi: 10.1136/bmjopen-2016-015758
39. Lee SY, Lim JS, Oh DJ, Kong IG, Choi HG. Risk of ischaemic stroke in patients with migraine: a longitudinal follow-up study using a national sample cohort in South Korea. *BMJ Open.* (2019) 9:e027701. doi: 10.1136/bmjopen-2018-027701
40. Tzourio C, Tehindrazanarivelo A, Iglésias S, Alpérovitch A, Chedru F, d'Anglejan-Chatillon J, et al. Case-control study of migraine and risk of ischaemic stroke in young women. *BMJ.* (1995) 310:830–3. doi: 10.1136/bmj.310.6983830
41. McCullough LD, Hurn PD. Estrogen and ischemic neuroprotection: an integrated view. *Trends Endocrinol Metab.* (2003) 14:228–35. doi: 10.1016/S1043-2760(03)00076-6
42. Hendrix SL, Wassertheil-Smoller S, Johnson KC, Howard BV, Kooperberg C, Rossouw JE, et al. Effects of conjugated equine estrogen on stroke in the women's health initiative. *Circulation.* (2006) 113:2425–34. doi: 10.1161/CIRCULATIONAHA.105594077
43. Gillum LA, Mamidipudi SK, Johnston SC. Ischemic stroke risk with oral contraceptives: a meta-analysis. *JAMA.* (2000) 284:72–8. doi: 10.1001/jama.284.172
44. Brenner B. Haemostatic changes in pregnancy. *Thromb Res.* (2004) 114:409–14. doi: 10.1016/j.thromres.2004.08.004
45. Park TH, Ko Y, Lee SJ, Lee KB, Lee J, Han MK, et al. Gender differences in the age-stratified prevalence of risk factors in Korean ischemic stroke patients: a nationwide stroke registry-based cross-sectional study. *Int J Stroke.* (2014) 9:759–65. doi: 10.1111/ijs.12146

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