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Identification of Biomarkers in Patients with Thrombotic Thrombocytopenic Purpura Presenting with Large and Small Ischemic Stroke

Chen Lin[a] Raima Memon[b] Jingrui Sui[b] X. Long Zheng[b, c]

[a]Department of Neurology, The University of Alabama at Birmingham, Birmingham, AL, USA; [b]Department of Pathology, The University of Alabama at Birmingham, Birmingham, AL, USA; [c]Department of Pathology and Laboratory Medicine, University of Kansas Medical Center, Kansas City, KS, USA

Keywords
Stroke etiology · Neuroimaging of stroke · Thrombotic thrombocytopenic purpura · ADAMTS13 · Neutrophil extracellular trap · Inflammation

Abstract
Background: Thrombotic thrombocytopenic purpura (TTP) is a rare blood disorder resulting in organ damage including ischemic strokes. We sought to characterize the neuroimaging patterns of stroke in a large cohort of patients with immune-mediated TTP (iTTP) and determined their associations with clinical and laboratory parameters and outcomes. ***Methods:*** We analyzed the Alabama TTP Registry who had laboratory confirmation of acute iTTP. We reviewed the neuroimaging patterns of those with ischemic stroke on MRI, clinical information, and laboratory results. Small ischemic strokes were ≤20 mm in their maximum diameter in the axial plane. Large ischemic strokes were >20 mm. Student *t* test, Mann-Whitney U test, and χ^2 test were all used for data analysis. ***Results:*** Of 108 iTTP patients, 21 had ischemic stroke on neuroimaging. The median platelet count in these patients was 12×10^9/L (interquartile range, IQR, 8.8–21 × 10^9/L), plasma ADAMTS13 activity 1.8 U/dL (IQR 0–4.5 U/dL), and the mean plasma level of anti-ADAMTS13 IgG was 6,595.8 U/mL (SD 3,448.9 U/mL). Comparison between patients with large ischemic strokes ($n = 10$) and small ischemic strokes ($n = 11$) revealed that patients with small stroke were older ($p = 0.043$) and had higher plasma levels of citrullinated histone 3 ($p = 0.006$) and histone/DNA complex ($p = 0.014$) than those with large strokes. There were no significant differences between 2 stroke groups in mortality or exacerbation. ***Conclusions:*** iTTP patients can present with large ischemic strokes and are usually younger. Further research should be performed in assessing different etiologies of iTTP-associated stroke based on neutrophil extracellular trap formation biomarkers (e.g., histone markers) seen in small ischemic stroke.

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Introduction

Thrombotic thrombocytopenic purpura (TTP) is a rare blood disorder, leading to multisystem organ damage and mortality [1, 2]. The incidence ranges from 3 to 13 cases per million residents per year [1, 3]. The pathophysiology of immune-mediated TTP (iTTP) is the result of an acquired inhibition of ADATMS13 activity due to autoantibodies against ADAMTS13 [4, 5], while con-

Chen Lin
Department of Neurology
The University of Alabama at Birmingham, RWUH M226
1813 6th Avenue South, Birmingham, AL 35294 (USA)
clin@uabmc.edu

X. Long Zheng
Department of Pathology and Laboratory Medicine
The University of Kansas Medical Center
3901 Rainbow Blvd, 5016 Delp, Kansas City, KS 66160 (USA)
xzheng2@kumc.edu

karger@karger.com
www.karger.com/cee

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genital TTP is caused by an inherited deficiency of ADAMTS13 due to mutations in *ADAMTS13* [6–8]. ADAMTS13 is a plasma metalloproteinase responsible for cleaving ultra-large von Willebrand factor, released from endothelial cells [9, 10]. A reduction of plasma ADAMTS13 activity leads to an accumulation of highly adhesive ultra-large von Willebrand factor multimers on endothelial surface [9, 10] and in circulation [11–13]. This ultra-large von Willebrand factor captures flowing platelets in circulation, resulting in excessive thrombus formation in areas of endothelial activation and/or injury [14–16]. If left unrecognized or untreated, TTP carries an exceedingly high mortality (90–95%) [1, 2, 17]. Therapeutic plasma exchange (TPE) or plasma infusion has dramatically reduced mortality and morbidity rates [2, 14, 17]. Recent advances in therapeutic strategy including the use of rituximab [18–20] and caplacizumab [21–23] have further improved survival rate and long-term outcome.

The typical presentation of TTP incudes thrombocytopenia, microangiopathic hemolytic anemia, and various degrees of organ injury [2, 24]. At initial diagnosis, roughly half of patients report neurological symptoms, but ultimately 90% of TTP patients develop some sort of central nervous system involvement [25, 26]. The most common symptoms include fluctuating mental status or persistent encephalopathy, aphasia, paresthesia, motor deficits, and seizure [27]. The presumptive mechanism is from thrombi in small vessels that lead to transient occlusion or small infarcts [14]. The typical small ischemic strokes seen in TTP are thought to be largely reversible in most patients if they survive the systemic issues related to TTP [25]. Large ischemic strokes are thought to be very rare neurological presentations in TTP. The literature on stroke in TTP is limited to only case reports or small series [14, 26, 28–31]. To our knowledge, a comparison of stroke neuroimaging findings and its association with laboratory values and clinical outcomes has never been reported. In this study, we sought to carefully characterize the neuroimaging patterns of stroke in a large cohort of patients with TTP and determined their associations with patient clinical characteristics, laboratory values, and clinical outcomes.

Methods

Patient Selection and Data Collection

The study protocol was approved by the International Review Committee at the University of Alabama at Birmingham (UAB). All the patients with suspected TTP (presenting with one or more of the diagnostic criteria for TTP mentioned below) who were referred to the UAB Apheresis service and captured in the Alabama TTP Registry between the period April 2006 and December 2019 for TPE were included in this study. A total of 109 patients were diagnosed with acute TTP. Of these patients, we reviewed whether they had complaints of neurological symptoms (i.e., headache, slurring of speech, forgetfulness, dizziness, visual changes, numbness or weakness of the extremities, coma or seizures) and whether neuroimaging was available to confirm ischemic stroke. Three patients were excluded because they were transferred from outside hospitals and imaging was not available for the review. While the Registry contains both congenital TTP ($n = 1$) and iTTP ($n = 108$), only iTTP patients who had ischemic strokes were included in the analysis. TTP was determined to be the etiology of all strokes included in the analysis.

The diagnostic criteria for TTP in this study were the following: severe thrombocytopenia and microangiopathic hemolytic anemia (e.g., low hemoglobin, low hematocrit, elevated lactate dehydrogenase, and presence of schistocytes on peripheral blood smear), with or without evidence of end-organ damage, including fluctuating neurological symptoms and fever or renal impairment [20, 32]. Plasma ADAMTS13 activity were all <5 IU/dL with inhibitor >0.4 U/mL, and/or anti-ADAMTS13 IgG >15 U/mL [33]. Patient clinical and routine laboratory information was extracted from the electronic medical record and Alabama TTP registry database.

Neuroimaging Studies and Stroke Classification

Neuroimaging studies performed at the time of presentation for stroke or transient ischemic attack were included for analysis. The magnetic resonance imaging (MRI) was performed using the UAB stroke protocol. MRI was done using a Philips 1.5-T scanner. Cerebral MRI was done including T1- and T2-weighted sequences, diffusion-weighted imaging (DWI), and FLAIR. The scan was done with a slice thickness of 6 mm and at a slice interval of 1 mm with DWI b-value = 0, 1,000 s/mm^2. All neuroimaging studies were independently reviewed by 2 of the investigators (C.L. and R.M.) to verify imaging results. One of the investigators (C.L.) was blinded to the clinical data. Radiological changes were categorized by large or small ischemic strokes if they exhibited a high-intensity signal on DWI and a corresponding low diffusion coefficient on the apparent diffusion coefficient map. The infarcts were classified into small or large ischemic strokes. Small ischemic strokes were classified by the Standards for Reporting Vascular changes on Neuroimaging (STRIVE) position statement on small vessel disease [34]. We considered small ischemic strokes to be ≤20 mm in their maximum diameter in the axial plane [34]. Large ischemic strokes were >20 mm. We further differentiated small strokes as being territorial (cortical), lacunar (deep), or a combination of both [35, 36]. By consensus, clinical and radiographic data were reviewed to determine Trial of Org 10172 in Acute Stroke Treatment (TOAST) subtype for each confirmed case [37, 38].

Laboratory Assessment

Routine and special laboratory analyses were performed on the initial blood samples as part of patient care, including complete blood count, creatinine, prothrombin time, partial thromboplastin time, fibrinogen, D-dimer, lactate dehydrogenase, troponin, and so on. These data were available from patients' electronic medical

Table 1. Demographic features of TTP patients with neuroimaging (n = 21)

Parameters	Values
Age, years	49.9±10.9
Gender, *n* (%)	
Female	12 (57.1)
Male	9 (42.9)
Race, *n* (%)	
Black	15 (71.4)
White	5 (23.8)
Hispanic	1 (4.8)
Disease status, *n* (%)	
Initial	14 (66.7)
Relapse	7 (33.3)
Blood group, *n* (%)	
O	10 (47.6)
Non-O	11 (52.4)
Comorbidities, *n* (%)	
Hypertension	16 (76.2)
Diabetes mellitus	3 (14.3)
Symptoms, *n* (%)	
Fever	3 (14.3)
Chest pain	8 (38.1)
Abdominal pain	2 (9.5)

Age is expressed as mean ± SD. All other parameters are expressed as number and percentage of patients. TTP, thrombotic thrombocytopenic purpura; O, blood type O.

Table 2. Presenting laboratory parameters of iTTP patients with neuroimaging (n = 21)

Parameters	Values
Routine laboratory tests	
White blood cells, $\times 10^9$/L	10.9±4.4
Hemoglobin, g/dL	8.7±1.9
Hematocrit, %	25.5±5.2
Platelet count, $\times 10^9$/L	12.0 (8.8–21.0)
Lactate dehydrogenase, U/L	963.6±466.9
Creatinine, mg/dL	1.4 (0.90–1.68)
Prothrombin time, s	14.85 (14–16.1)
Partial thromboplastin time, s	32.0±6.5
Fibrinogen, mg/dL	420.6±108.6
D-dimer, ng/mL (n = 16)	1,369.5 (711.8–2,920.5)
Troponin, ng/mL	0.6 (0.2–2.2)
Special laboratory tests	
ADAMTS13 activity, U/dL	<5.0
Anti-ADAMTS13 IgG, U/mL (n = 17)	6,595.8±3,448.9
ADAMTS13 inhibitor, BU (n = 15)	1.4 (0.6–3.2)
Citrullinated histone H_3, ng/mL (n = 15)	3.6 (3.1–7.1)
Histone/DNA complex, U/mL (n = 18)	71.6 (31.3–171.2)
Cell-free DNA, ng/mL (n = 15)	987.6 (818.0–1,195.5)

White blood cells, hemoglobin, hematocrit, lactate dehydrogenase, partial thromboplastin time, fibrinogen, and anti-ADAMTS13 IgG are expressed as the mean ± SD. All other parameters are expressed as the median and interquartile range. TTP, thrombotic thrombocytopenic purpura; BU, Bethesda unit.

records [39]. Additionally, all patients with TTP had blood drawn at the time of presentation for investigational assays. In this case, whole-blood samples were collected from an apheresis catheter before the initiation of TPE and anticoagulated with 0.39% sodium citrate. After being centrifuged (1,500 × g for 15 min) within 2 h of collection, platelet-poor plasma was separated from the cellular components, aliquoted, and stored at –80 °C until assays.

Plasma ADAMTS13 activity, inhibitor, and anti-ADAMTS13 IgG were determined in a reference laboratory (Versiti, Milwaukee, WI) and in-house assays as previously described [33].

Plasma Histone DNA Complex, Cell-Free DNA, and Citrullinated Histone

Plasma levels of histone/DNA complex (Millipore Sigma) and citrullinated histone H_3 (Cayman Chemicals, Ann Arbor, MI, USA) were determined by the commercial ELISA kits according to the manufacturers' instructions. Cell-free DNA was determined using the Quant-IT PicoGreen dsDNA assay (Thermo Fisher Scientific) according to the manufacturer's instructions.

Statistical Analysis

Means, median, and percentage of ordinal variables were calculated using SPSS software. Student t test and Mann-Whitney U test or χ^2 test was used for data with normal distribution and categorical data, respectively. A p value <0.05 and 0.01 was statistically significant and highly significant, respectively.

Results

Clinical and Laboratory Characteristics of the Patients with Reviewable Neuroimaging in the Alabama TTP Cohort

Out of 108 iTTP patients in our Alabama Registry, 21 of the iTTP patients had ischemic stroke on neuroimaging. Of these, the mean age was 49.9 (SD 10.9) years at the time of admission. Twelve (57.1%) patients were female and 9 (42.9%) were male. Fifteen (71.4%) of these patients were African Americans. Fourteen (66.7%) were initial and 7 (33.3%) were relapsed presentations of iTTP. In this cohort, blood group O patients comprised 47.6% of the patients. The majority of the patients had comorbidities, including hypertension (76.2%) and diabetes mellitus (14.3%). All the patients had neurological symptoms, 14.3% had fever, 38.1% had chest pain, and 9.5% had abdominal pain (Table 1).

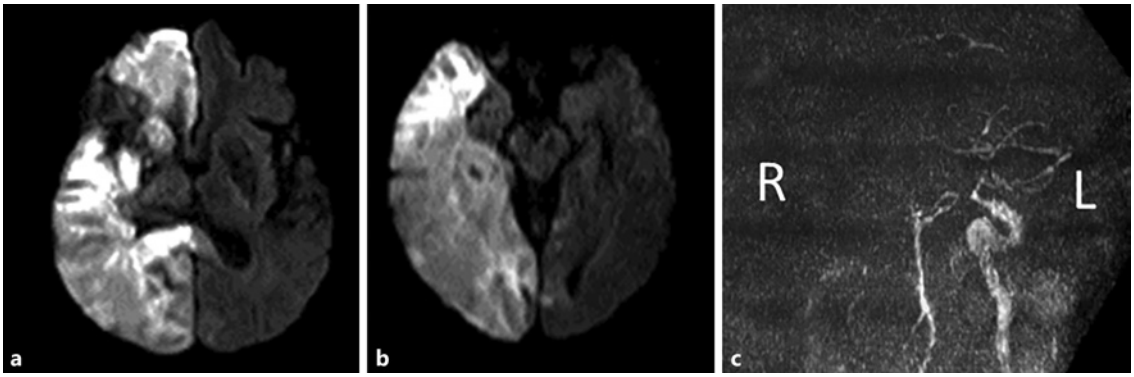

Fig. 1. Example of large territorial infarct with diffusion restriction of the anterior cerebral and middle cerebral arteries (**a**) and middle cerebral and posterior cerebral arteries (**b**). **c** Magnetic resonance angiography in the same patient with complete occlusion of the right internal carotid and posterior cerebral arteries.

Table 3. Treatment and outcomes of iTTP patients with neuroimaging (n = 21)

Parameters	Values
Who received TPE	21 (100)
Who received rituximab	14 (66.7)
Outcome	
Death	4 (19.1)
Remission	7 (33.3)
Recurrence	9 (42.9)
Exacerbation	7 (33.3)
Relapse	2 (9.5)

All the parameters are expressed as number and percentage of patients. TPE, therapeutic plasma exchange; n, total number of patients with imaging.

Of the 21 patients, 17 had "other" as the TOAST subtype, attributed directly to TTP. Four patients are considered cryptogenic because more than 1 etiology could have contributed to their stroke but all 4 were noted to have TTP as one of those contributing etiologies. Seventeen patients had neurology formally consulted in the hospital though all eventually followed with a neurologist. During index hospitalization for the stroke, 10 had extracranial carotid duplex ultrasonography. Nineteen had a transthoracic echocardiogram, 7 had transesophageal echocardiogram, and 13 had either CT or MR angiography. Of the 10 patients with small strokes, we identified 7 cortical, 3 mixed, and no patients with only lacunar infarcts.

Laboratory parameters in all 21 iTTP patients at the time of admission are shown in Table 2. The laboratory results fully supported the diagnosis of iTTP, with the median platelet count 12 × 10^9/L (interquartile range, IQR, 8.8–21 × 10^9/L) and plasma ADAMTS13 activity 1.8 U/dL (IQR 0–4.5 U/dL). The mean hematocrit was 25.5% (SD 5.2%) and lactate dehydrogenase 963.6 U/L (SD 466.9 U/L). The mean plasma level of anti-ADAMTS13 IgG was 6,595.8 U/mL (SD 3,448.9 U/mL). All 21 patients received TPE plus corticosteroid therapy, and 14 (66.7%) were treated with rituximab. Of 21 patients, 4 (19.1%) did not survive from the acute episode, 8 (38.1%) were followed by sustained remission, and 9 (42.9%) exhibited a disease recurrence (Table 3).

Correlations between Ischemic Stroke Size and Demographic and Laboratory Parameters in iTTP Patients

Based on the criteria we described in the method, the infarcts of the 21 iTTP patients were classified into small (n = 10) and large (n = 11) ischemic strokes. The correlations between stroke size and demographics, clinical presentation, laboratory parameters, and patient outcomes were determined. Compared with patients with large ischemic strokes, those with small ischemic strokes were much older (p = 0.043) and had higher levels of citrullinated histone 3 (p = 0.006) and histone/DNA complex (p = 0.014; Table 4). Using the log-rank test, we determined the size of the ischemic stroke and the outcome of the patients. Patients with small ischemic stroke did not have a higher risk of death than those with large ischemic stroke (hazard ratio, HR 1.17, 95% CI 0.16–8.36; p = 0.88; Table 4). It looked like that patients with small ischemic

Table 4. Association between the stroke size and demographic or laboratory parameters in patients with iTTP ($N = 21$)

Variables	Large ($n = 11$)	Small ($n = 10$)	p value
Demographics			
Age (mean ± SD), years	45.4±11.2	54.8±8.4	*0.043*[a]
Female, *n* (%)	6 (54.5)	6 (60)	0.58[b]
African American, *n* (%)	7 (63.6)	7 (70)	1.00[b]
Disease status, initial, *n* (%)	6 (54.5)	8 (80)	0.22[b]
Blood group O, *n* (%)	4 (36.4)	6 (60)	0.26[b]
Comorbidities			
Hypertension, *n* (%)	9 (81.8)	7 (70)	0.64[b]
Diabetes mellitus, *n* (%)	3 (27.3)	0 (0)	0.12[b]
Fever, *n* (%)	2 (18.2)	1 (10)	1.00[a]
Chest pain, *n* (%)	3 (27.3)	5 (50)	0.39[b]
Abdominal pain, *n* (%)	1 (9.1)	1 (10)	1.00[b]
Laboratory results			
White blood cells (mean ± SD), $\times 10^9$/L	11.6±5.1	10.2±3.7	0.50[a]
Hemoglobin (mean ± SD), g/dL	9.1±1.5	8.3±2.1	0.32[a]
Hematocrit (mean ± SD), %	26.3±4.6	24.6±5.9	0.48[a]
Platelet count (median, IQR), $\times 10^9$/L	14.1 (8.8–27.9)	11.5 (6.4–19.9)	0.22[a]
LDH (mean ± SD), IU/L	838.1±365.4	1,103.1±546.5	0.23[a]
Creatinine (median, IQR), mg/dL	0.9 (0.9–4.3)	1.6 (1.5–1.8)	0.39[c]
PT (median, IQR), s	15.2 (13.9–16.9)	14.8 (13.9–16.0)	0.68[c]
PTT (mean ± SD), s	33.7±8.0	29.9±4.0	0.21[a]
Fibrinogen (mean ± SD), mg/dL	431.2±100.8	405.1±129.5	0.67[a]
D-dimer (median, IQR), ng/mL ($n = 16$)	708.0 (460.5–5,966.0)	2,155.0 (1,768.3–6,485.3)	0.07[c]
Troponin (median, (IQR), ng/mL	1.1 (0.4–11.3)	0.42 (0.25–1.16)	1.00[c]
ADAMTS13 activity, U/dL	<5	<5	n.d.
Anti-ADAMTS13 IgG (mean ± SD), U/mL ($n = 17$)	6,750.1±4,737.7	5,485.6±1,924.7	0.99[a]
Inhibitor (median, IQR), IU ($n = 15$)	3.2 (0.7–6.0)	0.8 (0.5–1.6)	0.12[c]
Citrullinated H_3 (median, IQR), ng/mL ($n = 15$)	3.2 (2.6–3.5)	7.1 (4.2–9.7)	*0.009*[c]
Histone/DNA complex (mean ± SD), U/mL ($n = 18$)	60.3±46.0	165.8±94.2	*0.014*[a]
Cell-free DNA (median, IQR), ng/mL ($n = 18$)	863.3 (753.6–1,143.6)	1,076.7 (894.5–1,580.2)	0.30[c]

N, total number of patients with imaging; *n*, number of patients tested; LDH, lactate dehydrogenase; PT, prothrombin time; PTT, activated thromboplastin time; SD, standard deviation; IQR, interquartile range; n.d., not determined. [a] *t* test. [b] χ^2 test. [c] Mann-Whitney U test. Italicized *p* values are statistically significant.

strokes had higher risk of exacerbation, but it did not reach statistical significance (HR 2.38, 95% CI 0.54–13.1; $p = 0.23$; Table 4). See Figure 1 for an example of an iTTP patient with a large ischemic stroke.

Discussion

In this study of a large cohort of consecutive patients with acute iTTP, we found that many of these patients with neurologic symptoms who had a stroke suffered from large ischemic strokes as opposed to the classically described small ischemic strokes associated with iTTP. Patients with larger strokes attributed to iTTP tended to be younger than patients with the classic small strokes on presentation. This highlights that iTTP is a potential stroke etiology to consider for patients with unknown causes of large and small ischemic strokes with otherwise no significant vascular risk factors and lack of atherosclerosis of the large vessels on their vessel imaging. In our population, there were no differences between size of stroke and recurrence of iTTP or mortality rates. The early diagnosis of iTTP as a potential etiology of large ischemic strokes could potentially be a reason for this lack of difference though our sample size was small.

Nevertheless, our study is the largest cohort study so far in the literature to analyze neuroimaging in patients with stroke caused by iTTP. Most prior studies have

been either case reports or small series [14, 28, 30, 31]. The largest neuroimaging review of iTTP patients performed by Burrus et al. [26] screened over 200 patients to identify 47 patients with neuroimaging available. Of those, 5 patients had stroke on CT scan, and 10 patients had small ischemic stroke on MRI. In our cohort, we were able to identify 65 patients with neurological symptoms, of which 21 had reviewable neuroimaging that demonstrated stroke. Fifty-two percent (n = 11) had what are considered large ischemic strokes while 48% (n = 10) had the classic small ischemic stroke associated with iTTP. Interestingly, Burrus et al. [26] did not report any large ischemic strokes in their cohort. Previous literature reviews have only identified a total of 10 reported cases of strokes in TTP caused by large ischemic strokes [14]. This is the first systematic study to identify that large ischemic strokes can be common in iTTP patients with stroke. Having a large ischemic stroke on neuroimaging should not eliminate the possibility of iTTP being an etiology.

Prior studies have not consistently reported their TTP-associated laboratory values. By not reporting the level of ADAMTS13, we do not know how many of these cases were in fact having a diagnosis of TTP [14]. The large neuroimaging cohort from Burrus et al. [26] did not report their laboratory values either. Therefore, they could not associate the large ischemic stroke presentations to the TTP severity. While we did not find any difference in plasma levels of ADAMTS13 activity, ADAMTS13 inhibitor, and anti-ADAMTS13 IgG between the large and small ischemic stroke groups, this is quite expected as these parameters are critical for establishing a diagnosis of iTTP. Interestingly, the ADAMTS13 inhibitor levels appeared to be borderline higher in the large stroke group.

Intriguingly, we did find a significant difference between the 2 groups in both citrullinated histone H_3 and the histone-DNA complex levels. Patients with large ischemic strokes had lower levels of citrullinated histone H_3 (3.0 vs. 7.8 ng/mL) and lower levels of histone-DNA complex (60.3 vs. 165.8 U/mL) than patients with small ischemic stroke. Citrullinated histone H_3 and histone-DNA complex are parts of neutrophil extracellular traps (NETs), released from acute inflammation and neutrophil apoptosis and necrosis. These NETs are formed after citrullination of histones by activation of protein arginine deiminase 4, which has been associated with thrombosis in patients with cancers [40, 41]. Leukocytes are known to release nuclear contents that form these extracellular traps, which are mainly composed of the histone-DNA complex [42]. These biomarker levels that lead to extracellular trap formation have been shown to be higher in serum circulation in patients who receive peripheral angioplasty with stent implementation that then develop strokes [43] and higher in stroke patients with internal artery occlusions [44]. Our data are the first to report these biomarkers are also associated with iTTP-induced stroke. Interestingly, the small ischemic stroke had higher levels of the NET biomarkers (i.e., citrullinated histone H_3 and histone-DNA complex, etc.). Since leukocytic levels are increased from an inflammatory response, it is possible that iTTP may be triggered by and/or leads to a significant inflammatory response, which results in a secondary microscopic polyangiitis, a vasculitis of small ischemic disease, in addition to thrombosis. Further research is needed to classify whether there are different mechanisms of stroke etiology in patients with iTTP-induced stroke and the role of NET biomarkers.

Similar to other retrospective cohort analyses, our study has several limitations. While neuroimaging was obtained for neurological symptom evaluation, exact timing of the examinations was variable ranging from hours to a week. Unfortunately, since these patients presented medically ill, the clinical severity of the stroke was not routinely recorded in these patients. They were often admitted to nonstroke units and so an NIH Stroke Scale was not obtained. This was a registry collected from a single center, though the patients come from across multiple states. Since many of these acute patients presenting with iTTP have other causes for neurological disorders and potential neuroimaging changes, we could not perform a more detailed analysis of potential confounders, including TOAST stroke subtype criteria and location of the small strokes, because of the relatively limited sample size.

We conclude that iTTP-induced stroke patients can commonly present with large ischemic strokes. These patients tend to be younger than cases with the classically associated small ischemic strokes. Further research should be performed in assessing different etiologies of iTTP-induced stroke based on NET formation biomarkers seen in small ischemic stroke. Targeted therapeutics aiming at eliminating NETs such as the use of DNase I may be considered in preclinical animal models and in iTTP patients with stroke to reduce neurocognitive sequelae.

Statement of Ethics

The present study conforms to the guidelines issued in the Declaration of Helsinki. This study was approved by the Institutional Review Committee (100811004). Subjects (or their parents or guardians) have given their written informed consent.

Conflict of Interest Statement

X.L.Z. is a speaker or consultant for Alexion, Sanofi, and Takeda. X.L.Z. is also the Founder of Clotsolution Inc. All other authors have declared no relevant conflict. All other authors report no conflicts of interest concerning the materials or methods used in this study or the findings specified in this paper.

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Author Contributions

C.L., R.M., J.S., and X.L.Z. designed research, performed experiments, and analyzed the results, as well as wrote the manuscript. All other nonauthors in the acknowledgement contributed to patient recruitment, informed consents, and sample collections. All authors have reviewed and approved the final version of the paper for submission.

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