Thrombosis Research 219 (2022) 60–69

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

Age and composition of the thrombus retrieved by mechanical thrombectomy from patients with acute ischemic stroke are associated with revascularization and clinical outcomes

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ARTICLE INFO

Keywords:
Thrombus age
Mechanical thrombectomy
Acute ischemic stroke
Revascularization

ABSTRACT

Introduction: Understanding the composition of stroke thrombi retrieved by mechanical thrombectomy is essential to clarify the pathogenesis of stroke. However, it is difficult to evaluate thrombus composition precisely and objectively. Immunohistochemical staining was used to evaluate thrombus composition and age.

Materials and methods: Consecutive thrombi (n = 108) retrieved from patients who underwent mechanical thrombectomy for acute large-vessel ischemic stroke were retrospectively analyzed. Lytic features of granulocytes and CD163 were estimated as indicators of the age of the cardioembolic (CE) thrombus.

Results: The stroke subtypes were as follows: CE, 74 cases; large artery atherosclerosis, 11; undetermined etiology, 12; and other determined etiology, 11. There were no statistical differences in thrombi composition according to stroke subtypes. The fibrin area was positively correlated with the red blood cell (RBC) and platelet areas. The following analysis was performed using CE only. Regarding age, the thrombus was judged as fresh in 30.0 % and older in 70.0 % based on the lytic features. The RBC areas of older thrombi were smaller than those of fresh thrombi. The puncture-to-reperfusion time of older thrombi was longer than that of fresh thrombi. Platelet-rich thrombi were associated with a greater number of maneuvers, a smaller prevalence of TICI 3, and unfavorable functional outcomes compared to platelet-poor thrombi. The number of CD163 positive cells in thrombi with anticoagulants was higher than in those without anticoagulants.

Conclusion: Thrombus composition correlated with revascularization and clinical outcomes. The composition of an acute ischemic thrombus may reflect the pathophysiology of stroke and influence treatment efficacy.

Abbreviations: CE, cardioembolism; tPA, tissue-type plasminogen activator; ICA, internal carotid artery; MCA, middle cerebral artery; PCA, posterior cerebral artery; mRS, modified Rankin Scale; NIHSS, National Institutes of Health Stroke Scale; TOAST, Trial of Org 10172 in Acute Stroke Treatment; TICI, thrombolysis in cerebral infarction; RBCs, red blood cells; VWF, von Willebrand factor.

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https://doi.org/10.1016/j.thromres.2022.09.004
Received 1 June 2022; Received in revised form 24 August 2022; Accepted 5 September 2022
Available online 7 September 2022

1. Introduction

Acute ischemic stroke is the most common cause of persistent physical dysfunction [1]. Novel therapies such as mechanical thrombectomy (MT) using catheter devices have become available in recent years. MT is effective and improves outcomes after acute large vessel occlusion stroke [2]. Additionally, MT has made it possible to evaluate the thrombus in cerebral embolism. The treatment of ischemic stroke is based on the expected pathophysiology, which is classified into five subtypes by the International Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria: large-artery atherosclerosis (LAA), cardioembolism (CE), small-vessel occlusion, undetermined etiology, and other determined etiology [3]. Thrombus studies in acute myocardial infarction (AMI) and deep venous thrombosis (DVT) have been frequently reported, but little is known about acute ischemic stroke thrombi. It is essential to understand the pathogenesis of acute ischemic stroke for its treatment and prevention. Understanding thrombi could help clarify the pathogenesis of stroke [3].

The thrombus is primarily composed of red blood cells (RBCs), platelets, fibrin focally with von Willebrand factor (VWF), white blood cells, neutrophil extracellular traps (NETs), and extracellular DNA [4,5]. Generally, arterial thrombi are formed by platelet aggregation, including VWF, due to endothelial injury or dysfunction (primary hemostasis), and propagated by cross-linking of fibrin involving RBCs (secondary hemostasis). Recent studies regarding the quantification of thrombus composition retrieved from ischemic stroke patients have been based on conventional methods using hematoxylin and eosin (HE) stain [6–8]. However, it is difficult to evaluate each component separately and objectively and determine thrombus composition using HE staining only because the blood cell components overlap. Immunohistochemical staining and image analysis software were used to quantitatively evaluate the thrombus [9]. Several studies have evaluated the relationship between the etiology and thrombus composition in ischemic stroke. While some studies show a higher proportion of RBCs in thrombi from LAA and fibrin in thrombi from CE, others report different results. Therefore, it remains inconclusive [10].

Recently, the time course (age) of thrombi has been reported [9,11,12]. Primary and secondary hemostasis are layered repeatedly on an embolic source. An embolized thrombus occludes the blood vessels and forms a secondary thrombus at the occlusion site. The lytic features of granulocytes and CD163 (phagocytosis of erythrocytes by macrophages) have been used as indicators of thrombus organization to examine the time course after onset. To date, lytic features have been identified in patients with AMI [9,11] and stroke [13], whereas CD163 analysis has been performed in patients with DVT [12] and stroke [13].

In this study, we investigated the correlation between four main factors of hemostasis (RBCs, fibrin, platelets, and VWF) in ischemic stroke thrombi. Furthermore, thrombus age based on lytic features and CD163-positive cells was analyzed in ischemic stroke thrombi. Finally, the relationship between the thrombus components and clinical data, such as stroke etiology, revascularization outcomes, and clinical outcomes, was clarified.

2. Materials and methods

2.1. Patient selection

This retrospective study included patients from two stroke centers in Japan (Nara City Hospital and Nara Medical University Hospital). We examined the data of 166 patients who underwent MT for acute ischemic stroke between December 2016 and January 2021. Thrombus specimens were available for 112 patients. The exclusion criterion was thrombus material that was unsuitable for histopathological analyses ($n = 4$). This study included 108 patients with thrombi retrieved during MT for cerebral embolisms (Supplemental Fig. 1). Written informed consent was obtained from all patients or relatives if the patient had communication problems that prevented the direct provision of consent. Consent to participate in this clinicopathological study was obtained using an opt-out approach. This study was approved by the Research Ethics Committee of Nara Medical University (approval number 2533, 2536).

In this study, most thrombi retrieved by MT were CE. The CE thrombus subtype is formed in the heart and moves; therefore, it is believed to exhibit a wide age range. As such, we only performed thrombus age analysis in the CE subtype. In addition, the relationship between clinical outcomes and thrombus components regarding RBCs, platelets, and VWF was also analyzed in CE only for uniformity (Supplemental Fig. 1).

2.2. Clinical data of patients

Clinical data of the patients were collected, including the National Institutes of Health Stroke Scale (NIHSS) scores [14] for the objective quantification of the impairment on admission, modified Rankin Scale (mRS) for the assessment of handicap [15] at discharge, tissue plasminogen activator (tPA) use, puncture-to-reperfusion time (time from groin puncture to final procedure), number of MT maneuvers, and the modified thrombolysis in cerebral infarction (mTICI) system score to evaluate angiographic intracranial flow [16]. Subtypes of acute ischemic stroke were determined according to the TOAST classification [3]. Cardioembolism (CE) was defined as a cardiac source without ipsilateral stenosis. Large artery atherosclerosis (LAA) was defined as angiographic findings of either significant (>50 %) stenosis or occlusion of the extracranial carotid artery or intracranial artery without evidence of potential sources of CE in other diagnostic studies.

2.3. Mechanical thrombectomy procedure

The treating neuro-interventionalist selected the strategies implemented to treat acute large-vessel occlusion stroke. Thrombi were retrieved using a stent retriever and aspiration devices, according to the judgment of the neuro-interventionalist. tPA was administered to eligible patients. We assessed the total number of thrombectomy device passes attempted before and at the end of the procedure. Successful angiographic reperfusion was identified as grade 3 using the mTICI system. Thrombus material collected from multiple passes in one patient was pooled, and one thrombus was further analyzed.

2.4. Histopathological procedure

The retrieved thrombus was immediately fixed in 4 % paraformaldehyde and then paraffin-embedded sections (4 μm thick) were stained with hematoxylin and eosin (HE) for morphological evaluation [9]. To confirm the composition of the thrombi, we immunohistochemically examined serial sections. Primary antibodies against fibrin (mouse monoclonal, clone 59D8; MILLIPORE, Germany), Glycophorin A (mouse monoclonal, clone JC159; DAKO, CA, United States) for RBCs, CD163 (mouse monoclonal, clone 10D6; Leica, Germany) for thrombus age, VWF (mouse monoclonal, clone F8/86; DAKO, CA, United States) and IIb/IIIa (donkey monoclonal; Affinity Biologicals, ON, United States) for platelets were used in this study [9,12,17–19].

2.5. Quantitative methods of thrombus components

Thrombus materials were photographed under a 4× objective lens using an Olympus BX53 microscope and digital camera (Olympus, DP26). Areas immuno-positive for fibrin, IIb/IIIa, glycophorin A, and VWF were quantified using a color imaging analysis system (Cellsense, Olympus, Japan; Fig. 1).

We counted the percentage of granulocytes exhibiting lytic feature in the five most cellular fields under a 20× objective lens and the number of immune-positive cell for CD163 in the five most heavily stained fields under a 20× objective lens. The CD163 cell density was expressed as the

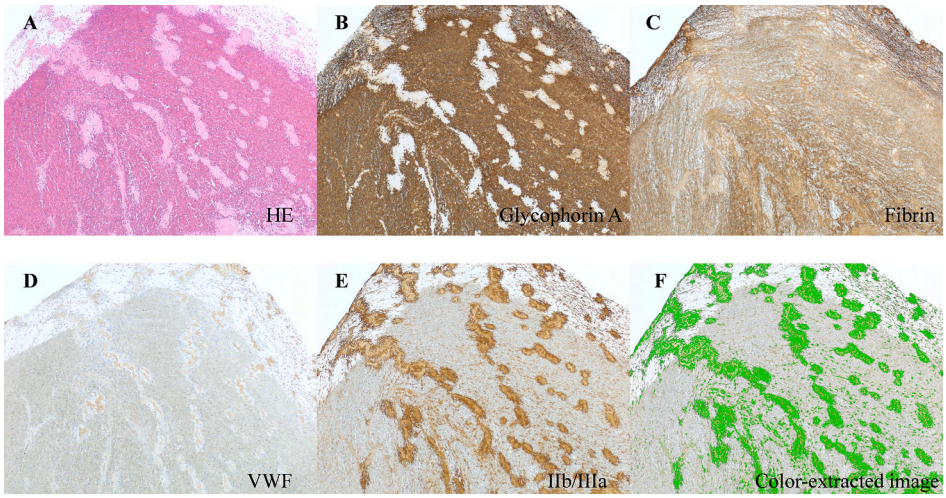

Fig. 1. Hematoxylin and eosin and immunohistochemical staining of thrombus retrieved from stroke patients. Examples of thrombus component quantification.
Serial sections of thrombus were stained with hematoxylin and eosin (HE) staining (A) and primary antibodies against Glycophorin A (B), fibrin (C), von Willebrand factor (VWF) (D), IIb/IIIa (E), color-extracted image (F). The positive area is expressed as the ratio of the extracted light green areas of the immunopositive areas of the thrombus. Original magnification ×40.

number of immune-positive cells per mm^2 (Fig. 2) [7,9,12].

2.6. Determination of thrombus age

We evaluated 74 cases of the subtype CE thrombus using lytic features (HE stain) and CD163 positive cell count (Fig. 2). The retrieved thrombus was pathologically classified using HE staining employing the accepted definition. Lytic thrombus (1–5 days) was characterized by areas with lysis of inflammatory cells and granulocyte karyorrhexis. An older thrombus was defined as a thrombus with >20 % area in a section with lysis of inflammatory cells and granulocyte karyorrhexis (Fig. 2B). A thrombus was considered fresh when intact granulocytes were >80 % (Fig. 2A) [9,20,21]. We also evaluated the number of CD163-positive cells to assess the age of the thrombus (Fig. 2C, D). CD163 is a scavenger receptor for the hemoglobin-haptoglobin complex and expressed exclusively on circulating monocytes and tissue macrophage subpopulations. The number of CD163-positive cells has been shown to increase with time after onset in deep vein thrombosis. Therefore, the number of CD163-positive cells is a useful indicator of the thrombus age. [12]

2.7. Statistical analysis

Values are presented as mean ± SD or median and interquartile range (IQR) unless specified otherwise. Fisher's exact test was used to determine a significant association between the two categorical variables. The Mann–Whitney *U* test and Kruskal–Wallis test were applied to determine the significant difference between 2 groups and more, respectively. Correlations between two continuous variables were evaluated using Spearman analysis. Adjusted common odds ratios (ORs) were calculated to reveal associations between functional outcomes and thrombus composition using multivariable ordinal logistic regression (proportional odds regression model). The adjusted variables were age, sex, TICI 3 reperfusion, and puncture-to-reperfusion time. Functional outcomes were examined based on the shift in the distribution of the mRS on hospital discharge, with scores of 5 (bed-bound with severe disability) and 6 (death) combined. Boxplot diagrams were generated to illustrate the group differences and similarities. All tests were two-tailed, and $p < 0.05$ was considered statistically significant. EZR software version 1.55 (Saitama Medical Center, Jichi Medical University, Saitama, Japan) was used for statistical analysis [22].

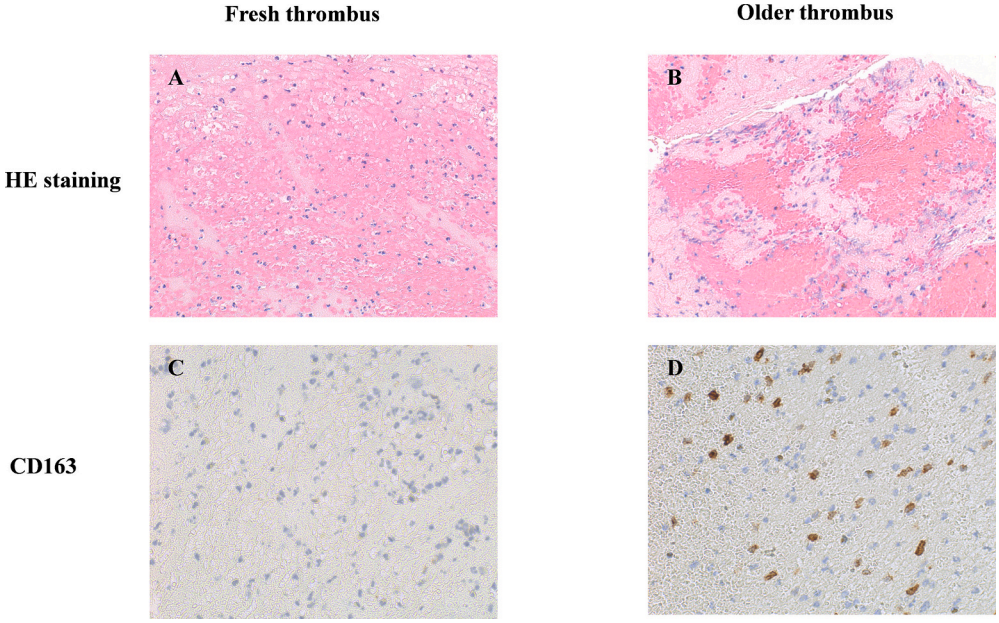

Fig. 2. Representative results of histopathological findings of thrombus age.
Hematoxylin and eosin (HE) staining. High-power view of a fresh thrombus (A) and an older thrombus (B). Granulocytes with lytic features such as karyorrhexis and nuclear dust. (C) CD163-positive macrophages are rarely observed in the thrombus. (D) Focal aggregation of CD163-positive macrophages was observed in the thrombus. Original magnification ×200.

3. Results

3.1. Baseline characteristics

The clinical characteristics of 108 patients analyzed in the initial study are summarized in Table 1.

The median patient age was 77.8 years. NIHSS scores on admission were 19.6 ± 8.1. A history of stroke was observed in 24 patients (22.0 %). The main occlusion site was the middle cerebral artery (MCA) in 60 patients (55.5 %). Vertebrobasilar occlusion accounted for 11 patients (10.2 %). Twenty patients (18.5 %) were classified as mRS grade 0–2 on discharge. The stroke subtypes were as follows: CE, 74 cases; LAA, 11 cases (extracranial carotid artery stenosis: 9, intracranial artery stenosis: 2); undetermined etiology, 12 cases; and other determined etiology, 11 cases (tumor-associated, 2; after intra- or extracranial artery dissection, 4; trauma, 1; cardiomyopathy, 1; carotid web, 1; cholesterol crystal embolization, 1; and at the time of myocardial infarction treatment, 1).

Regarding revascularization outcomes in all 108 patients, the number of maneuvers was 1.8 ± 1.1 (mean ± SD). The puncture-to-reperfusion time was 60.0 min (median). TICI grade 3 was identified in 47 patients (43.6 %).

Table 1
Clinical, angiographic, procedural, functional outcomes, and thrombus component characteristics of the patients.

Variables	n = 108
Age, years (means ± SD)	77.8 ± 11.0
Sex, n (%)	
Female	51 (47.2)
Baseline NIHSS (means ± SD)	19.6 ± 8.1
Stroke history, n (%)	24 (22.0)
Anticoagulants, n (%)	29 (27.4)
Antiplatelet drug, n (%)	16 (14.8)
Blood type non O, n (%)	65 (73.9)
tPA, n (%)	50 (46.3)
Vascular risk factors, n (%)	
Hypertension	76 (71.0)
Hyperlipidemia	33 (30.6)
Diabetes mellitus	20 (18.5)
Atrial fibrillation	75 (69.4)
Occlusion site, n (%)	
ICA including carotid-T	37 (34.3)
MCA m1	47 (43.5)
MCA m2	13 (12.0)
Basilar artery/PCA	11 (10.2)
Clinical outcomes	
mRS (at discharge), n (%)	
0–2	20 (18.5)
>2	88 (81.5)
Stroke cause (TOAST), n (%)	
1 = large artery atherosclerosis	11 (10.2)
2 = cardioembolism	74 (68.5)
3 = small vessel occlusion	0 (0)
4 = other determined etiology	11 (10.2)
5 = undetermined etiology	12 (11.1)
Revascularization outcomes	
No. of maneuvers, (mean ± SD)	1.8 ± 1.1
Onset-to-reperfusion time (min), median (IQR)	238 (192–324)
Puncture-to-reperfusion time (min), median, (IQR)	60.0 (37.0–102.3)
TICI grade, n (%)	
2b or 3	83 (76.9)
3	47 (43.6)
Components of thrombi, median, (IQR)	
Fibrin (%)	40.8 (29.4–46.4)
RBCs (%)	34.7 (27.2–45.7)
Platelets (%)	23.1(14.8–29.1)
VWF (%)	4.2 (2.3–8.0)

tPA, intravenous tissue-type plasminogen activator; ICA, internal carotid artery; MCA, middle cerebral artery; PCA, posterior cerebral artery; mRS, modified Rankin Scale; NIHSS, National Institutes of Health Stroke Scale; TOAST, Trial of Org 10172 in Acute Stroke Treatment; TICI, thrombolysis in cerebral infarction; RBCs, red blood cells; VWF, von Willebrand factor.

3.2. Correlation of thrombus components

Table 1 (bottom row) shows the results of quantitative analysis of the 108 patients. Thrombi are quantitatively dominated by RBCs and fibrin, with a mixture of platelets and VWF.

Fig. 1 shows serial sections of the thrombus stained with each immunohistochemical stain. These serial sections reveal the location of each component. The morphological and immunohistological distribution trends of these components indicated that the RBCs and platelet regions were located separately in these thrombi (Fig. 1B and E). In addition, fibrin tended to be present in both platelet and RBCs regions but was more abundant in the platelet region (Fig. 1B, C and E). Furthermore, VWF and platelets were always present in the same regions (Fig. 1D and E). Most thrombi were RBC-dominated, but some thrombi were dominated by platelets. These findings are similar to those previously reported by a Belgian group [23].

To accurately confirm these trends, we examined the correlation between the proportions of each thrombus component. Fig. 3 shows the correlations among the areas with the four main components of the thrombus: fibrin, VWF, platelets, and RBCs. Fibrin showed positive correlations with RBCs and platelets and had a stronger correlation with platelets than with RBCs (Fig. 3A, B). These results suggest that fibrin is present in both platelet and RBCs regions but is more abundant in the platelet region. Furthermore, VWF showed a stronger correlation with platelets than fibrin (Fig. 3C, D). This finding supports the hypothesis that VWF and platelets exist in the same region. There was no correlation between RBCs and platelet regions (data not shown), indicating that RBCs and platelets exist independently.

3.3. The components of the thrombus in stroke subtypes

Table 2 shows a comparison between the subtypes CE and LAA. The thrombus composition of each subtype was almost the same, and the difference was not statistically significant. Regarding the background of patient characteristics, patients with subtype CE were older than those with other subtypes. The patients with the LAA subtype were men. Our study evaluated the differences in blood cell components objectively using immunohistological staining, but there was no difference in thrombus components by stroke subtype.

3.4. Thrombus age and pathological features

Next, we further analyzed two indices regarding thrombus age in 74 patients with subtype CE. Lytic features on HE staining were observed in 35.0 % (IQR: 17.3–54.0). The number of CD163-positive cells was 155 cells/mm^2 (114–271). According to the lytic features on HE staining, 22 cases were classified as fresh thrombi (30.0 %) and 52 cases were classified as older thrombi (70.0 %). The baseline characteristics of the patients according to their lytic features are shown in the left panel of Table 3.

RBC areas of older thrombi were smaller than those of fresh thrombi (34.1 vs. 42.2 %, $p = 0.015$). The puncture-to-reperfusion time of the older thrombus was longer than that of the fresh thrombus (median [IQR]: 62.0 [45.0–105.0] vs. 46.0 [33.0–79.0] min, $p = 0.036$). In addition, the RBC areas tended to decrease with increasing lytic features ($p = 0.026$; Fig. 4A). However, the fibrin area did not correlate with the lytic features (Fig. 4B). In contrast, the number of CD163 positive cells was correlated with the fibrin area ($p < 0.001$; Fig. 4D). No relationship was observed between lytic features and CD163-positive cells (data not shown).

3.5. Relation between thrombus components (platelet, RBC, and VWF) and clinical outcomes

The patients with thrombus of subtype CE ($n = 74$) were divided into two categories with an equal number of patients ($n = 37$) using the

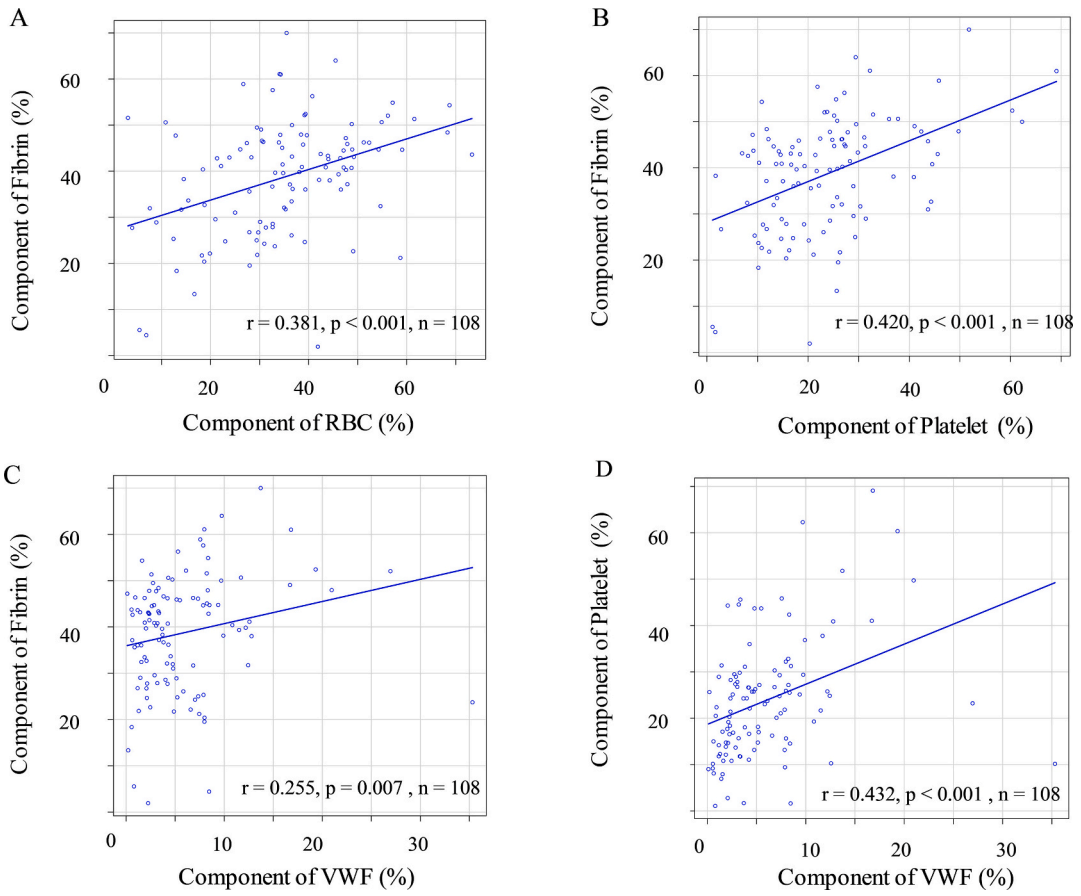

Fig. 3. Correlations of the thrombus components. Fibrin, RBCs, platelets, and VWF were quantitively analyzed for each thrombus.
(A) A positive linear association was found between fibrin and RBC areas. (B) A positive linear association was found between fibrin and platelets area. (C) A weak positive linear association was found between fibrin and VWF area. (D) A positive linear association was found between platelets and VWF area. RBC, red blood cells; VWF, von Willebrand factor.

Table 2
Differences among CE, LAA, and strokes of undetermined etiology and other determined etiology.

	CE (n = 74)	LAA (n = 11)	Etiology undetermined (n = 12)	Other determined etiology (n = 11)	CE vs LAA (p value)	CE vs etiology undetermined (p value)	CE vs other determined etiology (p value)
Fibrin (%)	42.8 (32.0–47.9)	41.1 (34.4–44.7)	37.6 (31.7–41.3)	28.6 (20.4–61.1)	0.763	0.213	0.079
RBC (%)	34.7 (27.5–44.2)	32.7 (30.2–48.8)	44.3 (28.7–47.3)	33.1 (4.0–39.2)	0.573	0.411	0.209
Platelet (%)	24.3 (15.8–29.4)	21.9 (15.4–28.5)	18.0 (14.4–28.1)	17.0 (10.2–43.7)	0.824	0.558	0.283
VWF (%)	4.3 (2.3–7.8)	7.9 (2.9–10.3)	3.1 (2.5–3.8)	5.2 (1.9–35.3)	0.295	0.39	0.421
Age, years (means ± SD)	81.0 ± 8.7	74.1 ± 7.3	73.0 ± 13.1	69.0 (42.0–83.0)	0.014	0.007	< 0.001
Female sex, n (%)	43 (58.1)	0 (0)	5 (41.7)	3 (27.3)	<0.001	0.355	0.102
tPA, n (%)	36 (48.6)	4 (33.3)	6 (50.0)	7 (63.6)	0.367	1	0.529
mRS on discharge (median, IQR)	4.0 (3.0–5.0)	4.0 (3.5–4.0)	4.5 (4.0–5.3)	4.0 (0.0–6.0)	0.376	0.194	0.915

tPA, tissue-type plasminogen activator; mRS, modified Rankin Scale; CE, cardioembolism; LAA, large artery atherosclerosis; RBC, red blood cell; VWF, von Willebrand factor. Values are presented as mean ± SD or median (interquartile range) or number (percentage).

percentage of platelet components (cut-off 24.3 %): platelet-rich thrombus and platelet-poor thrombus (Table 3, 2nd panel from left). Similarly, these patients were divided into two groups with equal numbers of patients using the components of RBCs (cutoff, 34.7 %) and VWF (cutoff, 4.3 %) (Table 3). Platelet-rich thrombi were associated with larger fibrin and VWF areas, a larger number of recanalization maneuvers, and a lower prevalence of TICI 3 reperfusion than platelet-poor thrombus (Table 3, 2nd panel from right). RBC-rich thrombi were associated with larger fibrin areas and shorter puncture-to-reperfusion times than RBC-poor thrombi (Table 3, right panel). Both platelet and RBC areas were associated with revascularization outcomes. In addition, platelet-rich thrombi were associated with poor clinical prognosis regarding mRS on discharge.

Table 3
Differences between thrombosis component and clinical outcomes.

	By HE staining		p value	Platelet-poor thrombus	Platelet-rich thrombus	p value	RBC-poor thrombus	RBC-rich thrombus	p value	VWF-poor thrombus	VWF-rich thrombus	p value
	Fresh thrombus	Older thrombus										
	(n = 22)	(n = 52)		(n = 37)	(n = 37)		(n = 37)	(n = 37)		(n = 37)	(n = 37)	
Thrombus component												
Fibrin (%)	43.4 (37.0–48.1)	40.9 (31.5–47.9)	0.478	37.1 (26.1–43.1)	46.3 (38.0–50.7)	0.001	36.7 (26.7–46.1)	45.9 (38.0–50.7)	0.006	40.9 (32.7–46.2)	45.9 (31.7–51.6)	0.106
RBCs (%)	42.2 (32.7–51.2)	34.1 (23.7–39.9)	0.015	34.8 (27.9–46.1)	34.7 (27.4–43.9)	0.935	27.4 (15.5–30.5)	44.3 (39.3–51.2)	<0.001	34.8 (29.5–44.0)	34.6 (19.9–44.3)	0.292
Platelets (%)	23.5 (17.8–28.5)	24.7 (15.5–30.0)	0.864	15.7 (10.8–20.3)	29.4 (26.6–42.4)	<0.001	24.8 (13.2–30.3)	24.3 (17.1–27.4)	0.812	17.7 (11.8–26.6)	26.3 (23.0–37.8)	<0.001
VWF (%)	3.6 (2.3–7.4)	4.4 (2.3–8.0)	0.714	2.5 (1.3–5.2)	6.8 (3.8–8.4)	<0.001	4.3 (2.1–7.6)	4.2 (2.4–8.4)	0.709	2.3 (1.2–3.2)	7.9 (5.8–9.8)	<0.001
CD 163 number/mm^2	144.0 (133.0–213.3)	164.0 (111.5–285.5)	0.786	171.0 (119.0–255.0)	142.0 (118.3–259.0)	0.88	132.0 (86.0–187.8)	207.0 (142.0–324.5)	<0.001	144.0 (109.5–229.3)	176.0 (132.0–308.0)	0.258
Lytic features (%)	8.8 (2.5–15.5)	46.0 (34.8–59.0)	<0.001	36.0 (18.0–54.0)	35.0 (15.6–50.0)	0.758	43.0 (24.0–58.0)	30.0 (15.0–47.5)	0.04	36.0 (17.0–57.5)	35.0 (20.0–50.0)	0.646
Age, y	80.8 ± 9.0	81.1 ± 8.7	0.895	79.5 ± 9.3	82.5 ± 7.9	0.137	82.5 ± 7.9	79.6 ± 9.3	0.159	80.6 ± 8.7	81.4 ± 8.8	0.692
Female sex, n (%)	9 (40.9)	34 (65.4)	0.072	21 (56.8)	22 (59.5)	1	26 (70.3)	17 (45.9)	0.059	21 (56.8)	22 (59.5)	1
Preanticoagulant, n (%)	11 (50.0)	17 (33.3)	0.2	18 (48.6)	10 (27.8)	0.093	13 (36.1)	15 (40.5)	0.811	13 (36.1)	15 (40.5)	0.811
Blood type non O, n (%)	13 (81.2)	28 (68.3)	0.513	24 (80.0)	17 (45.9)	0.238	18 (69.2)	23 (74.2)	0.771	20 (69.0)	21 (75.0)	0.77
tPA, n (%)	13 (59.1)	23 (44.2)	0.311	19 (51.4)	17 (45.9)	0.816	16 (43.2)	20 (54.1)	0.486	22 (59.5)	14 (37.8)	0.103
NIHSS	20.3 ± 7.0	21.2 ± 8.0	0.641	19.7 ± 7.5	22.3 ± 7.8	0.174	20.0 ± 7.7	21.8 ± 7.7	0.342	19.5 ± 7.9	22.5 ± 7.2	0.106
Risk factors, n (%)												
Diabetes mellitus	4 (18.2)	6 (11.5)	0.471	4 (10.8)	6 (16.2)	0.736	5 (13.5)	5 (13.5)	1	4 (10.8)	6 (16.2)	0.736
Hyper lipidemia	4 (18.2)	16 (30.8)	0.392	9 (24.3)	11 (29.7)	0.794	8 (21.6)	12 (32.4)	0.433	8 (21.6)	12 (32.4)	0.433
Hypertension	18 (81.8)	39 (75.0)	0.763	30 (81.1)	27 (73.0)	0.581	29 (78.4)	28 (75.7)	1	27 (73.0)	30 (81.1)	0.581
Stroke history	7 (31.8)	13 (25.0)	0.576	11 (29.7)	9 (24.3)	0.794	7 (18.9)	13 (35.1)	0.19	13 (35.1)	7 (18.9)	0.19
Radiological parameters												
Occlude vessel, n (%)												
ICA	9 (40.9)	18 (34.6)	0.71	15 (40.5)	12 (32.4)	0.279	11 (29.7)	16 (43.2)	0.251	11 (29.7)	16 (43.2)	0.609
M1	10 (45.5)	25 (48.1)		16 (43.2)	19 (51.4)		19 (51.4)	16 (43.2)		20 (54.1)	15 (40.5)	
M2	1 (4.5)	6 (11.5)		4 (10.8)	3 (8.1)		9 (24.3)	3 (8.1)		3 (8.1)	4 (10.8)	
Basilar artery/PCA	2 (9.0)	3 (5.8)		2 (5.4)	3 (8.1)		3 (8.1)	2 (5.4)		3 (8.1)	2 (5.4)	
Revascularization outcomes												
No. of maneuvers	1.0 (1.0–2.0)	1.0 (1.0–2.0)	0.74	1.0 (1.0–2.0)	1.5 (1.0–3.0)	0.019	1.0 (1.0–2.0)	1.0 (1.0–2.0)	0.375	1.0 (1.0–2.0)	1.0 (1.0–2.0)	0.85
Multiple-pass, n (%)	7 (35.0)	19 (38.8)	1	10 (27.8)	16 (48.5)	0.088	15 (41.7)	11 (33.3)	0.62	14 (40.0)	12 (35.3)	0.805
TICI grade 3, n (%)	11 (50.0)	23 (44.2)	0.799	22 (59.5)	12 (32.4)	0.035	16 (43.2)	18 (48.6)	0.816	15 (40.5)	19 (51.4)	0.484
Puncture-to-reperfusion time, min	46.0 (33.0–79.0)	62.0 (45.0–105.0)	0.036	53.0 (35.0–85.0)	71.0 (41.3–108.0)	0.16	75.0 (50.0–107.0)	45.0 (34.0–80.0)	0.007	54.0 (37.0–83.0)	62.0 (40.0–109.5)	0.309
Clinical outcomes												
mRS. (discharge)	4.0 (3.0–4.0)	4.0 (3.0–5.0)	0.204	4.0 (3.0–4.0)	5.0 (3.0–5.0)	0.014	4.0 (3.0–5.0)	4.0 (3.0–5.0)	0.226	4.0 (3.0–4.0)	4.0 (3.0–5.0)	0.15
>mRS 4, n (%)	5 (22.7)	21 (40.4)	0.187	7 (18.9)	19 (51.4)	0.007	15 (40.5)	11 (29.7)	0.465	9 (24.3)	17 (45.9)	0.087

RBCs, red blood cells; VWF, von Willebrand factor; IV tPA, intravenous tissue-type plasminogen activator; ICA, internal carotid artery; MCA, middle cerebral artery; TICI, thrombolysis in cerebral infarction; mRS, modified Rankin Scale; NIHSS, National Institutes of Health Stroke Scale; PCA, posterior cerebral artery. Values are presented as mean ± SD or median (interquartile range) or number (percentage).

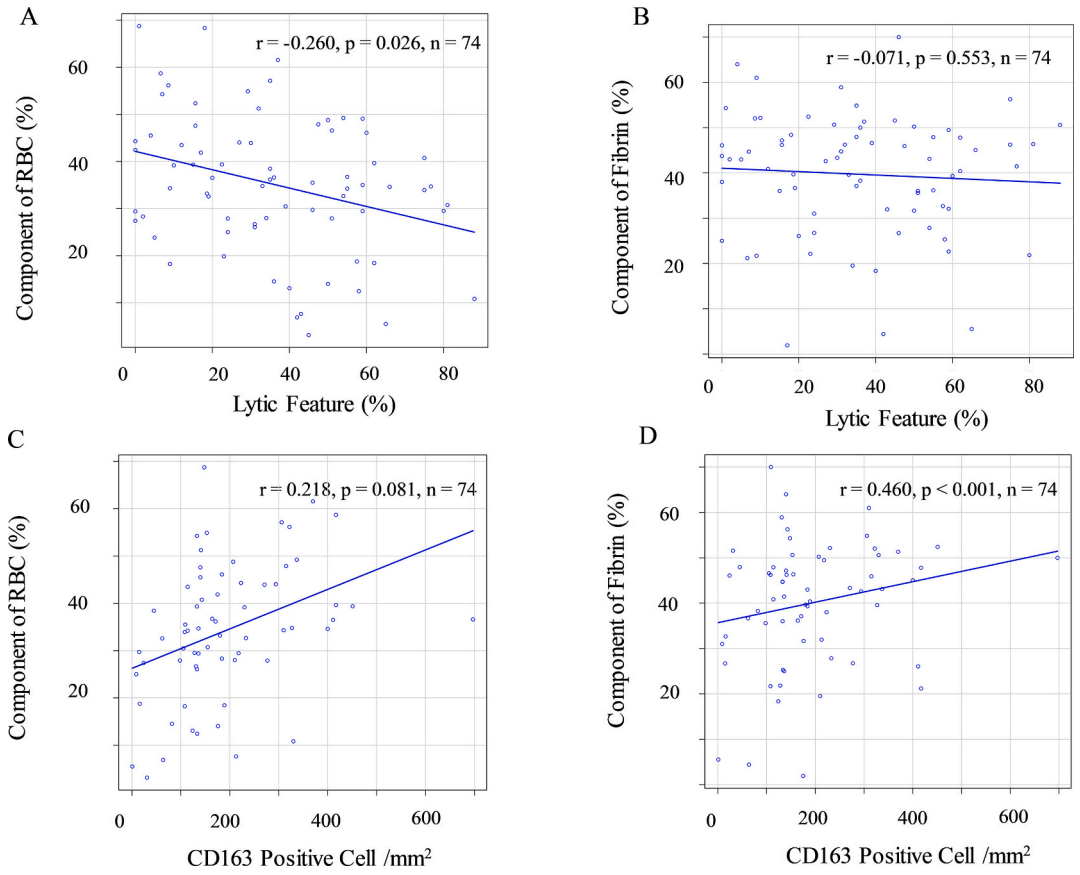

Fig. 4. Association of thrombus age with pathological features.
Two metrics were used to evaluate thrombus age in the cardioembolic group: Lytic features of thrombus percentage on hematoxylin and eosin stains and CD163 positive cells number of thrombus. (A) A weak negative linear association was found between the RBCs area and lytic features. (B) There was no correlation between the fibrin area and lytic features. (C) There was no correlation with CD163 the number and RBCs content. (D) A positive linear association was found between CD163 positive number and fibrin content.

3.6. Thrombus age and anticoagulant drug use

As shown in Table 1, 29 of 108 stroke patients received anticoagulants. Therefore, we analyzed the relationship between thrombus age and anticoagulant use in patients with subtype CE. Lytic features were not associated with anticoagulant use (data not shown).

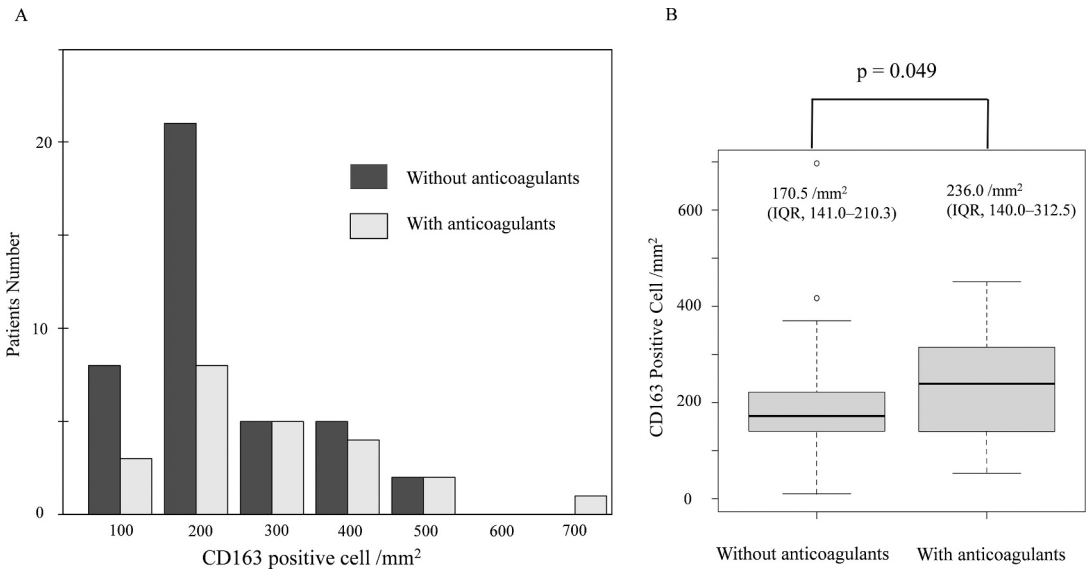

Fig. 5. Relationship between CD163 positive cell count and anticoagulant use.
(A) Histograms illustrating CD163 positive cell numbers per 100/mm^2 with or without anticoagulant use. (B) Boxplots represent the median and interquartile range between the different subgroups. The number of CD163 positive cells was higher in patients with anticoagulants than those without anticoagulants.

However, Fig. 5 shows the relationship between CD163-positive cell counts and anticoagulant use in patients with stroke subtype CE. In most CE cases, the number of CD163 positive cells was <200/mm^2. This tendency was apparent in the patients who did not receive anticoagulants. The number of CD163 positive cells was higher in patients treated with anticoagulants than in those not treated ($p = 0.049$; Fig. 5B). These results indicate that patients without oral anticoagulants developed ischemic stroke after thrombus formation at the embolic source earlier than those on oral anticoagulants. In other words, an older thrombus was found in patients with acute ischemic stroke taking anticoagulants.

3.7. Functional outcomes and thrombus composition

We investigated functional outcomes of stroke in subtype CE using mRS. Fig. 6A shows that an older thrombus by HE staining tends to have unfavorable outcomes compared with a fresh thrombus, but not the statistical difference (common odds ratio: 2.30, 95 % confidence interval [CI]: 0.74–7.21; $p = 0.15$). Fig. 6B shows a comparison of the functional outcomes between platelet-rich and platelet-poor thrombi. The frequency of the worse prognosis group (mRS 5 and 6) in platelet-rich thrombus group was significantly higher than that in the platelet-poor group (common odds ratio: 4.426, 95 % CI: 1.437–15.097; $p = 0.007$). These results indicate that platelet-rich thrombi are associated with unfavorable functional outcomes.

4. Discussion

In this study, we analyzed thrombus composition, focusing on the subtype CE of ischemic stroke. We identified a relationship between thrombus composition and clinical outcomes, including revascularization in acute ischemic stroke. Pathological analysis of thrombus composition, including thrombus age, may contribute to the elucidation of the pathogenesis of thrombosis and to the improvement of its treatment.

Concerning the impact of thrombus composition on the clinical course, our results revealed that platelet-rich thrombi were associated with unfavorable revascularization and clinical outcomes (Table 3). Conventionally, the critical factors that affect the prognosis of MT are

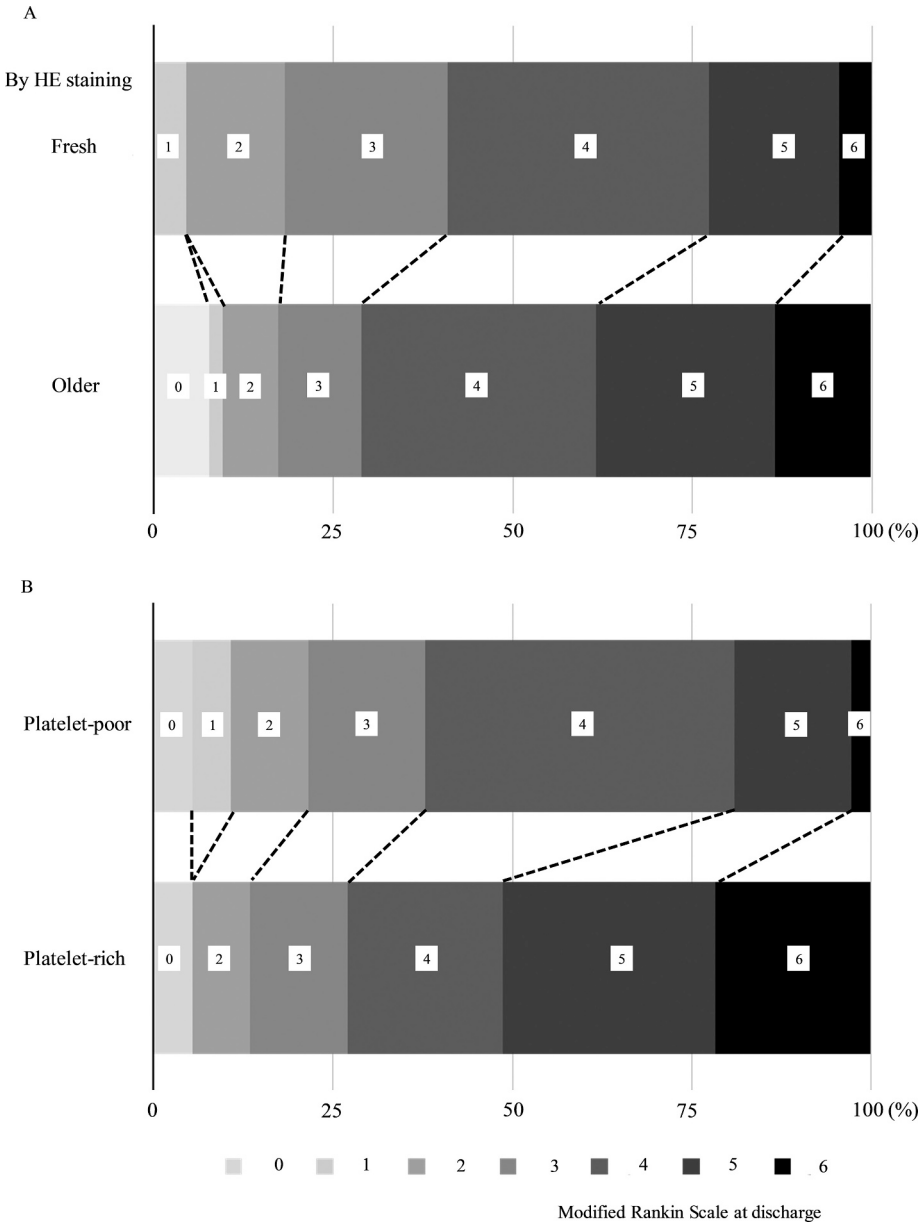

Fig. 6. Distribution of the modified Rankin Scale score at hospital discharge.
(A) Comparison of the modified Rankin Scale between fresh and older thrombi (common odds ratio: 2.30, 95 % confidence interval [CI]: 0.74–7.21; p = 0.15). (B) Comparison of the modified Rankin Scale between platelet-rich and platelet-poor thrombi (common odds ratio: 4.426, 95 % CI: 1.437–15.097; p = 0.007). Fisher's exact test was performed.

onset-to-recanalization time and TICI grade. Rapid and complete reperfusion is crucial for favorable outcomes [2,24–26]. However, many patients still have poor prognoses even after adequate revascularization. We speculated that two factors related to platelet-rich thrombi contributed to unfavorable outcomes. One is the environment in which platelet-rich thrombi are formed. Blood coagulability is important for thrombus formation in subtype CE, in addition to changes in blood flow. A previous study reported that high mean platelet volume (MPV) is an independent predictor of poor outcomes in patients with acute ischemic stroke undergoing MT [27]. Platelets in the blood affect thrombus formation and clinical outcomes. In a subsequent study, we propose to analyze the relationship between blood platelet counts and platelet composition in thrombi. The other factor was distal embolism after revascularization. A previous study reported that the thrombectomy maneuver results in thousands of small thrombus fragments that may not be visible on digital subtraction angiography and magnetic resonance imaging [24,28]. Platelet-rich thrombi may induce many distal embolisms after revascularization, leading to unfavorable outcomes. Platelet areas complicated by platelets, fibrin, WBCs, and NETs are likely more resistant to antithrombotics and MT therapy. It is necessary to consider the age and components of the thrombus when selecting the approach to manage stroke.

Our quantitative evaluation showed that VWF and platelets were present in the same regions of the thrombus. Fibrin is present in both platelet and red blood cell regions but is more abundant in platelet regions. The distribution of thrombus compositions suggests that thrombus formation occurs because blood cell components shape the ischemic stroke thrombus according to the theory of hemostasis. Staessens et al. [23] reported that a cerebral thrombus is primarily composed of two regions, the RBC and platelet areas. Platelet areas consist of dense fibrin, VWF, white blood cells, and platelets. The RBC areas are composed of packed RBCs within a fibrin meshwork. Furthermore, we observed several characteristics of thrombus morphology. When considering resistance to antithrombotic therapy and MT, both the composition and morphology of the thrombus are important factors. Using electron microscopy, Di Meglio et al. [29] reported that an ischemic stroke thrombus has a thin outer shell made of fibrin, VWF, and aggregated platelets. Therefore, thrombi surrounded by platelet areas may be considered resistant to antithrombotics and MT therapy.

With respect to thrombus age, Furukoji et al. [12] reported that CD163-positive cells were positively correlated with the time after onset in patients with DVT, whereas the RBC area was negatively correlated with the time after onset. However, in our study, the number of CD163-positive cells was positively correlated with RBC count (Fig. 4C). On the other hand, the lytic features observed on HE staining were negatively correlated with the RBC area (Fig. 4A). The number of CD163-positive cells did not correlate with the percentage of lytic features, and CD163-positive cell counts did not show a relationship with revascularization or clinical outcomes (data not shown). The two indicators used to evaluate the time course of thrombus exhibited conflicting results. Lytic features assess the nuclei of leukocytes, whereas CD163 assesses the macrophages that phagocytose RBCs. The reason for this discrepancy is unclear; however, the location and circumstances of thrombus formation may determine the progression of the organization process.

In our study, fresh and RBC-rich thrombi were associated with good revascularization outcome (Table 3). A platelet-rich thrombus was associated with poor revascularization and clinical outcomes (Table 3). A previous study on lytic features reported that older thrombi required more device passes and were associated with worse functional outcomes than fresh thrombi [13]. Maekawa et al. [30] reported that RBC-rich thrombi were correlated with fewer recanalization maneuvers and shorter procedure times. Douglas et al. [31] reported that a low platelet thrombus level was associated with TICI3 reperfusion. These findings suggest that fresh, RBC-rich, platelet-poor thrombi are easier to retrieve by MT. We identified that thrombus age and composition were associated with revascularization outcomes.

The number of CD163-positive cells was higher in CE patients treated with anticoagulants compared to those of patients not treated with anticoagulants. Most thrombus of CE may develop stroke at a relatively early stage of thrombus propagation. In patients without anticoagulants, early-stage thrombi develop ischemic stroke. In contrast, late-stage thrombi developed ischemic stroke in patients on anticoagulants. Manning et al. [32] reported that left atrial thrombi were identified in >40 % of the patients with acute ischemic stroke and newly recognized atrial fibrillation. Anticoagulants prevent thrombus formation. However, in some patients taking anticoagulants, the thrombus may enlarge slowly and gradually, and a small number of patients develop a stroke. Late-stage thrombus was considered resistant to antithrombotic and MT therapies, but there were no differences between patients with and without anticoagulants in terms of thrombus composition, revascularization outcomes, and functional outcomes. In this study, patients with stroke despite anticoagulant use were 27.4 %, therefore another prophylaxis in addition to anticoagulants is necessary for these patients with recurrent stroke.

This study had some limitations. In the pathological evaluation of cerebral thrombi, the retrieved thrombus is not the same as the thrombus formed in the embolic source. An entire thrombus formed in the embolic source, such as the left appendage, left atrium, left ventricle, and the site of cerebral artery stenosis, should be analyzed in comparison with the retrieved thrombus. In addition, we did not analyze the components in the blood, such as RBCs, platelets, fibrin, and VWF. The relationship between thrombus composition and blood levels of components gives us a further understanding of not only the pathophysiology, but also of the prevention and treatment of ischemic stroke. We plan to investigate this relationship further.

5. Conclusion

In conclusion, the analysis of thrombi retrieved by MT, including composition and thrombus age, showed a relationship with both revascularization and clinical outcomes. Therefore, neurointerventionalists should submit the retrieved thrombi for pathological analysis. The results of thrombus composition might reveal the pathophysiology of acute ischemic stroke and treatment to prevent a second stroke.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2022.09.004.

Declaration of competing interest

All authors state that there are no conflicts of interest to declare.

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