DNA content in ischemic stroke thrombi can help identify cardioembolic strokes among strokes of undetermined etiology

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Abstract

Background and purpose. Identification of acute ischemic stroke (AIS) etiology is crucial for guidance of secondary prevention. Previous studies have yielded inconsistent results regarding possible correlations between AIS etiology and thrombus composition, as assessed by semiquantitative histological analysis. Here, we performed a correlation analysis between AIS etiology and AIS thrombus cellular composition and content, as assessed using quantitative biochemical assays.

Methods. Homogenates of 250 AIS patient thrombi were prepared by mechanical grinding. Platelet, red blood cell, and leukocyte content of AIS thrombi were estimated by quantification of glycoprotein (GP)VI, heme, and DNA in thrombus homogenates. AIS etiology was defined as cardioembolic, non-cardioembolic, or embolic stroke of undetermined source (ESUS), according to the TOAST classification.

Results. Cardioembolic thrombi were richer in DNA (35.8 vs 13.8 ng/mg, $p<0.001$) and poorer in GPVI (0.104 vs 0.117 ng/mg, $p=0.045$) than non-cardioembolic ones. The area under the receiver operating characteristic curve of DNA content to discriminate cardioembolic thrombi from non-cardioembolic was 0.72 (95% CI, 0.63 to 0.81). With a threshold of 44.7 ng DNA/mg thrombus, 47% of thrombi from undetermined etiology would be classified as cardioembolic with a specificity of 90%.

Conclusions. Thrombus DNA content may provide an accurate biomarker for identification of cardioembolic thrombi in AIS patients with ESUS.

Clinical Trial Registration-URL: http://www.clinicaltrials.gov. Unique identifier: NCT03268668.

Non-standard Abbreviations and Acronyms: AIS: acute ischemic stroke, ESUS: embolic stroke of undetermined source; EVT: endovascular therapy; GPVI: glycoprotein VI; LVO:

large vessel occlusion, MRI: magnetic resonance imaging; MSD: MesoScale Discovery; NETs: neutrophil extracellular traps, RBCs: red blood cells

Introduction

Acute ischemic stroke (AIS) can result from various mechanisms, such as large artery atherosclerosis or cardioembolism[1]. Determining AIS etiology is crucial for optimal patient management. Stroke etiology is indeed a key factor for secondary prevention decisions. Yet, in 30 to 40% of AIS patients, a specific stroke etiology cannot be determined[2]. In the case of AIS due lo large vessel occlusion (LVO), it has been proposed that thrombus composition could help determine thrombus origin. Although AIS thrombi causing LVO have been shown to share the same basic components and structure[3], they are highly heterogeneous in that they contain highly variable amounts and proportions of red blood cells (RBCs)[4], platelets[5], leukocytes[5], fibrin[6], and von Willebrand factor[4]. This heterogeneity in thrombus composition has been suggested to reflect that in AIS etiology. Nevertheless, previous studies have reported conflicting results regarding possible correlations between thrombus composition and AIS etiology. The lack of consistency in conclusions on this issue might be related, at least in part, to the fact that the vast majority of studies on thrombus composition have been based on semiquantitative histological analyses using nonspecific staining methods of thrombus components[4–7]. In addition, considering the large inter- and/or intra-observer variability inherent to histological scoring strategies, such approaches may not allow for the development of accurate diagnostic tools. In order to explore possible alternative methods for AIS thrombus analysis and etiology identification, we compared AIS thrombus composition according to AIS etiology using cell-type specific quantitative assays performed on whole-thrombus homogenates.

Methods

Data Availability

The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request and with permission of all contributing authors.

Standard Protocol Approvals, Registrations, and Patient Consents

Thrombi were collected in two centers at the end of endovascular therapy (EVT). The EVT procedure was chosen at the interventionalist's discretion, using a stent-retriever and/or a contact aspiration technique. AIS etiology was classified as described[1] and determined based on cerebral magnetic resonance imaging (MRI), computed tomography or MRI angiography, transcranial and extracranial duplex sonography, coagulation tests, 1 to 3 days electrocardiography recording, and transthoracic and/or transesophageal echocardiography. Patient data were collected prospectively using a standardized questionnaire (Endovascular Treatment in Ischemic Stroke -ETIS- registry NCT03776877). All patients were provided with a written explanation of the study. The patients or their representatives were given the opportunity to refuse participation. The local Ethics Committee approved this research protocol (CPP Nord Ouest II, ID-RCB number: 2017-A01039-44).

Preparation of thrombus homogenates

Thrombus homogenates were prepared with stainless steel beads (5 mm, Qiagen, 69989) in cold PBS (30 µL/mg thrombus) supplemented with protease inhibitor (1%, Sigma, P8340), using a tissue lyser (25Hz, 4 minutes, TissueLyser II, Qiagen). Thrombi not completely grinded went through a second passage in the tissue lyser. The thrombus homogenates were then recovered

after centifugation (14 000g x 20 minutes, 4°C) to eliminate non-soluble debris. Homogenates of initially cut thrombi were pooled before analysis.

Quantification of red blood cell and DNA

RBC content was estimated by measurement of heme concentration in thrombus homogenates using a formic acid-based colorimetric assay, as described previously[8]. DNA was quantified using the Molecular Probes Quant iT Picogreen dsDNA Assay kit (Life Technologies).

Quantification of platelet content

Soluble GPVI levels were measured by immunoassay according to the following protocol. Ninety-six wells standard binding plate from MesoScale Discovery (MSD, Rockville, MD) were coated overnight at 4˚C with 2 µg/mL sheep anti human GPVI polyclonal antibody (Bio Techne, France, AF3627). After 1 hour of incubation at room temperature with 5% MSD Blocker A (R93AA-1) and 3 washes with 150 µL PBS / 0.05% Tween, 25 µL of thrombus homogenate or standard were added and the plate was incubated for 1 hour at room temperature, 500 rpm. Standard curve was obtained with Recombinant Human GPVI protein (Bio techne, France, 3627-GP, 0,097-25 ng/ml). After 3 PBS Tween washes, 25 µL of biotinylated sheep anti-human GPVI antibody (Bio Techne, France, BAF3627, 0,5 µg/mL in 1% MSD Blocker A) was added to each well and the plate was incubated 1 hour at room temperature. Finally, 25 uL of streptavidin Sulfo-TAG/well was added after 3 PBS Tween washes and the plate was incubated 1 hour at room temperature. A MesoScale Quickplex Plate Scanner was used of quantification.

Statistical Analysis

Categorical variables were expressed as frequencies and percentages. Quantitative variables were expressed as mean (standard deviation, SD), or median (interquartile range, IQR) for non-normal distribution. Normality of distributions was assessed graphically and by using the Shapiro-Wilk test. We compared the different proportions of components of thrombi (heme, DNA, platelet, and DNA/platelet ratio) between the 3 AIS etiology subgroups (cardioembolic, non cardioembolic and ESUS) using one-way analysis of variance (ANOVA); post-hoc pairwise comparisons were done using linear contrast after Bonferroni correction. Primary comparison covered the overall study sample and was further performed according to use of IV alteplase prior to EVT. For thrombus content which were significant between the two group of interest (cardioembolic vs. non cardioembolic), we assessed the performance of thrombus content to determine cardioembolic from noncardioembolic etiology by calculating the area under the ROC curves (AUCs) and their 95% confidence intervals (CIs). From the ROC curves, we determined the optimal threshold value by maximizing the Youden index as well as the threshold values to reach a sensitivity and specificity of 0.90, respectively. We applied these threshold value in the cryptogenic patients. Statistical testing was conducted at the two-tailed α-level of 0.05. Data were analyzed using the SAS software version 9.4 (SAS Institute, Cary, NC).

Results

From June 2016 to November 2018, a total of 1209 consecutive AIS patients with LVO were treated by EVT in our institutions. Thrombi from 250 of these patients selected randomly were homogenized and analyzed for RBC, platelet, and leukocyte content, as estimated by quantification of heme, GPVI, and DNA, respectively. Patient and treatment characteristics of the study sample are reported in table 1. Stroke etiology was cardioembolic in 142 (56.8%) patients, non-cardioembolic in 33 patients (13.2%), and undetermined in 75 patients (30.0%).

147

148 ***Thrombus cellular content and AIS etiology***

149 There was no significant difference in the heme content between thrombi from cardioembolic
150 and non-cardioembolic origin (Figure 1A).

151 Non-cardioembolic thrombi had reduced DNA content, and increased GPVI content as
152 compared to cardioembolic thrombi (Figure 1B and C). As a consequence, the DNA/GPVI ratio
153 (Figure 1D) was higher in cardioembolic thrombi than in non-cardioembolic ones (median IQR
154 : 322 (151 to 1132) vs 114 (73 to 341), $p<0.001$). Together, these results indicate that
155 cardioembolic thrombi contain significantly more leukocytes and less platelets than non-
156 cardioembolic ones.

157 Thrombi from undetermined etiology had increased heme content compared to cardioembolic
158 thrombi (Figure 1A), but showed no significant differences in DNA or platelet content as
159 compared to either of the other groups of thrombi (Figure 1B-D).

160

161 ***Thrombus DNA content to discriminate cardioembolic versus non-cardioembolic AIS***

162 The area under the receiver operating characteristic curve (AUC) for thrombus DNA content
163 used for differentiating thrombi of cardioembolic and non-cardioembolic origins was of 0.72
164 (95% CI, 0.63 to 0.81). A similar AUC value was obtained for the DNA/GPVI ratio (Figure 2
165 and table 2). These data suggest that both thrombus DNA content and DNA/GPVI ratio hold
166 potential usefulness for identification of cardioembolic thrombi. In contrast, the AUC for the
167 GPVI thrombus content was of 0.65 (95% CI, 0.54 to 0.77) (Figure 2 and table 2), indicating a
168 poor diagnostic potential. The specificity and sensitivity of thrombus DNA content for
169 discriminating cardioembolic thrombi from non-cardioembolic thrombi was calculated for
170 various thresholds of DNA thrombus content (table 2). For a threshold of 44.7 ng DNA/mg

thrombus, nearly 50% of ESUS thrombi would be classified as cardioembolic with a specificity of 90%.

Discussion

In the present study conducted on 250 AIS thrombi responsible for LVO, we have explored possible relationships between AIS etiology and thrombus cell composition. In order to avoid the inherent limitations of semi-quantitative immunohistological methods[7], we have analyzed cell composition using quantitative assays for markers of RBCs, platelets, and leukocytes. Our results show that cardioembolic thrombi are richer in DNA and poorer in platelets compared to non-cardioembolic thrombi. From a pathophysiological perspective, the increased DNA content of thrombi from cardioembolic origin suggests a more prominent role of leukocytes in the formation of those thrombi. Leukocytes, especially neutrophils, are indeed the primary source of DNA in blood and are now widely recognized as active players of thrombosis[9,10]. Interestingly, previous studies have shown that elevated neutrophil-lymphocyte ratios in patients with nonvalvular atrial fibrillation were independently associated with the presence of left atrial thrombus[11], as well as with an increased risk of thromboembolic stroke[12]. Also consistent with our results, patients with cardioembolic stroke were reported to have increased plasma cell-free DNA levels compared to stroke patients of other etiologies[13].

The increased DNA content of cardioembolic thrombi might also reflect their previously reported higher leukocyte and neutrophil extracellular traps (NETs) content compared to thrombi of other origins[14]. Additionally, the high proportion of DNA content found in cardioembolic thrombi and the pivotal role of neutrophils and NETs in thrombosis give additional arguments for a potential benefit of DNAse 1 in AIS treatment[14,15]. It should be noted, however, that the lack of specificity of DNA for a particular cell type might be a source of variability hindering the drawing of more definitive correlations between thrombus DNA

content and stroke etiology. Besides leukocytes, endothelial cells, which can be extracted together with the thrombus during EVT, represent a potential non-etiology-specific source of contaminating DNA[16]. Moreover, while there is converging evidence that cardioembolic thrombi are enriched in neutrophils and NETs, immunohistological analyses have indicated that thrombi from atherosclerotic origin have an increased T cell content[17].

Still, despite the lack of cell specificity of DNA, our results indicate that both the thrombus DNA content and the thrombus DNA/GPVI ratio could provide biomarkers for identification of cardioembolic thrombi among thrombi of undetermined origin. In fact, specificity/selectivity calculations revealed that, by adjusting the DNA thrombus content threshold, one could classify nearly 50% of ESUS thrombi as cardioembolic with a specificity of 90%. Given that ESUS represents 20-25% of all AIS, there is a clear interest in developing new diagnostic tools to better identify ESUS patient subgroups. A recent major secondary prevention trial found no superiority of rivaroxaban over aspirin for prevention of recurrent stroke in the overall ESUS patient population[18]. Identifying the subgroup of ESUS patients requiring more active cardiac screening and which could benefit from anticoagulant therapy could help to improve both patient management and design of secondary prevention studies. Notably, the specificity and sensitivity of stroke classification systems have been reported to be variable[19]. This variability represents a potential challenge for prospective studies aimed at validating the use of quantitative measurement of thrombus-derived biomarkers like DNA as adjunctive assays for determination of stroke etiology. Prospective studies focusing on the impact of such adjunctive assays for patient selection on secondary stroke prevention efficacy could also help to validate their clinical utility.

In addition to be inexpensive, thrombus homogenization as performed in our study requires only moderate skills and is fairly easily feasible with common laboratory and hospital equipment, and so is the subsequent measurement of DNA in thrombus homogenates. The main

limitation of this method based on mechanical grinding of AIS thrombi is that non-soluble components such as fibrin could not be directly quantified. Another limitation may arise from the fact that thrombus components measured in thrombus homogenates may not strictly reflect the composition of the initial culprit thrombus causing LVO. In fact, it is well accepted that thrombus expansion occurs secondary to arterial occlusion. As a consequence, thrombus parts building up from and on top of the original thrombus enrich it with components unrelated to stroke etiology. Because of this variable dilution effect, the sole quantitative analysis of thrombus composition is unlikely to allow accurate determination of stroke etiology in all cryptogenic cases. A more global approach combining this quantitative method and classical investigation strategies (i.e cardiac, hemostasis, and vascular screenings) may thus prove more efficient for this purpose.

Our data need reproduction and confirmation in other cohorts. Nonetheless, to date, and to our knowledge, it is the largest study on thrombus composition based on biochemical quantitative analysis of their cellular content. Our results provide a potential basis for the development of new tools and strategies for identification of ESUS patient subgroups and improved secondary prevention.

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Conflict of interest: None

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Appendix

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Figure legends

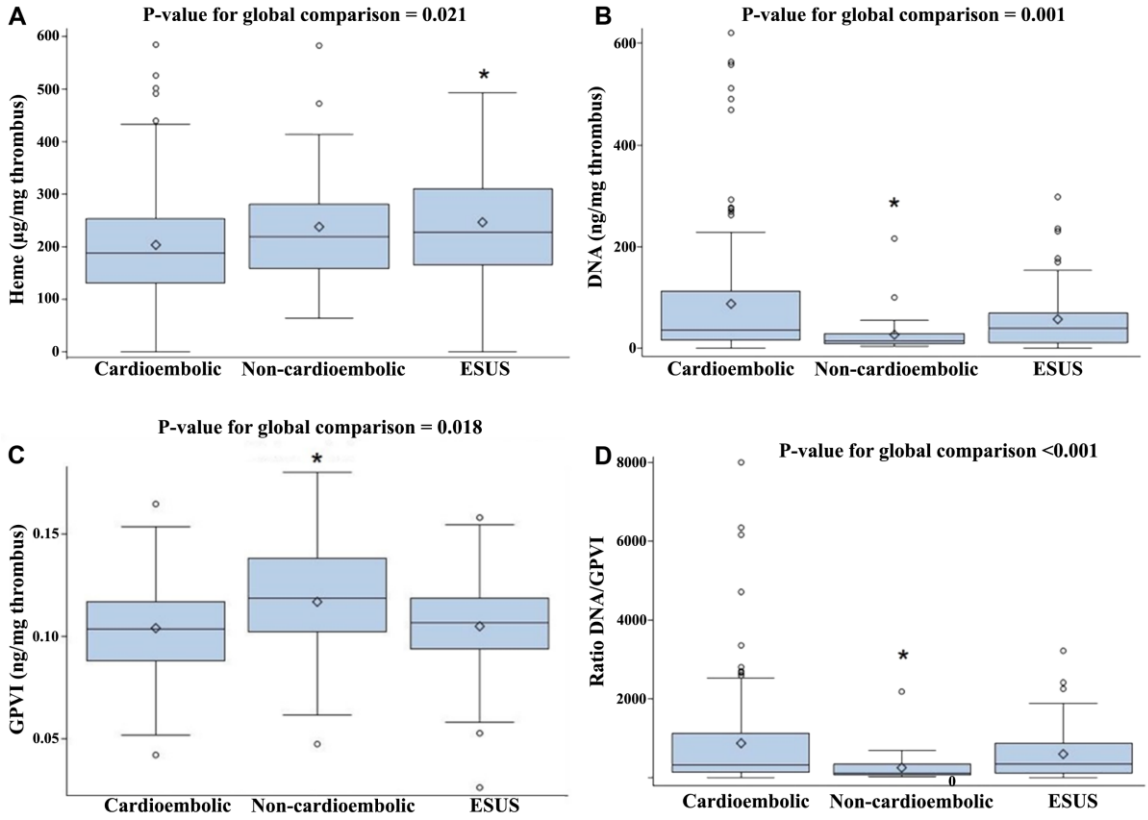
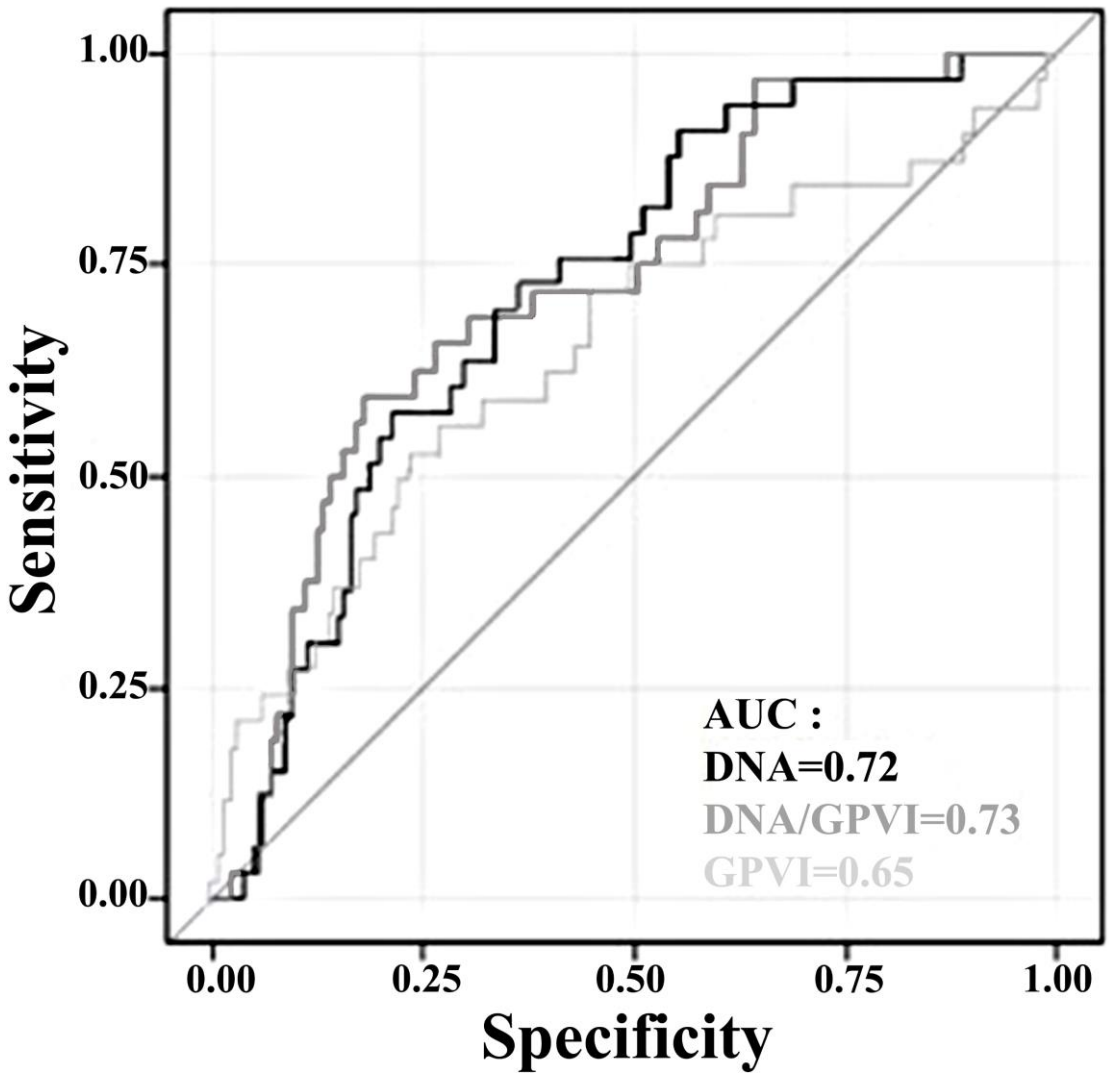


Figure 1. Distribution of biochemical features of AIS thrombi according to etiology. Boxes show the 25th, 50th, and 75th, and whiskers indicate values outside the lower and upper quartile with a length equal to 1.5 interquartile range; diamond indicates the mean values. P-values for global comparison (one-way ANOVA) are reported after a log-transformation for DNA, and ratio DNA/GPVI; * indicated *P*-values <0.05 for post-hoc pairwise comparison between cardioembolic stroke and each other stroke subgroups (adjusted for multiple comparison using Bonferroni correction).



317 **Figure 2. Receiver operating characteristic (ROC) curve for differentiation of**
318 **cardioembolic and non-cardioembolic strokes according to DNA and GPVI thrombus**
319 **content, and to the DNA/GPVI thrombus content ratio**.

320

Table 1. Patients and treatment characteristics, in overall and according to suspected acute ischemic stroke etiology

Characteristics	Overall	Suspected AIS etiology		
		Cardioembolic	Non-cardioembolic	ESUS
Number of patients	*250*	*142*	*33*	*75*
Demographics				
Age, years, mean (SD)	70.1 (15.5)	74.4 (14.6)	62.2 (12.9)	65.3 (15.5)
Men, n (%)	129/250 (51.6)	66/142 (46.5)	24/33 (72.7)	39/75 (52.0)
Medical history				
Hypertension	144/247 (58.3)	92/141 (65.2)	14/32 (43.8)	38/74 (51.4)
Diabetes	42/248 (16.9)	25/142 (17.6)	6/32 (18.8)	11/74 (14.9)
Hypercholesterolemia	79/247 (32.0)	52/141 (36.9)	9/32 (28.1)	18/74 (24.3)
Current smoking	50/238 (21.0)	22/134 (16.4)	7/32 (21.9)	21/72 (29.2)
Coronary artery disease	32/245 (13.1)	21/139 (15.1)	3/33 (9.1)	8/73 (11.0)
Previous stroke or TIA	36/246 (14.2)	23/139 (16.5)	5/33 (15.2)	7/74 (9.5)
Previous antithrombotic medications	103/244 (42.2)	81/140 (57.9)	7/31 (22.6)	15/73 (20.5)
Antiplatelet	47/244 (19.3)	29/140 (20.7)	5/31 (16.1)	13/73 (17.8)
Anticoagulant	48/244 (19.7)	44/140 (31.4)	2/31 (6.5)	2/73 (2.7)
Current stroke event				
NIHSS score, median (IQR)[a]	17 (12 to 20)	18 (14 to 21)	16 (9 to 19)	16 (12 to 20)
Pre-stroke mRS≥1	23/248 (9.2)	30/141 (21.3)	5/33 (15.2)	8/74 (10.8)
ASPECTS, median (IQR)[b]	7 (5 to 8)	7 (6 to 8)	6 (5 to 8)	6 (5 to 8)
Site of occlusion				
M1-MCA	134/246 (54.5)	80/139 (57.6)	7/33 (21.2)	47/74 (63.5)
M2-MCA	20/246 (8.1)	14/139 (10.1)	0 (0.0)	6/74 (8.1)
Intracranial ICA or tandem	53/246 (21.5)	28/139 (20.1)	7/33 (21.2)	18/74 (24.3)
Tandem	19/246 (7.7)	5/139 (3.6)	14/33 (42.4)	0 (0.0)

extracranial ICA	6/246 (2.4)	4/139 (2.9)	1/33 (3.0)	1/74 (1.4)
Vertebro-Basilar	12/246 (4.9)	6/139 (4.3)	4/33 (12.1)	2/74 (2.7)
Others	2/246 (0.8)	2/139 (1.4)	0 (0.0)	0 (0.0)
Treatment characteristics				
Intravenous Alteplase	131/250 (52.4)	62/142 (43.7)	20/33 (60.6)	49/75 (65.3)
General anesthesia	38/242 (15.7)	22/138 (15.9)	7/30 (23.3)	9/74 (12.2)
Onset to groin puncture time, min, median (IQR)[c]	240 (186 to 286)	222 (170 to 279)	262 (217 to 308)	250 (205 to 295)

Values expressed as no/total no. (%) unless otherwise indicated. [a]3 missing data (2 in cardioembolic group and 1 in Non-cardioembolic group) [b]18 missing data (12 in cardioembolic group, 1 in Non-cardioembolic group and 5 in Cryptogenic group) [c]7 missing data (4 in cardioembolic group, 1 in Non-cardioembolic group and 2 in Cryptogenic group).

Abbreviations: ASPECTS= Alberta stroke program early computed tomography score; ICA=internal carotid artery; IQR=interquartile range; MCA=middle cerebral artery; NIHSS=National Institutes of Health Stroke Scale; rt-PA=recombinant tissue plasminogen activator; TIA=transient ischemic attack; mRS=modified Rankin scale, SD=standard deviation.

Table 2. Accuracy of thrombus cell marker content for identification of cardioembolic thrombi

	AUC (95%CI)	Threshold	Sensitivity (95%CI)	Specificity (95%CI)	% of patients with ESUS
DNA	0.72 (0.63 to 0.81)	>22.4[1]	66.0 (57.5 to 73.7)	69.7 (51.3 to 84.4)	62.5
		>8.9	90.0	27.3 (13.3 to 45.5)	84.7
		>44.7	44.0 (35.6 to 52.3)	90.0	47.2
GPVI	0.65 (0.54 to 0.77)	<11.5[1]	56.2 (37.7 to 73.6)	89.2 (82.6 to 94.0)	71.9
		<13.4	90.0	28.1 (13.7 to 46.7)	90.6
		<7.7	10.0 (5.4 to 16.5)	90.0	12.5
DNA/GPVI	0.73 (0.63 to 0.82)	>161[1]	72.9 (64.3 to 80.3)	65.6 (46.8 to 81.4)	65.6
		>81	90.0	34.4 (18.6 to 53.2)	81.2
		>614	36.4 (28.1 to 45.4)	90.0	31.2

[1] cut-value who maximize the Youden index.

Abbreviations: AUC=area under the Receiver Operating Curve; CI=confidence interval.