Stroke

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DNA Content in Ischemic Stroke Thrombi Can Help Identify Cardioembolic Strokes Among Strokes of Undetermined Cause

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

METHODS: Homogenates of 250 patients with AIS thrombi were prepared by mechanical grinding. Platelet, red blood cell, and leukocyte content of AIS thrombi were estimated by quantification of GP (glycoprotein) VI, heme, and DNA in thrombus homogenates. AIS cause was defined as cardioembolic, noncardioembolic, or embolic stroke of undetermined source, according to the TOAST classification (Trial of ORG 10172 in Acute Stroke Treatment).

RESULTS: Cardioembolic thrombi were richer in DNA (35.8 versus 13.8 ng/mg, $P<0.001$) and poorer in GPVI (0.104 versus 0.117 ng/mg, $P=0.045$) than noncardioembolic ones. The area under the receiver operating characteristic curve of DNA content to discriminate cardioembolic thrombi from noncardioembolic was 0.72 (95% CI, 0.63–0.81). With a threshold of 44.7 ng DNA/mg thrombus, 47% of thrombi from undetermined cause 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 patients with AIS with embolic stroke of undetermined source.

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Acute ischemic stroke (AIS) can result from various mechanisms, such as large artery atherosclerosis or cardioembolism.[1] Determining AIS cause is crucial for optimal patient management. Stroke cause is indeed a key factor for secondary prevention decisions. Yet, in 30% to 40% of patients with AIS, a specific stroke cause cannot be determined.[2] In the case of AIS due to large vessel occlusion (LVO), it has been proposed that

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†A list of all compoCLOT study group members is given in the Appendix and the Data Supplement.

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Nonstandard Abbreviations and Acronyms

AIS	acute ischemic stroke
ESUS	embolic stroke of undetermined source
EVT	endovascular therapy
GPVI	glycoprotein VI
LVO	large vessel occlusion
RBC	red blood cell

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 cause. Nevertheless, previous studies have reported conflicting results regarding possible correlations between thrombus composition and AIS cause. 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 intraobserver variability inherent to histological scoring strategies, such approaches may not allow for the development of accurate diagnostic tools. To explore possible alternative methods for AIS thrombus analysis and cause identification, we compared AIS thrombus composition according to AIS cause using cell-type specific quantitative assays performed on whole-thrombus homogenates.

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METHODS

Data Availability

The data sets generated during and 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 2 centers at the end of endovascular therapy (EVT). The EVT procedure was chosen at the interventionalist's discretion, using a stent-retriever and a contact aspiration technique. AIS cause was classified as described[1] and determined based on cerebral magnetic resonance imaging, computed tomography or magnetic resonance imaging angiography, transcranial and extracranial duplex sonography, coagulation tests, 1 to 3 days electrocardiography recording, and transthoracic and transesophageal echocardiography. Patient data were collected prospectively using a standardized questionnaire (Endovascular Treatment in Ischemic Stroke registry, URL: https://www.clinicaltrials.gov; Unique identifier: 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 (25 Hz, 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 centrifugation (14 000*g*×20 minutes, 4°C) to eliminate nonsoluble debris. Homogenates of initially cut thrombi were pooled before analysis.

Quantification of RBC 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 (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% MesoScale Discovery 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% MesoScale Discovery Blocker A) was added to each well and the plate was incubated 1 hour at room temperature. Finally, 25 µL 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 (SD), or median (interquartile range) 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 cause subgroups (cardioembolic, noncardioembolic, and embolic stroke of undetermined source [ESUS]) using 1-way 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 intravenous alteplase before EVT. For thrombus content which were significant between the 2 group of interest (cardioembolic versus noncardioembolic), we assessed the performance of thrombus content to determine cardioembolic from noncardioembolic cause by calculating the area under the receiver operating characteristic curve and their 95% CIs. From the receiver operating characteristic 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 2-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 patients with consecutive AIS 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 cause

Table 1. Patients and Treatment Characteristics, in Overall and According to Suspected Acute Ischemic Stroke Cause

Characteristics	Overall	Suspected AIS Cause		
		Cardioembolic	Noncardioembolic	ESUS
No. of patients	250	142	33	75
Demographics				
Age, y, 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 mellitus	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)*	17 (12–20)	18 (14–21)	16 (9–19)	16 (12–20)
Prestroke mRS ≥1	23/248 (9.2)	30/141 (21.3)	5/33 (15.2)	8/74 (10.8)
ASPECTS, median (IQR)†	7 (5–8)	7 (6–8)	6 (5–8)	6 (5–8)
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)‡	240 (186–286)	222 (170–279)	262 (217–308)	250 (205–295)

Values expressed as n/total n (%) unless otherwise indicated. AIS indicates acute ischemic stroke; ASPECTS, Alberta Stroke Program Early CT Score; ESUS, embolic stroke of undetermined source; ICA, internal carotid artery; IQR, interquartile range; MCA, middle cerebral artery; mRS, modified Rankin Scale; NIHSS, National Institutes of Health Stroke Scale; r-tPA, recombinant tissue-type plasminogen activator; and TIA, transient ischemic attack.

*Three missing data (2 in cardioembolic group and 1 in noncardioembolic group).

†Eighteen missing data (12 in cardioembolic group, 1 in noncardioembolic group, and 5 in cryptogenic group).

‡Seven missing data (4 in cardioembolic group, 1 in noncardioembolic group, and 2 in cryptogenic group).

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was cardioembolic in 142 (56.8%) patients, noncardioembolic in 33 patients (13.2%), and undetermined in 75 patients (30.0%).

Thrombus Cellular Content and AIS Cause

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

Noncardioembolic thrombi had reduced DNA content and increased GPVI content as compared with cardioembolic thrombi (Figure 1B and 1C). As a consequence, the DNA/GPVI ratio (Figure 1D) was higher in cardioembolic thrombi than in noncardioembolic ones (median interquartile range: 322 [151–1132] versus 114 [73–341], P<0.001). Together, these results indicate that cardioembolic thrombi contain significantly more leukocytes and less platelets than noncardioembolic ones.

Thrombi from undetermined cause had increased heme content compared with cardioembolic thrombi (Figure 1A) but showed no significant differences in DNA or platelet content as compared with either of the other groups of thrombi (Figure 1B through 1D).

Thrombus DNA Content to Discriminate Cardioembolic Versus Noncardioembolic AIS

The area under the receiver operating characteristic curve for thrombus DNA content used for differentiating thrombi of cardioembolic and noncardioembolic origins was of 0.72 (95% CI, 0.63–0.81). A similar area under the receiver operating characteristic curve value was obtained for the DNA/GPVI ratio (Figure 2 and Table 2). These data suggest that both thrombus DNA content and DNA/GPVI ratio hold potential usefulness for identification of cardioembolic thrombi. In contrast, the area under the receiver operating characteristic curve for the GPVI thrombus content was of 0.65 (95% CI, 0.54–0.77; Figure 2 and Table 2), indicating a poor diagnostic potential. The specificity and sensitivity of thrombus DNA content for discriminating cardioembolic thrombi from noncardioembolic thrombi was calculated for various thresholds of DNA thrombus content (Table 2). For a

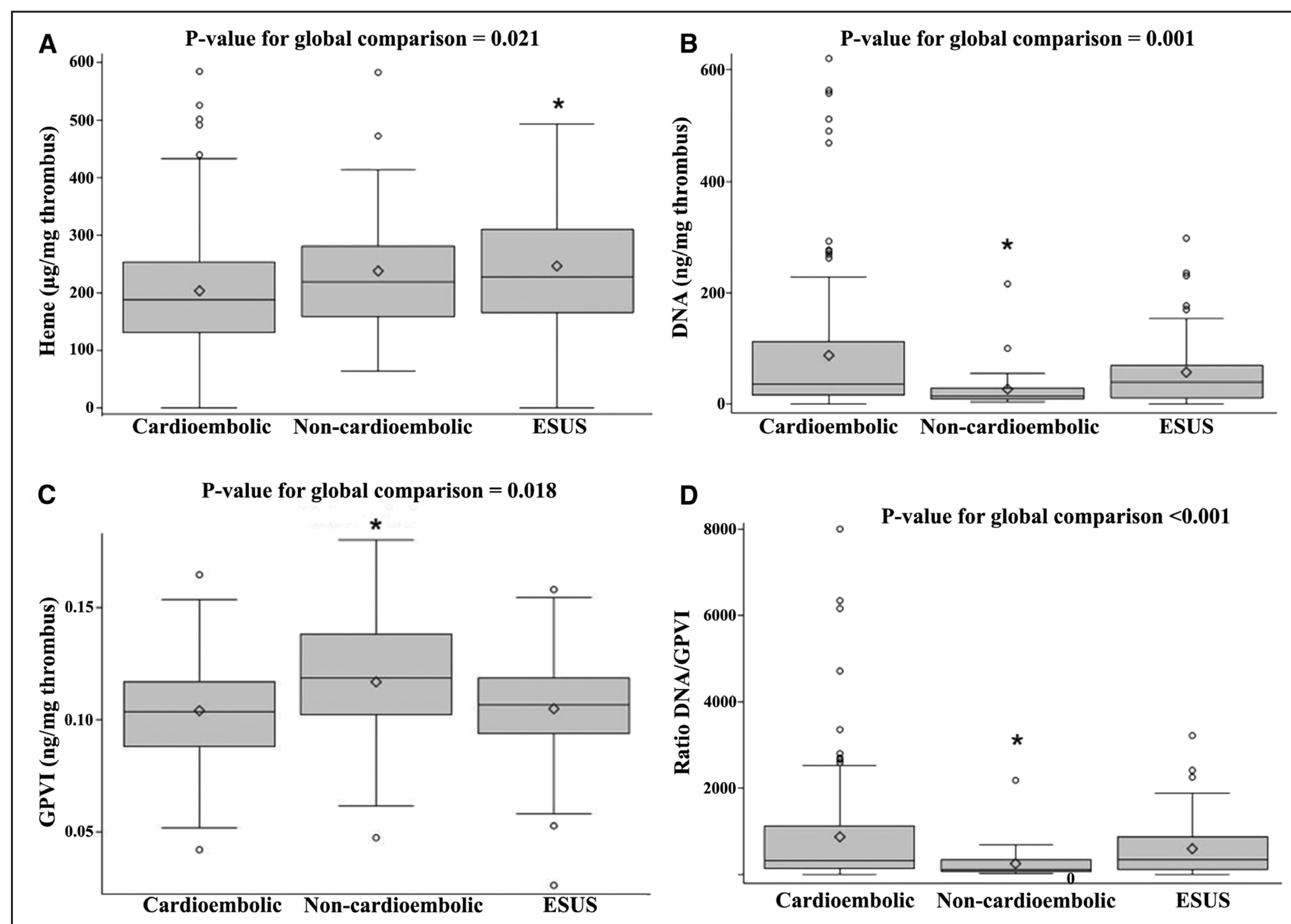

Figure 1. Distribution of biochemical features of acute ischemic stroke (AIS) thrombi according to cause.
Boxes show the 25th, 50th, and 75th, and whiskers indicate values outside the lower and upper quartile with a length equal to 1.5 interquartile range; diamond indicates the mean values. P values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

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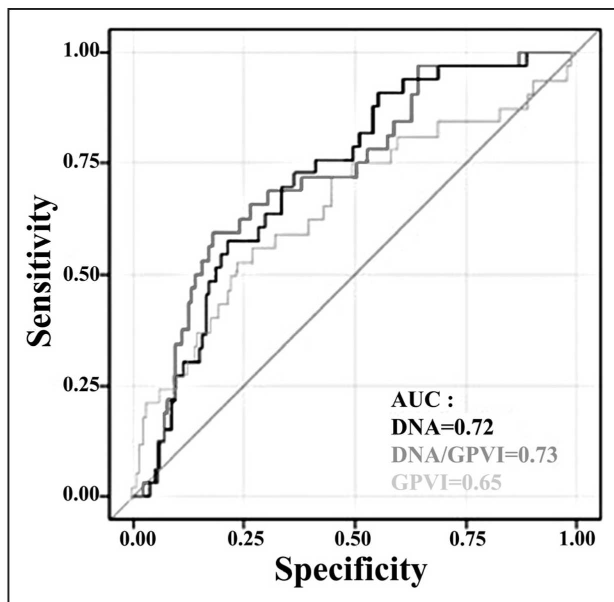

Figure 2. Receiver operating characteristic (ROC) curve for differentiation of cardioembolic and noncardioembolic strokes according to DNA and GPVI (glycoprotein VI) thrombus content, and to the DNA/GPVI thrombus content ratio. AUC indicates area under the ROC.

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 cause and thrombus cell composition. To avoid the inherent limitations of semiquantitative 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 with noncardioembolic 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 with stroke patients of other causes.[13]

The increased DNA content of cardioembolic thrombi might also reflect their previously reported higher leukocyte and neutrophil extracellular traps content compared with thrombi of other origins.[14] Additionally, the high proportion of DNA content found in cardioembolic thrombi and the pivotal role of neutrophils and neutrophil extracellular traps 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 cause. Besides leukocytes, endothelial cells, which can be extracted together with the thrombus during EVT, represent a potential noncause-specific source of contaminating DNA.[16] Moreover, while there is converging evidence that cardioembolic thrombi are enriched in neutrophils and neutrophil extracellular traps, 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

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 (0.63–0.81)	>22.4*	66.0 (57.5–73.7)	69.7 (51.3–84.4)	62.5
		>8.9	90.0	27.3 (13.3–45.5)	84.7
		>44.7	44.0 (35.6–52.3)	90.0	47.2
GPVI	0.65 (0.54–0.77)	<11.5*	56.2 (37.7–73.6)	89.2 (82.6–94.0)	71.9
		<13.4	90.0	28.1 (13.7–46.7)	90.6
		<7.7	10.0 (5.4–16.5)	90.0	12.5
DNA/GPVI	0.73 (0.63–0.82)	>161*	72.9 (64.3–80.3)	65.6 (46.8–81.4)	65.6
		>81	90.0	34.4 (18.6–53.2)	81.2
		>614	36.4 (28.1–45.4)	90.0	31.2

AUC indicates area under the receiver operating curve; ESUS, embolic stroke of undetermined source; and GPVI, glycoprotein VI.
*Cut-value who maximize the Youden index.

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of 90%. Given that ESUS represents 20% to 25% of all AIS, there is a clear interest in developing new diagnostic tools to better identify patient with ESUS subgroups. A recent major secondary prevention trial found no superiority of rivaroxaban over aspirin for prevention of recurrent stroke in the overall patient with ESUS population.[18] Identifying the subgroup of patients with ESUS 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 cause. 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 nonsoluble 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 cause. Because of this variable dilution effect, the sole quantitative analysis of thrombus composition is unlikely to allow accurate determination of stroke cause in all cryptogenic cases. A more global approach combining this quantitative method and classical investigation strategies (ie, 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 patient with ESUS subgroups and improved secondary prevention.

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Disclosures

Dr Mazighi has relevant financial activities outside the submitted work with the following companies: Acticor Biotech, Air liquide, Boerhinger Ingelheim, Medtronic, Amgen. Dr Lapergue has relevant financial activities outside the submitted work with the following companies: Microvention, Stryker, and Penumbra. The other authors report no conflicts.

APPENDIX

List of compoCLOT Research Investigators

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