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

3

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Cover title: DNA content in AIS thrombi and etiology

Tables 2, Figures 2.

23 **Key words:** stroke etiology – ischemic stroke thrombi – secondary prevention

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25 **Abstract**

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

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

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

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

45 **Clinical Trial Registration-URL:** <http://www.clinicaltrials.gov>. Unique identifier:
46 NCT03268668.

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

49 large vessel occlusion, MRI: magnetic resonance imaging; MSD: MesoScale Discovery;

50 NETs: neutrophil extracellular traps, RBCs: red blood cells

51

52 **Introduction**

53 Acute ischemic stroke (AIS) can result from various mechanisms, such as large artery
54 atherosclerosis or cardioembolism¹. Determining AIS etiology is crucial for optimal patient
55 management. Stroke etiology is indeed a key factor for secondary prevention decisions. Yet,
56 in 30 to 40% of AIS patients, a specific stroke etiology cannot be determined². In the case of
57 AIS due to large vessel occlusion (LVO), it has been proposed that thrombus composition
58 could help determine thrombus origin. Although AIS thrombi causing LVO have been shown
59 to share the same basic components and structure³, they are highly heterogeneous in that they
60 contain highly variable amounts and proportions of red blood cells (RBCs)⁴, platelets⁵,
61 leukocytes⁵, fibrin⁶, and von Willebrand factor⁴. This heterogeneity in thrombus composition
62 has been suggested to reflect that in AIS etiology. Nevertheless, previous studies have
63 reported conflicting results regarding possible correlations between thrombus composition
64 and AIS etiology. The lack of consistency in conclusions on this issue might be related, at
65 least in part, to the fact that the vast majority of studies on thrombus composition have been
66 based on semiquantitative histological analyses using nonspecific staining methods of
67 thrombus components⁴⁻⁷. In addition, considering the large inter- and/or intra-observer
68 variability inherent to histological scoring strategies, such approaches may not allow for the
69 development of accurate diagnostic tools. In order to explore possible alternative methods for
70 AIS thrombus analysis and etiology identification, we compared AIS thrombus composition
71 according to AIS etiology using cell-type specific quantitative assays performed on whole-
72 thrombus homogenates.

73

74

75 **Methods**

76 *Data Availability*

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

80

81 *Standard Protocol Approvals, Registrations, and Patient Consents*

82 Thrombi were collected in two centers at the end of endovascular therapy (EVT). The EVT
83 procedure was chosen at the interventionalist's discretion, using a stent-retriever and/or a
84 contact aspiration technique. AIS etiology was classified as described¹ and determined based
85 on cerebral magnetic resonance imaging (MRI), computed tomography or MRI angiography,
86 transcranial and extracranial duplex sonography, coagulation tests, 1 to 3 days
87 electrocardiography recording, and transthoracic and/or transesophageal echocardiography.
88 Patient data were collected prospectively using a standardized questionnaire (Endovascular
89 Treatment in Ischemic Stroke -ETIS- registry NCT03776877). All patients were provided
90 with a written explanation of the study. The patients or their representatives were given the
91 opportunity to refuse participation. The local Ethics Committee approved this research
92 protocol (CPP Nord Ouest II, ID-RCB number: 2017-A01039-44).

93

94 *Preparation of thrombus homogenates*

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

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

101

102 *Quantification of red blood cell and DNA*

103 RBC content was estimated by measurement of heme concentration in thrombus homogenates
104 using a formic acid-based colorimetric assay, as described previously⁸. DNA was quantified
105 using the Molecular Probes Quant iT Picogreen dsDNA Assay kit (Life Technologies).

106

107 *Quantification of platelet content*

108 Soluble GPVI levels were measured by immunoassay according to the following protocol.
109 Ninety-six wells standard binding plate from MesoScale Discovery (MSD, Rockville, MD)
110 were coated overnight at 4°C with 2 µg/mL sheep anti human GPVI polyclonal antibody (Bio
111 Techne, France, AF3627). After 1 hour of incubation at room temperature with 5% MSD
112 Blocker A (R93AA-1) and 3 washes with 150 µL PBS / 0.05% Tween, 25 µL of thrombus
113 homogenate or standard were added and the plate was incubated for 1 hour at room
114 temperature, 500 rpm. Standard curve was obtained with Recombinant Human GPVI protein
115 (Bio techne, France, 3627-GP, 0,097-25 ng/ml). After 3 PBS Tween washes, 25 µL of
116 biotinylated sheep anti-human GPVI antibody (Bio Techne, France, BAF3627, 0,5 µg/mL in
117 1% MSD Blocker A) was added to each well and the plate was incubated 1 hour at room
118 temperature. Finally, 25 uL of streptavidin Sulfo-TAG/well was added after 3 PBS Tween
119 washes and the plate was incubated 1 hour at room temperature. A MesoScale Quickplex
120 Plate Scanner was used of quantification.

121

122 *Statistical Analysis*

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

140

141 **Results**

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

148

149 ***Thrombus cellular content and AIS etiology***

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

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

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

161

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

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

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

174

175 **Discussion**

176 In the present study conducted on 250 AIS thrombi responsible for LVO, we have explored
177 possible relationships between AIS etiology and thrombus cell composition. In order to avoid
178 the inherent limitations of semi-quantitative immunohistological methods⁷, we have analyzed
179 cell composition using quantitative assays for markers of RBCs, platelets, and leukocytes.
180 Our results show that cardioembolic thrombi are richer in DNA and poorer in platelets
181 compared to non-cardioembolic thrombi. From a pathophysiological perspective, the
182 increased DNA content of thrombi from cardioembolic origin suggests a more prominent role
183 of leukocytes in the formation of those thrombi. Leukocytes, especially neutrophils, are
184 indeed the primary source of DNA in blood and are now widely recognized as active players
185 of thrombosis^{9,10}. Interestingly, previous studies have shown that elevated neutrophil-
186 lymphocyte ratios in patients with nonvalvular atrial fibrillation were independently
187 associated with the presence of left atrial thrombus¹¹, as well as with an increased risk of
188 thromboembolic stroke¹². Also consistent with our results, patients with cardioembolic stroke
189 were reported to have increased plasma cell-free DNA levels compared to stroke patients of
190 other etiologies¹³.

191 The increased DNA content of cardioembolic thrombi might also reflect their previously
192 reported higher leukocyte and neutrophil extracellular traps (NETs) content compared to
193 thrombi of other origins¹⁴. Additionally, the high proportion of DNA content found in
194 cardioembolic thrombi and the pivotal role of neutrophils and NETs in thrombosis give
195 additional arguments for a potential benefit of DNase 1 in AIS treatment^{14,15}. It should be
196 noted, however, that the lack of specificity of DNA for a particular cell type might be a source

197 of variability hindering the drawing of more definitive correlations between thrombus DNA
198 content and stroke etiology. Besides leukocytes, endothelial cells, which can be extracted
199 together with the thrombus during EVT, represent a potential non-etiology-specific source of
200 contaminating DNA¹⁶. Moreover, while there is converging evidence that cardioembolic
201 thrombi are enriched in neutrophils and NETs, immunohistological analyses have indicated
202 that thrombi from atherosclerotic origin have an increased T cell content¹⁷.

203 Still, despite the lack of cell specificity of DNA, our results indicate that both the thrombus
204 DNA content and the thrombus DNA/GPVI ratio could provide biomarkers for identification
205 of cardioembolic thrombi among thrombi of undetermined origin. In fact,
206 specificity/selectivity calculations revealed that, by adjusting the DNA thrombus content
207 threshold, one could classify nearly 50% of ESUS thrombi as cardioembolic with a specificity
208 of 90%. Given that ESUS represents 20-25% of all AIS, there is a clear interest in developing
209 new diagnostic tools to better identify ESUS patient subgroups. A recent major secondary
210 prevention trial found no superiority of rivaroxaban over aspirin for prevention of recurrent
211 stroke in the overall ESUS patient population¹⁸. Identifying the subgroup of ESUS patients
212 requiring more active cardiac screening and which could benefit from anticoagulant therapy
213 could help to improve both patient management and design of secondary prevention studies.

214 Notably, the specificity and sensitivity of stroke classification systems have been reported to
215 be variable¹⁹. This variability represents a potential challenge for prospective studies aimed at
216 validating the use of quantitative measurement of thrombus-derived biomarkers like DNA as
217 adjunctive assays for determination of stroke etiology. Prospective studies focusing on the
218 impact of such adjunctive assays for patient selection on secondary stroke prevention efficacy
219 could also help to validate their clinical utility.

220 In addition to be inexpensive, thrombus homogenization as performed in our study requires
221 only moderate skills and is fairly easily feasible with common laboratory and hospital

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

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

239

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240 **Appendix**

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242 Jean-Philippe Désilles; Mikael Mazighi; Michel Piotin; Raphael Blanc; Hocine Redjem,
243 Stanislas Smajda; Gabriele Ciccio; Simon Escalard; Francois Delvoye; Benjamin Maier;
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250 Denis Vivien; Emmanuel Touze; Maxime Gauberti; Gaetane Blaizot.

251

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312

313

314

315 **Figure legends**

316 **Figure 1. Distribution of biochemical features of AIS thrombi according to etiology.**

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

323

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

327

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

| Characteristics | Overall | Suspected AIS etiology | | |
|--|----------------|------------------------|-------------------|---------------|
| | | Cardioembolic | Non-cardioembolic | ESUS |
| <i>Number of patients</i> | 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 \geq 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) |

330 Values expressed as no/total no. (%) unless otherwise indicated. ^a3 missing data (2 in cardioembolic group and 1
331 in Non-cardioembolic group) ^b18 missing data (12 in cardioembolic group, 1 in Non-cardioembolic group and 5
332 in Cryptogenic group) ^c7 missing data (4 in cardioembolic group, 1 in Non-cardioembolic group and 2 in
333 Cryptogenic group).

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

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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 ESU |
|-----------------|---------------------|--------------------|---------------------|---------------------|------------------------|
| 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 |

¹cut-value who maximize the Youden index.

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