

ORIGINAL ARTICLE

Cancer-associated stroke: a comparative analysis of thrombus composition, resistance to lysis, and plasma biomarkers

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Abstract

Background: Acute ischemic stroke (AIS) is a significant complication of cancer and may reveal occult malignancy. Whether cancer-related AIS (CAS) provides specific biological features remains unclear.

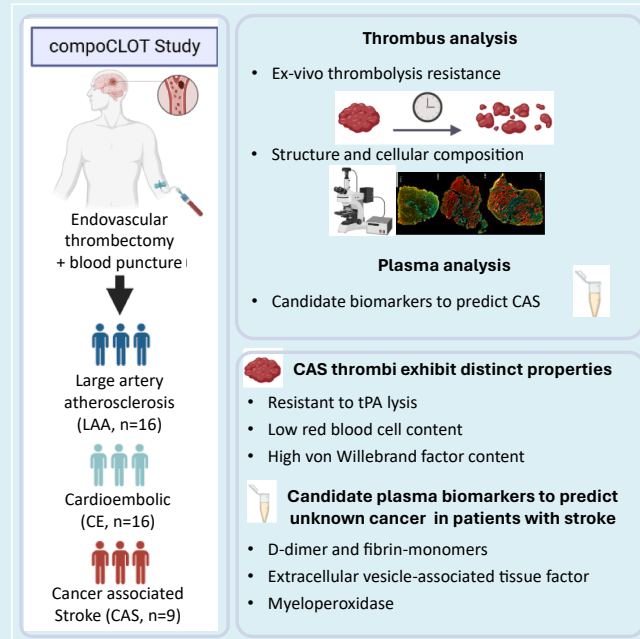
Objectives: This case-control study aimed to characterize thrombi and plasmas from patients with CAS.

Methods: Nine consecutive patients with CAS, including 3 nonbacterial thrombotic endocarditis (NBTE), and age- and sex-matched AIS controls with cardioembolism (CE, $n = 16$) or large artery atherosclerosis (LAA, $n = 16$) were included from the compoCLOT study. Thrombi retrieved after endovascular thrombectomy underwent *ex vivo* thrombolysis using recombinant tissue-type plasminogen activator and were analyzed for composition. Plasma biomarkers were also assessed.

Results: CAS thrombi were resistant to tissue-type plasminogen activator lysis compared with LAA thrombi (median thrombus weight conservation, 92% vs 9%; $P = .0002$) and, to a lesser extent, compared with CE thrombi (92% vs 43%; $P = .06$). Thrombolysis resistance correlated negatively with red blood cell content ($P < .0001$) but positively with platelet factor-4 ($P = .0014$), von Willebrand factor ($P = .0002$) and DNA content ($P < .0001$). A subgroup of macroscopically white thrombi ($n = 6$), characterized by low red blood cell and high von Willebrand factor content, was identified in CAS, encompassing all 3 NBTE cases. Plasma D-dimer, fibrin monomers, extracellular vesicle-associated tissue factor and myeloperoxidase were significantly higher in patients with CAS than in the combined LAA and CE control groups (31,290 vs 1900 $\mu\text{g/L}$; 143 vs 9 $\mu\text{g/mL}$; 143 vs 9 fM; and 51 vs 25 ng/mL, respectively).

* compoCLOT study group: all collaborators are listed in [Supplementary Table 1](#).

Conclusions: CAS thrombi exhibit distinct properties, with white thrombi as a hallmark of undiagnosed NBTE. Furthermore, plasma biomarkers such as D-dimer, fibrin monomers, extracellular vesicle-associated tissue factor and myeloperoxidase could help in cancer screening in AIS of unknown etiology.



KEYWORDS

biomarkers, cancer, stroke, thrombus, von Willebrand Factor

Essentials

- Cancer-associated stroke (CAS) may result from cancer-specific pathophysiological mechanisms.
- Thrombi and plasma from patients with CAS were compared with those from stroke patients without cancer.
- CAS thrombi were resistant to thrombolysis, especially if they were poor in red blood cells.
- CAS plasma contained high levels of several biomarkers of coagulation or leukocyte activation.

1 | INTRODUCTION

Acute ischemic stroke (AIS) is a major complication in patients with cancer, often associated with a poor prognosis [1]. In some cases, AIS can be the first manifestation of an occult malignancy [2]. Approximately 25% to 40% of AIS are classified as cryptogenic, with no specific cause identified after diagnostic workup, and cancer-related AIS (CAS) may account for a significant proportion of these cases [3,4]. Notably, 10% of patients with AIS may have an underlying cancer, and up to 50% of AIS in cancer patients are considered cryptogenic [5,6]. While cancer and AIS share several risk factors (eg, smoking and obesity) [7], increasing evidence suggests that CAS may involve specific pathophysiological mechanisms [8].

The risk of AIS is especially increased in the year leading up to cancer diagnosis, particularly in the preceding month, highlighting the

possibility that AIS may be the first manifestation of an occult cancer [2]. Despite this evidence, CAS is not currently recognized as a distinct subtype of AIS, and there is no clear consensus on the best approach for cancer screening in patients presenting with AIS [9]. Given that CAS is associated with worse outcomes, a better understanding of its specific pathophysiological mechanisms is essential to improve both patient management and treatment strategies, including thrombolysis [1].

In this context, the joint analysis of thrombectomy-recovered thrombi and plasma from patients with CAS may provide valuable insights into their specific characteristics and improve our understanding of the underlying mechanisms involved in CAS. However, studies investigating thrombus composition in CAS remain scarce [10–15], and none have assessed thrombus susceptibility to *ex vivo* thrombolysis. Furthermore, no study to date has simultaneously analyzed both thrombus characteristics and plasma biomarkers in

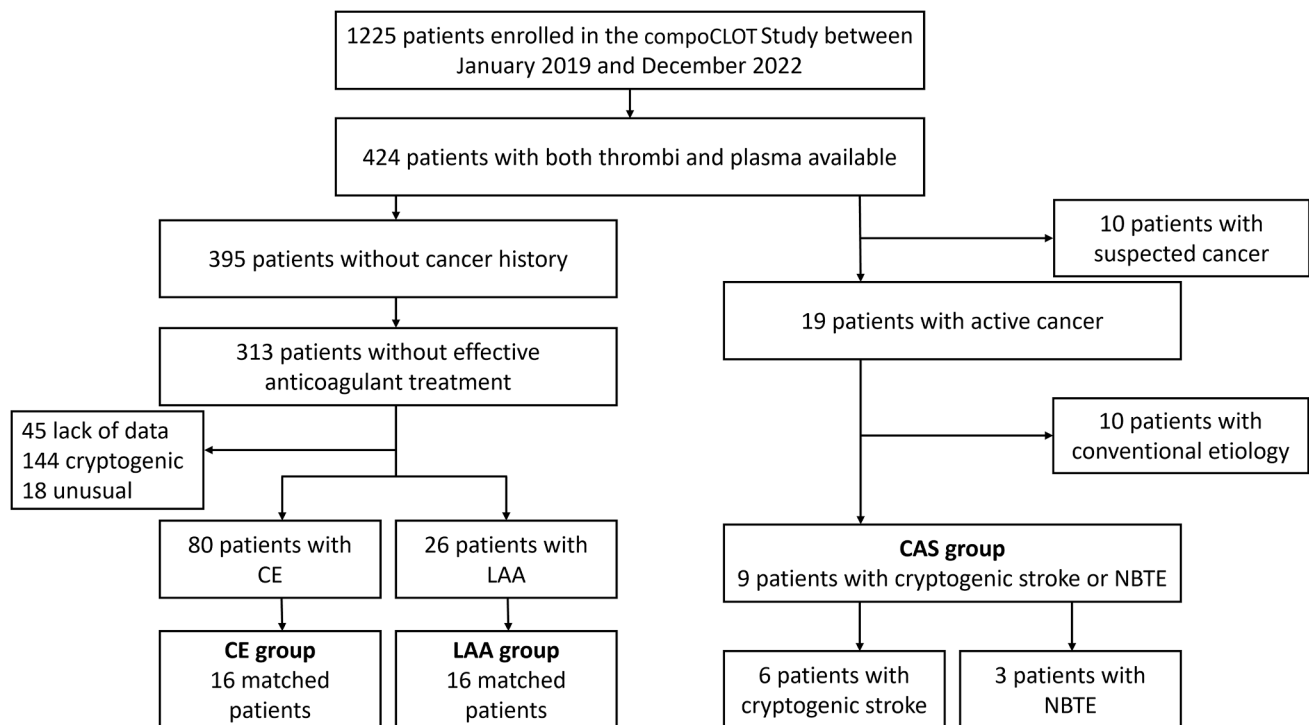


FIGURE 1 Flowchart of the study population. Patients were categorized into a cancer-associated stroke (CAS) group and 2 control groups matched by age and sex to the CAS group: cardioembolism (CE) and large artery atherosclerosis (LAA) groups. Active cancer was defined as cancer diagnosis or treatment within 6 months prior to acute ischemic stroke (AIS) or known recurrent or metastatic cancer. Ineffective anticoagulant treatment was defined as vitamin K antagonist therapy with an international normalized ratio of <1.5 or direct oral anticoagulant therapy with a plasma concentration of <50 ng/mL. AIS etiologies were classified according to TOAST (Trial of Org 10172 in Acute Stroke Treatment) classification criteria: unusual stroke etiologies included carotid dissection ($n = 12$), carotid artery dissection ($n = 3$), carotid artery thrombus ($n = 1$), cerebral venous thrombosis ($n = 1$), COVID-associated stroke ($n = 1$). Conventional etiologies included CE and LAA. NBTE, nonbacterial thrombotic endocarditis.

this population. This study aimed to identify the characteristics of thrombi from patients diagnosed with CAS and to explore several candidate plasma biomarkers that could help predict the presence of an occult cancer in patients with AIS.

2 | METHODS

2.1 | Study population

This retrospective case-control study derives from the compoCLOT cohort from the Adolphe de Rothschild Foundation Hospital (ethics committee: North-West II; RCB ID: 2017-A01039-4; ClinicalTrials.gov: NCT03268668), which prospectively included patients with AIS treated with endovascular thrombectomy (EVT) and successful thrombus retrieval. Retrieved thrombi were frozen immediately at -80°C . Blood samples were collected at EVT initiation, after intravenous thrombolysis when performed, in citrated tubes. Platelet-poor plasma was obtained immediately after 2 successive centrifugations at 2500 g for 15 minutes at room temperature and then stored at -80°C . Patient inclusion process is illustrated in Figure 1.

We included patients who underwent EVT between January 2019 and December 2022, with available thrombus and plasma samples. Patients were classified into a CAS group and 2 noncancer control groups. The CAS group included patients with AIS of unidentified etiology or nonbacterial thrombotic endocarditis (NBTE) and active cancer (defined as cancer diagnosis or treatment within 6 months prior to AIS or known recurrent or metastatic cancer). Control groups consisted of patients with AIS with cardioembolism (CE) or large artery atherosclerosis (LAA) without cancer history and without cancer diagnosis within the 3-month follow-up. Among men, a 3:1 matching ratio (controls:CAS) was applied. Patients were ordered chronologically, and the 3 individuals with the closest age to each CAS case were selected, with a maximum age difference of 5 years. Among women, only 4 were eligible for the LAA control group, and all were included. To ensure consistency across control groups, 5 women matching to the CAS group based on the closest available age were included in the CE control group. AIS etiologies were classified by a neurologist according to TOAST classification criteria [16].

2.2 | Ex vivo thrombolysis assay

Thrombi of >15 mg ($n = 30/41$) were divided for separate analyses, while those from 9 to 15 mg ($n = 6/41$) underwent only the ex vivo thrombolysis

TABLE Baseline characteristics of patients by study group.

Characteristic	LAA (n = 16)	CE (n = 16)	CAS (n = 9)	P
Demographics				
Age (y)	63 ± 6	66 ± 9	61 ± 10	.284
Sex, men	12 (75)	11 (69)	4 (45)	.288
Initial NIHSS	15 (1-20)	11 (1-25)	14 (4-24)	.517
Clinical risk factors				
Hypertension	10 (63)	4 (25)	3 (33)	.098
Diabetes	3 (19)	4 (25)	2 (22)	NA
Smoking	12 (75)	3 (19)	4 (45)	NA
Hyperlipidemia	5 (31)	1 (6)	2 (22)	NA
Coronary artery disease	2 (13)	0 (0)	0 (0)	NA
Atrial fibrillation	0 (0)	7 (44)	0 (0)	NA
Prior stroke	2 (13)	1 (6)	0 (0)	NA
Antithrombotic treatments				
Antiplatelet	4 (25)	3 (19)	2 (22)	NA
Anticoagulant	0	3 (19)	2 (22)	NA
Basic laboratory data (at admission)				
Hemoglobin (g/dL)	14.1 ± 1.2	13.7 ± 1.6	11.6 ± 2.0	.004
White blood cells (10 ⁹ /L)	11.2 ± 3.6	9.5 ± 2.7	8.4 ± 3.6	.148
Neutrophils (10 ⁹ /L)	8.0 ± 3.5	7.0 ± 2.9	6.2 ± 3.8	.491
Platelets (10 ⁹ /L)	255.7 ± 58.8	224.7 ± 36.6	156.6 ± 54.32	.000
Fibrinogen (g/L)	4.45 ± 1.18	3.60 ± 1.78	3.72 ± 1.25	.308
Occlusion site				
MCA	11 (69)	15 (94)	6 (67)	
ICA	1 (6)	1 (6)	3 (33)	
BA	1 (6)	0 (0)	0 (0)	
Tandem	2 (13)	0 (0)	0 (0)	
Other/multiple	1 (6)	0 (0)	0 (0)	
Intravenous thrombolysis	6 (38)	10 (63)	2 (22)	.089
No. of manipulations	2 (1-3)	1.5 (1-5)	2.5 (1-5)	.244
Recanalization success (TICI 2b/2c/3)	14 (87.5)	16 (100)	9 (100)	NA
mRS at 3 mo after stroke				.089
mRS0-2	8 (50)	7 (44)	2 (22)	
mRS3-5	6 (38)	8 (50)	2 (22)	
mRS6	0 (0)	1 (6)	5 (56)	
NA	2 (12)	0 (0)	0 (0)	

Data are represented as mean ± SD, n (%), or median (range).

BA, basilar artery; CAS, cancer-associated stroke; CE, cardioembolic; ICA, internal carotid artery; LAA, large artery atherosclerosis; MCA, middle cerebral artery; mRS, modified Rankin Scale; NA, not applicable; NIHSS, National Institutes of Health Stroke Scale; TICI, thrombolysis in cerebral infarction.

assay. Thrombi were treated at 37 °C (500 rpm; Thermomixer) in phosphate-buffered saline supplemented with Glu-plasminogen (25 µg/mL; Technoclone) and tissue-type plasminogen activator (tPA; 1 µg/mL alteplase; Actilyse; Boehringer Ingelheim). Incubation volume was

adjusted to a 40 mL/g ratio. Thrombus weight was measured before lysis and at 10, 30, 60, 90, 120, and 180 minutes. After thrombolysis, lysis supernatants and residual thrombi were collected and stored at −80 °C.

2.3 | Biochemical analysis of lysed thrombi

Lysis supernatants and residual thrombi were sonicated to measure myeloperoxidase (MPO; DuoSet Human Myeloperoxidase; R&D Systems), DNA (Quant iT Picogreen dsDNA, Molecular Probes; Life Technologies), von Willebrand factor (VWF; Asserachrom VWF:ag, Diagnostica Stago) and platelet factor-4 (PF4; DuoSet Human CXCL4/PF4; R&D Systems) using commercial kits. Red blood cell (RBC) content was estimated by heme concentration using a formic acid-based colorimetric assay, as previously described [17]. D-dimer (DDi) were measured in lysis supernatants at 180 minutes using an immunoturbidimetric assay (INNOVANCE D-Dimer; Siemens) on CN-6000 analyzer (Sysmex).

2.4 | Immunostaining

Immunostaining was performed on thrombi weighing <9 mg ($n = 5/41$) and on divided ones. Thrombi were embedded in Tissue-Teck O.C.T compound medium (CellPath, ref KMA-0100-00A), frozen at -80°C and cryosectioned ($7\text{ }\mu\text{m}$; CM1900; Leica). Sections were air dried, fixed in 4% paraformaldehyde (10 minutes), rinsed in phosphate-buffered saline, permeabilized (0.1% Triton), and blocked (3% bovine serum albumin). After incubation with primary antibodies, sections were washed, treated with fluorophore-conjugated secondary antibodies (Supplementary Table 2) and mounted. Staining specificity was assessed by omitting primary antibody on control slices. Positive surface area was quantified as a percentage of total thrombus area. Images were captured (Leica DMI8; LasX) and analyzed with ImageJ software (National Institutes of Health).

2.5 | Analysis of plasma biomarkers

MPO, DNA, and VWF were measured in plasma using the same commercial kits as in lysis supernatants. Fibrin monomers and DDi were quantified via immunoturbidimetric assay using FM-Liatest (Diagnostica Stago) and INNOVANCE D-Dimer (Siemens) on CN-6000 analyzer (Sysmex). Extracellular vesicle-associated tissue factor (EV-TF) activity was determined by a chromogenic assay measuring factor (F)Xa generation on extracellular vesicles [18].

2.6 | Statistical analysis

Data normality was assessed with Kolmogorov–Smirnov test. Results are presented as mean $\pm$ SD, median (range), or number (%). Categorical variables were tested for independence with the chi-square or Fisher's exact test. For quantitative variables, if the data were normally distributed, the 3 groups were compared using a 1-way analysis of variance (ANOVA), followed—when significant—by pairwise *t*-tests. For variables that were not normally distributed, a Kruskal–Wallis

test was performed, followed by post hoc Dunn tests with Bonferroni correction. When only 2 groups were compared (plasma analyses), the Mann–Whitney U-test was used. Correlations were analyzed with Spearman rank test. Receiver-operating characteristic analysis determined optimal plasma biomarker cutoffs for CAS discrimination. Statistical analyses were performed with GraphPad Prism 8.0 (GraphPad Software 2020), with significance set at $P < .05$.

3 | RESULTS

During the study period, 1225 consecutive patients with AIS were enrolled in the compoCLOT study. Thrombus and citrated plasma samples were both available for 424 patients, 19 of whom had active cancer. Of these, 10 patients were excluded due to having an AIS with a concomitant conventional etiology. The remaining 9 patients were included in the CAS group, including 3 NBTE and 6 cryptogenic strokes. Thirty-two age- and sex-matched patients without a history of cancer were included in 2 control groups: 16 had CE with atrial fibrillation, and 16 had LAA (Figure 1).

3.1 | Patient characteristics

Patient characteristics are presented in the Table. Among the 9 patients with cancer, lung cancer was the most common diagnosis ($n = 3$), followed by pancreatic cancer ($n = 2$), breast cancer ($n = 2$), esophageal cancer ($n = 1$), and myeloma ($n = 1$). Metastasis was identified in 5 patients (56%). At the time of AIS, 3 patients were undergoing chemotherapy, and 1 was on hormone therapy. For 4 patients, cancer was diagnosed concurrently with AIS. Time from cancer diagnosis to stroke was >12 months for 3 patients and between 1 and 3 months for 2 patients. Additionally, 4 patients experienced venous thrombosis either within the 3 months preceding the AIS or concurrently (Supplementary Table 3).

3.2 | Resistance to thrombolysis of CAS thrombi

Ex vivo thrombolysis assay was performed on all thrombi from the CAS group, 14 thrombi from the CE group and 13 thrombi from the LAA group. Within the CAS group, 6 thrombi were macroscopically distinct (cases 1–6); they were small, dense and white (Figure 2). Thrombi from the CAS group were resistant to *ex vivo* thrombolysis compared to those from the control groups. After 180 minutes of thrombolysis, median residual thrombus weight tended to be higher in the CAS group compared to the CE group (92% vs 43%; $P = .0601$) and was significantly higher compared to the LAA group (92% vs 9%; $P = .0002$; Figure 3). All 6 white thrombi exhibited a very similar profile in the *ex vivo* lysis assay, with residual thrombus weight remaining close to 100% at 60 min, 120 min and 180 min

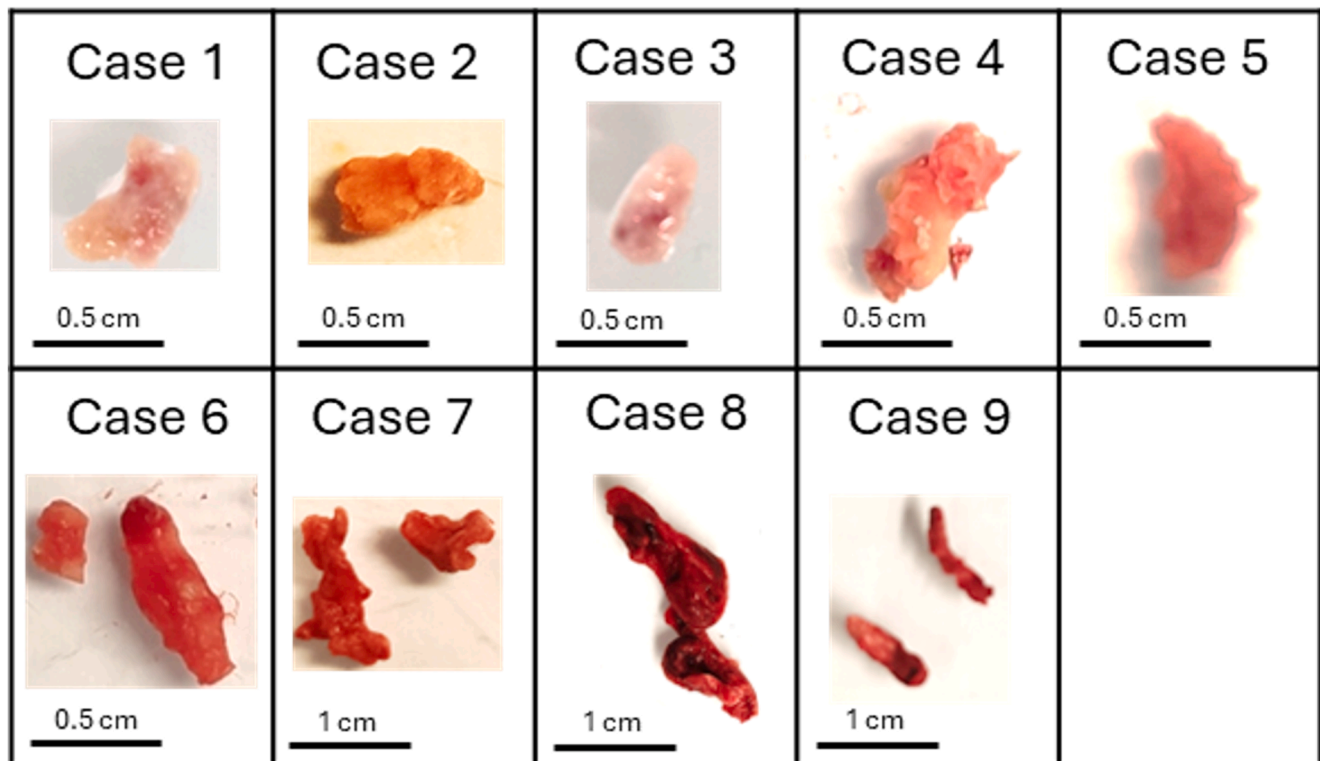


FIGURE 2 Macroscopic aspect of retrieved thrombi from patients with cancer-associated stroke (CAS; $n = 9$); 6 thrombi were macroscopically distinct (cases 1 to 6): small, dense, and white.

([Supplementary Figure 1](#)). Interestingly, all 3 thrombi associated with NBTE were in this subgroup (cases 1-3).

3.3 | Composition and structure of CAS thrombi

Thrombus composition was evaluated by biochemical assays after sonication of lysis supernatants and residual thrombi. Additionally, thrombus structure was analyzed by immunofluorescence and a semiquantitative analysis was performed for each staining.

First, regarding RBC content, biochemical assays of thrombus lysates revealed that thrombi from the CAS group had a lower heme content (21 [4-188] $\mu\text{g}/\text{mg}$) than thrombi from the CE (203 [12-331] $\mu\text{g}/\text{mg}$; $P = .0111$) and LAA (244 [22-351] $\mu\text{g}/\text{mg}$; $P = .0005$) groups ([Figure 4A](#)). These findings were further supported by quantification of RBCs using immunostaining, as glycophorin A-positive surface area was smaller in the CAS group (2.1% [1.0%-20.7%]) than that in the CE (14.5% [0.9%-44.7%]; $P = .0111$) and the LAA (21.3% [2.6%-46.4%]; $P = .0037$) groups ([Supplementary Figure 2A](#)). Glycophorin A-positive surface was positively correlated with heme content ($r = 0.662$; $P < .0001$, data not shown). Representative immunofluorescence images of fibrin(ogen), RBC, and DNA staining clearly illustrate the poor RBC content of CAS thrombi, particularly from the white thrombi subgroup (CAS 1-6), compared with CE and LAA thrombi ([Figure 5](#); [Supplementary Figure 3](#)).

Second, VWF content was evaluated. In thrombus lysates, it was significantly higher in CAS thrombi (210% [18%-554%]) than that in CE

(47% [28%-103%]; $P = .0436$) and LAA thrombi (48% [15%-138%]; $P = .0315$), with particularly elevated levels observed in the 6 thrombi from the white thrombi subgroup ([Figure 4B](#)). No significant differences were found in the quantification of VWF-positive surfaces ([Supplementary Figure 2B](#)). However, interestingly, immunofluorescence staining revealed distinct distribution of VWF among groups. In CAS thrombi, VWF staining, especially in white thrombi, exhibited a diffuse distribution throughout the thrombus, with significant colocalization with fibrinogen staining. In contrast, in the CE and LAA groups, VWF displayed a granular and localized pattern, forming discrete VWF-enriched areas ([Figure 5B](#)).

Third, to evaluate platelet content, we measured PF4 levels in thrombus lysates. PF4 levels tended to be higher in the CAS group, although the differences were not statistically significant ([Figure 4C](#)). Platelet quantification based on CD42b-positive surface area showed no significant difference among the CAS, LAA, and CE groups ([Supplementary Figure 2C](#)).

Fourth, we measured DDi concentrations in lysis supernatants after 180 minutes of thrombolysis. As fibrin degradation products, DDi indirectly reflect the initial fibrin content within thrombi. No significant differences were observed between the 3 groups ([Figure 4D](#)).

Finally, we measured DNA and MPO levels from thrombus lysates, which reflect the presence of polynuclear neutrophils and neutrophil extracellular traps (NETs) in thrombi. No significant differences in DNA from thrombus lysates were observed between thrombi from CAS and control groups ([Figure 4E](#)), but percentage of DNA-positive surface area was significantly higher in CAS thrombi

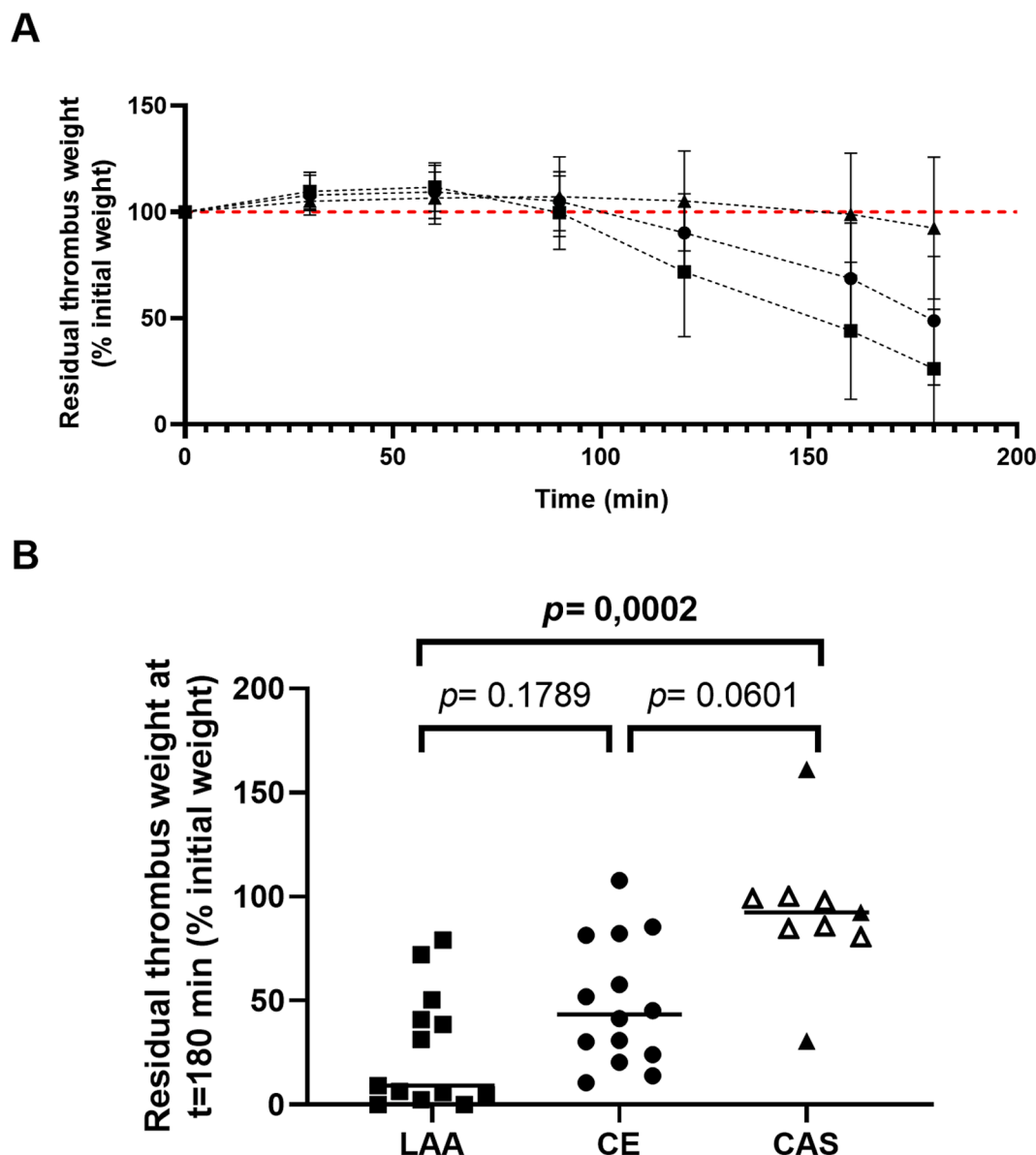


FIGURE 3 Resistance to thrombolysis of cancer-associated stroke (CAS) thrombi. Comparison of *ex vivo* thrombolysis rates across large artery atherosclerosis (LAA; squares), cardioembolic (CE; circles), and CAS (triangles) groups, quantified by measuring thrombus weight change relatively to initial thrombus weight. Red dotted line indicates 100% residual weight, representing no lysis. (B) Comparison of residual thrombus weight across groups at $t = 180$ minutes. P values for comparison (post hoc Dunn test with Bonferroni correction). Empty triangles, white thrombi subgroup.

than that in CE ones ($P = .0092$) (Supplementary Figure 2D). Notably, 1 thrombus showed extremely large and dense DNA-positive surface areas (CE12; Supplementary Figure 3). There were no significant differences in MPO levels in thrombus lysates among CAS, CE, and LAA groups (Figure 4F).

We also explored the potential presence of tumor cells within the thrombi. DAPI staining revealed no cells with atypical nuclear size or morphology suggesting a malignant origin. In addition, EpCAM (CD236) immunostaining was performed to detect cancer cells or tumor-derived microvesicles, but no significant staining could be observed.

3.4 | Correlation between thrombus composition and resistance to thrombolysis

Correlating biochemical measurements from thrombus lysates with *ex vivo* thrombolysis assay results, we investigated which elements could be responsible for thrombolysis resistance. Resistance to thrombolysis showed a significant negative correlation with RBC content ($r = -0.722$; $P < .0001$) but a positive correlation with PF4 ($r = 0.525$; $P = .0014$), VWF ($r = 0.589$; $P = .0002$), and DNA content ($r = 0.757$; $P < .0001$) (Figure 6).

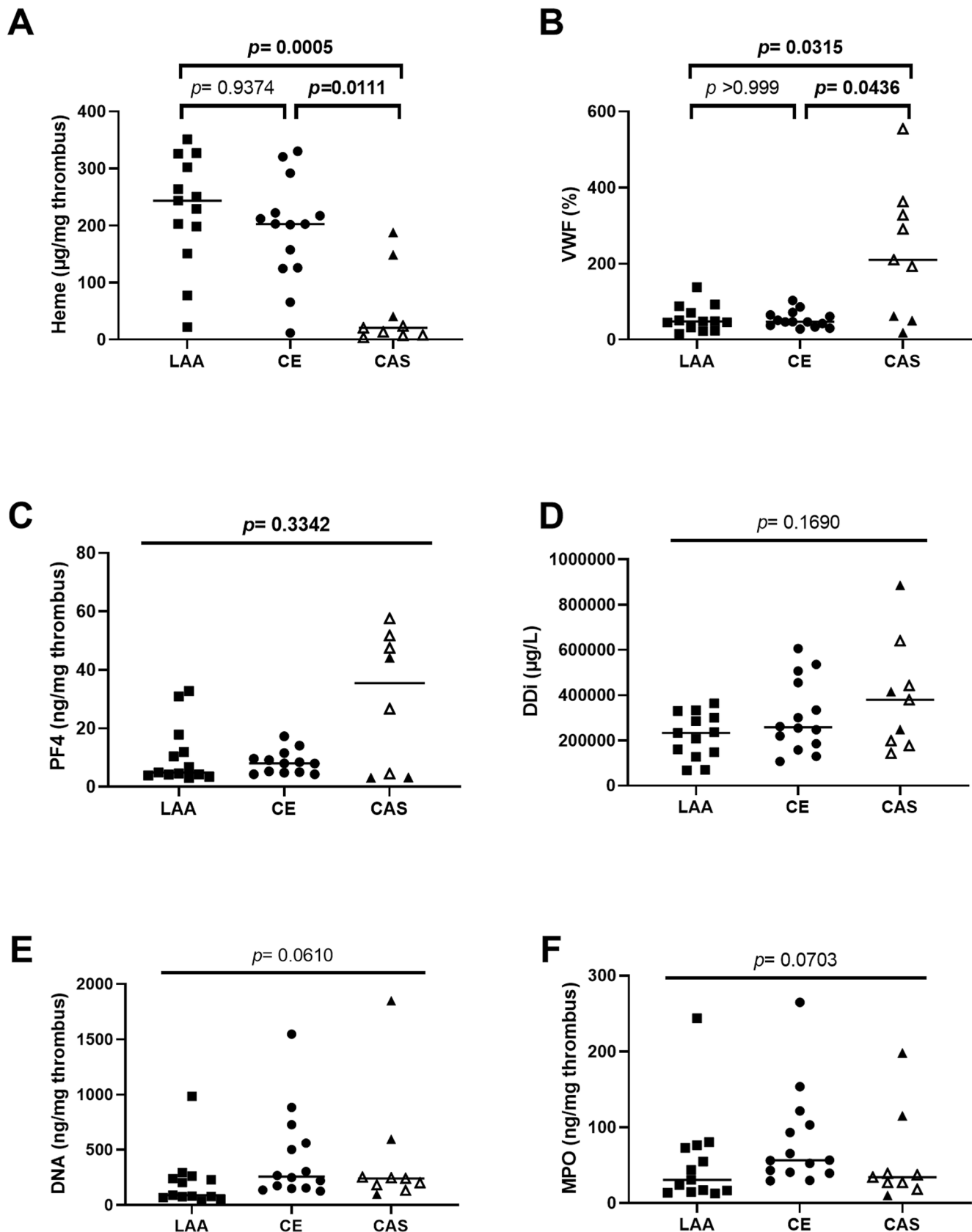


FIGURE 4 Thrombus composition according to stroke etiology. Biochemical analysis of thrombus lysates: (A) red blood cell content assessed by heme quantification; (B) von Willebrand factor (VWF) content; (C) platelet content measured by platelet factor-4 (PF4) quantification; and (D) D-dimer (DDi) levels in lysis supernatants at 180 minutes. (E) DNA content and (F) myeloperoxidase (MPO) levels were

3.5 | Plasma biomarkers

To identify plasma biomarkers potentially associated with the presence of cancer, CE and LAA groups were combined to form a unique control group. Plasma levels of DDi and fibrin monomers, reflecting coagulation activation, were more elevated in the CAS group (31,290 [1350-35,000] vs 1900 [320-25,130] $\mu\text{g/L}$; $P = .0033$, and 143 [6-3173] vs 9 [3-176] $\mu\text{g/mL}$; $P = .0199$, respectively) (Figure 7A, B). Particularly high DDi levels were observed in the white thrombi subgroup, exceeding 25,000 $\mu\text{g/L}$. EV-TF levels (78 [0-251] vs 15 [0-93] fM; $P = .0316$) (Figure 7C) and MPO concentrations (51 [22-121] vs 25 [10-130] ng/mL; $P = .0121$) (Figure 7D) were higher in the CAS group. According to the receiver-operating characteristic analysis, optimal cutoff values for predicting CAS were 27,770 $\mu\text{g/L}$ for DDi (sensitivity, 67%; specificity, 100%; area under the curve [AUC], 0.85), 257 $\mu\text{g/mL}$ for fibrin monomers (sensitivity, 50%; specificity, 100%; AUC, 0.77), and 112 fM for EV-TF (sensitivity, 50%; specificity, 100%; AUC, 0.75). No statistically significant differences were observed between the groups regarding plasma DNA or VWF concentrations (Figure 7E, F).

4 | DISCUSSION

A major strength of this study is the direct comparison of thrombus composition and plasma biomarkers in the same patients with CAS, enabling a comprehensive evaluation of both local and systemic prothrombotic mechanisms. We demonstrated that CAS thrombi had specificities that could distinguish them from other etiologies. To our knowledge, this is the first study to assess *in vitro* thrombolysis resistance in thrombi from patients with CAS, providing new insights into their limited responsiveness to standard thrombolytic therapy. CAS thrombi were resistant to thrombolysis compared with LAA and CE ones and were characterized by a low RBC and high VWF content. Hence, RBC and VWF content was significantly correlated with thrombolysis resistance, suggesting that thrombus composition in CAS may be responsible for resistance to thrombolysis, particularly in patients with white thrombi. Finally, we identified 4 potential plasma biomarkers for CAS, as plasma levels of DDi, fibrin monomers, EV-TF, and MPO were significantly higher in the CAS group.

AIS thrombi have a common framework made of RBC, fibrin, platelets, VWF, neutrophils, and NETs. Both the distribution and relative proportions of these components represent major distinguishing factors and can provide useful information to predict stroke etiology, treatment efficiency and further complications [19]. Multiple studies have provided converging evidence that AIS thrombi exhibit different susceptibilities to tPA-mediated thrombolysis according to their composition in RBC, VWF, platelets, and DNA [20-32]. RBC-rich thrombi contain sparse thin fibrin fibers and

respond effectively to tPA, whereas platelet-rich thrombi contain leukocytes, mostly neutrophils, but no or few RBCs, feature a dense fibrin network interspersed with VWF and NETs and are resistant to thrombolysis [21-23,26,33]. In this study, CAS thrombi were resistant to *ex vivo* thrombolysis compared with LAA and CE ones and could be differentiated from control groups based on their RBC-poor but VWF-rich content. Of interest, lysis resistance was negatively correlated with RBC content but positively correlated with PF4, VWF, and DNA content. Thus, the strong resistance to lysis observed in CAS thrombi can likely be explained by their distinct platelet- and VWF-rich composition.

The association between white thrombi and active cancer has been reported in several studies [10-15,34,35] and a fibrin/platelet proportion >65% in thrombi has been shown to be predictive of CAS [11]. In a recent study, thrombi from patients with CAS were characterized by very low RBC content and higher proportions of platelets ($n = 23$, including 7 NBTE) [14]. Similar results were reported in a larger cohort of cancer patients ($n = 50$) without excluding coexisting etiologies such as atrial fibrillation [12]. The identification of this subgroup of white thrombi, which included all NBTE cases and presented with similar characteristics in terms of composition and resistance to lysis, suggests that the remaining thrombi within this group could be associated with undiagnosed NBTE. Similarly, Park et al. [13] showed that patients with cryptogenic stroke and active cancer ($n = 7$) displayed thrombus characteristics similar to those of patients with NBTE ($n = 4$), suggesting that NBTE might be the underlying cause of thrombus formation in most cancer patients with strokes of unknown etiology and that NBTE frequency is most likely underestimated. Indeed, in an autopsy study of cancer patients, NBTE was found to be the most frequent etiology of AIS in patients with carcinoma [36]. NBTE vegetations consist of deposits of degenerating platelets interwoven with stands of fibrin on healthy heart valves. These vegetations are typically small and friable, making them difficult to confirm, even with transesophageal echocardiography [37,38]. The pathogenesis of NBTE remains incompletely understood, but malignancy-associated hypercoagulability is considered a major contributing factor [38]. Cancer cells release tumor necrosis factor and interleukin-1 β into the circulation, which may induce endothelial injury. Platelet adhesion to damaged endothelium, followed by aggregation, is facilitated by elevated VWF preferentially released from activated endothelial cells under high-shear stress conditions, such as those found in cardiac valves. In parallel, the prothrombotic state driven by cancer notably via the release of EV-TF promotes activation of the coagulation cascade, thrombin generation, and fibrin formation. These combined mechanisms may account for the development of thrombi rich in platelets, fibrin, and VWF but poor in RBCs. Consistent with this hypothesis, both our study and that of Woock et al. [10] (64 thrombi from patients with CAS, without excluding coexisting etiologies) demonstrated a significantly higher

also analyzed in thrombus lysates. P values for comparison (Kruskal-Wallis or post hoc Dunn test with Bonferroni correction). Squares, LAA; circles, CE; triangles, CAS; empty triangles, white thrombi subgroup. CAS, cancer-associated stroke; CE, cardioembolic; LAA, large artery atherosclerosis.

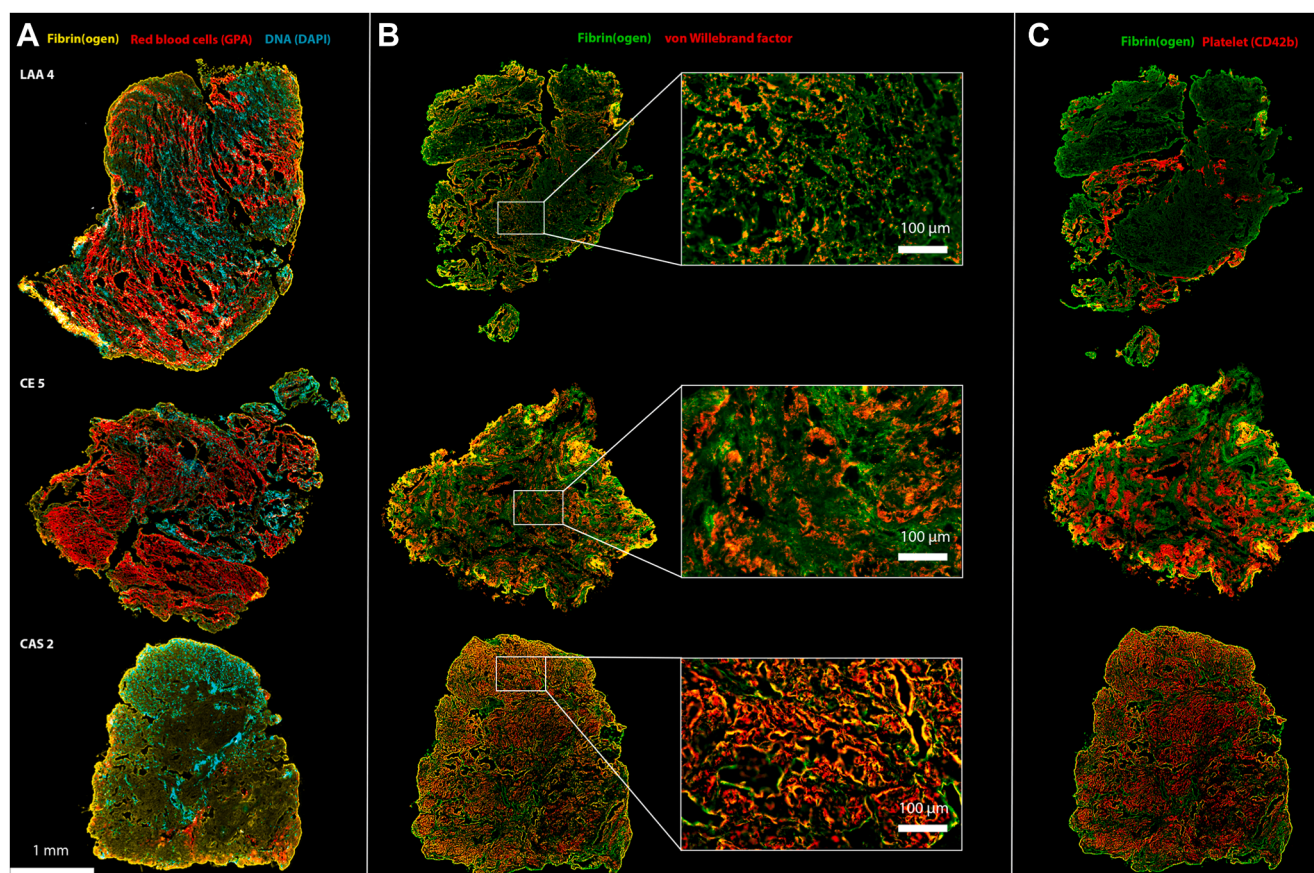


FIGURE 5 Structure of representative thrombi from the 3 groups according to stroke etiology. (A) Panoramic overviews of red blood cell, fibrin(ogen), and DNA immunostaining. (B) Panoramic and magnified views of VWF and fibrin(ogen) immunostaining, highlighting distinct VWF distribution among groups. (C) Panoramic overviews of fibrin(ogen) and platelet immunostaining. CAS, cancer-associated stroke; CE, cardioembolic; LAA, large artery atherosclerosis.

VWF content in CAS thrombi than that in control groups. A higher proportion of thrombin and tissue factor (TF) in CAS thrombi has been reported, further supporting the central role of cancer-associated prothrombotic pathways in their formation [14]. Such thrombi are often friable and prone to fragmentation, making them likely to embolize and cause ischemic complications, including stroke [39–43].

Interestingly, alongside our analysis of thrombi from patients with CAS, we observed elevated plasma levels of EV-TF activity. TF is a key initiator of the coagulation cascade and is frequently expressed by cancer cells. Notably, patients with metastatic pancreatic cancer exhibit increased EV-TF activity compared with healthy individuals [44]. To date, only 1 study has demonstrated higher levels of cancer cell-derived EVs in patients with CAS than in those with cancer-unrelated AIS [45]. These findings are consistent with the mechanistic hypothesis discussed earlier and further highlight the central role of TF in CAS pathogenesis.

AIS can be the first manifestation of an occult cancer, but the optimal approach for cancer screening after AIS remains unclear [9]. In this context, there is a crucial need to identify reliable biological markers that could help predict the presence of an occult cancer in

patients with AIS, especially those with unknown etiology. A meta-analysis reported that the most consistent laboratory features associated with cancer after AIS were low hemoglobin, high C-reactive protein, high DDi, and high fibrinogen levels [46]. In our study, hemoglobin levels were lower in the CAS group, but some patients were undergoing chemotherapy, which may have contributed to reduced hemoglobin levels. We did not find any significant difference in fibrinogen levels between groups. Still, markers indicating a hypercoagulable state and coagulation activation, such as DDi, fibrin monomers, and EV-TF were significantly elevated in the CAS group, especially in the white thrombi group. In the literature, DDi levels have been reported elevated in patients with CAS, together with other markers of coagulation activation, such as thrombin-antithrombin complexes [47]. DDi have also been identified as an independent factor associated with white thrombi, with a cutoff value of 35,000 μg/L, yielding 83.3% sensitivity and 100% specificity [34]. Similarly, in our study, DDi emerged as the best biomarker, offering the highest sensitivity and specificity for identifying CAS. A cutoff value of 27,770 μg/L yielded a sensitivity of 67% and a specificity of 100%. Notably, 100% of patients with DDi of >27,770 μg/L had a white thrombus. Regarding fibrin monomers, a threshold of

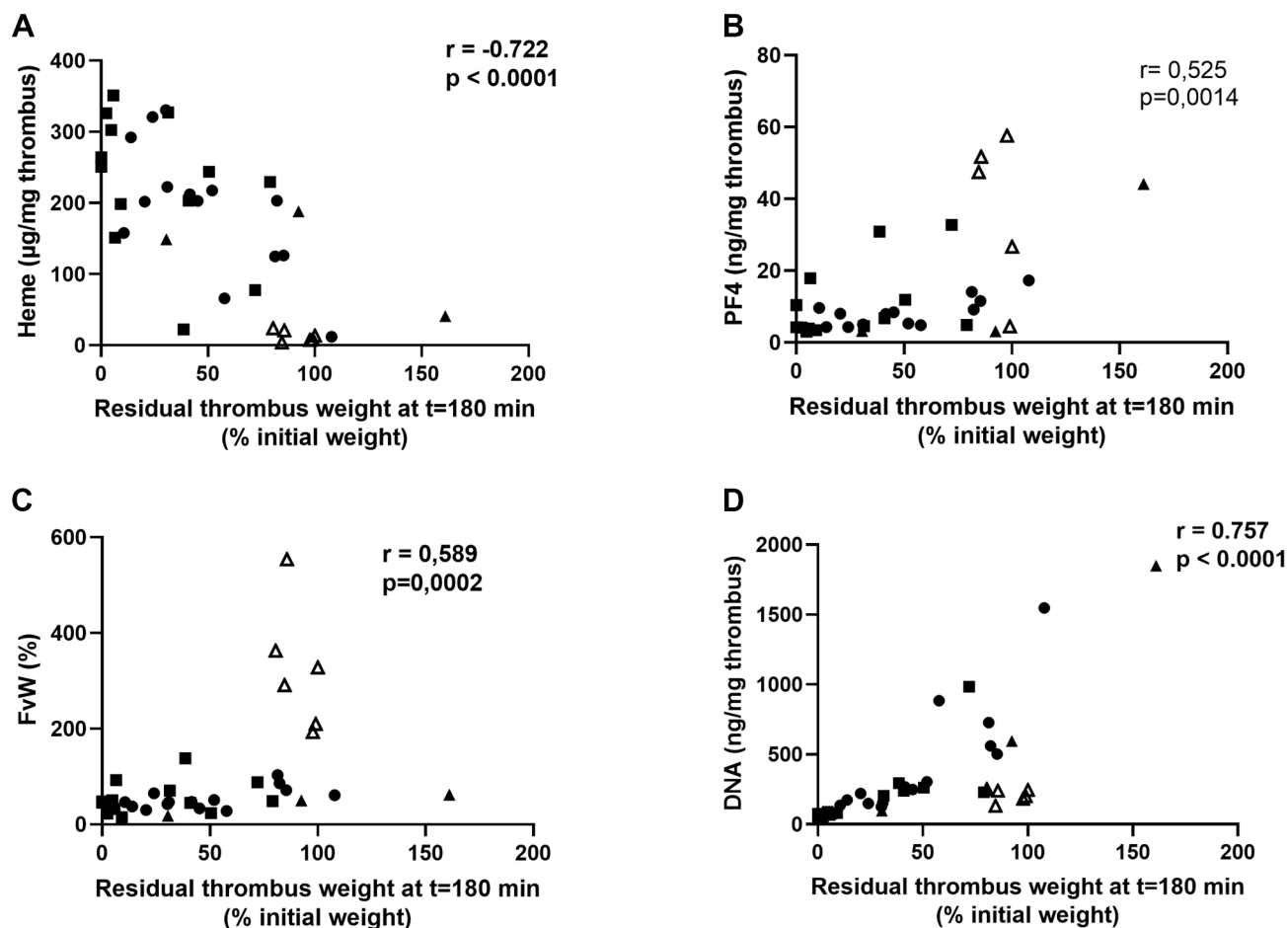


FIGURE 6 Correlation of thrombus composition with thrombolysis resistance. Correlation between resistance to lysis (residual thrombus weight) and heme (A), platelet factor-4 (PF4) (B), von Willebrand factor (VWF) (C), or DNA content (D). *P* values for correlation (Spearman rank test). Squares, LAA; circles, CE; triangles, CAS; empty triangles, white thrombi subgroup. CAS, cancer-associated stroke; CE, cardioembolic; LAA, large artery atherosclerosis.

142.6 µg/mL provided 50% sensitivity and 100% specificity for CAS identification. Importantly, both biomarkers are easily measurable as part of routine laboratory tests and are cost-effective. As for EV-TF activity, a threshold of 91.4 fM discriminated patients with CAS, with 50% sensitivity and 96.8% specificity. Importantly, some patients received intravenous thrombolysis prior to EVT, which may have influenced the plasma biomarkers, particularly DDi. Although DDi levels were significantly higher in thrombolysed patients (3680 [780-35,000] vs 1395 [320-35,000] µg/L; *P* = .0192), markedly elevated DDi levels (>27,000 µg/L) were observed exclusively in the CAS group, irrespective of prior thrombolysis, suggesting that these extreme elevations cannot be attributed solely to systemic thrombolytic treatment.

We also observed a significant elevation in the levels of MPO, a major granular component of neutrophils, in the plasma of patients with active cancer, while there was no significant difference in neutrophil counts between groups. This finding may suggest an increased release of MPO and NETs by activated neutrophils, although no significant difference between groups regarding circulating plasma DNA levels was found. Conversely, the

OASIS-Cancer (Optimal Anticoagulation Strategy in Stroke Related to Cancer) study demonstrated an association between increased circulating DNA and CAS, suggesting that NETosis may be one of the molecular mechanisms driving CAS [48]. To confirm this hypothesis, more specific assays targeting NETs, such as measurement of MPO-DNA complexes, would be necessary.

Other studies have explored additional plasma biomarkers that appeared promising in the context of CAS, such as markers of platelet activation (P-selectin) and endothelial dysfunction (soluble intercellular adhesion molecule-1 and soluble vascular cell adhesion molecule-1) [47] or carcinomatous mucins, such as CA-125 [49,50]. Our study had several limitations. First, regarding patient selection, among eligible patients without a cancer history and without effective anticoagulant treatment (*n* = 313), ~46% had a stroke of unknown etiology, 25% had a CE-related stroke, 8% had an LAA stroke, 5% had an unusual stroke etiology, and data were missing for 14% of patients. In our cohort, stroke of unknown etiology was likely overestimated, as some diagnostic evaluations were not completed or access to the full data necessary to establish the etiology was lacking. Moreover, LAA strokes appear to be

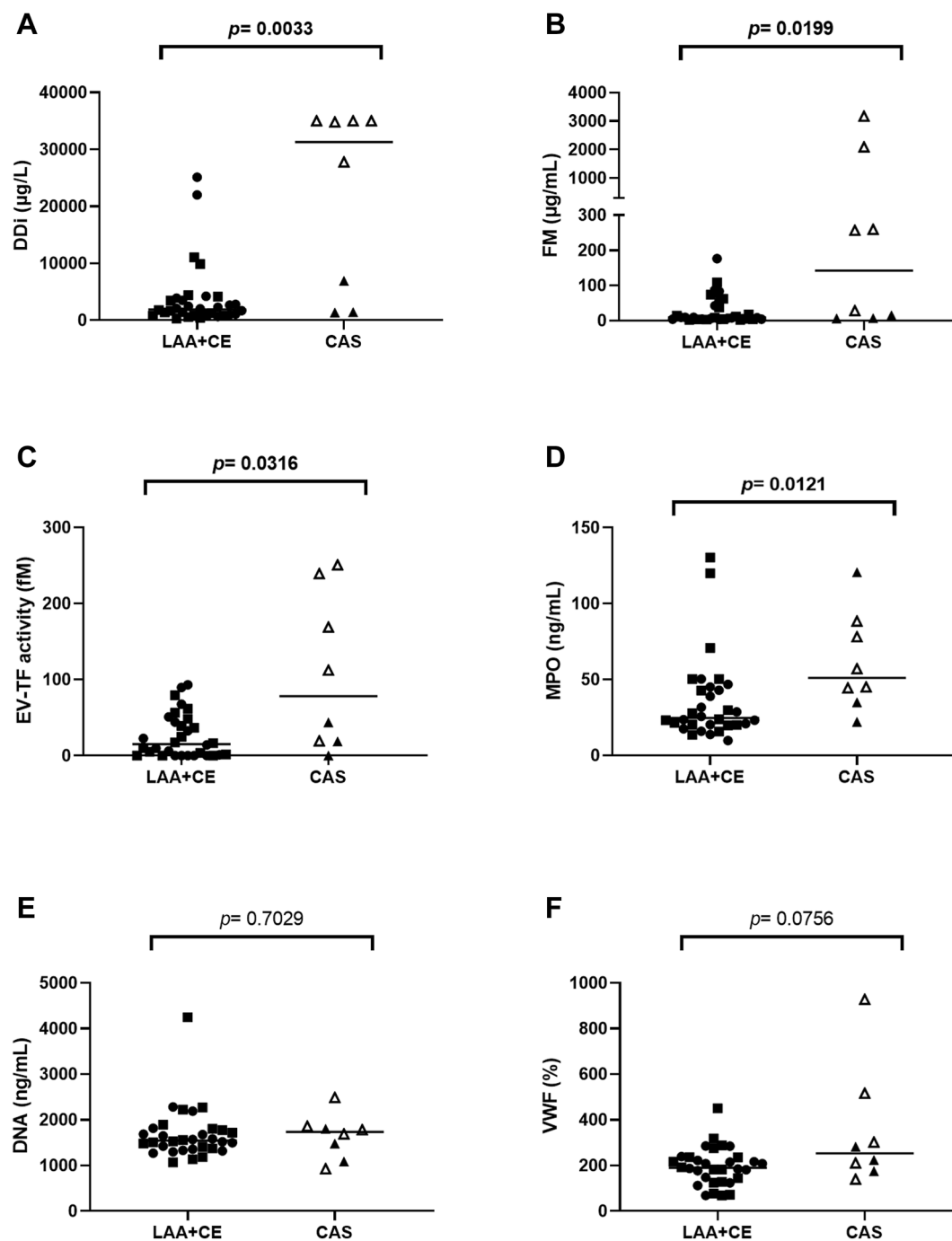


FIGURE 7 Plasma biomarkers in cancer-related ischemic stroke (CAS). (A) Plasma levels of D-dimer (DDi), (B) fibrin monomers (FM), (C) extracellular vesicle-associated tissue factor activity (EV-TF), (D) myeloperoxidase (MPO), (E) DNA, and (F) von Willebrand factor (VWF). *P* values for comparison (Mann–Whitney test). Squares, LAA; circles, CE; triangles, CAS; empty triangles, white thrombi subgroup. CAS, cancer-associated stroke; CE, cardioembolic; LAA, large artery atherosclerosis.

underestimated, representing only 8% of our cohort compared with ~25% in the literature. This discrepancy may be due to a selection bias, as all included patients underwent EVT, while data from patients with thrombi that were inaccessible, resistant to EVT, or completely dissolved by pharmacologic thrombolysis were not available for analysis.

To minimize confounding factors, we deliberately excluded patients with cancer and an identified stroke etiology unrelated to NBTE, such as CE and LAA, although we acknowledge that cancer may contribute to AIS in these settings. The limited sample size of the CAS group reflects our strict inclusion criteria, focusing only on cryptogenic strokes without competing causes such as atrial

fibrillation or significant atherosclerosis. This approach allowed for a more robust biological interpretation. Due to the small sample size of the CAS group, we were unable to investigate thrombus structure according to each cancer type. Moreover, we did not exclude patients on anticoagulants in this group, and only 1 patient (case 1) was on effective direct oral anticoagulant therapy at the time of EVT, potentially underestimating the observed differences. Finally, we divided each thrombus into 2 parts: one to assess resistance to lysis and biochemical composition and the other for immunofluorescence analysis. Considering that thrombi have a heterogeneous composition, this division may have led to differences in the results, especially when comparing biochemical and immunofluorescence results for thrombus composition analysis.

Due to the limited number of patients in the CAS group, we were unable to analyze potential correlation between thrombus characteristics and plasma biomarkers or to establish their potential predictive value for detecting cancer when used combined in patients with stroke of unknown etiology. Five of 6 patients with white thrombi (including 2 of the 3 NBTE) had known metastatic cancer, either previously diagnosed or newly identified at the time of stroke. In these cases, the underlying cancer was rapidly uncovered following stroke event. However, the ultimate goal of our work would be to detect cancer in patients who are not yet at an advanced stage. Expanding the size of the CAS group and the number of patients with either localized or metastatic cancer is thus needed.

Furthermore, the small number of matched controls makes this case-control study vulnerable to selection biases. The number of controls was limited by the availability of plasma and thrombi samples from the compoCLOT biobank.

Another unresolved question is whether cancer can contribute to ischemic strokes of conventional causes, such as CE or LAA. Based on the results observed in this well-defined population, it would now be of interest to investigate whether the same thrombus and plasma biomarkers are present in patients with LAA or CE strokes and active cancer. This could help determine whether cancer plays a contributory role in these more common stroke subtypes and potentially allow earlier cancer detection in affected patients.

5 | CONCLUSION

Thrombi from CAS display specific features including thrombolysis resistance, reduced RBC but increased VWF content. This VWF-rich structure suggests a significant role for VWF, and possibly platelets, in thrombus formation during AIS, as well as, in the resistance to thrombolysis. The identification of a homogeneous subgroup of white thrombi with similar characteristics, including all NBTE thrombi, suggests that the remaining thrombi in this group may be associated with undiagnosed NBTE. The elevation of plasma biomarkers such as DDi, fibrin monomers, and EV-TF underline the major role of hypercoagulability in the pathophysiology of CAS. Further prospective studies are needed to evaluate their predictive value, alone or in

combination, in identifying patients with occult malignancy in patient with AIS of unknown etiology.

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AUTHORS CONTRIBUTIONS

C.H., B.H., D.F., and J.-P.D. designed the study. C.H., M.S.N., V.O., I.A., and L.-R.K. performed research. C.H. and D.F. performed data analysis and wrote the manuscript. B.H., J.-P.D., N.A., and M.M. reread the manuscript. M.M. and N.A. supervised the study. All authors read and approved the final manuscript.

RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

DATA AVAILABILITY

Data are available upon reasonable request to the corresponding author.

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SUPPLEMENTARY MATERIAL

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