UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Medicina - DIMED

CORSO DI DOTTORATO DI RICERCA IN ARTERIAL HYPERTENSION AND VASCULAR BIOLOGY
CICLO XXXVI

ASSOCIATION BETWEEN HISTOLOGY OF THROMBI RETRIEVED DURING MECHANICAL THROMBECTOMY AND ATRIAL CARDIOMYOPATHY MARKERS IN ESUS PATIENTS

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SUMMARY

ABSTRACT

Background. Data that could be obtained thanks to histological analyses of the thrombi retrieved during mechanical thrombectomy is still controversial; therefore, these analyses have not yet been included in the diagnostic work up after an ischemic stroke. Embolic strokes of undetermined source (ESUS) are a subtype of ischemic stroke whose therapeutic approach has not yet been defined. According to recent literature, atrial cardiomyopathy (a complex of pathological changes affecting the atria) could be the trigger of an ESUS in those patients with no evidence of atrial fibrillation. Aim of the study is to demonstrate a correlation between histological features and atrial cardiomyopathy biomarkers in ischemic stroke, to better stratify ESUS patients.

Materials and Methods. We enrolled a series of 114 consecutive subjects, over a period of three years. Demographic and clinical data was collected, besides specific data of interest for the study (presence of atrial cardiomyopathy biomarkers, HAVOC score). All the clots retrieved were stained and analyzed to quantify major components (red blood cells, platelets and fibrin, neutrophils). Univariate and multivariate analyses were performed.

Results. Cerebral clot composition (in terms of red blood cells, platelets and fibrin) seem not to depend on the stroke etiology; conversely, thrombi retrieved from cardioembolic strokes present a higher presence of neutrophils (OR 3.25; 95% confidence interval 1.29 – 8.24; p= 0.01). In ESUS subjects, the presence of a first degree interatrial block as a marker of atrial cardiomyopathy seems to be related to a higher presence of neutrophils in the thrombus (p= 0.02); clots retrieved in ESUS patients with this characteristic, therefore, seem to be more similar to those retrieved in cardioembolic strokes.

Conclusion. Cardioembolic strokes show cerebral thrombi richer in neutrophils when compared to strokes of other etiologies. No differences were observed regarding other clot components (red blood cells, platelets and fibrin). Presence of a first degree interatrial block seems to be a possible marker of atrial cardiomyopathy that could be used to stratify ESUS patients. Further studies with larger sample sizes are needed to confirm these findings.

BACKGROUND

A stroke is a medical condition consisting in a neurological deficit with a sudden development. This condition is caused by an abrupt neuronal death, due to a lack of nutrients and oxygen to the cerebral cells.

Strokes are classified in three big categories:

1. Ischemic stroke: a cerebral infarction, due to an occlusion of a cerebral artery.
2. Intracerebral hemorrhage: a hemorrhage in the brain parenchyma caused by a rupture of a cerebral blood vessel.
3. Subarachnoid hemorrhage: a hemorrhage occurring in the subarachnoid space.

Stroke: epidemiological impact

According to the 2019 Global Burden of Disease Study, 12.2 million incident cases of stroke were registered worldwide that year, with a prevalence of 101 million cases of stroke, 6.55 million deaths due to stroke and 143 million DALYs due to this pathology (GBD 2019 Stroke Collaborators, 2019).

Considering such data, in 2019, stroke is the second-highest cause of death in the world and the third-highest cause of death and disability combined. In the last 30 years, more and more cases were diagnosed (+70% incidence, +85% prevalence, +43% deaths due to stroke, +32% DALYs from stroke).

Ischemic stroke contributed to 62.4% of all strokes in 2019, intracerebral hemorrhage to 27.9% of the total cases, while subarachnoid hemorrhages were just 9.7% of the total.

According to this epidemiological analysis, five principal risk factors for stroke were identified: high systolic blood pressure, high body mass index, high fasting plasma glucose, environmental pollution and smoking. Other key risk factors, especially for ischemic stroke, are diabetes (Weinberger, 1983) and atrial fibrillation (Goldstein, 2011).

Regional and between-country variations were observed: low-income countries have a higher (3.6 times) stroke-related mortality rate and a higher (3.7 times) stroke-related DALY rate compared to high-income countries.

Considering the global relevance of the impact, the prevention and the treatment of stroke has to be considered as a primary question in the medical and the scientific community.

Ischemic stroke and ESUS

About the 60% of all strokes are ischemic strokes. The pathophysiological mechanism of an ischemic stroke is the occlusion of a cerebral vessel, that can involve both a small or a large cerebral artery. Several causes can lead to this situation; the evaluation of the causes is a crucial point in the clinical work-up of the patient, because different etiologies need different therapeutical approaches. To better categorize the different causes of ischemic strokes, Adams et al. proposed an etiological classification of ischemic stroke (the TOAST classification) that is widely used both in clinical practice and in research trials (Adams, 1993). According to this classification, ischemic stroke etiology could be divided in five groups:

- Large artery atherosclerosis
- Cardioembolism
- Small-vessel occlusion
- Rare causes, other determined etiology

- Stroke of undetermined etiology

Defining the correct etiology of an ischemic stroke plays a crucial role in choosing the correct therapy for secondary prevention (for example, the anticoagulant therapy in cardioembolic stroke due to atrial fibrillation). Nevertheless, this goal is not always easy to reach.

According to the American Heart Association and the American Stroke Association guidelines published in 2019, after the evaluation of a patient with a stroke in the emergency setting (to decide whether to start a reperfusion therapy or not), several exams are required to investigate the etiology of the stroke (Kleindorfer, 2021). An electrocardiogram, basic laboratory tests, an echocardiographic examination and non-invasive intracranial and extracranial imaging are mandatory in all stroke patients. If the cause of the stroke is not clear after these exams, further diagnostic tests should be performed (long-term cardiac rhythm monitoring, tests for genetic stroke syndromes, tests for infectious vasculitis, transesophageal echocardiography, cardiac CT or cardiac MRI).

Nowadays, increasingly more attention is paid to stroke of undetermined etiology, or cryptogenetic strokes. The main reasons for this are mainly two: this subtype constitutes about the 25% of all ischemic strokes; a clear therapeutic strategy in the secondary prevention of cryptogenic strokes has not yet been established.

According to the TOAST classification, the etiology is undetermined if: (1) two or more causes of ischemic stroke are identified; (2) a complete, negative evaluation of potential stroke causes has been conducted or (3) an incomplete evaluation of potential stroke causes has been performed.

In 2014, Hart et al. concluded that the majority of cryptogenic strokes are thromboembolic and proposed the definition of embolic stroke of undetermined source (ESUS) (Hart, 2014). ESUS are not lacunar (subcortical infarct with a dimension that is ≤ 1.5 cm on CT or ≤ 2 cm on MRI diffusion weighted image) cryptogenic strokes, with no evidence of significant intracranial or extracranial atherosclerosis in the area of the stroke and no evidence of a major cardioembolic source of embolism.

Many authors agree that an occult paroxysmal atrial fibrillation could be the triggering cause in the majority of ESUS (Sajeev, 2020). Therefore, the risk of not detecting atrial fibrillation after a cryptogenic stroke should be avoided. For this reason, several trials have been designed to assess whether a prolonged cardiac monitoring should be performed in these patients.

The 30-Day Cardiac Event Monitor Belt for Recording Atrial Fibrillation after a Cerebral Ischemic Event (EMBRACE) study was designed to compare a long cardiac monitoring (30 days) vs a short one (24 hours) in patients with cryptogenic stroke within six months of the onset and no history of atrial fibrillation (Gladstone, 2014). Short run of atrial fibrillation (less than 2.5 minutes) was detected in 16.1% of subjects who underwent a prolonged cardiac monitoring and only in 3.2% of subjects in the other group (with a significant statistical difference between the two groups). Moreover, a longer run of atrial fibrillation (more than 2.5 minutes) was detected in 9.9% of patients from the first group and only in 2.5% of patients from the second group.

Moreover, detecting occult atrial fibrillation plays a crucial role also for other subtypes of ischemic strokes. Detection of Silent Atrial Fibrillation aFter Ischemic StrOke (SAFFO) is an Italian multicentric study that is currently ongoing (Toni, 2016). In this trial, patients with an atherothrombotic or a lacunar ischemic stroke are enrolled and randomly assigned to the standard of care (a 24-hour cardiac monitoring after 3 months

from the index event) or to the implant of an implantable loop recorder. The aim of the study is to demonstrate that the detection of atrial fibrillation is higher with a prolonged monitoring, even in patients with an atherothrombotic or a lacunar stroke.

Therapeutical approach to ESUS

As previously said, the correct therapy for the secondary prevention of ESUS has not yet been established (Johansen, 2023). Several studies have tried to figure out whether anticoagulant therapy could be a good choice for this type of patients, with one clinical trial that is currently ongoing (Vassiliki' Coutsoumbas, 2022).

The first study of this type was the WARFARIN–ASPIRIN RECURRENT STROKE STUDY (WARSS), in which investigators tried to compare whether anticoagulant therapy and antiaggregant therapy have a difference in efficacy and safety among all patients who suffered from an ischemic stroke (Mohr, 2001). A total of 2206 patients were enrolled in this study, they were randomly assigned to receive warfarin or acetylsalicylic acid for the secondary prevention after an ischemic cerebral event and they were followed up for 24 months. The study failed to demonstrate the superiority of the anticoagulant therapy; no differences, in fact, were observed between the two groups in terms of efficacy and safety outcomes. According to the results of the trial, 176 patients in the warfarin group (17.8% of the total) had a new ischemic stroke or a fatal event during the follow up period, while 176 subjects in the aspirin group (16.0% of the total) reached the same primary end point (P=0.25; hazard ratio comparing 1.13; 95% confidence interval, 0.92 to 1.38). Regarding the safety outcomes, major bleedings were similar in both groups (2.22% in the warfarin group and 1.49% in the acetylsalicylic acid group). Moreover, investigators found no significant differences in the subgroup analysis based on the etiology of the index event (1237 patients with a small-vessel disease, 576 patients with cryptogenic strokes and 259 patients with atherothrombotic infarcts).

A more extensive post hoc analysis of the WARSS study was later published (Sacco, 2006). At first, authors analyzed some clinical characteristics of the enrolled patients, finding out that warfarin was correlated to a greater rate of recurrent strokes and fatal events in some patients subgroups (patients with a history of arterial hypertension at the baseline, patients with a moderate stroke, subjects with a posterior circulation ischemic event involving the brainstem).

Moreover, according to the WARSS study and the post hoc analysis, 576 patients were classified as cryptogenic strokes according to the TOAST classification, and they constituted 26% of the total subjects enrolled. In this patients subgroup, the primary efficacy outcome (recurrent stroke or death during the follow up period) was reached in 15% of subjects treated with warfarin and in 16.5% of patients treated with acetylsalicylic acid. As showed in the previous analyses, warfarin was related to a worse outcome in patients with moderate strokes and in patients with infarction involving the brainstem; on the other hand, the anticoagulant therapy showed a benefit in patients with a cryptogenic stroke and no history of arterial hypertension and in subjects with posterior circulation infarctions that did not involve the brainstem.

In the New Approach Rivaroxaban Inhibition of Factor Xa in a Global Trial vs. ASA to Prevent Embolism in Embolic Stroke of Undetermined Source (NAVIGATE ESUS), the efficacy and safety of rivaroxaban vs aspirin were compared, for the prevention of recurrent stroke in patients with a previous ESUS (Hart, 2018). 7213 patients were enrolled to randomly receive warfarin or aspirin and they were followed up for a median time of 11 months (due to an early termination of the study, because of an increased bleeding risk in the warfarin group). The trial concluded that warfarin was not superior to aspirin in preventing recurrent stroke, but it is associated with a greater bleeding risk. Interestingly, a secondary analysis of this trial concluded that a subgroup of patients (those with a left atrial enlargement) could benefit from the anticoagulant therapy when

compared to aspirin, showing a risk of ischemic stroke of 1.7% per year versus a risk of 6.5% per year (hazard ratio, 0.26; 95% confidence interval, 0.07-0.94; P for interaction=0.02) (Healey, 2019).

The Randomized, Double-Blind, Evaluation in Secondary Stroke Prevention Comparing the Efficacy and Safety of the Oral Thrombin Inhibitor Dabigatran Etexilate Versus Acetylsalicylic Acid in Patients With ESUS (RE-SPECT ESUS) trial compared another anticoagulant drug, Dabigatran, versus aspirin (Diener, 2019). 5390 subjects with a recent history of ESUS were enrolled, assigned to one of the two therapeutical groups and followed-up for a median time of 19 months. No differences in the two groups were obtained from an efficacy endpoint, so Dabigatran was not superior to aspirin in preventing recurrent strokes. Moreover, the incidence of major bleeding was equal in the two groups. Nevertheless, in the Dabigatran group more nonmajor bleeding events were registered.

The Apixaban for treatment of embolic stroke of undetermined source (ATTICUS randomized trial) is the third study that compares a direct oral anticoagulant (DOAC) vs acetylsalicylic acid in cryptogenic stroke (Geisler 2017). In this study, the primary endpoint was different compared to other previously mentioned trials; the primary endpoint of the trial, in fact, was to determine the superiority of apixaban, compared to aspirin, in preventing the occurrence of new ischemic lesions observed in a brain magnetic resonance image performed one year after the index event. Another interesting aspect of this study was that every patient enrolled in the trial underwent an implantation of an insertable cardiac monitor to detect atrial fibrillation. The results of this trial have not yet been published; nevertheless, the ATTICUS investigators presented preliminary data analysis, on a subgroup of 352 patients enrolled, at the 2023 International Stroke Conference (Poli, 2023). Similar to the previous trials, also this DOAC seems not to be superior when compared to aspirin (the rate of new ischemic lesions at the follow up brain

MRI, in fact, was not significantly different in the two groups); no difference was found between the two groups for the rate of observed adverse events. Interestingly, authors reported that about 25% of the subjects developed an atrial fibrillation, detected by prolonged cardiac monitoring.

A very interesting trial of secondary prevention in cryptogenic stroke is The AtRial Cardiopathy and Antithrombotic Drugs In prevention After cryptogenic stroke (ARCADIA) randomized trial (Kamel 2019). This phase 3 clinical trial is currently ongoing and it focuses on the concept that atrial cardiomyopathy could be the cause of a stroke in patients with no evidence of atrial fibrillation. According to this hypothesis, the trial wants to demonstrate that Apixaban 5 mg twice daily is more effective than acetylsalicylic acid 81 mg quam die to prevent the recurrence of other ischemic strokes in patients who suffered a previous ESUS and have clinical characteristics that suggest the presence of an atrial cardiomyopathy. The estimated sample size consists of 1100 participants, who are being enrolled in 120 centers located in the United States of America. In this study, investigators will also focus on both efficacy and safety outcomes. The primary efficacy endpoint will be the recurrence of both ischemic and hemorrhagic stroke; meanwhile, the secondary efficacy endpoints are the composite recurrence of ischemic stroke or systemic embolism, and the composite recurrence of any type of stroke and death. On the other hand, the primary safety outcomes are the occurrence of symptomatic intracranial bleeding and other major bleeding; the secondary safety outcome is all-cause mortality. Major bleeding in this trial will be considered an hemorrhage with one of the following characteristics: a bleeding that causes a loss of at least 2 g/dL in hemoglobin value in a period of 24 hours; a bleeding that needs a transfusion of 2 or more units of red blood cells concentrates; an hemorrhage that involves a critical non-intracranial site (intraspinal, intraarticular, intraocular, retroperitoneal pericardial, intramuscular complicated by a compartment syndrome); a fatal bleeding. Inclusion and exclusion criteria for this clinical trial are reported in the following table.

Inclusion Criteria	*Exclusion Criteria*
Clinical diagnosis of ischemic stroke with brain imaging to rule out hemorrhagic stroke	Atrial fibrillation of any duration prior to randomization
Embolic stroke of undetermined source	Clear indication for treatment-dose anticoagulant therapy
Ability to be randomized no later than 120 days after stroke onset	Left ventricular ejection fraction <30%
Modified Rankin Scale score ≤4	Definite indication for antiplatelet agent
Age ≥45 years	History of spontaneous intracranial hemorrhage
Presence of atrial cardiopathy, atrial cardiopathy, defined as ≥1 of the following: PTFV1 >5,000 µV*ms on 12-lead ECG; Serum NT-proBNP >250 pg/mL; Left atrial diameter index ≥3 cm/m2 on echocardiogram	Chronic kidney disease with serum creatinine ≥2.5 mg/dL
	Active hepatitis or hepatic insufficiency with Child-Pugh score B or C
	Pregnancy risk
	Known allergy or intolerance to aspirin or apixaban
	Concomitant participation in another clinical trial involving a drug or intervention
	Any condition that precludes follow-up or safe participation in the trial
	Inability to obtain written, informed consent from patient or surrogate for trial participation

Figure 1. Inclusion and Exclusion criteria of the ARCADIA trial (modified from Kamal, 2019).

Atrial cardiomyopathy and ischemic stroke

Association between ischemic stroke and atrial fibrillation is clear, considering that atrial fibrillation increases about 5 times the risk of having an ischemic stroke (Wolf, 1978). Nevertheless, the mechanism of association of these two diseases is still being debated (Kamel, 2016). According to Kamel et al, it is not correct to think that atrial fibrillation is simply the cause of the stroke; rather, we should consider the presence of atrial fibrillation in a more complex model of disease. The presence of cardiovascular risk factors can be the trigger that generates atrial abnormalities (atrial cardiomiopathy) which are the cause of both atrial fibrillation and ischemic stroke. On one hand, the presence of atrial fibrillation causes blood stasis and, over the time, this leads to a remodeling of the atrial structure which increases the risk of an ischemic stroke; on the other hand, an ischemic

stroke itself could be the trigger for an autonomic disfunction that could lead to establish a condition of atrial fibrillation.

This model could explain not only why an ischemic stroke and an atrial fibrillation could be present with a temporal dissociation, but also why, in some cases, an atrial fibrillation is detected only after the ischemic event (Brambatti, 2014).

According to this model, in 2016, an expert consensus came to the definition of a new entity, called atrial cardiomyopathy, which is “any complex of structural, architectural, contractile or electrophysiological changes affecting the atria with the potential to produce clinically-relevant manifestations” (Goette, 2016).

However, it is still not clear how to make a reliable diagnosis of atrial cardiomyopathy. Several studies were published, where different clinical characteristics were taken into account to reach this diagnosis (Kreimer, 2022). Furthermore, it is not clear if this disease could show itself in different stages, each one with specific clinical features (Kariki, 2023). Hence, a sensitive and specific diagnostic work up has not yet been validated. It is reasonable to start the evaluation with simple and accessible diagnostic tests (such as electrocardiogram and echocardiographic evaluation) and to reserve secondary imaging (such as cardiac magnetic resonance) for selected patients (Kariki, 2023).

Diagnostic methodic	Atrial cardiomyopathy features	Association
Electrocardiogram	PTFV1 P wave duration P wave area Abnormal P wave axis P wave dispersion	Ischemic stroke, AF Ischemic stroke Ischemic stroke Ischemic stroke AF
Transtoracic ecocardiogram	LA diameter LA volume index	Ischemic stroke, AF AF
Cardiac MRI	LA strain Delayed enhancement in LA wall	AF Ischemic stroke, AF
Serum Biomarkers	Inflammation markers Fibrosis markers Immunothrombosis markers proB natriuretic peptide	AF Ischemic stroke, AF AF AF

Figure 2. Clinical biomarkers of atrial cardiomyopathy (modified from Kreimer, 2022).

Electrocardiographic features of atrial cardiomyopathy.

- Interatrial block (Bayés de Luna, 2012). It is a marker of delayed conduction between the two atria. In the first degree interatrial block, the P wave duration is above 120 msec; the second degree interatrial block presents with transient, intermediate characteristics between the two other degrees; in the third degree interatrial block, the P wave is longer than normal and it presents a biphasic morphology in leads II, III and aVF. This pattern has been associated with supraventricular arrhythmias and ischemic stroke occurence.
- Abnormal P wave axis. The normal range of values of this parameter is from 0 to 75 degrees. It has been demonstrated that the presence of an abnormal P wave axis increases the risk of having an ischemic stroke, independently from the presence of atrial fibrillation (Maheshwari, 2017).

- Abnormal terminal force in V1 (PTFV1). It is the product of the terminal, negative portion of the P wave and its depth in the lead V1. An increase in this parameter is related to a high risk of having an ischemic stroke (Kamel, 2015).
- Maximum P wave area. It is calculated as the area under the P wave in all the leads. Only few data are available about this parameter, but it seems to be related with a higher risk of ischemic stroke (He, 2017).
- P wave dispersion. It is the difference between the longest and the shortest P wave duration, in all 12 ECG leads. This parameter presents a correlation with atrial fibrillation (Pérez-Riera, 2016).
- Reduced P-wave Voltage in Lead I. It is the depth in mV of the P wave, from the isoelectric to its peak. A reduction of this parameter has been associated to a higher risk of developing atrial fibrillation (Alexander, 2017).

Ecocardiographic features of atrial cardiomyopathy. The most common parameter of atrial cardiomyopathy to be calculated with an echocardiographic evaluation is the left atrium volume. The left atrium volume has replaced the anteroposterior length of the atrium in the parasternal long axis view because it reflects the atrium enlargement with more precision (Kariki, 2023). An increased atrium volume is correlated with atrial fibrillation and other cardiac arrhythmias (Kranert, 2020).

Cardiac magnetic resonance and atrial cardiomyopathy. Cardiac magnetic resonance imaging could give precious information about atrial disease; however, this type of imaging should be performed only in centers with a high experience. The most powerful information produced by such method is the quantification of atrial wall fibrosis, which is correlated to atrial cardiomyopathy (Shen, 2019). Cardiac MRI is also more accurate than echocardiography in measuring the atrial volume (Mandoli, 2021). Furthermore, cardiac MRI could give information about atrial flow velocity, defining patients with a low atrial flow who are, therefore, more susceptible to blood stasis (Spartera, 2021).

ESUS and atrial cardiomyopathy. The importance of diagnosing atrial cardiomyopathy in a patient with an ischemic stroke is particularly relevant in patients with ESUS, to define the best medical treatment for the secondary prevention. As previously described, the biomarkers of atrial cardiomyopathy are associated with ischemic stroke and they are often found in patients with ESUS (Yaghi, 2016). Yaghi et al conducted a study on 40 subjects with an embolic stroke of undetermined source and they concluded that 63% of them (25 patients) had one or more biomarker of atrial cardiomyopathy.

Kwong et al tried to resolve this issue, formulating a clinical risk score that could predict the future development of atrial fibrillation in patients with ESUS, named HAVOC score (Kwong, 2017). These researchers studied a cohort of 9589 patients with cryptogenic stroke and they took into account seven different conditions that could increase the risk of developing atrial fibrillation: age (≥ 75 years), obesity, congestive heart failure, hypertension, coronary artery disease, peripheral vascular disease, valvular disease. Authors managed to define a 0-14 points clinical score that could divide patients in 3 groups, according to their risk of developing atrial fibrillation: low risk (0-4 points), medium risk (5-9 points), high risk (10-14 points). This score proves to be more specific and accurate than the CHA2DS2-VASc score in predicting atrial fibrillation. The utility of this score was confirmed in further studies (Bahit, 2021) and it was also tested in patients with no history of ischemic stroke (Elkind, 2021). Nevertheless, a confirmation study for this score, conducted on an external cohort of 658 ESUS patients, failed to demonstrate a correlation between HAVOC score and the risk of atrial fibrillation (Ntaios, 2020). As a result, this score is still not widely used in clinical practice, and further analyses on the topic are mandatory.

Histological analysis of thrombi in ischemic stroke

According to Italian and international guidelines, mechanical thrombectomy plays a leading role in the therapy of acute ischemic stroke due to a large intracranial vessel occlusion (ISA-AII, 2023; Powers, 2019). The clinical impact of this therapeutic strategy on survival and good outcome is an interesting field of research for neurologists and interventional radiologists. Many clinical and procedural aspects deserve further investigation.

One crucial point is the possibility of performing a histological analysis on the clot retrieved from a mechanical thrombectomy. No definitive data is available about the composition of clots and how it can help in clinical practice, so further study on this topic are strongly recommended (De Meyer, 2017). Moreover, the histological analysis of thrombi has not yet been introduced in clinical practice and this could cause a lack of information in the general approach of a patient who suffered from an ischemic stroke.

In 2019, Staessens and colleagues decided to share their advice on how to retrieve, handle and analyze clots (Staessens, 2019). In particular, thrombus should be, at first, fixated in 10% formalin and then embedded in paraffin. Then, about 20 sections (3-5 μm thick) should be prepared and then stained via several procedures to determine the general structure of the clot and the presence of fibrin, reb blood cells, platelets, fibrin, white blood cells, von Willebrand factor. More detailed information are given in Figure 3.

Step	Recommendation
Retrieval	Remove clot from the device and transfer to saline at 4°C for 24 hours
Fixation	Fixate clot in 10% formalin for 24 hours
Paraffin embedding	Embed in parafin using dehydratation steps
Sectioning	Prepare sections of the thrombus (3-5 µm)
Staining	Stain sections using H&E and MSB
Analysis	Quantify clot components

Figure 3. Information about thrombus analysis (modified from Staessens, 2019).

Red blood cells, platelets and fibrin. Several studies tried to find a correlation between the stroke etiology and the quantitative composition of blood cells in the clot (Nicolini, 2022). In the majority of these studies, cardioembolic strokes were related to greater levels of platelets and fibrin in the thrombus. Goebel et al found that the percentage of macrophages and platelets in cardioembolic thrombi is higher when compared to both atherosclerotic clots and those retrieved in ESUS (Goebel, 2020). Analogue results were presented in a previous study, wrote by Boeckh-Behrens et al in 2016 (Boeckh-Behrens, 2016). In this large study, authors collected 145 clots that were analyzed using the hematoxylin and eosin-stain to determine the percentage of main constituents. Interesting data was found regarding all clot components: in cardioembolic strokes the percentage of fibrin, platelets and white blood cells were higher; on the other hand, in non-

cardioembolic clots the proportion of erythrocytes were higher when compared to other etiologies. Furthermore, authors also studied embolic strokes of undetermined source and they found that, in these patients, the composition of the thrombus is similar to cardioembolic strokes but not to non-cardioembolic ones.

The same conclusion was also obtained in a different study. Nouh et al, in fact, focused too their attention on embolic strokes of undetermined source (Nouh, 2020). Despite the lower number of subjects enrolled in their case study (only 33 clots were analyzed), the authors found that the percentage of platelets is higher in cardioembolic strokes while atherosclerotic thrombi are richer in red blood cells. Furthermore, clots retrieved in ESUS patients are similar to those retrieved in cardioembolic patients.

As previously mentioned, no definitive data is yet available on this topic; data obtained in other studies, in fact, is completely different. Kim et al in 2015 presented a study on a small cohort of 37 subjects (22 cardioembolic, 8 large artery atherosclerotic and 7 cryptogenic strokes) (Kim, 2015). These authors found that cardioembolic strokes were related to clots richer in red blood cells (37.8% versus 8.5%) and with a lower percentage of fibrin (32.3% versus 52.1%) when compared to atherothrombotic strokes. Other major components of clots (platelets and white blood cells) did not present significant differences in their proportion (26.6% versus 34.5% for platelets; 3.3% versus 4.9% for white blood cells). Regarding patients with an embolic stroke of undetermined source, authors did not find significant differences in the composition of thrombi retrieved in such patients and in those with cardioembolic or atherosclerotic strokes.

Fitzgerald et al in 2019 presented a study on a bigger cohort of patients, in which 105 patients were included (Fitzgerald, 2019). In this study, clots retrieved in large artery atherosclerosis were associated to a higher percentage of platelets (55% of these clots, in fact, were considered platelet-rich; on the other hand, only 21.2% of non-LAA strokes

showed this characteristic). Furthermore, these authors tried to focus on cryptogenic strokes; they found that ESUS clots were similar to LAA patients, showing a higher proportion of platelets in the thrombus (50% of ESUS clots, in fact, were platelet-rich). No significant differences in other major clot components were observed. Nevertheless, authors underlined that in their protocol platelets and fibrin were counted separately while other studies considered them together.

Furthermore, not only the quantitative analysis of the thrombus, but also the morphology of its components seems to have a potential relevance. Khismatullin et al, in fact, studied the composition of the clots using electron microscopy, to better analyze morphological characteristics of blood cells and fibrin (Khismatullin, 2020). These authors found that clots are compact structures (only 6% of empty space) made of blood cells (73% of the total) and fibrin (20% of the total). They did not find any difference between cardioembolic and atherothrombotic thrombi, in terms of quantitative composition. Regarding blood cells, the major components of the clot are red blood cells (about 80% of all the cells) and, in particular, deformed ones (such as polyhedral red blood cells, that originate after the compression of the clot in the artery). Platelets constituted about 18% of the whole cells; interestingly, authors found that platelets aggregates were more represented in clots retrieved from patients whose stroke severity was higher. Moreover, morphological characteristics of platelets seemed to be different according to the stroke etiology: in cardioembolic strokes the percentage of balloon-like platelets were higher when compared to atherothrombotic ones (3% versus 1%). Lastly, among fibrin types, fibrin bundles were the structures more represented (about 40% of the total) both in cardioembolic and atherothrombotic thrombi; cardioembolic clots seemed to be richer in fibrin when compared to atherothrombotic ones (23% versus 13%).

Cell types	Fibrin types
Biconcave RBCs (4- to 5-μm disc-shaped flattened cells; both sides of the surface curve inward forming a dimple) **Mainly biconcave intermediate-shaped RBCs** (Similar to biconcave RBCs with a dimple in the middle, but the surface deviates from discoid and is partially rounded) **Mainly polyhedral intermediate-shaped RBCs** (Polyhedral-like RBCs with more or less flat sides except areas where the sides are distorted by either protrusions or indentations) **Polyhedral RBCs** (Polyhedral cells with surface made up of intersecting polygonal sides) **Spherocytes** (Convex-shaped RBCs ≈5 μm in diameter with a smooth round surface without protrusions, indentations, or dimples) **Echinocytes** (RBCs with multiple small, evenly spaced thorny projections) **Balloon-like platelets** (Bloated single or doubled platelets with a plicate surface of a size comparable or larger than RBCs (≥5–6 μm); multiple wrinkles and lines on the surface and no bumps, which makes them distinct from spherocytes and leukocytes) **Platelet aggregates** (Clusters of deformed irregular-shaped platelets with outgrowths and multiple filopodia) **Leukocytes** (Spherical or irregular-shaped cells varying from ≈5 up to ≈10–12 μm in diameter with much membrane folding and a tuberous rough surface with multiple short bumps)	**Fibrin bundles** (Thick fibrillar structures made up of several laterally aggregated fibers) **Fibrin fibers** (Thin fibrillar structures single or usually arranged into a network) **Fibrin sponge** (Porous amorphous structure) **Fibrin debris** (Unstructured detritus and separate fibrin pieces)

Figure 4. Morphological characteristics of clots components (modified from Khismatullin, 2020).

Regarding the presence of fibrin in clots, data is still controversial. According to Khismatullin et al, atherothrombotic clots are richer in the fibrillar type of fibrin more than the sponge type, whereas cardioembolic thrombi present more fibrin bundles and more young type fibrin (Khismatullin, 2020). Nevertheless, a previous study did not find any differences in the fibrin structure among the various stroke subtypes (Sallustio, 2016).

Thrombin activity. Another study focused on thrombin activity in the clots, using fluorometric assays repeated after serial washing procedures of the thrombotic sample (Itsekson Hayosh, 2021). In this study, 68 patients were enrolled and divided in subgroups according to their stroke etiology. Authors found that in cardioembolic strokes the thrombin activity tends to decrease after serial washes; on the other hand, in atherothrombotic clots, thrombin activity shows a steady trend. According to the authors, this difference could be explained by the fact that cardioembolic clots originate in a low-

flow cardiac atrium whereas atherothrombotic thrombi originate on an arterial plaque, in a high-flow situation. Hence, cardioembolic clots are richer in thrombin, that could be easily washed out, while atherothrombotic clots are poorer in thrombin, due to the high velocity of blood flow in the artery.

White blood cells. After a vessel occlusion, the inflammatory cascade is strongly activated (De Meyer, 2016). Hence, inflammation cells and mediators are present in the clots. Moreover, the inflammatory cascade seems to be more involved in ischemic strokes than in thrombosis of other body districts. In this regard, in fact, a study found that the composition of cerebral and coronary clots is different (Novotny, 2020). These researchers collected a relatively large sample size of clots (143 clots, 71 from patients with ischemic stroke and 72 from patients with acute myocardial infarction). Analyzing the thrombi and comparing the two groups, authors found no difference in terms of total leukocytes and neutrophils. In contrast, other inflammatory cells (monocytes, eosinophils, B cells and T cells) were more represented in ischemic strokes when compared to coronary thrombosis. Furthermore, the presence of neutrophil extracellular traps (that are networks of neutrophils DNA and other extracellular fibers) was clearly higher in ischemic strokes (100% of clots analyzed) when compared to myocardial infarction (20.8% of clots analyzed) and their quantity in the thrombus was correlated to a worse clinical outcome, both in strokes and in myocardial infarctions. The quantity of NETs in the clots seemed also to be related to the stroke etiology (they were richer in atherothrombotic and cryptogenic strokes when compared to cardioembolic ones).

Schuhmann et al too, in 2016, focused their attention on inflammatory biomarkers involved in cerebral clots (Schuhmann, 2016). These researchers collected 37 thrombi and they analyzed both macroscopic and microscopic characteristics (after staining specimens with hematoxylin and eosin, Martius scarlet blue and von Willebrand factor staining). According to the macroscopic features of the thrombi, they divided the clots

into three subgroups: erythrocytic, layered and serpentine. Then, regarding the percentage of red blood cells and fibrin, they classified thrombi as red (higher percentage of red blood cells), white (lower percentage of red blood cells) or mixed. At last, they analyzed the presence of white blood cells (CD4+ T cells, CD68+ monocytes) and the von Willebrand factor and they correlated each subgroup with the quantity of these inflammatory biomarkers. Authors found that erythrocytic thrombi are richer in CD4+ T cells and CD68+ monocytes and they did not find a specific pattern in the distribution of these cells within the thrombus. On the other hand, white clots are richer in the von Willebrand factor, and it is localized in the same region of fibrin and collagen. Finally, researchers studied the clinical characteristics of patients enrolled in this study, finding that the quantity of CD68+ monocytes and von Willebrand factor correlates with the severity of the stroke at the admission.

Several studies, then, tried to demonstrate the correlation between the presence of inflammatory cells and the stroke etiology. According to Dargazanli et al, clots retrieved from strokes due to large artery thrombosis present a larger number of T cells, when compared to cardioembolic and cryptogenic ones (Dargazanli, 2016). On the other hand, Laridan et al focused their attention on the presence of NETs in the thrombi; these authors found that H3Cit-positive areas (that are a hallmark of NETs) are higher in cardioembolic clots compared to thrombi of other etiologies (Laridan, 2017).

In a recent study, Di Meglio et al collected 250 thrombi from patients with an ischemic stroke (Di Meglio, 2020). These researchers tried to investigate the presence of DNA and glycoprotein VI in the clots, besides the presence of blood cells. They found that cardioembolic clots present more DNA and less glycoprotein VI when compared to clots of other etiology (DNA 35.8 ng/mg versus 13.8 ng/mg; glycoprotein VI 0.104 ng/mg versus 0.117 ng/mg). Hence, considering that DNA in thrombi could derive mostly from white blood cells and NETs, authors concluded not only that cardioembolic clots have a

higher presence of leukocytes and that they are involved in the cerebral thrombosis, but also that measuring the quantity of DNA in the thrombus could be a useful tool in clinical practice for the management of ESUS patients.

Other correlations with thrombus histology. Several attempts to correlate the composition of thrombi and various other variables were made in recent years.

Regarding the correlation between thrombus histology and endovascular procedure, it is reported that clots richer in red blood cells are associated to a shorter duration of the procedure, requiring less passages (Sporns, 2017; Maekawa, 2018; Fitzgerald, 2020). On the contrary, clots with less red blood cells and richer in fibrin are associated with a major rate of secondary embolism during the mechanical thrombectomy (Sporns, 2017). Nevertheless, this last point is still controversial, considering that another study found that patients with secondary embolism present a higher proportion of erythrocytes in the clot (Ye, 2020), whereas another study found no correlation (Kaesmacher, 2017).

Controversial data is also available showing a possible influence of intravenous thrombolysis on the thrombus composition. Some articles, in fact, report that, after intravenous thrombolysis, the clots tend to have more fibrin and less red blood cells (Choi, 2018; Horie, 2019); on the contrary, other researches did not find any differences (Ahn, 2016; Kaesmacher, 2017).

Regarding the radiological features, several studies have demonstrated that the presence of the hyperdense middle cerebral artery sign in the non-contrast computed tomography of the brain is related to thrombi richer in red blood cells (Liebeskind, 2011; Simons, 2015), while its absence is correlated to a higher percentage of platelets in the clot (Brinjikji, 2017; Fitzgerald, 2019). In magnetic resonance imaging, the presence of the susceptibility vessel sign (a hypointense signal in the occluded artery on the gradient echo

sequence) is related to a higher proportion of red blood cells, while its absence is related to a higher fibrin content in the clot (Liebeskind, 2011; Kim, 2015).

Finally, a difference between thrombi retrieved in anterior and in posterior cerebral circulation has been reported. A recent study, in fact, concluded that basilar thrombi show a higher percentage of red blood cells and a lower amount of platelets and fibrin (Berndt, 2021).

Hypothesis and aims of the study

Histological analysis of thrombi is not generally performed in clinical practice. However, histological information obtained thanks to this procedure could be relevant and could help to define the best diagnostic work up in patients with an ischemic stroke.

Embolic strokes of undetermined source constitute a variegate subgroup of ischemic strokes and only a portion of them is related to an occult atrial fibrillation. Several studies have theorized the existence of a clinical situation, the atrial cardiomyopathy, that could be involved in the physiopathology of ESUS.

Clinical trials on ESUS failed to demonstrate the superiority of the anticoagulant therapy. Hence, the correct therapeutical approach to ESUS is still not clear. Histological analysis of the thrombi retrieved during mechanical thrombectomy could give useful information especially in this subtype of ischemic stroke.

The aim of this study is to find an association between clinical and histological markers that could be used in the clinical practice. These tools could not only help understanding

the etiology of the stroke, but also help defining which patients with ESUS have a higher risk of cardioembolism.

MATERIALS AND METHODS

This is a retrospective analysis of prospectively collected patients. We enrolled consecutive patients with an acute ischemic stroke and a large vessel occlusion, treated with mechanical thrombectomy, aged ≥ 18 years.

The presence of a large vessel occlusion was evaluated using a contrast-enhanced CT scan of the brain or a magnetic resonance angiography (according to the Italian national guidelines).

The data collection period lasted three years; it started on the 1st of January 2020 and it ended on the 31st of December 2022.

The study was monocentric, involving patients treated in the Emergency Department of Policlinico Umberto I – La Sapienza University in Rome.

The exclusion criteria were the following:

- Patients with an ischemic stroke with no evidence of a large vessel occlusion.
- Patients who did not undergo mechanical thrombectomy, according to the national guidelines.
- Patients who did not accept to participate in the study.
- Patients who, for any reason, were treated in the acute phase at the Emergency Department of Policlinico Umberto I and then transferred to another hospital.
- Patients resulted positive for SARS-CoV-2 infection at the hospital admission.

Every subject signed a written informed consent to be enrolled in the study.

We enrolled a series of 114 consecutive subjects.

Clinical data was collected at the baseline (age; presence of cardiovascular and cerebrovascular risk factors; history of systemic arterial hypertension, known atrial fibrillation and other arrhythmias, history of cardiac heart failure).

After the mechanical thrombectomy procedure, all patients enrolled in the study were admitted to the "Unità di Terapia Neurovascolare" of the Policlinico Umberto I. There, all subjects underwent an extensive and complete diagnostic workup to evaluate the etiology of the ischemic event. Furthermore, all subjects underwent an electrocardiographic study and an echocardiographic imaging study and the data obtained was evaluated by an independent cardiologist. Specific data of interest for the study was collected (presence of left atrium enlargement; electrocardiographic data such as PR length, P wave duration, amplitude negative P in V1 in mm, duration of Negative P wave in V1 in ms, abnormal P wave terminal force in lead V1, reduced P-wave Voltage in Lead I).

HAVOC score was calculated for every enrolled subject, based on clinical and anamnestic data.

At the end of the diagnostic evaluation, patients were classified in three groups, according to their stroke etiology and to the TOAST classification: cardioembolic, atherothrombotic, embolic stroke of undetermined source.

Clots were retrieved by expert interventional radiologists during the mechanical thrombectomy procedure. Material retrieved was initially stored in a saline solution and then transferred to a solution of 10% formalin. Both these phases lasted no longer than 24 hours each. Then, the measures of the thrombus were obtained by an expert

pathologist. After this, thrombi were embedded in paraffin via standard dehydration steps and finally sectioned, with an average section thickness of 5 μm. Sections were then stained with both hematoxylin and eosin stain (to evaluate the general structure of the clot) and Martius Scarlet Blue stain (to evaluate the quantity of fibrin and red blood cells). All sections obtained were independently evaluated by two expert pathologists. Data of interest was collected (measures of the clots; quantity of red blood cells, platelets e fibrin expressed as a percentage; quantity of neutrophils, expressed as abundant or not abundant).

Statistical analysis

At first, a descriptive statistical analysis of the population enrolled in the study was performed. Mean, median and standard deviation were calculated for continuous variables. Frequency count and proportion (or percentage) were calculated for categorical variables.

Subgroups (cardioembolic, atherothrombotic and embolic stroke of undetermined source) characteristics were compared using the T-test or the chi-squared test, when appropriate.

Hence, inferential statistical analyses were performed. To compare variables between groups, T- tests or Mann-Whitney tests were used, as appropriate.

Correlations between variables of interest were evaluated using the Pearson correlation coefficient (for quantitative variables) or the Spearman's rank correlation coefficient (for qualitative variables), as appropriate.

Multivariate analyses were performed using binary logistic regressions models or linear regression models, to adjust the univariate analyses results for possible confounding factors.

$P \leq 0.05$ was accepted as an indicator of a statistically significant difference in all the analyses performed.

All analyses were performed using the IBM SPSS software.

RESULTS AND DISCUSSION

Analysis of the population

In the study, a series of 114 consecutive patients were enrolled.

Clinical characteristics of the patients enrolled in the study are reported in table 1.

	All patients (n = 114)	
Age		74 ± 11 years
Male sex		49 (43%)
Arterial hypertension		89 (78.1%)
Atrial fibrillation		54 (47.4%)
Diabetes		18 (15.8%)
Previous ACS		17 (14.9%)
Dyslipidemia		38 (33.3%)
Smoking habit		17 (14.9%)
Obesity		26 (22.8%)
History of CAD		24 (21.1%)
History of PAD		6 (5.3%)
Carotid stenosis		
≤50%		44 (38.6%)
>50%		19 (16.7%)
Cardiac valvular disease		20 (17.7%)
History of malignant neoplasm		22 (19.3%)
mRS pre-Stroke (0-1)		103 (90.4%)
NIHSS at admission		15 ± 6
Intravenous r-tPA		45 (40.2%)
Thrombus site		
ICA		18 (15.9%)
MCA - M1		57 (50.4%)
MCA - M2		18 (15.9%)
Thrombus characteristics		
%RBC		50 ± 17
%PF		50 ± 17
Neutrophils (abundant)		38 (35.5%)
Ventricular hypertrophy		41 (36.6%)
Cardiac heart failure		13 (11.6%)
Atrial cardiomyopathy features		
Interatrial block		7 (11.5%)
Abnormal PTFV1		30 (26.3%)
Reduced P Wave voltage in Lead I		25 (41.7%)
Left atrium enlargment		68 (60.7%)
HAVOC score > 4		43 (37.7%)
CHADsVaSC2		5 ± 1.5

Table 1. Clinical characteristics of the study population.

Regarding the anamnestic characteristics of the study population, the mean age was 74 years (± 11 SD), 49 patients were male (43% of the total), 89 patients suffered of arterial hypertension (78.1% of the total), 54 patients had atrial fibrillation (47.4% of the total), 18 patients had diabetes (15.8% of the total); 103 subjects (90.4% of the total) had no pre-Stroke disability (with a modified Rankin Scale pre-Stroke value of 0-1). The mean value of CHADSVASc score was 5 (±1.5 SD); regarding the HAVOC score, 43 patients (37.7% of the total) presented a score higher than 4.

At the admission, the mean NIHSS value was 15 (± 6); 45 patients (40.2% of the total) underwent intravenous r-tPA before mechanical thrombectomy. In 18 subjects (15.9% of the total) the site of the occlusion was the Internal Carotid Artery, in 57 subjects (50.4% of the total) it was the Middle Cerebral Artery in the M1 tract, in 18 subjects (15.9% of the total) it was the Middle Cerebral Artery in the M2 tract.

Regarding atrial cardiomyopathy features, 7 patients (11.5% of the total) presented a first grade interatrial block; 30 patients (26.3% of the total) had an abnormal PTFV1 value; 25 patients (41.7% of the total) presented with a reduced P wave voltage in Lead I; 68 patients (60.7% of the total) presented a left atrium enlargement.

At first, we performed an univariate analysis (using the Pearson correlation coefficient or the Spearman's rank correlation coefficient, as appropriate) to determine which clinical characteristics of the subjects are correlated to the thrombus composition (in terms of quantity of platelets and of presence of neutrophils). Results of this analysis are summarized in table 2.

	%FP		Neutrophils	
	r	p	r	p
Age	0.15	0.13	0.15	0.13
Male sex	0.02	0.85	0.05	0.59
Arterial hypertension	0.03	0.78	0.03	0.80
Atrial fibrillation	0.04	0.72	0.23	0.02
Diabetes	0.14	0.16	0.07	0.46
Previous ACS	0.14	0.16	0.16	0.10
Dyslipidemia	0.12	0.22	0.15	0.13
Smoking habit	-0.06	0.55	-0.08	0.45
History of CAD	0.12	0.24	0.18	0.06
History of PAD	-0.06	0.53	0.07	0.45
Carotid stenosis ≤50%	0.01	0.95	0.12	0.21
Carotid stenosis >50%	0.05	0.63	-0.06	0.57
Cardiac valvular disease	0.15	0.14	-0.01	0.93
History of malignant neoplasm	-0.10	0.29	-0.09	0.37
mRS pre-Stroke (0-1)	0.13	0.17	0.08	0.41
NIHSS at admission	0.04	0.71	0.16	0.10
Intravenous r-tPA	0.02	0.88	0.14	0.14
TOAST	0.01	0.96	-0.24	0.01
Thrombus site	-0.16	0.11	-0.04	0.68
Ventricular hypertrophy	-0.06	0.53	0.12	0.23
Cardiac heart failure	0.05	0.58	0.05	0.62
HAVOC score > 4	0.27	0.004	0.23	0.02

Table 2. Correlations between clinical characteristics and thrombus composition (on all the subjects).

According to the percentage of platelets in the clot, this univariate analysis showed that only an HAVOC score > 4 presented a correlation with platelets (p= 0.004, r= 0.27). We did not find association with the etiology of the ischemic stroke, in terms of TOAST classification (p= 0.96, r= 0.01).

According to the presence of abundant neutrophils in the thrombus, the analysis showed that this variable is correlated to the TOAST classification (p= 0.01, r= -0.24), the presence of atrial fibrillation (p= 0.02, r= 0.23) and to an HAVOC score > 4 (p= 0.02, r = 0.23).

We, therefore, performed a multivariate analysis on this data (with a multiple linear regression for platelets and a logistic regression for neutrophils), showed in table 3.

	Neutrophils		
	Adjusted OR	**p**	**95% C.I.**
Age	1.02	0.51	0.97 - 1.07
Male sex	1.51	0.39	0.60 - 3.82
Arterial hypertension	0.95	0.93	0.31 - 2.90
Cardioembolic etiology	3.25	0.01	1.29 - 8.24

	%PF		
	Adjusted OR	**p**	**95% C.I.**
Age	0.15	0.34	-0.16 - 0.47
Male sex	1.49	0.66	-5.13 - 8.10
NIHSS at admission	-0.19	0.47	-0.70 - 0.32
TOAST	2.59	0.16	-1.04 - 6.22
HAVOC > 4	7.55	0.04	0.43 - 14.67

Table 3. Multivariate analysis on platelets and neutrophils in the thrombus (on all the subjects).

In the multivariate analysis for the percentage of platelets in the clot, the correlation with an HAVOC score > 4 remained statistically significant (p= 0.04; OR 7.55; 95% confidence interval 0.43 – 14.67) after the adjustment for potential confounding factors (age, sex, NIHSS at admission, TOAST classification).

In the multivariate analysis for the presence of abundant neutrophils in the thrombus, the correlation with the cardioembolic etiology seemed to be independently related, reaching the statistical significance (p=0.01; OR 3.25; 95% confidence interval 1.29 – 8.24) after the correction for potential confounding factors (age, sex, arterial hypertension).

Analysis based on etiological classification

Patients were then divided in three subgroups, according to the TOAST classification (cardioembolic, atherothrombotic, ESUS) and a univariate analysis was performed to compare these subgroups. Results are shown in table 4.

	CE (n=54)	LAA (n=13)	ESUS (n=46)	p
Age	78 ± 9.5	70 ± 9.5	72 ± 12.5	0.002
Male sex	40.7%	69.2%	37%	0.12
Arterial hypertension	79.6%	92.3%	73.9%	0.13
Diabetes	18.5%	7.7%	13%	0.09
Previous ACS	16.7%	15.4%	13%	0.93
Dyslipidemia	37%	38.5%	28.3%	0.68
Smoking habit	9.3%	30.8%	17.4%	0.23
Obesity	27.8%	30.8%	15.2%	0.39
History of CAD	27.8%	23.1%	13%	0.32
History of PAD	5.6%	15.4%	2.2%	0.31
Cardiac valvular disease	24.5%	23.1%	8.7%	0.19
History of malignant neoplasm	18.5%	15.4%	21.7%	0.91
NIHSS at admission	17 ± 6	11.5 ± 7	14.5 ± 6	0.04
Intravenous r-tPA	40.7%	41.7%	40%	0.88
Thrombus characteristics				
%RBC	50.2%	51.2%	49.2%	0.69
%PF	49.8%	48.8%	50.8%	0.69
Neutrophils (abundant)	49%	15.4%	26.2%	0.04
Ventricular hypertrophy	42.3%	0	39.1%	0.02
Cardiac heart failure	15.4%	0	10.9%	0.46
Left atrium enlargment	88.5%	46.2%	32.6%	<0.001
HAVOC score > 4	53.7%	30.8%	21.7%	0.01

Table 4. Univariate analysis between subgroups based on the TOAST classification.

54 subjects with a cardioembolic stroke, 13 subjects with large artery atherosclerosis and 46 subjects with an ESUS were enrolled.

Patients with a cardioembolic strokes showed a higher age (the mean age was 78 years for CE vs 70 years for LAA vs 72 years for ESUS; p= 0.002) and a higher NIHSS at admission (the mean NIHSS was 17 for CE vs 11.5 for LAA vs 14.5 for ESUS; p= 0.04). Furthermore, in this subgroup, ventricular hypertrophy was more frequent (42.3% for CE vs 0% for LAA vs 39.1% for ESUS; p= 0.02); analogue results were found for the presence of a left atrium enlargement (88.5% for CE vs 46.2% for LAA vs 32.6% for ESUS; p< 0.001). Furthermore, a HAVOC score > 4 was more frequent in the cardioembolic stroke patients (53.7% for CE vs 30.8% for LAA vs 21.7% for ESUS; p= 0.01).

No other statistically significant differences were found, in the comparison with other variables.

Analysis on ESUS subjects

In the second part of the statistical analysis, the focus was only on the ESUS subgroup of subjects. Clinical characteristics of these patients are reported in table 5.

	ESUS (n=46)
Age	72 ± 12.5
Male sex	37%
Arterial hypertension	73.9%
Diabetes	13%
Previous ACS	13%
Dyslipidemia	28.3%
Smoking habit	17.4%
Obesity	15.2%
History of CAD	13%
History of PAD	2.2%
Carotid stenosis ≤50%	32.6%
Cardiac valvular disease	8.7%
History of malignant neoplasm	21.7%
mRS pre-Stroke (0-1)	93.3%
NIHSS at admission	14.5 ± 6
Intravenous r-tPA	40%
Thrombus site	
ICA	15.6%
MCA - M1	48.9%
MCA - M2	13.3%
Thrombus characteristics	
%RBC	49.2%
%PF	50.8%
Neutrophils (abundant)	26.2%
Ventricular hypertrophy	39.1%
Cardiac heart failure	10.9%
Atrial cardiomyopathy features	
Interatrial block	13.3%
Abnormal PTFV1	66.7%
Reduced P Wave voltage in Lead I	42.2%
Left atrium enlargment	32.6%
HAVOC score > 4	20%
CHADsVaSC2	5 ± 1.5

Table 5. Clinical characteristics of ESUS subjects.

The mean age was 72 years (± 12.5 SD), 17 patients were male (37%), the mean NIHSS at the admission was 14.5 (± 6 SD). As for the cerebrovascular risk factors, 73.9% of

subjects had arterial hypertension, 13% of them had diabetes, 28.3% presented with dyslipidemia. 20% of the subjects had a HAVOC score higher than 4. 48.9% presented with an occlusion of the Middle Cerebral Artery in the M1 tract. Regarding the thrombus composition, the mean percentage of red blood cells was 49.2%, the mean percentage of platelets and fibrin was 50.8%; abundant neutrophils were presented in 26.2% of subjects. Regarding atrial cardiomyopathy features, 13.3% of the subjects presented a first grade interatrial block; 66.7% of the patients had an abnormal PTFV1 value; 42.2% of the subjects presented with a reduced P wave voltage in Lead I; 32.6% of them presented a left atrium enlargement.

At first, ESUS patients were analyzed based on the presence or the absence of a marker of atrial cardiomyopathy (PTFV1 $\geq$ 5000, I degree interatrial block, reduced P wave voltage in Lead I, left atrium enlargement), on the HAVOC score and on the presence of cardiac heart failure. The results of the univariate analysis of these subgroups are shown in table 6.

	PTFV1≥5000 (n=30)	PTFV1<5000 (n=13)	p
%RBC	48,17 ± 15,89	51,54 ± 15,05	0.51
%FP	51,83 ± 15,89	48,46 ± 15,05	0.51
Neutrophils	27.59%	23.08%	1.00
	Interatrial block (n=6)	**No Interatrial block (n=36)**	**p**
%RBC	32.5 ± 17.82	51.94 ± 13.75	0.04
%FP	67.50 ± 17.82	48.06 ± 13.75	0.04
Neutrophils	66.67%	20.00%	0.04
	Reduced P wave voltage (n=18)	**Normal P wave voltage (n=24)**	**p**
%RBC	46.11 ± 13.35	51.46 ± 17.23	0.26
%FP	53.89 ± 13.35	48.54 ± 17.23	0.26
Neutrophils	34.78%	16.67%	0.29
	LA enlargment (n=14)	**LA normal (n=29)**	**p**
%RBC	52.14 ± 17.62	47.76 ± 14.55	0.43
%FP	47.86 ± 17.62	52.24 ± 14.55	0.43
Neutrophils	28.57%	25.00%	1.00
	HAVOC>4 (n=10)	**HAVOC≤4 (n=32)**	**p**
%RBC	38.00 ± 17.51	52.58 ± 13.41	0.23
%FP	62.00 ± 17.51	47.42 ± 13.41	0.23
Neutrophils	40.00%	21.88%	0.41
	CHF (n=5)	**no CHF (n=38)**	**p**
%RBC	42.0 ± 16.43	50.13 ± 15.40	0.34
%FP	58.00 ± 16.43	49.87 ± 15.40	0.34
Neutrophils	40.00%	24.32%	0.59

Table 6. Comparison between ESUS subgroups.

In these analyses, the statistical significance was reached only in the comparison between ESUS with a first grade interatrial block and those with a normal interatrial conduction. In the first group, in fact, clots appeared to be richer in platelets and fibrin (67.50% ± 17.82 SD vs 48.06% ± 13.75 SD in the second group; p= 0.04) and in neutrophils (66.67% vs 20% in the second group; p= 0.04).

A univariate analysis was then performed in order to predict the quantity of platelets and fibrin and of neutrophils in the clots of ESUS patients, reported in table 7.

	%FP		Neutrophils	
	r	p	r	p
Age	0.41	0.01	0.04	0.80
Male sex	0.18	0.25	0.31	0.04
Arterial hypertension	0.07	0.68	-0.02	0.93
Diabetes	0.24	0.12	-0.05	0.75
Previous ACS	0.33	0.03	0.38	0.01
Dyslipidemia	0.44	0.003	0.34	0.03
Smoking habit	-0.13	0.42	0.02	0.88
Obesity	0.29	0.06	0.12	0.47
History of CAD	0.37	0.01	0.38	0.01
History of PAD	-0.11	0.49	-0.09	0.56
Carotid stenosis ≤50%	0.09	0.57	0.12	0.45
Carotid stenosis >50%	0.15	0.35	-0.01	0.96
Cardiac valvular disease	0.19	0.22	0.18	0.27
History of malignant neoplasm	-0.07	0.68	0.05	0.76
mRS pre-Stroke (0-1)	-0.06	0.73	-0.06	0.72
NIHSS at admission	0.29	0.06	0.36	0.02
Intravenous r-tPA	0.24	0.12	0.23	0.16
Thrombus site	0.25	0.11	-0.13	0.41
Ventricular hypertrophy	-0.11	0.50	0.12	0.23
Cardiac heart failure	0.17	0.28	0.12	0.47
HAVOC score > 4	0.40	0.01	0.22	0.17
Atrial cardiomyopathy features				
Interatrial block	0.44	0.04	0.37	0.02
Abnormal PTFV1	-0.04	0.78	-0.14	0.38
Left atrium enlargment	-0.13	0.39	0.04	0.81
Reduced P Wave voltage in Lead I	0.17	0.28	0.20	0.20

Table 7. Correlations between clinical characteristics and thrombus composition (on ESUS subjects).

In this univariate analysis, the quantity of platelets in the thrombus of ESUS subjects appeared to be correlated with age (p= 0.01, r= 0.41), the presence of dyslipidemia (p= 0.03 , r= 0.44), a history of coronary artery disease (p= 0.01, r= 0.37), a HAVOC score >4 (p=0.01, r= 0.40). Furthermore, the presence of abundant neutrophils in the thrombus was correlated with sex (p=0.04, r=0.31), a history of coronary artery disease (p=0.01, r= 0.38), the NIHSS at the admission (p=0.02, r=0.36) and the presence of a first degree interatrial block (p=0.02, r=0.37).

Despite the small sample size, a multivariate analysis was performed to confirm the results obtained. This analysis, however, did not find any variable independently related to the thrombus composition (results of the models are reported in table 8).

	%FP		
	Adjusted OR	**p**	**95% C.I.**
Age	0.49	0.06	-0.02 - 1.00
Male sex	6.98	0.33	-7.34-21.29
Dyslipidemia	4.08	0.43	-6.31 - 14.47
HAVOC score > 4	-0.21	0.97	-13.03 - 12.60
Interatrial block	11.16	0.06	-0.35 - 22.67

	Neutrophils	
	OR	**p**
Age	0.98	0.58
Male sex	1.71	0.63
Dyslipidemia	2.72	0.30
NIHSS at admission	1.11	0.25
Interatrial block	6.83	0.09

Table 8. Multivariate analysis on platelets and neutrophils in the thrombus (on ESUS subjects).

Discussion

Acute ischemic stroke due to a large vessel occlusion is a frequent and life-threatening condition in the emergency setting. The best therapeutic treatment, in the first hours of this condition, consists of performing a mechanical thrombectomy to remove the thrombus in the occluded cerebral artery, according to all national and international guidelines. Besides its leading role in the therapy for this condition, this procedures allows also to collect thrombi and perform histological analyses. Nevertheless, the histological examination of thrombi retrieved during the mechanical thrombectomy has not yet been included in the general diagnostic work up of the patients who suffered an ischemic stroke. The main reason for that is a lack of clear information that this practice could give to the clinicians, due to the controversial data available in literature (Nicolini, 2022).

One first question is regarding the composition of the thrombus, in terms of percentage of red blood cells and of percentage of platelets and fibrin. Some studies, in fact, concluded that cardioembolic strokes are related to clots richer in platelets (Boeckh-Behrens, 2016; Goebel, 2020; Nouh, 2020); on the other hand, other studies concluded that this etiology is related to thrombi richer in platelets (Kim, 2015; Fitzgerald, 2019). Another study, however, did not find any difference in the clot composition of cardioembolic patients (Khismatullin, 2020). Furthermore, other studies tried to investigate the presence of neutrophils in the clots and their relation to the stroke etiology. Even for these studies, results were not conclusive: in general, the majority of the researchers agreed on the fact that cardioembolic clots are richer in neutrophils (Laridan, 2017; Di Meglio, 2020), although other studies obtained different results (Dargazanli, 2016). One possible explanation for these controversial results could be the relative small sample size of subjects enrolled in these studies (some of them included less than 40 subjects).

In our study, we enrolled 114 subjects with an ischemic stroke with a large vessel occlusion who underwent mechanical thrombectomy. In our cohort, cardioembolic strokes seem to be related to a higher age and to a worse clinical presentation, in terms of NIHSS at the admission. This data is in line with the previous literature.

The histological analysis revealed that the composition of the clots is not influenced by the etiological classification, in terms of quantity of red blood cells, platelets and fibrin. Hence, cardioembolic strokes did not seem to be different from other stroke subtypes, in regards to these histological features. Interestingly, cardioembolic patients present thrombi richer in neutrophils, when compared to atherothrombotic and ESUS ones. These results were confirmed also in the multivariate analysis, after the adjustment for potential confoundable factors. These results are in line with previous studies if we take into account those ones whose sample size is larger. According to this data, the composition of the thrombi (in terms of presence of inflammatory cells) seems to be different in various stroke subtypes and this suggests that the inflammatory cascade could play an important role in the genesis of the clots.

Regarding the quantity of red blood cells and platelets in the clots, analysis showed that these parameters were related to the HAVOC score. In particular, patients whose HAVOC score was higher than 4 appeared to have thrombi richer in platelets. Considering the composition of the score (hypertension, age, valvular heart disease, peripheral vascular disease, obesity, congestive heart failure, coronary artery disease), this data suggests that the clot composition could be influenced by the general cardiovascular status of the patients and by the presence of comorbidity. To our knowledge, considering that the HAVOC score is a relatively new risk score (implemented only in the last years), there are no previous studies on this topic so further investigations are mandatory.

The focus was then shifted on to the ESUS subgroups of subjects. Among our patients, 46 of them were classified as ESUS. In particular, we tried to investigate if the presence of atrial cardiomyopathy could be related to a specific pattern in the thrombus composition. According to the literature, several markers of atrial cardiomyopathy has been proposed (Kreimer, 2022). However, reliable diagnostic criteria for this condition have not yet been defined and it is not clear if this disorder could present itself in different stages (Kariki, 2023). We decided to take into account some electrocardiographic features (I degree interatrial block, abnormal PTFV1, reduced P wave voltage in Lead I), the presence of a left atrium enlargement, the presence of a cardiac heart failure and the HAVOC score. Among these features, only the presence of a I degree interatrial block resulted to be correlated to the presence of abundant neutrophils in the thrombus. This data suggests that this characteristic could be used to stratify ESUS patients and to detect subjects with a higher risk of cardioembolism. However, the multivariate analysis did not reach a statistical significance. In this regard, the sample size of our study must be taken into account; only 6 patients of our case study, in fact, present an interatrial block and this sample is not adequate to obtain definitive data. Further studies are, therefore, mandatory.

A HAVOC score higher than 4 resulted to be correlated to the percentage of platelets and fibrin in the thrombus also in ESUS patients (together with age, the presence of dyslipidemia, a history of coronary heart disease and the presence of I degree interatrial block). Even in this case, the multivariate model did not reach the statistical significance and the sample size should be enlarged.

In the end, these results suggest that a histological analysis of thrombus and a specific research of atrial cardiomyopathy should be implemented to better categorize ESUS patients. This could help both in clinical practice and in further researches, to design

more accurate inclusion criteria for pharmacological trials on the secondary prevention of ESUS patients.

Limitations of the study

The first limitation of this study is the sample size that was obtained. We enrolled, in fact, 114 patients (54 cardioembolic strokes, 13 atherothrombotic strokes, 46 ESUS, 1 rare cause of stroke). Hence, the sample size should be incremented in the future to reach more statistical significance.

Furthermore, the study has included only patients with a large vessel occlusion who underwent a mechanical thrombectomy. Considering this point, there is no available information about patients with a small vessels disease or patients with subacute strokes who received just the medical treatment.

Another aspect, that has to be taken into account, is related to the histological analysis of the thrombi. Only a quantitative analysis of the clot composition was performed; qualitative analysis of the cells, presence of NETs and structural information about the thrombus were not available. These aspects, according to the most recent published studies, could give important information and should be considered in further investigations.

Lastly, to better categorize ESUS patients (especially those with a suspected atrial cardiomyopathy), a clinical cardiological follow up should be performed to detect atrial fibrillation in the months that follow an ischemic stroke; this information could be used to enhance the results obtained in the study.

Conclusions

The histological analysis of the thrombi retrieved during a mechanical thrombectomy could be an important diagnostic tool in the management of patients with an acute ischemic stroke. Results of this study, in fact, suggest that cardioembolic patients present a higher expression of inflammatory cells in the clots, when compared to strokes of other etiologies. This type of analysis, along with a correct definition of atrial cardiomyopathy biomarkers (such as the presence of a I degree interatrial block), could help in a more accurate stratification of ESUS patients. However, further studies on this topic should be performed.

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