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Novel Insights into Thrombolysis Resistance: Neutrophil Extracellular Traps and Targeting Therapies

Nové poznatky o odolnosti vůči trombolýze: Neutrofilní extracelulární pasti a cílené terapie

Supervisor: prof. MUDr. Petr Widimský, DrSc.

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ABSTRACT

Studies on thrombus composition in acute stroke or acute myocardial infarction may help to elucidate clot etiology and understand reperfusion success or failure. Such studies may certainly aid in the development of new technologies aimed at treating specific subtypes of thrombi. Thrombus composition is suggested to influence the choice of techniques used during mechanical thrombectomy and plays a role in potential device and thrombus interaction. Histological analysis on the composition of thrombi causing ischemic stroke has proved to be a powerful tool to set standard prevention and treatment protocols. Based on clot components, it is possible to provide a more accurate diagnosis and distinguish different stroke subtypes. Studies on histological clot composition support the theory that cryptogenic stroke can have a cardiogenic origin. Components found in thrombi extracted from stroke patients support the importance of antithrombotic therapy in preventing and treating cerebral ischemia; however, more studies are needed to improve results in all types and subtypes of stroke. Thrombosis is linked to neutrophil release of Neutrophil Extracellular Traps (NETs). NETs are proposed as a mechanism of resistance to thrombolysis.

My original study intends to analyze the composition of thrombi retrieved after mechanical thrombectomy, estimate the age and organization of thrombi and evaluate associations with the use of thrombolysis, antiplatelets and heparin. The samples retrieved (44 from cerebral and 28 coronary arteries) were stained with Hematoxylin Eosin (HE), anti-Neutrophil Elastase antibody (Anti-NE) and anti-histone H2B antibody (Anti-H2B), representing different components in NETs formation, all detectable during the later stages of NETosis, for histochemical and digital quantification of NETs content. The histological and morphological evaluations of the specimens were correlated, through univariate and mediation analysis, with clinical information and therapy administered before intervention. The results demonstrated that the composition of cerebral and coronary thrombi differs and that there were significantly more lytic cerebral thrombi than coronary thrombi (66% vs 14%, p-value=0.005). There was a considerably higher expression of NETs in the cerebral thrombi as testified by the higher expression of H2B (p-value=0.031). Thrombolysis was remarkably associated with higher NE positivity, (AME 6.461, 95% CI 0.7901-12.13, p-value=0.02555), regardless of the origin of thrombi. There was no notable association between the administration of antiaggregant therapy/heparin and H2B/NE amount when adjusted for the thrombus location. Importantly, the age of thrombus was the only independent predictor of NETs content without any mediation of the thrombolytic treatment (p- value=0.014). The age of thrombus is the driving force for NETs content, which correlate with impaired clinical outcomes. The therapy that is currently administered does not modify NETs content. This original study supports the need to investigate new pharmacological approaches added to thrombolysis to prevent NETs formation or enhance their disruption, such as recombinant human DNase I (deoxyribonuclease I).

ABSTRAKT

Studie o složení trombů při akutní cévní mozkové příhodě nebo akutním infarktu myokardu mohou přispět k objasnění etiologie trombu a porozumění úspěšnosti či neúspěšnosti rekanalizace. Tyto studie mohou nepochybně pomoci při vývoji nových technologií zaměřených na léčbu specifických podtypů trombů. Předpokládá se, že složení trombu ovlivňuje volbu technik používaných při mechanické trombektomii a hraje roli v možné interakci mezi zařízením a trombem. Histologická analýza složení trombů způsobujících ischemickou cévní mozkovou příhodu se ukázala jako účinný nástroj pro stanovení standardních preventivních a léčebných protokolů. Na základě složení trombu je možné přesněji diagnostikovat a rozlišit různé podtypy cévních mozkových příhod. Studie o histologickém složení trombů podporují teorii, že kryptogenní cévní mozková příhoda může mít kardiogenní původ. Složky nalezené v trombech extrahovaných od pacientů s cévní mozkovou příhodou zdůrazňují význam antitrombotické terapie při prevenci a léčbě mozkové ischemie; nicméně, je zapotřebí více studií k dosažení lepších výsledků u všech typů a podtypů cévních mozkových příhod. Trombóza je spojena s uvolňováním neutrofilních extracelulárních sítí (NETs) neutrofily. NETs jsou považovány za mechanismus rezistence na trombolýzu.

Má studie si klade za cíl analyzovat složení trombů získaných po mechanické trombektomii, odhadnout stáří a organizaci trombů a zhodnotit souvislosti s použitím trombolýzy, antiagregantů a heparinu. Odebírané vzorky (44 z mozkových a 28 z koronárních tepen) byly barveny hematoxylinem eosinem (HE), anti-neutrofilní elastázovou protilátkou (Anti-NE) a anti-histonovou protilátkou H2B (Anti-H2B), představující různé složky tvorby NETs, všechny detekovatelné v pozdních stádiích NETózy, za účelem histochemického a digitálního kvantifikování obsahu NETs. Histologická a morfologická hodnocení vzorků byla korelována prostřednictvím jednorozměrné a mediační analýzy s klinickými údaji a terapií podávanou před zákrokem. Výsledky ukázaly, že složení mozkových a koronárních trombů se liší a že mozkové tromby byly významně více lytické než koronární (66 % vs 14 %, p-hodnota = 0,005). Výrazně vyšší exprese NETs byla nalezena u mozkových trombů, což dokládá vyšší exprese H2B (p-hodnota = 0,031). Trombolýza byla pozoruhodně spojena s vyšší pozitivností NE (AME 6,461, 95% CI 0,7901–12,13, p-hodnota = 0,02555), bez ohledu na původ trombů. Nebyla nalezena významná souvislost mezi podáváním antiagregační terapie/heparinu a množstvím H2B/NE při zohlednění lokalizace trombu. Důležité je, že stáří trombu bylo jediným nezávislým prediktorem obsahu NETs bez jakékoli mediace trombolytickou léčbou (p-hodnota = 0,014). Stáří trombu je hnací silou obsahu NETs, které korelují s horšími klinickými výsledky. Terapie, která je aktuálně podávána, obsah NETs nemění. Tato studie podporuje potřebu zkoumání nových farmakologických přístupů přidaných k trombolýze za účelem prevence tvorby NETs či zlepšení jejich destrukce, jako je rekombinantní lidská DNáza I (deoxyribonukleáza I).

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LIST OF ABBREVIATIONS

DNase I deoxyribonuclease I

IS, AIS ischemic stroke/acute ischemic stroke

MI myocardial infarction

MT mechanical thrombectomy

NET neutrophil extracellular trap

PCI percutaneous coronary intervention

rt-PA recombinant tissue-type plasminogen activator

mRS modified Ranking Scale

NIHSS National Institutes of Health Stroke

AME average marginal effect

CI confidence interval

rms regression modeling strategies

IHC immunohistochemistry

VWF von Willebrand Factor

NE neutrophil elastase, **Anti-NE** anti-Neutrophil Elastase

Anti-H2B anti-histone H2B

HE Hematoxylin and Eosin

INTRODUCTION

This dissertation thesis is based on the original research study conducted by the doctoral candidate, throughout the entirety of his doctoral program, on Neutrophil Extracellular Traps and the role they play in acute ischemic stroke and myocardial infarction. Such original study gave origin to mainly two different publications on international journals with high impact factor where the doctoral candidate is listed as the first author. These publications provided him with some of the material and data utilized in this dissertation thesis.

Firstly, this dissertation thesis will unravel the importance of histological analysis of the composition of emboli/thrombi retrieved from patients in the settings of acute ischemic stroke and myocardial infarction. Secondly, this thesis will cover the current state of therapeutic interventions involved in the treatment of the aforementioned acute conditions, both from a pharmacological and a procedural point of view. Subsequently, this thesis will analyze the role played by Neutrophil Extracellular Traps (NETs) both in the formation of thrombi/emboli and in the resistance mechanisms to the present treatment options in the settings of acute ischemic stroke and myocardial infarction. As a matter of fact, not only do they provide an involvement in the resistance to pharmacological thrombolysis, but thrombi/emboli rich in Neutrophil Extracellular Traps seem to hinder the outcomes of also procedural techniques such as mechanical thrombectomy (MT) and percutaneous coronary intervention (PCI). Lastly, this dissertation thesis will propose a medical solution in order to target Neutrophil Extracellular Traps and improve clinical outcomes.

1. Ischemic Stroke

The great majority of ischemic strokes is caused by an occlusion of a cerebral artery due to thrombosis or embolization. The result is a decrease in perfusion pressure and cerebral flow in the area of the affected artery, which initially is mitigated by autoregulatory mechanisms.

Physiologically speaking, the value of brain perfusion is 50-60 ml of blood per 100 grams of tissue each minute. The progression of the decrease in cerebral flow to values of 12-18 ml per 100 grams each minute leads to the arising of the so-called ischemic penumbra. At this perfusion value, the function of neurons is interrupted and the clinical manifestations of an acute stroke occur according to the affected artery. Neurons in the penumbra remain vital from minutes to hours after arterial occlusion, therefore ischemic changes are reversible with timely reperfusion. When the perfusion of the brain tissue drops below 10 ml per 100 grams each min, a so-called necrotic core is formed and the impairment is irreversible. It is estimated that 2 million neurons die every 60 seconds. The goal of the treatment is timely recanalization of the affected artery and saving the potentially viable penumbra.

Ischemic strokes are an urgent condition. Early identification of a patient with stroke, prompt transport to a stroke unit and the early initiation of adequate therapy are decisive not only for the life of the patient, but also for their subsequent quality of life. The rule "time is brain" applies here. Any patient with rapidly developing clinical signs of focal brain involvement within the last 24 hours should be evaluated as a potential acute stroke patient and a candidate for ischemic stroke recanalization.

The goal of the initial clinical examination by an experienced neurologist is to evaluate the state of consciousness and the severity of the neurological deficit using the NIHSS classification (National Institute of Health Stroke Scale). For instance, patients with ischemic strokes caused by occlusion of a large cerebral artery usually achieve an NIHSS of equal or more than 10 points.

Furthermore, the level of self-sufficiency before the stroke is determined using the modified Rankin scale (mRS), where 0 means full self-sufficiency and 5 means immobility with full dependence on another person. At the same time, laboratory diagnostics (biochemical analysis, blood count, coagulation factors) are performed, as well as monitoring of basic vital functions and possibly correction of pathological values (blood pressure, glycemia).

A thorough clinical examination can predict the type of stroke, but the definitive diagnosis and extent of the stroke are determined only on the basis of imaging, most often CT.

Head CT is widely available, rapid, and has a high sensitivity to rule out intracranial hemorrhage as one of the possible causes of ongoing neurological symptoms. The presence of a "high density sign" at the location of a large cerebral artery on native CT confirms its thrombotic occlusion with high sensitivity.

Native CT examination of the brain is also used to display early ischemic changes, which are classified using the ASPECT score (Alberta stroke programme early CT). The presence of early ischemic changes in more than 4 regions increases the risk of hemorrhagic transformation and poses a contraindication to the administration of thrombolysis.

CT/MRI angiography is also part of the ischemic stroke diagnostic protocol, which confirms the occlusion of a large cerebral artery in up to 60% of patients with acute ischemic stroke. The result of CT/MRI angiography is one of the entry criteria used in all recent studies that have demonstrated the benefit of mechanical thrombectomy in patients with occlusion of a large cerebral artery. Using CT angiography, we can also visualize the collateral supply, the presence of which reduces the extent and speed of infarction and increases the chances of a more favorable prognosis for the given patient. In patients with an unknown onset time or after more than 6 hours from the onset of the stroke, it is recommended to evaluate early ischemic changes or the presence of a penumbra using a CT perfusion examination or a diffusion weighted MRI of the brain, which is currently not routinely performed due to the limited availability and time-consuming nature of these examinations.[1–5]

2. Myocardial Infarction

Acute myocardial infarction occurs in the settings of a focal ischemic necrosis of the heart muscle caused by the occlusion or extreme narrowing of a coronary artery.

In most cases, its cause is atherosclerosis of the coronary arteries. Less commonly, myocardial ischemia can be caused by coronary spasms, arteritis, trauma, dissection, recreational drug use, congenital abnormalities or complications associated with cardiac catheterization, only rarely by coronary embolism. The whole process is triggered by risk factors such as smoking, hypertension, diabetes mellitus, dyslipidemia, metabolic syndrome, renal failure and others. Atherosclerotic plaques are created by the migration of blood cells into the subendothelial spaces and their transformation into foam cells. The plaque, which is thus more prone to rupture, has a large lipid core, a thin superficial fibrous layer, and is infiltrated by inflammatory cells, mainly macrophages and neutrophils.

The plaque actively ruptures in the settings of the production of proteolytic enzymes and it is mediated by the immune system, in particular macrophages. The so called passive rupture of the plaque is caused by physical forces that act on the thinnest layer of the fibrotic cap of the plaque, most often at the junction of the plaque with the adjacent healthy wall of an artery.

When an unstable atherosclerotic plaque ruptures, subendothelial structures containing tissue factor and collagen come into contact with blood and the thrombus, which is rich in fibrin, neutrophils and platelets, begins to form. Further activation of the coagulation cascade leads to a progression of thrombus size with possible distal embolization of its fragments.

The growth of the thrombus at the site of damage to the coronary artery endothelium can lead to rapid changes in the severity of coronary artery stenosis with subsequent subtotal or complete vascular occlusion. A thrombus can cause complete occlusion of the epicardial part of the coronary artery in the case of an acute myocardial infarction with ST segment elevations.

Partial or intermittent coronary artery occlusion leads to acute myocardial infarction without ST segment elevations. Complete necrosis of the myocyte occurs in approximately two to four hours, depending on factors such as the collateral circulation, the type of the occlusion of the artery, the sensitivity of myocytes to ischemia, comorbidities and individual requirements of the myocyte for oxygen and nutrients.[6,7]

3. The Importance of Histological Analysis of Thrombi

Myocardial infarction is the leading cause of death in high- or middle-income countries and the second most relevant cause of disability worldwide[8], while stroke is the second cause of death worldwide and the second leading cause of disability in Western countries.[9] For decades, percutaneous coronary intervention has been the gold standard for the treatment and prevention of acute myocardial infarction, and although its combined use with thrombus extraction is a matter of discussion among experts, it has surely opened new doors in terms of research.

Extracted thrombi are stained, analyzed, and employed to support new advancements in the diagnosis, treatment, prevention, and prognosis of myocardial infarctions. Furthermore, thrombus extraction devices were introduced for the treatment of acute ischemic stroke many years later, and from 2004 onward most Western countries began to include this technique in the standard care of acute cerebral ischemia.[10] Stent retrievers not only represented an improvement in the clinical outcome of stroke therapy, but also inspired research in finding new answers to questions that most certainly would have been left unanswered.

The treatment techniques of mechanical thrombectomy in acute ischemic stroke are aimed to reperfuse cerebral tissue and minimize neurological damage.[11] Similarly to histological and immunohistochemical analyses on thrombi retrieved from coronary arteries, stroke research has supported clarifications in terms of diagnosis, prevention, prognosis, and treatment throughout the past decade, by utilizing specimens retrieved from the cerebral circulation. Many studies focused on stroke and myocardial infarctions have concluded that analyzing thrombus composition can provide insights into their etiology and predict successful reperfusion following intravenous thrombolysis and mechanical thrombectomy. Moreover, such studies may certainly assist in the development of new technologies aimed at retrieving specific subtypes of thrombi. In fact, thrombus composition is suggested to influence the choice of techniques used during mechanical thrombectomy and plays a role in potential device and clot interaction.[12] Here, we review the importance of histological and immunohistochemical analyses of thrombi extracted during mechanical thrombectomy in the diagnosis, prevention, and treatment of acute ischemic stroke.

3.1. Etiology of Stroke According to Composition

The etiology of ischemic stroke can also be studied through the composition of retrieved thrombi. A better understanding of thrombus origin is a powerful tool for prevention, treatment, and prognosis of ischemic stroke. Stroke subtypes are classified into 5 categories based on etiology, using the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) classification: (1) large artery atherosclerosis (LAA), (2) small vessel occlusion (SVO), (3) cardioembolism (CE), (4) stroke of other determined etiology, and (5) stroke of undetermined etiology.[13] A newer system called ASCO (A for atherosclerosis, S for small vessel disease, C for cardiac pathology, and O for other causes) to categorize ischemic stroke patients was introduced in 2009. This system distinguishes patients in more detail the differences between diseases underlying a cerebral ischemic event in a stroke patient.

The Oxford classification only considers size and location of cerebral infarction, whereas TOAST and CCS classifications involve the disease directly related to ischemic stroke, neglecting underlying causes.

ASCOD, proposed in 2013, introduces a fifth element – "D" for dissection – and similarly to the ASCO classification, three degrees of causality between the index ischemic stroke and each category are mentioned. Certainly, the biggest difference and one of the main advantages of the ASCOD system is the lack of definitions such as "undetermined," "cryptogenic," or "embolic stroke of unknown source." These categories are too difficult to define and may bring discomfort to patients when such definitions differ from one specialist to another. For example, in the event where no ASCOD 1 category is found, specific diseases (grades 2 or 3) can be mentioned, and these diseases can be clinically addressed according to guidelines to reduce recurrence even if a causal relationship between these diseases and ischemic stroke cannot be established yet (Table 1).

TABLE 1. *TOAST and ASCOD classification systems*

	TOAST	**ASCOD**
SUBTYPES	Large artery atherosclerosis	Atherosclerosis
	Cardioembolism	Cardioembolism
	Small vessel disease	Small vessel disease
	Other determined	Dissection
	Undetermined	Other
CHARACTERISTICS	Classification based on stroke mechanisms	Phenotypic classification system
	Most widely used worldwide	Each phenotype is graded 1, 2, 3, 0 or 9
ADVANTAGES	Accurate prediction of prognosis	Takes into consideration non-causative factors
	Convenient	
	Simpler	
DISADVANTAGES	Large pool of strokes of undetermined origin	More intricate for interpretation
	Less reliable in specific subtypes	Large number of subtypes

Grade 1 defines diseases that can potentially be a cause. Grades 2 and 3 are those that have an uncertain and unlikely causal link, respectively. Grade 0 is assigned when no disease is found, whereas grade 9 occurs if there is no sufficient data to grade a disease.[13]

Atherosclerosis of large vessels (aortic arch, internal carotid artery, vertebral artery, and stems of the main arteries of Willis' circle) constitutes approximately 25% of all causes of acute cerebral ischemia. Small vessel disease accounts for about 25% of all causes of cerebral ischemia and lacunar infarcts. Despite appearing as small lesions (<15 mm on CT and <20 mm on MRI), small vessel disease is associated with severe neurological deficit and poor clinical outcome.

Cardioembolism is known to cause approximately 25% of all strokes, with atrial fibrillation being the trigger in 90% of cases. Thrombi in the left atrium and ventricle are additional high risk causes, whereas atrial septum abnormalities, including the patent foramen ovale, are considered low risk or "unclear" depending on individual cases. In the remaining 25% of causes, the trigger remains unclear despite extensive diagnostic tests, and thus, these are classified as cryptogenic. In younger patients, more than 70% of cases are cryptogenic.

However, there is increasingly higher evidence to having a vast cardiac origin also for the cryptogenic subtypes, and recently, the concept of embolic stroke of undetermined source (ESUS) was introduced as a cryptogenic stroke subgroup. For the majority of patients with ESUS, undetected paroxysmal atrial fibrillation is considered to be the cause. Common causes are present in approximately 70%–75% of all ischemic strokes.[14]

Thrombi can be sampled, stored in formalin, embedded in paraffin, and subsequently cut into slices ranging between 3 and 10 µm. Staining methods can involve basic histological procedures with hematoxylin and eosin, and red blood cells (RBCs), platelets, and white blood cells (WBCs) can be identified and quantified by means of focal microscopy. Identification of fibrin is possible using Mallory's phosphotungstic acid-hematoxylin stains, whereas glycophorin A is an example to isolate RBCs; CD31 immunostains can help in quantifying platelets. Percent-ages of RBCs, platelets, and fibrin should be quantitatively determined in consensus. Subsequently, thrombi can be classified as red if RBCs outnumber platelets and fibrin by at least 15% and as white if platelets outnumber RBCs and fibrin also by at least 15%; in any other case, the thrombus is classified as mixed.[15] Pathologically speaking, thrombi can be classified into 3 groups according to published definitions of thrombus age.

Literature on thrombus age already exists for thrombi retrieved from coronary arteries in myocardial infarcts patients, and most subsequent literature on thrombus age in ischemic stroke patients owes to cardiac research. A thrombus is considered fresh when formed within 24 hours and appears to be composed of layered patterns of platelets, fibrin, erythrocytes, and intact granulocytes. Lytic thrombi generally occur between 1 and 5 days and exhibit areas of necrosis and karyorrhexis of granulocytes. Eventually, an organized thrombus (>5 days) is characterized by areas of smooth muscle cell ingrowth, with or without depositions of connective tissue and capillary vessel ingrowth. Between 65% and 75% of retrieved thrombi are fresh, whereas lytic thrombi account for approximately 15% and 20%, leaving organized thrombi a total of 5% to 15%.

There are no fundamental differences in age of thrombi between stroke subtypes. However, cryptogenic thrombi tend to present more similar composition features as cardioembolic thrombi.[16]

Most thrombus components show significant differences in percentages between cardioembolic and non-cardioembolic stroke causes. Cardioembolic thrombi consist of higher mean proportions of fibrin, less RBCs, and more WBCs.

The mean proportion of fibrin in cryptogenic stroke patients is almost identical to that in cardioembolic stroke patients but much higher than those with non-cardioembolic stroke. Similarly, RBCs are about as high in cryptogenic stroke as in cardioembolic stroke but definitely lower than in non-cardioembolic stroke. WBC content does not differ substantially in thrombi of cryptogenic, non-cardioembolic, and cardioembolic stroke patients.[6]

Over all, in terms of thrombus composition, there are no significant differences between anterior and posterior circulation strokes and between patients with or without thrombolysis. Thrombus size is directly proportional to the size of the occluded vessel (internal carotid artery>M1/BA>M2/A2), showing no relevant differences between TOAST categories. LAA and stroke of other determined etiology thrombi consist of larger amounts of RBCs with sparse fibrin-platelet complexes and fewer WBCs rather than cardioembolic and cryptogenic stroke thrombi with erythrocytes located and entrapped in the center and large amounts of fibrin/platelets.

Thus, cryptogenic thrombi demonstrate very similar patterns to cardioembolic thrombi, which is clearly different from non-cardioembolic thrombi, supporting the fact that most strokes of undetermined etiology may indeed present a cardiogenic nature.[17] In the TOAST classification, uncommon stroke causes are classified as "other determined causes" and as "other causes" in the ASCOD classification. Uncommon stroke causes represent less than 5% of all ischemic strokes and are more frequent in younger individuals. Uncommon causes are categorized according to pathological conditions, such as infection, inflammation, genetic malformations, coagulopathies, vasospasm, and other miscellaneous vasculopathies. Histopathological analysis of thrombi retrieved from such patients can most certainly be beneficial for the diagnosis of the primary diseases, which require dedicated and at times rather different treatments than standard protocols to prevent reoccurrence in ischemic stroke.[18]

3.2. Prevention and Treatment of Stroke According to Composition

The analysis of components found in extracted thrombi has proved to be an effective tool in studying the efficacy of modern treatments, including mechanical thrombectomy. Histological staining (Hematoxylin and Eosin, Prussian blue, and Elastica van Gieson) and immunohistochemistry for CD3, CD20, and CD68/ KiM1P make it possible to determine the fibrin, erythrocyte, and WBC components and compare the results to intervention time, frequency of secondary embolisms, and additional clinical and interventional parameters. Moreover, extra useful data can be obtained by assessing pre-interventional CT attenuation of the thrombi in relation to the unaffected side and their association with histological features.

Most fibrin-rich thrombi with low erythrocyte content are associated with longer intervention times, and thrombi with low rate of RBCs and low CT density frequently cause complications such as embolism in the thrombectomy procedure, suggesting that these thrombi have higher fragility.[19] Besides the duration of the interventional procedure in treating cerebral ischemia patients, it is possible to estimate through thrombus composition how many passes are necessary for the reperfusion of the cerebral vasculature. Certainly, such results may also depend on different factors, i.e., the instrument used or the experience of the medical specialist. Thrombus fragments retrieved from patients with acute ischemic stroke obtained in each pass can be collected as individual samples and maintained throughout the histological analysis as independent samples.

All samples can be stained with hematoxylin and eosin and Martius scarlet blue to quantify the composition of RBCs, fibrin, and WBCs in thrombus fragments in each pass. It has been shown that the number of passes required generally to complete a mechanical thrombectomy ranges from 1 to 6 passes. The analysis of thrombus fragments retrieved in each pass provides a great insight into the thrombectomy procedure progression. Generally, RBC content of thrombus fragments retrieved in passes 1 and 2 is significantly higher than that retrieved in passes 3 to 6. The removal of thrombus material in 1, 2, or 3 passes is normally associated with the highest rate of final TICI 2b–3. Fragments retrieved in passes 1 and 2 are also associated with a much lower fibrin composition compared with fragments retrieved in passes 3 to 6.

These notions may be a useful consideration in determining the treatment strategy as a case evolves and may be useful for the development of new devices to increase rates of 1-pass recanalization.[20] Treatment is focused on fast and efficient removal of the occluding thrombus, either via intravenous thrombolysis or via endovascular thrombectomy. However, recanalization is not always successful and factors contributing to failure are not completely understood. Bright field and fluorescence microscopy is used to histologically analyze thrombi retrieved from stroke patients for fibrin, RBCs, von Willebrand factor (vWF), platelets, leukocytes, and DNA. This would show how thrombi are composed of two main types of zones: RBC-rich and platelet-rich areas. RBC-rich areas are characterized by limited complexity because they consist of RBC trapped in a meshwork of fibrin, whereas platelet-rich areas consist of dense structures of fibrin, vWF, and a great amount of leukocytes and DNA.

These findings are important to better understand why platelet-rich thrombi tend to be more resistant to thrombolysis and more complicated to retrieve through thrombectomy. It is clear since recently that there is a consistent presence of leukocytes in stroke thrombi, and other than their presence, not much is known about their specific cellular or molecular distribution. Notably, when staining for RBC-rich and platelet-rich areas, leukocytes are primarily found at the interface between RBC-rich and platelet-rich areas.

Leukocytes are also consistently present within platelet-rich zones and do not seem to be found generally in RBC-rich areas, which are homogenously spread throughout the erythrocytes.

Leukocytes have also been shown to induce thrombus formation by means of extracellular DNA traps. When Feulgen stain is performed on thrombi, large extracellular DNA networks, appearing as extracellular smears, can be seen throughout the majority of retrieved specimens. Relevant amounts of extracellular DNA are observed particularly in platelet-rich areas and boundary zones between platelet-rich and RBC-rich regions, whereas no DNA is found within the RBC-rich regions.

Currently, in many Western countries, recombinant tissue plasminogen activator (rt-PA) is the only approved medication for thrombolysis in patients with cerebral ischemia, but it is effective in less than half of the patients. For this reason, it is practical to assume the reality of the so-called “rt-PA resistance,” even if the mechanisms of actions are not completely understood. RBC-rich thrombi generally respond better to rt-PA rather than platelet-rich thrombi. It is safe to state that by inducing fibrin degradation, rt-PA can have a direct and efficient thrombolytic effect on RBC-dominant areas, with thin fibrin as the main extracellular skeleton. Platelet-dominant thrombi contain denser fibrin scaffolds mixed with high amounts of other components such as vWF and extracellular DNA.

For this reason, it is possible to assume that fibrin, vWF, and DNA form the structural basis of platelet-rich thrombi and that vWF and DNA could characterize the so called rt-PA resistance. Extracellular DNA and histones have indeed been shown to modify the structure of fibrin, making it more resistant to degradation.

Leukocytes, more specifically neutrophils, may support thrombosis through the formation of neutrophil extracellular traps.

Furthermore, ex vivo studies have demonstrated how rt-PA in combination with DNAse-1 can be more effective. vWF is associated with thrombus formation by interacting with fibrin in platelet-rich regions. In vitro studies demonstrate how fibrin and vWF interact with each other through factor XIII or by means of thrombin-dependent incorporation, enhancing thrombus formation. Histological results on vWF and fibrin clarify that fibrin degradation with only plasmin is not enough to obtain thrombolysis of platelet-dominant thrombi. By adding the vWF-cleaving enzyme ADAMTS13, it may be possible to target vWF present in thrombi, thus improving the effectiveness of thrombolysis in rt-PA-resistant thrombi. This is very similar to the use of DNAse-1 in combination with rt-PA to tackle extracellular DNA. Recent studies indicate that erythrocyte-rich thrombi are more easily extracted through endovascular procedures compared with more complex fibrin-/platelet-rich thrombi. [21-22]

Current retriever devices and available techniques are more efficient with fresher thrombi; however, mechanisms that render platelet-rich thrombi more resistant to interventional procedures are not completely understood. Platelet-dominant thrombi from ischemic stroke patients include dense fibrin/vWF structures, leukocytes, and DNA. These aforementioned thick fibrin fibers trigger higher thrombus rigidity and affect the coefficient of friction and level of physical compression.[23] In addition, extracellular DNA is believed to strengthen fibrin structure, making it more resistant to mechanical forces. As a matter of fact, recently a direct proportion between the amount of neutrophil extracellular DNA traps and the number of device passes needed to achieve successful recanalization has been found.[24,25]

3.3. Limitations of Histopathological Analysis of Thrombi

After a careful reading of the current literature on the composition of thrombi retrieved from the cerebral vasculature, a few limitations were found that require significant attention. Ischemic strokes treated with mechanical thrombectomy represent only a percentage of total cases, and in all studies the only available thrombi were obtained from those patients in whom the clot did not dissolve spontaneously or during thrombolysis treatment and in whom the thrombus could be successfully retrieved.

Most studies present a relatively small patient population size; thus, some comparisons are not possible with small sample sizes. Furthermore, the variation in procedural techniques and the combination of devices applied to retrieve the thrombus fragments is a confounding factor that may potentially affect the rates of composition and histological distribution of the components.

Another important limitation in many studies is that the use of intravenous rt-PA might have already altered the specimens and manipulation with catheters might have produced some thrombi or fragmented them. The retrieved thrombus fragment does not always reflect the whole occlusive thrombus; thus, a certain bias toward more stable clot components is common.

Moreover, given the broad variation of clot composition in the evaluated sections of individual specimens, quantitative component assignments may not always be perfectly representative of the entire clot volume. Another important limitation, other than the limited number of case studies, is the predominance of specific types of ischemic stroke. As a matter of fact, most retrieved specimens are extracted from the middle cerebral arteries, rendering the results of a study a mere generalization for all the less represented subtypes.

Certain studies involve the use of only hematoxylin and eosin stains for the quantitative analysis, which surely permits a direct comparison of the different components without the risk of methodological differences. However, important components such as platelets and fibrin may not be quantified successfully, and quantification can be subjected to bigger human and statistical error due to difficulties in reaching a consensus. Moreover, the use of immunological techniques may instead improve precision, meanwhile involving greater costs and requiring better equipped laboratories (Table 2).

Over the years, histological analysis aimed to study the composition of thrombi causing ischemic stroke has proved to be a powerful tool to set standard prevention and treatment protocols. By isolating the clot components, it is possible to provide a more accurate diagnosis and distinguish different stroke subtypes.

Studies on histological clot composition support the theory that cryptogenic stroke can have a cardiogenic origin too. Components found in thrombi extracted from stroke patients confirm the importance of antithrombotic therapy in preventing and treating cerebral ischemia, but more studies are needed to improve results for all types and subtypes of stroke.

Thus, it is clear that more research and investigations are definitely required to further comprehend the role platelets, fibrin, vWF, and DNA play in relation to mechanical thrombectomy and recombinant rt-PA resistance and to overcome the limitations faced by existing literature.

TABLE 2. *Limitations of studies and possible solutions*

LIMITATION	MAIN BIAS	POTENTIAL MITIGATIONS
• **Small patient population size** • **Predominance of specific types of ischemic strokes (MCA)**	Sampling bias Faulty generalization	• Increase population size • Cooperation with other centres (stroke units) • Extend time of collection
Mechanical-thrombectomy-treated-strokes represent only a percentage of the total	Exclusion bias	Include patients treated only with intravenous thrombolysis when possible
• **Variation in procedural techniques for retrieval** • **Combination of devices applied for retrieval**	Confounding bias	Apply categories according to the device used
• **Already altered specimens by intravenous rt-PA** • **Manipulation with catheters**	Confounding bias	• Distinguish and compare specimens in which patients were subjected to intravenous thrombolysis

		• Count and minimize where possible per-pass retrieval of specimens
Retrieved thrombus material not always reflects the whole occlusive thrombus	Survivorship bias Faulty generalization	• Minimize need for more stable thrombi for the purpose of the study • Enlarge patient population size • Use of immunological detection procedures
Exclusive use of haematoxylin and eosin stains	Observer bias	• Enlarge pool of expert raters • Strengthen inter-rater reliability score
Immunological procedures	Funding bias	• Cooperation with more centres and laboratories • Grant application

4. Thrombolysis

Currently, two main formulations are used for systemic reperfusion therapy of acute ischemic stroke: alteplase (fibrinolytic of the 2nd generation) and tenecteplase (3rd generation).

Tenecteplase is a genetically modified rt-PA. It is a 3rd generation fibrinolytic with a higher affinity for fibrin. Its longer half-life (17 ± 7 min) allows for bolus administration in the recommended dose of 0.25 mg/kg, maximum 25 mg.

Alteplase is the most widely used selective thrombolytic to date. It is a recombinant human tissue plasminogen activator (rt-PA) with a short half-life (4-6 min), therefore it is necessary to administer it as a continuous infusion with an initial bolus in a total dose of 0.9 mg/kg, maximum 90 mg.

According to current recommendations[26,27], administration of tenecteplase should be considered as a first line thrombolytic in patients with acute ischemic stroke due to occlusion of the large cerebral arteries, who are indicated for mechanical thrombectomy and for whom intravenous thrombolysis is indicated before mechanical thrombectomy within a time window of up to 4.5 hours since the onset of symptoms. This guideline is based on the results of the Extend-IA TNK study. In other cases, the treatment of first choice is the administration of alteplase.[28]

4.1. Intravenous Thrombolysis

Intravenous thrombolysis is the only evidence-based systemic reperfusion therapy for acute ischemic stroke. Based on the results of the 2008 European Cooperative Acute Stroke Study III (ECASS 3), the time window for performing intravenous thrombolysis was extended from 3 hours to 4.5 hours from the onset of symptoms. For more than 10 years, this strict therapeutic window was considered an insurmountable limit. The exception was acute occlusion of the basilar artery, in which case intravenous thrombolysis was accepted after 4.5 hours from the onset of symptoms as a lifesaving treatment. In 2018 and 2019, multicenter randomized placebo-controlled clinical trials were published that provided evidence of the clinical efficacy and safety of intravenous thrombolysis in the treatment of ischemic stroke in selected patients in a time window longer than 4.5 hours or in patients with an unknown onset time.[29,30]

The advantage of intravenous thrombolysis is its wide availability, fast administration and low price. Disadvantages are a narrow therapeutic window (generally up to 4.5 hours), low efficiency for larger thrombi, risk of intracerebral or systemic bleeding, and numerous contraindications, although gradually, many of them have become relative. Data on the success rate of recanalization vary in the literature. However, the larger the thrombus, the lower the effectiveness. Intravenous thrombolysis alone achieves complete recanalization in only about a quarter of ischemic strokes.[31]

4.2. The indication Criteria for Intravenous Thrombolysis

I. Age. In general, the lower limit for intravenous thrombolysis administration is 18 years. In children and adolescents, there is only limited clinical experience with the use of alteplase in the treatment of acute stroke. According to the consensus of expert opinions, intravenous thrombolysis - alteplase - can be administered in the treatment of acute ischemic stroke even in adolescents aged 16–17 years, while the same entry criteria and contraindications as for adult patients must be observed. It is recommended to proceed individually and always consider the benefits and risks for a specific patient. Intravenous thrombolysis in children under the age of 16 is an "off-label" procedure.

II. Modified Rankin scale (mRS) and functional neurological deficit at admission (NIHSS). A patient with acute ischemic stroke indicated for intravenous thrombolysis should be able to walk independently before the event and may require the assistance of a second person only in some activities, corresponding to modified Rankin scale 0-3. In cases of strokes with a longer time window than 4.5 hours, the entry criterium is a modified Rankin scale with a maximum value of 2, that is the patient is fully selfsufficient. The lower limit score of NIHSS in patients indicated for intravenous thrombolysis is equal or above 2. In the case of pauci-symptomatic occlusions of a large cerebral artery, the procedure is individualized.

III. Time window (from onset of symptoms). Based on the results of studies published in 2018 and 2019, the therapeutic window for administering intravenous thrombolysis has been extended when certain criteria are met. In clinical practice there are mainly 4 situations.

a. Symptoms lasting less than 4.5 hours.

When this time window is met, intravenous thrombolysis is given if the native brain CT is negative or demonstrates early ischemic changes of small to moderate extent, for example less than one third of the Middle Cerebral Artery, or the Alberta Stroke Program Early CT Score (ASPECTS) value is ≥ 7. If MRI was performed at baseline, the extent of demonstrated ischemia on diffusion weighted MRI must not be greater than one third of the Middle Cerebral Artery.

b. Wake-up stroke within 4.5 hours of waking up.

If the symptoms were already present upon waking up and at the same time no more than 4.5 hours have passed since waking up, the requirements for the result of a native CT brain are the same as in the case of strokes with symptoms lasting up to 4 .5 hours. When performing an MRI, a negative FLAIR, that is fluid-attenuated inversion recovery with suppression of the cerebrospinal fluid signal, on T2-weighted sequences is another indication criterium.

c. Duration of symptoms between 4.5hours and 9 hours at a known time of onset, or wake-up stroke after 4.5-9 hours after waking up.

If the symptoms were present when waking up and last between 4.5 hours and 9 hours after waking up, in this case, intravenous thrombolysis can only be performed based on evidence of ischemic penumbra on multimodal imaging examinations (CT perfusion, diffusion weighed MRI). If these examinations cannot be performed, intravenous thrombolysis is allowed only in patients with symptomatic occlusion of the basilar artery verified by CT or MR angiography after exclusion of extensive complete ischemia of the brainstem as a lifesaving.

d. Unknown time of onset.

In patients where the time of onset is unclear or this data cannot be obtained, because of aphasia, the same indication criteria apply as in the previous situation. However, if multimodal neuroimaging, such as diffusion weighed MRI, perfusion weighed MRI or CT perfusion, is not available, intravenous thrombolysis may be considered based on a negative native CT scan or early small to moderate ischemic changes (< 1/3 Middle Cerebral Artery or ASPECTS $\geq$ 7)), without the presence of clear hypodensity.[32–35]

4.3. Contraindications of Intravenous Thrombolysis

4.3.1. Absolute Contraindications to Intravenous Thrombolysis

An absolute contraindication to intravenous thrombolysis is active bleeding, such as intracranial bleeding confirmed on CT/MRI of the brain or other internal bleeding. Intravenous thrombolysis is also contraindicated in extensive early ischemic changes (≥ 1/3 of the Middle Cerebral Artery or ASPECTS < 7) on native CT brain, or in the presence of evidence of extensive ischemia on diffusion weighed MRI (in the extent of ≥ 1/3 of the Middle Cerebral Artery) with a positive finding in FLAIR (fluid-attenuated inversion recovery sequences). Intravenous thrombolysis must also not be administered in uncontrolled hypertension unresponsive to adequate treatment (systolic blood pressure above 185 mmHg or diastolic blood pressure above 110 mmHg).

A large group of contraindications to intravenous thrombolysis consists of anamnestic data and comorbidities of serious diseases such as craniocerebral and spinal injuries in the last 3 months, intracranial bleeding in the last 6 months, severe of bleeding in the last 21 days, including bleeding in the chest, gastrointestinal or genitourinary tract, known hemorrhagic diathesis, known arteriovenous malformation and known symptomatic aneurysms, known aneurysm of the thoracic aorta, abdominal aorta or peripheral arteries, intracranial or intraspinal surgery in the last 4 weeks, arterial puncture at a non-compressible site in the last 7 days, delivery in the last 10 days or third trimester with imminent delivery, known infective endocarditis, aortic arch artery dissection, intracranial tumor or metastatic central nervous system tumors.

According to laboratory findings, intravenous thrombolysis is contraindicated for INR > 1.7 (this also applies to patients taking Warfarin), thrombocytopenia with a value < 100,000/µl and aPTT above the upper limit of norm.

In patients receiving direct oral anticoagulants, intravenous thrombolysis can be initiated after administration of an available antidote after monitoring of coagulation parameters (aPTT, TT).

4.3.2. Relative Contraindications of Intravenous Thrombolysis

According to available scientific studies, intravenous thrombolysis is effective and safe even in selected patients with acute stroke with one or more relative contraindications. However, individual risks and clinical benefit must be carefully assessed.

a. Myocardial infarction in the last 3 months. In a patient with a recent myocardial infarction, intravenous thrombolysis can be performed for non-ST elevation myocardial infarction. In the event of ST-elevation myocardial infarction, intravenous thrombolysis may be considered taking into account time interval from myocardial infarction (>7 days), extent of necrosis, current echocardiographic findings, and whether percutaneous coronary intervention has been performed. In a patient with acute myocardial infarction, intravenous thrombolysis may be considered with subsequent coronary angioplasty.

b. Disability with modified Rankin scale ≥ 4 before the acute event.

c. In the event of epileptic seizure at the beginning of the development of symptoms with persistent focal neurological deficit, intravenous thrombolysis should be performed if the focal neurological deficit is related to acute cerebral ischemia, especially when symptomatic occlusion of the intracranial artery is demonstrated or brain ischemia is found on diffusion weighed MRI or CT perfusion, and there is no suspicion of severe head injury.

d. Glycemia should not be < 48 mg/dL. Conversely, in case of hyperglycemia (>400 mg/dL), insulin should be administered at the same time during intravenous thrombolysis.

e. Lumbar puncture in the last 10 days.

f. According to published cases on pregnant women, intravenous thrombolysis was not associated with a higher risk of intracranial bleeding or death compared to women who were not pregnant and had no contraindications to intravenous thrombolysis treatment. According to published data, the risk of abortion related to intravenous thrombolysis is <10%. Taking into account the rate of serious bleeding complications and the fact that rt-PA does not cross the placenta, pregnancy is not an absolute contraindication to intravenous thrombolysis. Pregnant women capable of consenting to intravenous thrombolysis have the right to refuse treatment based on the information that uterine bleeding and fetal death have been rarely reported in published cases.

g. Intracranial tumor (including meningioma, neurinoma).

h. A neurological evaluation of NIHSS > 25 points and a time window of up to 4.5 hours, thus a stroke with severe neurological deficit, is a significant predictor of worse clinical outcome and is associated with a higher risk of intracranial bleeding. Auxiliary criteria for deciding on the indication of intravenous thrombolysis are previous functional status (modified Rankin scale ≤ 2), age (≤ 80 years) and the absence of serious comorbidity (among others, advanced dementia or oncological disease with an expected survival time of < 6 months).

i. In patients with a history of hemorrhagic retinopathy or other hemorrhagic eye comorbidity, intravenous thrombolysis may be performed if the clinical benefit of intravenous thrombolysis treatment clearly outweighs the risk of progression of vision impairment with hemorrhagic eye complications.[29,36,37]

4.4. Management of Intravenous Thrombolysis

If a patient with acute ischemic stroke meets the appropriate entry criteria after the initial examinations and shows no contraindication, intravenous thrombolysis must be administered as soon as possible after arrival at the hospital, considering that the median target value of the so called “door-to-needle time" is 20 minutes.

A patient treated with thrombolysis should be hospitalized in the intensive care unit. Regular monitoring of the patient's clinical conditions and continuous monitoring of physiological and vital functions, including the state of consciousness, are required.

A control CT or MRI of the brain is recommended, within a day after thrombolysis. The administration of antithrombotic or anticoagulant therapy should only be started according to the result of the imaging examination, which excludes bleeding.

According to current recommendations, intravenous thrombolysis should also be administered to patients indicated for mechanical thrombectomy, if the patient meets the indication criteria and obviously has no contraindications to thrombolysis administration. At the same time, the administration of thrombolysis should not delay the initiation of mechanical thrombectomy in patients with confirmed symptomatic occlusion of a large intracranial artery.[35,38]

4.5. Complications of intravenous thrombolysis

The following are reported as the most common complications of intravenous thrombolysis:

a. Minor bleeding at the injection site, from the gums, the gastrointestinal tract or the urogenital tract.

b. Symptomatic intracranial hemorrhage. Determining the incidence of this serious complication is difficult, as there are a variety of definitions used in studies. In general, however, intracranial hemorrhage is defined as hemorrhage demonstrated on CT or MRI of the brain associated with clinical deterioration of the general condition (differential of NIHSS points of more than 4) or death. The incidence of intracranial hemorrhage in connection with the administration of intravenous thrombolysis varies between 3-8% of cases. The incidence of any intracranial hemorrhage is up to 30%.

c. Systemic extracranial bleeding. The incidence of systemic bleeding in association with the administration of intravenous thrombolysis is around 1.5% according to the available literature.

d. Orolingual angioedema. The incidence of this complication is 1-5%. It is more common in patients taking ACE-inhibitors and in patients with frontal lobe lesions.

e. Less common complications are nausea, vomiting, chills, fever, urticaria, mild headache, convulsions, confusion. Treatment is symptomatic.[36]

4.6. Intra-arterial Thrombolysis (IAT)

The use of intra-arterial thrombolysis in the treatment of acute ischemic stroke has been almost entirely abandoned. In clinical practice, intra-arterial thrombolysis can be used as a complementary method to mechanical thrombectomy, for instance to treat distal embolization or when the thrombus is unreachable for mechanical thrombectomy instruments. Alteplase is recommended as the first line thrombolytic.[39]

5. Antiplatelet Therapy during Acute Ischemic Stroke

It is recommended to administer acetylsalicylic acid (initial dose 160 - 325 mg) within 48 hours after an acute ischemic stroke (class I, level A). Intravenous thrombolysis should not be initiated if planned to administer, or already administered, acetylsalicylic acid or other antiplatelet therapies during the first 24 hours. The use of other antiplatelet agents (alone or in combination) is not recommended for acute ischemic stroke (class III, level C). Administration of glycoprotein IIb-IIIa inhibitors is not recommended (Class I, Level A).[40,41]

5.1. Antiplatelet Therapy in Primary Prevention of Acute Ischemic Stroke

It is recommended that a low-dose acetylsalicylic acid be considered in men for primary prevention of myocardial infarction. However, acetylsalicylic acid does not reduce the risk of ischemic stroke (Class I, Level A). Currently, according to good clinical practice, antiplatelet agents other than acetylsalicylic acid are not recommended in the primary prevention of stroke. Acetylsalicylic acid may be recommended in patients with non-valvular atrial fibrillation younger than 65 years, in which vascular risk factors are not present (class I, level A). In patients with non-valvular atrial fibrillation aged 65-75 years who do not have vascular risk factors, acetylsalicylic acid or oral anticoagulation (INR 2.0 – 3.0) is recommended (class I, level A).

In female patients aged 45 and above, not at increased risk of intracerebral hemorrhage and with good gastrointestinal tolerance, a low dose of acetylsalicylic acid is recommended. However, its effectiveness is low (class I, level A).

It is recommended that patients with atrial fibrillation who cannot take anticoagulants be given acetylsalicylic acid (Class I, Level A). In patients with asymptomatic stenosis of the internal carotid artery > 50%, low dose of acetylsalicylic acid is recommended to reduce the risk of cerebrovascular events (class II, level B).[30,40]

5.2. Antiplatelet Therapy in Secondary Prevention of Acute Ischemic Stroke

After an ischemic cerebral event it is recommended that patients receive antithrombotic therapy (Class I, Level A). It is recommended that antiplatelet agents be given to patients not requiring anticoagulation therapy (Class I, Level A). A combination of acetylsalicylic acid and dipyridamole or clopidogrel alone should be administered. Alternatively, acetylsalicylic acid or “triflusal” alone can be used (Class I, Level A).

The combination of acetylsalicylic acid and clopidogrel is not recommended in patients with a recent ischemic stroke, with the exception of patients with specific indications (e.g. unstable angina pectoris, NSTEMI or recent stenting). Treatment should be administered for up to 9 months after the acute event (class I, level A).

In patients with the development of a stroke while taking antiplatelet therapy, it is recommended to reassess the pathophysiology of the ischemia and the influence of risk factors. Treatment of patients with recurrent strokes on antiplatelet therapy remains unclear. The therapeutic plan may include maintaining the treatment unchanged, switching to another antiplatelet agent, adding another antiplatelet agent or using oral anticoagulation.[42]

6. Anticoagulation Therapy

Warfarin is recommended for the prevention and treatment of venous, arterial and intracardiac thrombosis and to reduce the risk of ischemic stroke in patients with atrial fibrillation or atrial flutter or with valvular heart disease.

DOAC or NOAC include the factor Xa inhibitors apixaban, rivaroxaban or the direct thrombin inhibitor dabigatran. They are indicated for secondary prevention of ischemic stroke in patients with chronic or paroxysmal atrial fibrillation.

Anticoagulants after acute ischemic stroke do not demonstrate any long- or short-term benefit. Anticoagulation therapy reduced recurrent ischemic stroke, deep vein thrombosis and pulmonary embolism, however hemorrhagic risk increased. Presently, data do not support the routine use of any of the available anticoagulants for acute ischemic stroke.[43]

In acute ischemic stroke with non-cardioembolic origin, for instance in large-artery stenosis, low molecular weight heparin can reduce the incidence of early neurological deterioration and recurrent ischemic stroke and improve the likelihood of independence (modified Rankin scale 0–1) at 6 months compared with those with aspirin treatment. Heparin use, although was related to an increased risk of extracranial hemorrhage among all patients, the difference in major extracranial hemorrhage and spontaneous intracerebral hemorrhage was not significant. The choice of the appropriate cases and the start time and duration of administered treatment are very important in the use of anticoagulation therapy.[44]

7. Mechanical Thrombectomy

Until 2014, intravenous thrombolysis was the only reperfusion therapy available. The approach to the treatment of ischemic stroke caused by occlusion of large cerebral arteries changed completely in 2015 with the publication of five important randomized multicenter clinical trials (MR CLEAN, SWIFT PRIME, EXTEND-IA, ESCAPE, REVASCAT).[39,45–48] Based on the results of these studies, mechanical thrombectomy is considered the most effective treatment for ischemic stroke caused by occlusion of large cerebral arteries.[30,49–53]

One important event in the treatment of acute ischemic stroke was the publication of the DEFUSE-3 and DAWN studies in 2018, which extended the therapeutic window to 24 hours in selected patients.[52,53]

The DAWN study uses the so-called mismatch between clinical and imaging features, which in practice is a combination of the severity of the neurological deficit (NIHSS above or equal to 10) and the extent of ischemic changes according to the imaging examination (diffusion-weighted MRI or perfusion CT). After 90 days, 49% of patients achieved functional independence (modified Rankin scale 0-2) compared to 13% in the control group. Only 12% of all strokes had a known time of onset.

The DEFUSE-3 trial (Endovascular Therapy Following Imaging Evaluation for Ischemic Stroke-3) enrolled patients with acute stroke within 6 to 16 hour time window based on ischemic core and penumbra volume. After 90 days, 45% of patients achieved functional independence (modified Rankin scale 0-2) compared to 17% in the control group. The time of onset was known only in one third of the patients.

7.1. Advantages and Disadvantages of Mechanical Thrombectomy

The advantage of mechanical thrombectomy is generally a longer therapeutic window and a high recanalization potential compared to intravenous thrombolysis monotherapy. At the same time, mechanical thrombectomy is the only possible treatment method in patients in whom intravenous thrombolysis is absolutely contraindicated or where intravenous thrombolysis has failed.

The disadvantage is the high costs and requirements for equipment and personnel. The results of the intervention depend on the training and experience of the intervening doctor. A disadvantage compared to the administration of intravenous thrombolysis may be the later initiation of therapy with regard to patient preparation. Complications associated with endovascular procedures may occur (dissection, perforation, thrombus fragmentation with multiple distal embolization).

Even though mechanical thrombectomy in the treatment of acute ischemic strokes caused by occlusion of a large cerebral artery has a class IA recommendation according to current guidelines, the availability of this therapy is very limited. The unavailability of mechanical thrombectomy is caused either by a lack of trained medical personnel or by a lack of fundings to ensure continuous availability of this treatment.

According to the analysis of data from randomized trials and national registries, it is estimated that up to 16% of all patients hospitalized with stroke are indicated for MT.[54] However, an analysis of ischemic stroke treatment across European countries found that only 1.9% of stroke patients were treated with mechanical thrombectomy.[55]

7.2. Indications for Mechanical Thrombectomy

Mechanical thrombectomy is a key reperfusion therapy in patients with acute ischemic stroke due to occlusion of the large cerebral arteries. The current mechanical thrombectomy indication criteria are based on the guidelines issued by the European Stroke Organization (ESO) from 2019[49] and the American Heart Association/American Stroke Association guidelines from 2019.[30] The basic factor that affects the indication criteria for performing mechanical thrombectomy is the time interval from the onset of symptoms to the start of intervention.

8. Neutrophil Extracellular Traps

Intravenous thrombolytic therapy has proved to be effective only in a relatively small percentage of patients with stroke and recombinant tissue-type plasminogen activators (rt-PAs) may become an obsolete and ineffective treatment if results are not improved.[56]

While there is still considerable debate on the use of thrombolytics among clinicians, one clear explanation of their inefficacy in coronary ischemia and cerebral infarctions lies in the composition of the thrombi, which shows resistance to medical therapy due to the nature of their cellular and extracellular components.

According to histological studies, cardioembolic thrombi showed a higher amount of neutrophils, a higher proportion of fibrin/platelets, and fewer erythrocytes than non-cardioembolic thrombi.[18] Thrombosis has recently been linked to neutrophil activation and release of neutrophil extracellular traps (NETs) via a process called NETosis.[57]

Neutrophils promote thrombus formation by 2 different mechanisms, from one side by localizing procoagulant factors including tissue factor and factor XII and on the other side by the formation of NETs, which are created when activated neutrophils release decondensed DNA fragments into the extracellular space.[58] This process is called NETosis. NETs are networks of extracellular fibers, primarily composed of DNA from neutrophils.[59] NETs are composed of a processed type of chromatin, tethered to specific cytoplasmic proteins with granular features.[60] In naive neutrophils, granular and nuclear antigens are separated, and within the process of NETosis, some granular proteins, such as myeloperoxidase and NE (neutrophil elastase), gradually migrate to the nucleus.[61] In addition, histones are citrullinated during NETosis.[62,63] The position of nuclear granular proteins and the citrullination of histones offer unique characteristics for neutrophils undergoing NETosis that can be used for their identification in specimens.[61] NETs can be identified by the presence of extracellular DNA–histone complexes (H2B) and NE.

The age of thrombi was extensively studied in acute myocardial infarctions (MIs); however, less is known about the age of thrombi in acute ischemic strokes (ISs). In the literature,[58,63] the presence of NETs varies according to the age of the thrombus, with fewer NETS in the fresh thrombus during its formation, increasing progressively in the lytic phase and disappearing in the organized thrombus, relating the age of the thrombus with the presence and number of NETs. Therefore, a clearer understanding of the time of clots formation, also according to the number of NETs within the retrieved specimen, could set the basis for risk assessments and better therapeutic treatment in clinical scenarios.

In this original study, the doctoral candidate analyzed in depth the composition of thrombi retrieved after mechanical thrombectomy (MT) in patients treated at 2 stroke units in Prague. By means of pathological characterization of NETs, ensuring their proper detection and confirming their origin from leucocytes, he selected 2 antibodies targeting NE and H2B and representing different components in NET formation, both detectable during the later stages of NETosis. The number of NETs were quantified, and compared to thrombi retrieved during coronary artery thrombus aspiration in MI. The age and organization of thrombi were assessed and NETs were quantified in relation to the use of anticoagulants (heparin), antiplatelet, and thrombolytics.

METHODS

9. Study Population Selection, Therapy, Sample Collection, and Processing

Thrombi were retrieved over 6 months from patients undergoing MT or percutaneous coronary intervention (PCI) at the Cardiac Center of the University Hospital Královské Vinohrady in Prague and the Cerebrovascular Center of the Military University Hospital in Prague.

All specimens were derived from patients providing informed consent to the analysis of biological material retrieved during the required intervention, and the study had been formally approved by the ethics committee of the University Hospital Královské Vinohrady.

No exclusion criterion was adopted in the selection of patients for the purpose of comparing as many specimens as possible and to obtain a real-world sample of a 6-month time frame medical practice. Patients requiring MT for acute IS or PCI for MI received standard care, and no alternative protocol was used. Specimens were retrieved only if standard care allowed it. Only patients without contraindications to the use of thrombolysis were treated with rt-PA. Anticoagulant and antiaggregant therapies were administered only if necessary and to patients without contraindications.

All patients with acute MI received the acute phase 3 antithrombotic drugs: intravenous lysin salicylate, 500 mg; unfractionated heparin, 100 units/kg; and ticagrelor, 180 mg. All patients with stroke receiving thrombolytic treatment did not receive any other antithrombotic drug during the acute phase before thrombus removal. Patients with stroke, undergoing direct MT (without bridging thrombolysis), received a minimal dosage of unfractionated heparin (10–25 units/kg) during the procedure. Specimens retrieved after MT or PCI were immediately preserved in a 10% formalin solution at 4 °C and carried within 2 hours to the laboratory for paraffin embedding.

10. Histology and Estimation of Thrombus Age

Thrombi specimens were formalin-fixed, paraffin-embedded, and cut in 5 μm with an LEICA SM2010R microtome. Samples were fixed by using the tissue processor VIP 6 (SAKURA). Tissues were deparaffinated, rehydrated, and stained with hematoxylin and eosin to assess the morphology and the age of the sample. Two expert pathologists conducted a detailed blinded morphological semiquantitative analysis, by assessing the presence of leucocytes, Zahn lines, fibrosis, atheromatous material, and fibrin-platelet complexes.[64,65] The semiquantification of each component was expressed as a percentage of the single component on the total area of the thrombus.

Specimens were classified based on their age, according to the criteria reported by Rittersma et al,[6] which represent the criteria followed afterward in all the papers dealing with thrombus material retrieved by coronary and cerebral arteries. We, too, have endorsed their classification, which we are reporting as follows. Fresh thrombi (<1 day) are characterized by layered patterns of platelets, fibrin, erythrocytes, and intact granulocytes. Lytic thrombi (1–5 days) present an area of colliquative necrosis and Karyorrhexis of granulocytes. Organized thrombi (>5 days) show ingrowth of smooth muscle cells, with or without depositions of connective tissue and capillary vessel ingrowth. As suggested by these authors, samples that have a heterogeneous composition are scored according to the age of the older part.

11. Immunohistochemistry and NET Quantification

Two tissue sections for each sample were immunoassayed to identify and quantify NETs through anti-NE and anti-H2B antibodies. They were selected because they represent different components in NET development, ensuring proper detection of NETs and confirming their origin from leucocytes. NE is a serine protease that, during NETosis, translocates into the nucleus and cleaves histones, such as H2B, and facilitates the homogenization of euchromatin and heterochromatin.[66]

Sections were cut on a LEICA SM2010R slide microtome and were deparaffinized and rehydrated, and 1 section for each sample was incubated in anti-NE (Millipore 481001: Rabbit pAb, 1 mL). The immunohistochemical staining was performed with the automated Ventana BenchMark ULTRA from ROCHE, and all reagents used, except the primary antibody, are from ROCHE. The primary antibody was diluted by 1:50 in an antibody diluent from DAKO (Dako-Agilent S0809 ready-to-use). Antigen retrieval was carried out at 37 °C with a citrate buffer at pH 6. The secondary antibody is from the ROCHE OptiView DAB Detection Kit (protocol U Optiview DAB IHC, v6). The second section was stained with an Anti-Histone H2B antibody (ab134211: Abcam, 100 µg). The primary antibody was diluted by 1:500 in an antibody diluent from DAKO (Dako-Agilent S0809 ready-to-use). The secondary antibody (Goat-Chicken IgY ab97135) was diluted by 1:2000 (Dako-Agilent S0809 ready-to-use). Heat-mediated (60 °C) antigen retrieval was performed with a citrate buffer solution at pH 6. Stained slides were scanned with Aperio ScanScope CS and analyzed with QuPath software,[67] v0.3.0, to quantify NE and H2B content, expressed as the percentage of the total area that stains positive for the NE or H2B.

12. Statistical Analysis

Descriptive statistics were reported as median (I quartile; III quartile) for continuous variables and as absolute numbers as corresponding percentages for categorical variables. Wilcoxon and $\chi 2$ tests were performed to compare the distribution of continuous and categorical variables, respectively.

P values underwent Benjamini-Hochberg correction to account for the multiplicity of testing. The association between the type of therapy administered and the NET content was evaluated using gamma regression because NETs were found to be not normally distributed according to the Shapiro-Wilk test.

The marginal effect was computed considering the partial derivatives of the marginal expectation. Results were reported as an average marginal effect, 95% CI, and P value. Mediation analysis[68] was performed to disentangle the effect of thrombosis site and age on NET content. CIs were computed using a nonparametric bootstrap with 5000 draws. Analyses were performed using the R software within the packages mediation,[69] regression modeling strategies, and margins.

13. Mediation analysis

Mediation analysis is a statistical approach used to investigate the process by which one variable exerts an effect on another through an intermediary variable, often referred to as a mediator. The fundamental goal of mediation analysis is to determine whether the relationship between an independent variable (X) and a dependent variable (Y) is either fully or partially explained by a third variable (M). In this context, the independent variable influences the mediator, which in turn influences the dependent variable.

The concept of mediation analysis was formalized to enhance our understanding of causal mechanisms, allowing researchers to quantify both direct effects - the influence of the independent variable on the dependent variable without the mediator - and indirect effects where the mediator accounts for part of the relationship. This technique is valuable in medical research because biological pathways are often complex and influenced by multiple factors. By employing mediation analysis, we can gain deeper insights into the roles specific biological components play in disease mechanisms and therapeutic responses.[70]

RESULTS

14. Population Risk Factors and Pharmacological Therapy in Cerebral Versus Coronary Thrombi

The study included 72 thrombotic/embolic specimens: 28 retrieved from coronary and 44 from cerebral arteries. We evaluated the clinical data in both groups based on the thrombosis site (cerebral versus coronary).

This analysis showed differences in cardiovascular risk factors prevalence (body mass index, patients' age, smoking, hypertension, and previously reported coronary disease) but no major differences in terms of chronic therapy before acute events (Table 3).

Conversely, the therapy administered during thrombectomy differs, according to guidelines, with a higher prevalence of heparin and antiaggregant in coronary thrombosis and a definite more frequent administration of thrombolysis in patients with cerebral stroke. Vascular risk factors are different between coronary and cerebral thrombi. This reflects the differences in the cause and pathophysiology of MI and IS. While myocardial infarction is typically caused by atherothrombosis (i.e., in situ thrombus on an unstable plaque), large vessel occlusion ischemic stroke is typically caused by cardioembolism (e.g., in atrial fibrillation) and, thus, unrelated to atherosclerosis. This difference may explain why most cerebral thrombi are older and less fresh than coronary thrombi.

TABLE 3. Patients' clinical characteristics of coronary vs cerebral thrombi. Data are shown as median [Q1;Q3] or n (%).

	Coronary thrombi (n=28)	**Cerebral thrombi (n=44)**	**p-value**
Age	63.0 [55.5;73.5]	74.0 [63.7;81.0]	0.033
Sex			
Male	11 (39%)	24 (55%)	0.343
Risk factors			
BMI	30.7 [27.3;33.0]	26.2 [24.2;34.4]	0.022
Smoker	19 (68%)	10 (23%)	0.003
Alcohol	9 (32%)	18 (41%)	0.646
Diabetes	7 (25%)	8 (18%)	0.624
Hypertension	27 (96%)	33 (75%)	0.053
Hyperlipidemia	25 (89%)	31 (70%)	0.133
Coexisting conditions			
Renal failure	5 (18%)	5 (11%)	0.589
History of atrial fibrillation	9 (32%)	19 (32%)	0.58
Atrial fibrillation *de novo*	5 (18%)	8 (18%)	1
Prior Stroke	5 (18%)	16 (36%)	0.196
Previously known coronary artery disease	13 (46%)	7 (16%)	0.027

Therapy			
Antiaggregant prior to the acute event	12 (43%)	11 (25%)	0.196
Anticoagulant prior to the acute event	9 (32%)	10 (23%)	0.566
Antiaggregant during intervention	26 (93%)	13 (30%)	0.003
Heparin during intervention	24 (86%)	9 (20%)	0.003
Thrombolysis during intervention	1 (4%)	30 (68%)	0.003

15. Histological Classification, Age Assessment, and NET Quantification

Specimens were histologically evaluated to classify their age, according to Rittersma et al,[6] and 54% of them were fresh (84% of the coronary and 34% of the cerebral thrombi), while 46% were lytic (14% of the coronary and 66% of the cerebral thrombi; P=0.005).

There were only 4 organized thrombi that were discarded from the analysis. In fact, we wanted to guarantee the robustness and reliability of the results, as their limited sample size could potentially introduce undue bias in our findings. Focusing on the typical morphological and cellular component of the samples, the analysis showed a higher presence of leucocytes (P<0.001), fibrin platelets (P=0.032), and Zahn lines (P=0.027) in cerebral thrombi compared with coronary specimens. Moreover, the cerebral samples revealed an increase in H2B positivity (P=0.031), lower erythrocyte aggregates (P=0.032), and the absence of atheromatous material (P=0.01; Table 4; Figure 1).

TABLE 4. Histomorphological characteristics of coronary vs cerebral thrombi. Data are shown as median [Q1;Q3] or n (%).

	Coronary thrombi (n=28)	**Cerebral thrombi (n=44)**	**p-value**
Morphological characteristics			
Leucocytes (%)	5.0 [4.5;10.0]	10.0 [10.0;20,0]	<0.001
Zahn lines (%)	0 [0;5]	5 [0;10]	0.027
Fibrosis (%)	0 (0%)	0 (0%)	
Atheromatous material (%)	0 [0;8.7]	0 [0;0]	0.01
Erythrocytes (%)	70.0 [45.0;80.0]	51.5 [30.0;70.0]	0.032
Fibrin-platelets (%)	12.0 [10.0;20.0]	20.0 [10.0;41.25]	0.032
Age of thrombus			
Fresh (<1 day)	24 (86%)	15 (34%)	
Lytic (1-5 days)	4 (14%)	29 (66%)	0.005
Immunohistochemistry for NETs			
H2B (%)	26.5 [17.0; 37.5]	38.9 [21.8;56.9]	0.031
NE (%)	8.5 [5.4;12.3]	7.8 [3.5;15.2]	0.873

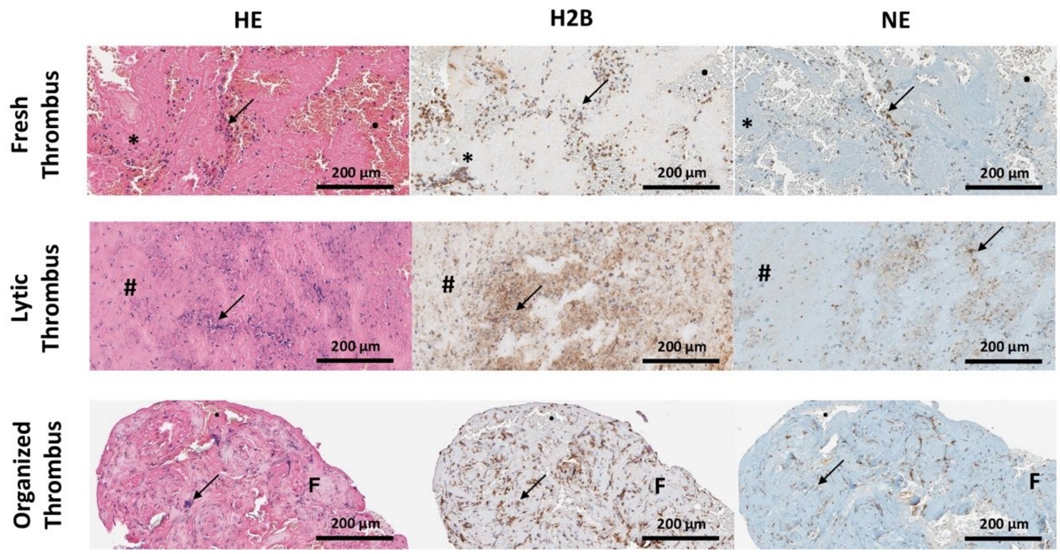
FIGURE 1. Three differently aged thrombi.

A, Fresh thrombus (aged <1 d). B, Lytic thrombus (aged 1–5 d). C, Organized thrombus (aged >5 d). Each specimen was stained with hematoxylin and eosin (HE) to identify each component and immunoassayed with anti-H2B and anti-NE (neutrophil elastase) antibodies for detecting neutrophil extracellular traps (NETs).

Note in (A) Layered patterns of platelets, fibrin, intact erythrocytes, and granulocytes, and a low NET presence.

Note in (B) colliquative necrosis and Karyorrhexis of granulocytes with a higher number of NETs.

Note in (C) an organized thrombus (>5 d) showing ingrowth of fibroblasts and capillary vessels. Asterisks indicate Zahn lines, arrows indicate leucocytes, black dots indicate erythrocytes, hashtags indicate fibrin-platelet complexes, and F indicates fibrosis. Scale bar: 200 µm.



16. Association of Therapy with NET Content

The main aim of this study was to assess the association of the pharmacological therapy administered before and during procedural interventions with NET content. The univariable analysis of the impact of the drugs administered during the interventional procedures on the number of NETs is shown in Table 5.

The results showed a statistically significant association between thrombolysis and a higher level of NE positivity (average marginal effect, 6.461 [95% CI, 0.7901–12.13]; *P*=0.02555).

In addition, the administration of an antiaggregant is associated with a lower level of H2B (*P*=0.0311) and NE positivity (*P*=0.035), whereas the use of heparin is associated with a lower level of H2B (*P*=0.03574). Because the pharmacological approach is significantly different in cerebral and coronary thrombi, we adjusted the univariate analyses for the thrombosis site (cerebral versus coronary thrombi) to account for potential confounding factors. All the associations were no more significant after the adjustment for the thrombosis site, except for thrombolysis and NETs (average marginal effect, 7.896 [95% CI, 0.2095–15.58]; *P*=0.04407).

TABLE 5. Univariable analysis of the association between NE and therapy, and univariable analysis of the association between H2B and therapy.

	AME	95% CI	p-value
NE and Thrombolysis	6.461	[0.7901;12.13]	0.02555
NE and Antiaggregant	-5.825	[-11.26;-0.3932]	0.03557
NE and Heparin	-1.702	[-7.273;3.868]	0.5492
H2B and Thrombolysis	9.225	[-0.5386;18.99]	0.06405
H2B and Antiaggregant	-10.56	[-20.17-0.9564]	0.03115
H2B and Heparin	-9.762	[-18.87;-0.65]	0.03574

17. Association among Age, Thrombolysis, and NET Content

Thrombolysis, as a variable, resulted to be significantly associated with a higher content of NETs even after adjustment for the thrombosis site; thus, we deeply investigated the clinical and morphological characteristics of our population, comparing the thrombolysis-treated group (n=31) with the patients not treated with thrombolysis (n=41; table S1; figure 2).

This analysis of clinical and morphological characteristics did not show significant differences, except for the age of the thrombus, which is the only variable acting on the NET content (Tables S1 and S2). Thus, there was a higher prevalence of lytic thrombi (>1 day) in the thrombolytic group (*P*=0.03). These results suggested that the ages of the thrombus and rt-PA were both associated with the NET content.

For this reason, we performed a further evaluation of the association between thrombolysis and NE positivity adjusting for the age of the thrombus.

The age of the thrombus proved to be the only independent predictor of the NE, while thrombolysis lost its significance on the NET contents. Furthermore, we tested whether thrombolysis had a mediation effect on the association between the age of the thrombus and the content of NETs (Figure 3). The mediation analysis revealed that the age of the thrombus had a direct effect on the NET content (*P*=0.014), without any mediation of the thrombolysis.

FIGURE 2. Morphology of a thrombus treated and a thrombus not treated with thrombolytics.

Each specimen is stained with hematoxylin and eosin (HE) and immunoassayed with anti-H2B and anti-NE (neutrophil elastase) antibodies for detecting neutrophil extracellular traps (NETs).

Asterisks indicate Zahn lines, arrows indicate leucocytes, black dots indicate erythrocytes, and hashtags indicate fibrin-platelet complexes. The comparison among these 2 groups does not reveal a significantly different morphology. Scale bar: 200 μm.

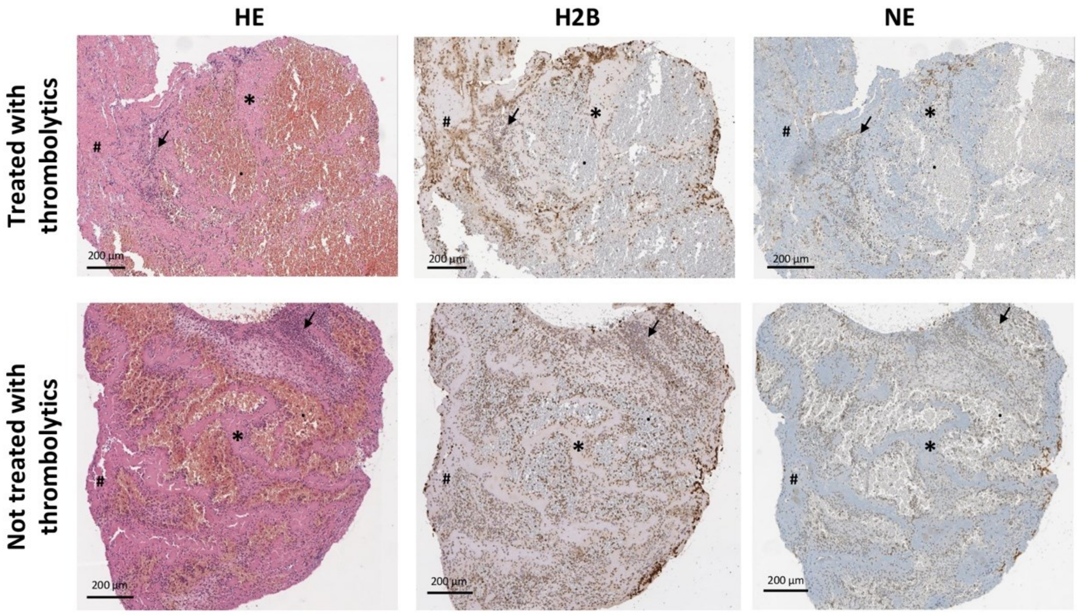


TABLE S1. Patients' clinical characteristic of not treated with thrombolysis group vs thrombolysis treated group. Data are shown as median [Q1;Q3] or n (%).

	Not treated with thrombolysis (n=41)	**Treated with thrombolysis (n=31)**	**p-value**
Age	64.0 [57.0;75.0]	73.0 [63.5;81.5]	0.207
Sex			
Male	20 (49%)	15 (48%)	1
Risk factors			
BMI	29.3 [25.5;32.9]	26.3 [24.2;34.4]	0.215
Smoker	22 (54%)	7 (23%)	0.092
Alcohol	13 (32%)	14 (45%)	0.653
Diabetes	10 (24%)	5 (16%)	0.907
Hypertension	37 (97%)	23 (74%)	0.316
Hyperlipidemia	35 (85%)	21 (68%)	0.267
Coexisting conditions			
Renal failure	35 (85%)	27 (87%)	1
History of atrial fibrillation	15 (37%)	13 (42%)	1
Atrial fibrillation *de novo*	6 (15%)	7 (23%)	0.907
Stroke	9 (22%)	12 (39%)	0.498
Coronary artery disease	17 (41%)	3 (10%)	0.029

Therapy			
Antiaggregant prior to the acute event	14 (34%)	9 (29%)	1
Anticoagulant prior to the acute event	14 (34%)	5 (16%)	0.316
Antiaggregant during PCI	37 (90%)	2 (6%)	0.007
Heparin during PCI	29 (71%)	4 (13%)	0.007

TABLE S2. Histomorphological characteristics of not treated with thrombolysis group vs thrombolysis treated group. Data are shown as median [Q1;Q3] or n (%).

	Not treated with thrombolysis (n=41)	**Thrombolysis treated (n=31)**	**p-value**
Morphological characteristics			
Leucocytes (%)	10 [5;15]	10 [7;15]	0.266
Zahn lines (%)	5 [0;5]	5 [0;0]	0.217
Fibrosis (%)	0 (0%)	0 (0%)	
Atheromatous material (%)	0 [0;0]	0 [0;0]	0.394
Erythrocytes (%)	65 [40;78]	60 [30;70]	0.394
Fibrin-platelets (%)	15.0 [10.0;25.0]	20.0 [11.5;42.5]	0.217
Age of thrombus			
Fresh (<1 day)	28 (68%)	11 (35%)	
Lytic (<1 day; >5days)	13 (32%)	20 (65%)	0.03
Immunohistochemistry for NETs			
H2B (%)	26.1 [17.5;41.4]	38.2 [25.2;54.3]	0.06405
NE (%)	7.94 [4.3;11.1]	8.16 [3.4;24.9]	0.02555

DISCUSSION

18. Histopathological Aspects

The main results of this study demonstrated that the composition of cerebral and coronary thrombi differs, and there were significantly more lytic cerebral thrombi than coronary thrombi. Moreover, there were more leukocytes, fibrin/platelets, and Zahn lines and fewer erythrocytes in cerebral thrombi compared with coronary thrombi. No atheromatous material was found in cerebral thrombi, confirming the main embolic origin of cerebral thrombi.

Interestingly, there was a considerably higher expression of NETs in the cerebral thrombi as testified by the higher expression of H2B. Thrombolysis was associated with higher NE positivity, regardless of the origin of the thrombi (ie, cerebral versus coronary thrombi). There was no notable association between the administration of antiaggregant/heparin and H2B/NE amount when adjusted for the thrombus location. Importantly, the age of the thrombus was the only independent predictor of NET content without any mediation of the thrombolytic treatment.

Our samples were fixed, and the NET components were marked by immunohistochemistry staining with anti-H2B to highlight the histone elements and anti-NE to determine the neutrophil origin of these extracellular structures. Our study includes samples retrieved from different thrombotic sites, allowing us to evaluate the possible effects of the thrombotic site itself on morphology and NET content, as suggested in the literature.[71]

19. Translational Aspects

First, we directed our attention to the evaluation of the age of retrieved thrombi, and we observed that, in our population, the cerebral specimens presented the largest level of thrombus maturation and a higher presence of leucocytes, in agreement with previous studies on IS thrombi with less NETs in fresh thrombi and a progressive increase during the lytic phase. Indeed, Laridan et al[58] quantified neutrophils in stroke thrombi, demonstrating that their amount is considerably higher when compared with coronary or stent thrombi.[72] We assessed the differential effect of the various therapies on the composition of the specimens. The difference in the pharmacological treatment between the 2 groups led us to perform an adjusted analysis to remove confounding factors and strengthen the results.

Many studies demonstrated that NETs have a relevant role in thrombosis, with a procoagulant effect,[73,74] and their presence in retrieved thrombotic/embolic material is associated with impaired clinical outcome.[71] Thrombolysis sensitivity is associated with fibrin-clot architecture in the thrombus. Fresh thrombi are typically associated with improved outcomes such as higher Thrombolysis in Cerebral Infarction scores, shorter procedural time, and increased sensitivity to thrombolysis.

Conversely, fibrin-rich thrombi, because of their higher stiffness, show increased resistance to MT and thrombolysis. Recent studies[75–77] claim that the outer layer of thrombi retrieved from patients with stroke is composed mainly of RBC, fibrin, von Willebrand Factor, and leukocytes that all play a role against rt-PA. An improvement in the rate of rt-PA effectivity is directly proportional to this external shell being compromised. Thus, targeting components other than fibrin can cause breaches into the outer layer of thrombi, which can enhance the efficacy of thrombolytics.

20. Clinical Aspects

An important consideration for clinical practice is that for every 5.4 patients treated with MT, 1 less patient will be dependent at 90 days, whereas for thrombolysis, the number needed to treat is 18, that is, 18 patients with stroke have to be administered thrombolysis to obtain a clinical benefit for 1 patient. In brief, MT is 3.3 times more effective than thrombolysis.[78]

The risk of complications related to stroke thrombectomy is higher in small distal arteries (e.g., M2 segment), and the procedural success rate is smaller in these distal segments; thus, the difference against thrombolysis is minimal. While for large vessel occlusion stroke (typically M1 segment of medial cerebral artery), the number needed to treat by thrombectomy to achieve functional independence is around 5,[79] for small vessels (e.g., M2), it is not known; for primary PCI in STEMI to prevent death, the number needed to treat is 29.[80] Although unfractionated heparin, along with other intravenous anticoagulants, holds a key role in the treatment of acute coronary syndromes, it is contraindicated, for the most part, in acute IS.

There are several reasons for contraindication of full therapeutic anticoagulants in stroke: hemorrhagic stroke (15% of all strokes, frequently difficult to differentiate based only on clinical signs), risk of hemorrhagic transformation of IS (especially large IS, i.e., those suitable for thrombectomy are at risk), uncontrolled hypertension, preexisting anticoagulant therapy, etc.[81] Reperfusion damage is closely related to sudden vigorous blood flow restoration to an ischemically damaged tissue; thus, it could be considered as reperfusion hemorrhage. This is rarely clinically relevant in acute MI, where the connective tissue network is robust enough; thus, in the myocardium, large reperfusion benefits almost always overcome small reperfusion damage.

The situation in the brain is different, mainly due to far less robust connective tissue; thus, reperfusion damage is a serious concern for the brain. This difference is reflected in the heparin use during the acute phase of myocardial infarction (dosage of 70–100 units/kg is standard) as opposed to acute ischemic stroke, where heparin is considered a class III indication, that is, it should not be used and certainly not in full therapeutic dose. When heparin is used in acute IS mainly to prevent catheter clots, the dose should be only around 20 to 30 units/kg. The difference in thrombolysis use in myocardial infarction versus ischemic stroke involves a complex explanation. In brief, in MI, thrombolysis does not add any benefit on top of mechanical reperfusion, and the so-called facilitated PCI was demonstrated to be inferior to simple direct PCI.

This may be related to procedural techniques (high-pressure balloon dilatation and permanent stent implantation). Trials comparing direct MT versus bridging thrombolysis before MT demonstrated similar outcomes or even better outcomes with bridging thrombolysis. The differences in the use of thrombolysis may explain (at least partially) the clot features in Table 4 and Table S1.

Many studies reported that heparin has an inhibitory effect on the interaction between NET histone components and platelets.[74,82,83] Accordingly, we detected a higher presence of NETs, marked with H2B, in the thrombolysis-treated group. Because these results suggested that the ages of the thrombus and rt-PA were both associated with a higher number of NETs, we tested the impact of thrombolysis on the association between the age of the thrombus and the NET specimen content. We demonstrated that the age of the thrombus has a direct effect on NET content, without any mediation of thrombolysis.

21. Study Limitations

The main limitations of the study are related to the small sample size and the absence of organized thrombi, older than 5 days. Unfortunately, the cases are not representative of older thrombi morphological features; however, our main interest was evaluating the morphology and the therapeutical effects in the acute and subacute phases.

Samples are equally distributed in the 2 classes of fresh (<1 day) and lytic (1–5 days) thrombi. For the first time, these mediation analysis results indicate a causal effect of the age of the thrombus on the NET bulk.

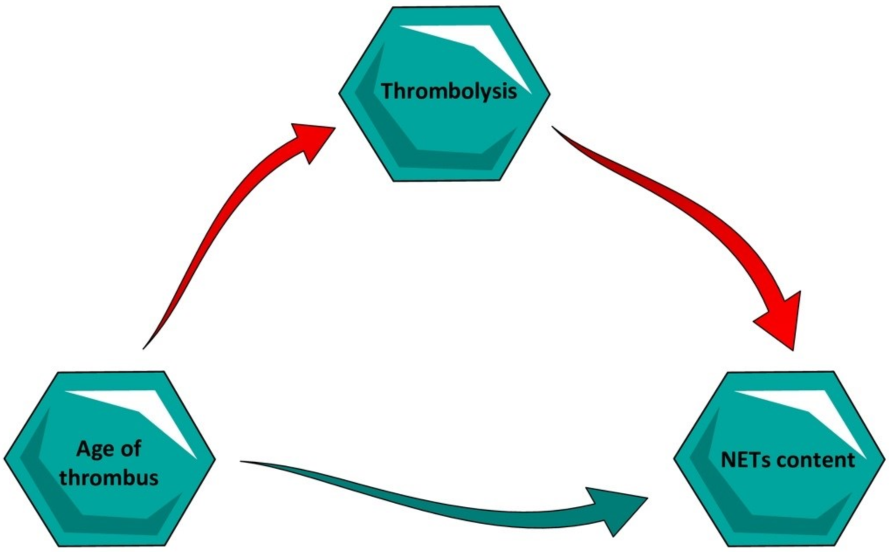
The small availability of organized thrombi is caused mainly by the fact that patients entering the study were only those who, in a specific time frame, experienced an IS or MI and required an MT or PCI.

Specimens were retrieved over a period of 6 months and represent the real-world sample of a specific time frame alongside probability factors.

The city of Prague offers a high density of stroke units along with tertiary and quaternary care facilities, thus, the time needed to treat a symptomatic patient is considerably reduced.[76]

Another possible limitation of such a study is the content of NETs retrieved after MT, thus, analyzed and the amount that could have been left within the vasculature of the patients; nevertheless, for patients with IS, each individual in this study scored between 2b and 3 on the Thrombolysis in Cerebral Infarction scale, when evaluated after MT, determining, then, an angiographic success in the whole group.[77,84]

FIGURE 3. Schematic representation of the mediation analysis hypothesis. Thrombolysis does not have any mediation effect on the association between the age of the thrombus and neutrophil extracellular trap (NET) content.



These results provide evidence of the requirement to improve the current pharmacological protocols during acute stroke.

CONCLUSION

22. Future Research, Recombinant Human DNase I

Although it is known that heparin has a partial inhibitory effect on NET maturation, the current therapy formulation is not sufficient to contrast their procoagulant effect and resistance to thrombolysis.

Adding new drugs with a specific action to disaggregate chromatin and enzymatic components of NETs can enhance the lytic effects of the therapy and improve patients' outcomes. Presently, DNase I (deoxyribonuclease I) has shown remarkable results in targeting NETS and extracellular DNA ex vivo lysis, thus demonstrating pro-thrombolytic potential when coupled with current thrombolytics.[58,85,86]

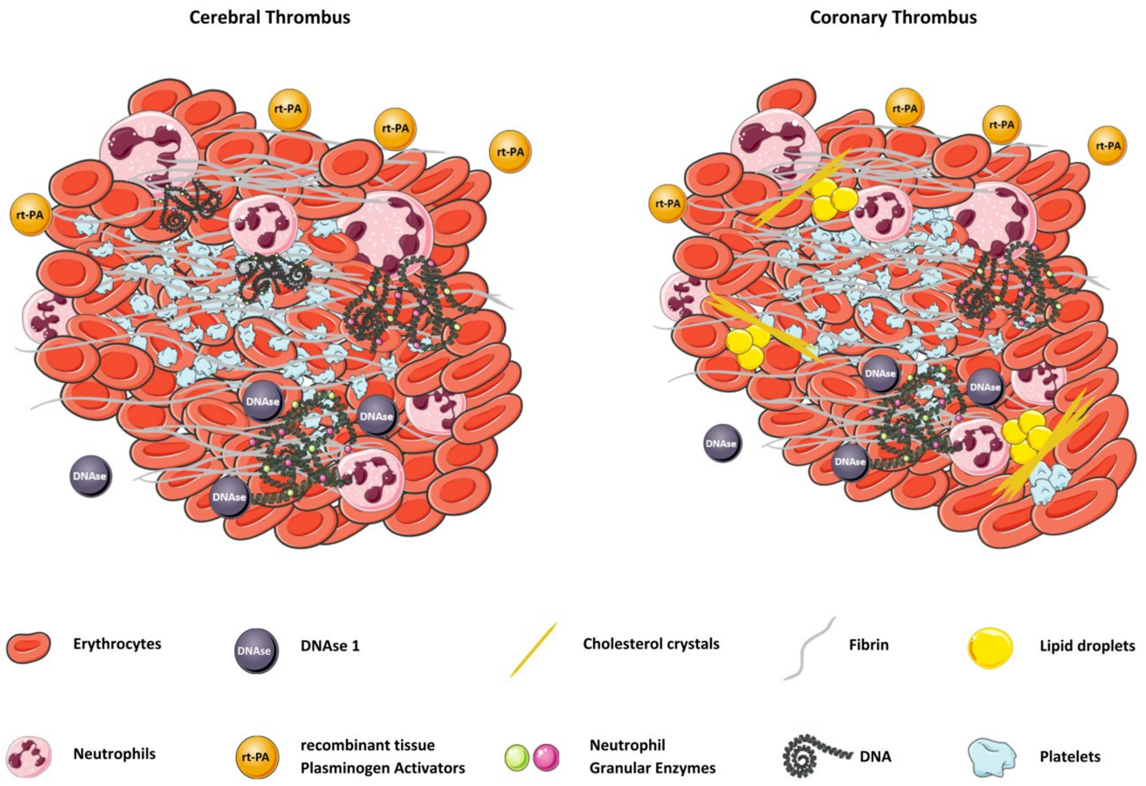
Recombinant human DNase I, under the name of dornase alfa, is currently labeled as a treatment in cystic fibrosis; dornase alfa hydrolyzes DNA present in the secretions of the airways in patients with cystic fibrosis, reducing viscosity, improving quality of life, and reducing mortality by infection.[85,87]

In conclusion, this study provides valuable insights into the complex relationship between thrombus composition, age, pharmacological therapy, and NET content and demonstrates that the age of the thrombus is the only independent predictor of NET content without any mediation of the thrombolytic treatment in thrombotic/embolic specimens retrieved during thrombectomy in patients with stroke.

This study supports the need for further investigations in also pharmacologically targeting NETs found in patients with acute cerebral ischemia, possibly, with recombinant human DNase I.[87]

23. Schematic Representation of the Conclusions of the Study

The age of the thrombus is the driving force for NET content, which correlates with impaired clinical outcomes. The therapy that is currently administered does not modify NET content. This study supports the need to investigate new pharmacological approaches added to thrombolysis to prevent NET formation or enhance their disruption, such as recombinant human DNase I (deoxyribonuclease I).



- Neutrophil Extracellular Traps cause thrombosis and constitute an important part of the architecture of thrombi.
- The presence of Neutrophil Extracellular Traps is related to thrombus age and triggers thrombolysis-resistance.
- The current therapy should be improved (e.g. adding DNAse1).
- Regardless of the location of the thrombus (cerebral vs coronary arteries), there are no significant differences in relation to response to therapy.
- Cerebral thrombi show a higher amount of Neutrophil Extracellular Traps compared to Coronary thrombi

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ATTACHMENTS

Attachment No. 1

Collection Protocol of the samples: thrombi retrieved from coronary arteries during PCI in acute myocardial infarction and thrombi retrieved during mechanical thrombectomy in acute ischemic stroke. Over the set time period of six months there were no intracardiac thrombi retrieved during cardiac surgery.

SBĚR VZORKŮ

K získání po:

- **mechanické trombektomii při akutní cerebrální ischémii**
- **aspiraci trombů při infarktu myokardu**
- **vyjmutí z levé síně při operaci srdce**

1. Sraženinu je nutno bezprostředně po vytažení ošetřit sterilními nástroji a rukavicemi.
2. Každá sraženina se rozdělí na dvě zdánlivě stejné části čistým skalpelem (čepel velikosti 10 nebo 11).
3. Každá polovina musí být **preservovaná ve formalinu** (obě dohromady v jedné nádobě): tyto poloviny se ponoří do roztoku formalinu a musí být uchovávány při teplotě 4 ° C. Etiketa musí obsahovat PŘESNÝ ČAS A DATUM PONOŘENÍ ve formalinu, název konzervačního materiálu (FORMALIN) a jeden z následujících kódů:

- CODE: 104368-T (pro akutní mrtvici)
- CODE: 104368-A (pro akutní infarkt myokardu)
- CODE: 104368-C (pro operaci srdce na oddělení kardiochirurgie)

Ve formalínu preservované sraženiny musí být bezpečně uchovávány v suchém prostředí, v ledničce při teplotě 4°C a následně nejdéle do 2 dnů – ne později - zaslány na oddělení patologie Fakultní nemocnice Královské Vinohrady pro “embedding” do parafínu. Takto finálně zpracované vzorky budou následně analyzovány.

Postup:

A. Umístíme čerstvě vyňaté sraženiny do formalínu udržovaném při teplotě 4°C, množství formalínu musí být 20 krát větší než velikost vyňaté sraženiny.
B. Sraženina musí být ponechán ve formalínu a uložena v ledničce při teplotě 4°C. Nejpozději do 48 hodiny musí být sraženina ve formalínu předána na oddělení patologie.
C. Příprava formalínu je následující: přimícháme 10 ml formaldehydu (37-40%) do 90 ml PBS (fosfátový pufr / phosphate-buffered saline) a uchováváme při teplotě 4°C.

4. Pokud nebylo z důvodu velikosti sraženiny nebo její křehkosti možné získat poloviny, sraženina bude zpracována vcelku dle ad. 3.

Attachment No. 2

Stroke

CLINICAL AND POPULATION SCIENCES

Neutrophil Extracellular Traps and Thrombolysis Resistance: New Insights for Targeting Therapies

Luca Mengozzi, MD; Ilaria Barison, BSc; Martin Malý, PhD; Giulia Lorenzoni, PhD; Marny Fedrigo, PhD; Chiara Castellani, PhD; Dario Gregori, PhD; Petr Malý, MD; Radoslav Matěj, PhD; Petr Toušek, PhD; Petr Widimský, DrSc; Annalisa Angelini, MD

BACKGROUND: Thrombosis is linked to neutrophil release of neutrophil extracellular traps (NETs). NETs are proposed as a mechanism of resistance to thrombolysis. This study intends to analyze the composition of thrombi retrieved after mechanical thrombectomy, estimate the age and organization of thrombi, and evaluate associations with the use of thrombolysis, antiplatelets, and heparin.

METHODS: This retrospective observational study involved 72 samples (44 from cerebral and 28 coronary arteries), which were stained with hematoxylin and eosin, anti-NE (neutrophil elastase) antibody, and anti-histone H2B (histone H2B) antibody, representing different components in NET formation, all detectable during the later stages of NETosis, for histochemical and digital quantification of NET content. The histological and morphological evaluations of the specimens were correlated, through univariate and mediation analyses, with clinical information and therapy administered before intervention.

RESULTS: The results demonstrated that the composition of cerebral and coronary thrombi differs, and there were significantly more lytic cerebral thrombi than coronary thrombi (66% versus 14%; P=0.005). There was a considerably higher expression of NETs in the cerebral thrombi as testified by the higher expression of H2B (P=0.031). Thrombolysis was remarkably associated with higher NE positivity (average marginal effect, 6.461 [95% CI, 0.7901–12.13]; P=0.02555), regardless of the origin of thrombi. There was no notable association between the administration of antiaggregant therapy/heparin and H2B/NE amount when adjusted for the thrombus location. Importantly, the age of the thrombus was the only independent predictor of NET content without any mediation of the thrombolytic treatment (P=0.014).

CONCLUSIONS: The age of the thrombus is the driving force for NET content, which correlates with impaired clinical outcomes. The therapy that is currently administered does not modify NET content. This study supports the need to investigate new pharmacological approaches added to thrombolysis to prevent NET formation or enhance their disruption, such as recombinant human DNase I (deoxyribonuclease I).

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: extracellular traps ■ immunohistochemistry ■ ischemic stroke ■ myocardial infarction ■ pathology

Strokes remain one of the leading causes of death and disability worldwide.[1] Intravenous thrombolytic therapy has proved to be effective only in a relatively small percentage of patients with stroke and recombinant tissue-type plasminogen activators (r-tPAs) may become an obsolete and ineffective treatment if results are not improved.[2] While there is still considerable debate on the use of thrombolytics among clinicians, one clear explanation of their inefficacy in coronary ischemia and cerebral infarctions lies in the composition of the thrombi, which shows resistance to medical therapy due to the nature of their cellular and extracellular components. According to histological studies,

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Stroke is available at www.ahajournals.org/journal/str

Nonstandard Abbreviations and Acronyms

DNase I	deoxyribonuclease I
IS	ischemic stroke
MI	myocardial infarction
MT	mechanical thrombectomy
NE	neutrophil elastase
NET	neutrophil extracellular trap
PCI	percutaneous coronary intervention
r-tPA	recombinant tissue-type plasminogen activator

cardioembolic thrombi showed a higher amount of neutrophils, a higher proportion of fibrin/platelets, and fewer erythrocytes than noncardioembolic thrombi.[3]

Thrombosis has recently been linked to neutrophil activation and release of neutrophil extracellular traps (NETs) via a process called NETosis.[4]

Neutrophils promote thrombus formation by 2 different mechanisms, from one side by localizing procoagulant factors including tissue factor and factor XII and on the other side by the formation of NETs, which are created when activated neutrophils release decondensed DNA fragments into the extracellular space.[5] This process is called NETosis. NETs are networks of extracellular fibers, primarily composed of DNA from neutrophils.[6] NETs are composed of a processed type of chromatin, tethered to specific cytoplasmic proteins with granular features.[7] In naive neutrophils, granular and nuclear antigens are separated, and within the process of NETosis, some granular proteins, such as myeloperoxidase and NE (neutrophil elastase), gradually migrate to the nucleus.[8] In addition, histones are citrullinated during NETosis.[9,10] The position of nuclear granular proteins and the citrullination of histones offer unique characteristics for neutrophils undergoing NETosis that can be used for their identification in specimens.[8] NETs can be identified by the presence of extracellular DNA–histone complexes (H2B) and NE.

The age of thrombi was extensively studied in acute myocardial infarctions (MIs); however, less is known about the age of thrombi in acute ischemic strokes (ISs). In the literature,[5,10] the presence of NETs varies according to the age of the thrombus, with fewer NETS in the fresh thrombus during its formation, increasing progressively in the lytic phase and disappearing in the organized thrombus, relating the age of the thrombus with the presence and number of NETs. Therefore, a clearer understanding of the time of clots formation, also according to the number of NETs within the retrieved specimen, could set the basis for risk assessments and better therapeutic treatment in clinical scenarios.

In this study, we analyzed in depth the composition of thrombi retrieved after mechanical thrombectomy (MT) in patients treated at 2 stroke units in Prague. By means of pathological characterization of NETs, ensuring their proper detection and confirming their origin from leucocytes, we selected 2 antibodies targeting NE and H2B and representing different components in NET formation, both detectable during the later stages of NETosis. We quantified the number of NETs, and we compared them to thrombi retrieved during coronary artery thrombus aspiration in MI. We also assessed the age and organization of thrombi and quantified NETs in relation to the use of anticoagulants (heparin), antiplatelet, and thrombolytics.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study Population Selection, Therapy, Sample Collection, and Processing

Thrombi were retrieved over 6 months from patients undergoing MT or percutaneous coronary intervention (PCI) at the Cardiac Center of the University Hospital Královské Vinohrady in Prague and the Cerebrovascular Center of the Military University Hospital in Prague. All specimens were derived from patients providing informed consent to the analysis of biological material retrieved during the required intervention, and the study had been formally approved by the ethics committee of the University Hospital Královské Vinohrady. No exclusion criterion was adopted in the selection of patients for the purpose of comparing as many specimens as possible and to obtain a real-world sample of a 6-month time frame medical practice. Patients requiring MT for acute IS or PCI for MI received standard care, and no alternative protocol was used. Specimens were retrieved only if standard care allowed it. Only patients without contraindications to the use of thrombolysis were treated with r-tPA. Anticoagulant and antiaggregant therapies were administered only if necessary and to patients without contraindications. All patients with acute MI received the acute phase 3 antithrombotic drugs: intravenous lysin salicylate, 500 mg; unfractionated heparin, 100 units/kg; and ticagrelor, 180 mg. All patients with stroke receiving thrombolytic treatment did not receive any other antithrombotic drug during the acute phase before thrombus removal. Patients with stroke, undergoing direct MT (without bridging thrombolysis), received a minimal dosage of unfractionated heparin (10–25 units/kg) during the procedure. Specimens retrieved after MT or PCI were immediately preserved in a 10% formalin solution at 4°C and carried within 2 hours to the laboratory for paraffin embedding.

Histology and Estimation of Thrombus Age

Thrombi specimens were formalin-fixed, paraffin-embedded, and cut in 5 μm with an LEICA SM2010R microtome. Samples were fixed by using the tissue processor VIP 6 (SAKURA). Tissues were deparaffinated, rehydrated, and stained with hematoxylin and eosin to assess the morphology and the age of the sample. Two expert pathologists conducted a detailed blinded morphological semiquantitative analysis, by assessing the presence of leucocytes, Zahn lines, fibrosis, atheromatous material, and fibrin-platelet complexes[11,12] The semiquantification of each component was expressed as a percentage of the single component on the total area of the thrombus.

Specimens were classified based on their age, according to the criteria reported by Rittersma et al,[13] which represent the criteria followed afterward in all the papers dealing with thrombus material retrieved by coronary and cerebral arteries. We, too, have endorsed their classification, which we are reporting as follows. Fresh thrombi (<1 day) are characterized by layered patterns of platelets, fibrin, erythrocytes, and intact granulocytes. Lytic thrombi (1–5 days) present an area of colliquative necrosis and Karyorrhexis of granulocytes. Organized thrombi (>5 days) show ingrowth of smooth muscle cells, with or without depositions of connective tissue and capillary vessel ingrowth. As suggested by these authors, samples that have a heterogeneous composition are scored according to the age of the older part.

Immunohistochemistry and NET Quantification

Two tissue sections for each sample were immunoassayed to identify and quantify NETs through anti-NE and anti-H2B antibodies. They were selected because they represent different components in NET development, ensuring proper detection of NETs and confirming their origin from leucocytes. NE is a serine protease that during NETosis translocates into the nucleus and cleaves histones, such as H2B, and facilitates the homogenization of euchromatin and heterochromatin.[14]

Sections were cut on a LEICA SM2010R slide microtome and were deparaffinized and rehydrated, and 1 section for each sample was incubated in anti-NE (Millipore 481001: Rabbit pAb, 1 mL). The immunohistochemical staining was performed with the automated Ventana BenchMark ULTRA from ROCHE, and all reagents used, except the primary antibody, are from ROCHE. The primary antibody was diluted by 1:50 in an antibody diluent from DAKO (Dako-Agilent S0809 ready-to-use). Antigen retrieval was carried out at 37 °C with a citrate buffer at pH 6. The secondary antibody is from the ROCHE OptiView DAB Detection Kit (protocol U Optiview DAB IHC, v6). The second section was stained with an Anti-Histone H2B antibody (ab134211: Abcam, 100 µg). The primary antibody was diluted by 1:500 in an antibody diluent from DAKO (Dako-Agilent S0809 ready-to-use). The secondary antibody (Goat-Chicken IgY ab97135) was diluted by 1:2000 (Dako-Agilent S0809 ready-to-use). Heat-mediated (60 °C) antigen retrieval was performed with a citrate buffer solution at pH 6. Stained slides were scanned with Aperio ScanScope CS and analyzed with QuPath software,[15] v0.3.0, to quantify NE and H2B content, expressed as the percentage of the total area that stains positive for the NE or H2B.

Statistical Analysis

Descriptive statistics were reported as median (I quartile; III quartile) for continuous variables and as absolute numbers as corresponding percentages for categorical variables. Wilcoxon and χ^2 tests were performed to compare the distribution of continuous and categorical variables, respectively. *P* values underwent Benjamini-Hochberg correction to account for the multiplicity of testing. The association between the type of therapy administered and the NET content was evaluated using gamma regression because NETs were found to be not normally distributed according to the Shapiro-Wilk test. The marginal effect was computed considering the partial derivatives of the marginal expectation. Results were reported as an average marginal effect, 95% CI, and *P* value. Mediation analysis[16] was performed to disentangle the effect of thrombosis site and age on NET content. CIs were computed using a nonparametric bootstrap with 5000 draws. Analyses were performed using the R software[17] within the packages mediation,[18] regression modeling strategies, and margins.

RESULTS

Population Risk Factors and Pharmacological Therapy in Cerebral Versus Coronary Thrombi

The study included 72 thrombotic/embolic specimens: 28 retrieved from coronary and 44 from cerebral arteries. We evaluated the clinical data in both groups based on the thrombosis site (cerebral versus coronary). This analysis showed differences in cardiovascular risk factors prevalence (body mass index, patients' age, smoking, hypertension, and previously reported coronary disease) but no major differences in terms of chronic therapy before acute events (Table 1). Conversely, the therapy administered during thrombectomy differs, according to guidelines, with a higher prevalence of heparin and antiaggregant in coronary thrombosis and a definite more frequent administration of thrombolysis in patients with cerebral stroke. Vascular risk factors are different between coronary and cerebral thrombi. This reflects the differences in the cause and pathophysiology of MI and IS. While MI is typically caused by atherothrombosis (ie, in situ thrombus on an unstable plaque), large vessel occlusion IS is typically caused by cardioembolism (eg, in atrial fibrillation) and, thus, unrelated to atherosclerosis. This difference may explain why most cerebral thrombi are older and less fresh than coronary thrombi.

Table 1. Patients' Clinical Characteristics of Coronary Versus Cerebral Thrombi

	Coronary thrombi (n=28)	Cerebral thrombi (n=44)	*P* value
Age	63.0 (55.5–73.5)	74.0 (63.7–81.0)	0.033
Sex			
Male	11 (39%)	24 (55%)	0.343
Risk factors			
Body mass index	30.7 (27.3–33.0)	26.2 (24.2–34.4)	0.022
Smoker	19 (68%)	10 (23%)	0.003
Alcohol	9 (32%)	18 (41%)	0.646
Diabetes	7 (25%)	8 (18%)	0.624
Hypertension	27 (96%)	33 (75%)	0.053
Hyperlipidemia	25 (89%)	31 (70%)	0.133
Coexisting conditions			
Renal failure	5 (18%)	5 (11%)	0.589
History of atrial fibrillation	9 (32%)	19 (32%)	0.58
Atrial fibrillation de novo	5 (18%)	8 (18%)	1
Prior stroke	5 (18%)	16 (36%)	0.196
Previously known coronary artery disease	13 (46%)	7 (16%)	0.027
Therapy			
Antiaggregant before the acute event	12 (43%)	11 (25%)	0.196
Anticoagulant before the acute event	9 (32%)	10 (23%)	0.566
Antiaggregant during intervention	26 (93%)	13 (30%)	0.003
Heparin during intervention	24 (86%)	9 (20%)	0.003
Thrombolysis during intervention	1 (4%)	30 (68%)	0.003

Data are shown as median (Q1, Q3) or n (%).

Histological Classification, Age Assessment, and NET Quantification

Specimens were histologically evaluated to classify their age, according to Rittersma et al,[13] and 54% of them were fresh (84% of the coronary and 34% of the cerebral thrombi), while 46% were lytic (14% of the coronary and 66% of the cerebral thrombi; *P*=0.005). There were only 4 organized thrombi that were discarded from the analysis. In fact, we wanted to guarantee the robustness and reliability of the results, as their limited sample size could potentially introduce undue bias in our findings. Focusing on the typical morphological and cellular component of the samples, the analysis showed a higher presence of leucocytes (*P*<0.001), fibrin platelets (*P*=0.032), and Zahn lines (*P*=0.027) in cerebral thrombi compared with coronary specimens. Moreover, the cerebral samples revealed an increase in H2B positivity (*P*=0.031), lower erythrocyte aggregates (*P*=0.032), and the absence of atheromatous material (*P*=0.01; Table 2; Figure 1).

Association of Therapy With NET Content

The main aim of this study was to assess the association of the pharmacological therapy administered before and during procedural interventions with NET content. The univariable analysis of the impact of the drugs administered during the interventional procedures on the number of NETs is shown in Table 3. The results showed a statistically significant association between thrombolysis and a higher level of NE positivity (average marginal effect, 6.461 [95% CI, 0.7901–12.13]; *P*=0.02555). In addition, the administration of an antiaggregant is associated with a lower level of H2B (*P*=0.0311) and NE positivity (*P*=0.035), whereas the use of heparin is associated with a lower level of H2B (*P*=0.03574). Because the pharmacological approach is significantly different in cerebral and coronary thrombi, we adjusted the univariate analyses for the thrombosis site (cerebral versus coronary thrombi) to account for potential confounding factors. All the associations were no more significant after the adjustment for the thrombosis site, except for thrombolysis and NETs (average marginal effect, 7.896 [95% CI, 0.2095–15.58]; *P*=0.04407).

Association Among Age, Thrombolysis, and NET Content

Thrombolysis, as a variable, resulted to be significantly associated with a higher content of NETs even after adjustment for the thrombosis site; thus, we deeply investigated the clinical and morphological characteristics of

Table 2. Histomorphological Characteristics of Coronary Versus Cerebral Thrombi

	Coronary thrombi (n=28)	Cerebral thrombi (n=44)	*P* value
Morphological characteristics			
Leucocytes, %	5.0 (4.5–10.0)	10.0 (10.0–20,0)	<0.001
Zahn lines, %	0 (0–5)	5 (0–10)	0.027
Fibrosis, %	0 (0%)	0 (0%)	
Atheromatous material, %	0 (0–8.7)	0 (0–0)	0.01
Erythrocytes, %	70.0 (45.0–80.0)	51.5 (30.0–70.0)	0.032
Fibrin platelets, %	12.0 (10.0–20.0)	20.0 (10.0–41.25)	0.032
Age of thrombus			
Fresh (<1 d)	24 (86%)	15 (34%)	
Lytic (1–5 d)	4 (14%)	29 (66%)	0.005
Immunohistochemistry for NETs			
H2B, %	26.5 (17.0–37.5)	38.9 (21.8–56.9)	0.031
NE, %	8.5 (5.4–12.3)	7.8 (3.5–15.2)	0.873

Data are shown as median (Q1, Q3) or n (%). NE indicates neutrophil elastase; and NET, neutrophil extracellular trap.

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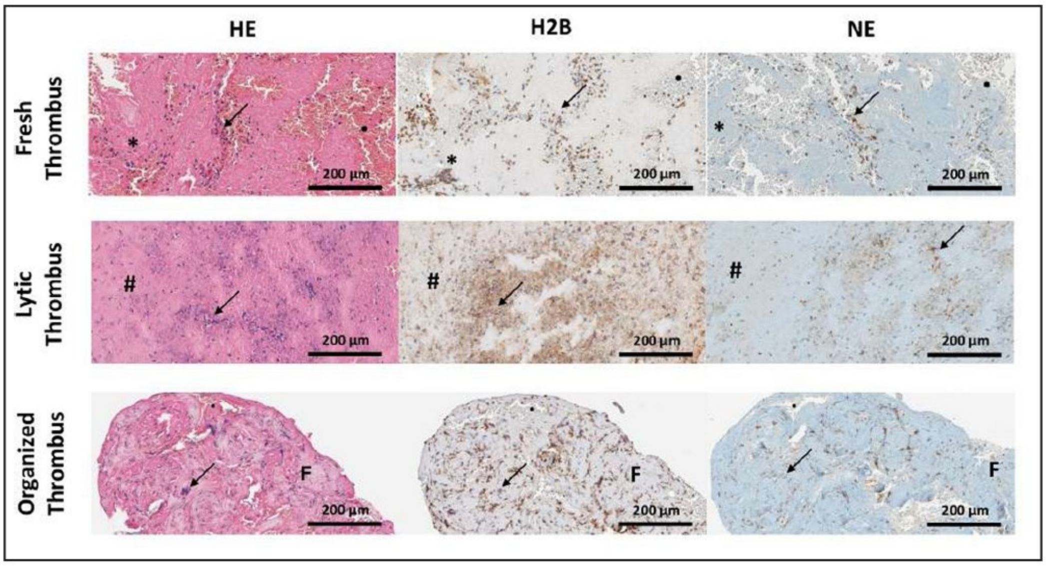

Figure 1. Three differently aged thrombi.

A, Fresh thrombus (aged <1 d). **B**, Lytic thrombus (aged 1–5 d). **C**, Organized thrombus (aged >5 d). Each specimen was stained with hematoxylin and eosin (HE) to identify each component and immunoassayed with anti-H2B and anti-NE (neutrophil elastase) antibodies for detecting neutrophil extracellular traps (NETs). Note in (**A**) Layered patterns of platelets, fibrin, intact erythrocytes, and granulocytes, and a low NET presence. Note in (**B**) colliquative necrosis and Karyorrhexis of granulocytes with a higher number of NETs. Note in (**C**) an organized thrombus (>5 d) showing ingrowth of fibroblasts and capillary vessels. Asterisks indicate Zahn lines, arrows indicate leucocytes, black dots indicate erythrocytes, hashtags indicate fibrin-platelet complexes, and F indicates fibrosis. Scale bar: 200 µm.

our population, comparing the thrombolysis-treated group (n=31) with the patients not treated with thrombolysis (n=41; Table S1; Figure 2). This analysis of clinical and morphological characteristics did not show significant differences, except for the age of the thrombus, which is the only variable acting on the NET content (Tables S1 and S2). Thus, there was a higher prevalence of lytic thrombi (>1 day) in the thrombolytic group (P=0.03). These results suggested that the ages of the thrombus and r-tPA were both associated with the NET content. For this reason, we performed a further evaluation of the association between thrombolysis and NE positivity adjusting for the age of the thrombus. The age of the thrombus proved to be the only independent predictor of the NE, while thrombolysis lost its significance on the NET contents. Furthermore, we tested whether thrombolysis had a mediation effect on the association between the age of the thrombus and the content of NETs (Figure 3). The mediation analysis revealed that the age of the thrombus had a direct effect on the NET content (P=0.014), without any mediation of the thrombolysis.

DISCUSSION

The main results of this study demonstrated that the composition of cerebral and coronary thrombi differs, and there were significantly more lytic cerebral thrombi than coronary thrombi. Moreover, there were more leukocytes, fibrin/platelets, and Zahn lines and fewer erythrocytes in cerebral thrombi compared with coronary thrombi. No atheromatous material was found in cerebral thrombi, confirming the main embolic origin of cerebral thrombi. Interestingly, there was a considerably higher expression of NETs in the cerebral thrombi as testified by the higher expression of H2B. Thrombolysis was associated with higher NE positivity, regardless of the origin of the thrombi (ie, cerebral versus coronary thrombi). There was no notable association between the administration of antiaggregant/heparin and H2B/NE amount when adjusted for the thrombus location. Importantly, the age of the thrombus was the only independent predictor of

Table 3. Univariable Analysis of the Association Between NE and Therapy and Univariable Analysis of the Association Between H2B and Therapy

	AME	95% CI	*P* value
NE and thrombolysis	6.461	0.7901 to 12.13	0.02555
NE and antiaggregant	−5.825	−11.26 to −0.3932	0.03557
NE and heparin	−1.702	−7.273 to 3.868	0.5492
H2B and thrombolysis	9.225	−0.5386 to 18.99	0.06405
H2B and antiaggregant	−10.56	−20.17 to 0.9564	0.03115
H2B and heparin	−9.762	−18.87 to −0.65	0.03574

AME indicates average marginal effect; and NE, neutrophil elastase.

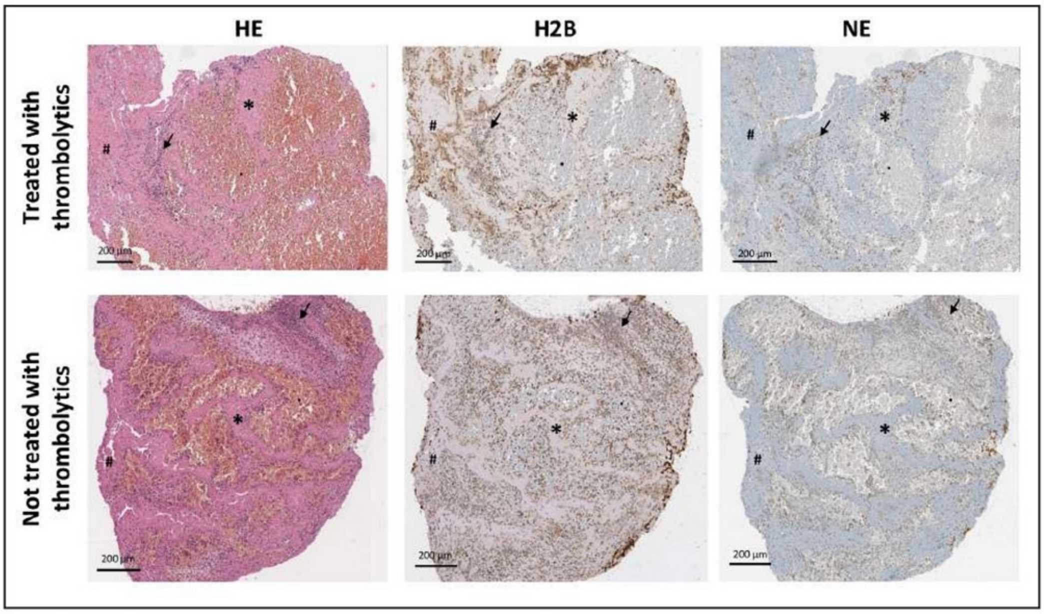

Figure 2. Morphology of a thrombus treated and a thrombus not treated with thrombolytics.
Each specimen is stained with hematoxylin and eosin (HE) and immunoassayed with anti-H2B and anti-NE (neutrophil elastase) antibodies for detecting neutrophil extracellular traps (NETs). Asterisks indicate Zahn lines, arrows indicate leucocytes, black dots indicate erythrocytes, and hashtags indicate fibrin-platelet complexes. The comparison among these 2 groups does not reveal a significantly different morphology. Scale bar: 200 µm.

NET content without any mediation of the thrombolytic treatment.

Our samples were fixed, and the NET components were marked by immunohistochemistry staining with anti-H2B to highlight the histone elements and anti-NE to determine the neutrophil origin of these extracellular structures. Our study includes samples retrieved from different thrombotic sites, allowing us to evaluate the possible effects of the thrombotic site itself on morphology and NET content, as suggested in the literature.[19]

First, we directed our attention to the evaluation of the age of retrieved thrombi, and we observed that, in our population, the cerebral specimens presented the largest level of thrombus maturation and a higher presence of leucocytes, in agreement with previous studies on IS thrombi with less NETs in fresh thrombi and a progressive

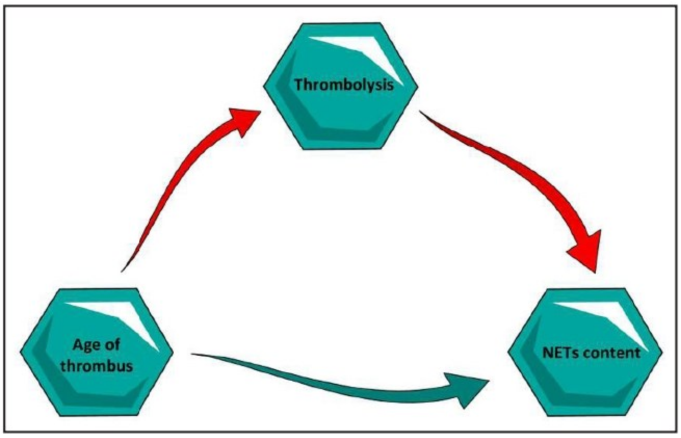

Figure 3. Schematic representation of mediation analysis hypothesis.
Thrombolysis does not have any mediation effect on the association between the age of the thrombus and neutrophil extracellular trap (NET) content.

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increase during the lytic phase. Indeed, Laridan et al[5] quantified neutrophils in stroke thrombi, demonstrating that their amount is considerably higher when compared with coronary or stent thrombi.[20] We assessed the differential effect of the various therapies on the composition of the specimens. The difference in the pharmacological treatment between the 2 groups led us to perform an adjusted analysis to remove confounding factors and strengthen the results.

Many studies demonstrated that NETs have a relevant role in thrombosis, with a procoagulant effect,[21,22] and their presence in retrieved thrombotic/embolic material is associated with impaired clinical outcome.[19] Thrombolysis sensitivity is associated with fibrin-clot architecture in the thrombus. Fresh thrombi are typically associated with improved outcomes such as higher Thrombolysis in Cerebral Infarction scores, shorter procedural time, and increased sensitivity to thrombolysis. Conversely, fibrin-rich thrombi, because of their higher stiffness, show increased resistance to MT and thrombolysis. Recent studies[23–25] claim that the outer layer of thrombi retrieved from patients with stroke is composed mainly of RBC, fibrin, von Willebrand Factor, and leukocytes that all play a role against r-tPA. An improvement in the rate of r-tPA effectivity is directly proportional to this external shell being compromised. Thus, targeting components other than fibrin can cause breaches into the outer layer of thrombi, which can enhance the efficacy of thrombolytics. An important consideration for clinical practice is that for every 5.4 patients treated with MT, 1 less patient will be dependent at 90 days, whereas for thrombolysis, the number needed to treat is 18, that is, 18 patients with stroke have to be administered thrombolysis to obtain a clinical benefit for 1 patient. In brief, MT is 3.3× more effective than thrombolysis.[26] The risk of complications related to stroke thrombectomy is higher in small distal arteries (eg, M2 segment), and the procedural success rate is smaller in these distal segments; thus, the difference against thrombolysis is minimal. While for large vessel occlusion stroke (typically M1 segment of medial cerebral artery), the number needed to treat by thrombectomy to achieve functional independence is around 5,[27] for small vessels (eg, M2), it is not known; for primary PCI in STEMI to prevent death, the number needed to treat is 29.[28] Although unfractionated heparin, along with other intravenous anticoagulants, holds a key role in the treatment of acute coronary syndromes, it is contraindicated, for the most part, in acute IS. There are several reasons for contraindication of full therapeutic anticoagulants in stroke: hemorrhagic stroke (15% of all strokes, frequently difficult to differentiate based only on clinical signs), risk of hemorrhagic transformation of IS (especially large IS, ie, those suitable for thrombectomy are at risk), uncontrolled hypertension, preexisting anticoagulant therapy, etc.[29] Reperfusion damage is closely related to sudden vigorous blood flow restoration to an ischemically damaged tissue; thus, it could be considered as reperfusion hemorrhage. This is rarely clinically relevant in acute MI, where the connective tissue network is robust enough; thus, in the myocardium, large reperfusion benefits almost always overcome small reperfusion damage. The situation in the brain is different, mainly due to far less robust connective tissue; thus, reperfusion damage is a serious concern for the brain. This difference is reflected in the heparin use during the acute phase of MI (dosage of 70–100 units/kg is standard) as opposed to acute IS, where heparin is considered a class III indication, that is, it should not be used and certainly not in full therapeutic dose. When heparin is used in acute IS mainly to prevent catheter clots, the dose should be only around 20 to 30 units/kg. The difference in thrombolysis use in MI versus IS involves a complex explanation. In brief, in MI, thrombolysis does not add any benefit on top of mechanical reperfusion, and the so-called facilitated PCI was demonstrated to be inferior to simple direct PCI. This may be related to procedural techniques (high-pressure balloon dilatation and permanent stent implantation). Trials comparing direct MT versus bridging thrombolysis before MT demonstrated similar outcomes or even better outcomes with bridging thrombolysis. The differences in the use of thrombolysis may explain (at least partially) the clot features in Table 2 and Table S1.

Many studies reported that heparin has an inhibitory effect on the interaction between NET histone components and platelets.[22,30,31] Accordingly, we detected a higher presence of NETs, marked with H2B, in the thrombolysis-treated group. Because these results suggested that the ages of the thrombus and r-tPA were both associated with a higher number of NETs, we tested the impact of thrombolysis on the association between the age of the thrombus and the NET specimen content. We demonstrated that the age of the thrombus has a direct effect on NET content, without any mediation of thrombolysis.

The main limitations of the study are related to the small sample size and the absence of organized thrombi, older than 5 days. Unfortunately, the cases are not representative of older thrombi morphological features; however, our main interest was evaluating the morphology and the therapeutical effects in the acute and subacute phases. Samples are equally distributed in the 2 classes of fresh (<1 day) and lytic (1–5 days) thrombi. For the first time, these mediation analysis results indicate a causal effect of the age of the thrombus on the NET bulk. The small availability of organized thrombi is caused mainly by the fact that patients entering the study were only those who, in a specific time frame, experienced an IS or MI and required an MT or PCI. Specimens were retrieved over a period of 6 months and represent the real-world sample of a specific time frame alongside probability factors. The city of Prague offers a high density of stroke units, and tertiary and quaternary care facilities, and the time needed to treat a symptomatic patient is considerably reduced.[24] Another possible limitation of such a study is the content of NETs retrieved after MT, thus, analyzed and the amount that could have been left within the vasculature of the patients; nevertheless, for patients with IS, each individual in this study scored between 2b and 3 on the Thrombolysis in Cerebral Infarction scale, when evaluated after MT, determining, then, an angiographic success in the whole group.[25,32]

These results provide evidence of the requirement to improve the current pharmacological protocols during acute stroke. Although it is known that heparin has a partial inhibitory effect on NET maturation, the current therapy formulation is not sufficient to contrast their procoagulant effect and resistance to thrombolysis. Adding new drugs with a specific action to disaggregate chromatin and enzymatic components of NETs can enhance the lytic effects of the therapy and improve patients' outcomes. Presently, DNase I (deoxyribonuclease I) has shown remarkable results in targeting NETS and extracellular DNA ex vivo lysis, thus demonstrating prothrombolytic potential when coupled with current thrombolytics.[5,33,34] Recombinant human DNase I, under the name of dornase alfa, is currently labeled as a treatment in cystic fibrosis; dornase alfa hydrolyzes DNA present in the secretions of the airways in patients with cystic fibrosis, reducing viscosity, improving quality of life, and reducing mortality by infection.[33,35]

In conclusion, this study provides valuable insights into the complex relationship between thrombus composition, age, pharmacological therapy, and NET content and demonstrates that the age of the thrombus is the only independent predictor of NET content without any mediation of the thrombolytic treatment in thrombotic/embolic specimens retrieved during thrombectomy in patients with stroke. This study supports the need for further investigations in also pharmacologically targeting NETs found in patients with acute cerebral ischemia, possibly, with recombinant human DNase I.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

STROBE Checklist
Tables S1–S2

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Attachment No. 3

The potential value of histological analysis of thrombi extracted through mechanical thrombectomy during acute ischemic stroke treatment

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Abstract

Studies on thrombus composition in acute stroke or acute myocardial infarction may help elucidate clot etiology and understand reperfusion success or failure. Moreover, such studies may certainly aid in the development of new technologies aimed at retrieving specific subtypes of thrombi; as a matter of fact, thrombus composition is suggested to influence the choice of techniques used during mechanical thrombectomy and plays a role in potential device and thrombus interaction. Over the years, histological analysis on the composition of thrombi causing ischemic stroke has proved to be a powerful tool to set standard prevention and treatment protocols. By isolating clot components, it is possible to provide a more accurate diagnosis and distinguish different stroke subtypes. Studies on histological clot composition support the theory that cryptogenic stroke can have a cardiogenic origin too. Components found in thrombi extracted from stroke patients support the importance of antithrombotic therapy in preventing and treating cerebral ischemia; however, more studies are needed to improve results in all types and subtypes of stroke. Hence, more research is required to further comprehend the role that platelets, fibrin, von Willebrand factor (vWF), and DNA play in relation to mechanical thrombectomy and recombinant tissue plasminogen activator (rtPA) resistance and to overcome certain limitations.
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Keywords: thrombus, composition, ischemic stroke, thrombectomy, thrombolysis, recombinant tissue plasminogen activator

Introduction

Myocardial infarction is the leading cause of death in high- or middle-income countries and the second most relevant cause of disability worldwide (1), while stroke is the second cause of death worldwide and the second leading cause of disability in Western countries (2). For decades, percutaneous coronary intervention has been the gold standard for the treatment and prevention of acute myocardial infarction, and although its combined use with thrombus extraction is a matter of discussion among experts, it has surely opened new doors in terms of research. Extracted thrombi are stained, analyzed, and employed to support new advancements in the diagnosis, treatment, prevention, and prognosis of myocardial infarctions. Furthermore, thrombus extraction devices were introduced for the treatment of acute ischemic stroke many years later, and from 2004 onward most Western countries began to include this technique in the standard care of acute cerebral ischemia (3). Stent retrievers not only represented an improvement in the clinical outcome of stroke therapy, but also inspired research in finding new answers to questions that most certainly would have been left unanswered. Treatment of mechanical thrombectomy in acute ischemic stroke aimed to reperfuse cerebral tissue and minimize neurological damage (4). Similarly to histological and immunohistochemical analyses on thrombi retrieved from coronary arteries, stroke research has supported clarifications in terms of diagnosis, prevention, prognosis, and treatment throughout the past decade, by utilizing specimens retrieved from the cerebral circulation. Many studies focused on stroke and myocardial infarctions have concluded that analyzing thrombus composition can provide insights into their etiology and predict successful reperfusion following intravenous thrombolysis and mechanical thrombectomy. Moreover, such studies may certainly assist in the development of new technologies aimed at retrieving

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specific subtypes of thrombi. In fact, thrombus composition is suggested to influence the choice of techniques used during mechanical thrombectomy and plays a role in potential device and clot interaction (5). Here, we review the importance of histological and immunohistochemical analyses of thrombi extracted during mechanical thrombectomy in the diagnosis, prevention, and treatment of acute ischemic stroke.

Etiology of stroke according to composition

The etiology of ischemic stroke can also be studied through the composition of retrieved thrombi. A better understanding of thrombus origin is a powerful tool for prevention, treatment, and prognosis of ischemic stroke. Stroke subtypes are classified into 5 categories based on etiology, using the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) classification: (1) large artery atherosclerosis (LAA), (2) small vessel occlusion (SVO), (3) cardioembolism (CE), (4) stroke of other determined etiology, and (5) stroke of undetermined etiology (5). A newer system called ASCO (A for atherosclerosis, S for small vessel disease, C for cardiac pathology, and O for other causes) to categorize ischemic stroke patients was introduced in 2009. This system distinguishes patient in more detail the differences between diseases underlying a cerebral ischemic event in a stroke patient. The Oxford classification only considers size and location of cerebral infarction, whereas TOAST and CCS classifications involve the disease directly related to ischemic stroke, neglecting underlying causes. ASCOD, proposed in 2013, introduces a fifth element – "D" for dissection – and similarly to the ASCO classification, three degrees of causality between the index ischemic stroke and each category are mentioned. Certainly, the biggest difference and one of the main advantages of the ASCOD system is the lack of definitions such as "undetermined," "cryptogenic," or "embolic stroke of unknown source." These categories are too difficult to define and may bring discomfort to patients when such definitions differ from one specialist to another. For example, in the event where no ASCOD 1 category is found, specific diseases (grades 2 or 3) can be mentioned, and these diseases can be clinically addressed according to guidelines to reduce recurrence even if a causal relationship between these diseases and ischemic stroke cannot be established yet (Table 1). Grade 1 defines diseases that can potentially be a cause. Grades 2 and 3 are those that have an uncertain and unlikely causal link, respectively. Grade 0 is assigned when no disease is found, whereas grade 9 occurs if there is no sufficient data to grade a disease (6). Atherosclerosis of large vessels (aortic arch, internal carotid artery, vertebral artery, and stems of the main arteries of Willis' circle) constitutes approximately 25% of all causes of acute cerebral ischemia. Small vessel disease accounts for about 25% of all causes of cerebral ischemia and lacunar infarcts. Despite appearing as small lesions (<15 mm on CT and <20 mm on MRI), small vessel disease is associated with severe neurological deficit and poor clinical outcome. CE is known to cause approximately 25% of all strokes, with atrial fibrillation being the trigger in 90% of cases. Thrombi in the left atrium and ventricle are additional high-risk causes, whereas atrial septum abnormalities, including the patent foramen ovale, are considered low risk or "unclear" depending on individual cases. In the remaining 25% of causes, the trigger remains unclear despite extensive diagnostic tests, and thus, these are classified as cryptogenic. In younger patients, more than 70% of cases are cryptogenic. However, there is increasingly higher evidence to also have a vast cardiac origin for the cryptogenic subtypes, and recently, the concept of embolic stroke of undetermined source (ESUS) was introduced as a cryptogenic stroke subgroup. For the majority of patients with ESUS, undetected paroxysmal atrial fibrillation is considered as the cause. Common causes are present in approximately 70%–75% of all ischemic strokes (7). Thrombi can be sampled, stored in formalin, embedded in paraffin, and subsequently cut into slices ranging between 3 and 10 μm. Staining methods can involve basic histological proce-

Table 1. TOAST and ASCOD classification systems

	TOAST	ASCOD
Subtypes	Large artery atherosclerosis	Atherosclerosis
	Cardioembolism	Cardioembolism
	Small vessel disease	Small vessel disease
	Other determined	Dissection
	Undetermined	Other
Characteristics	Classification based on stroke mechanisms	Phenotypic classification system
	Most widely used worldwide	Each phenotype is graded 1, 2, 3, 0, or 9
Advantages	Accurate prediction of prognosis	Takes into consideration noncausative factors
	Convenient	
	Simpler	
Disadvantages	Large pool of strokes of undetermined origin	More intricate for interpretation
	Less reliable in specific subtypes	Large number of subtypes

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dures with hematoxylin and eosin, and red blood cells (RBCs), platelets, and white blood cells (WBCs) can be identified and quantified by means of focal microscopy. Identification of fibrin is possible using Mallory's phosphotungstic acid-hematoxylin stains, whereas glycophorin A is an example to isolate RBCs; CD31 immunostains can help in quantifying platelets. Percentages of RBCs, platelets, and fibrin should be quantitatively determined in consensus. Subsequently, thrombi can be classified as red if RBCs outnumber platelets and fibrin by at least 15% and as white if platelets outnumber RBCs and fibrin also by at least 15%; in any other case, the thrombus is classified as mixed (8). Pathologically speaking, thrombi can be classified into 3 groups according to published definitions of thrombus age. Literature on thrombus age already exists for thrombi retrieved from coronary arteries in myocardial infarcts patients, and most subsequent literature on thrombus age in ischemic stroke patients owes to cardiac research. A thrombus is considered fresh when formed within 24 hours and appears to be composed of layered patterns of platelets, fibrin, erythrocytes, and intact granulocytes. Lytic thrombi generally occur between 1 and 5 days and exhibit areas of necrosis and karyorrhexis of granulocytes. Eventually, an organized thrombus (>5 days) is characterized by areas of smooth muscle cell ingrowth, with or without depositions of connective tissue and capillary vessel ingrowth. Between 65% and 75% of retrieved thrombi are fresh, whereas lytic thrombi account for approximately 15% and 20%, leaving organized thrombi a total of 5% to 15%. There are no fundamental differences in age of thrombi between stroke subtypes. However, cryptogenic thrombi tend to present more similar composition features as cardioembolic thrombi (9). Most thrombus components show significant differences in percentages between cardioembolic and noncardioembolic stroke causes. Cardioembolic thrombi consist of higher mean proportions of fibrin, less RBCs, and more WBCs. The mean proportion of fibrin in cryptogenic stroke patients is almost identical to that in cardioembolic stroke patients but much higher than those with noncardioembolic stroke. Similarly, RBCs are about as high in cryptogenic stroke as in cardioembolic stroke but definitely lower than in noncardioembolic stroke. WBC content does not differ substantially in thrombi of cryptogenic, noncardioembolic, and cardioembolic stroke patients (10). Overall, in terms of thrombus composition, there are no significant differences between anterior and posterior circulation strokes and between patients with or without thrombolysis. Thrombus size is directly proportional to the size of the occluded vessel (internal carotid artery>M1/BA>M2/A2), showing no relevant differences between TOAST categories. LAA and stroke of other determined etiology thrombi consist of larger amounts of RBCs with sparse fibrin-platelet complexes and fewer WBCs rather than cardioembolic and cryptogenic stroke thrombi with erythrocytes located and entrapped in the center and large amounts of fibrin/platelets. Thus, cryptogenic thrombi demonstrate very similar patterns to cardioembolic thrombi, which is clearly different from noncardioembolic thrombi, supporting the fact that most strokes of undetermined etiology may indeed present a cardiogenic nature (11). In the TOAST classification, uncommon stroke causes are classified as "other determined causes" and as "other causes" in the ASCOD classification. Uncommon stroke causes represent less than 5% of all ischemic strokes and are more frequent in younger individuals. Uncommon causes are categorized according to pathological conditions, such as infection, inflammation, genetic malformations, coagulopathies, vasospasm, and other miscellaneous vasculopathies. Histopathological analysis of thrombi retrieved from such patients can most certainly be beneficial for the diagnosis of the primary diseases, which require dedicated and at times rather different treatments than standard protocols to prevent reoccurrence in ischemic stroke (12).

Prevention and treatment of stroke according to composition

The analysis of components found in extracted thrombi has proved to be an effective tool in studying the efficacy of modern treatments, including mechanical thrombectomy. Histological staining (hematoxylin and eosin, Prussian blue, and Elastica van Gieson) and immunohistochemistry for CD3, CD20, and CD68/KiM1P make it possible to determine the fibrin, erythrocyte, and WBC components and compare the results to intervention time, frequency of secondary embolisms, and additional clinical and interventional parameters. Moreover, extra useful data can be obtained by assessing preinterventional CT attenuation of the thrombi in relation to the unaffected side and their association with histological features. Most fibrin-rich thrombi with low erythrocyte content are associated with longer intervention times, and thrombi with low rate of RBCs and low CT density frequently cause complications such as embolism in the thrombectomy procedure, suggesting that these thrombi have higher fragility (13). Besides the duration of the interventional procedure in treating cerebral ischemia patients, it is possible to estimate through thrombus composition how many passes are necessary to reperfuse the cerebral vasculature. Certainly, such results may also depend on different factors, i.e., the instrument used or the experience of the medical specialist. Thrombus fragments retrieved from patients with acute ischemic stroke obtained in each pass can be collected as individual samples and maintained throughout the histological analysis as independent samples. All samples can be stained with hematoxylin and eosin and Martius scarlet blue to quantify the composition of RBCs, fibrin, and WBCs in thrombus fragments in each pass. It has been shown that the number of passes required generally to complete a mechanical thrombectomy ranges from 1 to 6 passes. The analysis of thrombus fragments retrieved in each pass provides a great insight into the thrombectomy procedure progression. Generally, RBC content of thrombus fragments retrieved in passes 1 and 2 is significantly higher than that retrieved in passes 3 to 6. The removal of thrombus material in 1, 2, or 3 passes is normally associated with the highest rate of final TICI 2b–3. Fragments retrieved in passes 1 and 2 are also associated with a much lower fibrin composition compared with fragments retrieved in passes 3 to 6. These notions may be a useful consideration in determining the treatment strategy as a case evolves and may be useful for the development of new devices to increase rates of 1-pass recanalization (14). Treatment is focused on fast and efficient removal of the occluding thrombus, either via intravenous thrombolysis or via endovascular thrombectomy. However, recanalization is not always successful and factors contributing to failure are not completely understood. Bright field and fluorescence microscopy is used to histologically analyze thrombi retrieved from stroke patients for fibrin, RBCs, von Willebrand factor (vWF), platelets, leukocytes, and DNA. This would show how thrombi are composed of two main types of zones: RBC-rich and platelet-rich areas. RBC-rich areas are characterized by limited complexity because they consist of RBC trapped in a meshwork of fibrin, whereas platelet-rich areas consist of dense structures of fibrin, vWF, and a great amount of leukocytes and DNA. These findings are important to better understand why platelet-rich thrombi tend to be more resistant to thrombolysis and more complicated to retrieve through thrombectomy. It is clear since recently that there is a consistent presence of leukocytes in stroke thrombi, and other than their presence, not much is known about their specific cellular or molecular distribution. Notably, when staining for RBC-rich and platelet-rich areas, leukocytes are primarily found at the interface between RBC-rich and platelet-rich areas. Leukocytes are also consistently present within platelet-rich zones and do not seem to be found generally in RBC-rich areas, which are homogenously spread throughout the erythrocytes. Leukocytes have also been shown to induce thrombus formation by means of extracellular DNA traps. When Feulgen stain is performed on thrombi, large extracellular DNA networks, appearing as extracellular smears, can be seen throughout the majority of retrieved specimens. Relevant amounts of extracellular DNA are observed particularly in platelet-rich areas and boundary zones between platelet-rich and RBC-rich regions, whereas no DNA is found within the RBC-rich regions. Currently, in many Western countries, recombinant tissue plasminogen activator (rtPA) is the only approved medication for thrombolysis in patients with cerebral ischemia, but it is effective in less than half of the patients. For this reason, it is practical to assume the reality of the so-called "rtPA resistance," even if the mechanisms of actions are not completely understood. RBC-rich thrombi generally respond better to rtPA rather than platelet-rich thrombi. It is safe to state that by inducing fibrin degradation, rtPA can have a direct and efficient thrombolytic effect on RBC-dominant areas, with thin fibrin as the main extracellular skeleton. Platelet-dominant thrombi contain denser fibrin scaffolds mixed with high amounts of other components such as vWF and extracellular DNA. For this reason, it is possible to assume that fibrin, vWF, and DNA form the structural basis of platelet-rich thrombi and that vWF and DNA could characterize the so-called rtPA resistance. Extracellular DNA and histones have indeed been shown to modify the structure of fibrin, making it more resistant to degradation. Leukocytes, more specifically neutrophils, may support thrombosis through the formation of neutrophil extracellular traps. Furthermore, ex vivo studies have demonstrated how rtPA in combination with DNAse-1 can be more effective. vWF is associated with thrombus formation by interacting with fibrin in platelet-rich regions. In vitro studies demonstrate how fibrin and vWF interact with each other through factor XIII or by means of thrombin-dependent incorporation, enhancing thrombus formation. Histological results on vWF and fibrin clarify that fibrin degradation with only plasmin is not enough to obtain thrombolysis of platelet-dominant thrombi. By adding the vWF-cleaving enzyme ADAMTS13, it may be possible to target vWF present in thrombi, thus improving the effectiveness of thrombolysis in rtPA-resistant thrombi. This is very similar to the use of DNAse-1 in combination with rtPA to tackle extracellular DNA. Recent studies indicate that erythrocyte-rich thrombi are more easily extracted through endovascular procedures compared with more complex fibrin-/platelet-rich thrombi (15, 16). Current retriever devices and available techniques are more efficient with fresher thrombi; however, mechanisms that render platelet-rich thrombi more resistant to interventional procedures are not completely understood. Platelet-dominant thrombi from ischemic stroke patients include dense fibrin/vWF structures, leukocytes, and DNA. These aforementioned thick fibrin fibers trigger higher thrombus rigidity and affect the coefficient of friction and level of physical compression (17). In addition, extracellular DNA is believed to strengthen fibrin structure, making it more resistant to mechanical forces. As a matter of fact, recently a direct proportion between the amount of neutrophil extracellular DNA traps and the number of device passes needed to achieve successful recanalization has been found (18, 19).

Limitations of histopathological analysis of thrombi

After a careful reading of the current literature on the composition of thrombi retrieved from the cerebral vasculature, a few limitations were found that require significant attention. Ischemic strokes treated with mechanical thrombectomy represent only a percentage of total cases, and in all studies the only available thrombi were obtained from those patients in whom the clot did not dissolve spontaneously or during thrombolysis treatment and in whom the thrombus could be successfully retrieved. Most studies present a relatively small patient population size; thus some comparisons are not possible with small sample sizes. Furthermore, the variation in procedural techniques and the combination of devices applied to retrieve the thrombus fragments is a confounding factor that may potentially affect the rates of composition and histological distribution of the components. Another important limitation in many studies is that the use of intravenous rtPA might have already altered the specimens and manipulation with catheters might have produced some thrombi or fragmented them. The retrieved thrombus fragment does not always reflect the whole occlusive thrombus; thus a certain bias toward more stable clot components is common.

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Moreover, given the broad variation of clot composition in the evaluated sections of individual specimens, quantitative component assignments may not always be perfectly representative of the entire clot volume. Another important limitation, other than the limited number of case studies, is the predominance of specific types of ischemic stroke. As a matter of fact, most retrieved specimens are extracted from the middle cerebral arteries, rendering the results of a study a mere generalization for all the less represented subtypes. Certain studies involve the use of only hematoxylin and eosin stains for the quantitative analysis, which surely permits a direct comparison of the different components without the risk of methodological differences. However, important components such as platelets and fibrin may not be quantified successfully, and quantification can be subjected to bigger human and statistical error due to difficulties in reaching a consensus. Moreover, the use of immunological techniques may instead improve precision, meanwhile involving greater costs and requiring better equipped laboratories (Table 2).

Conclusion

Over the years, histological analysis aimed to study the composition of thrombi causing ischemic stroke has proved to be a powerful tool to set standard prevention and treatment protocols. By isolating the clot components, it is possible to provide a more accurate diagnosis and distinguish different stroke subtypes. Studies on histological clot composition support the theory that cryptogenic stroke can have a cardiogenic origin too. Components found in thrombi extracted from stroke patients confirm the importance of antithrombotic therapy in preventing and treating cerebral ischemia, but more studies are needed to improve results for all types and subtypes of stroke. In conclusion, more research and investigations are definitely required to further comprehend the role platelets, fibrin, vWF, and DNA play in relation to mechanical thrombectomy and recombinant rtPA resistance and to overcome the limitations faced by existing literature.

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Table 2. Limitations of studies and possible solutions

Limitation	Main bias	Potential mitigations
• Small patient population size • Predominance of specific types of ischemic stroke (MCA)	Sampling bias Faulty generalization	• Increase population size • Cooperation with other centers (stroke units) • Extend time of collection
Mechanical thrombectomy-treated stroke represent only a percentage of total cases	Exclusion bias	Include patients treated only with intravenous thrombolysis when possible
• Variation in procedural techniques for retrieval • Combination of devices applied for retrieval	Confounding bias	Apply categories according to the device used
• Already altered specimens by intravenous rtPA • Manipulation with catheters	Confounding bias	• Distinguish and compare specimens in which patients were subjected to intravenous thrombolysis • Count and minimize where possible per-pass retrieval of specimens
Retrieved thrombus material not always reflects the whole occlusive thrombus	Survivorship bias Faulty generalization	• Minimize need for more stable thrombi for the purpose of the study • Enlarge patient population size • Use of immunological detection procedures
Exclusive use of hematoxylin and eosin stains	Observer bias	• A large pool of expert raters • Strengthen inter-rater reliability score
Immunological procedures	Funding bias	• Cooperation with more centers and laboratories • Grant application

Attachment No. 4

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Long-term outcomes of thrombectomy for acute ischaemic stroke by occluded artery and stroke aetiology: a PRAGUE-16 substudy

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KEYWORDS

- ischaemic stroke
- miscellaneous
- stent retrievers
- thrombectomy

Abstract

Background: Thrombectomy is an effective treatment for acute ischaemic stroke (AIS).

Aims: The aim of this study was to compare clinical outcomes with intracranial artery occlusion site among AIS patients treated in the setting of a cardiology cath lab.

Methods: This was a single-centre, prospective registry of 214 consecutive patients with AIS enrolled between 2012 and 2018. All thrombectomy procedures were performed in a cardiology cath lab with stent retrievers or aspiration systems. The functional outcome was assessed by the modified Rankin Scale (mRS) after three months.

Results: Ninety-three patients (44%) had middle cerebral artery (MCA) occlusion, 28 patients (13%) had proximal internal carotid artery (ICA) occlusion, 27 patients (13%) had tandem (ICA+MCA) occlusion, 39 patients (18%) had terminal ICA (T-type) occlusion, and 26 patients (12%) had vertebrobasilar (VB) stroke. Favourable clinical outcome (mRS ≤2) was reached in 58% of MCA occlusions and in 56% of isolated ICA occlusions, but in only 31% of T-type occlusions and in 27% of VB stroke. Poor clinical outcome in T-type occlusions and VB strokes was influenced by the lower recanalisation success (mTICI 2b-3 flow) rates: 56% (T-type) and 50% (VB) compared to 82% in MCA occlusions, 89% in isolated ICA occlusions and 96% in tandem occlusions.

Conclusions: Catheter-based thrombectomy achieved significantly better clinical results in patients with isolated MCA occlusion, isolated ICA occlusions or tight stenosis and tandem occlusions compared to patients with T-type occlusion and posterior strokes.

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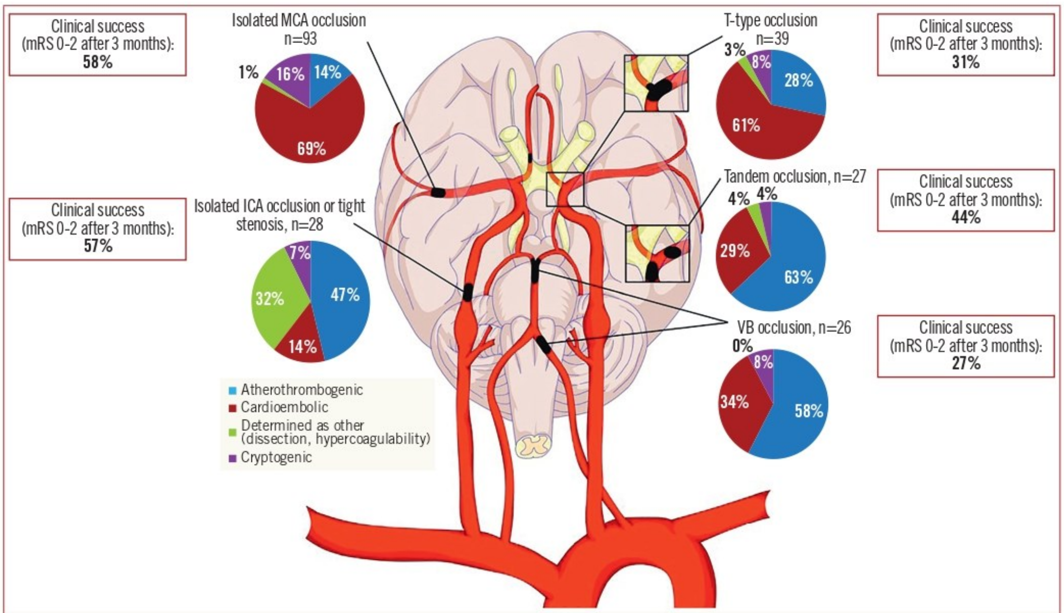

Visual summary. *Endovascular intervention of isolated MCA or ICA occlusions provides greatest clinical benefit, while interventions in posterior circulation have lower chance for clinical success.*

Abbreviations

AIS	acute ischaemic stroke
BA	basilar artery
CBT	catheter-based thrombectomy
ICA	internal carotid artery
MCA	middle cerebral artery
mRS	modified Rankin Scale
NIHSS	National Institutes of Health Stroke Scale
pts	patients
VB	vertebrobasilar

Introduction

According to estimates by the World Health Organization, stroke is the second leading cause of preventable death worldwide and the leading cause of serious, long-term disability[1]. It is also the second most common cause of dementia, the most frequent cause of epilepsy in the elderly, and a frequent cause of depression[2,3]. The majority of strokes (87%) are classified as ischaemic, the rest (13%) as haemorrhagic[4].

Death or severe disability occurs in patients with emergent large vessel occlusion. Approximately one third of all ischaemic strokes are caused by middle cerebral artery (MCA) occlusion. Vertebrobasilar (VB) strokes represent about 5-6% of all ischaemic strokes, but prognosis may be fatal[5]. A prompt and complete restoration of blood flow in the affected artery is the key factor for a favourable neurological outcome – "time is brain". Until recently, intravenous tissue plasminogen activator (t-PA) was the only proven treatment for large vessel occlusion. However, the landscape of ischaemic stroke treatment has changed with the publication of five randomised multicentre controlled clinical trials in 2015. According to these trials, catheter-based mechanical thrombectomy (CBT) was proven to be an effective treatment for proximal occlusions of the major intracranial arteries in acute stroke patients, and became a class IA indication[6-10]. Approximately 30% of ischaemic strokes are caused by large vessel occlusion and should be considered for intervention[11].

The aim of this study was to compare clinical outcomes of patients treated by CBT for acute ischaemic stroke based on intracranial artery occlusion site and stroke aetiology.

Methods

STUDY PATIENTS

This was a single-centre, prospective, observational registry of consecutive patients (pts) treated by CBT for an acute ischaemic stroke. A total of 214 consecutive pts (mean age 67.4±12.2, range 21-92 years, male 53.7%) were enrolled between October 2012 and June 2018. The study was based on the pre-specified protocol approved by the ethics committee and was designed in collaboration among cardiologists, neurologists and radiologists[12]. All participants (or their legal representatives) provided written informed consent. On admission, all patients were examined by a neurologist, including basic laboratory screening. Afterwards, a computed tomography (CT) scan with angiography was performed to confirm large vessel occlusion and to rule out intracranial haemorrhage. On the basis of a comprehensive examination and compliance with inclusion criteria, an endovascular intervention was indicated. Intravenous thrombolytic pretreatment was administered by a neurologist based on CT scan and coagulation results. Inclusion criteria were: moderate to severe acute ischaemic stroke (National Institutes of Health Stroke Scale [NIHSS] score ≥6), time interval <6 hours from symptom onset (except for basilar artery occlusion, where the therapeutic window was not strictly limited and the treatment strategy was assessed individually) or CT scan <2 hours from time of "wake-up stroke" diagnosis, no or only small ischaemia visible on the admission CT scan, CT evidence for an occluded major artery (either CT angio or dense artery sign on CT scan), expected ability to start intervention within <60 minutes from CT and age ≥18 years. Exclusion criteria were previously known neurologic symptoms (modified Rankin Scale [mRS] score 2-5), known severe hypoglycaemia, unmeasurable international normalised ratio (INR), intracranial bleeding and CT evidence of large ischaemia.

INTERVENTIONS

All interventions were performed in the cardiology cath lab either by a board-certified interventional radiologist or by a cardiologist with a licence for interventional angiology (Czech Medical Chamber – licence no. F021), mostly under local anaesthesia. The decision concerning the revascularisation strategy was made by the interventionist. In patients with proximal internal carotid artery (ICA) occlusion or tight stenosis, the default strategy was carotid stenting in the acute phase; we did not perform deferred stenting in this study. The dose of heparin administered was at the operator's discretion and depended on the patient's physical constitution, type of procedure and preprocedual INR value. In patients with an effective INR value or in those who had been demonstrably taking any of the new oral anticoagulants, the heparin dose was either reduced or not administered at all.

FOLLOW-UP

The success of recanalisation was assessed immediately post procedure by follow-up angiography. Patients were subsequently monitored in the intensive care unit in either the Department of Cardiology or the Department of Neurology. Most cases in our study group had a control CT scan on day 1 to evaluate infarction size and possible haemorrhage. Details have been described previously[12]. During the remaining hospitalisation, at least 24-hour electrocardiography monitoring, sonography of the carotid arteries and transthoracic/transoesophageal echocardiography were performed in all patients to exclude other possible sources of embolisation. In indicated cases, prolonged ECG monitoring (48 hours, 7 days, 30 days) was carried out during three-month follow-up. The aetiology of strokes was determined based on the TOAST classification[13].

ANTITHROMBOTIC REGIMEN

After the acute phase the antithrombotic regimen was individualised based on stroke aetiology and stroke severity. In atherothrombotic strokes (e.g., ICA lesions), antiplatelet treatment was initiated, while in cardioembolic or cryptogenic strokes anticoagulation treatment was initiated if no contraindication was present. The onset of these antithrombotic treatments varied between day 2 and day 14 after stroke onset, based on the extent of final ischaemia and presence/absence of haemorrhagic transformation.

STUDY ENDPOINTS

The primary endpoint was a functional neurologic outcome in three months assessed by board-certified neurologists. The secondary endpoints were angiographic recanalisation success rate, change of the NIHSS (ΔNIHSS) score from admission to discharge and symptomatic intracranial bleeding. The degree of dependence after a stroke was measured using the mRS after three months of follow-up. Two patients (foreign tourists) were lost during three-month follow-up. The study registry included patients' demographic and epidemiological data – age, sex, body mass index (BMI), prior stroke/transient ischaemic attack (TIA), known coronary and peripheral arterial disease, arterial hypertension, hyperlipidaemia, diabetes mellitus, chronic kidney injury, current smoking (defined as ≥1 month prior to admission), atrial fibrillation (AF; known or *de novo* identified either during the hospitalisation or during three-month follow-up) and chronic antithrombotic therapy; periprocedural data – time intervals (symptoms onset – door time – CT scan – needle time [if] – groin puncture time – recanalisation time [if]), change of NIHSS score from admission to discharge and angiographic findings (type of occluded artery, preprocedural and post-procedural modified Thrombolysis In Cerebral Infarction [mTICI] flow).

STATISTICAL ANALYSIS

Age, BMI and scores as continuous variables were described by a mean including standard deviation. All other variables were dichotomous, expressed as number and proportion. The normal distribution of continuous variables enabled their comparison among occlusion sites using one-way ANOVA with Tukey's multiple comparisons test. The difference in NIHSS score at admission and discharge was assessed by paired t-test. Furthermore, dichotomous variables among occlusion sites were statistically evaluated using the χ^2 test. All tests were two-tailed, and the level of significance was set at 0.05. Statistical analyses were performed using Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA) and Stata version 16 software (StataCorp, College Station, TX, USA).

Results

BASELINE CHARACTERISTICS

A total of 214 patients were enrolled in the study. Mean age of this cohort was 67.4±12.2 years, with VB stroke (71.9±8.8) patients being significantly older than others. The prevalence of active smoking, hypertension, diabetes mellitus, dyslipidaemia, renal insufficiency, prior stroke/TIA and known coronary and peripheral artery disease did not differ significantly among the occlusion sites. Intravenous thrombolytic pretreatment was administered in 88 pts (41.1%). General anaesthesia was used in a total of 32 pts, significantly more often in T-type and VB strokes (p=0.031). Details are presented in **Table 1**.

Table 1. Detailed baseline characteristics and stroke aetiology per occlusion site.

	Total n=214	Isolated MCA occlusion, n=93	Isolated ICA occlusion, n=28	Tandem occlusion, n=27	T-type occlusion, n=39	VB occlusion n=26	*p*-value
Male	115 (53.7%)	49 (52.7%)	15 (53.6%)	19 (70.4%)	16 (41%)	15 (57.7%)	NS
Age, years	67.4±12.2	68.7±12.5	62.9±11.1	65.1±12.8	66.5±12.6	71.9±8.8	0.047
Smokers	69 (32.2%)	28 (30.1%)	10 (35.7%)	10 (37%)	11 (28.2%)	9 (34.6%)	NS
BMI (kg/m^2)	29.7±22.5	27.6±5.6	26.5±3.9	29.9±5.9	28.4±8.1	28.3±4.2	NS
Hypertension	150 (70.1%)	63 (67.7%)	20 (71.4%)	19 (70.4%)	26 (66.7%)	22 (84.6%)	NS
Diabetes mellitus	53 (24.8%)	18 (19.4%)	11 (39.3%)	8 (29.6%)	8 (20.5%)	8 (30.8%)	NS
Dyslipidaemia	72 (33.6%)	29 (31.2%)	10 (35.7%)	9 (33.3%)	12 (30.8%)	12 (46.2%)	NS
Chronic kidney disease	50 (23.4%)	22 (23.7%)	7 (25%)	5 (18.5%)	10 (25.6%)	6 (23.1%)	NS
Prior stroke or TIA	39 (18.2%)	20 (21.5%)	6 (21.4%)	2 (7.4%)	8 (20.5%)	3 (11.5%)	NS
Known coronary and peripheral arterial disease	59 (27.6%)	25 (26.9%)	8 (28.6%)	9 (33.3%)	9 (23.1%)	8 (30.8%)	NS
Atrial fibrillation – known, de novo	98 (45.8%)	55 (59.1%)	6 (21.4%)	8 (29.6%)	20 (51.3%)	9 (34.6%)	<0.001
Chronic antithrombotic therapy	101 (47.2%)	51 (54.8%)	10 (35.7%)	11 (40.7%)	18 (46.2%)	10 (38.5%)	NS
NIHSS score at admission	15.6±6.5	15.2±5.4	12.3±6.5	16.0±5.3	16.5±4.6	18.6±12.1	0.023
IV pretreatment	88 (41.1%)	38 (40.9%)	11 (39.3%)	13 (48.1%)	12 (30.8%)	13 (50%)	NS
General anaesthesia	32 (15%)	8 (8.6%)	4 (14.3%)	2 (7.4%)	10 (25.6%)	8 (30.8%)	0.031
Stroke aetiology							
Atherothrombogenic	69 (32.2%)	13 (14%)	13 (46.4%)	17 (63%)	11 (28.2%)	15 (57.7%)	<0.001
Cardioembolic	109 (50.9%)	64 (68.8%)	4 (14.3%)	8 (29.6%)	24 (61.5%)	9 (34.6%)	<0.001
Determined as other	13 (6.1%)	1 (1.1%)	9 (32.1%)	1 (3.7%)	1 (2.6%)	0 (0%)	<0.001
Cryptogenic	23 (10.7%)	15 (16.1%)	2 (7.1%)	1 (3.7%)	3 (7.7%)	2 (7.7%)	NS

BMI: body mass index; ICA: internal carotid artery; IV: intravenous; MCA: middle cerebral artery; NIHSS: National Institutes of Health Stroke Scale; TIA: transient ischaemic attack; VB: vertebrobasilar artery

OCCLUDED ARTERIES

The distribution of intracranial artery occlusion sites was non-uniform. Ninety-three pts (43.5%) had MCA occlusion, 28 pts (13.1%) had isolated ICA occlusion/tight stenosis, 27 pts (12.6%) had tandem occlusion (ICA+MCA), 39 pts (18.2%) had terminal internal carotid artery (T-type) occlusion, 26 pts (12.1%) had VB stroke, and one patient had an isolated anterior cerebral artery (ACA) occlusion.

STROKE AETIOLOGY

The AIS aetiology was assumed to be cardioembolic in 50.9%, atherothrombogenic in 32.2%, cryptogenic in 10.7% and determined as other in 6.1% of pts. Isolated MCA occlusion and T-type strokes were significantly more frequently caused by cardioembolism (68.8% and 61.5%), which was also reflected by more frequent occurrence of AF in these patients (59.1% and 51.3%). VB strokes, tandem occlusion and isolated ICA occlusion/tight stenosis strokes were mostly atherothrombogenic (57.7%, 63.0% and 46.4%). Details are presented in **Figure 1**.

ANGIOGRAPHIC OUTCOMES

The overall recanalisation success rate (mTICI 2b-3 flow) was 76.2%. The proportion of successful recanalisation was significantly higher in MCA (81.7%), ICA (89.3%) and tandem (96.3%) occlusions compared to T-type (56.4%) and VB (50%) occlusions ($p<0.001$). A total of 13 patients (6.1%) suffered from symptomatic intracranial bleeding (defined as ΔNIHSS $\geq$4 within 48 hours after intervention) with uniform distribution among all occlusion sites. Detailed primary and secondary endpoints are shown in **Table 2**. Examples of thrombectomy procedures per anatomic stroke type are shown in **Figure 2-Figure 4**. The only patient with isolated ACA occlusion was a 50-year-old man, smoker. His NIHSS score decreased from 12 (at admission) to 0 (at discharge). The recanalisation was successful with final mTICI 3 and the patient reached full neurological recovery (mRS 0) in three-month follow-up. The stroke aetiology was assumed to be due to known hypercoagulability.

CLINICAL OUTCOMES

The primary study endpoint was three-month clinical outcome with a favourable clinical outcome defined as mRS $\leq$2, which was reached in 47.6% (101 of 212 pts). Patients' clinical outcomes varied significantly among occlusion sites. A favourable clinical outcome was achieved significantly more often in MCA occlusions (56.7%) and isolated ICA occlusions or tight stenosis (55.6%) compared to others (p=0.010). In addition, patients

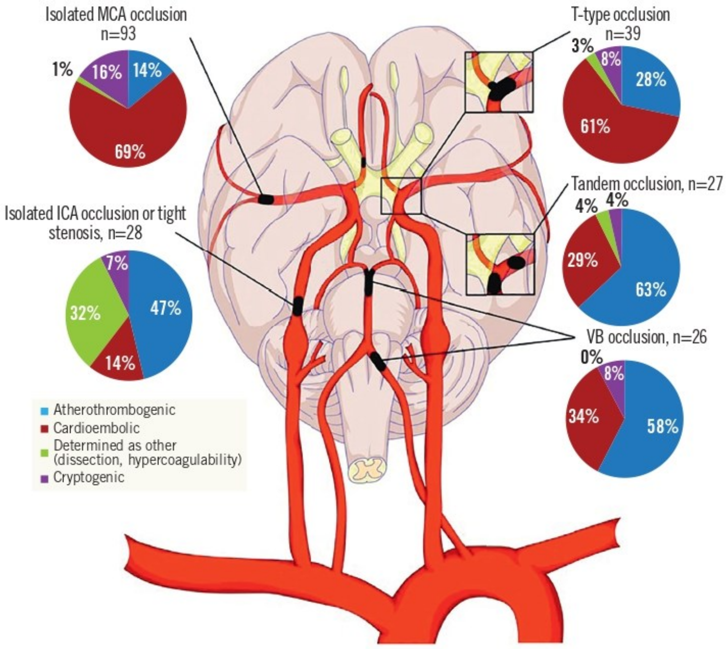

Figure 1. *Stroke aetiology by occlusion site. Isolated MCA occlusion is most frequently caused by cardioembolism, while proximal ICA and VB lesions are most frequently caused by atherothrombosis. ICA: internal carotid artery; MCA: middle cerebral artery; VB: vertebrobasilar artery*

Table 2. Primary and secondary endpoints.

	Total n=212	Isolated MCA occlusion, n=92	Isolated ICA occlusion or tight stenosis, n=27	Tandem occlusion, n=27	T-type occlusion, n=39	VB occlusion, n=26	*p*-value
mRS score 0-2 after 90 days	101 (47.6%)	53 (57.6%)	15 (55.6%)	13 (48.1%)	12 (30.8%)	7 (26.9%)	0.010
Mortality rate in 90 days	68 (32.1%)	24 (26.1%)	4 (14.8%)	9 (33.3%)	18 (46.2%)	13 (50%)	0.015
Angiographic success overall (mTICI 2b-3 flow, %)	163 (76.2%)	76 (81.7%)	25 (89.3%)	26 (96.3%)	22 (56.4%)	13 (50%)	<0.001
Symptomatic intracranial bleeding	13 (6.1%)	3 (3.3%)	1 (3.7%)	2 (7.4%)	4 (10.3%)	3 (11.5%)	NS

without diabetes had twice as much chance of achieving a favourable clinical outcome as patients with diabetes, and not using general anaesthesia increased the chance of a favourable clinical outcome almost three times. The overall mortality rate was 32.1% with significantly higher rates in T-type and VB occlusions (46.2% and 50%, p=0.015). The distribution of mRS score based on occluded artery site is presented in **Figure 5**. The distribution of NIHSS scores at admission was not uniform among occlusion sites. Patients with VB strokes had the highest NIHSS scores (18.6±12.1); on the other hand, patients with isolated ICA occlusion or tight stenosis had the lowest scores (12.3±6.5). Comparing NIHSS scores at admission and discharge based on occlusion site (unfortunately determined in only 121 patients), NIHSS scores decreased significantly in almost all stroke types except VB stroke. Details are shown in **Table 3**.

Discussion

COMPARISON WITH TREVO 2000 REGISTRY[14] IN NON-SELECTED ALL-COMERS

In our study group of patients treated for anterior and posterior strokes with mechanical thrombectomy for large vessel occlusions, a favourable clinical outcome (mRS score ≤2 at three months) was reached in 47.6% (101 of 212 pts). Despite the fact that the angiographic success rate was higher in the Trevo 2000 study (92.8%)

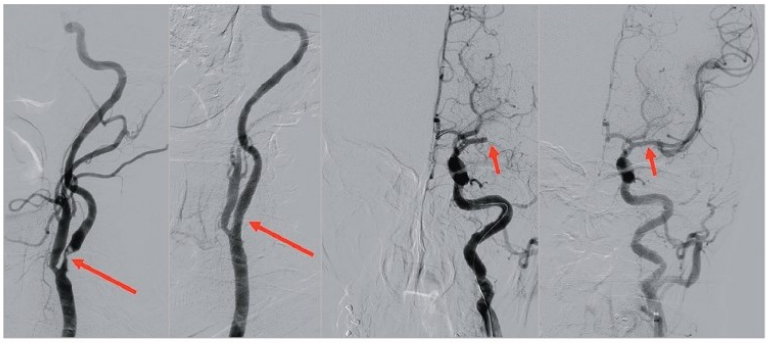

Figure 2. *Typical angiographic findings in proximal ICA occlusion and in MCA occlusion. From left to right: (1) proximal ICA severe stenosis with intraluminal thrombus, (2) after stent implantation, (3) MCA occlusion, (4) after thrombectomy.*

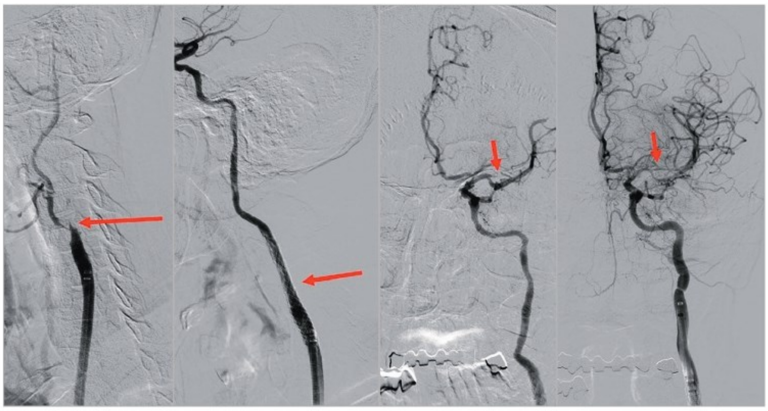

Figure 3. *Typical angiographic findings in the tandem occlusion (proximal ICA and MCA occlusion). From left to right: (1) proximal ICA occlusion, (2) after stent implantation, (3) MCA occlusion, (4) after thrombectomy.*

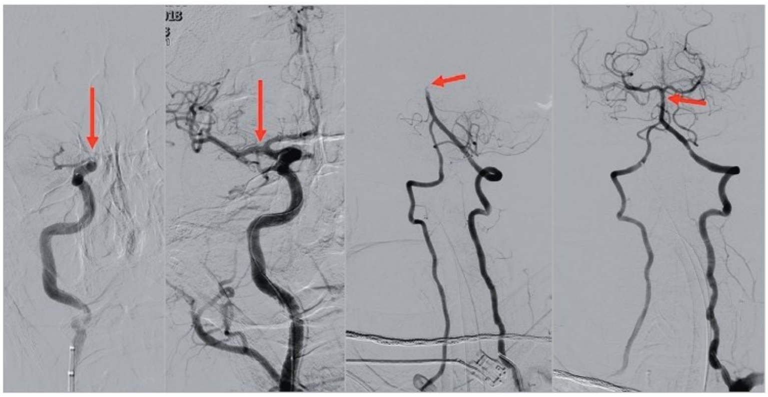

Figure 4. *Typical angiographic findings in ICA T-occlusion and in BA occlusion. From left to right: (1) terminal ICA occlusion (T-type), (2) after thrombectomy, (3) BA occlusion, (4) after thrombectomy. BA: basilar artery*

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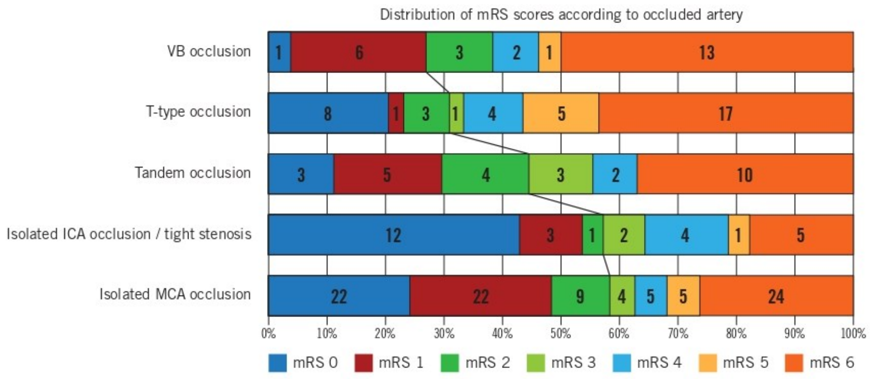

Figure 5. *Distribution of mRS scores based on occluded artery site. From the lowest clinical success rates (VB occlusion) to the highest rates (isolated MCA occlusion). ICA: internal carotid artery; MCA: middle cerebral artery; mRS: modified Rankin Scale; VB: vertebrobasilar artery*

Table 3. Comparing NIHSS scores at admission and at discharge.

	Total, n=121	Isolated MCA occlusion, n=60	Isolated ICA occlusion or tight stenosis, n=15	Tandem occlusion, n=20	T-type occlusion, n=20	VB occlusion, n=5	*p*-value
NIHSS score at admission	14.8±5.6	14.7±5.5	13.1±6.0	15.9±5.7	16.6±4.3	9.2±6.4	NS
NIHSS score at discharge	6.9±7.4	6.4±7.4	5.8±7.6	7.8±7.1	7.7±7.5	10.4±9.3	NS
NIHSS score difference	7.9±7.0	8.3±5.9	7.3±9.8	8.1±6.3	8.9±7.2	1.2±9.0	NS
p-value (difference)	<0.001	<0.001	0.012	<0.001	<0.001	NS	-

versus our study (76.2%), the resulting favourable clinical outcomes (mRS score ≤2) were comparable (55.3% in Trevo versus 47.6% in PRAGUE-16). This may be explained by the different distribution of occlusion sites. Patients' baseline characteristics (age, diabetes mellitus, AF, NIHSS score at admission) did not differ in the other compared data. Details are shown in **Table 4**.

Table 4. Comparison of PRAGUE-16 data with Trevo 2000 registry and HERMES meta-analysis.

	Trevo 2000	PRAGUE-16	HERMES	PRAGUE-16
Number of patients	2,008	212	634	184
Stroke location	Anterior + posterior	Anterior + posterior	Anterior only	Anterior only
Age (mean±SD; median)	68.3±14.4	67.4±12.2	68	69
Diabetes mellitus	23.8%	24.8%	13.0%	24.2%
Atrial fibrillation	36.1%	45.8%	33.0%	48.4%
NIHSS score at admission (mean±SD; median)	15.5±6.8	15.6±6.5	17	16
mRS score 0-2 after 90 days	55.3%	47.6%	46.0%	50.5%
Angiographic success overall (mTICI 2b-3 flow, %)	92.8%	76.2%	71.0%	79.6%
Symptomatic intracranial bleeding	11.7%	6.1%	4.4%	5.4%

COMPARISON WITH FIVE RANDOMISED TRIALS[15] IN ANTERIOR STROKE

A previous meta-analysis of five randomised trials (MR CLEAN, ESCAPE, REVASCAT, SWIFT PRIME, and EXTEND IA) found favourable clinical outcomes (mRS score 0-2 after 90 days) in 46% of pts and an angiographic success rate of 71% in cases treated by thrombectomy for anterior stroke. In our study subgroup with anterior stroke only (184 patients), neurological recovery was achieved in 50.5% of cases and successful recanalisation in 79.6%, which is consistent with expert neurointerventional centre data. Furthermore, the risk of symptomatic intracerebral haemorrhage in our study (5.4%) was comparable with that reported in the meta-analysis (4.4%). Baseline characteristics of patients differed only by the presence of AF, which was more common in our study. Details are shown in **Table 4**.

OUTCOMES BASED ON OCCLUSION SITE

The best clinical outcomes (mRS score ≤2 at three months, mortality rate) were reached in patients with MCA and isolated ICA occlusions/tight stenosis. In these patients, the recanalisation success rate was high (81.7%, 89.3%) and blood flow was restored in less than four hours from symptom onset. The incidence of symptomatic intracranial bleeding was the lowest in these two groups as well. Better clinical outcome in these patients might also be influenced by lower initial NIHSS scores as a powerful predictor for long-term outcome[16,17]. T-type occlusion and VB strokes were associated with the poorest outcomes. Untreated VB strokes are associated with almost 100% mortality. Therefore, endovascular treatment is strongly recommended, although, according to a meta-analysis of 45 clinical trials, three patients with this life-threatening diagnosis have to be treated to prevent one death or dependency[5,18]. In our study, patients with T-type occlusion or VB strokes had a higher initial NIHSS score, lower recanalisation success rate and higher incidence of intracranial haemorrhage. VB stroke patients were the oldest subgroup. The North American Solitaire Stent Retriever Acute Stroke (NASA) registry found that age, occlusion site, high NIHSS score, diabetes, no t-PA, ≥3 passes, and use of rescue therapy were associated with poor 90-day outcome despite successful recanalisation[19].

OUTCOMES BASED ON STROKE AETIOLOGY

AF is associated with a 4-5 times increased risk of ischaemic stroke[20]. It is known that patients who suffered from cardioembolic ischaemic stroke tend to have worse outcomes after any (not only endovascular) treatment compared to patients with other aetiologies of stroke[21]. Generally, patients with AF are older, have a worse functional pre-stroke condition and appear to have different occlusion locations and larger infarct volumes[22]. In half of all ischaemic strokes (50.9%), stroke aetiology was assumed to be cardioembolic, mostly due to known or *de novo* identified AF (81.7%). Isolated MCA occlusion and T-type strokes were caused mostly by cardioembolism (68.8% and 61.5%, respectively); VB strokes, isolated ICA occlusion/tight stenosis and tandem occlusion strokes were mostly atherothrombogenic (57.7%, 46.4% and 63%, respectively).

OUTCOMES OF STROKE THROMBECTOMY PERFORMED IN CARDIOLOGY CATH LABS

The main limitation of the widespread use of mechanical thrombectomy for the treatment of acute ischaemic stroke is the lack of experienced interventionalists in many countries and regions. Thus, in some regions, cardiologists in close cooperation with neurologists, offer stroke thrombectomy services. The first published data provided outcomes comparable to those of stroke thrombectomy performed by neuroradiologists[10,23-25]. Our study presents the largest series of consecutive patients with acute ischaemic stroke treated in the cardiology cath lab.

Study limitations

The main study limitation is the single-centre design. However, the consecutive enrolment of all thrombectomy patients treated in this institution during the study period and the complete three-month follow-up formed a good base for this analysis, which does not compare two treatments, but rather several anatomic and aetiologic variants of the same disease treated by the same method.

Conclusions

Catheter-based thrombectomy achieved significantly better clinical results in patients with isolated MCA occlusion, isolated ICA occlusions and tandem occlusions compared to patients with T-type occlusion and posterior strokes.

Impact on daily practice

Stroke aetiology and location of the thrombotic occlusion play a very important role in the angiographic and clinical outcomes of acute ischaemic stroke patients treated by thrombectomy. Patients with posterior circulation strokes and anterior strokes caused by carotid artery terminus occlusion have worse outcomes, and techniques for these stroke subtypes should be further refined.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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Attachment No. 5

ACC22
APRIL 2 - 4 | WASHINGTON, DC

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JACC March 8, 2022
Volume 79, Issue 9, suppl A

Interventional and Structural

NEUTROPHIL EXTRACELLULAR TRAPS (NETS) AND THROMBOLYSIS RESISTANCE - COMPARATIVE ANALYSIS OF THROMBOTIC/EMBOLIC SPECIMENS, RETRIEVED FROM CEREBRAL ARTERIES AFTER MECHANICAL THROMBECTOMY AND CORONARY ARTERY THROMBUS ASPIRATION IN MYOCARDIAL INFARCTION

Poster Contributions
For exact presentation time, refer to the online ACC.22 Program Planner at https://www.abstractsonline.com/pp8/#!/10461

Session Title: Interventional and Structural Flatboard Poster Selections: Basic and Translational Science
Abstract Category: 12. Interventional and Structural: Basic and Translational Science

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Authors: *Luca Mengozzi, Petr Widimsky, Annalisa Angelini, Ilaria Barison, Martin Augustin Maly, Marny Fedrigo, Giulia Lorenzoni, Dario Gregori, Lea Anna Cozzucoli, Radoslav Matej, Charles University: Third Faculty of Medicine, Charles University: project GA UK No. 940120, Prague, Czech Republic, University of Padua: Department of Pathology, Cardiac, Thoracic and Vascular Sciences, Public Health, Padua, Italy*

Background: Thrombosis is linked to neutrophil release of NETs. This immune response, a bactericidal tool, triggers thrombosis. NETs are proposed as a mechanism of resistance to thrombolysis in acute ischemia. This study intends to analyse the composition of thrombi retrieved after mechanical thrombectomy, estimate the age and organization of clots and compare the results with the use of thrombolysis, antiplatelets and anticoagulants in acute phases.

Methods: The samples retrieved from 77 patients (29 CAD and 48 CVA) were cut into 4 slices, then stained with H&E and 3 antibodies: H2B, NE and H3cit. H&E stained sections were evaluated by two pathologists after agreeing on the semiquantitative assessment. All slides were scanned by an Aperio ScanScope CS and analysed with a QuPath software by applying positive cell detection functions with default parameters. Variable distribution was compared using Wilcoxon test and a simplified Monte Carlo significance test. P-values underwent Benjamini-Hochberg corrections.

Results: Specimens were classified as fresh (40), lytic (33) and organized (4 CVA only thrombi). Fresh intracoronary thrombi were 86% of all CAD samples, 31% of all intracerebral samples were fresh and the lytic group of CVA thrombi was 60%. Atherosclerotic plaques were found in 12 patients (3 strokes). The antibody staining estimated the presence of NETs, with anti-NE representing late stage NETosis, anti-H3cit indicating NETosis potential and anti-H2B confirming colocalization. Median values for anti-NE are similar for CAD and CVA samples (8,95 -7,48), with a peak of 10,9 for lytic and 6,8 for fresh thrombi. The median value for anti-NE is practically unchanged among the 34 patients treated with thrombolysis before intervention and the untreated 43 patients. A regression model was used among anti-NE, fibrin-platelet complexes and thrombolysis use and while the increase of positivity for anti-NE is in direct proportion to fibrin-platelets, there is no differential effect of the therapy.

Conclusion: No difference is found in NETs and fibrin-platelets in treated and untreated patients. Thrombolysis may need to include a NET targeting agent (e.g. DNase) in the treatment of acute ischemia.

Attachment No. 6

NEUTROPHIL EXTRACELLULAR TRAPS (NETs) AND THROMBOLYSIS RESISTANCE

COMPARATIVE ANALYSIS OF THROMBOTIC/EMBOLIC SPECIMENS, RETRIEVED FROM CEREBRAL ARTERIES AFTER MECHANICAL THROMBECTOMY AND CORONARY ARTERY THROMBUS ASPIRATION IN MYOCARDIAL INFARCTION

Luca Mencozzi[1], Ilaria Barison[2], Martin Augustin Maly[1], Marny Fedrigo[2], Giulia Lorenzoni[2], Dario Gregori[2], Lisa Anna Cozzuol[2], Radoslav Matej[1], Annalisa Angelini[2], Petr Widimsky[1]

1: Charles University, Third Faculty of Medicine, Charles University, project GA UK No. 940120, Prague, Czech Republic.
2: University of Padua: Department of Cardiac, Thoracic and Vascular Sciences, Public Health, Padua, Italy

BACKGROUND

Thrombosis is linked to neutrophil release of **NETs.** This immune response, a bactericidal tool, triggers thrombosis. NETs are proposed as a mechanism of resistance to thrombolysis in acute ischemia. This study intends to analyse the **composition of thrombi** retrieved after mechanical thrombectomy, estimate the **age** and **organization** of clots and compare the results with the use of **thrombolysis (recombinant tissue-type Plasminogen Activator, *rtPA*)**, antiplatelets and anticoagulants in acute phases.

METHODS

The samples retrieved from 77 patients (29 CAD and 48 CVD) were cut into 4 slices, then **stained with H&E and 3 antibodies: H2B, NE and H3cit (Figure 1**). H&E stained sections were evaluated by two pathologists after agreeing on the semiquantitative assessment. All slides were scanned by an Aperio ScanScope CS and analysed with a QuPath software by applying positive cell detection functions with default parameters. Variable distribution was compared using Wilcoxon test and a simplified Monte Carlo significance test. P-values underwent Benjamini-Hochberg corrections.

RESULTS

Specimens were classified as **fresh (n=40), lytic (n=33) and organized (n=4 CVD only thrombi).** Fresh intracoronary thrombi were 86% of all CAD samples. 31% of all intracerebral samples were fresh and the lytic group of CVD thrombi was 60%. Atherosclerotic plaques were found in 12 patients (3 strokes). **The antibody staining estimated the presence of NETs, with anti-NE representing late stage NETosis, anti-H3cit indicating NETosis potential and anti-H2B confirming colocalization (Figure 2**). Median values for anti-NE are similar for CAD and CVD samples (8,95 -7,48), with a peak of 10,9 for lytic and 6,8 for fresh thrombi. The median value for anti-NE is practically unchanged among the 34 patients treated with thrombolysis before intervention and the untreated 43 patients (Figure 3). A regression model was used among anti-NE, fibrin-platelet complexes and thrombolysis use and while the increase of positivity for anti-NE is in direct proportion to fibrin-platelets, there is no differential effect of the therapy (Table 1).

CONCLUSION

No difference is found in NETs and fibrin-platelets in treated and untreated patients. Thrombolysis may need to include a NET targeting agent (e.g. DNase) in the treatment of acute Ischemia.

THE MORPHOLOGY OF THROMBI, IN PATIENTS TREATED AND NOT TREATED WITH THROMBOLYSIS (rtPA) RETRIEVED AFTER ISCHEMIC EVENTS, SHOW NO SIGNIFICANT VARIATIONS

Table 1: Statistical analysis of the characteristics of thrombi based on the administration of thrombolysis (rtPA) before mechanical thrombectomy/PCI. Values are expressed in absolute numbers, percentages or lower quartile, median and upper quartile.

Characteristics	Not treated with rtPA (N=43)	Treated with rtPA (N=34)	Combined (N=77)	P-value
CORONARY ARTERIES	65% (28)	3% (1)	38% (29)	<0.001
CEREBRAL ARTERIES	35% (15)	97% (33)	62% (48)	
FRESH	67% (29)	32% (11)	52% (40)	0.063
LYTIC	30% (13)	59% (20)	43% (33)	
ORGANIZED	2% (1)	9% (3)	5% (4)	
PMN	5/10/15	7/10/15	5/10/15	0.46
LINES OF ZAHN	0.0/ 5.0/ 7.5	0.0/ 5.0/10.0	0.0/ 5.0/10.0	0.401
RBC	40.0/65.0/76.5	30.0/57.0/70.0	30.0/60.0/75.0	0.401
FIBRIN-PLATELETS	10.0/12.0/25.0	13.5/20.0/45.0	10.0/15.0/35.0	0.132
H2B_IHC	17.2850/24.7600/40.7300	23.1450/37.1100/55.5125	19.6200/32.2700/48.8200	0.132
NE_IHC	4.220/ 7.940/11.155	3.265/ 7.785/22.975	3.640/ 7.900/13.870	0.835
H3-CIT_IHC	18.9850/37.3100/50.7900	18.2450/27.6200/48.4625	18.3500/35.7600/50.1300	0.579

For more information, scan the QR code or go to https://orcid.org/0000-0001-6845-3856

ACC22

NETs

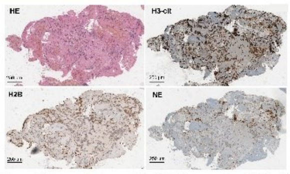

Figure 1: The specimens are stained with Haematoxylin and Eosin (HE) to evaluate the morphology and the age of the thrombus, immunostained with anti-H3 citrullined antibody (H3-cit), anti-H2B (H2B) and anti-Neutrophil Elastase (NE) to evaluate the presence of Neutrophil Extracellular Traps (NETs).

AGE OF THROMBI

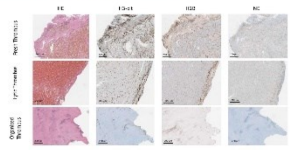

Figure 2: Thrombi of three different age groups: fresh (age <1day), lytic (age between 1 day and 5 days) and organized thrombi (age >5 days). Each specimen is stained with HE. and immunostained with anti-H3cit antibody, anti-H2B antibody and anti-NE antibody. The comparison among different thrombi shows a different positivity level for each individual marker of NETosis: fresh thrombi show an higher level of NETosis activation compared to more organized thrombi.

THROMBOLYSIS

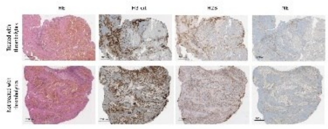

Figure 3: Morphology of a thrombus from a patient treated with thrombolytics and a thrombus from a patient not treated with thrombolytics. Each specimen is stained with HE and immunostained with anti-H3-cit antibody, anti-H2B antibody and anti-NE antibody. The comparison among these two groups does not reveal a significantly different morphology.

Attachment No. 7

MECHANICAL THROMBECTOMY RESULTS VARIABILITY PER OCCLUDED ARTERY IN ISCHAEMIC STROKE.

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Mechanical thrombectomy results variability per occluded artery in ischaemic stroke.

Background

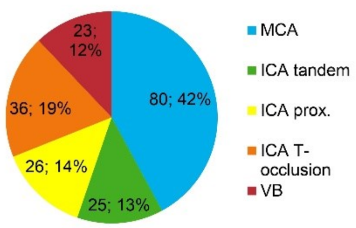
Although mechanical thrombectomy is a rapidly developing technique worldwide in ischaemic stroke, the success rate according to the affected vessel is still not fully established.

Overview

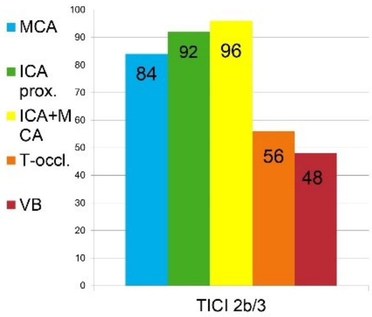
A total of **190 patients** presenting with acute ischaemic stroke undergoing mechanical thrombectomy were divided into **5 subgroups**: (a) isolated occlusion of the middle cerebral artery (MCA), (b) isolated occlusion of the proximal internal carotid artery (ICA prox.), (c) isolated occlusion of the distal ICA (T-occlusion), (d) tandem occlusion of ICAprox and MCA, (e) basilar or vertebral artery occlusion. Immediate reperfusion success rate and 3-months clinical outcomes were analysed according to the occluded artery involved.

190 patients

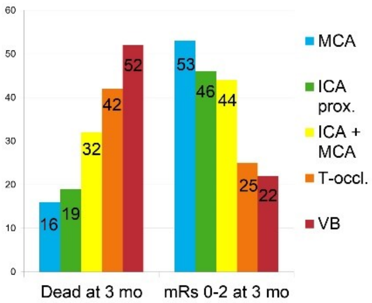
Artery



The success of the intervention depends on the localization of the blockage



Clinical scenario 90 days following a stroke



Isolated occlusions of the MCA represented alone one of the largest subgroups with 42% of the patients, whereas 46% presented with ICA occlusions, of which 19% ICA distal occlusions (T-occlusion), 14% ICA proximal occlusions and 13% ICA proximal / MCA tandem occlusions; only 12% of cases manifested ischaemia of the vertebral/basilar artery (VB). The TICI grading system was used to assess the success rate of the thrombectomy and grade 2b/3 was achieved in 84% of patients with obstructions of the MCA and 92% of cases with ICA proximal occlusions. Altogether, the success rate at TICI 2b/3 for MCA and ICA occlusions reached 96%. Although not the least common, ICA distal occlusions had a post-thrombectomy success rate of 56% and mortality at 3 months of 42%, not quite unlike vertebral/basilar occlusions (VB) with a 48% reperfusion success and a 52% mortality in the first trimester, the latter being the rarest encountered type. Overall, 16% and 19% of patients died within 3 months, previously presenting with respectively MCA and ICA proximal occlusions, of which 53% and 46% scored between 0-2 with the modified Rankin scale (mRs). Only approximately ¼ of patients treated for VB and ICA distal occlusions manifested no significant disability despite some symptoms at 3 months (mRs 0-2).

Results

	MCA (n=80, 42%)	**ICA prox** (n=26, 14%)	**ICA T-occlusion** (n=36, 19%)	**MCA/ ICA tandem occl.** (n=105, 55%)	**Basilar/ vertebral** (n=23, 12%)
TICI IIb/3 rate	84%	92%	56%	96%	48%
mRs 0-2 at 3 months	53%	46%	25%	44%	22%
3-months mortality	16%	19%	42%	32%	52%

Conclusion

Timely thrombectomy provided best outcomes in MCA and/or extra-cranial segment of the ICA. Intracranial ICA (T-occlusion) and vertebrobasilar occlusions offer less favourable outcomes, however endovascular intervention is generally the only chance for these patients due to their grave prognosis with best medical treatment.

Additional information

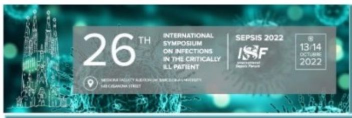
University Hospital Královské Vinohrady and Third Faculty of Medicine, Charles University, Prague, Czech Republic.

Funding : Charles University in Prague.

Data obtained from the database of the stroke unit at the University Hospital Královské Vinohrady in Prague.

The authors have no conflict of interest to declare.

Attachment No. 8



Derivation of a clinical prediction rule to predict bacterial etiology of sepsis at ED admission.

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Background

The latest sepsis guidelines suggest the start of antibacterial therapy in all patients with suspected community acquired sepsis (CAS).Therefore, at least at the moment when sepsis is suspected, the patients with nonbacterial CAS are inappropriately treated with antibiotics. In the age of resistances, it would be advisable to support physicians in the early discrimination of bacterial from nonbacterial CAS.

Materials and Methods

This study is a secondary analysis of a previous multicenter prospective study that aimed to derive and validate a nomogram, based on several serum biomarkers of infection, to discriminate sepsis from sepsis mimickers at ED admission[1].

Only the patients with microbiologically documented sepsis (SOFA>=2) were included in this work.

The objectives of this secondary analysis were to:

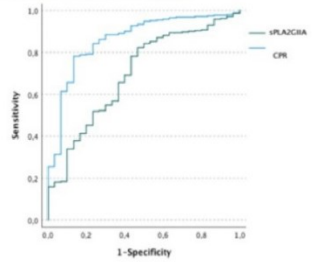
1. Identify the independent serum biomarkers of bacterial CAS among those previously assayed in the original study
2. Derive a robust clinical prediction rule (CPR) for bacterial CAS (logistic regression).

The discrimination power was quantified using C statistics.

Results

The patients with CAS due to bacterial and nonbacterial pathogens were 262 (92%) and 24 (8%), respectively. The median serum concentrations of procalcitonin, soluble phospholipase A2 group IIA (sPLA2GIIA), and soluble interleukin-2 receptor alpha were significantly higher in CAS due to bacteria ($p<0.001$, $p=0.007$, and $p=0.038$, respectively). According to multivariable analysis, the independent predictors of bacterial etiology of sepsis were sPLA2GIIA and non-respiratory source of infection. They were used to build the CPR. The area under the receiver operating characteristic curve of sPLA2GIIA and that of sPLA2GIIA combined with non-respiratory source of infection (i.e. the CPR) to discriminate between bacterial from non bacterial CAS were 0.69 (95% CI: 0.57-0.81) and 0.89 (95% CI: 0.83-0.96), respectively (Figure1).

Figure1. sPLA2GIIA and CPR: receiver operating characteristic curves to discriminate bacterial from non bacterial etiology of sepsis.



Conclusions

Out of the several biomarkers assayed in this study at ED admission, only sPLA2GIIA resulted independently correlated with the bacterial etiology of sepsis. Notably, when sPLA2GIIA was included in multivariable analysis, procalcitonin was excluded.

We derived a CPR that exhibited an high performance in discriminating bacterial from nonbacterial etiology of infection among patients with microbiologically documented sepsis.

References

1-Mearelli et al. Derivation and Validation of a Biomarker-Based Clinical Algorithm to Rule Out Sepsis From Noninfectious Systemic Inflammatory Response Syndrome at Emergency Department Admission: A Multicenter Prospective Study. Crit Care Med. 2018 Sep;46(9):1421-1429.

Attachment No. 9

XXXVII Congresso Nazionale FMSI
"Età biologica, età anagrafica 2.0. Una longevità in salute"
Roma, 20-22 luglio 2023

Evaluation of a suspected Marfan Syndrome in a basketball player: a case report

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Introduction

Marfan syndrome (MFS) is a highly variable systemic connective tissue disease with clinical features similar to a variety of other inherited disorders from which it ought to be distinguished. The purpose of this case report is to draw the attention to the clinical features of MFS.

Clinical case

A young 16-year-old basketball athlete presents himself for annual preparticipation evaluation. At a first anthropometric evaluation we note the **high height** (197 cm) with normal BMI. In the family history, only one paternal uncle with aortic bicuspid is reported. The young man reports anamnestic vertebral kyphoscoliosis for which he was followed by pediatric orthopedics. The athlete is asymptomatic, on inspection there is **pectus excavatum** at the lower third of the sternum, **mild kyphosis** of the right hemithorax and also has an **asymmetry of the chest** with evidence of prominence of the antero-inferior left costal arches. The rest of the physical examination is normal. Visual acuity testing and spirometry are normal. The electrocardiogram at rest and after step test does not present significant abnormalities. The findings of pectus excavatum, chest asymmetry, kyphosis, and mild scoliosis together constitute multiple systemic signs of suspected connective tissue disease. These data suggest an echocardiographic assessment of the size of the ascending aorta and mitral valve. Echocardiographic examination shows **mitral prolapse (MVP)** of both leaflets, thickened, without associated regurgitation. Since no contraindications have emerged, the athlete is eligible for competitive basketball activity.

Revised Ghent criteria	Box 1
In the absence of family history	**In the presence of family history**
Ao (z>=2) and EL=MFS	EL and FH of MFS=MFS
Ao (z>=2) and FBN1=MFS	Syst>=7pts and FH of MFS=MFS
Ao (z>=2) and Syst >=7pts=MFS	Ao (z>=2 above 20 years old or >=3 below 20 years old) + FH of MFS=MFS
EL and FBN1 with known Ao=MFS	
Ao aortic diameter Valsalva, EL ectopia lentis, MFS Marfan Syndrome, Syst systemic score, z z-score	

Box 2 Scoring of systemic features

- Wrist AND thumb sign — 3 (wrist OR thumb sign — 1)
- Pectus carinatum deformity — 2 (pectus excavatum or chest asymmetry — 1)
- Hindfoot deformity — 2 (plain pes planus — 1)
- Pneumothorax — 2
- Dural ectasia — 2
- Protrusio acetabuli — 2
- Reduced US/LS AND increased arm/height AND no severe scoliosis — 1
- Scoliosis or thoracolumbar kyphosis — 1
- Reduced elbow extension — 1
- Facial features (3/5) — 1 (dolichocephaly, enophthalmos, downslanting palpebral fissures, malar hypoplasia, retrognathia)
- Skin striae — 1
- Myopia > 3 diopters - 1
- Mitral valve prolapse (all types) — 1

Maximum total: 20 points; score ≥7 indicates systemic involvement; US/LS, upper segment/lower segment ratio.

Diagnostic criteria

The clinical suspicion of MFS is based on clinical findings which, alone or in combination, should indicate the possible presence of disease. Diagnosis is based on the **Ghent criteria** (Box 1). The following steps are required: identification of any family history (a relative certainly affected by the syndrome or carrier of mutation), study of the dilation of the aortic root by means of echocardiographic examination with the application of international normograms (Z-score) and evaluation of the mitral valve pathology as well as the possible degree of associated valvular insufficiency; identification of the position of the lens and the degree of myopia if present; calculation of the systemic score (Box 2).

In the reported clinical case, absence of familial history, calculated systemic score of 4 points, normal parameters of ascending aorta size, normal visual acuity and absence of visual disturbances does not meet Ghent's criteria for MFS. The primary objective is to formulate the diagnosis as early as possible to identify patients with dilatation of the aortic root at risk of **sudden death** and the possible surgical timing.

Differential diagnoses for MFS include a range of disorders with phenotypic features that partially overlap with the Marfan phenotype, including disorders associated with FBN1/2 or TGFBR1/2 mutations, as well as a variety of other genetic disorders .

In this particular case we consider Mitral valve prolapse syndrome, the revised Ghent criteria for **mitral valve prolapse syndrome** (MVPS) are mitral valve prolapse and systemic features (score <5) and aortic diameter Z <2 and absence of ectopia lentis. Some common systemic features are pectus excavatum, scoliosis and mild arachnodactyly. It is possible to suspect that this athlete may be suffering from mitral prolapse syndrome.

Conclusion

This clinical case highlights how some clinical-anamnestic characteristics are strategic to suspect a connective tissue disease. The diagnosis of MFS is predominantly clinical, so clinical suspicion and instrumental insight are imperative in identifying patients with MFS for early diagnosis. In fact, MFS is a common and predictable cause of sudden death in athletes from aortic dissection.

MFS is the main connective tissue disease and the use of Ghent criteria is fundamental in diagnosis and orientation in differential diagnostics. In the event that there is no familiarity with MFS or Ghent's criteria are not met, the suspicion of connective tissue disease opens up numerous pathologies characterized by a wide clinical presentation with important repercussions in the follow-up of these patients and in family screening. **In our case, the clinical suspicion of MFS allowed us to diagnose an otherwise unknown mitral prolapse.**

References: The revised Ghent nosology for the Marfan syndrome. Loeys BL, Dietz HC, et all. J Med Genet. 2010 Jul;47(7):476-85.