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In vitro clot lysis assays for the prediction of outcomes in acute ischemic and hemorrhagic strokes

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INTRODUCTION

Cerebrovascular diseases are among the leading causes of death in developed countries. About 80% of all stroke cases are ischemic strokes (AIS), about 15-18% are intracerebral hemorrhagic strokes (ICH) and about 3-5% are subarachnoid haemorrhages. The cause of stroke is often unknown, and the prognosis is difficult to predict. The only effective drug currently registered in Hungary for the treatment of AIS is recombinant tissue-type plasminogen activator (rt-PA). Although this treatment has been shown to be effective in clinical studies, only about 30-35% of patients improve and 6-8% of patients develop intracranial hemorrhagic complications as a consequence of therapy. Mortality rates are high for both AIS and ICH, but the latter is much higher than the former. Mortality for ICH is estimated to be 40% in the first month and 54% in the first year after the event. Independent of the type of stroke, the majority of studies in the literature primarily attempt to estimate the risk of stroke by specific biomarkers, with far fewer studies investigating hemostasis tests that potentially predict stroke outcome. Several clinical factors are known to potentially increase the risk of unfavorable stroke outcome (e.g., advanced age, stroke severity at admission, arterial hypertension, etc.), but accurate prediction of clinical outcome at admission is not yet possible. As the coagulation and fibrinolytic system plays a key role in the pathogenesis of both AIS and ICH, the identification of appropriate specific hemostasis laboratory markers or tests may theoretically provide the opportunity to predict stroke outcome. Early prediction of stroke prognosis and potential complications may facilitate the selection of appropriate therapy as early as in the acute phase of the disease, improving the patient's chances of survival and the likelihood of developing a smaller neurological deficit. However, due to logistical reasons, in most studies, biomarkers are not measured in blood samples collected at the time of admission, but in blood samples taken a in a period of a few days following the event. During this period, however, significant changes can occur that affect the balance of hemostasis. The number of publications in the literature on specific markers of fibrinolysis or methods for the global assessment of fibrinolysis is still scarce, although it may seem obvious that there may be methods used for the assessment of fibrinolysis that could have predictive value regarding the outcome of cerebrovascular pathologies.

LITERATURE REVIEW

Hemostasis system

Hemostasis is the physiological defence mechanism against vascular injury. Both platelets and the coagulation system are required for coagulation to occur and to minimise bleeding at the site of vascular injury and to create a haemostatic plug composed of platelets and fibrin.

The cascade-like activation system of blood clotting was first described by MacFarlane and colleagues in 1964. The cascade-like activation process they outlined was divided into two stages; the intrinsic and extrinsic pathways, which continued along a common pathway. Today, the cascade system described by MacFarlane and his research team has been replaced by a cell-based model of hemostasis defined by Hoffman and Monroe in 2001. The system they describe divides hemostasis into three overlapping phases: initiation, amplification, and propagation.

The initiation step is localized to the cells expressing tissue factor (endothelium, subendothelial structures). At this stage, the activated factor VII (FVIIa)-tissue factor complex activates small amounts of factors IX and X. Subsequently, activated factor X (FXa), together with activated factor V (FVa), forms a small amount of prothrombinase complex on the surface of tissue factor–bearing cells, which converts prothrombin to thrombin. The process remains localized by keeping cell surface–bound FXa protected from plasma protease inhibitors. If FXa dissociates from tissue factor–bearing cells, its activity is rapidly inhibited in the fluid phase by tissue factor pathway inhibitor (TFPI) or antithrombin. Accordingly, only a few active coagulation proteins are formed during the initiation phase.

The amplification step involves the activation of platelets and their cofactors by small amounts of thrombin formed on tissue factor–bearing cells. Although platelets are already adhered and partially activated at the site of injury, the presence of thrombin may induce higher procoagulant activity than adhesion interactions alone. Another function of thrombin formed during the initiation phase is to activate cofactors V and VIII and factor XI, now present in high amounts on the platelet surface. Adenosine diphosphate (ADP) released from platelet dense granules further enhances platelet activation and aggregation. By the end of the amplification phase, enhanced thrombin generation occurs.

The propagation phase takes place on the surface of activated platelets and is formed by several coagulation proteins. In this step, FIX (FIXa), activated during initiation, binds to FVIIIa on the platelet surface, generating substantial amounts of the tenase complex through a positive feedback mechanism. FXa, when combined with its cofactor FVa, subsequently forms the

prothrombinase complex in large amounts. At this stage, prothrombinase already converts substantial amounts of prothrombin into thrombin. Thus, during the propagation phase, the activity of the procoagulant complexes results in explosive thrombin formation, which generates fibrin from fibrinogen by fibrinopeptide cleavage. The thrombin formed also activates factor XIII (FXIII) in the final step of coagulation. Activated FXIII (FXIIIa) cross-links fibrin chains and binds α2-plasmin inhibitor (α2-PI, anti-plasmin), a major inhibitor of fibrinolysis, to the clot that has formed, thus stabilising the clot.

Previous studies have shown that the quality of the clot formed during coagulation is strongly influenced by the presence of blood cells, cellular elements and cell-derived components at the site of injury. In addition to the role of platelets and red blood cells, recent studies have revealed previously unknown effects of neutrophil extracellular traps (NETs) on fibrin formation, structure, and stability. The results of this work may be important for the future understanding of blood coagulation disorders, particularly thrombotic events.

Neutrophil extracellular trap (NET)

The neutrophil extracellular trap, also known as NET, was first described in 2004 by Brinkmann and his research team as an extracellular network consisting of mainly DNA, histones and granular proteins released from activated neutrophils in response to an inflammatory stimulus. The results of Brinkmann and his coworkers showed that NETs contained some proteins from azurophilic (primary) granules, such as neutrophil elastase and myeloperoxidase, as well as proteins from specific (secondary) and tertiary granules. Based on the results of DNA intercalating staining procedures, it was also described that DNA is a major structural component of NETs. Double immunostaining of the globular domains of NETs confirmed colocalization of neutrophil elastase and histones. Early observations by Brinkmann's team suggested that NET formation is part of the immune response against bacteria introduced into the human body, i.e., NET formation is a newly described form of early neutrophil cell death. Results from further studies have also demonstrated that NETs are the result of a unique form of cell death, morphologically characterised by the loss of intracellular membranes before the integrity of the plasma membrane is compromised. Steinberg and Grinstein, in a study published in 2007, coined the term NETosis, which is morphologically quite different from apoptosis and other forms of cell death.

Brinkman and Zychlinsky, in a summarising study published in 2012, described that NETs can fight various pathogens and diseases depending on location, time and activator dose, but they can also contribute to the pathomechanism of diseases, such as chronic inflammatory diseases

and thrombotic diseases. In 2012, Manzenreiter and colleagues detected the presence of NETs in the lung secretions of patients with cystic fibrosis, contributing to an increase in viscosity. Previously published studies have also shown the presence of NETs in the placenta of pregnant women with preeclampsia and have linked it to the pathomechanism of the disease. Further studies have shown the presence of NETs in plasma samples from patients with autoimmune diseases such as systemic lupus erythematosus (SLE). Their results have shown that patients with SLE, particularly women, often produce antibodies against DNA, histone and neutrophil proteins, components of NETs. One of the most important effects of NETs is hypothesised to be on the hemostasis system. The role of NETs in blood coagulation is an example of how the amount of NETs formed modulates the pathophysiology of a particular disease. NETs are physiologically involved in clot formation in small amounts, but when NET formation is excessive, massive coagulation and thrombotic complications can develop, causing ischaemia. The role of NETs in thrombosis was first reported by Fuchs et al. Their study showed that NETs bind to platelets, promoting their activation and aggregation. They hypothesised that the DNA-histone complex backbone of NETs stabilises fibrin and showed that the clot formed in the presence of the DNA-histone complex is a denser network of thinner fibrin fibres that is more resistant to fibrinolysis.

The presence of NETs in thrombi has been demonstrated in both arterial (myocardial infarction, stroke) and venous (deep vein thrombosis, pulmonary embolism) thrombotic events. Several studies have reported the presence of NETs in catheter-removed thrombi from patients with acute ischemic stroke (AIS). A few small studies have shown an association between the amount of NETs and unfavorable outcome in AIS patients undergoing thrombectomy.

Fibrinolysis

The fibrinolytic system is a proteolytic enzyme system with many physiological functions, the best known and most important of which is the breakdown of fibrin clot formed during excessive coagulation. Functional disturbances in the fibrinolytic system may be the result of overactivation (bleeding tendency) or impaired activation (thrombotic complications, fibrin deposition). The fibrinolytic system consists of an inactive proenzyme, plasminogen, and its activators and inhibitors, whose specific interactions with each other ensure efficient clot dissolution during fibrinolysis.

Plasminogen is converted into plasmin, the central protease of the fibrinolytic system, by plasminogen activators. Two physiological plasminogen activators are known; the most important one is the tissue-type plasminogen activator (t-PA) and the other is the urokinase-

type plasminogen activator (u-PA). t-PA efficiently converts plasminogen to plasmin on the surface of fibrin, so t-PA-mediated plasminogen activation is primarily involved in the dissolution of fibrin in the vasculature. u-PA binds to a specific u-PA receptor (u-PAR), resulting in increased activation of cell-bound plasminogen, thus u-PA does not require the fibrin surface for its function. Plasmin can cleave both t-PA and u-PA, converting them from single-chain polypeptides to more active double-chain polypeptides. The presence of fibrin can increase the slow activation of plasminogen by t-PA by up to three orders of magnitude and it protects plasmin from specific inhibitors.

Fibrinolysis is a tightly regulated process that is controlled at several levels by potent inhibitors. Inhibition of fibrinolysis can be achieved by direct inhibition of plasmin activity, inhibition of plasmin activation by direct inhibition of plasminogen activators, or inhibition of plasminogen and t-PA binding to fibrin. Direct inhibition of plasmin activity is one of the main and most effective regulatory mechanisms. The main physiological inhibitor of plasmin is α2-PI, which is cross-linked by FXIIIa to the α-chains of fibrin, thus effectively protecting the newly developed clot from fibrinolysis. Free, un-bound α2-PI inactivates free plasmin very rapidly, forming a plasmin-antiplasmin (PAP) complex, thereby preventing systemic spread of fibrinolysis. A further important level of inhibition of fibrinolysis is the prevention of plasminogen-to-plasmin conversion by inhibition of plasminogen activators. The most important inhibitor of plasminogen activators is plasminogen activator inhibitor-1 (PAI-1). PAI-1 is a serine protease inhibitor that effectively inactivates t-PA and u-PA in the circulation. The concentration of PAI-1 in the circulation always predominates over that of plasminogen activators, and therefore t-PA is predominantly present in the circulation in the form of t-PA-PAI-1 complex. Another important level of regulation of fibrinolysis is the inhibition of the binding of plasminogen and t-PA to fibrin. Thrombin-activated fibrinolysis inhibitor (TAFI) removes C-terminal lysine binding sites from fibrin following activation, thereby reducing the affinity of fibrin for plasminogen and t-PA. This process reduces the extent of plasminogen activation and thus TAFI provides effective protection against fibrinolysis.

Global tests for fibrinolysis

The role of fibrinolysis in the maintenance of haemostatic balance was recognised relatively early, but the development of fibrinolysis tests, both at individual and global levels, has lagged significantly behind the development and standardisation of coagulation tests. Both in routine laboratory diagnostics and in research laboratory practice, there are only a few methods

available for the complex investigation of the process of fibrinolysis as compared to the assessment of coagulation. This is mainly due to the fact that, unlike coagulation, fibrinolysis is a very slow process, under physiological conditions hours or even days are required to study lysis, and therefore the speed of the reactions in the tests used must be modified. In most fibrinolysis assays, either inhibitors are removed from, or activators are added to the system, which means that the test results do not fully reflect reality. Most fibrinolysis assays are technically cumbersome to perform, making the majority of tests unsuitable for automation. However, fibrinolysis testing can be important for several reasons. Fibrinolysis imbalances may be associated with bleeding or excessive fibrin deposition, and effective drug treatments are available for these conditions. Increased fibrinolysis (hyperfibrinolysis) can be associated with bleeding complications and even life-threatening blood loss. Hypofibrinolysis has been associated in some reports with the development of thrombotic events, in particular with an increased risk of venous thromboembolism.

Individual tests (e.g., determination of plasminogen, plasminogen activators, plasminogen activator inhibitors, and plasmin inhibitor levels, or measuring the levels of certain markers indicating activation of fibrinolysis) and global tests can be used to test fibrinolysis. Global tests for fibrinolysis can be performed on whole blood, for example by viscoelastic methods such as thromboelastography (TEG) and rotational thromboelastometry (ROTEM). Plasma-based tests for global fibrinolysis tests are also known, such as the plasma clot lysis time, the fibrin plate method, or the simultaneous thrombin and plasmin generation test. In the euglobulin lysis time measurement, the euglobulin fraction of the plasma is used to test global fibrinolysis, although the test is now of historical significance. Currently, there is no gold standard method for the global assessment of fibrinolysis, each test has its advantages and disadvantages. Some tests are more sensitive to detect hypo- or hyperfibrinolysis, thus it is important to choose the right type of test according to the clinical/experimental situation.

In vitro clot lysis assay (CLA)

The *in vitro* clot lysis assay (CLA) is a global test for fibrinolysis. In this test, clot formation is induced in plasma samples by the addition of recombinant tissue factor and phospholipids or thrombin, followed by the addition of rt-PA to induce lysis, which is monitored in real time by turbidimetry. However, the procedure is very time-consuming and labour-intensive and is not currently a routine laboratory test. Although an international process of standardisation of the method has started in recent years, the conditions for performing CLA vary from laboratory to laboratory. The differences in methods are due to the different compositions of the buffers used,

different plasma dilutions, varying concentrations of the reagents and solutions (t-PA, thrombin, calcium chloride, tissue factor-phospholipid) added to the system, and the fact that some research groups induce clot formation by adding recombinant tissue factor, while others do the same with thrombin. However, the principle of the method is the same for all, i.e., fibrinogen-to-fibrin conversion leads to the formation of a fibrin clot, the light that passes through the fibrin meshwork is scattered and the resulting turbidity is proportional to the extent of fibrin formation. The formation of the clot is associated with an increase in absorbance, whereas clot lysis is associated with a decrease in absorbance. The method of evaluating the turbidimetric curves obtained during CLA may vary between research groups, but in general the parameters used are: maximum absorbance, time to reach maximum absorbance, 10-, 50- and 90% clot lysis time (10%CLT, 50%CLT, 90%CLT) and area under the curve (AUC). To evaluate the curves and determine the parameters described above, a software called Shiny Apps ClotlysisCL was developed in 2017 and it is available online for free.

To make the CLA method more reflective of reality, different research groups add different cellular elements, activators, inhibitors to the system. Kolev and his group modified the CLA method to mimic NETs in the thrombus in vivo by cell-free DNA (cfDNA) and histones. Their research results showed that the rigidity, viscoelastic parameters and lysis of purified fibrin or plasma clots formed by the addition of cfDNA or histones were significantly modified. Prolonged clot lysis was observed as a combined effect of cfDNA and histone added at concentrations mimicking in vivo conditions. Based on their results, it was suggested that studying the anti-fibrinolytic effect of NETs may be of interest in a number of clinical pathologies, in particular with regard to thrombolytic therapy.

Stroke

Stroke is the second leading cause of death after ischemic heart disease and the most common cause of disability worldwide. The incidence of stroke has increased significantly in recent years, with stroke-related deaths rising by around 30% globally. Approximately 80% of all stroke cases are ischemic, about 15-18% are non-traumatic intracerebral haemorrhages, the remaining 3-5% are subarachnoid haemorrhages. The proportion of aetiological subtypes of stroke varies between populations of different ages and ethnic backgrounds.

The risk factors for stroke can be divided into controllable and non-controllable risk factors. Clinical studies have shown that the most important risk factor for ischemic and hemorrhagic stroke is high blood pressure, and the most important non-controllable risk factor is advanced

age. Other well-known risk factors include hypercholesterolaemia, atrial fibrillation, smoking, excessive alcohol consumption, diabetes mellitus, and risk from inappropriate diet, obesity, physical inactivity, sleep disturbance, chronic inflammation, chronic kidney disease, hormonal therapy, stress, and depression.

According to the WHO definition, stroke is "rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than of vascular origin". Typical symptoms of stroke include sudden onset of unilateral weakness, numbness, visual disturbances, altered speech, ataxia, and dizziness. It can be detected by the Face Arm and Speech Test (F.A.S.T.), which can be used even by people without medical training to identify the most characteristic symptoms of stroke. To diagnose a stroke, it is essential to take a proper history, perform clinical neurological examinations and brain imaging studies, all of which are complemented by laboratory tests. When taking the history, it is important to clarify the background of the onset of symptoms, underlying medical conditions and the medications the patient is taking. Imaging techniques such as CT and MRI scans play a primary role in the diagnosis. Cranial CT is highly sensitive for the detection of acute intracranial haemorrhage. The purpose of imaging studies is to assess the extent of the lesion in addition to the detection of haemorrhage.

Acute ischemic stroke

Approximately 80% of strokes are ischemic in origin, occurring due to the blockage of a cerebral vessel, causing ischemic damage to the brain tissue in the area supplied by the vessel and resulting in loss of neurological function. The aim of treating ischemic stroke is to rapidly restore blood flow in order to limit the developing physical and mental disability.

Acute ischemic stroke (AIS) is divided into two main subtypes, thrombotic and embolic. Thrombotic strokes, which account for about 45% of all ischemic strokes, can occur in large and small vessels of the brain. Embolic strokes account for about 20% of all ischemic strokes. In this case, the thrombus does not originate in the brain but travels through the bloodstream to an artery in the brain. The heart is the most common source of such emboli, particulary in patients with atrial fibrillation. The remaining proportion of AIS are the so-called cryptogenic strokes. Cryptogenic stroke is a type of ischemic stroke with an uncertain or unknown cause. The etiological subtypes of ischemic stroke are classified according to the TOAST (Trial of ORG 10172 in Acute Stroke Treatment) criteria system.

To assess the clinical severity of stroke, neurologists use the National Institutes of Health Stroke Scale (NIHSS). The NIHSS is an 11-item scale that provides a quantitative measure of key

components of standard neurological tests. The scale system assesses alertness, extraocular movements, facial muscle function, limb strength, sensory function, coordination and speech. The maximum score is 42, the better the patient performs, i.e., the milder the stroke, the lower the score.

In international practice, the most common method for the assessment of the radiological severity of stroke is the ASPECTS (Alberta Stroke Program Early CT Score) scoring system. ASPECTS is used to assess the severity of early ischemic lesions in the region supplied by the middle cerebral artery. It is a 10-point scale, with a maximum of 10 points representing normal physiological status. The scoring system divides the region supplied by the middle cerebral artery into 10 areas, with 1 point deducted for ischaemia in each area.

The modified Rankin Scale (mRS) is the most commonly used way to assess the long-term functional outcome of AIS. The mRS is a scoring system ranging from 0 to 6, which provides an assessment of mobility following an event. mRS can be used to determine the degree of disability or ability to care for oneself. A score of mRS=0 indicates complete self-sufficiency, while a score of 6 indicates death.

The only drug currently registered in Hungary for the effective, targeted treatment of AIS is intravenous thrombolysis with rt-PA. The limitation of this treatment is that it has a narrow therapeutic window, i.e., it should be initiated within 3-4.5 hours after the onset of symptoms. The dose of intravenous rt-PA is 0.9 mg/kg, and it must not exceed 90 mg. The first half of the treatment, i.e., 10% of the dose, is administered as a bolus to AIS patients, followed by the remaining 90% administered as continuous intravenous infusion over 60 minutes. However, therapy only leads to clinical improvement in 30-40% of cases and in the majority of cases the hoped-for improved outcome is not achieved. An important complication is the hemorrhagic transformation of ischemic stroke, which can also occur spontaneously without thrombolytic therapy, especially in the setting of embolic infarction and elevated blood glucose. However, it is difficult to predict the outcome before starting thrombolytic therapy, which is due to the non-specificity and low predictive value of currently available risk assessment scales. It can be assumed that individual differences in the balance between coagulation and fibrinolysis, which determine the structure and solubility of the clot, may play a key role in recanalization failure and in the development of hemorrhagic complications. Nevertheless, there are currently no known hemostasis or fibrinolysis biomarkers that have been shown to provide useful information on the outcome of therapy at admission. As there may be numerous factors affecting the balance of hemostasis and fibrinolysis, it can be assumed that in clinical practice,

the use of global tests may be the appropriate choice to rapidly assess outcome and to guide clinical decision-making.

Nowadays, catheter-directed (mechanical) thrombectomy is another important option for the rapid restoration of circulation in AIS patients, which is only possible in the case of large vessel occlusion. Mechanical thrombectomy is most successfully used in patients with good collateral circulation, anterior circulation involvement, severe neurological deficit, but without extensive early ischemic signs, no severe comorbidities and adequate pre-stroke functional status. The therapeutic window for mechanical thrombectomy is 6 hours, i.e., it can be used after a failed thrombolysis. The risk of thrombectomy is related to hemorrhagic complications after the procedure, as is the case with thrombolysis using rt-PA. Based on current international recommendations, the effectiveness of mechanical thrombectomy is enhanced if thrombolysis is performed in eligible AIS patients before the procedure.

Non-traumatic intracerebral hemorrhagic stroke

Intracerebral hemorrhagic stroke (ICH) accounts for about 15-18% of all strokes, but the mortality rate is much higher. It is about 40% in the first month and 54% in the first year. The global incidence of ICH has increased in recent decades. Hemorrhagic stroke is classified based on its anatomical location or presumed aetiology. The most common site of ICH is supratentorial (85-95%), with is further divided into extensive hemispheric, medial and lateral basal ganglia, thalamic and lobar haemorrhages. Infratentorial haemorrhages include brainstem haemorrhage and cerebellar haemorrhage. The most common causes of ICH are hypertension (30-60%), cerebral amyloid angiopathy or arteritis (10-30%), anticoagulation or coagulation disorders (1-20%), vascular abnormalities such as aneurysms, angiomas, arterio-venous malformations (3-8%) and in about 5-20% of cases the cause is undetermined. Risk factors include diabetes mellitus, smoking, alcohol and certain drugs. Stroke may also occur at the site of an existing cerebral lesion, e.g., central nervous system tumour, granuloma, inflammation, e.g., encephalitis, abscess. Although ICH of hypertensive origin remains the most common form, due to the high risk of recurrent bleeding and the available treatment options, it is important to consider the possibility of underlying vascular abnormalities in all cases.

ICH patients should ideally be treated in a comprehensive hospital setting involving a multidisciplinary healthcare team, consisting of neurology, neurosurgery, neuroradiology, intensive care, emergency care and internal medicine specialists. Following hospital admission, it is important to take a medical history and perform detailed neurological and imaging examinations. It is important to clarify whether the patient has an elevated systolic blood

pressure, is taking anticoagulants, has intraventricular extension, shows signs of hydrocephalus, has evidence of macrovascular haemorrhage, and is at risk of haematoma expansion. All these data can be critical for establishing a rapid diagnosis and treatment protocol for ICH. ICH is a life-threatening medical emergency requiring rapid diagnosis. Radiological imaging is essential for reliable differentiation of ICH from AIS. CT angiography, non-contrast CT or MR angiography may also be suitable for the detection of macrovascular sources of bleeding. Early intensive care in hospital settings has been shown to have a direct impact on morbidity and mortality following ICH. Recommendations for non-surgical management of bleeding are not uniform and, based on the available small, randomized trials, the benefits of neither surgical nor medical treatment have been clearly demonstrated. The immediate goal after diagnosis is to minimise the risk of rebleeding and hematoma spread within the first 24-72 hours. It is important to stabilise blood pressure, as persistently elevated blood pressure is a potential consequence of haematoma growth. Correction of the coagulation abnormality, including suspension of anticoagulants, is an important part of conservative management.

The outcome of ICH is influenced by many factors, one of which is the volume of the haematoma. The volume of hematoma formed during ICH is most often determined with the ABC/2 method based on the results of CT scans. The ICH score is a scoring system ranging from 0 to 6 and is the most commonly used clinical scale system in international practice for predicting 30-day mortality in ICH. The ICH score includes age, estimated haemorrhage volume, presence of intraventricular haemorrhage, localisation of ICH (supra/infratentorial origin), and Glasgow Coma Scale (GCS) score. The higher the patients' calculated ICH score, the higher the likelihood of mortality is within the first month after the event. An ICH score= 6 is associated with a mortality rate of 100%.

Understanding the extent, evolution and impact of intracerebral haematoma on cerebral physiology may be critical for the development of better, state-of-the-art treatment protocols and improved patient survival in the future. Based on current treatment guidelines, it is clear that, in contrast to the management of acute ischemic stroke, the management of hemorrhagic stroke has changed little over the past decades. Our knowledge about changes in hemostasis and fibrinolysis in ICH patients is very limited. We have only a few studies describing how high D-dimer levels may predict mortality, whereas a global study of fibrinolysis in a cohort of ICH patients has not yet been performed.

AIMS

Our aim was to investigate whether a newly-developed, modified *in vitro* CLA assay (mCLA), that incorporates the effect of cfDNA and histones to mimic the presence of NETs, is able predict short- and long-term functional outcome in patients with acute ischemic stroke undergoing thrombolytic treatment and in patients with non-traumatic hemorrhagic stroke.
In details, by means of two prospective observational studies, we aimed to investigate whether:

1. cfDNA levels, parameters of the *in vitro* CLA and the modified CLA in plasma samples from patients with acute ischemic stroke undergoing intravenous thrombolytic therapy are associated with stroke severity and whether the results predict outcomes and hemorrhagic complications.
2. Different parameters of the *in vitro* CLA and modified CLA in plasma samples collected at admission from patients with non-traumatic intracerebral hemorrhagic stroke are associated with stroke severity, haematoma volume, stroke mortality and long-term functional outcomes.

Examination of acute ischemic stroke patients undergoing intravenous thrombolysis

In this observational study, AIS patients were enrolled in a single stroke center (Department of Neurology, University of Debrecen, Hungary). Patient enrollment started in September 2016 and finished in April 2019. Inclusion and exclusion criteria of patients were identical to the standard criteria of rt-PA administration according to 2008 ESO guideline. All patients underwent thrombolysis within the 4.5 h therapeutic time window using rt-PA (Boehringer Ingelheim, Germany) according to standard protocols. Patients receiving mechanical thrombectomy in addition to thrombolysis were not included in the study. The presence of AIS was diagnosed based on clinical symptoms, brain imaging using non-contrast computerized tomography (CT) scan, and CT angiography (CTA). A control CT was performed for every patient 24 h after the event. CT images taken on admission and 24 h post-lysis was analyzed simultaneously by 3 independent investigators and the Alberta Stroke Program Early CT Scores (ASPECTS) were calculated. For each patient, the time of symptom onset, demographic and clinical characteristics (age, sex, BMI, previous medications, history of cerebrovascular and cardiovascular diseases, cerebrovascular risk factors including smoking) were registered on admission. Stroke severity was determined by the National Institutes of Health Stroke Scale (NIHSS) on admission and day 7 after therapy. Trial of ORG 10172 in Acute Stroke Treatment (TOAST) criteria was used to identify the etiology of stroke. Patients were followed and long-term functional outcomes were determined at 3 months after the stroke event using the modified Rankin Scale (mRS).

The following outcomes and safety endpoint were registered:

1/ Short-term outcome at 7 days post-event: a decrease in NIHSS score by at least 4 points or to 0 was defined as favorable outcome, while an increase in NIHSS score by at least 4 points was defined as unfavorable outcome.

2/ Long-term outcome at 90 days post-event: mRS 0-1 was defined as favorable long-term outcome.

3/ Hemorrhagic transformation: symptomatic or asymptomatic intracranial hemorrhage (ICH) using the European Cooperative Acute Stroke Study (ECASS) II criteria

Examination of non-traumatic intracerebral hemorrhagic stroke patients

In this prospective observational study, consecutive patients with non-traumatic intracerebral hemorrhage stroke (ICH) were enrolled in a single stroke center (Department of Neurology, University of Debrecen, Hungary). Patient enrollment started in June 2017 and finished in September 2020. Inclusion criteria were: patients over 18 years of age with acute non-traumatic intracerebral hemorrhage, verified with non-contrast computerized tomography (NCCT) scan. Exclusion criteria included the presence of cerebral aneurysm, AV malformation, epidural hemorrhage, subdural hemorrhage, malignancy, severe hepatic- and renal insufficiency, hemorrhagic diathesis and SARS-CoV-2 infection at hospital admission or during follow-up. The presence of ICH was diagnosed by complex neurological examination based on clinical symptoms, brain imaging using NCCT scan. Follow-up NCCT scans were performed 14 days and 3 months after the event. CT images were analysed simultaneously by 3 independent investigators and a comprehensive list of radiographic features and estimated ICH volume was recorded. For each patient, the time of symptom onset, demographic and clinical characteristics (age, sex, BMI, previous medications, history of cerebrovascular and cardiovascular diseases, cerebrovascular risk factors including smoking) were registered on admission. Stroke severity was determined by the National Institutes of Health Stroke Scale (NIHSS) on admission and on day 7. Risk stratification of each patient was performed using the ICH score (based on GCS score, age, infratentorial origin, intraventricular hemorrhage and ICH volume). Patients were followed and long-term functional outcomes were determined at 3 months after the stroke event using the modified Rankin Scale (mRS). As from March 2020, all patients were investigated about potential acquiration and symptoms of SARS-CoV-2 infection on admission and during follow-up. In case of a suspected infection, the diagnosis was confirmed by a routine method of reverse transcriptase polymerase chain reaction testing of RNA extracted from nasopharyngeal/oropharyngeal swabs.

The study also examined a group of healthy volunteers. All selected volunteers were 18 years of age or older with no history of underlying disease other than arterial hypertension and no history of venous or arterial thrombotic events in the family history.

The following outcomes were investigated:

1/ Mortality by day 14 and day 90.

2/ Long-term outcome at 90 days post-event: mRS 0-1 was defined as favorable long-term outcome.

Informed consent

The study designs were in accordance with the guiding principles of the Declaration of Helsinki and were approved by the Institutional Ethics Committee of the University of Debrecen and the Ethics Committee of the National Medical Research Council. All patients or their relatives provided written informed consent.

Blood sampling and laboratory measurements

Peripheral blood samples were taken from all patients on admission. Routine laboratory tests (ions, glucose level, renal and liver function tests, high-sensitivity C-reactive protein measurement, complete blood count) were carried out immediately by standard laboratory methods (Roche Diagnostics, Mannheim, Germany and Sysmex Europe GmbH, Hamburg, Germany). For the examination of hemostasis tests and cfDNA, blood samples were collected to vacutainer tubes containing 0.109 M sodium citrate (Becton Dickinson, Franklin Lane, NJ) and were processed immediately to gain platelet free plasma (centrifugation twice at 2690 RPM, room temperature for 15 min). Screening tests of coagulation (prothrombin time, activated partial thromboplastin time, and thrombin time) were performed immediately on a BCS coagulometer using routine methods (Siemens Healthcare Diagnostic Products, Marburg, Germany). For the execution of *in vitro* CLA, other specific hemostasis tests, and cfDNA, aliquots of citrated plasma were labelled with a unique code and stored at −80°C until analysis.

Measurement of cell-free DNA (cfDNA)

Plasma cfDNA levels were quantified using fluorescent nucleic acid stain, Quant-iT PicoGreen dsDNA reagent and kit assay (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions. Briefly, a 5-point standard from 1 ng/mL to 1 μg/ml was prepared by serial dilutions. Standards and samples (100 μl) were loaded into black 96-well plates (Greiner Bio-One, Kremsmünster, Austria) followed by the addition of 100 μl working solution of the Quant-iT PicoGreen reagent to each sample, incubation for 5 minutes at room temperature, protected from light. The fluorescence intensity was quantified at 480 nm using a TECAN Infinite p200 PRO microplate reader (TECAN Trading AG, Männedorf,

Switzerland). Based on the measured values, the cfDNA concentration of each plasma sample was determined using a standard curve.

In vitro clot lysis assays

Recombinant t-PA-driven lysis of tissue factor-induced plasma clots was studied in 96-well microtiter plates by monitoring changes in turbidity. Final assay conditions were set based on previous studies, with some modifications, optimized for reliable high-throughput analysis of patient samples. Two assay conditions were used, and plasma samples were run in quadruplicates in both assay conditions. All concentrations provided refer to final concentrations in the 100 μL final well volume. Plasma samples were thawed in a water bath at 37°C. In the first assay condition, a clot induction and lysis mix were prepared, where citrated plasma was mixed with 1000-fold diluted human tissue factor (Innovin, Siemens, Marburg, Germany) and 100 ng /ml rt-PA (Alteplase, Boehringer Ingelheim, Ingelheim, Germany) in HEPES buffer (10mM HEPES, 150mM NaCl, 0.05% Tween20, pH:7.4). In another set of experiments, in order to imitate the effect of NETs, 150 μg/ml pure and cell-free DNA (cfDNA; calf thymus DNA, Sigma-Aldrich, Darmstadt, Germany) and 50 μg/ml calf thymus histone (TIII S, Calbiochem, La Jolla, CA, USA) were added to the clot induction and lysis mixtures. Optimal concentrations of cfDNA and histones were adopted based on previous literature where the combined effect of histones (50 μg/ml) and various concentrations of cfDNA (50-250 μg/ml) were studied on fibrinolysis kinetics in purified experimental conditions. Dilution of plasma samples with buffer was 1.2-fold. Clotting and subsequent lysis were induced with automated sample pipetting of HEPES buffer, containing 21 mM $CaCl_2$, to each sample well. Optical density was measured at 340 nm, 37 °C every minute for 300 min in a TECAN Infinite m200 microplate reader (TECAN Trading AG, Männedorf, Switzerland). Curves were analyzed using the Shiny app software tool. Clot formation and lysis were defined using the following variables calculated from the turbidimetric curves: maximum absorbance, time to maximum absorbance, various points of clot lysis time (CLT): 10% clot lysis time (10%CLT), 50%CLT, 90%CLT and area under the curve (CLA AUC). Clot lysis times were defined as the time from the 10%, 50% or 90% point, from clear to maximum turbidity, to the 10%, 50% or 90% point in the transition from maximum turbidity to the final baseline turbidity, respectively (resulting in 10%CLT, 50%CLT and 90%CLT parameters, respectively). Analytical precision of both assay conditions was evaluated according to the guidelines of Clinical and Laboratory

Standards Institutes (CLSI document EP05-A3). Precision was tested using healthy control plasmas, each run in quadruplicate, for 20 days. Coefficients of variation (CVs) of the within-run and total (within-laboratory) precision assessments were 8.6% and 8.9%, respectively. Precision results were essentially similar in both assay conditions.

Specific hemostasis assays

Quantitative D-dimer levels were measured using a particle-enhanced, immuno-turbidimetric assay (Innovance D-dimer) on a BCS coagulometer according to the manufacturer's instructions (Siemens Healthcare Diagnostic Products, Marburg, Germany). α2-plasmin inhibitor (α2-PI) activity and plasminogen activity were determined by commercially available methods (Siemens Healthcare Diagnostic Products, Marburg, Germany). Fibrinogen levels were analysed according to the method of Clauss using standard methods. Plasma levels of FXIII activity were determined by ammonia release assay using a commercially available reagent kit (REA-chrom FXIII kit, Reanalker, Budapest, Hungary).

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, Version 26.0, Chicago, IL), and GraphPad Prism 8.0 (GraphPad Prism Inc., La Jolla, CA). The number of samples in the study was determined using the Casagrande, Pike, and Smith's method. The study was powered to have a 90% chance of detecting 10% true difference between two subgroups, setting the value of α (type I error rate) to 0.05, based on previous CLA assay results. Shapiro-Wilk test was used to assess the normality of the data. Student's t test or Mann–Whitney U test was performed for two-group analyses. In case of paired data, paired t-test or Wilcoxon signed-rank test was applied. ANOVA with Bonferroni post hoc test or Kruskal–Wallis analysis with Dunn–Bonferroni post hoc test was applied for multiple comparisons. Pearson's or Spearman's correlation coefficient was used to determine the strength of correlation between continuous variables. Differences between categorical variables were assessed by $\chi 2$ test or by Fisher's exact where appropriate. Receiver operating characteristic (ROC) curves were built by plotting sensitivity vs. 1-specificity and calculating the area under the curve (AUC). Optimal threshold values of the CLA parameters were calculated based on Youden's J statistics. Test characteristics of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated using contingency tables and

$\chi 2$ test or Fisher's exact at statistically optimal threshold values. The Kaplan-Meier method was applied to plot survival vs. non-survival of patients, based on the calculated optimal test parameter cut-off. Survival curves were compared using the log-rank test. Binary backward logistic regression models were used to determine independent predictors of short-term functional outcome and therapy-associated intracerebral bleeding. Adjustments of the models were based on the results of preliminary statistical analyses of baseline characteristics between groups (Student's t test or Mann–Whitney U test, χ^2 test or Fisher's exact), previous literature, and methodological principles (dichotomized variables when possible). Results of the logistic regression analysis were expressed as odds ratio (OR) and 95% confidence interval (CI). A p-value of <0.05 was considered statistically significant.

RESULTS

In vitro clot lysis assay results in acute ischemic stroke patients undergoing intravenous thrombolysis

Study population

A total of 231 AIS patients receiving intravenous thrombolysis with rt-PA according to standard protocols were included in the study. Median age of the cohort was 67 (IQR: 50-76) years, 54.6% were men. Median NIHSS on admission was 7 (IQR: 4-11). Median time from symptom onset to treatment with rt-PA was 150 (IQR: 111-206) min. Arterial hypertension was at the highest rate of the cerebrovascular risks factors in this population. Favorable short- and long-term outcome was achieved in 44.1% and 45.5% of patients, respectively. Intracerebral bleeding occurred in 18 patients (7.8%), in 6 cases it was symptomatic.

Cell free DNA (cfDNA) levels, AIS severity and outcomes

We examined the relationship between pre-thrombolysis cfDNA levels, AIS severity and outcomes. On admission of patients, stroke severity demonstrated a step-wise association with cfDNA levels. Patients with more severe stroke had significantly higher cfDNA levels as compared to patients with milder strokes (NIHSS: 0-5) on admission. Stroke etiology did not show an association with cfDNA levels. cfDNA levels on admission did not differ in patients with favorable or unfavorable short term outcomes of stroke. On the other hand, patients with

favourable long-term outcomes of stroke (mRS 0-1) demonstrated significantly lower on admission cfDNA levels as compared to those with unfavorable outcomes at 90 days post-event. Admission cfDNA levels in patients with therapy-associated intracerebral hemorrhage did not differ significantly from the results of those without such complication.

Association of clot lysis assay (CLA) parameters with cfDNA levels, AIS severity and outcomes

In this cohort, stroke severity on admission demonstrated a step-wise association with 50%CLT parameter. AIS stroke patients who suffered more severe stroke on admission presented significantly shorter 50%CLT parameter. Similar significant associations were found in case of 10%CLT and CLA AUC parameters. Despite a similar step-wise but inverse association of 50%CLT with stroke severity as seen in case of cfDNA and stroke severity, surprisingly, 50%CLT and cfDNA parameters did not show a significant correlation (Spearman r=0.072; 95%CI: -0.069-0.209, p=0.301). Moreover, CLA parameters did not show any association with short- or long-term outcomes. We also found no association between bleeding complications and the 50%CLT parameter, although CLA AUC was significantly lower in patients experiencing post-lysis ICH. Stroke etiology according to TOAST classification, treatment specifications including the time from symptom-onset-to-treatment and radiological severity of strokes based on ASPECTS did not show any association with CLA results.

Association of the modified clot lysis assay (modified CLA) parameters with AIS severity and outcomes

In order to better imitate conditions present in AIS thrombi, we modified the CLA by adding cfDNA and histones to the clot induction and lysis mixtures (modified CLA). Concentrations of cfDNA and histones used in the assay were selected based on their presumed concentration within the thrombi according to previous literature and based on biochemical studies where the combined effect of histones and cfDNA were studied on fibrinolysis kinetics in purified experimental conditions. As expected, based on previous reports using purified proteins, the presence of cfDNA and histones affected clot formation and prolonged clot lysis significantly in the total cohort. Median time to reach 50% clot lysis was delayed by 4 min when cfDNA and histones were present in the assay mixture. Similarly to the conventional CLA assay, stroke severity on admission demonstrated a step-wise association with 50%CLT parameter of the

modified assay, and similar significant associations were found in case of 10%CLT and CLA AUC parameters of the modified CLA. Notably, also in the case of modified CLA, patients with more severe stroke presented significantly shorter clot lysis. In order to explain this finding, specific hemostasis proteins suggestive of consumption or excessive fibrinolysis were measured from all samples. While fibrinogen levels did not suggest extensive consumption related to stroke severity, interestingly, plasminogen activity showed a significant step-wise decrease in case of more severe strokes. This finding, however, does not explain shorter clot lysis in case of more severe strokes. Although it did not reach the level of significance, a decreasing trend was observed for α2-PI activity in case of most severe strokes (NIHSS>15). As compared to those who did not benefit from thrombolysis, patients with favorable short-term outcomes showed significantly shorter 50%CLT in the modified CLA. This association was not significant in case of the original assay when cfDNA and histones were not present in the assay mixture. Long-term outcomes, however, showed no association with modified CLA results. The occurrence of therapy-associated intracerebral hemorrhage showed a significant association with clot lysis parameters, particularly when the assay was modified using cfDNA and histones. In patients who suffered post-lysis intracerebral bleeding, 50%CLT was significantly shorter in the modified assay as compared to those without such complications. No difference was observed between patients experiencing symptomatic hemorrhage as compared to those with asymptomatic ICH.

Diagnostic performance of the modified CLA test related to treatment-associated intracerebral hemorrhage and short-term outcome in AIS patients

We compared the diagnostic efficiency of the modified CLA method with the diagnostic efficacy of the traditional CLA method, in terms of treatment-associated bleeding and short-term outcome of ischemic stroke. ROC analysis indicated that the addition of cfDNA and histones to the assay mixture considerably improved the diagnostic performance of the CLA. Improvement of the diagnostic performance was most prominent for the 50%CLT parameter for the prediction of ICH (ROC AUC in the absence of DNA and histones: 0.56; 95%CI: 0.43-0.69; p=0.371; ROC AUC in the presence of DNA and histones: 0.66; 95%CI: 0.54-0.78; p=0.024). Of all test parameters, the CLA AUC parameter of the modified assay showed the best diagnostic performance measures to predict ICH (ROC AUC in the presence of cfDNA and histones: 0.69; 95%CI: 0.59-0.80; p=0.006). Based on the optimal threshold value as defined by ROC analysis (<29.9 OD*min), the CLA AUC parameter of the modified assay

provided a remarkably high negative predictive value for the occurrence of post-lysis ICH (97.9%; 95%CI: 92.7-99.8%). Regarding the prediction of short-term outcomes, the addition of cfDNA and histones improved the diagnostic parameters significantly, but the performance of the assay remained modest (ROC AUC for 50%CLT: 0.57; 95%CI: 0.49-0.65; p=0.082; ROC AUC for 50%CLT in the presence of DNA and histones: 0.61; 95%CI: 0.53-0.69; p=0.008). A binary backward logistic regression model (including age, sex, increased NIHSS on admission, specific hemostasis/fibrinolysis proteins: fibrinogen, D-dimer, plasminogen activity, α2PI activity level and 50%CLT and CLA AUC parameters of the CLA in the absence or the presence of DNA and histones) revealed that a prolonged 50%CLT of the modified CLA (>44 min) is a modest, independent predictor of recanalization failure as determined by the change in NIHSS by day 7 post-lysis. On the other hand, in another regression model, a low CLA AUC parameter (<29.9 OD*min) of the modified CLA proved to be a significant, independent predictor of post-lysis ICH (OR: 5.85; 95%CI: 1.24-27.7; p=0.026). Besides this parameter, only NIHSS>15 on admission remained in the stepwise backward regression analysis model as a significant, independent predictor of ICH (OR: 5.32; 95%CI: 1.69-16.75; p=0.004).

In vitro clot lysis assay in non-traumatic hemorrhagic stroke patients

Study population

In the IRONHEART study, 89 consecutive patients with non-traumatic, spontaneous ICH were enrolled. One patient was excluded from the study due to SARS-CoV-2 infection on admission. One patient acquired SARS-CoV-2 infection on day 25 after the event, thus long-term follow-up results were excluded in this case. The assumed cause of ICH was hypertension in all patients, as based on the exclusion criteria, other causes, including cerebral aneurysm, AV malformation, malignancy, severe liver insufficiency, hemorrhagic diathesis, amyloidosis or vasculitis were excluded. The mean age of the cohort was 68 (± 11.6) years, 64% of patients were men. Median NIHSS on admission was 14 (IQR: 8-20), median ICH score was 1 (IQR:1-3). The most frequent cerebrovascular risk factor was hypertension (96.6%). Screening tests of coagulation and fibrinogen levels did not indicate a hemorrhagic defect in any of the patients. The median volume of hemorrhage was 20.0 (IQR: 3.7-48.0) cm^3 on admission and 46 (51.7%) of patients had intraventricular hemorrhage extension. Mortality was 29.0 % within the first 14 days after event and 43.8 % by day 90.

Examination of *in vitro* CLA and modified CLA in ICH patients

As expected, clot lysis parameters (max. absorbance, 10%CLT, 50% CLT and AUC) became significantly prolonged in the total cohort when cfDNA and histones were added to the sample solutions. Next we compared the CLA and modified CLA parameters of ICH patients with the CLA parameters determined from plasma samples from healthy volunteers. As expected, patients with ICH showed significantly shorter clot lysis times as compared to a healthy reference group, indicating faster fibrinolysis, that was independent of the addition of cfDNA and histones. As a next step, we examined the relationship between traditional CLA parameters of ICH patients and the stroke severity and the outcomes as defined above. However, stroke severity showed no association with the conventional CLA in the absence of DNA and histones. Examining the relationship between the traditional CLA parameters and mortality at day 14 and the long-term functional outcome of stroke, we found no significant difference between different groups.

Association between modified CLA parameters and ICH outcomes

ICH patients with more severe stroke (NIHSS>10) showed significantly shorter clot lysis (10%CLT) in the modified test as compared to patients with milder stroke (NIHSS 0-10). Similarly, significantly shorter clot lysis was observed using the modified CLA in patients with higher ICH score (2-5) as compared to those with ICH 0-1. Mortality by day 14 was associated with significantly shorter 10%CLT of the modified CLA. The median 10%CLT was 9.5 min shorter in those patients who died by day 14 as compared to those who survived (p=0.037). Besides CLA parameters, admission NIHSS, INR, smoking and platelet count showed association with mortality by day 14. Long-term functional outcomes showed significant association with the parameters of the modified assay as well. Those patients, who died or had unfavorable outcomes (mRS≥2) by the end of the 3rd month, demonstrated significantly shorter modified CLA parameters on admission as compared to those with good functional outcomes. Besides modified CLA parameters, admission NIHSS, BMI, INR and platelet count were associated with functional outcomes by day 90. In addition, estimated hemorrhage volume on admission showed strong association with day 14 and day 90 mortality. Notably, modified CLA parameters correlated significantly with estimated intracerebral hemorrhage volume. Modified CLA parameters indicating faster clot formation and lysis (shorter 10%CLT and time to maximal absorbance parameters, lower CLA AUC) showed significant association with larger hemorrhage volumes. ROC analysis was performed for all outcomes to investigate the

diagnostic performance of modified CLA parameters. The best AUC of ROC was 0.73 (95%CI: 0.57-0.89) for the parameter 10%CLT for predicting mRS 0-1 as outcome. Based on the optimal threshold value as defined by the Youden index (32.25 min), best sensitivity and specificity was provided by the 10%CLT parameter (77.0% and 67.7%, respectively). When performing ROC analysis for mortality by day 14 and day 90, similar optimal threshold values were defined (10%CLT cut-off: >38.5 min for 90 day survival, curves not shown). In a Kaplan-Meier survival analysis, those patients who presented with a 10%CLT result of >38.5 min on admission showed significantly better survival as compared to those with shorter clot lysis results (p=0.010). Compared to the results observed in the Kaplan-Meier survival study, in a binary backward logistic regression model (including age, sex, NIHSS on admission, hypertension, INR, platelet count, smoking status, cerebral hemorrhage volume, 10%CLT and BMI), 10%CLT of the modified CLA did not prove to be an independent predictor of mortality by 14 days and 90 days post-event. On the other hand, another binary backward logistic regression model (including age, sex, NIHSS on admission, INR, BMI, platelet count, 10%CLT, hemorrhage volume, hypertension) revealed that a shorter 10%CLT of the mCLA (<32.25 min) is a significant, independent predictor of unfavorable long-term functional outcome (mRS≥2) (OR: 6.14, 95%CI: 1.11-34.02, p=0.038).

DISCUSSION

Recent studies indicated a new model of stroke thrombus evolution, where, as the last step in the process of thrombi ageing, neutrophils infiltrate the thrombus by forming NETs and stabilize the thrombus with much smaller pores. In fact, clot dissolution by rt-PA is the easiest in the early stages of thrombus formation, when the cross-linking of fibrin and fibrinolysis inhibitors to fibrin by activated factor XIII has not yet taken place, and the clot is less compact with larger pores. Although these events are likely to be crucial in the response to rt-PA, no such test exist that takes the effect of NET components into consideration. In this study, we demonstrate that a modified CLA supplemented with cfDNA and histones might be a promising tool to predict short-term outcomes and post-lysis intracerebral hemorrhagic complications in AIS patients undergoing i.v. thrombolysis. Moreover, when choosing a different threshold, the test might be useful to identify a considerable fraction of patients as potential non-bleeders. This could be an important aspect in clinical scenarios when thrombolysis is applied before mechanical thrombectomy. Recent guidelines propose that intravenous rt-PA should be considered for eligible patients even if mechanical thrombectomy is used. To improve the safety of this approach, novel tests, such as the modified CLA might prove to be useful in the future. Despite the clear benefit of diagnostic tests with acceptable predictive value regarding thrombolysis outcomes in AIS patients, surprisingly few studies are available on this topic. In a recent meta-analysis, where over 6400 records were screened, only four papers were found where hemostasis biomarkers were tested from a relatively large (>100 patients) cohort of AIS patients before the start of reperfusion therapy. Most studies collected blood samples within 24 h after stroke onset, which is a fairly wide interval. Ideally, a hemostasis biomarker of AIS thrombolysis outcome should be assessed before the initiation of treatment. Given the short time-window of i.v. thrombolysis, sample collection of relatively large cohorts could be a challenging task. In this study, we were able to enroll 231 AIS patients, all tested before thrombolysis and followed for specific outcomes and safety at days 1, 7 and 90 post-event. Studies on predictive biomarkers of thrombolysis outcomes in AIS are often limited to investigating one or few hemostasis or fibrinolysis factors. As the end result of thrombolysis is thought to depend on a sensitive balance and interaction between a series of factors and their inhibitors, the benefit of using a global assay for predicting therapy outcomes instead of measuring individual factors is biologically plausible. In particular, CLA is a theoretically optimal test for this purpose. As rt-PA concentrations used in this assay are much higher than

endogenous t-PA concentrations, the CLA can be considered as a measure of fibrin resistance to therapeutical doses of exogenous rt-PA, rather than a marker of endogenous fibrinolytic capacity. On the other hand, the CLA is a laborious test which suffers from several weaknesses. Firstly, it is poorly standardized, despite efforts to generate a standardized assay. In our study, assay conditions were chosen based on available literature and a series of preliminary experimental conditions performed on healthy individuals. We optimized the assay conditions for a semi-automated testing of a relatively large set of patient samples, with an acceptable assay precision. Secondly, the assay is performed using plasma and therefore potential cellular contributors of thrombolysis resistance are not incorporated in the test. As an effort to improve the diagnostic performance of the assay, we supplemented the test with cfDNA and histones, mimicking the effect of NETs. It has been shown in several elegant studies that fibrin and NETs form a composite network within cerebral thrombi, and the effect of NETs is surmised to contribute to the overall lysability of clots in vivo. Here we found that the addition of cfDNA and histones to the *in vitro* CLA mixture, as expected based on the literature, resulted in significantly prolonged clot formation and lysis. Interestingly, the prolongation of clot lysis by cfDNA and histones showed inter-individual differences. Significant increase in circulating cfDNA levels have been previously reported as a result of stroke-induced damage to the neurovascular unit in animal models and in few clinical studies as well. In line with our report, cfDNA levels were found to be associated with stroke severity and post-stroke mortality in a handful of papers. On the other hand, circulating cfDNA might not only be a stroke biomarker, but, in theory, potentially influence clot lysis. Our results showed that this is not the case as the cfDNA levels detected in this cohort did not show any association with CLA parameters. The presence of cfDNA within the thrombus, as a result of NETosis, however, could potentially influence lysis susceptibility and a test that imitates this effect might better predict treatment outcomes. Here we show that the diagnostic performance of the conventional CLA was considerably improved by the presence of cfDNA and histones in the assay mixture. It must be emphasized that the incorporation of cfDNA and histones to the test is an oversimplification of the effect of NETs within thrombi. The release of NETs is a finely tuned process that constitutes not only of the release of DNA and histones, but of other proteins, including neutrophil granule proteins (human neutrophil elastase, myeloperoxidase, etc.), leading to a variety of complex interactions within the thrombus. Although the effect of cfDNA and histones are far from identical from the effect of intact NETs, DNA and histones have been shown to have important clot stabilizing and antifibrinolytic effects. Among other mechanisms, cfDNA has been shown to accelerate tPA-PAI-1 complex formation, slow down t-PA mediated plasmin generation,

modulate clot structure and delay plasmin-mediated lysis by intercalating into fibrin fibers. Histones bind fibrinogen and fibrin and as a result of histone incorporation into polymerized fibrin, more stable clots are formed. Conditions in these models and in our assay reflect pathologically high DNA concentrations that occur within thrombi. It is difficult to estimate the amounts of DNA that might be found in blood clots, but very high concentrations are likely as observed in previous studies. In healthy individuals, cfDNA circulates at low levels (0.02-1.7 μg/ml) but elevated levels (5 μg/ml or above) have been detected in a variety of disease states, including sepsis. In the modified CLA used in this study, optimal concentrations of DNA and histones were adapted from previous *in vitro* studies. Here we showed that results of the modified CLA was associated with short-term thrombolysis outcomes related to unsuccessful reperfusion. A prolonged (>44 min) 50%CLT parameter of the modified assay was found to be a significant, independent predictor of therapy failure at 7 days. Significant association with short-term outcome was found only in case of the modified CLA, suggesting that the addition of cfDNA and histones to the assay mixture is crucial to obtain an improved diagnostic performance. Furthermore, the modified CLA showed a high negative predictive value (97.9%) for the occurrence of ICH. Logistic regression analysis showed that the CLA AUC parameter of 29.9 <OD*min of the modified CLA is a significant, independent predictor of post-lysis ICH, similarly to increased NIHSS on admission. Intracerebral hemorrhagic complication is the most feared side-effect of rt-PA therapy, limiting its widespread use in less-experienced centers. Nevertheless, only a handful of studies are available on hemostasis or fibrinolysis biomarkers predicting post-lysis ICH, as the number of patients with ICH in the investigated cohorts is often too low to draw any conclusions. Currently, the risk/benefit profile of thrombolysis prior to endovascular thrombectomy cannot be accurately predicted in individual cases, thus, incorporation of the CLA results into clinical predictive models could improve patient selection. Ideally, given the high positive predictive value of the modified CLA to predict ICH, it could be used to select those patients with proximal arterial occlusion who are at considerable risk of developing post-lysis hemorrhagic complications and thus would more likely benefit from thrombectomy alone. Moreover, even in a case when the modified CLA yields increased ICH risk and the patient have already received thrombolysis within the shortest timeframe, the information could be relevant in the clinical practice, as the patient could be strongly monitored to reduce potential damage (e.g. longer ICU stay, aggressive control of hypertension, extensive neurologic follow-up, personalized post-lysis therapeutic approach, etc.). It must be noted, however, that our results did not show a difference between aSICH and SICH patients, which

is most likely due to the fact that symptoms related to intracerebral bleeding are strongly influenced by the localization of the hemorrhage. Results of the CLA on admission did not show an association with long-term functional outcomes, which might be explained by the fact that the level of disability at 90 days post-lysis is driven by series of factors, often independent of the hemostasis/fibrinolysis system at the occurrence of the event (co-morbidities, socio-economic status, post-event infections, stc.). Notably, the 50%CLT parameter of both assay conditions showed a significant, step-wise negative association with stroke severity. The negative association between stroke severity and 50%CLT is a puzzling result that was not associated with significant consumption of the few individual hemostasis and fibrinolysis proteins measured to test this possibility. Interestingly, plasminogen activity also showed a significant, step-wise negative association with stroke severity in the acute ischemic stroke cohort- the biological relevance of which needs to be investigated in further studies. After the modified CLA method proved to be a predictor of hemorrhagic transformation in the thrombolytic treatment of ischemic stroke, we examined whether the new method correlated with the size of the hematoma and the outcome of bleeding of non-traumatic hemorrhagic stroke patients. To our knowledge, the current study shows for the first time that a CLA might be a promising tool to predict the outcome of intracerebral hemorrhagic stroke. Despite the clear benefit of diagnostic tests with acceptable predictive value regarding outcomes in acute ICH stroke patients, surprisingly few studies are available on this topic. Here we show that mCLA parameters of patients on admission correlate with the estimated size of hematoma on admission, which is an important predictor of outcomes. Shorter clot formation and lysis times, indicating faster break-down of the newly formed clot were associated with larger hematoma volume, more severe stroke, and worse outcomes in this cohort. These results are in line with previous studies revealing that an elevated D-dimer, indicating a more extensive break-down of clots, is associated with adverse outcomes in patients with ICH. Our results clearly show a potential effect of the distortion of fibrinolytic balance on the evolution of the intracerebral hematoma. Fibrinogen levels were within the normal range in this cohort, suggesting that fibrinolytic factors rather than fibrinogen itself might be involved in this process. On the other hand, FXIII activity, plasminogen and α2-PI activity did not show an association with outcomes, suggesting the presence of other fibrinolytic alterations. Gaining knowledge on the factors that drive the enlargement or the dissolution of the bleeding are potentially important when designing future pharmacological therapies. As a first step, it is crucial to understand the underlying pathomechanism leading to poor outcomes in patients, and adequate diagnostic tools are a pre-requisite of such approaches.

The CLA is a method that has been shown to be potentially useful to predict outcomes in a wide range of pathologies where the fibrinolytic balance has been tilted. In our second study, using the new assay conditions already established in the studies of patients with ischemic stroke, the diagnostic performance of the modified assay was found to be particularly good to predict unfavorable long-term outcomes. In a binary logistic regression model, a shorter 10%CLT of the modified CLA (<32.25 min) proved to be an independent predictor of unfavorable long-term outcomes (mRS≥2).

In conclusion, our data implicate that the modified CLA using pre-thrombolysis plasma of AIS patients might be a useful tool to predict short-term outcomes and post-lysis intracerebral hemorrhagic complications after i.v. rt-PA therapy. Parameters of the modified CLA correlate well with ICH bleeding volume and could suggest unfavorable outcomes and mortality in spontaneous, non-traumatic ICH. Future studies including large cohorts of patients with AIS or ICH are warranted to further study the relevance of fibrinolysis alterations in the evolution of intracerebral hematoma and patient outcomes. Further modifications of the test might allow better diagnostic performance and easier implementation, which might be necessary for its potential clinical utilisation in the future.

Limitations

Results of the present study should be interpreted in the context of its limitations and strengths. In both studies the sample size is limited, however, as compared to other published studies measuring hemostasis or fibrinolysis biomarkers in stroke patients from pre-thrombolysis samples, it is the largest study as yet. Due to the limited number of patients with post-lysis ICH, despite the significant associations found, results presented here must be confirmed and validated by larger studies. Being single-centered, our studies had the advantages of uniform sample handling and uniform patient care. The Stroke Center of the University of Debrecen recruits patients from a relatively large geographic area, thus, unfortunately, a proportion of patients were lost to follow-up due to the transfer of patients. In case of the AIS patient cohort the lost to follow up was 8.7%, while in the ICH population, it was only 3.4% over the three-month follow-up period (including one patient that acquired SARS-CoV-2 infection post-event, and in this case long-term follow-up results were excluded). This percentage of follow-up drop-out is comparable or even lower to that observed in other studies involving post-stroke patients, however, it might have influenced the results to a certain extent and thus larger clinical studies are needed to confirm and to validate our data.

SUMMARY

Stroke is a leading cause of death and the most common disabling disease in all developed countries. Approximately 80% of all stroke cases are of ischemic origin (AIS), while about 15-18% are hemorrhagic strokes (ICH). In both subtypes, prognosis is difficult to estimate. We aimed to investigate whether a modified *in vitro* clot lysis assay incorporating neutrophil extracellular trap (NET) components could predict the clinical outcomes of AIS and ICH.

In two prospective observational studies, 231 AIS patients undergoing intravenous thrombolysis and 89 non-traumatic ICH patients were enrolled. Conventional clot lysis assay (CLA) and a modified CLA (mCLA) containing cfDNA and histones were performed using platelet-poor plasma samples of patients taken on admission. Stroke severity was determined by NIHSS on admission. Thrombolysis-associated bleeding complications were classified according to ECASSII criteria. Short- and long-term outcomes were determined at 7 and 90 days post-event based on ΔNIHSS and the modified Rankin Scale (mRS).

The presence of cfDNA and histones prolonged clot lysis significantly in both studied cohorts. A step-wise association was observed between stroke severity and the 50% clot lysis time (50%CLT) in case of AIS patients. ROC analysis showed improved diagnostic performance of the mCLA. Logistic regression analysis proved that a prolonged 50%CLT is a significant, independent predictor of unfavorable short-term outcome (OR: 2.19; 95%CI: 1.17-4.11; p=0.015), and a low AUC parameter is an independent predictor of hemorrhagic transformation (OR:5.85; 95%CI: 1.24-27.7; p=0.026) in AIS patients receiving thrombolysis. In the ICH cohort, patients with more severe stroke showed significantly shorter 10% clot lysis time (10%CLT) of the mCLA (10%CLT: NIHSS 0-10: median 31.5 [IQR: 21.0-40.0] min vs. NIHSS>10: median 24.0 [18.0-31.0] min, p=0.032). Shorter clot lysis parameters of the mCLA showed significant association with mortality by day 14 and with unfavorable long-term outcomes (mRS 0-1: 36.0 [22.5-51.0] min vs. mRS: 2-5: 23.5 [18.0-36.0] min vs. mRS 6: 22.5 [18.0-30.5] min, p=0.027). In a Kaplan-Meier survival analysis, patients presenting with an mCLA 10%CLT result of > 38.5 min on admission demonstrated significantly better survival (p= 0.010).

Conclusions. The modified CLA incorporating the effect of cfDNA and histones, may be a promising method to predict short-term functional outcomes and therapy-associated bleeding complications in AIS patients receiving thrombolysis, and to predict long-term outcomes and mortality in ICH patients.

MAIN SCIENTIFIC RESULTS, OBSERVATIONS

1. We developed a novel, modified clot lysis assay (mCLA) in which the addition of cfDNA and histones to the conventional CLA method increased the diagnostic efficacy of the assay to predict outcomes of stroke.

2. Here we found that the addition of cfDNA and histones to the *in vitro* CLA mixture resulted in significantly prolonged clot formation and lysis.

3. The 50%CLT parameter of the conventional and modified assay conditions showed a significant, step-wise negative association with ischemic stroke severity.

4. Here we showed that results of the modified CLA was associated with short-term thrombolysis outcomes related to unsuccessful reperfusion. A prolonged (>44 min) 50%CLT parameter of the modified assay was found to be a significant, independent predictor of therapy failure at 7 days.

5. The modified CLA showed a high negative predictive value (97.9%) for the occurrence of ICH. Logistic regression analysis showed that the CLA AUC parameter of 29.9 <OD*min of the modified CLA is a significant, independent predictor of post-lysis ICH, similarly to increased NIHSS on admission.

6. Here we show that mCLA parameters of ICH patients on admission correlate with the estimated size of hematoma on admission, which is an important predictor of outcomes.

7. Shorter clot formation and lysis times, indicating faster break-down of the newly formed clot were associated with larger hematoma volume, more severe stroke, and worse outcomes in the cohort of ICH patients.

8. The diagnostic performance of the modified assay was found to be particularly good to predict unfavorable long-term outcomes in ICH patients. In a binary logistic regression model, a shorter 10%CLT of the modified CLA (<32.25 min) proved to be an independent predictor of unfavorable long-term outcomes (mRS≥2).

LIST OF PUBLICATIONS

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List of publications related to the dissertation

1. **Orbán-Kálmándi, R. A.**, Szegedi, I., Sarkady, F., Fekete, I., Fekete, K., Molnárné Vasas, N., Berényi, E., Csiba, L., Bagoly, Z.: A modified in vitro clot lysis assay predicts outcomes and safety in acute ischemic stroke patients undergoing intravenous thrombolysis.
Sci. Rep. 11 (1), 1-14, 2021.
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Front. Neurol. 12, 1-11, 2021.
DOI: http://dx.doi.org/10.3389/fneur.2021.613441
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List of other publications

3. Szegedi, I., **Orbán-Kálmándi, R. A.**, Nagy, A. C., Sarkady, F., Molnárné Vasas, N., Sik, M., Lánczi, L., Berényi, E., Oláh, L., Crișan, A., Csiba, L., Bagoly, Z.: Decreased clot burden is associated with factor XIII Val34Leu polymorphism and better functional outcomes in acute ischemic stroke patients treated with intravenous thrombolysis.
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5. Árokszállási, T., Héja, M., Bagoly, Z., Kovács, K. B., **Orbán-Kálmándi, R. A.**, Sarkady, F., Tóth, J., Fekete, K., Fekete, I., Csiba, L.: Prognostic value of various hemostasis parameters and neurophysiological examinations in spontaneous intracerebral hemorrhage: the IRONHEART study protocol.
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