SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

# Associations of fibrinolytic parameters and clot burden with the outcome of intravenous thrombolysis in patients with ischemic stroke

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# ASSOCIATIONS OF FIBRINOLYTIC PARAMETERS AND CLOT BURDEN WITH THE OUTCOME OF INTRAVENOUS THROMBOLYSIS IN PATIENTS WITH ISCHEMIC STROKE

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| Head of the **Examination Committee**: | Prof. Miklós Antal, MD, PhD, Dsc |
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The Examination takes place at Room 312 of Department of Neurology, University of Debrecen, 30th January, 2019 at 13:00

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| Head of the **Defense Committee**: | Prof. Béla Fülesdi, MD, PhD, Dsc |
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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 2nd November, 2021 at 13:00

# Introduction

Cerebrovascular disease is one of the leading causes of mortality and disability in developed countries, with several thousands of new cases of stroke diagnosed in Hungary every year, according to data published a few years ago, the annual incidence is 43.3/10,000 people. The most common type of this disease is ischemic stroke (IS), which is caused by the blockage of an artery supplying the brain (less commonly the spinal cord or the retina), resulting in an infarct. Many risk factors for stroke are known (e.g., diabetes mellitus, hypertension, etc.) and considerable progress has been made in recent decades in understanding the pathophysiology of the disease, but many questions remain to be answered.

The best available treatment of acute ischemic stroke (AIS) is the fast recanalization the blocked vessel. Currently, there are two treatments available: intravenous thrombolysis using recombinant tissue plasminogen activator (rt-PA) remains the mainstay treatment, and mechanical thrombectomy is increasingly used, but it can only be attempted when the stroke is caused by the blockage of a large artery.

Intravenous thrombolysis has been routinely used for more than 20 years, but it cannot be considered a panacea, as clinical improvement is only achieved in about one third of patients, and hemorrhagic complications can develop in about 6-8% of patients.

The relative inefficacy of thrombolysis and the development of hemorrhagic complications are probably related to certain components of the hemostasis system, and the size and structure of the clot formed. Despite the importance of the topic, relatively few studies have investigated markers of the coagulation and/or fibrinolytic system in acute stroke patients, and most of the studies have only examined a relatively small cohort of patients. By studying the hemostasis system, we have the opportunity to gain a deeper understanding of the pathophysiological processes leading to the development of stroke and its treatment failure, which may help to predict therapeutic outcomes and provide the basis for individualized therapy in the future.

# Literature overview

## Ischemic stroke

### Epidemiology

Stroke is the second leading cause of death and the first cause of disability worldwide. In Hungary, the prevalence of ischemic stroke in the total population is 40 cases per 100,000 people.

### Definition, types

Stroke is defined by the WHO as “rapidly developing clinical signs of focal or global disturbance of cerebral function, lasting more than 24 hours or leading to death, with no apparent cause other than that of vascular origin”. In 80% of patients the stroke is ischemic, the remaining 20% is hemorrhagic. Within the hemorrhagic group, two further subgroups are distinguished: spontaneous parenchymal hemorrhages (15%) and non-traumatic subarachnoid hemorrhages (5%).

Another type of ischemic cerebral dysfunction is transient ischemic attack (TIA). According to the old definition, it is a group of transient neurological symptoms caused by focal cerebral, spinal, or retinal ischemia lasting less than 24 hours. The current definition of TIA is when the development of symptoms does not result in acute infarction. Symptoms usually last for minutes. About one-third of TIAs are followed by stroke within a month. Using the ABCD2 scale, the risk of a potential future stroke can be estimated.

Within the group of ischemic strokes, several subtypes are distinguished according to their etiology. In our studies, we used the classic TOAST (Trial of Org 10172 in Acute Stroke Treatment) classification system to distinguish the five subtypes.

1. Large vessel atherosclerosis

   In these patients, imaging studies show a >50% narrowing or blockage of the main arteries supplying the brain which is thought to be caused by atherosclerosis. Cerebral cortical lesions, cerebellar ischemic lesions, and subcortical lesions larger than 15 mm are likely to be of large vessel origin. Clinical features include cortical, brainstem or cerebellar symptoms.

2. Small vessel disease (lacunar stroke)

There is no significant large vessel stenosis in these patients. No acute infarction is detected by imaging, or the relevant lesion is smaller than 15 mm. Clinically, this subtype includes lacunar syndromes (purely motor or purely sensory symptoms). Concomitant hypertension and/or diabetes mellitus supports the clinical diagnosis.

3. Cardiogenic stroke

This category consists of patients in whom the occlusion of the vessel was caused by an embolus from the heart. A cardiological disease predisposing to thrombus formation, such as atrial fibrillation or dilated cardiomyopathy, is known in the history. They have similar clinical and imaging characteristics to patients in the large vessel atherosclerosis group. Previous stroke or TIA involving multiple vascular regions supports the clinical diagnosis. Stroke and cortical symptoms occurring during daily activity also support the cardiogenic origin.

4. Stroke of other origin

Various rare conditions are included in this group: thrombophilia, vasculitis, hematological diseases, etc. Imaging and laboratory tests can be used to confirm an acute infarction, regardless of its size.

5. Stroke of unknown origin (cryptogenic stroke)

If, despite thorough investigations, no etiological factor can be found causing a cerebral infarction, the stroke is considered of unknown origin, also known as a cryptogenic stroke.

**Risk factors**

Ischemic stroke has many modifiable and non-modifiable risk factors. Stroke is essentially a disease of old age, and its incidence increases steadily with age, doubling for every decade after the age of 55. There are also sex differences in the risk of stroke. In the elderly population, the relative risk is slightly higher among men. Nevertheless, the disease is more common in women because they have a longer life expectancy. Genetic studies have found several mutations that increase the risk of stroke.

Of the modifiable risk factors, the most common is hypertension that has a linear association with the development of the disease. Diabetes is an independent risk factor of stroke with diabetic patients having twice the risk of stroke. Stroke is responsible for about 20% of the mortality of diabetic individuals. Dyslipidemia also influences the risk

of ischemic stroke: elevated total cholesterol levels increase, while higher HDL levels decrease its risk. Atrial fibrillation, valve defects are also significant risk factors of stroke. Lifestyle also contributes to the development of ischemic stroke: sedentary lifestyle, obesity and the consequent metabolic syndrome are serious risk factors. The relationship between alcohol consumption and stroke risk can be characterized by a J-shaped curve: light alcohol consumption reduces the risk of ischemic stroke, whereas heavy consumption increases the risk. Smoking is an important risk factor of stroke, and the risk is proportional to the number of cigarettes smoked.

**Diagnostics**

The diagnosis of stroke is based on data from the patient's medical history, and clinical and cerebral imaging findings.

When taking anamnestic data, it is important to try to assess the circumstances of the onset of symptoms as accurately as possible. The patient or family member should be asked whether the patient is taking any medications that may cause low blood sugar, has a history of epileptic seizures, has had a trauma, or may have overdosed on any medications or other toxic substances. If the possibility of ischemic stroke cannot be ruled out, finding out when the symptoms developed is of paramount importance, as acute treatments of various kinds are only available within a certain time-window from the onset of symptoms. Clarification of anticoagulant treatment is of paramount importance: it is necessary to find out what type of anticoagulant medication the patient is using (vitamin K antagonist, low molecular weight heparin, direct oral anticoagulant), and when was the last time the anticoagulant was used, as in some cases the treatment may contraindicate thrombolysis.

During a detailed neurological examination, two main groups of symptoms can be distinguished: carotid (anterior) and vertebrobasilar (posterior) circulation disorders. To assess stroke severity, the NIHSS (National Institutes of Health Stroke Scale) system is used, which has 11 items, with the different items scored between 0 and 4 points. The maximum score is 42. Similarly to other scoring scales, NIHSS is difficult to apply if the patient had neurological symptoms prior to the current stroke (e.g., hemi or tetraparesis, severe visual impairment or language barrier, dementia, limit the value of NIHSS).

The aim of the physical examination and recording an accurate medical history is to distinguish the possibility of cerebral ischemia from stroke mimics and to assess whether

the patient is suitable for some form of desobliterative treatment. Stroke mimics include hypoglycemia, encephalitis, migraine, transient paralysis following an epileptic seizure, central nervous system tumor, etc.

The next step in the care of a patient with suspected acute stroke is to perform an imaging study of the brain, which may be a cranial CT or MRI scan, either native or contrast enhanced. The purpose of imaging is to rule out central nervous system hemorrhage and to assess the extent of the damage that has occurred. The Alberta stroke program early CT score (ASPECTS), a 10-point quantitative system, is routinely used to determine the extent of middle cerebral artery (MCA) territory stroke. The system divides vascular territory of the ACM into 10 segments: 1 point is deducted in the presence of an ischemic lesion in a given segment. A score of 7 or less on an admission CT scan is a predictor of unfavorable functional outcome in case of intravenous thrombolysis.

An important part of the diagnostic process is performing angiographic studies to confirm the presence of a large vessel occlusion. The clot burden score (CBS) can be used to estimate the size of the clot blocking the vessels in the anterior circulation. This is a 10-point system that allows the anterior circulation to be assessed on CTA images based on the presence of contrast agent filling in different vessel segments: supraclinoid internal carotid artery, proximal MCA M1, distal MCA M1 (2-point vessel segments), MCA M2 branches, ACA A1 branch, and infraclinoid internal carotid artery (1-point vessel segments). ACM M1 and ACA are the two end branches of ACI. The divison of ACM M1 is variable, most commonly divided into two, one upper and lower branch (M2 branches). It is generally accepted that the more distal a vessel section is to the heart, the less chance there is for sufficient collaterals. The patient is assigned 10 points if all vessel segments show contrast agent filling.

Before the initiation of acute desobliteration treatment, additional tests are required: pulse oximetry, blood glucose, blood count with platelet count and hemostasis screening tests (according to the latest guidelines, treatment should be initiated as soon as possible and should not be delayed by waiting for the results of the latter two tests, unless there is a reasonable suspicion of low platelet count or a blood clotting disorder, e.g., due to a medication).

Following the acute phase, further diagnostic steps include complex cardio- and cerebrovascular investigations to determine the cause of the stroke.

## Treatment

The standard treatment of acute ischemic stroke is the desobliteration of the blocked vessel by intravenous thrombolysis. This treatment can be used within 4.5 hours (12 hours for basilar artery occlusion) of the onset of symptoms if the patient meets all of the inclusion criteria and none of the exclusion criteria. The latest ESO guideline, released in 2021, recommends venous lysis within 9 hours and mechanical thrombectomy within 24 hours in selected cases where a sufficient intact brain tissue can be demonstrated using special imaging tests (perfusion CT or MRI). The therapeutic agent is alteplase (rt-PA; recombinant tissue plasminogen activator). The dose is 0.9 mg/kg, with a maximum dose of 90 mg. Ten percent of the dose is administered in bolus and 90% is infused over the course of 60 minutes. The efficacy and safety of intravenous thrombolysis has been demonstrated in several studies, but it cannot be considered a panacea, as only 30-40% of patients will have a favorable outcome. The early recanalization rate with rt-PA is approximately 25% in patients with proximal MCA occlusion and only 10% in patients with internal carotid artery (ICA) occlusion. In addition, the chance of recurrent occlusion can be as high as 30%. In addition to recanalization failure, intracranial hemorrhage develops as a side effect, even if the bleeding risk is reduced to the minimum. Several baseline clinical factors are associated with the outcome of rt-PA therapy, including male sex, stroke severity at admission, infarct size, advanced age, hyperglycemia, etc. However, given their low predictive value, they cannot be used for individualized therapeutic decisions.

An alternative form of thrombolytic therapy is intra-arterial lysis, which can be performed within 6 hours of the onset of symptoms in the event of a large vessel occlusion. The procedure is invasive, requires an interventionalist and advanced instruments, making it a less commonly used treatment. There is no uniformly accepted intraarterial dose (~10-80 mg rt-PA) and the rate of hemorrhagic complications also exceeds the complications of intravenous therapy.

Another important treatment for ischemic stroke is interventional clot removal using a catheter (mechanical thrombectomy), which is only available in case of large vessel occlusions. The procedure should be performed within 6 hours of the onset of symptoms. An extended, 24-hour therapeutic time window might be an option in selected patient groups, but this requires special imaging (perfusion CT or MRI).

**Outcome**

An important complication of ischemic stroke is hemorrhagic transformation, which may occur spontaneously without thrombolytic therapy, particularly in cases of embolic infarction and elevated blood glucose. The frequency detected on CT alone ranges from 13 to 43%, and the frequency of symptomatic cases varies from 0.6 to 20% according to the literature. According to the observations of our colleagues during brain dissection, the frequency of major or minor hemorrhagic transformation is 38%.

The risk of recurrent stroke in patients without hemorrhagic transformation is not negligible either. The risk of recurrence 7 days after the first ischemic stroke is 2% – it is 4% at 30 days, 12% after 1 year and 29% after 5 years. After the first ischemic stroke, the 30-day mortality rate ranges between 16% and 23%. A significant proportion of neurological symptoms persist beyond 6 months in the majority of patients: 40-50% of patients have residual hemiparesis and some cognitive deficit, and 15-20% of patients have some form of sensory deficit, hemianopsia or aphasia. Persistent symptoms usually result in disability, with a large proportion of patients requiring long-term care and about a quarter requiring institutionalization. The modified Rankin scale is used to assess long-term functional outcome in patients.

Prognostic studies are important in clinical practice for the following reasons. Ischemic stroke, even without thrombolysis, tends to spontaneously undergo hemorrhagic transformation, which is usually associated with a worse outcome. Thrombolytic therapy is often performed in multimorbid, multi-drug-treated patients who are at increased risk of systemic or cerebral hemorrhage. The indication of thrombolysis is not always clear in the everyday practice, several factors support intervention, others are in favor of the traditional treatment. In such cases, new knowledge is very important, that can help us to choose between watch-and-wait and active therapy. Since in many cases the patient is aphasic, consent to the lysis is the responsibility of the relative. It is important to estimate the chances of improvement or a worse outcome as accurately as possible when educating a relative.

## Short overview of the hemostatic system

The hemostasis system has three overlapping parts: the initiation, amplification, and propagation phases. The initiation phase occurs on cells carrying tissue factor

(endothelium, subendothelial structures), during which few active coagulation proteins are produced. In the amplification phase, platelet and cofactor activation occurs, leading to extensive thrombin generation. The propagation phase takes place on the surface of the activated platelets, during which large amounts of thrombin and fibrin clot are formed. The amplification phase of clotting and the propagation of the resulting thrombus involve several coagulation proteins.

## Fibrinolysis

Fibrinolysis is a tightly regulated enzymatic process that prevents the excess accumulation of intravascular fibrin and allows the removal of blood clots from the vascular bed. The central enzyme in fibrinolysis is plasmin, a serine protease. The inactive form of the enzyme is the single-chain glycoprotein plasminogen, which is mainly produced in the liver, but is also found in the extravascular spaces of many other tissues and in the α-granules of platelets. Plasminogen-plasmin conversion is mediated by tissue-type and urokinase-type plasminogen activators (t-PA and u-PA). In the body, t-PA is formed in endothelial cells and, at the right concentration, efficiently converts plasminogen to plasmin on the surface of fibrin, and its recombinant form is used to dissolve thrombi.

The fibrinolytic system is tightly regulated. One of the main regulators of the system is the fibrin clot itself, which localizes the lysis being the major substrate for fibrinolysis. Fibrin is not only a substrate, but also a regulator of fibrinolysis, as it binds both plasminogen and t-PA to its surface and thereby promotes plasmin formation. Fibrin clearance is accelerated by the increased formation of C-terminal lysine residues during fibrinolysis, providing new binding sites for plasminogen.

Fibrin degradation produces various fibrin degradation products (FDPs), including D-dimer, which is commonly tested in the diagnostic practice. As a cross-linked fibrin degradation product, D-dimer provides useful information on the progress of coagulation and fibrinolysis. D-dimer levels are of predictive and prognostic value in the diagnosis of a number of diseases such as disseminated intravascular coagulation (DIC), deep vein thrombosis, pulmonary embolism.

The structure of the clot also plays an important role in the breakdown of fibrin. Compact clots with thinner fibrin fibers break down more slowly than looser clots with thicker fibers. At high thrombin concentrations, it has been observed that more branched but

thinner fibrin clots are formed, whereas at low thrombin concentrations, a looser clot with thicker fibrin fibers is formed. The former carries a higher risk of thrombosis, the latter a higher risk of bleeding.

Endogenous fibrinolysis inhibitors play an important role in regulating the balance of fibrinolysis.

The main physiological inhibitor of plasmin is α2-plasmin inhibitor (α2-PI), a glycoprotein synthesized by the liver. It inhibits the fibrinolytic pathway in three ways: it forms a complex with plasmin; it inhibits the binding of plasminogen to fibrin; and it makes the newly formed fibrin more resistant to the effect of plasmin by being cross-linked to fibrin via activated factor XIII (FXIIIa).

TAFI (thrombin-activated fibrinolysis inhibitor) is a single-chain plasma protein that is activated by proteolysis catalyzed by thrombin or thrombin/thrombomodulin complex. TAFIa suppresses fibrinolysis by removing C-terminal lysine residues from fibrin, thereby preventing t-PA or plasminogen binding to fibrin and thus plasmin generation.

The primary plasminogen activator inhibitor is plasminogen activator inhibitor-1 (PAI-1).

In addition to the thrombolytic effect, the local, organ-specific function of some components of the fibronolytic system is also known. In the brain parenchyma, t-PA is involved in several central nervous system activities that are not necessarily associated with plasmin formation. t-PA has been shown to play a key role in modulating neurons, astrocytes, microglia, and pericytes, either directly or via plasmin, with remarkable effects on a variety of central nervous system conditions, such as IS, neurodegenerative diseases, and traumatic brain injury. The results of animal models are promising, but human studies affecting clinical conclusions are yet to come.

**The biochemistry of plasminogen activator inhibitor-1 (PAI-1)**

Plasminogen activator inhibitor-1 (PAI-1) is a single-chain glycoprotein belonging to the serine protease inhibitor family and plays a prominent role in the regulation of fibrinolysis. PAI-1 is produced in endothelial cells, smooth muscle cells, adipocytes, as well as in the liver and the spleen. The expression of the PAI-1 gene is induced by TNF-alpha, TGF-beta, other cytokines, insulin and other hormones, proteases, and hypoxia. PAI-1 is the major inhibitor of t-PA and u-PA. The detectable levels of PAI-1 in the circulation are regulated by a number of factors, such as inflammatory processes, obesity, circadian rhythm and genetic factors. Elevated levels of PAI-1 have been identified as a risk factor

for cardiovascular and cerebrovascular pathologies in several studies.

**PAI-1 4G/5G polymorphism**

PAI-1 protein is encoded by the SERPINE 1 gene, which is located on chromosome 7 in humans. The most studied inversion/deletion variation of the gene is the 4G/5G polymorphism, located at site -675 of the promoter region. This region may contain 4 or 5 guanine bases (4G and 5G alleles, respectively). Both alleles of the polymorphism are able to bind transcription factors, but the promoter with the 5G allele is also able to bind a repressor protein. As a result, the 5G allele will have lower PAI-1 transcriptional activity than the 4G allele. Individuals carrying the 4G allele will therefore have higher plasma levels of PAI-1, whereas individuals carrying the 5G allele will have lower PAI-1 levels. Previous studies have also associated the effect of the 4G allele with increased susceptibility to the development of thrombotic processes, increased mortality following myocardial infarction and increased mortality following sepsis.

**The biochemistry of factor XIII**

Factor XIII (FXIII) is a pro-transglutaminase found in the circulation in a tetrameric form: it consists of two catalytic A subunits and two carrier/inhibitor B subunits (FXIII-$A_2B_2$). The A subunit (FXIII-A) is produced in cells of bone marrow origin, mainly monocytes/macrophages, while the B subunit (FXIII-B) is produced in hepatocytes. The cellular form of the protein is the FXIII-$A_2$ dimer, which is found in many cells (monocytes, macrophages, platelets, etc.). FXIII is activated in two steps, by the concerted action of thrombin and $Ca^{2+}$. In the first step, cleavage by thrombin results in the detachment of the activation peptide from the FXIII-A subunit, and in the second step, in the presence of $Ca^{2+}$, the B subunits are detached, and FXIII-A subunits assume an active conformation (FXIIIa). FXIIIa is a key regulator of fibrinolysis, affecting fibrinolysis by two main mechanisms, including 1) making the clot more resistant to fibrinolysis by cross-linking fibrin chains and 2) protecting the fibrin clot by cross-linking α2-PI or other plasma components to fibrin and preventing its immediate elimination by plasmin or the fibrinolytic system.

**FXIII-A Val34Leu polymorphism**

The most studied common genetic alteration of FXIII-A is the Val34Leu polymorphism (allele frequency in Caucasian population: ~25%). The polymorphism results in a G->T substitution at codon 103 of the gene, resulting in a valine-to-leucine substitution at position 34 of the protein. The affected region is located only 3 amino acids away from the thrombin cleavage position, thus it is not surprising that the speed of thrombin cleavage is significantly affected. The FXIII-A Leu34 allele is cleaved about 2.5 times faster than the Val34 allele, with the consequence that the rate of FXIII activation is increased in the presence of the Leu34 allele. As the polymorphism affects the rate of FXIII activation, it affects the molecular structure of the cross-linked fibrin network. In the presence of the Leu34 allele, thicker fibrin fibers and a looser mesh structure are formed, which significantly affects the features of the clot, including its resistance to fibrinolysis. These structural differences in the fibrin structure can be detected by biochemical studies depending on fibrinogen concentration. At higher fibrinogen concentrations, the effect of the Leu34 allele is particularly striking, with homozygous individuals having increased fibrin clot permeability and reduced resistance to fibrinolysis compared to Val34 homozygous wild-type individuals. “Higher” in this case is indeed a relative term and is not aligned with the reference range of fibrinogen based on biochemical experiments. In an early paper investigating this issue in in vitro experiments, the fibrinogen concentration, above which the effect of the FXIII-A Leu34 allele on fibrin structure and permeability began to prevail under purified conditions, was 3.4 g / L. These biochemical experimental results are consistent with the mild protective effect of the polymorphism against myocardial infarction and venous thromboembolic events shown in clinical studies and described in several studies and meta-analyses. Recent *in vitro*, biochemical results suggest that the protective effect of the Leu34 allele against thrombotic events may be mediated by the formation of a significantly smaller clot containing cellular elements (red blood cells and platelets) in Leu34 allele carriers compared to individuals homozygous for the wild-type allele, in the presence of fibrinogen concentrations higher than 3,5 g/L. These recent *in vitro* experimental results have not yet been confirmed in clinical studies.

# Objectives

We investigated the association of various fibrinolytic parameters and the size of clots blocking cerebral blood vessels with short- and long-term outcomes in patients undergoing intravenous thrombolysis for AIS. Two prospective observational clinical studies were conducted:

1. In the first one, we aimed to investigate PAI-1 activity and antigen levels in consecutive AIS patients multiple times during thrombolysis. Our goal was to investigate the association between PAI-1 levels, PAI-1 4G/5G polymorphism and treatment outcome and safety.
2. In the second study, our objective was to assess the effect of thrombus size on the outcome of intravenous thrombolysis. We aimed to study the relationship between clot burden score (CBS), which describes the size of the clot, and various hemostasis parameters, including FXIII activity and FXIII-A Val34Leu polymorphism, and their impact on the success of lysis.

# Patients and methods

## Patients

In the two conducted prospective, observational studies, we selected patients with acute ischemic stroke at the Department of Neurology of the University of Debrecen Clinical Centre. In the first study, 131 consecutive patients were enrolled regardless of the vascular territory of the stroke. In the second, case-control type study, only patients with AIS involving the anterior circulation were selected. For the case-control study, each LVO (large vessel occlusion) patient (CBS 0-9) was matched with an age- and sex-matched patient without LVO (CBS 10). Patients were selected from 519 consecutive AIS patients admitted to the Department of Neurology over 59 months between 2011 and 2020.

All patients were selected using the inclusion and exclusion criteria of the 2008 ESO (European Stroke Organization) guidelines. All patients underwent intravenous thrombolysis according to a standard protocol with rt-PA within the 4.5-hour therapeutic window. Mechanical thrombectomy was not performed in any of the patients, either because it was not yet available in routine practice or because the patient was not suitable

for the procedure. The diagnosis of AIS was based on clinical symptoms and NCCT and CTA. Control CT scans were performed in all patients 24 hours after thrombolysis. CBS for the admission CTA scan and ASPECTS scores for the admission and control CT scans were determined independently by four radiologists.

In case of all patients detailed clinical history, including basic anthropometric data (age, gender, BMI, etc.), cerebrovascular risk factors (arterial hypertension, smoking, etc.), and medications were collected. In terms of cerebrovascular risk factors, patients were assigned to the risk group, if they had a positive history for the risk factors in the past or on admission, and /or they had received medication targeted for the risk factor.

Stroke severity was determined using the NIHSS at admission, on the first day and seventh day after treatment. TOAST criteria were used to determine the etiology of stroke. The classification of hemorrhagic transformation was performed according to the ECASS II criteria, thus two groups were distinguished: symptomatic and asymptomatic hemorrhagic transformation. In case of hemorrhage, the estimated volume was determined from the control cranial CT as previously described. During both studies, the following outcomes were evaluated:

1. Short-term outcomes were defined according to the change in NIHSS score. A change of at least 4 points was used to define favorable or unfavorable outcomes.
2. To characterize long-term functional outcome, the modified Rankin Scale (mRS) was used 90 days after the onset of stroke. Long-term outcome was considered unfavorable when the mRS was between 3 and 6 points.
3. The presence or absence of hemorrhagic transformation was determined as a separate outcome variable. Based on the ECASS II criteria, we distinguished between symptomatic and asymptomatic hemorrhagic transformation.

The studies were approved by the Regional and Institutional Ethics Committee of the University of Debrecen, Clinical Center and by the Ethics Committee of the National Medical Research Council. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. All patients or their relatives provided written informed consent.

**Blood sample collections and laboratory tests**

Peripheral venous blood samples were collected three times for the first study: before, immediately after and 24 hours after thrombolysis. For the second study, blood samples were taken 24 hours before and 24 hours after thrombolysis. Admission samples were subjected to routine laboratory tests (electrolytes, glucose, renal and hepatic function, hsCRP, complete blood count) using standard methods (Roche Diagnostics, Mannheim, Germany and Sysmex Europe GmbH, Hamburg, Germany). Coagulation screening tests (prothrombin time, activated partial thromboplastin time and thrombin time) and fibrinogen measurement according to the Clauss method were performed on freshly separated, citrate-anticoagulated plasma samples using routine methods with a BCS coagulometer (Siemens Healthcare Diagnostic Products, Marburg, Germany). For PAI-1 assays, blood samples were collected into tubes containing Vacuetta CTAD (sodium citrate, theophylline, adenosine, and dipyridamole) platelet inhibitor, which prevented the subsequent release of PAI-1 from platelets during transport and storage of blood samples. Samples were processed immediately: they were centrifuged twice at 1500 g for 15 minutes at room temperature. The plasma aliquots were individually coded and stored at -80°C until further special hemostasis studies. PAI-1 activity and antigen levels were determined using sandwich ELISA assays (Technozym PAI-1 Actibind ELISA and Technozym PAI-1 Antigen ELISA, Technoclone, Vienna, Austria). The Actibind assay is suitable for the determination of free, active PAI-1 only (reference range 1-7 U/ml, according to the manufacturer's description). The Technozym PAI-1 antigen assay measures free PAI-1, PAI-1 complexed with t-PA and latent PAI-1 forms, as well (reference range: 7-43 ng/ml, according to the manufacturer's description). PAI-2 and other plasminogen activator inhibitors have no effect on the assays. PAI-1 activity and antigen levels were determined from blood samples taken at all three time points studied. In addition, in the first clinical study, PAI-1 4G/5G polymorphism was determined in all patients using the LightMix ®PAI-1 4G/5G kit (Roche Diagnostics GmbH, Mannheim, Germany). Genomic DNA was extracted from the buffy coat layer of blood samples using standard methods (QIAamp DNA Blood Mini Kit, Qiagen, Hilden, Germany). PAI-1 4G/5G polymorphisms were determined using LightMix on a ®LightCycler® 480 (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

For the special hemostasis measurements performed in the second clinical study,

standard methods were used and determinations were carried out using citrate-anticoagulated plasma samples from blood samples taken on two occasions (before and 24 h after thrombolysis) as described above. Chromogenic assays were used to measure plasminogen and α2-plasmin inhibitor (α2-PI) activity and measurements were performed on a BCS coagulometer (Siemens Healthcare Diagnostic Products, Marburg, Germany). D-dimer levels were determined by an immuno-turbidimetric test, also on a BCS coagulometer (Siemens Innovance D-dimer, Marburg, Germany). Plasma levels of FXIII activity were determined by ammonia release test using a commercially available reagent kit (Technochrome FXIII, Technochlone, Vienna, Austria). FXIII-A Val34Leu polymorphism was determined from genomic DNA isolated as described above by real-time PCR using our previously described method, fluorescence resonance energy transfer detection and melting curve analysis on a LightCycler® 480. All measurements were performed from coded aliquots, and the operators performing the measurements were blinded to patient identification and clinical data.

**Statistical analysis**

Statistical analyses were performed using SPSS (Statistical Package for Social Sciences, Release 22.0, Chicago, IL), GraphPad Prism 8.0 (GraphPad Prism Inc., La Jolla, CA), and Stata 12 (Stata Corp., College Station, TX). Shapiro-Wilk test was used to assess normality of the data. Student's t-test (parametric distribution) or Mann–Whitney U test (non-parametric distribution) was used to compare two groups. When comparing multiple groups, either ANOVA analysis with Bonferroni post hoc test or Kruskal–Wallis analysis with Dunn–Bonferroni post hoc test was used depending on the normality of the data. Pearson's or Spearman's correlation coefficients were used to determine the strength of correlations between continuous variables. To compare categorical variables, $\chi 2$ test or Fisher's exact test was used. A binary backward logistic regression model was used to determine independent predictors of poor short- and long-term outcome after thrombolysis. Individual elements of the multivariate model were selected for inclusion in the statistical model based on the results of univariate model calculations, and literature data. The results of the logistic regression were expressed as odds ratio (OR) and 95% confidence interval (CI). A p value less than 0.05 was considered statistically significant.

# Results

## Patient characteristics at admission according to their PAI-1 4G/5G genotype

Of the 131 patients included in the first study, 31 were homozygous for PAI-1 5G/5G. There were no significant differences between PAI-1 5G/5G homozygous patients and PAI-1 4G carriers in terms of clinical or laboratory characteristics on admission, except that post-lysis intracranial hemorrhage was significantly more frequent in PAI-1 5G/5G homozygotes (frequency of hemorrhage: 19.35% in PAI-1 5G/5G homozygotes vs 7% in PAI-1 4G carriers, $p = 0.036$).

## PAI-1 levels during thrombolysis

Compared to the patients' admission values, PAI-1 activity was transiently reduced immediately after thrombolysis in the entire cohort. A highly significant decrease was observed as compared to the admission PAI-1 activity levels; measured immediately after thrombolysis, the median value of PAI-1 activity was below the lower limit of the reference interval (median admission PAI-1 activity: 2.34, IQR: 1.46–5.17 U/ml; median PAI-1 activity immediately post-thrombolysis: 0.94, IQR: 0.73–1.18 U/ml). When measured 24 hours after thrombolysis, PAI-1 activity showed a significant increase (median: 3.44, IQR: 1.65–7.60 U/L). In contrast to PAI-1 activities, PAI-1 antigen levels remained unchanged during thrombolysis. The best correlation between PAI-1 activity and antigen levels was observed 24 hours after thrombolysis (Spearman r: 0.539, $p < 0.001$; r: 0.355, $p < 0.001$ and r: 0.752, $p < 0.001$, at admission, immediately after thrombolysis and 24 hours after thrombolysis, respectively).

PAI-1 4G/5G polymorphism had no effect on PAI-1 activity and antigen levels after thrombolysis. Among admission clinical and laboratory parameters, PAI-1 levels correlated well with BMI and CRP, especially in samples measured 24 hours after thrombolysis (PAI-1 activity 24 hours after thrombolysis and BMI: Spearman r: 0.338, $p < 0.001$; PAI-1 activity 24 hours after thrombolysis and CRP: Spearman r: 0.418, $p < 0.001$). PAI-1 levels showed negligible diurnal variation in the studied cohort (median admission PAI-1 activity between 0:00 and 12:00: 2.81, IQR: 1.48–5.97 U/ml, vs PAI-1 activity between 12:01 and 23:59: 2.09, IQR: 1.45–4.68 U/ml, $p = 0.363$).

**The association between PAI-1 activity and antigen levels with stroke severity, etiology, and outcome**

PAI-1 activity and antigen levels at admission were not associated with stroke severity or etiology. At admission PAI-1 activity levels were significantly higher in patients with worse ASPECTS (<7) scores on native CT scans 24 hours after thrombolysis. PAI-1 antigen levels were also significantly higher on admission and 1 hour after lysis in patients with worse ASPECTS scores at 24 hours post-event. Despite these associations, PAI-1 activity and antigen levels measured during thrombolysis showed no relation with short- or long-term functional outcome or post-lysis hemorrhagic transformation. PAI-1 activity and antigen levels did not show a significant correlation with estimated intracranial hematoma volume.

**PAI-1 4G/5G polymorphism is an independent predictor of hemorrhagic transformation**

Patients who developed intracranial hemorrhage after lysis (n=13) had significantly lower BMI. The prevalence of hypertension and hyperlipidemia was significantly lower in this group as compared to those without hemorrhagic complications. Using a binary, backward logistic regression model (including: age, sex, BMI, admission NIHSS, hypertension, hyperlipidemia, PAI-1 4G/5G genotype), PAI-1 5G/5G genotype was found to be a significant independent risk factor for post-lysis hemorrhagic transformation. The risk of hemorrhage was almost fivefold in PAI-1 5G/5G homozygotes (OR: 4.75, 95% CI: 1.18–19.06, p = 0.028). Patients with the PAI-1 5G/5G genotype had a higher median post-lysis intracranial hemorrhage volume as compared to PAI-1 4G carriers, although presumably due to the relatively small number of cases, the difference was not significant (median: 16.82, IQR: 1.46–58.16 $cm^3$ vs. median: 0.67, IQR: 0.26–13.55 $cm^3$, p = 0.09, respectively).

**Association of clot burden score (CBS) with fibrinolytic markers and FXIII-A Val34Leu polymorphism in patients with acute ischemic stroke treated with intravenous thrombolysis**

In our second case-control prospective observational study, we included a total of 200 patients with anterior circulation AIS who underwent intravenous thrombolysis with rt-PA: 100 AIS patients with LVO (CBS 0-9) and 100 control AIS patients without LVO (CBS 10), matched by age and sex. Smoking was significantly more frequent and NIHSS was significantly higher in the CBS 0-9 group. There were significant differences in both radiological and clinical outcomes between the two groups. Patients in the CBS 0-9 group had a significantly higher incidence of unfavorable outcomes in both short- and long term and lower ASPECTS scores on 24-hour control CT scans. Among routine laboratory parameters, hsCRP was significantly higher in the CBS 0-9 group. Plasminogen activity at admission was significantly higher in the CBS 0-9 group, while other hemostasis parameters did not differ significantly between the two groups. Allele frequencies of the FXIII-A Val34Leu polymorphism were in Hardy-Weinberg equilibrium when the entire cohort was examined (FXIII-A Val34Val: n = 112 [56%], FXIII-A Val34Leu: n = 78 [39%] and FXIII-A Leu34Leu: n = 10 [5%]). Allele frequencies of the FXIII-A Val34Leu polymorphism were not significantly different in either CBS group compared to a large population control group.

The Leu34 allele of FXIII-A was significantly more prevalent in the CBS 10 group, consistent with the effect of the allele described in *in vitro* experiments, resulting in smaller whole blood cell containing clots. In univariate analysis, the Leu34 allele showed a significant protective effect against the development of larger clots (CBS 0-9) (OR: 0.519; 95% CI: 0.298–0.922, p = 0.0227). In the CBS 0-9 group, 11 patients developed hemorrhagic transformation (11%), whereas in the CBS 10 group this complication occurred in 7 patients (7%). Our results showed no significant difference in the rate of intracranial hemorrhage in the two CBS groups when classified according to ECASS II (aSICH or SICH), suggesting that clot size is not associated with post-lysis hemorrhagic complications in the studied cohort.

To investigate possible associations between CBS and some key fibrinolysis markers, CBS patient groups 0-9 were divided into further subgroups. None of the hemostasis markers tested showed a significant correlation with CBS at admission or 24 hours after lysis, ruling out the possibility of significant consumption of these hemostasis factors in case of larger thrombi. Accordingly, D-dimer levels measured 24 hours after thrombolysis showed no significant difference between the different CBS subgroups. Taken together, this suggests that the magnitude of CBS is not related to the levels of hemostasis or fibrinolytic markers tested, and thus these measurements would not be useful in clinical

practice for estimating thrombus size.

**The association of CBS with short and long-term functional outcomes of thrombolysis**

To explore which clinical or laboratory parameters are associated with thrombolysis outcomes, a binary, backward, multivariate logistic regression statistical model was used Univariate analyses showed that low CBS, higher admission NIHSS, previous stroke and higher admission plasminogen levels were significantly associated with poor short-term outcome. Although the FXIII-A Leu34 allele was significantly more prevalent in the CBS 10 patient group, the polymorphism did not show an association with short-term outcome. Based on a multiple logistic regression model (components: age, sex, previous stroke, admission NIHSS, admission plasminogen activity, and CBS), only age, NIHSS, and CBS were found to be independent predictors of poor short-term outcome.

Univariate analyses examining long-term outcome revealed that older age, diabetes mellitus, lower CBS, higher admission NIHSS, elevated serum glucose, elevated CRP, elevated creatinine, higher admission D-dimer, higher admission fibrinogen levels and lower 24-hour FXIII activity were significantly associated with poor long-term outcome. As observed for short-term outcome, FXIII-A Val34Leu polymorphism did not show an association with long-term outcome in this patient group. In a binary backward multiple regression model, CBS (CBS 0-9 vs. 10: OR: 2.501; 95% CI: 1.179-5.306, $p = 0.017$), as well as age, admission NIHSS and admission creatinine were shown to be independent predictors of long-term functional outcome.

# Discussion

Despite PAI-1 is the most effective physiological t-PA inhibitor, its role in the thrombolytic therapy of ischemic stroke is not fully understood. A key finding of our first study is that the presence of the PAI-1 5G/5G genotype is associated with a significant risk of post-lysis hemorrhagic transformation. According to a recently published systematic review of biomarkers potentially predictive of ischemic stroke outcome, that summarized the results of more than 6000 publications, PAI-1 may be one of the most promising prognostic biomarkers when measured before the initiation of thrombolytic therapy. In our study cohort, 46.15% of patients with post-ischemic ICH had PAI-1 5G

homozygous genotype. In a multiple logistic regression model including all possible common risk factors for post-lysis hemorrhage, the presence of PAI-1 5G/5G genotype was the strongest independent risk factor for the development of hemorrhagic transformation (OR: 4.75 95% CI: 1.18–19.06, p = 0.028). In addition, patients with PAI-1 5G/5G genotype showed a tendency to develop larger intracerebral hematomas, also supporting a possible role of this polymorphism in the pathophysiology of post-lysis hemorrhage.

The contributing effect of PAI-1 5G/5G genotype to post-lysis hemorrhage might be in theory related to its effect on plasma PAI-1 levels regulating intravascular fibrinolysis or to a local effect of PAI-1 levels in the brain parenchyma limiting excessive t-PA activity. In our study, PAI-1 4G/5G polymorphism did not seem to have a major influence on plasma PAI-1 levels, which is in line with few previous reports. Theoretically, the PAI-1 5G/5G genotype may contribute to the development of ICH locally through lower PAI-1 levels at the site of the intracerebral lesion. Local PAI-1 levels in the thrombus may differ substantially from peripheral levels. PAI-1 levels may also show significant variation during the course of stroke: in an animal model, intravascular PAI-1 levels showed an increasing trend after acute stroke – the same phenomenon was confirmed in this study in acute stroke patients. Known determinants of PAI-1 levels (e.g., BMI and inflammation) were also confirmed by our study. Interestingly, BMI was significantly lower in patients with post-lysis ICH. Although there is a potential association between low PAI-1 levels, low BMI and post-lysis bleeding, the backward regression model used in this study did not confer BMI as an independent risk factor for hemorrhage. Further studies with larger cohorts of patients are needed to confirm this result.

The link between PAI-1 genotype 5G/5G and hemorrhagic transformation presumably extends beyond the biochemical process of intravascular fibrinolysis. PAI-1 released from astrocytes may locally reduce toxicity and neuronal damage by limiting excessive t-PA activity in the parenchyma. Besides astrocytes, cerebral endothelial cells and pericytes also express PAI-1. PAI-1 released from these cells may prevent damage to the blood-brain barrier. Since the PAI-1 4G/5G polymorphism affects PAI-1 transcriptional activity in human astrocytes, the effect of the PAI-1 5G/5G genotype on post-lysis hemorrhage might be related to a shift in the fibrinolytic balance expressed at the local tissue level rather than to plasma PAI-1 levels. Given the complex role of PAI-1 in stroke pathophysiology, these theories warrant further experimental studies.

Our study showed that PAI-1 activity was significantly reduced during thrombolytic

treatment, while no change in PAI-1 antigen levels (including free, PAI-1–t-PA-complex and latent forms) was observed. Median PAI-1 activity in the cohort decreased below the lower reference limit immediately after thrombolysis, indicating that PAI-1 effectively inhibits excess rt-PA activity during thrombolytic therapy. Nevertheless, absolute values of PAI-1 activity and antigen levels showed no significant correlation with treatment efficacy or safety. It should be noted, however, that PAI-1 antigen levels measured at admission and 1 hour after lysis were significantly higher in patients with more severe, larger ischemic lesions (ASPECTS <7) on 24-hour control CT scans. In these patients, the median PAI-1 antigen level at admission and 1 hour after lysis was twice as high as in patients with a favorable score. Similar significant differences in admission PAI-1 activity levels were observed between the two groups with different radiological stroke severity. These results indicate that elevated PAI-1 activity/antigen levels at admission may predict the development of more severe ischemic lesions 24 hours after lysis. This type of association of PAI levels with imaging findings has not been previously described. The median value of PAI-1 activity at 24 hours after lysis tended to be higher in patients with unfavorable long-term functional outcome compared with patients with good outcome (mRS 3-6 vs 0-2); however, the magnitude of the difference in this patient group did not reach the threshold for statistical significance ($p = 0.091$).

In our second study, we investigated the association of thrombus mass with the FXIII-A Val34Leu polymorphism and the outcome of thrombolytic therapy. CBS accurately classifies thrombus size and length, and its utility in identifying potentially adverse responders to intravenous thrombolysis has been demonstrated in previous studies. In our study, we demonstrate that higher CBS is associated with better short- and long-term outcomes, and that CBS can be used as a prognostic marker for the thrombolytic treatment outcome of AIS. Our results suggest that the outcome of recanalization and thrombolysis is highly dependent on thrombus size. Although a number of studies have proposed CBS to be useful in predicting stroke outcomes, our study is among the first to measure key hemostasis parameters that could be associated with thrombus size in AIS patients. We hypothesized that thrombus size may be related to the levels of key coagulation or fibrinolytic proteins that regulate clot structure and lysis. Interestingly, here we show that with the exception of plasminogen levels at admission, there is no direct relation between thrombus size and the investigated fibrinolysis parameters at admission or 24 h later. In this patient cohort, plasminogen levels were significantly lower in patients with a higher CBS score and smaller thrombus size, that might be a result of significantly higher

incorporation of plasminogen to thrombi and as a consequence, the consumption of the protein.

Lower plasminogen levels were associated not only with a higher CBS score (smaller thrombus size), but also with a more favorable short-term functional outcome in the univariate statistical model. In a multivariate model, however, we could not demonstrate an independent effect of admission plasminogen levels on short-term outcomes. There is only one study in the literature in which the relationship of hemostasis proteins with CBS was investigated. In this study, only fibrinogen was measured in a small group of AIS patients, and higher fibrinogen levels were associated with smaller thrombus size. In our study of much larger sample size, fibrinogen levels on admission and 24 hours after lysis were virtually identical in groups of patients with different CBS, i.e., we could not confirm this association. Apart from the fibrin and fibrinolytic network, thrombus size might be influenced by red blood cells and platelets that are important components of thrombi. Here we show that slightly, but significantly decreased red blood cell count and hemoglobin concentration was found in patients with higher thrombus burden (CBS<10). Without further experiments, it is difficult to interpret whether this difference might be the result of consumption, but overall, red blood cell count was not associated with outcomes in this cohort. Platelet count did not show an association with thrombus burden or outcomes.

Our study demonstrated for the first time that the FXIII-A Val34Leu polymorphism had a significant effect on clot size *in vivo*. FXIII has been implicated in limiting thrombus size by various mechanisms, including the regulation of the retention of red blood cells in thrombi or by down-regulating the accumulation of platelets on fibrin, among others. Of the major polymorphisms of FXIII, FXIII-A Val34Leu polymorphism has been studied most extensively in relation to the risk of thrombotic pathologies. Meta-analyses have confirmed that the FXIII-A Leu34 allele has a mild protective effect against the development of coronary artery disease and venous thromboembolism, but no evidence is yet available for ischemic stroke. The effect of the FXIII-A Val34Leu polymorphism has been shown to be influenced by gene-environment interactions. Several studies have shown that the protective effect of Leu34 is exerted at high fibrinogen concentrations. *In vitro* experimental studies have demonstrated that fibrin clot formed in the presence of the FXIII-A Leu34 allele is less resistant to fibrinolysis as compared to the FXIII-A Val34 allele. In a recently published collaborative study by our group, we were to first to demonstrate that in *in vitro* experimental conditions, reconstituted whole blood clot mass

is greatly determined by the FXIII-A Val34Leu polymorphism. Using plasma samples with high fibrinogen levels (~3.5 g/L), clot mass was significantly higher in clots formed from FXIII-A Val34 genotype blood samples as compared to homozygous Leu34 genotype blood samples. In the current cohort of AIS patients, the median fibrinogen at admission was above 3.5 g/L in both groups studied (CBS 0-9 vs CBS 10: 3.88 g/L vs. 3.91 g/L), and therefore the effect of the Leu34 allele could prevail in both groups. Further statistical analysis of the FXIII-A Leu34 allele's effect on clot burden with respect to fibrinogen levels did not reveal statistically significant differences among groups, which might be explained by the influence of the acute event on fibrinogen levels. However, our results clearly demonstrated that the presence of the FXIII-A Leu34 allele showed significant protection against the development of larger (CBS 0-9) clots (OR: 0.519; 95% CI: 0.298-0.922, p = 0.0227) in the studied cohort. Interestingly, the polymorphism did not prove to be an independent predictor of functional outcome in multivariate statistical analysis. Given that thrombolysis outcome is influenced by several factors, most importantly by stroke severity and its location, it is plausible that the FXIII-A Val34Leu polymorphism does not contribute significantly to the overall functional outcome in patients. This is in line with our previous study where FXIII-A Val34Leu had no effect on outcomes in a small group of AIS patients treated with thrombolysis.

In multivariate models, CBS was found to be an independent predictor of short- and long-term outcomes in the studied cohort (CBS 0-9 vs. 10: OR: 2.777; 95% CI: 1.439–5.361, p = 0.002 and OR: 2.501; 95% CI: 1.179–5.306, p = 0.017). Previous studies have already suggested that patients with lower CBS (larger thrombus size) are more likely to benefit from endovascular treatment as compared to intravenous thrombolysis alone. On the other hand, a large body of previous evidence suggests that, in addition to thrombus length, the structure and functional properties of the clot may also play an important role in recanalization during intravenous thrombolysis and/or thrombectomy. Here we show that thrombus size is indeed among the most important factors for the outcome of intravenous thrombolysis, and potential differences between cellular composition or the levels of key fibrinolysis factors in larger or smaller thrombi cannot be detected using peripheral blood samples of patients on admission and 24 h post-lysis. In univariate models, higher admission D-dimer levels, higher admission fibrinogen levels and lower 24-hour FXIII activity were significantly associated with unfavorable long-term outcome. However, these associations could not be confirmed in the multiple logistic regression model.

# Conclusions

Although many advances have been made in understanding the mechanisms leading to unfavorable clinical outcome and hemorrhagic complications following intravenous rt-PA treatment of acute ischemic stroke, individualized risk assessment is not yet possible. The clinical inefficacy of thrombolysis and hemorrhagic complications are thought to be related to key elements and regulators of the hemostasis system. A number of studies are available on coagulation and/or fibrinolytic markers in acute stroke patients, but most include relatively few patients and thus have low statistical power. A further problem is that in the majority of studies the timing of blood sample collection was not precisely defined in relation to the time of lysis, or blood was only collected after lysis. Based on these previous results, clinical recommendations for treatment and monitoring cannot be made at this stage. Nevertheless, some markers appear to have potential for predicting therapeutic outcomes. Recently, we have published a literature summary of the prognostic value of markers of the hemostasis system in predicting the outcome of thrombolysis.

In our first study, we focused our attention on the potential role of PAI-1 in thrombolysis outcome and demonstrated that PAI-1 4G/5G polymorphism is an independent predictor of hemorrhagic transformation. This result could be the starting point for intervention studies in which individualized hemorrhagic transformation risk could be established through point-of-care diagnostics and could all lay the groundwork for individualized stroke therapy in the future.In our second study, we investigated the FXIII-A Val34Leu polymorphism and concluded that the presence of the FXIII-A Leu34 allele has a significant protective effect against the formation of larger clots. Confirming the findings of previous studies, larger thrombus size (low CBS) was found to be an independent predictor of poor short- and long-term outcome.

In the future, further well-designed prospective clinical trials involving large patient cohorts are needed to better understand the coagulation and fibrinolytic system during rt-PA therapy and to confirm the prognostic value of markers that currently appear promising for clinical decision-making. Conclusions drawn from such studies may lead to improvements in the efficacy and/or safety of thrombolytic therapy and may provide the basis for individualized thrombolytic therapy.

# Limitations

Similarly to most observational clinical trials, results of our studies should be interpreted in the context of their limitations and strengths. Patients were enrolled in a single center, therefore our sample size is limited. However, as compared with other published studies that have investigated biomarkers of hemostasis and/or fibrinolysis in patients with acute ischemic stroke in pre- and post-thrombolysis samples, our studies are among those with the largest reported sample sizes in the literature. Being single-centered, our studies had the advantages of uniform sample handling and uniform patient care, moreover, the proportion of patients that were lost to follow-up was remarkably low (PAI-1 study: 17.5% for long-term outcome; CBS study: 2% for short and 3.5% for long-term outcome). It must be noted, however, that the relatively low sample size, together with this drop-out rate at follow-up might have influenced the results to a certain extent and thus larger clinical studies are needed to confirm and to validate our data. A further limitation is that the levels of hemostasis and fibrinolytic markers studied in our studies were determined from peripheral blood samples, and local hemostasis conditions in the brain may be significantly different or their effects may not be reflected at peripheral sites. Therefore, further studies are needed to assess the exact role of the examined proteins in the outcome of thrombolysis.

# Summary

Background: Thrombolysis by recombinant tissue plasminogen activator (rt-PA) is the main pharmacological therapy in acute ischemic stroke (AIS); however, it is only effective in a subset of patients. In two prospective observational studies, we investigated the association between various fibrinolytic parameters, cerebral occlusive clot burden and therapy outcomes in AIS patients receiving intravenous thrombolysis.

Methods: In the first study, associations between plasminogen activator inhibitor-1 (PAI-1) levels, PAI-1 4G/5G polymorphism, and therapeutic outcome and safety were studied in 131 consecutive AIS patients receiving rt-PA treatment. In the second, case-control study of 200 AIS patients, associations between clot burden score (CBS) describing thrombus size, various hemostasis parameters, including factor XIII (FXIII) activity and FXIII-A Val34Leu polymorphism, and their effect on the success of thrombolysis were examined. Peripheral venous blood samples were taken on three occasions: before, immediately after (only in the first study), and 24 hours after the thrombolysis. Short-term functional outcome was defined as a change of at least 4 points in the NIHSS. Long-term outcome was defined according to the modified Rankin Scale at 90 days.

Results: PAI-1 activity significantly decreased upon thrombolytic treatment, while no change was observed in PAI-1 antigen levels. PAI-1 levels showed no association with therapy outcomes. In a binary backward logistic regression model, PAI-1 5G/5G genotype was revealed as a significant, independent risk factor for post-lysis intracranial hemorrhage (OR: 4.75, 95%CI: 1.18–19.06, $p = 0.028$). In a univariate analysis, a significant protective effect of the FXIII-A Leu34 allele against developing larger clots (CBS 0–9) could be demonstrated (OR:0.52; 95%CI:0.30–0.92, $p = 0.023$). Based on multivariate models, CBS was found to be a significant independent predictor of short-term and long-term outcomes (CBS 0–9 vs. 10: OR: 2.78; 95%CI: 1.44–5.36, $p = 0.002$ and OR: 2.50; 95%CI: 1.18–5.31, $p = 0.017$, respectively), while such effect of the studied hemostasis parameters could not be demonstrated.

Conclusions: In the studied cohort, PAI-1 5G/5G genotype was revealed as a significant, independent risk factor for post-lysis intracerebral hemorrhage. The presence of FXIII-A Leu34 allele provided a significant protective effect against developing larger (CBS 0–9) clots. The presence of larger thrombi (lower CBS) was found to be a significant independent predictor of poor short-term and long-term thrombolysis outcomes.

# New scientific findings

- In our prospective observational study of 131 acute ischemic stroke patients receiving intravenous thrombolysis, we found that PAI-1 activity was significantly reduced during thrombolytic treatment, while no change in PAI-1 antigen levels (including free, PAI-1–t-PA-complex and latent forms) was observed.
- PAI-1 activity levels measured at admission, PAI-1 antigen levels measured at admission and 1 hour after lysis were significantly higher in patients with more severe, larger ischemic lesions (ASPECTS <7) as observed on 24-hour control CT scans. However, PAI-1 activity and antigen levels at any of the time points studied failed to show significant association with the efficacy or safety of therapy.
- In a multiple logistic regression model including all possible common risk factors for post-lysis hemorrhage, the presence of PAI-1 5G/5G genotype was the strongest independent risk factor for the development of hemorrhagic transformation (OR: 4.75 95% CI: 1.18–19.06, p = 0.028).
- In our prospective observational case control study of 200 acute ischemic stroke patients receiving intravenous thrombolysis, we found that admission plasminogen levels were significantly lower in patients with a higher CBS score and smaller thrombus size, that might be a result of significantly higher incorporation of plasminogen to thrombi and as a consequence, the consumption of the protein.
- In univariate model, the presence of the FXIII-A Leu34 allele showed significant protection against the development of larger (CBS 0-9) clots (OR: 0.519; 95% CI: 0.298-0.922, p = 0.0227) in the studied cohort, which is consistent with the clot size-reducing effect of the allele that has been shown earlier in biochemical experiments.
- In univariate models, higher admission D-dimer levels, higher admission fibrinogen levels and lower 24-hour FXIII activity were significantly associated with unfavorable long-term outcome, but in multivariate models, the effects of these hemostasis factors on the studied outcomes could not be confirmed.
- In multivariate models, clot burden (CBS) was found to be among the most important independent predictors of short- and long-term outcomes in the studied cohort (CBS 0-9 vs. 10: OR: 2.777; 95% CI: 1.439–5.361, p = 0.002 and OR: 2.501; 95% CI: 1.179–5.306, p = 0.017).

# Publication list

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## A PhD értekezés alapjául szolgáló közlemények

1. **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.
   *PLoS One. 16* (7), 1-16, 2021.
   DOI: http://dx.doi.org/10.1371/journal.pone.0254253
   IF: 3.24 (2020)
2. Bagoly, Z., **Szegedi, I.**, Orbán-Kálmándi, R. A., Tóth, N. K., Csiba, L.: Markers of Coagulation and Fibrinolysis Predicting the Outcome of Acute Ischemic Stroke Thrombolysis Treatment: a Review of the Literature.
   *Front. Neurol. 10*, 1-13, 2019.
   DOI: http://dx.doi.org/10.3389/fneur.2019.00513
   IF: 2.889
3. **Szegedi, I.**, Nagy, A. C., Székely, E. G., Czuriga-Kovács, K. R., Sarkady, F., Lánczi, L., Berényi, E., Csiba, L., Bagoly, Z.: PAI-1 5G/5G genotype is an independent risk of intracranial hemorrhage in post-lysis stroke patients.
   *Ann. Clin. Trans. Neurol. 6* (11), 2240-2250, 2019.
   DOI: http://dx.doi.org/10.1002/acn3.50923
   IF: 3.66

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## További közlemények

4. 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.
DOI: http://dx.doi.org/10.1038/s41598-021-92041-1
IF: 4.379 (2020)

5. Kalmár, J. P., Tárkányi, G., Nagy, B. C., Csécsei, P., Lenzser, G., Bosnyák, E., Kárádi Nozomi, Z., Annus, Á., **Szegedi, I.**, Büki, A., Szapáry, L.: Comparing Endovascular Treatment Methods in Acute Ischemic Stroke Due to Tandem Occlusion Focusing on Clinical Aspects.
*Life (Basel). 11* (5), 1-10, 2021.
DOI: http://dx.doi.org/10.3390/life11050458
IF: 3.817 (2020)

6. Bagoly, Z., Baráth, B., Orbán-Kálmándi, R. A., **Szegedi, I.**, Kissné Bogáti, R., Sarkady, F., Csiba, L., Katona, É.: Incorporation of [alfa]2-Plasmin Inhibitor into Fibrin Clots and Its Association with the Clinical Outcome of Acute Ischemic Stroke Patients.
*Biomolecules. 11* (3), 1-13, 2021.
DOI: http://dx.doi.org/10.3390/biom11030347
IF: 4.879 (2020)

7. Kešnerová, P., Školoudík, D., Herzig, R., Netuka, D., **Szegedi, I.**, Langová, K., ANTIQUE Trial Group: Peripheral Vascular Resistance in Cerebral Arteries in Patients With Carotid Atherosclerosis - Substudy Results of the Atherosclerotic Plaque Characteristics Associated with a Progression Rate of the Plaque and a Risk of Stroke in Patients With the Carotid Bifurcation Plaque Study (ANTIQUE).
*J. Ultrasound Med. [Epub ahead of print]*, 2021.
DOI: http://dx.doi.org/10.1002/jum.15703
IF: 2.153 (2020)

8. Tárkányi, G., Csécsei, P., **Szegedi, I.**, Fehér, E., Annus, Á., Molnár, T., Szapáry, L.: Detailed severity assessment of Cincinnati Prehospital Stroke Scale to detect large vessel occlusion in acute ischemic stroke.
*BMC Emerg Med. 20* (1), 1-6, 2020.
DOI: http://dx.doi.org/10.1186/s12873-020-00360-9
IF: 2.119

9. Tárkányi, G., Karádi, Z. N., Szabó, Z. S., **Szegedi, I.**, Csiba, L., Szapáry, L.: Relationship between leukocyte counts and large vessel occlusion in acute ischemic stroke.
*BMC Neurol. 20* (1), 1-8, 2020.
DOI: http://dx.doi.org/10.1186/s12883-020-02017-3
IF: 2.474

10. **Szegedi, I.**, Orbán-Kálmándi, R. A., Csiba, L., Bagoly, Z.: Stroke as a Potential Complication of COVID-19-Associated Coagulopathy: a Narrative and Systematic Review of the Literature.
*J Clin Med. 9* (10), 3137-3148, 2020.
DOI: http://dx.doi.org/10.3390/jcm9103137
IF: 4.241

11. Finnsdóttir, H., **Szegedi, I.**, Oláh, L., Csiba, L.: The applications of transcranial doppler in ischemic stroke.
*Ideggyogy. Szle. 73* (11-12), 367-378, 2020.
DOI: http://dx.doi.org/10.18071/isz.73.0367
IF: 0.427

12. **Szegedi, I.**, Szapáry, L., Csanádi, Z., Csiba, L.: Potential biological markers of atrial fibrillation: a chance to prevent cryptogenic stroke.
*Biomed Res. Int. 2017* (8153024), 1-10, 2017.
IF: 2.583

**A közlő folyóiratok összesített impakt faktora: 36,861**
**A közlő folyóiratok összesített impakt faktora (az értekezés alapjául szolgáló közleményekre): 9,789**

A DEENK a Jelölt által az iDEa Tudóstérbe feltöltött adatok bibliográfiai és tudománymetriai ellenőrzését a tudományos adatbázisok és a Journal Citation Reports Impact Factor lista alapján elvégezte.

Debrecen, 2021.07.20.

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