Short Thesis for the Degree of Doctor of Philosophy (PhD)

Effect of fibrinolysis inhibitors on the outcome of intravenous thrombolysis in acute ischaemic stroke patients

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EFFECT OF FIBRINOLYSIS INHIBITORS ON THE OUTCOME OF INTRAVENOUS THROMBOLYSIS IN ACUTE ISCHAEMIC STROKE PATIENTS

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INTRODUCTION

Acute ischemic stroke (AIS) is a leading cause of death and the major cause of adult disability worldwide. Currently, the most effective pharmacological therapy for the restoration of the blood flow and lysis of the thrombi is recombinant tissue type plasminogen activator (rt-PA). Although rt-PA administration has a relatively short time window, it has immediately become a truly effective therapeutic option for a disease where previously the therapy was limited to rehabilitation and to avoid the secondary complications. Among the thrombolytic agents, streptokinase has been tested for this purpose since the 1970s, but the results have usually been disappointing due to severe haemorrhagic complications. In the 1980s, neurologists began to investigate rt-PA, and in a pathfinder study, Zivin and his group reported promising data on its use. Currently, the only registered thrombolytic agent for AIS remains rt-PA, which is safe and effective in the majority of cases, but the main side effect of this therapy is intracerebral haemorrhage, which can be fatal, occurring in about 3-40% of patients. On the other hand, in the majority of cases, the therapy is inefficient due to the failure of recanalization of the closed vessel, and no clinical improvement is observed. As of today, the inefficacy of treatment or therapy-associated intracranial hemorrhage (ICH) cannot be foreseen at the initiation of thrombolysis and their occurrence remains unexplained. In most cases, these complications cannot be foreseen at the initiation of therapy. It is plausible that the effectiveness as well as adverse effects of the thrombolytic agent could depend on hemostatic and fibrinolytic factors influencing the structure of thrombi and the susceptibility to lysis. The main fibrinolysis inhibitors that have a potential role in this process are coagulation factor XIII (FXIII) and α2-plasmin inhibitor (α2-PI), but the role of other proteins that inhibit fibrinolysis cannot be excluded (e.g. plasminogen activator inhibitor-1, thrombin-activatable fibrinolysis inhibitor). Despite the socio-economic burden of stroke and the relevance of the subject, this area of research is severely under-represented. This is evidenced by a recent systematic review of more than 6000 publications, which found only four where the authors included at least 100 AIS patients and analysed haemostasis factors in blood samples taken before thrombolysis. A better understanding of the role of fibrinolytic regulators in thrombolysis may be important for the future management of AIS patients: it may guide the development of new types of drug treatments and may also reveal factors that may allow the identification of patients with a high risk of developing haemorrhagic complications before therapy.

Nowadays, mechanical thrombectomy or its combination with rt-PA treatment for large vessel occlusions has opened up new perspectives, but is still only available in specialised centres for a small number of patients. The knowledge of which haemostasis factors influence the outcome of thrombolysis may in the long term contribute to the expansion of our diagnostic toolbox, helping clinical decisions towards a safer and more efficient therapeutic practice.

LITERATURE REVIEW

Fibrinolysis

Fibrin, as the primary product of the coagulation cascade and the final substrate for fibrinolysis, plays a central role in the process of haemostasis. The formation of fibrin is catalysed by thrombin, which cleaves fibrinopeptides from fibrinogen. Solubilized fibrin becomes insoluble by polymerization and is stabilized by cross-linking via activated factor XIII (FXIIIa). The timely degradation of the localised, cross-linked fibrin clot is the responsibility of the fibrinolytic system, whose central protease is the plasmin. The efficiency of fibrinolysis *in vivo* is strongly influenced by biochemical factors that are determined by the structure of the fibrin clot, the cellular elements present in the clot, and the general biochemical environment. The regulation of the fibrinolytic system, similarly to the coagulation cascade, is achieved through a finely tuned system of cofactors, receptors and inhibitors.

The central enzyme of fibrinolysis is plasmin, which is a member of the serine protease family. The inactive zymogenic form of plasmin is plasminogen, which is largely produced by the liver and circulates in plasma at concentrations of ~150 μg/ml. Plasminogen is structurally composed of an N-terminal Pan-apple domain (PAp), 5 kringle domains and a serine protease domain. The kringle domains contain a C-terminal lysine residue binding motif. These lysine-binding domains mediate the specific interactions of the protein with fibrin, cell surface receptors and other proteins, and inhibitors. The PAp domain, or activation peptide, plays an important role in regulating of plasmin activity and activation, in the presence of activation peptide the plasminogen is in a closed conformation. The open conformation can be stabilised by cleavage of the activation peptide by plasmin or by binding of lysine/lysine analogues to the lysine-binding domains. Activation of the plasminogen under physiological conditions is catalysed by plasminogen activators. During activation, by cleavage of the Arg-Val peptide bond at positions 560-561 in the C-terminal region of the plasminogen, the catalytic triad becomes available in the serine protease domain.

The physiological plasminogen activators of plasminogen are the urokinase-type plasminogen activator (u-PA), which belongs also to the group of serine proteases, and tissue-type plasminogen activator (t-PA). t-PA is synthesized and secreted mainly by endothelial cells, whereas u-PA is produced by monocytes, macrophages, renal epithelial cells and some tumor cells. Due to the high concentrations of specific inhibitors, both activators have extremely short half-lives in circulation, with half-lives of only 4-8 minutes.

In the absence of fibrin, t-PA binds to plasminogen with weak affinity. Interaction between the lysine-binding domain of plasminogen and lysines on the C-terminal of fibrinogen increases the efficiency of plasminogen activation by several hundred-fold. The fibrin degradation powdered by the formed plasmin results in additional C-terminal lysine residues, thus providing even more binding sites for plasminogen and t-PA, creating a positive feedback mechanism. Plasminogen activation by t-PA promotes intravascular fibrin degradation: it prevents unnecessary accumulation of intravascular fibrin and allows the removal of already formed clots. Unlike t-PA, u-PA does not have a fibrin binding site. u-PA, by binding to specific cell surface receptors, primarily activates cell surface-bound plasminogen. The cell-surface activated plasmin plays an important role in extracellular matrix degradation, in the activation of certain growth factors, therefore u-PA is not involved in intravascular fibrin degradation under physiological conditions.

The function of proteases in the fibrinolytic system is tightly regulated by several endogenous inhibitors. Plasminogen activators and plasmin itself are neutralised in the circulation by serine protease inhibitors, also known as serpins. The main physiological inhibitor of plasminogen activators is plasminogen activator inhibitor-1 (PAI-1), which is an effective inhibitor of both t-PA and u-PA. PAI-1 is a 45 kDa single-chain glycoprotein, which is the key regulator of the fibrinolytic system. It is produced in many tissue and cell types, including the liver, spleen, endothelial cells, smooth muscle cells, macrophages and adipocytes. Circulating platelets also synthesise PAI-1 continuously, therefore when PAI-1 is released during platelet activation, the local concentrations of PAI-1 can increase up to tenfold, reducing fibrinolysis at the onset of coagulation. The production and secretion of PAI-1 is influenced by a number of genetic factors and various conditions such as insulin resistance, obesity, plasma lipid levels and the circadian rhythm. Based on literature it is known, that several inflammatory cytokines, some growth factors, hormones, glucose and endotoxins stimulate PAI-1 expression. PAI-2, which was first extracted from the placenta, has weaker inhibitory properties, being mainly involved in the inhibition of u-PA. PAI-2 is essentially undetectable in healthy individuals, but its plasma concentration increases dramatically during pregnancy.

The main natural inhibitor of activated plasmin is α2-plasmin inhibitor (α2-PI). α2-PI binds rapidly and irreversibly to plasmin, forming a stable 1:1 inactive plasmin-antiplasmin complex. Plasmin, when linked to the fibrin clot is protected against the inhibitory effect of α2-PI, ensuring proteolytic degradation of fibrin.

The nonserpin-dependent regulation of fibrinolysis is mediated by, among others, the thrombin-activatable fibrinolysis inhibitor (TAFI). TAFI is synthesised by the liver and megakaryocytes

as an inactive zymogen. The proenzyme is converted into a functionally active form by thrombin, the thrombin-thrombomodulin complex, or plasmin. TAFI is present in the circulation in concentrations of 4-15 μg/mL. Its active form is a carboxypeptidase that removes the C-terminal lysine and arginine side chains of fibrin, thereby reducing the number of available plasminogen binding sites, slowing down the formation of plasmin and thereby stabilising the clot.

α2-PI and its major polymorphisms

α2-PI was first described in 1976 by three different research groups (Mullertz, Moroi and Collen and coworkers) as a fast-acting inhibitor of plasmin. As a result, three different names are used in the literature: α2-plasmin inhibitor, anti-plasmin and primary plasmin inhibitor. A significant part of α2-PI is synthetised by the liver, but it is also produced by the kidney, platelets, gastrointestinal tract, muscles, lungs, placenta and brain (cortex, hippocampus and cerebellum). In Alzheimer's disease patients the increased expression is associated with amyloid β plaques. α2-PI is secreted into the plasma as a single-chain polypeptide with a molecular mass of 70 kDa and composed of 464 amino acids. Structurally, α2-PI has a 12 amino acid N-terminal domain, a central serpin domain and a C-terminal tail ~55 amino acids in length.

In the circulation α2-PI undergoes both amino-terminal (N-terminal) and carboxyl-terminal (C-terminal) proteolytic modifications, leading to four heterogeneous α2-PI isoforms with modified activities. The secreted α2-PI carries methionine on its N-terminal side (Met-α2-PI). Approximately 70% of circulating α2-PI is N-terminally cleaved by antiplasmin cleaving enzyme (APCE), which removes an oligopeptide of 12 amino acids from the amino-terminal end. Lee and his group have shown that the cleaved (Asn-α2-PI) isoform is incorporated into fibrin filaments 13 times faster than the full-length Met-α2-PI protein. The cross-linking of α2-PI to fibrin is catalysed by FXIIIa, and the cross-linked inhibitor makes fibrin more resistant to degradation by plasmin. Since cross-linking of Met-α2-PI is slower, elevated levels of this isoform are associated with increased fibrinolysis.

The carboxy-terminal part of α2-PI also undergoes post-translational modifications, but the enzyme responsible for cleavage has not yet been identified. The located lysine side chains play a key role in interacting with the lysine-binding sites of the plasminogen. Based on this interaction, two isoforms are distinguished. The uncleaved, longer, plasminogen-binding form (PB-α2-PI) accounts for approximately 65% of the α2-PI in the circulation and is an effective inhibitor of plasmin through the formation of plasmin-antiplasmin complexes. In contrast, the

shorter, cleaved isoform is unable to bind to plasminogen (NPB-α2-PI). C-terminal cleavage of α2-PI may have important clinical implications, as the PB-α2-PI form is primarily required for α2-PI-fibrin cross-linking mediated by activated FXIII. Consequently, C-terminal cleavage of the circulating protein regulates α2-PI activity.

In addition to these modifications, the heterogeneity of α2-PI is affected by a common polymorphism of the SERPINF2 gene. In a study of a family with frequent bleeds, Lind and his group identified 3 polymorphisms in the α2-PI gene. One of these was a C/T single nucleotide polymorphism (SNP) affecting the nucleotides encoding amino acid 6 of the Met-α2-PI gene, which can result in arginine or tryptophan at position six. The study also demonstrated that both forms occur in healthy human plasma. Christiansen and his group were able to demonstrate that p.Arg6Trp is a functional polymorphism that affects the conversion of Met-α2-PI to the Asn-α2-PI form, thereby influencing the rate of incorporation of α2-PI into fibrin. It has been shown that APCE cleaves the Met-α2-PI (Arg6) form approximately eight times faster than Met-α2-PI (Trp6), and that the rate of N-terminal proteolysis is highest in homozygous (Arg6Arg) individuals. The pathophysiological and clinical significance of the p.Arg6Trp polymorphism is still under investigation. Our working group previously found that the presence of the Trp allele did not affect the risk of developing venous thrombosis. Bridge and his group investigated the association between the p.Arg6Trp polymorphism and the development of abdominal aortic aneurysms and concluded that carrying the Trp allele slightly, but not significantly reduces the risk of developing aneurysms. However, their results showed that another polymorphism of α2-PI (p.Arg407Lys) was significantly associated with abdominal aortic aneurysm development, with a 23% reduction in the risk of abdominal aortic aneurysm in Lys407 carriers.

FXIII and its major polymorphisms

FXIII, also known as fibrin stabilising factor, is the final clotting factor in the coagulation cascade and a major inhibitor of fibrinolysis. Its physiological function is to stabilize fibrin by cross-linking and making it resistant against the prompt fibrinolytic degradation by plasmin. FXIII in plasma is a heterotetrameric protein (FXIII-A_2B_2) with a molecular mass of 325 kDa. The tetramer is composed of two potentially catalytic A subunits (FXIII-A) and two carrier/inhibitory B subunits (FXIII-B). Under physiological conditions, its concentration in plasma is 14-28 mg/L, where the inactive protein circulates in a fibrinogen-bound form. FXIII-A is produced in cells of bone marrow origin, whereas FXIII-B is produced in hepatocytes, the subunits forming the tetrameric structure in plasma. Approximately 50% of the excess subunit

B circulates in the plasma in a free, noncomplexed form. The cellular form of FXIII consists only of the two potentially catalytic subunits (FXIII-A_2), which are found in the cytoplasm of many cell types (e.g. monocytes/macrophages, platelets, osteoblasts). In platelets, FXIII-A_2 is present in 46-82 fg/platelet, which corresponds to 3% of the total platelet protein. FXIII is a zymogen (protransglutaminase) of the transglutaminase family. The catalytic A subunit is composed of 5 domains: the N-terminal activation peptide, a β-sandwich, a catalytic central domain, and two β-barrel domains. The catalytic domain carries the catalytic triad responsible for the enzyme reaction, which is inaccessible to the substrate in the inactive enzyme. The B subunit is a glycoprotein containing about 8.5% carbohydrate, consisting of a total of ten so-called sushi domains, each stabilized by two disulfide bridges. The B subunit plays a protective role by inhibiting the degradation of the catalytic subunit, thus increasing its lifetime from 3 to 11 days. Based on previous research, the interaction between fibrinogen and FXIII-A_2B_2 zymogen is thought to be mediated by FXIII-B subunits. The activation of FXIII occurs in the last step of the clotting cascade by thrombin and Ca^{2+}. As a first step, thrombin cleaves the activation peptide from the amino-terminal of FXIII-A subunits. Subsequently, the B subunits are dissociated by Ca^{2+} and the cleaved catalytic subunit undergoes a conformational change. As a result, the active site becomes accessible, and the active form (FXIIIa) is formed. The presence of fibrin accelerates the activation process 80-100-fold, and FXIII is activated in the plasma on the newly formed fibrin surface. In the absence of FXIII-B, the cellular form of FXIII does not require proteolytic cleavage, and the increase of the intracellular Ca^{2+} concentration is sufficient for the active configuration to be formed. FXIIIa catalyses an acyl transfer reaction in which the acyl donor is a glutamine γ-carboxamide group in a peptide chain and the acyl acceptor is a primary amine. In the process, the primary amine is covalently linked to the γ-glutamyl side chain by an isopeptide bond. If the primary amine is the ε-amino group of a lysine in a peptide/protein, FXIIIa covalently cross-links the two polypeptide chains by intermolecular ε-N-(γ-glutamyl)-lysine cross-linking.

FXIIIa plays a crucial role in the regulation of fibrinolysis. Its antifibrinolytic effect is mediated by 3 major ways. Firstly, FXIIIa incorporates α2-PI and other plasma components to the forming fibrin clot through covalent cross-linking, thereby providing effective protection against premature plasmin-mediated proteolytic fibrin degradation. Second, FXIIIa cross-links fibrin α-chains into high molecular weight α-polymers, which most likely has a direct effect on the susceptibility of fibrin clots to lysis. Thirdly, cross-linking of C-terminal lysines of fibrin α-chains reduces the number of binding sites available for plasminogen and t-PA on fibrin, thus reducing the rate of plasminogen activation. FXIII deficiency results in bleeding, which can be

severe or even life-threatening depending on the degree of deficiency. In congenital forms, prolonged umbilical bleeding, slow wound healing, but also skin, muscle and mucosal haemorrhages and, as a feared consequence, severe and even fatal intracranial haemorrhage are typical.

The gene encoding human FXIII-A (F13A1) is over 160 kb long and is located on chromosome 6p24-25. The gene encoding FXIII-B (F13B) is 28 kb long; it is located on chromosome 1q31-32.1 and consists of 12 exons and 11 introns. Five common coding polymorphisms have been identified in the A subunit (p.Val34Leu, p.Tyr204Phe, p.Pro564Leu, p.Val650Ile, p.Glu651Gln) and two in the B subunit (p.His95Arg and c. 1952 + 144 C > G in intron K).

The most studied polymorphism in FXIII is p.Val34Leu. This SNP is a G>T substitution affecting codon 103 of the gene encoding the A subunit, resulting in the replacement of valine at position 34 with leucine. The p.Val34Leu polymorphism is located near the thrombin cleavage site within the activation domain, thereby affecting the rate of FXIII activation. In the Leu34 variant, thrombin cleavage occurs 2.5-fold faster, so the presence of the Leu34 allele is associated with increased FXIII activation. Accordingly, fibrin cross-linking occurs faster in the growing clot. The polymorphism also affects the structure of the cross-linked fibrin network, which is influenced by the concentration of fibrinogen. At high fibrinogen concentrations, individuals homozygous for the Leu34 allele develop a clot with thicker fibrin fibres, a looser structure and increased permeability, which is less resistant to fibrinolysis. The FXIII-A p.Val34Leu polymorphism and the risk of venous and arterial thrombosis have been the subject of numerous studies over the past two decades, the results of which have often been contradictory. A meta-analysis of these studies suggests that the presence of the 34Leu allele confers a small but significant protection against the development of coronary artery disease and venous thromboembolism, but does not appear to be a significant protective or risk factor for ischaemic stroke.

There is considerably less literature on the pathophysiological role of other common polymorphisms of the FXIII-A subunit. Pruissen and his group found a strong association between the p.Tyr204Phe variant and the risk of ischemic stroke in young women, the risk associated with carrying the Phe204 allele was further increased by the use of oral contraceptives. Their data, however, showed that the FXIII-A p.Pro564Leu polymorphism did not affect the risk of ischemic stroke in the studied population. However, Reiner and his group found that in women under 45 years of age, FXIII Phe204 and Leu564 variants may serve as markers of genetic susceptibility to hemorrhagic stroke.

Molecular genetic and biochemical techniques have revealed two major polymorphisms in the F13B gene. The A>G substitution within exon 3 leads to His-Arg amino acid substitution at position 95 of the mature protein. Arg95 is relatively rare (7.5%) in the Caucasian population. The p.His95Arg polymorphism has been identified as a risk factor for venous thromboembolism and has been shown to increase the risk of ischemic stroke 1.7-fold in young women.

The other polymorphism described in the FXIII-B gene is a C>G substitution at nucleotide position 29756 of intron K, which leads to a new splice acceptor site. The polymorphism results in an allele-specific splicing product in which the last 10 amino acids are replaced by an alternative sequence of 25 amino acids. The variant sequence contains two additional lysines and a glutamic acid. These amino acids change the isoelectric point of the protein. The polymorphism is typically found in Asians, and with an allele frequency of 14.2% in the Caucasian population.

Thrombolytic therapy for the treatment of acute ischemic stroke

Stroke is defined as a 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. Stroke is one of the most important and serious vascular diseases and is the leading cause of mortality worldwide. As the leading cause of adult disability, stroke has the highest socio-economic burden of all vascular diseases. Therefore, early detection and prompt treatment of stroke are of high importance to prevent or minimise morbidity and mortality. The pathomechanism of the most common acute cerebrovascular accidents is ischaemic in origin, accounting for 80% of all acute strokes. In the remaining approximately 20%, haemorrhagic pathology is behind the symptoms. Within haemorrhagic stroke, two main subtypes can be distinguished: intracerebral haemorrhage (ICH) (15%) and subarachnoid haemorrhage, which accounts for about 5% of all strokes.

An ischaemic stroke is a sudden functional impairment of the brain tissue that occurs due to the blockage of a cerebral vessel. 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. Approximately 45% of AIS cases develop at

the base of local atherosclerosis and are of thrombotic origin. In the majority of the cases the intracranial large vessels are involved, where the thrombus develops on the surface of ruptured atherosclerotic plaques. The other type of thrombotic stroke involves occlusion of small cerebral vessels. These so-called lacunar strokes usually develop from a precedent hypertension. If the occlusion of the artery is caused by thrombi from atrial fibrillation, embolization of thrombus from the carotid artery or from the ascending aorta, it is called a cardioembolic stroke, which accounts for about 20% of all ischemic strokes. About 20% to 30% of ischemic strokes are cryptogenic, i.e. the infarct cannot be linked to a definitive source of embolism, large vessel atherosclerosis or small vessel disease. On the basis of the above, the etiological subtypes of ischaemic stroke can be classified according to the classic TOAST (Trial of ORG 10172 in Acute Stroke Treatment) criteria system into the following groups: large artery atherosclerosis, cardioembolism, small vessel occlusion (lacunar stroke), other defined pathogenesis, and cryptogenic stroke.

The National Institute of Health Stroke Scale (NIHSS) is used to objectively assess the severity of ischemic strokes. An increase in NIHSS score indicates the degree of neurological deficit, correlates with stroke severity and reflects clinical status. The NIHSS score predicts clinical outcome and a high score indicates an increased risk of future complications. Several scoring systems are used to assess the radiological severity of stroke. The Alberta Stroke Program Early CT Score (ASPECTS) is a 10-point quantitative system used to determine the extent of ischemic lesions supplied by the middle cerebral artery. The scoring system divides the region supplied by the middle cerebral artery into 10 areas, with 1 point deducted for ischemic lesions in each area. The system divides the region supplied by the middle cerebral artery into 10 segments: 1 point is subtracted if an ischaemic lesion is observed in a given segment. A ≤ 7 score based on CT scan on admission, is a predictor of adverse functional outcome in intravenous thrombolysis.

The chance of recovery for patients with AIS depends on the successful opening of the blocked vessel in a relatively short time. Recanalisation can be achieved by rapid drug dissolution of the thrombi (thrombolysis) and/or surgical removal (mechanical thrombectomy). In Hungary, the only currently registered drug for use as a thrombolytic agent in AIS is rt-PA (Alteplase, Actilyse; Boehringer Ingelheim). Intravenous thrombolysis with rt-PA is most likely to be used within 4.5 hours after the onset of symptoms in stroke patients with suitable lysis candidates based on inclusion and exclusion criteria, with a window of 9 hours for specific imaging tests. Although this is now a routine therapy, the narrow time window means that the majority of patients with AIS do not receive this treatment. According to the guidelines, the dose of

intravenous rt-PA is 0.9 mg/kg, and it must not exceed 90 mg. Ten percent of the dose is administered as a bolus to AIS patients, followed by the remaining 90% administered as a continuous intravenous infusion over 60 minutes. Several clinical trials have demonstrated that intravenous thrombolysis within a time window has resulted in significant neurological improvement, but its efficacy is greater when elapsed time is shorter between symptom onset and rt-PA treatment. Despite the undoubted benefits of rt-PA in the treatment of AIS, favourable therapeutic outcomes are observed in only ~30-40% of patients, and its efficacy is greatly reduced in cases of large vessel occlusion. As a complication of thrombolytic therapy, despite the minimisation of bleeding risk, haemorrhagic transformation develops in about 3-40% of patients (depending on the study or definition), which may be symptomatic and therefore worsens the outcome in approximately half of the cases, and may even be fatal.

Today, mechanical thrombectomy is the standard therapy for large vessel occlusions. During mechanical recanalisation, the thrombi blocking the blood vessel is removed by catheterisation. The procedure is effective within a 6-hour time window, but can be extended to 24 hours in selected patient groups. A practical limitation of the treatment is that only ~20% of stroke patients suffer from a large vessel occlusion, and managing these patients within the time window is challenging as the procedure can only be performed in highly specialised centres.

AIMS

Our aim was to investigate whether the levels and common polymorphisms of FXIII and α2-PI, two key inhibitors of fibrinolysis, are associated with the outcome of intravenous thrombolysis in AIS patients.

In detail, by means of prospective observational studies, we aimed to investigate:

1. How FXIII and α2-PI activity and antigen levels change before and after thrombolytic therapy in plasma samples of AIS patients undergoing intravenous thrombolysis, and whether these results are associated with the outcome and the development of bleeding complications.
2. Whether common polymorphisms in FXIII-A and FXIII-B subunits (FXIII-A p.Val34Leu, p.Tyr204Phe and FXIII-B p.His95Arg, intron K c. 1952 + 144 C > G) and α2-PI p. Arg6Trp polymorphisms affect thrombolysis outcome and the development of bleeding complications.

PATIENTS AND METHODS

Acute ischemic stroke patients treated with intravenous thrombolysis

Patients

Two prospective observational studies were carried out. In both studies, AIS patients who received intravenous thrombolysis within 4.5 hours of stroke onset were selected in collaboration with the Department of Neurology, University of Debrecen. In the first study, patient enrolment was initiated in March 2011 and was completed in January 2013, in the second study, patient enrolment continued and additional patients were recruited between September 2016 and April 2019. Patient treatment was consistent throughout the study. Intravenous thrombolysis was administered according to the European Stroke Organisation (ESO) 2008 rt-PA (Alteplase, Boehringer Ingelheim, Ingelheim an Rhein, Germany) treatment guidelines. Inclusion and exclusion criteria of patients were identical to standard criteria of thrombolysis. None of the patients included in the cohort were eligible for mechanical thrombectomy: thrombectomy was either unavailable at the time of enrolment or the patient was unsuitable to perform the procedure. The presence of AIS was diagnosed based on clinical symptoms and imaging using noncontrast computerized tomography (CT) scan, and CT angiography (CTA). CT images taken on admission and 24 h post-lysis was analysed 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. TOAST criteria were 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 in this study:

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 (neurologic improvement), 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. Therapy-associated intracerebral hemorrhage (ICH): symptomatic (SICH) or asymptomatic (aSICH) using the European Cooperative Acute Stroke Study (ECASS) II criteria, as observed on CT scans at 24h post-lysis.

Informed consent

The study designs were in accordance with the guiding principles of the Declaration of Helsinki. The tests were approved by the Institutional Ethics Committee of the University of Debrecen and the Ethics Committee of the National Medical Research Council (approval number: 3287-2010 RKEB/IKEB, 4672-2016 RKEB/IKEB and 12698-1/2017/EKU). All patients or their relatives provided written informed consent.

Blood sampling and laboratory measurements

Peripheral blood samples were taken from all patients on admission, before the initiation of rt-PA infusion, and at 24 hours post-lysis. In a subset of patients (n=131), blood was also obtained immediately after administering full dose of rt-PA. Routine laboratory tests (ions, glucose level, renal and liver function tests, high-sensitivity C-reactive protein measurement, complete blood count) were measured by standard laboratory methods (Roche Diagnostics, Mannheim, Germany and Sysmex Europe GmbH, Hamburg, Germany) at the Department of Laboratory Medicine, University of Debrecen.

For studying specific hemostasis tests, blood samples were collected into vacutainer tubes containing 0.109 M sodium citrate (Becton Dickinson, Franklin Lane, NJ) respective into tubes containing sodium citrate, theophylline, adenosine and dipyridamole (Vacutte CTAD tubes, Greiner Bio-One, Austria) and were processed immediately (centrifugation twice at 1500 g, room temperature for 15 min). Screening tests of coagulation (prothrombin time, activated partial thromboplastin time, and thrombin time) and the determination of fibrinogen levels according to Clauss were performed on a BCS coagulometer using standard methods (Siemens Healthcare Diagnostic Products, Marburg, Germany). For the determination of FXIII activity and antigen levels, α2-PI activity and antigen levels, aliquots of citrated plasma were uniquely coded and stored at -80 ◦C until analysis in batches.

Determination of FXIII activity, FXIII-A_2B_2 antigen level and major FXIII polymorphisms

Plasma levels of FXIII activity were determined by ammonia release assay using a commercially available reagent kit (REA-chrom FXIII kit, Reanal-ker, Budapest, Hungary, reference range: 69-143%, CV: 3.8%). FXIII-A_2B_2 antigen levels were determined by a sandwich enzyme-linked immunosorbent assay (ELISA), comprising of a biotinylated monoclonal capture-antibody against the B-subunit and a peroxidase-labelled monoclonal tag-antibody against the A-subunit (reference range: 14-28 mg/l, CV: 2.0%). DNA isolation was performed from buffy coat of CTAD blood samples by QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). FXIII-A p.Val34Leu (c.103G>T; rs5985), FXIII-A p.Tyr204Phe (c.614A>T; rs3024477), FXIII-B p.His95Arg (c.344G>A; rs6003) and FXIII-B Intron K (IVS11 c.1952+144C>G; rs12134960) polymorphisms were determined by in-house developed real-time PCR methods using fluorescence resonance energy transfer (FRET) detection and melting curve analysis on a LightCycler® 480 instrument (Roche Diagnostics GmbH, Mannheim, Germany). In one patient with considerably low FXIII levels at admission (<50%) who developed a therapy-associated bleeding complication, Sanger sequencing was performed to identify mutations in the F13A1 exons, intron regions and promoter regions using ABI3130 Genetic Analyzer and Sequencing Analysis 5.4 software (Termo Fisher Scientifc, Carlsbad, CA).

Determination of α2-PI activity, α2-PI antigen level and α2-PI p.Arg6Trp polymorphism

Functional α2-PI activity was measured from stored plasma samples using the Berichrom α2-PI activity chromogenic assay on a BCS coagulometer following the manufacturer's instructions (Siemens Healthcare Diagnostic Products, Marburg, Germany). The assay principle is the following: patient plasma containing α2-PI is incubated with the reagent that contains plasmin in excess. Plasmin becomes rapidly inactivated by α2-PI and residual plasmin activity is measured by an amidolytic assay based on the cleavage of the plasmin-specific chromogenic substrate (D-norvalil-cyclohexylalanil-lysil-nitroanilid). α2-PI activity inversely correlates with the changes in absorption at 405 nm. Total α2-PI antigen levels were measured by an in-house ELISA test. This assay detects all forms of α2-PI and is not influenced by the presence of plasmin-antiplasmin complexes (reference range of plasma α2-PI antigen levels: 48-85 mg/L). The α2-PI Arg6Trp (rs2070863) polymorphism was identified by real-time PCR using fluorescence resonance energy transfer detection and melting curve analysis on a LightCycler® 480 instrument (Roche Diagnostics GmbH, Mannheim, Germany).

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). Shapiro-Wilk test was used to assess the normality of the data. For continuous variables, the Student's t-test or Mann-Whitney U test was used to determine the difference between two groups. In case of paired data, paired t-test or Wilcoxon signed-rank test used for paired data depending on normality. Depending on the normality of data, ANOVA with Bonferroni post-hoc test or Kruskal–Wallis analysis with Dunn–Bonferroni post hoc analysis was applied for multiple group comparisons. Strength of association between variables was tested using Pearson's or Spearman correlation test. Differences between categorical variables were assessed by $\chi 2$ test or by Fisher's exact where appropriate depending on case numbers. The Kaplan-Meier method was applied to plot survival vs. nonsurvival of patients based on their α2-PI levels. Survival curves were compared using the log-rank test. Binary backward logistic regression models were used to determine independent predictors of mortality and long-term functional outcomes. Adjustments of the models were based on the results of univariate statistical analyses of baseline characteristics between groups, previous literature, and methodological principles. 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

Association of FXIII levels and common FXIII polymorphisms with outcomes in acute ischemic stroke patients treated with intravenous thrombolysis

Study population

A total of 132 consecutive AIS patients undergoing thrombolysis were included in the study. Mean age was 69.0±12.2 years, and 58.3% were men. The median NIHSS score on admission was 8 (interquartile range: 5-14). According to the TOAST criteria, most patients suffered a large vessel thrombosis (n=49, 37.1%). Average time from symptom onset to treatment with rtPA was less than 3 h in the cohort and the duration of thrombolysis was approximately one hour for each patient. In case of 7 patients intravenous thrombolytic therapy was supplemented with intraarterial thrombolysis according to standard protocols; the final dose of rtPA and the duration of thrombolysis were not significantly different for these patients as compared to the rest of the study group. Favorable short-term and long-term functional outcomes were observed in 53 (40.2%) and 46 (34.8%) cases, respectively. Stroke-associated mortality by day 7, day 14 and by the end of the 3rd month post-event was observed in 5 (3.8%), 18 (13.6%), and 29 (22.0%) cases, respectively. Therapy-associated bleeding complication was detected in 13 cases (7 patients presented with aSICH, 6 patients with SICH).

The influence of thrombolysis on FXIII levels

On admission (before thrombolysis) a considerable number of patients (n=39, 29.5%) had FXIII levels above the upper limit of the reference interval (above 143% or 28 mg/l). FXIII levels showed a gradual decrease after thrombolysis; at 24 h post-lysis significantly lower FXIII levels were detected as compared to initial values. Strong correlation was observed between FXIII activity and FXIII antigen levels at all investigated occasions (before thrombolysis: Pearson $r=0.915$, $p<0.001$; after thrombolysis: $r=0.919$, $p<0.001$ and 24 hours post-lysis: $r=0.917$, $p<0.001$).

Association of FXIII levels on admission with stroke severity and etiology

FXIII levels on admission showed no association with the severity of the stroke if divided into categories according to NIHSS and no correlation was observed between FXIII levels and NIHSS scores on admission (Pearson $r=-0.09$, $p=0.28$). FXIII activity was significantly higher

in case of atherothrombotic stroke as compared to strokes of cardioembolic origin. FXIII activity and antigen levels showed a weak negative association with the age of the patients (Pearson r=-0.299, p<0.001 and r=-0.286, p<0.001). FXIII antigen levels were significantly higher in active smokers vs. never-smokers (24.60 mg/l vs. 21.15±0.9 mg/l, p<0.05). No correlation was observed between FXIII levels and any measured routine clinical chemistry, hemostasis and hematology parameters. No correlation was observed between FXIII levels measured at any time points and data obtained from CT imaging analysis (ASPECTS). No correlation was observed between FXIII activity/antigen levels measured on admission and the elapsed time between symptom onset to thrombolysis treatment (FXIII activity: r=0.081, p=0.186 and FXIII antigen: r=0.061, p=0.251).

Association of FXIII levels and outcome of thrombolytic therapy

FXIII levels before the initiation of the therapy or immediately after thrombolysis showed no association with short-term functional outcomes. On the contrary, at 24 h after thrombolysis significantly lower FXIII levels were detected in those patients who died within the first week following treatment. FXIII levels were remarkably low in these patients, approximately 50% as compared to values measured in patients with other outcomes. Mortality and low FXIII levels were not associated with bleeding complications. On the other hand, no difference was observed in the FXIII levels of patients with any other outcomes except for mortality (i.e. favorable outcome vs. no change or unfavorable outcome; data not shown). Patients experiencing bleeding after therapy were separately handled during the analysis due to different assumed underlying pathomechanisms.

Low FXIII levels were associated with mortality by day 14 post-event as well. When studying the baseline characteristics of patients grouped according to mortality by the end of the 2nd week, it was found that besides the NIHSS on admission, only FXIII levels measured before and 24 h after thrombolysis were significantly different in the two groups. Similar results were observed when FXIII levels were investigated in terms of functional outcomes at 3 months post-event. FXIII levels 24 h after lysis were significantly lower in those patients who died by the end of the 3rd month after the event (mRS 6). In order to test whether a low FXIII level measured 24 h after thrombolysis is an independent predictor of short- and long-term mortality of patients, backward multiple regression analysis for mortality was performed including all potentially relevant risk factors and confounders. NIHSS score on admission was found to be an independent predictor of mortality by 14 days (OR: 1.12; 95%CI: 1.02–1.23, p=0.013) and by the end of the 3rd month after the event (OR: 1.16; 95%CI: 1.05–1.28, p=0.004). FXIII levels

in the lowest quartile 24 h after thrombolysis were found to be an independent predictor of short-term mortality (OR: 4.95; 95%CI: 1.31–18.68, p=0.018). On the other hand, low FXIII levels 24 h after thrombolysis did not prove to be an independent predictor of mortality at 3 months post-stroke (OR: 1.88; 95%CI: 0.55–6.41, p=0.311).

In patients suffering therapy-associated intracerebral bleeding complications (n=13), FXIII levels during thrombolysis were surprisingly similar to that observed in patients without complications. Although a decreasing trend was observed immediately after thrombolysis, FXIII activity or antigen levels did not show a significant difference between patients without bleeding and patients with SICH or aSICH complications at any time point measured. In this cohort, only one patient with therapy-associated hemorrhage had low FXIII levels (FXIII activity before thrombolysis: 45.9%, immediately after thrombolysis: 50.2%, 24 h after thrombolysis: 39.9%). Heterozygous FXIII-A deficiency was ruled out in this patient by direct fluorescent sequencing of F13A1 gene. Except for this single patient, FXIII levels were within or above the reference range at all measured time points in patients suffering symptomatic or asymptomatic bleeding as side-effect. One patient had died due to therapy-associated SICH by day 1, in this case, FXIII levels were also within the reference interval (FXIII activity before thrombolysis: 107.7%, immediately after thrombolysis: 104.5%).

FXIII polymorphisms, stroke characteristics and thrombolytic outcomes

Allele frequencies of the common FXIII-A and FXIII-B polymorphisms were not significantly different in this AIS patient population as compared to a large cohort of population control group tested earlier. In agreement with previous findings, the FXIII-A p.Val34Leu, FXIII-A p.Tyr204Phe, FXIII-B p.His95Arg polymorphisms had no influence on FXIII levels. Carriers of the FXIII-B intron K nt29756 G allele showed significantly lower FXIII levels as compared to noncarriers (FXIII activity: 114.5±30.9% vs. 130.46±36.9%, p=0.021, respectively and FXIII antigen levels: 19.26±1.2 vs. 23.5±0.75, p=0.004, respectively), but after adjustment to confounders (age, CRP, smoking), differences were not statistically significant among the two groups. None of the investigated factor XIII polymorphisms were associated significantly with stroke severity, unfavorable short-term or long-term outcomes of therapy, therapy-associated symptomatic intracranial hemorrhage and mortality. In case of FXIII-A p.Val34Leu polymorphism, a trend could be observed showing that carriers of the Leu allele might be protected against unfavorable short-term outcomes (OR: 0.33; 95% CI: 0.09-1.10), but the association was not statistically significant (p=0.072).

Association of α2-PI levels and α2-PI p.Arg6Trp polymorphism with outcomes in acute ischemic stroke patients treated with intravenous thrombolysis

Study population

A total of 421 AIS patients receiving intravenous thrombolysis were included in the study. Median age of the cohort was 68 (IQR: 60-77) years, 57.2% were men. Median NIHSS on admission was 7 (IQR: 4-11). Median time from symptom onset to treatment with rt-PA was 150 (IQR: 115-185) min. The most frequent cerebrovascular risk factor was hypertension (82.2 %). Favorable short- and long-term outcome was achieved in 45.2% and 48.6% of patients, respectively, excluding cases with post-lysis intracranial hemorrhage. ICH occurred in 34 patients (8.1%), in 14 cases (3.3 % of total cohort) it was considered SICH, and in 20 patients (4.8% of total cohort) aSICH.

The effect of thrombolysis on α2-plasmin inhibitor levels

Admission α2-PI activity and antigen levels showed a surprisingly wide distribution, but the majority of patients had α2-PI levels within the reference range. Both α2-PI activity and antigen levels showed a highly significant decrease immediately after thrombolysis, indicating a rapid complex formation and consumption of the protein by the generated plasmin during the procedure. α2-PI activity and antigen levels of all patients were below the lower limit of reference when measured immediately after thrombolysis (α2-PI activity median: 8 [IQR: 1-29] %; α2-PI antigen median: 11.3 [IQR: 8.2-17.3] mg/L).

Twenty-four hours after thrombolysis, α2-PI activity and antigen levels were substantially increased but were still below admission values (α2-PI activity median: 76 [66-86] %; α2-PI antigen median: 39.4 [IQR: 34.1-46.1] mg/L). Strongest correlation between α2-PI activity and antigen levels ($r=0.770$, 95%CI: 0.723-0.808, $p<0.001$) were observed at 24 h after thrombolysis. Interestingly, the association between α2-PI activity and antigen levels was found to be the weakest on admission in this cohort ($r=0.560$, 95%CI: 0.486-0.627, $p<0.001$). Among the baseline clinical and laboratory parameters, both α2-PI activity and antigen levels showed a modest significant negative correlation with age (Spearman r: -0.2244; 95%CI: -0.3168 to -0.1278, $p<0.001$ and r: -0.3908; 95%CI: -0.4739 to -0.3008, $p<0.001$, respectively). α2-PI activity, but not α2-PI antigen, showed a fair significant positive correlation with admission fibrinogen levels (r: 0.3623; 95%CI: 0.2723 to 0.4460, $p<0.001$). α2-PI activity and antigen levels were significantly higher in active smokers compared to non-smokers on admission (α2-

PI activity: median: 106 [98-114] % vs. 101 [92-109.5] %, p=0.0024, respectively α2-PI antigen: median: 61.0 [56.4-70.4] mg/L vs. 58.7 [52.6-66.9] mg/L p=0.0135). The higher α2-PI levels seen in active smokers were also observed 24 hours after thrombolysis (α2-PI activity: median: 81 [69-90] % vs. 75 [65-83] %, p=0.0005, respectively α2-PI antigen: median: 43.4 [35.7-48.7] mg/L vs. 38.4 [33.2-44.8] mg/L, p=0.0011). α2-PI activity and antigen levels were significantly higher in women compared to men (α2-PI activity: median: 105 [97-114] % vs. 100 [92-108] %, p<0.001, α2-PI antigen: median: 60.9 [55.1-69.1] mg/L vs. 58.6 [52.9-66.7], p=0.0363).

Admission α2-plasmin inhibitor levels and stroke characteristics

α2-PI levels on admission showed a significant association with stroke severity. Those patients who suffered more severe stroke based on their NIHSS value on admission demonstrated significantly lower α2-PI levels. The stepwise inverse association with stroke severity was found to be more profound for α2-PI antigen levels, and a similar but weaker association was found again in samples obtained at 24h post-lysis. α2-PI levels after thrombolysis did not show a significant association with stroke severity. Admission α2-PI antigen levels were the highest in case of small vessel infarcts, and lowest in strokes of cardio-embolic origin (α2-PI antigen median: 61.8 [IQR: 56.3-72.8] mg/L vs. 56.6 [IQR: 52.3-64.2] mg/L, respectively, p=0.024).

α2-plasmin inhibitor levels during thrombolysis and stroke outcomes

Admission α2-PI antigen levels showed a significant association with short-term outcomes of stroke. Significantly lower α2-PI antigen levels were found on admission in patients with more severe stroke at 7 days post-lysis. In patients who demonstrated unfavorable outcome at 7 days post-lysis, α2-PI antigen levels on admission were significantly lower as compared to those improving or showing no change in their status (favorable outcome/no change group median: 60.4 [IQR: 54.5-68.8] mg/L vs. unfavorable outcome group median: 58.0 [IQR: 49.6-64.1] mg/L, p=0.045), although it must be noted that the difference between the median of the two groups was marginal.

α2-PI antigen levels after thrombolysis and α2-PI activity as measured at any time points were not associated with short-term outcomes. Similarly, long-term functional outcomes and mortality at 90 days were found to be associated only with α2-PI antigen levels on admission. The extent of decrease between pre- and post-thrombolysis activity or antigen α2-PI levels did not show an association with outcomes. Patients who died (mRS 6) or had unfavorable long-term outcomes (mRS 2-5) had a significantly lower α2-plasmin inhibitor antigen levels on admission as compared to patients with favorable long-term outcomes at 90 days after the event

(mRS 0-1 median: 61.6 [IQR: 55.9-70.5] mg/L vs. mRS 2-5 median: 59.7 [IQR: 54.5-69.1] mg/L vs. mRS 6 median: 56.0 [IQR: 48.5-61.0] mg/L, p<0.001). In a Kaplan-Meier survival analysis, those patients who presented with α2-plasmin inhibitor antigen level in the highest quartile on admission showed significantly better survival as compared to those with α2-plasmin inhibitor antigen level in the lowest quartile on admission (HR: 4.54; 95%CI: 1.92-10.8, p<0.001).

Binary backward logistic regression models (model 1: including hyperlipidemia, BMI, diabetes mellitus, sex and CRP) revealed that α2-PI antigen level in the lowest quartile on admission is a significant predictor of unfavorable long-term outcomes (mRS 3-6) (OR: 2.10; 95%CI: 1.21-3.66, p=0.008) and death (mRS=6) 3 months after thrombolysis (OR: 2.22, 95%CI: 1.147-4.312, p=0.018) (Table 2). However, when age and NIHSS were also introduced in the models (Models 2 and 3), the effect of α2-PI antigen level diminished and only age and NIHSS on admission remained as independent predictors of outcomes.

In patients with therapy-related ICH (n=32), admission α2-PI antigen levels were significantly lower as compared to those without hemorrhagic complications. No such association was observed in the post-lysis samples of patients and in case of α2-PI activity at any measured time points. Difference between median α2-PI antigen levels on admission of those without or with post-lysis ICH was marginal (no ICH median: 59.8 [IQR: 54.0-68.0] mg/L vs. ICH: 57.0 [IQR: 53.4-61.7] mg/L, p=0.036). No correlation was observed between estimated post-lysis hematoma volumes and α2-PI antigen levels on admission (r=0.207; 95%CI: -0.176 to 0.536, p=0.272). α2-PI antigen or activity levels did not differ in subgroups of SICH or aSICH at any time points investigated. As expected, NIHSS was significantly higher in patients with ICH as compared to those without post-lysis hemorrhage (median: 12 [IQR: 7-32] vs. 6.5 [IQR: 4-36], respectively, p<0.001).

α2-plasmin inhibitor Arg6Trp polymorphism, stroke characteristics and outcome

Genotype frequencies of α2-PI Arg6Trp polymorphism were consistent with Hardy-Weinberg equilibrium in the cohort (C=0.8046; T=0.1953) and were practically identical to allele frequencies reported in the 1000 Genomes project for the European subgroup (C=0.8012; T=0.1988) [40]. α2-PI Arg6Trp polymorphism had no effect on α2-PI activity or antigen levels at any measured time points and showed no association with stroke severity, etiology, or outcomes.

DISCUSSION

Understanding the reasons for thrombolysis failure is critical to improve acute stroke care. Although both activated FXIII and α2-PI play a key role in plasmin-mediated protection against fibrinolysis, the two observational studies we describe are the first two comprehensive studies to investigate the activity and antigen levels of these two proteins in AIS patients during thrombolysis and study the association of the major polymorphisms of these fibrinolysis inhibitors with the patient's clinical outcome. In our first study, we showed that FXIII activity and antigen levels gradually decrease during thrombolysis. The mechanism involved in such reduction of FXIII levels is not entirely clear. Plasmin has recently been shown to cleave and inactivate FXIIIa in vitro, but not the zymogen form of FXIII. FXIII activity and antigen measurements performed in our study reflect circulating zymogen FXIII levels, which are not expected to be cleaved by plasmin. This is confirmed in our study, as FXIII activity and antigen levels measured before and immediately after thrombolysis were not significantly different in this cohort. A significant decrease in FXIII levels occurred 24 hours after lysis, suggesting that plasmin has a negligible effect on zymogen FXIII *in vivo.* Furthermore, since FXIII activity and FXIII-A_2B_2 antigen levels showed a good correlation before and during thrombolysis, degradation of FXIIIa by plasmin or other proteases is presumably negligible. The most likely explanation for the significant decrease in FXIII levels after stroke is that the activated protein is continuously incorporated into the growing thrombus, leading to consumption due to the ongoing activity of the coagulation system. This hypothesis was suggested by an earlier pilot study, where FXIII-A subunit levels (but not FXIII activity) were measured in a group of AIS patients. In that cohort, 41 patients received thrombolysis by rtPA or urokinase and their results did not differ from AIS patients not receiving thrombolytic therapy (n=23).

Using a logistic regression model including all potentially relevant risk factors, we found that a low FXIII level 24 h post-event is an independent predictor of short-term mortality (by 14 days). This result suggests that the reduction in FXIII levels directly relates to the pathomechanism of fatal stroke. This effect was found to be independent of the severity of stroke as measured by the NIHSS. Remarkably, those patients who died within the first week after stroke had unusually low FXIII levels the day after thrombolysis; with FXIII levels reaching only approximately 50% as compared to patients with better outcomes. Low FXIII levels as measured 24 h post-lysis were found to be associated not only with short-term but with long-term mortality (by the end of the 3rd month post-event) as well. However, in the logistic

regression model, low FXIII levels 24 h post-lysis did not prove to be an independent predictor of long-term mortality. Post-stroke mortality at the long-term is influenced by a number of factors including age, co-morbidities, in the case of significant functional neurological deficit, social background, care conditions etc. which could explain the loss of significance in this case. Although our initial hypothesis suggested that low FXIII levels may play a role in the pathomechanism of intracranial haemorrhage following lysis, the results of our study did not confirm this. Our study suggests that the development of bleeding complications after thrombolysis is not related to low FXIII levels. This finding is consistent with the results of some previously published studies involving small cohorts. FXIII levels were not associated with stroke severity in our patient cohort. As it has been found earlier, we also showed that FXIII activity was significantly higher in atherothrombotic stroke compared to strokes of cardioembolic origin, which suggests a role for FXIII in atherothrombosis, but further studies are needed to confirm this.

In this study, no association was found between any of the investigated FXIII-A or FXIII-B polymorphisms with stroke severity, unfavorable outcomes of therapy, therapy-associated symptomatic intracranial hemorrhage and mortality. In case of the FXIII-A p.Val34Leu polymorphism, an interesting trend was observed showing a protective effect against unfavorable short term outcomes in carriers of the Leu allele, but results did not reach statistical significance, therefore, larger number of cases need to be tested to confirm or exclude the possibility of this association. Among the polymorphisms investigated in the study, only FXIII-B intron K nt29756 G allele was associated with lower FXIII levels, but after adjustment to confounders differences between carriers and non-carriers were not statistically significant. In a most recent study investigating patients with coronary sclerosis (CS) and/or myocardial infarction (MI), carriers of the FXIII-B intron K nt29756 G allele had significantly lower FXIII levels, and the presence of the allele provided significant protection against CS and MI in patients with fibrinogen in the upper tertile, which prevailed only in the presence of FXIII-A Leu34 allele. In our study, the presence of FXIII-B intron K nt29756 G allele did not seem to have any impact on therapeutic outcomes, although due to the limited number of patients, it was impossible to perform subgroup analysis to seek a synergistic effect between FXIII-A Leu34 and the FXIII-B intron K nt29756 G allele.

In a recent study, we have shown that the presence of the FXIII-A Leu34 allele provides significant protection against the development of larger clots (clot burden score: 0-9) in AIS patients. However, in multivariate analysis, this polymorphism was not shown to be an independent predictor of short- or long-term functional outcomes after thrombolysis. Given that

the outcome of thrombolysis is influenced by a number of factors, in particular stroke severity and location, in the light of these data it is likely that the modifying effect of the FXIII-A p.Val34Leu polymorphism alone is weak and does not contribute significantly to the overall functional outcome of patients.

Although α2-PI is among the key regulators of fibrinolysis, little is known about its role in AIS thrombolysis outcome as yet. In the literature only a few studies have been published on the potential association between α2-PI levels and AIS thrombolysis outcomes, with controversial results. In a study including 63 AIS patients, baseline levels of α2-PI activity correlated well with the rate of recanalization. Patients who recanalized had lower α2-PI activity levels and α2-PI level was described to be the only predictive variable of recanalization, although it didn't show an association with long-term outcomes. On the other hand, in studies by others, although a decrease in α2-PI levels was found after thrombolysis, α2-PI levels showed no association with therapy outcomes. As it was pointed out by a recent systematic review, controversial data on markers of hemostasis and fibrinolysis and the outcome in AIS thrombolysis may arise from numerous methodological issues. Firstly, most studies include relatively few patients lacking statistical power and only a handful of papers are published in the literature where at least 100 patients are included. Another critical factor is the time interval between stroke onset and blood sampling, as in the majority of studies a baseline blood sample is collected within 24 h after stroke onset, that is a fairly wide time interval. Moreover, in patients receiving thrombolysis, therapy is provided during this period thus it is essential to differentiate between results gained from pre- or post-thrombolysis blood samples. Ideally, a hemostasis biomarker should be assessed before the initiation of thrombolysis treatment in AIS patients. Finally, depending on the research question more than one sampling time-point could be necessary to draw sound conclusions.

In the second prospective observational study, we were able to enroll 421 AIS patients undergoing thrombolysis, which makes this cohort one of the largest published as yet. Blood samples were collected from all patients before and 24 h after thrombolysis, while in a subset of patients we also aimed at collecting samples immediately post-lysis. α2-PI levels were tested at all time points by a functional test and an antigen assay detecting all 4 isoforms. Due to the natural heterogeneity of this protein, data derived from both assays could shed light on the action of α2-PI during AIS thrombus formation and provide information on the inhibitory effect of α2-PI on the excess plasmin generated during rt-PA therapy. Similarly to previous reports, α2-PI activity and antigen results showed good correlation in this cohort. Interestingly, on admission, only moderately strong correlation was found between the activity and antigen assay

results, which then improved post-lysis. This might arise from the fact that the incorporation of α2-PI into the thrombus by FXIIIa primarily involves PB-α2-PI, moreover the extent of incorporation has its limit (45-50%). It has been suggested by previous reports that the commercially available chromogenic α2-PI activity used assay is mainly sensitive the levels of PB-α2-PI, the more active and kinetically faster plasmin inhibitor. On the other hand, the α2-PI antigen test used in this study captures all forms of α2-PI to an equal extent, therefore, it is surmised to fully demonstrate the extent of α2-PI consumption. This might be the reason for the stronger association between α2-PI antigen levels and stroke severity observed in this cohort as compared to α2-PI activity and might explain the link between admission α2-PI antigen levels and outcomes. Further studies are required to understand the extent of incorporation of various α2-PI isoforms into thrombi and their relation to thrombus burden and treatment outcomes in AIS patients.

Based on our results there is a considerable consumption of α2-PI in patients with more severe strokes, as a significant step-wise negative association was found between α2-PI antigen levels and stroke severity. Significantly lower admission α2-PI antigen levels were found in patients with unfavorable short- and long-term outcomes, moreover, admission α2-PI antigen levels in the lowest quartile showed a significant association with the long-term mortality of patients. However, in a multivariate analysis including all relevant factors determining long-term outcomes, the significant effect of α2-PI was diminished and only age and NIHSS remained in the model as most important predictors of long-term outcomes. These results suggest that although the incorporation of α2-PI into intracerebral thrombi and thus the consumption of the protein is associated with stroke severity and most probably with thrombus burden as well, apparently, α2-PI cannot be considered an independent biomarker of therapy outcome. Nevertheless, these results suggest that α2-PI could play a role in the pathophysiology of thrombolysis failure and may be an important factor contributing to the inefficacy of therapy in case of severe strokes. Consistent with the current findings, we have shown in previous work that the extent of α2-PI incorporation into fibrin clots was the lowest in AIS patients with favorable outcomes after thrombolysis. These results are in line with another recent work from our group where we showed that together with age and NIHSS, thrombus burden is the most important indicator of thrombolysis outcome, and the levels of a number of key hemostasis parameters measured on admission or 24 h later showed no association with treatment outcomes in a multivariate model.

Although it is biologically plausible that low α2-PI levels post-lysis could be responsible for therapy associated ICH events, such association was not found in this cohort. α2-PI levels

immediately post-lysis were below the lower limit of reference in all individuals tested in this cohort, yet bleeding complications were not directly related to levels of α2-PI at this time point. These results are in line with experimental studies suggesting that targeting α2-PI may be a novel paradigm for the dissolution of thrombi without compromising safety. Admission α2-PI levels were indeed significantly lower in patients with post-lysis ICH, however, the difference between groups was very modest. As patients with post-lysis ICH presented with more severe stroke on admission, it can be surmised that the significant difference observed between the α2-PI levels of the two groups is related to differences in stroke severity. As α2-PI levels in the groups with or without ICH showed a considerable overlap on admission, moreover, α2-PI activity levels did not differ among groups, it is unlikely that low α2-PI would be the only responsible factor for post-lysis ICH. α2-PI had no effect on the enlargement of the hematoma as α2-PI levels did not show a significant correlation with the estimated post-lysis hematoma volumes. On the other hand, it has been known from the literature that more severe strokes are associated with not only lysis failure, but with a higher chance of bleeding complications, although the reasons for this association have not been clarified.

Among the parameters influencing α2-PI heterogeneity, α2-PI Arg6Trp was tested in the cohort. Given the relatively large sample size, the effect of α2-PI Arg6Trp polymorphism on α2-PI levels, on stroke characteristics and treatment outcomes was assessed, but no associations were found. Only a few studies have investigated the relation between α2-PI Arg6Trp polymorphism and the risk or outcomes of arterial thrombotic events. Limited data based on these reports suggest that the polymorphism has no effect on the risk or outcomes of ischemic events. As we have shown most recently, besides α2-PI Arg6Trp polymorphism, an important modifying factor of the extent of α2-PI N-terminal cleavage is circulating APCE (sFAP) level. It can be surmised that the effect of α2-PI Arg6Trp should be considered in the light of APCE levels, however, this measurement was out of the scope of our study.

In conclusion, low FXIII levels 24 hours after thrombolysis is a significant independent predictor of AIS mortality on day 14 after the event. Examination of FXIII levels at 24 hours after lysis may help to identify patients at high risk of mortality, which may warrant reconsideration of therapeutic options. Further studies involving large numbers of patients are needed in the future to see if early selection of such patients can help improve outcomes by providing intensified treatment strategies.

In conclusion, α2-PI levels in AIS patients changed dramatically during thrombolysis, but post-lysis α2-PI levels were not associated with treatment outcome or safety. Significantly lower α2-PI levels were found in AIS patients with more severe stroke at admission, and significantly

lower admission α2-PI antigen levels were observed in patients with unfavourable short- and long-term outcomes, however, in a multivariate regression analysis including all relevant factors determining long-term outcomes, the significant effect of α2-PI was reduced, and only age and NIHSS remained in the model as the most important predictors of long-term outcome. Taken together, these data suggest that although α2-PI is a dominant inhibitor of physiological fibrinolysis, its level at admission is strongly associated with stroke severity and is not an independent predictor of outcome on thrombolytic therapy. However, it can be assumed that the rate of incorporation of α2-PI into thrombi is higher in more severe stroke, which may be an important mechanism for the limited success of rt-PA-induced thrombolysis in these cases.

LIMITATIONS

As with all observational clinical studies, our studies have limitations and the results should be interpreted in the context of these factors. First, the aim of our studies was to observe changes in FXIII and α2-PI levels during thrombolysis and compare them with the outcome of therapy, and thus we did not design a case-control study. Accordingly, we did not study patients who did not receive thrombolysis as controls. Theoretically, it would be possible to compare FXIII and α2-PI levels in AIS patients receiving and not thrombolysis, but the group of rt-PA-treated patients is a highly selected group of patients with strict inclusion criteria (e.g. narrow time window, lack of effective anticoagulation, etc.), whereas patients not receiving thrombolysis do not meet these inclusion criteria by definition. Given the significant baseline differences between the two groups and the heterogeneity of the non-thrombolysis group, we believe that comparisons between these groups may not be appropriate.

Secondly, the patients were enrolled in a single-centre study, therefore our sample size is limited, but compared to previously published studies on the subject, the number of patients we enrolled is among the largest. Due to the single-centre study design, our studies had the advantage of uniform patient and sample treatment; in addition, the proportion of patients lost during follow-up was relatively low compared to other studies with similar study designs.

Although we could have obtained a more complex picture of the pathophysiology of α2-PI by identifying specific isoforms of α2-PI, this was beyond the aim of our study.

Our studies provided information on the systemic haemostasis system during the treatment of acute stroke, thus indirectly reflecting only local processes.

Finally, as we did not include patients treated with mechanical thrombectomy in our studies, it is not possible to draw conclusions from the results regarding the current management of AIS patients with large vessel occlusion.

SUMMARY

Intravenous rt-PA treatment to dissolve the thrombi is unsuccessful in a proportion of patients with AIS, while in a significant proportion of cases ICH develops as a side effect. In two prospective observational clinical studies, our aim was to investigate FXIII and α2-PI levels during thrombolysis to see if they are associated with the outcome of therapy. Blood samples were taken from AIS patients (n=132 and n=421) treated with rt-PA within 4.5 hours of symptom onset before and 24 hours after thrombolysis, and in a subset of patients, immediately after lysis. FXIII activity and antigen were measured by ammonia release assay and ELISA, α2-PI activity and antigen by chromogenic assay and ELISA for all isoforms of α2-PI. Samples were analysed for FXIII-A p.Val34Leu, p.Tyr204Phe, FXIII-B p.His95Arg, intronK (IVS11+144) and α2-PI p.Arg6Trp polymorphisms. Stroke severity was determined by NIHSS at admission and day 7. Post-lysis ICH was classified according to ECASSII. Long-term outcome was graded according to mRS 3 months after the event. Results: FXIII levels showed a gradual decrease immediately after thrombolysis and 24 hours later, but this was not associated with post-lysis ICH. In a multiple logistic regression model, the lowest quartile FXIII level 24 hours after lysis was found to be an independent predictor of day 14 mortality (OR:4.95, 95%CI:1.31-18.68, $p<0.05$). α2-PI levels significantly decreased immediately after lysis and then increased again the day after the event. α2-PI levels at admission showed a significant negative correlation with stroke severity. In a Kaplan-Meier survival analysis, patients with α2-PI antigen in the highest quartile on admission had significantly better long-term survival compared with those with α2-PI antigen in the lowest quartile (HR:4.54; 95%CI:1.92-10.8, $p<0.001$); however, in multivariate analysis, low α2-PI antigen on admission was not an independent risk factor for poor long-term outcome. Patients with ICH had a slightly but significantly lower α2-PI antigen compared with patients without complications. No association was found between the tested FXIII or α2-PI polymorphisms and treatment outcome. Conclusion: Our results suggest that FXIII levels measured 24 hours after thrombolysis can help identify patients at higher risk of short-term mortality. Low α2-PI antigen levels at admission were associated with more severe stroke and poor long-term outcome in the study cohort. Our results suggest that in cases of more severe stroke, α2-PI may play a role in the limited efficacy of rt-PA thrombolysis.

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