

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

Investigation of factor VIII and von Willebrand factor levels in
patients with atrial fibrillation and ischemic stroke

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF KÁLMÁN LAKI

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Head of the **Examination Committee:** György Balla, MD, PhD, DSc, Member of
Hungarian Academy of Sciences

Members of the Examination Committee: Imre Bodó, MD, PhD
Péter Ilonczai, MD, PhD

The Examination takes place at the Library of Division of Clinical Laboratory Science,
Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen; at
11 a.m., 8th of March 2019

Head of the **Defense Committee:** György Balla, MD, PhD, DSc, Member of
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The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal
Medicine, Faculty of Medicine, University of Debrecen; at 1 p.m., 8th of March 2019

Introduction

Cardiovascular and cerebrovascular diseases are among the leading causes of death in developed countries. The classic risk factors for the development of atherosclerosis and atherothrombosis are now well known, e.g. hypertension, diabetes mellitus, smoking, obesity. However, the exact pathomechanism of arterial thrombosis is unknown in many respects, although better understanding of the factors leading to the development of thrombosis may serve to detect and treat cardiovascular and cerebrovascular diseases earlier in the future. A consensus paper published in 2018 by more than 60 internationally acknowledged researchers stated that more intense research is necessary on the pathomechanism of thrombosis and investigating new potential biomarkers as diagnostic and prognostic tool of atherothrombotic diseases.

One of the key factors in the development of arterial thrombosis is the disintegration of the balance of hemostasis system. However, the role of some markers of coagulation and fibrinolysis in the development of atherothrombotic events is less explored than in case of venous thromboembolic events. Meta-analyses and prospective studies have been found to have a positive correlation with the risk of coronary artery disease and stroke for only a few key factors (e.g. fibrinogen, von Willebrand factor, tissue plasminogen activator inhibitor) so far. These studies are far from complete, but the findings so far outline the idea that coagulation plays an important role in the progression of atherothrombotic processes. Studying the differences in the hemostasis system can not only provide a better understanding of the pathomechanism but hemostasis factors as biomarkers can provide useful information about the presence, severity and prognosis of the disease. In the Maastricht Consensus Statement experts also pointed out that studying certain factors (von Willebrand factor, factor VIII, factor IX, factor XI, factor XII) is also important in atherothrombotic diseases because they may become potential therapeutic targets in the future.

Among cardiovascular diseases atrial fibrillation is characterized by high-risk arterial thrombosis (leading to stroke). It is widely recognized that thromboembolism in AF is associated with a combination of pathophysiological mechanisms, which fulfill the requirement of Virchow's triad for thrombogenesis: stasis, abnormal change in the vessel wall and pathological imbalance of hemostasis and fibrinolysis. Several studies have been carried out to investigate the hypercoagulability in atrial fibrillation, but the

starting process that activates the coagulation cascade is not yet clear during atrial fibrillation. The examination of the overt hemostasis equilibrium can best be performed by analyzing intracardiac blood samples, since the starting point of the differences is the left atrium itself. By analyzing blood samples obtained from the left atrium, we can get to know the pathomechanism of enhanced arterial thrombosis propensity associated with atrial fibrillation and potentially identify markers whose examination can help to predict stroke.

In the case of an established stroke, the outcome of the therapy depends on the localization, size and structural features of the thrombus formed. The hemostasis changes accompanying the acute thrombotic process and the knowledge of hemostasis in the applied therapy allow a better understanding of the therapeutic outcomes. At present, intravenous thrombolysis with recombinant tissue plasminogen activator is the only registered drug for acute ischemic stroke therapy, but recently mechanical thrombectomy is also available. However, the rate of recanalization and clinical outcome in the treated patients is very different, and according to our current knowledge we can hardly predict it. Intravenous thrombolysis treatment has positive outcome only in 33-35% of patients and is provoking symptomatic intracranial hemorrhage in 6-8%. Similarly, only 36% of those who have undergone mechanical thrombectomy will be completely independent in their daily living, moreover, bleeding complications will occur in 2.5%. Studies that enhance our knowledge of certain factors regarding the tendency of thrombi to recanalize can make great efforts in the future to improve therapy. Understanding the changes in certain hemostasis factors under thrombolytic therapy is an opportunity to use biomarkers for the early detection of patients with worse prognosis in addition to knowing the pathomechanism.

Literature overview

Overview of the hemostasis system

The model described by MacFarlane, Ratnoff and Davie in 1964, which emphasized the cascade-like activation of coagulation, has now been replaced by the cell-based model described by Hoffman and Monroe in 2001. This model breaks down the coagulation pathway into three overlapping portions: an initiation phase on cells carrying tissue factor, in which only a small amount of active coagulation protein is generated, the amplification phase during which the platelet and cofactor activation occurs, in the interest of high thrombin generation, and the propagation stage on the surface of activated platelets, in which large amounts of thrombin and fibrin clot are formed.

At the beginning of coagulation, von Willebrand factor (VWF) binds to subendothelial matrix proteins, such as type I, III and VI collagen, and the bound VWF binds to the GP-Ib α receptor of circulating platelets initiating the process of platelet adhesion during which platelets are activated. Thrombin cleaves factor VIII from platelet-binding VWF-FVIII complex, thereby activating it (FVIIIa). FVIIIa serves FIXa as a cofactor on the surface of activated platelets, their complex converts FX to active FX (FXa).

Summary of fibrinolysis

The blood clot resulting from the coagulation process would inhibit the local blood flow after tissue healing and therefore it needs to be demolished. The fibrinolytic system is responsible for the localized, timely demarcation of the fibrin clot, the central protease of which is plasmin. Plasmin, generated from plasminogen by plasminogen activators, is a single-chain glycoprotein. As a first step in fibrinolysis, plasminogen binds to and activates the lysine residues on the fibrin surface. The resulting serine protease plasmin cleaves the fibrin clot into partially degraded fibrin, generating various, ever-smaller fibrin degradation products. D-dimer consists of two D-regions of the fibrin monomer, which is covalently linked to the γ chain, which may complex with the E fragment via non-covalent binding. **Plasminogen activators** include tissue-type plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA).

Although the thinner fibrin fibers are degraded faster, fibrin clot consisting of thin fibrin

fibers, is more compact and therefore degraded more slowly than the loose clot consisting of thicker fibrin fibers. In the case of high thrombin concentrations, the formation of denser, thinner fibrin fibers, which is associated with increased risk of thrombosis is observed. At low thrombin concentrations a clot of thicker fibrin fibers with a looser structure and a higher risk of bleeding is arisen.

Inhibitors of fibrinolysis

Fibrinolysis is regulated at several levels. One of the central elements of regulation is to prevent plasminogen and t-PA from binding to fibrin C-terminal lysine. The main inhibitor of plasmin is α 2-plasmin inhibitor (α 2-PI). α 2-PI is incorporated into the fibrin clot to effectively protect the forming clot against fibrinolysis. This is very important in inhibiting premature fibrin degradation.

The major plasminogen activator inhibitor is serpin-type plasminogen activator inhibitor-1 (PAI-1). It blocks both t-PA and u-PA. Because of the predominance of PAI-1, t-PA is mainly present in the circulation as a t-PA-PAI-1 complex.

The role of endothelium in hemostasis, endothelial damage and markers

The vascular endothelium provides an antithrombogenic environment by regulating coagulation, platelet adhesion, vessel tone and blood flow. The endothelium is heterogeneous, it has varying structures and functions depending on tissue and vessel type and the diameter of the vessel. The subendothelial surface is rich in collagen, heparan, von Willebrand factor and tissue factor, but their amount differs according to tissue type. Subcellular organelles of the endothelial cells, the Weibel-Palade bodies, are controlled by an appropriate stimulus to release their contents in inflammatory processes, when hemostasis is activated, during regulation of angiogenesis, and changes in rheological conditions. For the diagnosis of activated endothelium, both ultrasonic tests and measurements of different biomarkers (e.g. VWF) are used.

Characterization and pathophysiology of FVIII and VWF

Biochemistry of FVIII

The plasma concentration of FVIII is about 1 nM (100-250 ng / mL) and it has a half-

life of about 12 hours. FVIII is probably synthesized in hepatocytes and/or reticuloendothelial systems, but it can also be found in α -granules of platelets. In the circulation, it forms a 1:1 complex with VWF via non-covalent binding. However, the plasma concentration of VWF is approximately 50 times higher than that of FVIII. Approximately 2% of the FVIII circulates in a free form. The plasma FVIII and VWF levels are influenced by the blood type (zero-blood type causes 25% lower plasma concentration as compared to non-zero blood type), race (about 20% higher levels in African Americans than in the Caucasian population) and age (their levels elevating with aging). FVIII is activated by limited proteolysis. Its main activator is thrombin, followed by FXa, FXIa and FVIIa. VWF not only prolongs FVIII half-life and stabilizes its structure but also regulates its activity: VWF prevents FVIII from binding to phospholipids and activated platelets, moreover it protects FVIII from activation by FXa and from inactivation by APC. The VWF-FVIII complex binds to the injured subendothelium in the initial step in the coagulation process, it not only allows the adhesion and aggregation of platelets, but also locally increases the FVIII concentration so that FVIII can bind to the phosphatidyl serine of activated platelets.

Biochemistry of the VWF factor

VWF is one of the largest circulating multimeric glycoproteins. VWF is synthesized by endothelial cells, megakaryocytes and platelets. The VWF is stored in the Weibel-Palade bodies of endothelial cells and in the α -granules of platelets. Weibel-Palade bodies can secrete VWF and its propeptide both lumenally, into the plasma and ablumenally into the subendothelial matrix. From the Weibel-Palade bodies, VWF is secreted into the plasma in ultralarge-multimer form. Subsequently, the ultralarge VWF multimer remains bound to the endothelial cell surface. As a result of higher shear forces along the vascular wall, the VWF structure changes, becomes elongated, and the VWF proteolytic cleavage site on the A2 domain becomes available and is rapidly cleaved by the ADAMTS13 metalloprotease. If the highly thrombogenic ultralarge VWF multimer is secreted directly into the bloodstream, platelets spontaneously aggregate, resulting in thrombosis.

VWF plasma concentration is $\sim 10 \mu\text{g/mL}$, its half-life is 12 hours. The free propeptide has a plasma concentration of $\sim 1 \mu\text{g/mL}$, with a half-life of 2-3 hours. People with blood group A, B and AB have a VWF antigen level $\sim 25\%$ higher than people with zero

blood group. According to the literature, plasmin is involved in the shear force dependent degradation of VWF beside ADAMTS13.

Increased levels of FVIII and VWF in different pathologies

The reason for the persistent increase in FVIII and VWF levels is largely unknown, and the rate of contribution of the underlying genetic and acquired factors is not entirely clear. Several studies suggest that inherited factors play a more pronounced role in the determination of plasma levels of both parameters. It is important to note that both FVIII and VWF are positive acute phase proteins, i.e. their levels increase by more than 25% when the immune system is activated. IL-6, the central cytokine of the acute phase reaction, is responsible for elevation of FVIII level and IL-6, IL-1 β and TNF- α cytokines are responsible for VWF level increase. The elevated levels of FVIII and VWF have also been associated with the development of certain diseases and their prognosis.

Association of increased FVIII and VWF levels and venous and arterial thrombosis

Several studies have investigated the relationship between venous and arterial thrombosis and elevated levels of FVIII and VWF. It was first described by Leiden Thrombophilia Study (LETS) more than 20 years ago that elevated FVIII activity and VWF antigen levels, as well as non-zero blood group, are risk factors for venous thromboembolism. However, after multivariate analysis, only elevated FVIII activity remained a significant independent risk factor suggesting that VWF antigen levels contributed to the risk by regulating FVIII plasma levels. The relationship between elevated FVIII levels and thromboembolic events was subsequently confirmed in a number of case-control studies and similar odds ratio of 6.2 times higher thrombosis risk were reported as in LETS, in case of values over the reference range. Increased FVIII levels have been shown to be a risk factor for recurrent venous thromboembolism as well. FVIII levels have increased the risk of venous thromboembolism in several studies in a dose-response manner. Several studies have demonstrated that elevated FVIII activity combined with other thrombotic risk factors (e.g. oral anticoncipient, FV Leiden mutation, malignancy) results in a much higher thrombosis risk than expected for a simple additive model, assuming synergistic effects. Vormittag et al. draw attention to the fact that elevated levels of FVIII in tumor patients are independent risk factor for

venous thromboembolism. In the study of thrombogenic effects of elevated FVIII levels, various in vitro and in vivo experimental results demonstrated that elevated FVIII associated with increased thrombin generation may be responsible for high risk of thrombosis. To a lesser extent, other mechanisms may also contribute to the increased risk of thrombosis: there is a direct inverse relationship between plasma FVIII levels and APC resistance.

The relationship between VWF levels and the formation of venous thromboembolic disorders is less clear than for elevated FVIII levels, several controversial reports have been made on this subject. In some of the publications, instead of elevated VWF levels, only the associated FVIII levels could be justified as an independent risk factor. In a large population prospective study (LITE: Longitudinal Investigation of Thromboembolism Etiology), however, VWF's independent risk role was demonstrated for venous thromboembolism: the risk ratio represented by VWF levels in the highest quartile was 4.6.

Compared to venous thromboembolism a lot more studies have investigated the relationship between VWF levels and cardiovascular diseases. According to literature data, elevated VWF levels have a significant correlation with the size of atherosclerotic plaque in the coronary artery. Clinical studies have shown that VWF levels are higher in patients with acute myocardial infarction compared to controls. Those with vascular disease and elevated VWF levels have higher risk of future myocardial infarction. Several studies have found correlation between elevated VWF and FVIII levels and increased risk of cardiovascular disease. According to a number of research, VWF may indicate an increased risk of micro- and macrovascular complications (e.g. nephropathy, cardiovascular disease) in diabetes mellitus type I and II. In the relationship of elevated VWF levels and arterial thrombosis, the effects of high shear forces on VWF play a central role. Severe degrees of stenosis caused by atherosclerotic plaques in arteries has been associated with altered hemodynamic conditions and high shear forces where the VWF creates an important link between the platelet membrane glycoproteins and the subendothelium. High shear forces increase the release of VWF from the endothelium, which increases the risk of thrombus formation.

Compared to cardiovascular research, far fewer studies address the role of elevated FVIII and VWF levels in cerebrovascular diseases. The risk factors and

pathomechanism of coronary diseases and ischemic stroke are largely the same, and therefore, it can be hypothesized that elevated FVIII/VWF levels play a role in the risk of ischemic stroke. A significant part of the publications describe a positive correlation between high VWF levels and ischemic stroke risk. In a meta-analysis of 2532 patients, there was a minor but significant correlation between increased levels of VWF and the risk of ischemic stroke (OR: 1.55, 95% CI: 1.31-1.83), which was, particularly considerable in case of atherosclerosis involving major arteries. The association between stroke outcome and FVIII/VWF levels have been much less studied, but most of them have drawn attention to the relationship between high VWF levels and increased mortality.

Atrial fibrillation

Epidemiology

Atrial fibrillation is the most common persistent arrhythmia, affecting about 33.5 million people worldwide, with an estimated lifetime risk of 22-26%. Its prevalence is expected to double by 2050, largely due to the aging society.

Risk Factors for Atrial Fibrillation

Age is considered to be one of the most significant risk factors for atrial fibrillation. More than 70% of patients with atrial fibrillation are over 65 years old. Further risk factors for atrial fibrillation include physically inactive lifestyle, extreme physical activity, both prior and current smoking, alcohol consumption, obesity, diabetes mellitus and left-ventricular hypertrophy. Hypertension is considered to be an extremely important risk factor for atrial fibrillation.

Pathophysiology

The central factor of atrial fibrillation is very fast, unmanaged atrial activity, which can be arisen in two ways. On the one hand, it can be derived from one or more rapidly discharging nodules that cause irregular driving and atrial fibrillation in the rest of the atrium. On the other hand, it can be created by re-entry. For the production of atrial fibrillation, a trigger mechanism is required, usually focal spontaneous discharge, most commonly in the area of the pulmonary veins. Atrial fibrillation is maintained by structural remodelling, which may be caused by a neurohormonal effect (autonomous overweight and overactive thyroid function), aging, myocardial ischemia, hypertension, obesity or atrial fibrillation. Atrial reestablishment leads to changed ion currents (decreased Ca^{2+} and increased K^+ currents down- and upregulation), faster atrial rhythm and reduced action potential. As the time progresses, pulmonary fibrillation becomes more persistent and becomes more resistant to therapy.

Virchow's Triad

Changes in atrial fibrillation meet the mechanisms described in Virchow's Triad for thrombogenesis, discussed in more detail below, but it is important to note that knowledge of the exact pathomechanism of enhanced thromboembolic risk requires further research. Virchow's Triad consists of the following elements: 1/ endothelial or endocardial dysfunction (and related structural changes), 2/ abnormal stasis, 3/ abnormal coagulation, platelet and fibrinolysis.

The presence of endothelial damage in atrial fibrillation is supported by several pathophysiological processes and biomarkers. Rheological changes in atrial fibrillation and the reduced contraction of the wall of the left atrium, result in reduced NO synthesis of the left atrial endothelium. A further clinical sign of endothelial impairment is a reduced flow-induced dilation observed in atrial fibrillation patients. In atrial fibrillation, inflammatory processes associated with endothelial dysfunction are also directly related to thrombus formation. CRP and IL-6 increase the production of monocyte tissue factor in vitro. IL-6 increases platelet count and platelet sensitivity to thrombin. IL-6 also stimulates fibrinogen transcription.

Structural and functional left atrial changes observed in atrial fibrillation cause slow flow conditions and stasis in the left atrium. Increased hematocrit levels due to wall motion abnormalities and hemoconcentration in some patients promote stasis. Atrial fibrillation promotes the development of progressive left atrial dilatation and thus stasis. Left atrial dilation contributes to thrombogenicity, the size of the left atrium corrected to body surface is an independent risk factor for stroke. Thrombus is detected in the left atrium or in the left atrial appendage in about 10% of patients with atrial fibrillation. The structure of the left atrial atrial appendage, its narrow ostium, and the changes in the diameter of the atrium are particularly prone to the formation of stasis as well. In case of thromboembolism, more than 90% of the emboli origins from the left atrial appendage in non-valvular atrial fibrillation. In transesophageal echography (TEE) stasis appears as spontaneous echo contrast (SEC). In atrial fibrillation, the SEC calls attention to the increased risk of thromboembolism. Researches have shown that SEC has a positive correlation with prothrombin fragment 1+2 (F1+2), fibrinopeptide A and TAT complex levels.

There are a number of reports available on the activation of coagulation cascade in atrial fibrillation investigating mainly peripheral blood samples, but fibrinolytic activity in patients with atrial fibrillation has been less studied. In most studies investigating the pathophysiology of increased thromboembolic risk observed in atrial fibrillation, mainly endothelial cell damage markers, inflammatory markers, prothrombotic markers and plasmatic markers of platelet activation were investigated. Various clinical studies found a correlation between atrial fibrillation and elevated F1+2, TAT complex, fibrinogen levels and markers of endothelial dysfunction such as soluble thrombomodulin (sTM) and VWF levels.

Of the markers indicating the activation of hemostasis, most data relating to D-dimer

associated with predicting stroke risk in atrial fibrillation. Taking into account the clinical risk factors, elevated D-dimer levels are prognostic for stroke risk in non-valvular atrial fibrillation in patients receiving oral anticoagulant therapy. Wan et al. reported in their meta-analysis, that D-dimer could be useful as a prognostic marker for left atrial thromboembolism in atrial fibrillation patients as higher D-dimer levels were found in spontaneous echo contrast and even higher values for TEE-confirmed left atrial thrombus. Clinical studies suggest that VWF levels may also be useful as elevated VWF level may indicate an increased risk of left atrial appendage thrombus in atrial fibrillation. In the Rotterdam study, there was a positive correlation between plasma VWF levels and atrial fibrillation, which was particularly pronounced among women.

The few studies examining the relationship between atrial fibrillation and fibrinolytic system have yielded controversial results. Some studies found elevated levels of PAI-1 indicating hypofibrinolysis, while other studies suggested elevated levels of t-PA and PAP complexes leading to hyperfibrinolysis. However, other studies found no change or reduced levels of PAP complex and PAI-1. According to some studies, the background of elevated levels of t-PA and PAI-1 found in atrial fibrillation may be associated with endothelial dysfunction, inflammation and vascular disease. Abnormal changes in the fibrinolytic system can be related not only to thrombus formation, but also to the structural transformation of the atrium as it has strong relationship with extracellular matrix turnover. In patients with atrial fibrillation, unfavorable structural changes in the fibrin clot have also been demonstrated by Drabik et al. According to their results, the fibrin clot consisted of more compacted and thinner fibrin fibers detected in patients with paroxysmal and persistent atrial fibrillation leading to hypofibrinolysis which contributes to thromboembolism.

Instead of investigating peripheral blood samples, the examination of intracardiac blood samples deserves more attention because prothrombotic changes may only occur locally, in the intracardiac environment, without these abnormalities being manifested in the peripheral circulation. This could be assumed on the basis of earlier studies that have shown that prothrombotic changes are limited to the intracardiac environment in atrial fibrillation. However, probably because of the difficult collection of intracardiac blood samples, in the last decade, only a few studies have been investigating the hemostasis system from left atrial samples in atrial fibrillation. Publications investigating left atrial blood samples so far have only studied selected members of the hemostasis system (VWF, P-selectin, TAT complex, platelet factor 4, platelet-derived

sCD40), furthermore the fibrinolytic system has been less studied in this regard. We did not find any data in the literature regarding FXIII and α 2-PI, two key members of fibrinolysis, investigated in intracardiac blood samples. In most studies investigating intracardiac blood samples, the atrial fibrillation patient group was not compared to age and sex adjusted non-atrial fibrillation control group. In many studies investigating intracardiac blood samples, patients had received non-fractionated heparin prior to sampling, which had a major influence on the hemostasis system, thus limiting the parameters of hemostasis that could be investigated. Examination of blood samples from the left atrial appendage investigating hemostasis and fibrinolysis parameters is also rare in the literature, despite the fact that earlier clinical studies indicate that thrombus located in the left atrial appendage is the most common source of embolism, making it the most potent thrombogenic intracardiac area in atrial fibrillation.

Diagnosis

Atrial fibrillation is often asymptomatic or patients present non-specific symptoms: palpitations, fatigue, dizziness, dyspnea, chest pain and syncope. The severity of the symptoms can be estimated with the EHRA (European Heart Rhythm Association) scoring system. The diagnosis is usually based on a 12-lead ECG but in the case of paroxysmal cases the arrhythmia is not always detectable. In this case 24-hour Holter monitoring can be useful.

Classification

Atrial fibrillation can be divided into five groups (first diagnosed atrial fibrillation, paroxysmal, persistent, long standing persistent, and permanent atrial fibrillation groups) based on the existence and frequency of arrhythmia.

Therapy

Stroke prevention

Atrial fibrillation have increased mortality and morbidity as a result of increased thromboembolic complications and increased stroke risk. Prevention of stroke plays a central role in the treatment of atrial fibrillation, as stroke risk is increased fivefold in patients with atrial fibrillation. To assess the risk of stroke, various clinical scoring systems can be used to assist in therapeutic decision making. According to the latest recommendations, an improved version of the CHADS₂ scale, the CHA₂DS₂-VASc scoring system is most suitable for assessing stroke risk, taking risk factors into account

in weighted terms.

In the case of low stroke risk (0 point in men, 1 point in women) no antithrombotic therapy is required. For higher values, vitamin K antagonist or novel oral anticoagulant (NOAC) is recommended. Vitamin K antagonist (VKA) reduces stroke risk by 64% and total mortality by 26%. In contrast, the use of antiplatelet therapy reduces stroke risk by 22% and does not significantly reduce total mortality. The latest European guidelines recommend NOAC therapy instead of VKA therapy, based on the overall effect of NOAC.

Catheter ablation

Catheter ablation is indicated in case of non-tolerated or unsuccessful antiarrhythmic drug therapy when treating paroxysmal, persistent and long standing persistent atrial fibrillation. Catheter ablation is the first line therapy for symptomatic paroxysmal atrial fibrillation.

Professional guidelines for catheter ablation interventions have changed regarding periprocedural anticoagulant therapy in recent years. The 2008 and 2010 guidelines have suggested that oral anticoagulant therapy should be suspended before catheter ablation, before the day of intervention and during the procedure heparin administration is recommended. The latest guideline does not recommend the suspension of oral anticoagulants, but suggest that thrombosis prophylaxis should be continued before catheter ablation (VKA/NOAC), during ablation (heparin) and after the procedure (VKA/NOAC) because of increased periprocedural thromboembolic risk. According to these latest guideline, oral anticoagulation is recommended for at least 2-3 months after the intervention. Thereafter, the need for continued therapy should be judged based on stroke risk (CHA₂DS₂-VASc: ≥ 1 for men and ≥ 2 for women).

Prognosis

Odutayo et al. examined 104 cohort studies and a total of 587867 patients with atrial fibrillation in their meta-analysis. According to their results, atrial fibrillation is associated with an increased risk of the following diseases and conditions: all-cause mortality, major cardiovascular events, ischemic stroke, ischemic heart disease, sudden death, heart failure, chronic kidney disease, peripheral arterial disease.

Atrial fibrillation and biomarkers

Biomarker research in atrial fibrillation can be significant in many ways: on the one hand, they can contribute to a better understanding of the various pathophysiological

mechanisms and the possible complications, on the other hand biomarkers can be useful tools for risk assessment of various complications. According to some recommendations, the addition of biomarkers (pro-BNP, hs cardiac troponin, D-dimer, IGDF-15: inflammatory growth differentiation factor-15, micro-RNAs, galectin-3, CRP, creatinine, cystatin c) to clinical stroke risk scales may further refine their clinical value. According to Roldan et al. the addition of VWF levels to CHA₂DS₂-VASc and HAS-BLED scales may further increase their prognostic value.

Stroke

Epidemiology

According to the literature, stroke is considered to be the second most common cause of death and the most common cause of disability worldwide. Between 1990 and 2010, the incidence of stroke across the world increased by 68% and the death rate by 26%. The incidence of ischemic stroke during this period increased by 37% and its mortality by 21% worldwide.

Definition, subtypes

Acute stroke is a brain, retina or spinal cord focal dysfunction that lasts for more than 24 hours or longer if imaging procedures (CT or MRI) or autopsy can verify focal infarction or hemorrhage. In the case of transient ischemic attack (TIA), focal dysfunction lasts less than 24 hours, and infarction cannot be confirmed by imaging procedures. Within the five major groups of stroke (ischemic stroke, hemorrhagic stroke, subarachnoid hemorrhage, cerebral venous thrombosis, spinal ischemic or hemorrhagic stroke) further subgroups can be distinguished. The origin of ischemic stroke is usually multifactorial, so the ideal classification system should include the most likely etiologic and pathophysiological mechanisms. The etiologic scales include the most commonly used TOAST (Trial of ORG 10172 in Acute Stroke Treatment, large artery atherosclerosis, cardioembolism, small vessel occlusion, other known etiology, unknown etiology) published in 1993. In the 21 to 37% of cases of ischemic stroke, there is a cardioembolic source, in 15-48% atherothrombotic cause, in 10-34% small vessel disease, and in 30-38% unknown etiology. Of these, the worst outcome is expected for the cardioembolic type, with the highest mortality rate in the hospital (6-

27%). Studies have shown that collateral circulation is better in atherothrombotic stroke than in cardioembolic stroke, which can contribute to a less favorable outcome in case of cardioembolic stroke, as collateral circulation plays a significant role in the success of reperfusion therapies as well as in long-term outcome.

Risk factors

The risk of both ischemic and hemorrhagic stroke is increased in advanced age (for patients older than 75 years, the relative risk of stroke is fivefold compared to 55-64 years of age), hypertension (in case of 160/95 mm Hg blood pressure the relative risk of stroke is seven times higher compared to 120/80 mm Hg), smoking (the relative risk of active smokers is double) and diabetes mellitus (the relative risk of stroke is double). The risk factors for hemorrhagic stroke include excessive alcohol consumption, thrombolytic, anticoagulant, and antiplatelet therapy. Stroke risk factors include male sex, but more women being affected by stroke due to the fact that women have longer life expectancy. Obesity and dyslipidemia also increase the risk of ischemic stroke (primarily raising the risk of large artery disease). Ischemic heart disease is a triple relative risk of stroke, whereas heart failure, atrial fibrillation and previous TIA have a five-fold relative risk. Inflammation, infection, migraine with aura and oral contraceptives also increase the risk of stroke. Increased VWF levels contribute to the increased risk of cardioembolic stroke, elevated leukocyte increase the risk of lacunar stroke, and hyperhomocysteinemia may increase the risk of large artery disease.

Diagnosis

Typical stroke symptoms include sudden, half-side numbness, paralysis, loss of vision, altered speech, ataxia, and non-orthostatic dizziness. Typical symptoms summed up in the FAST campaign for the general public for easy recognizability and quick care: facial weakness, arm weakness, speech problems, calling emergency services. The purpose of diagnostic procedures is to identify the stroke subtype, to determine its localization, to exclude other stroke imitating diseases. To do this, the following information should be obtained first: time of symptoms onset, blood pressure and blood glucose measurement at hospital admission, determination of severity of the stroke by NIHSS (National Institute of Health Stroke Scale) and brain imaging procedures: primarily non-contrast CT, which is used to detect ischemic and hemorrhagic stroke and to detect early ischemic signs and arteriosclerosis. Multimodal CT (CT perfusion, CT angiography, CT venography) can provide information on the extent of ischemic injury, perfusion status,

vascular stenosis/occlusion, collateral flow and acute stroke differential diagnosis, furthermore intracranial hemorrhage can be excluded by multimodal CT.

With NIHSS, beyond the severity of stroke, stroke progression, response to therapy, short and long term outcome can also be estimated. NIHSS examines the level of alertness, eye movements, field of vision, the function of facial muscles (paralysis), the motoric function of the hands and feet, coordination (presence of ataxia), sensory function and speech (presence and extent of aphasia). Its benefits include its validity and reliability, also when performed by non-neurologist specialists. It is widely used in clinical trials.

The ASPECT (Alberta Stroke Program Early CT) score was developed and validated for systemic detection and quantification of early ischemic changes in CT on the territory of middle cerebral artery. On the 10-point scale, the value of 10 means the normal state. Clinical research shows that ASPECT score is independently associated with long-term functional outcome. The ASPECT value ≤ 7 has a strong correlation with poor functional status (modified Rankin scale: mRS ≥ 3) and the increased risk of intracerebral hemorrhage. A separate ASPECT score was created and validated for the basilar artery territory (pc-ASPECT: posterior circulation Alberta Stroke Program Early CT score). According to research, the pc-ASPECT < 8 value independently predicts poor functional outcome (mRS ≥ 4), despite the complete revascularization. The ASPECT score is widely used in clinical trials, it is considered as a useful diagnostic tool, but it is not recommended to determine patient prognosis or to exclude patients from a therapeutic procedure based on ASPECT score.

To evaluate the long-term functional status after acute stroke, the modified Rankin scale is widely used. On the scale of 0 to 6, mRS evaluates global functional status with a focus on mobility. MRS is not only used in everyday clinical practice, but is also used in clinical trials, e.g. when comparing the outcome of different study groups. The strengths of validated mRS include comprehensive measurement of functionality and having good correlation with lesion size, stroke type, neurological damage and other functional test measurements. At the same time, its subjectivity and reproducibility are considered to be weak.

Therapy

Intravenous thrombolytic therapy with alteplase initiated within 4.5 hours of symptom onset is considered standard specific treatment for acute ischemic stroke if the patient

meets the criteria for inclusion. Dose of intravenous alteplase (iv. rt-PA: recombinant tissue-type plasminogen activator) is 0.9 mg/kg which does not exceed 90 mg. 10% of the dose should be administered as a bolus and the remaining for 60 minutes as an infusion. The best results can be achieved by thrombolysis within the first 90 minutes of symptom onset. Thrombolytic therapy should be monitored for 24 hours and the patient's blood pressure should be monitored (below 180/105 mm Hg) as well. The safety and efficacy of rt-PA therapy was supported by several clinical studies and systematic summary reports, but less than half of the patients treated in this way achieved functionally independent status three months later with full or nearly complete recovery of neurological functions. The most serious complication of thrombolytic therapy is intracerebral hemorrhage, which occurs more frequently in large ischemia, older age, more serious stroke indicated by NIHSS, hyperglycemia, atrial fibrillation, congestive heart failure and renal insufficiency. Intracerebral hemorrhage occurs in approximately 7% of patients treated with rt-PA thrombolysis, significantly increasing morbidity and mortality. Although many risk factors for the adverse outcome of thrombolysis with rt-PA have been identified (e.g. old age, male gender, stroke severity, diabetes mellitus, hyperglycemia diagnosed at hospital admission), it is not easy to predict the outcome before thrombolysis, due to the fact that most risk assessment scales based on clinical and radiological data are not specific and have a moderate predictive value.

Mechanical thrombectomy is another novel option in the treatment of ischemic stroke within the first 6 hours. Thrombectomy can be applied after thrombolytic therapy in patients eligible for intravenous thrombolysis, as well as in patients who may not be eligible for intravenous thrombolysis due to increased bleeding risk.

Intraarterial thrombolysis therapy may be used within 6 hours after symptom onset in patients with large artery occlusion, in patients who are not eligible for iv. rt-PA therapy due to anticoagulant therapy or postoperative status, and in patients after iv. thrombolysis without any improvement.

Initial therapies for acute ischemic stroke prior to thrombolysis include reducing blood pressure below 185/110 mm Hg to avoid hemorrhagic complications of thrombolytic therapy. Treatment of hyperglycemia is also required, as clinical studies have indicated that >7.7 mmol/l blood glucose levels have associated with worse long-term outcome.

24 hours after hospital admission, control brain imaging (CT or MRI) is required to exclude hemorrhage and determine radiological changes that can help estimate

outcome.

Prognosis

The mortality of acute stroke is 15% after one month, 25% after one year and 50% after five years. 40% of stroke survivors have unfavorable functional outcome (mRS 3-5). The risk of stroke recurrence after ischemic stroke or TIA is 10% one week later, 15% one month later, 18% three months later, 10% one year later and 25% five years later. The risk of recurrence is greater in case of symptomatic atherosclerosis, vascular risk factors, active thrombosis sources and suspension of antiplatelet or antihypertensive therapy. In patients with atrial fibrillation the risk of stroke recurrence increases with the increase in CHA₂DS₂-VASc and ABC score.

Stroke biomarkers

To improve acute stroke treatment, it would be helpful to supplement risk assessment scales based on clinical and radiological data with biomarkers.

FVIII and VWF as stroke biomarkers

Several studies confirmed the role of FVIII and VWF in the pathophysiology of acute ischemic stroke. In animal experiments, plasma levels of FVIII and VWF showed direct correlation with the rate of formation of arterial thrombus and the extent of ischemia. Human research has shown that elevated levels of FVIII and VWF increase the risk of stroke. Elevated levels of FVIII increased the risk of stroke with atherothrombotic and cardioembolic etiology. However, there is much less information regarding the relationship between FVIII and VWF levels and the outcome of stroke. There was a significant association between low rate of recanalization and adverse outcome and elevated VWF levels following the therapy of acute myocardial infarction thrombolysis. Changes in the levels of FVIII and VWF during acute stroke thrombolytic therapy are less known, although experimental results on animal models suggest that FVIII degradation during thrombolysis may have a causal effect on bleeding complications.

In the past decades particular attention is drawn for the feasibility of VWF inhibition therapy for acute stroke. VWF antagonists used in combination with rt-PA may contribute to the lysis of thrombus and may limit the formation of consequent thrombo-inflammatory ischemia or reperfusion injury. The principle of VWF inhibition therapies is based on animal experiments which found that the inhibition of GPIb-VWF and VWF-collagen interaction are protective against the onset of ischemic brain damage in mice with acute stroke. According to the literature, platelets and VWF contribute to the

development of neurological lesions not only through the initiation of thrombus formation but also through the activation of inflammatory processes. The role of VWF is also supported by experimental results with ADAMTS13 metalloprotease. According to clinical observations, low ADAMTS13 activity was associated with higher risk of ischemic stroke. In animal experiments, use of recombinant ADAMTS13 showed a protective effect against reocclusion, reduced leukocyte migration to the site of injury and reduced bleeding complications of rt-PA therapy by the preservation of the blood-brain barrier. Given that most of these researches have been based on animal models, human studies on FVIII and VWF levels may provide important findings.

Objective

In the course of our work, two observational clinical studies were performed to investigate the correlation between FVIII and VWF levels and other hemostasis factors and the pathomechanism of increased thromboembolic risk in patients with atrial fibrillation, and to investigate the correlation between FVIII and VWF levels and the outcome of therapy after thrombolysis in patients with acute ischemic stroke.

In detail:

1. We aimed to identify local hemostasis and fibrinolysis abnormalities, which are associated with AF and increase the risk of thromboembolism. Intracardiac blood samples taken from the left atrium and left atrial appendage of AF patients and non-AF controls were tested for a comprehensive set of hemostasis and fibrinolytic factors in order to assess AF associated alterations.
2. We aimed to investigate the relation of FVIII and VWF levels to stroke severity and stroke etiology. Furthermore, we aimed to investigate FVIII and VWF levels during the course of thrombolysis in acute ischemic stroke (AIS) patients and to find out whether they predict long-term outcomes.

Patients and Methods

Intracardiac Hemostasis and Fibrinolysis Parameters in Patients with Atrial Fibrillation

Study Population

Consecutive patients undergoing radiofrequency ablation for symptomatic paroxysmal or persistent AF (AF group) as well as age- and sex-matched patients with any arrhythmia other than AF requiring left atrial access (non-AF control group) were enrolled in the study. Patients were enrolled between 2013 October and 2015 December. All AF patients were undergoing pulmonary vein isolation (PVI) with phased radiofrequency (RF) or cryoballoon ablation procedure. Non-AF controls were undergoing routine RF ablation of a left atrial substrate (mostly a left-sided accessory atrioventricular pathway).

Inclusion criteria for the AF group were the following: age 18–75 years, documented, symptomatic paroxysmal or persistent AF, failure of at least one antiarrhythmic drug, and patient being willing to sign a written informed consent. Inclusion criteria for the control group were age 18–75 years, documented non-AF arrhythmia including one of the following: left atrial tachycardia, paroxysmal supraventricular tachycardia (orthodromic or antidromic), or FBI (fast, broad, and irregular) tachycardia due to a left-sided accessory pathway, preexcitation on the 12-lead electrocardiogram in an asymptomatic individual in whom the electrophysiology study revealed a left-sided accessory pathway potentially resulting in significant arrhythmia based on its conduction properties, and patient being willing to sign a written informed consent. Exclusion criteria for the patient and control groups were previous heart surgery, valvular heart disease, left ventricular ejection fraction (LVEF) $\leq 30\%$, heart failure of New York Heart Association functional classification (NYHA) class III or IV, documented carotid stenosis, history of ischemic stroke or TIA, prior cardiac surgery, unstable angina or myocardial infarction within the last 3 months, severe chronic obstructive pulmonary disease, known bleeding or thrombotic disorders, acute inflammation, contraindication to oral anticoagulation or to diffusion weighted magnetic resonance imaging (DW MRI), and pregnancy. Additional exclusion criteria for the patient group were long-standing persistent AF, reversible cause of AF (e.g.

hyperthyroidism), and presence of AF thrombus. Risk factors for stroke (hypertension, diabetes mellitus, smoking, BMI, etc.) together with the list of current medications were assessed before the enrollment of patients.

The CHADS₂ score (congestive heart failure, hypertension, age ≥ 75 years, diabetes mellitus, and stroke/transient ischemic attack), CHA₂DS₂-VASC score (congestive heart failure, hypertension, age ≥ 75 years, diabetes mellitus, stroke/ transient ischemic attack/ thromboembolism, vascular disease (prior myocardial infarction, peripheral vascular disease, or aortic atherosclerosis), age (65–74 years), and sex category (female)) and EHRA score (European Heart Rhythm Association score) were recorded for every AF patient.

The study design was in accordance with the guiding principles of the Declaration of Helsinki, and was approved by the Institutional Ethics Committee of the University of Debrecen and the Ethics Committee of the National Medical Research Council (ETT-TUKEB). All patients signed a written informed consent form prior to inclusion.

Electrophysiology Procedure and Blood Drawing

Patients were hospitalized 1 or 2 days before the procedure. All medications with a potential effect on coagulation or platelet activity were discontinued for a period of at least three halflives (or a period needed for reaching complete decay of their action) before the procedure. Transesophageal echocardiography was carried out within 24 h prior to the procedure in order to rule out the presence of a cardiac thrombus in all AF patients. All procedures were carried out under conscious sedation, using midazolam and fentanyl. The ablation procedures were performed as described previously. Blood samples were taken before the ablation procedures from multiple sites: (1) peripheral femoral venous (FV) sheath, (2) left atrial (LA) sheath, and (3) left atrial appendage (LAA) sheath. Intracardiac blood samples were collected before the administration of unfractionated heparin.

Briefly, three punctures of the right femoral vein were performed using the Seldinger technique and introducers with side arms were placed in the vein. Forty-five ml blood sample was drawn through the side arm of a short introducer immediately after access to the vein, from which the first 5ml of blood was discarded in order to exclude intrasheath hemostasis activation (FV sample). Blood samples were collected into vacutainer tubes (tubes anticoagulated with K₃-EDTA for complete blood count, tubes

containing 0.109 M sodium citrate and CTAD (buffered citrate, theophylline, adenosine, and dipyridamole)) for hemostasis and fibrinolysis tests (Becton Dickinson, Franklin Lakes, NJ). After blood drawing, a decapolar catheter and an intracardiac echo (ICE) catheter were advanced from the femoral vein and positioned in the coronary sinus and in the right atrium, respectively. A single ICE-guided transseptal puncture was performed using a Mullins transseptal sheath and a Brockenborough needle (Medtronic, Kirkland, QC, Canada) under fluoroscopic and ICE guidance using standard technique. After crossing the septum, the dilator of the Mullins sheath was removed and 45 ml blood sample was drawn from the LA, from which the first 5 ml of blood was discarded (LA sample). LA blood samples were collected into vacutainer tubes as described above. After the blood drawing of LA samples, the LAA was accessed by using a 5 F pigtail catheter (Medtronic, Kirkland, QC, Canada) under fluoroscopy and ICE control. A blood sample of 45 ml was taken from the LAA, of which, again, the first 5 ml was discarded (LAA sample). LAA blood samples were collected into vacutainer tubes as described above. Immediately after blood samplings, 150 IU/kg body weight i.v. heparin was administered and ablations were performed according to standard protocols.

Laboratory Investigations

Blood samples anticoagulated with K₃-EDTA were immediately tested for complete blood count. Blood samples anticoagulated with citrate or CTAD were centrifuged twice at 1500 g at room temperature for 20 min and plasma samples were stored at -70 °C until further analysis. The measurement of plasminogen activator inhibitor-1 (PAI-1) activity was performed from plasma samples anticoagulated with CTAD; besides this measurement, all hemostasis and fibrinolysis tests were performed using citrated plasma. Hemostasis and fibrinolysis tests were performed from all sample types (FV, LA, and LAA samples). Screening tests of hemostasis (prothrombin time, activated partial thromboplastin time, and thrombin time) were performed using routine methods (Siemens Healthcare Diagnostic Products, Marburg, Germany). Fibrinogen concentrations were measured by the Clauss method. Commercially available ELISA tests were used to determine PAI-1 activity (Technozym PAI-1 Actibind, Technoclone, Vienna, Austria), plasmin- α 2-antiplasmin (PAP) complex (Technozym PAP complex ELISA kit, Technoclone, Vienna, Austria), and thrombin-antithrombin (TAT) complex (Enzygnost TAT micro, Siemens Healthcare Diagnostic Products, Marburg, Germany).

Factor VIII (FVIII) activity using a chromogenic assay, von Willebrand factor (VWF) antigen level, α 2- plasmin inhibitor (α 2-PI) activity, plasminogen activity and D-dimer levels were measured on a BCS coagulometer by standard methods (Siemens Healthcare Diagnostic Products, Marburg, Germany). Plasma levels of FXIII activity were determined by ammonia release assay using a commercially available reagent kit (REA-chrom FXIII kit, Reanalker, Budapest, Hungary). Soluble fibrin monomer levels (FM) were measured using the Liatest FM assay (Diagnostica Stago, Asnières, France). High sensitivity C-reactive protein (CRP) and a comprehensive lipid profile including total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and triglyceride levels were measured from antecubital vein blood samples of all patients upon hospital admission by routine methods (Roche Diagnostics, Mannheim, Germany).

Statistical Analysis

All data were analyzed using the GraphPad Prism Software version 5.0 (La Jolla, CA) and the Statistical Package for Social Sciences (SPSS, Release 22.0, Chicago, IL). Normality of the data was evaluated by the D'Agostino and Pearson omnibus normality test. A paired *t*-test or Wilcoxon matched pairs rank-sum test was applied for comparing results obtained from intracardiac and FV samples. In case of two-group analyses between AF patients and controls, unpaired *t*-test or in case of nonparametric data Mann–Whitney U test was used. ANOVA or Kruskal- Wallis test was applied for multiple comparisons. Pearson's or Spearman's correlation coefficient was used to determine the strength of correlation between variables. Differences between categorical variables were assessed by the Fisher's exact test. $P < 0.05$ was considered statistically significant.

Elevated Factor VIII and von Willebrand Factor Levels Predict Unfavorable Outcome in Stroke Patients Treated with Intravenous Thrombolysis

Patients

Consecutive AIS patients aged 18 years or more, eligible for thrombolysis, admitted to a single Stroke Center (Department of Neurology, Faculty of Medicine, University of Debrecen, Hungary) were enrolled in the study. All patients were within 4.5 h of their symptom onset at the time of admission. Patient enrollment lasted for 22 months starting in March 2011. Intravenous thrombolytic therapy was applied according to the European Stroke Organization (ESO) guidelines using rt-PA (Alteplase, Boehringer Ingelheim, Germany). Inclusion and exclusion criteria of patients were identical to that of thrombolysis eligibility as described in the ESO 2008 guideline. The diagnosis of IS was based on clinical symptoms and brain imaging using computer tomography (CT) scan and CT angiography (CTA). Admission CTA was used to identify the level of vessel occlusion in every patient. A control CT was performed for every patient 24 h after thrombolysis. All CT images were analyzed by four different investigators blinded to the clinical state of the patients and the Alberta Stroke Program Early CT Scores (ASPECTS) were calculated. Neurological deficit of patients was determined by the NIHSS at various time points: on admission and at 2 h, 24 h, and 7 days post-lysis. Stroke etiology was determined according to the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) criteria. Hemorrhagic events were classified as symptomatic or asymptomatic intracranial hemorrhage (SICH or aSICH, respectively) according to the European Cooperative Acute Stroke Study (ECASS) II criteria.

For each patient a detailed list of clinical parameters was recorded including demographic characteristics, neurological status, time of symptom onset, cardiovascular risk factors (arterial hypertension, atrial fibrillation, hyperlipidemia, diabetes mellitus, smoking status), history of previous cardiovascular events, and medications.

Patients were followed and long-term functional outcomes were determined at 90 days post-event using the modified Rankin Scale (mRS).

The following outcomes were investigated:

(1) short-term functional outcome at 7 days post-event: favorable outcome was defined as a decrease in NIHSS score by at least 4 points or to 0 by day 7, unfavorable outcome

was defined as an increase in NIHSS score by at least 4 points by day 7.

(2) The presence of therapy-associated SICH or aSICH was defined according to ECASS II criteria.

(3) Long-term functional outcome at 90 days post-event: poor long-term outcome was specified as an mRS greater than 2.

The study was approved by the Ethics Committee of the University of Debrecen, Hungary. All patients or their relatives provided written informed consent.

Blood Sampling and Laboratory Measurements

Peripheral blood samples were drawn from patients into vacutainer tubes on three different occasions: upon hospital admission (before thrombolysis), immediately after the administration of rt-PA infusion (~1 h after the initiation of thrombolysis) and approximately 24 h after the administration of thrombolytic therapy. Routine laboratory tests were performed from blood samples taken before thrombolysis and included the measurements of complete blood count, serum ions, glucose levels, basic kidney function tests, liver function test, high-sensitivity C-reactive protein (hsCRP), and screening tests of coagulation (prothrombin time, activated partial thromboplastin time and thrombin time). Blood samples anticoagulated with sodium citrate, theophylline, adenosine and dipyridamole (Vacurette CTAD Tubes, Greiner Bio-One, Austria) were centrifuged at 1,220 g, room temperature for 15 min. Plasma aliquots were labeled with a code and stored at -70°C until further analysis of FVIII activity and VWF antigen levels. FVIII activity, determined by chromogenic method and VWF antigen level, determined by immunoturbidimetric assay were measured from coded plasma samples on a BCS coagulometer by standard methods (Siemens Healthcare Diagnostic Products, Marburg, Germany).

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, Release 22.0, Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Prism Inc., La Jolla, CA, USA) softwares. Normality of the data was evaluated by the Shapiro–Wilk test. As FVIII activity and VWF antigen levels were not normally distributed at any time points measured, the Mann–Whitney U test was applied for all two-group analyses and the Kruskal–Wallis analysis with Dunn–Bonferroni post hoc test was used for multiple comparisons. Differences between categorical variables were assessed by the Fisher’s exact or χ^2 test. Friedman’s two-way ANOVA with Dunn–

Bonferroni post hoc test was applied to investigate the effect of thrombolysis on FVIII activity and VWF levels. Strength of association between FVIII activity and VWF antigen levels was tested using Spearman's correlation test. In order to test for differences between adjusted means, univariate analysis incorporating covariate testing (one-way ANCOVA) was performed after logarithmic transformation of data. Positive predictive values (PPVs) and negative predictive values (NPVs) of the studied parameters were assessed using contingency tables and the Fisher's exact test. A binary backward logistic regression model was used to determine whether elevated FVIII and VWF levels of different time points are independent predictors of poor functional outcomes at 90 days post-event. Adjustment of the models were based on the results of previous statistical analyses (Mann–Whitney U test, Fisher's exact, or χ^2 test), previous literature and methodological principles (dichotomized variables wherever possible). Results of the logistic regression analysis were expressed as odds ratio (OR) and 95% confidence interval (CI). A p-value of <0.05 was considered statistically significant.

Results

Intracardiac Hemostasis and Fibrinolysis Parameters in Patients with Atrial Fibrillation

Baseline Characteristics of AF Patients and Non-AF Controls

In total 32 AF patients and 18 controls were enrolled in the study. Unfortunately, 8 AF patients and 4 controls had to be excluded from the study population due to technical problems arising during the intracardiac blood drawing procedure (clot formation in the sample during the blood drawing procedure, clot formation on the sheath requiring instant heparin administration, etc.). In case of 12 AF patients and 8 non-AF controls an LAA sample was not possible to obtain due to technical/anatomic difficulties. The final numbers of AF patients and non-AF controls included in the study were 24 and 14, respectively. No significant differences were observed between the AF patients and non-AF controls regarding BMI and cerebrovascular risk factors except for smoking, which was more frequent in controls. Only two patients experienced paroxysmal AF periods during the procedure. Most AF patients had low or moderate risk for stroke according to the CHADS₂ and CHA₂DS₂-VASC score. A similar fraction of AF patients and non-AF controls received statins and antihypertensive drugs. CRP levels and lipid parameters, measured from peripheral venous blood samples, did not differ significantly between AF patients and non-AF controls.

Intracardiac Levels of Hemostasis Factors in AF Patients and Non-AF Controls

FVIII activity and VWF antigen levels were significantly higher in the AF patient group as compared to the control group in the samples obtained from the FV and from the LA. LAA levels of both proteins showed a marked elevation in AF patients as well; however, very likely due to the lower number of LAA samples, results were only borderline significance. Elevated levels were not due to acute phase reaction as CRP levels of all individuals were in the normal range. In case of AF patients, median values of VWF antigen levels were above the upper limit of the reference interval in all sample types (171% (IQR: 129.4–195.1%), 176.7% (IQR: 129.3–192.7%), and 164% (IQR: 114.8–189.8%) for FV, LA, and LAA sample types, resp.). The observed differences between

patients and controls remained significant after adjustments for ABO blood type in the statistical model. No local differences were found in the FVIII and VWF levels of intracardiac samples as compared to the FV samples in either group. FVIII and VWF levels showed good correlation in AF patients (Spearman $r = 0.808$, 95% CI: 0.691–0.884, $p < 0.0001$) as well as in non-AF controls (Pearson $r = 0.737$, 95% CI: 0.502–0.871, $p < 0.0001$), suggesting that they are in a complex form. No considerable differences were seen in the correlation of FVIII and VWF levels with respect to sampling sites (data not shown). No significant differences were found between sample types and patient groups in case of FXIII activity and fibrinogen levels.

Intracardiac Levels of Coagulation Activation Markers in AF Patients and Non-AF Controls

Median values of soluble FM and TAT complex levels exceeded the upper limit of reference interval in the FV samples of AF patients (18.16 $\mu\text{g/mL}$ (IQR: 5.83–33.91 $\mu\text{g/mL}$) and 15.17 $\mu\text{g/L}$ (IQR: 6.96–22.83 $\mu\text{g/L}$) for FM and TAT, respectively) and non-AF controls (23.05 $\mu\text{g/mL}$ (IQR: 9.55–51.41 $\mu\text{g/mL}$) and 16.36 $\mu\text{g/L}$ (IQR: 9.84–28.59 $\mu\text{g/L}$) for FM and TAT, respectively). Moreover, both parameters were significantly elevated in the samples obtained from the LA as compared to the FV samples in case of both groups, suggesting that the observed differences are not AF-specific and most probably the catheterization procedure itself has a major effect on the results. FM levels showed a decrease in the LAA samples as compared to the LA samples; this decrease was significant in case of the patients ($p < 0.001$, Wilcoxon matched pairs rank-sum test). TAT complex levels were also significantly lower in the LAA samples versus LA samples of AF patients ($p < 0.01$, Wilcoxon matched pairs rank-sum test), while such significant association was not observed in case of the non-AF control patients. TAT complex levels were significantly increased in the LAA samples of both AF patients and non-AF controls as compared to the FV samples. Surprisingly, a marginal but significant elevation was observed in the TAT complex levels of the LA samples of non-AF controls versus AF patients ($p < 0.05$).

Intracardiac Parameters of Fibrinolysis in AF Patients and Non-AF Controls

Plasminogen activity, $\alpha 2$ -PI activity, and PAI-1 activity levels showed no difference between AF patients as compared to non-AF controls. In general, no difference was

observed between the intracardiac and peripheral levels of these parameters, except for a small, but significant reduction of plasminogen level in the LAA versus FV sample of the AF patients. PAP complex and D-dimer levels were significantly increased in the LA samples of both AF patients and non-AF controls as compared to the respective FV samples, suggesting that the activation of the fibrinolytic system took place during the transcatheter procedure in both groups. In fact, approximately half of the AF patients and non-AF controls had D-dimer levels exceeding the cut-off value in the LA sample, while median values of D-dimer were well below the cut-off in the FV samples (0.26 mgFEU/L (IQR: 0.17–0.48 mgFEU/L) and 0.30 mgFEU/L (IQR: 0.18–0.48 mgFEU/L) in AF patients and controls, respectively).

Elevated Factor VIII and von Willebrand Factor Levels Predict Unfavorable Outcome in Stroke Patients Treated with Intravenous Thrombolysis

Study Population

During the study period, 131 consecutive AIS patients receiving intravenous rt-PA treatment were enrolled. In case of six patients, intravenous thrombolytic therapy was supplemented with intra-arterial thrombolysis using rt-PA according to standard protocol; the duration of thrombolysis and the final dose of rt-PA applied did not significantly differ for these patients. Median age of the patient cohort was 69 (IQR: 59–79) years, 60.3% were men. The most common cerebrovascular risk factor was arterial hypertension in this patient cohort (n= 100, 76.3%). Median time from symptom onset to treatment was 155 min (IQR: 125–180). Median NIHSS before stroke treatment was 8 (IQR: 5–14). According to the TOAST criteria, etiology of stroke was most commonly large vessel disease (n= 49, 37.4%), followed by 27 (20.6%) patients with cardioembolic stroke. As detected by CTA on admission, 70 patients (53.4%) had a vessel occlusion, and 27 patients (20.6%) stenosis. Poor functional outcome at 7 days post-event was observed in 20 cases (15.3%), while poor outcome at 90 days (mRS ≥ 3) was observed in case of 51 (38.9%) patients. Therapy-associated intracranial hemorrhage was detected in 13 cases, of which 6 cases (4.6%) were symptomatic according to ECASS II. Mortality rates by day 7, 14, and day 90 post-event were 3.8, 6.1, and 22.1%, respectively.

The Effect of Thrombolysis on FVIII Activity and VWF Antigen Levels

In the samples taken on admission, the median values of both hemostasis parameters were above the upper limit of the respective reference interval in the whole patient cohort (FVIII activity median: 188.0%, IQR: 153.0–242.0%, VWF antigen level median: 201.3%, IQR: 169.1–259.6%). FVIII activity dropped significantly in the samples obtained immediately after thrombolysis as compared to the initial values (median: 102.0%, IQR: 62.0–155.5%, $p < 0.001$) and showed an increase 24 h after the event (median: 166%, IQR: 130.0–209.0%, $p = 0.014$). On the contrary, VWF levels increased steadily post-lysis (median VWF levels immediately after lysis: 229.1%, IQR: 157.6–293.3%, at 24 h post-lysis: 231.6%, IQR: 176.8–284.8%, Friedman's two-way ANOVA $p = 0.002$). Notably, VWF median and IQR values were above the upper limit

of the reference interval at all investigated time points in the study cohort.

Factor VIII activity and VWF antigen levels showed good correlation on admission ($r=0.748$, $p < 0.001$), but no significant correlation was found immediately after thrombolysis ($r=0.093$, $p=0.299$), most probably due to plasmin mediated FVIII degradation. Fair correlation was observed between the two parameters in the samples obtained 24 h after thrombolysis ($r=0.420$, $p < 0.001$).

The Association of FVIII Activity and VWF Antigen Levels with Stroke Severity

Von Willebrand factor antigen levels were gradually and significantly elevated in case of more severe AIS (NIHSS 6–16 and NIHSS >16) at all investigated time points, but no such significant association was observed for FVIII activity levels. The association between VWF antigen levels and stroke severity remained significant after adjustments for confounders (current smoking, hsCRP, age) in the statistical model.

The association of elevated VWF levels and more severe AIS was also proved as significantly higher VWF levels were found at all investigated time points in patients presenting with worse 24 h post-lysis CT scans (ASPECTS score 7–0). Similar association was observed for FVIII levels, except for the samples investigated immediately after lysis. Associations for VWF levels and FVIII activity remained significant after adjustments for confounders (current smoking, hsCRP, age) in the statistical model. As CT scans on admission are not indicative of stroke severity and have less predictive values as the ASPECTS at 24 h post-lysis, it was not surprising that no significant association was found between the investigated hemostasis parameters and the ASPECTS on admission.

No association was found between FVIII activity and VWF antigen levels at any time points and stroke subtypes according to TOAST criteria (data not shown).

FVIII activity at 24 h post-lysis was significantly elevated in patients with vessel occlusion (median: 175.0%, IQR: 151.5–227.0%) as compared to those with stenosis only (median: 137.0%, IQR: 98.5–175.0%) or without occlusion/ stenosis (median: 142.0%, IQR: 115.0–177.0%) ($p=0.001$), while such association was not observed for VWF levels.

Elevated FVIII Activity and VWF Antigen Levels As Predictors of Thrombolysis Outcomes

Short-term outcome. Factor VIII activity and VWF antigen levels were not associated at any investigated time points with short-term therapy outcomes as assessed by the changes in NIHSS score by day 7 post-lysis.

Risk of Intracranial Hemorrhage. No association was found between FVIII activity or VWF antigen levels at any investigated time points and therapy-associated ICH, except for higher VWF at 24 h post-lysis in patients presenting with SICH (median: 226.8%, IQR: 176.5–279.4% vs. median: 347.5%, IQR: 263.3–372.1% for no bleeding or aSICH vs. SICH, $p=0.017$).

Long-term outcome. Poor functional outcome ($mRS \geq 3$) at 90 days post-event was associated with traditional risk factors including advanced age, increased NIHSS on admission, elevated hsCRP, and the presence of diabetes/diabetes treatment. Moreover, as expected, ASPECTS at 24 h post-lysis and the level of vessel occlusion as detected by CTA was also indicative of the long-term outcome. Among the hemostasis parameters investigated at various time points, elevated FVIII activity 24 h post-lysis and elevated VWF antigen level measured immediately after lysis and 24 h after therapy showed significant association with poor outcomes.

Both parameters, as measured immediately post-lysis and 24 h post-lysis conferred a significant PPV and NPV for poor functional outcomes (highest PPV: VWF 24 after thrombolysis: 0.83; 95% CI: 0.59–0.96, $p=0.009$ and highest NPV: FVIII immediately after thrombolysis: 0.73; 95% CI: 0.50–0.89, $p=0.009$).

A binary backward logistic regression model including age, gender, elevated hsCRP, active smoking, diabetes mellitus, and NIHSS >5 on admission revealed that a FVIII activity and VWF antigen level above the upper limit of the reference interval (168 and 160%, respectively) as measured immediately after lysis and 24 h after thrombolysis significantly and independently increase the risk of unfavorable functional outcomes at 90 days. In this model, FVIII activity and VWF antigen levels on admission did not prove to have an independent prognostic value regarding poor functional outcomes at 90 days, while elevated FVIII and VWF levels immediately after thrombolysis conferred an independent OR: 7.09 (IQR: 1.77–28.38, $p=0.006$) and OR: 6.31 (IQR: 1.83–21.7, $p=0.003$), respectively. Elevated levels of both factors 24 h after lysis were also found to have a significant predictive value (OR: 4.67, IQR: 1.42–15.38, $p=0.011$).

for FVIII activity and OR: 19.02, IQR: 1.39–187.0, $p= 0.012$ for VWF antigen level). Besides these hemostasis parameters, only hsCRP >5.2 mg/L and NIHSS >5 on admission remained in the stepwise backwards regression analysis model as independent risk factors for poor outcomes at 90 days (OR: 4.85, 95% CI: 1.64–14.33, $p= 0.004$ and OR: 3.51, 95% CI: 1.17–10.57, $p= 0.026$, respectively).

Discussion

Intracardiac Hemostasis and Fibrinolysis Parameters in Patients with Atrial Fibrillation

Although it is a general belief that in AF the intracardiac milieu is more thrombogenic than the peripheral blood, supporting pieces of evidence derived from measurements using intracardiac blood samples are scarce. In this study, we investigated the levels of a comprehensive list of hemostasis and fibrinolysis markers from intracardiac blood samples of AF patients and non-AF controls and failed to detect significant AF-specific alterations of hemostasis or fibrinolysis in intracardiac blood samples. It is to be noted, however, that only two patients experienced paroxysmal AF periods during the procedure, which means that most patients were on sinus rhythm during blood sampling. Our results suggest that as compared to peripheral samples, paroxysmal and persistent AF patients have no significant alterations in the intracardiac levels of the investigated hemostasis and fibrinolytic parameters, at least when they are not experiencing AF periods.

Although significant local differences were observed for certain coagulation activation and fibrinolytic markers (namely, for FM, TAT complex, PAP complex, and D-dimer levels) in the intracardiac samples as compared to the FV samples, the same differences were found in non-AF control individuals. Moreover, in the LAA sample of both groups, a general tendency of decrease was observed in the level of most investigated markers as compared to LA samples. In earlier studies in which non-AF control population was not investigated, these differences were attributed to AF pathophysiology. However, our results imply that changes in the level of these markers are not specific to AF and are likely to be attributed to the invasive nature of the catheterization procedure, including transseptal puncture and tissue damage.

Among all investigated hemostasis and fibrinolysis parameters, only the elevation of FVIII and VWF levels was found to be AF-associated in our study. Interestingly, FVIII and VWF levels were significantly elevated in both peripheral and intracardiac blood samples of AF patients as compared to controls. Elevation of VWF levels was particularly considerable in the AF patient group as the medians of VWF levels were at the upper limit of the reference interval in all sample types. Although the levels of VWF in AF patients have been studied earlier using peripheral samples, the relationship

between intracardiac and peripheral VWF levels has been obscure. An elevation of FVIII and VWF has been described earlier in the peripheral samples of AF patients and it has been proposed to be attributed to endothelial damage. Moreover, elevated levels of VWF have been associated with increased stroke risk and poor prognosis. Only few papers enrolling a limited number of patients have investigated the levels of VWF in AF patients from both intracardiac and peripheral blood samples, but in these studies FVIII levels were not determined. In line with our findings, in these earlier reports it was found that VWF levels were similar in the intracardiac samples and in samples obtained from the peripheral sampling site. In our study FVIII and VWF levels showed good correlation in all sample types, suggesting that they were in complexed form. As both proteins are stored in the Weiber-Palade bodies of the endothelium, these results imply that the elevation of VWF and FVIII levels are the consequence of endothelial damage and not necessarily restricted to the LA. It has to be noted that in the LAA of patients a similar tendency of FVIII and VWF elevation was observed as in case of FV and LA samples, but this is most likely due to the limited number of LAA samples since differences were not proved to be significant between patients and controls for this sample type.

Despite the important role of the fibrinolytic system in preventing intravascular thrombosis, previous studies have paid little attention to the investigation of fibrinolytic abnormalities associated with AF. Moreover, little is known about the levels of important regulators of fibrinolysis in intracardiac samples in AF. Here we assessed a series of fibrinolytic markers from both peripheral and intracardiac blood samples of AF patients and non-AF controls. Besides a small but significant decrease in the levels of plasminogen in the LAA samples of AF patients as compared to the FV samples, no significant differences were observed between AF patients and non-AF controls and among sample types concerning FXIII activity, α 2-plasmin inhibitor, PAI-1 activity, and plasminogen activity measurements. There were no differences between PAP complex and D-dimer levels in AF patients and non-AF controls either. These findings suggest that the investigated components of the fibrinolytic system are mostly unaltered in AF.

Limitations

Our study has some limitations. First, the number of patients enrolled in the study was limited, which was obviously due to the highly invasive nature of blood sampling,

during which technical difficulties were often encountered. We would like to highlight, however, that the number of patients enrolled in our study is still more than the average number of patients undergoing this kind of blood sampling as published so far. Moreover, in our study a non-AF control patient group was also enrolled, which was often missing from earlier studies. Despite the particularly difficult and potentially risky technique of LAA sampling, a considerable number of patients were sampled from the LAA as well, which is a rarity in the literature as of yet. Based on our findings larger studies are warranted to corroborate our observations.

Second, most patients enrolled in the study had low or moderate stroke risk according to the CHADS₂ or CHA₂DS₂-VASC score, which limits the extrapolation of our findings to the general AF patient population. It has to be noted, however, that the stroke risk of our patient population reflects the current practice of most ablation centers, which offer ablation for younger patients with mostly paroxysmal AF, structurally normal heart, and no significant comorbidity. In addition, the necessity and safety of the discontinuation of anticoagulation preablation (which was a requirement in our study in order to carry out certain measurements) are only evident in low-risk patients.

Third, only 2 patients experienced a paroxysmal AF period during the catheterization and blood drawing procedure. Naturally, more patients having AF period during sampling could have supplemented our results with a further interesting aspect.

Conclusion

AF patients have elevated FVIII and VWF levels, most likely due to endothelial damage, which is present in the intracardiac and peripheral environment as well. Intracardiac activation of hemostasis and fibrinolysis was demonstrated in AF patients and in non-AF controls to a similar extent, indicating that this might be a consequence of the catheterization procedure itself rather than a footprint of AF pathophysiology.

Elevated Factor VIII and von Willebrand Factor Levels Predict Unfavorable Outcome in Stroke Patients Treated with Intravenous Thrombolysis

In this study, we examined the levels of FVIII and VWF during thrombolysis in 131 consecutive AIS patients and studied the relationship between the hemostasis factor

levels and stroke characteristics and therapy outcomes. Only few papers are found in the literature studying the changes of certain hemostasis factors during the course of thrombolysis following ischemic stroke and to our knowledge, none of them studied the levels of FVIII and VWF comprehensively in this respect. It has been known for almost 40 years that in vitro plasmin degrades and inactivates FVIII. Studies in animal models also suggested such effect of plasmin on FVIII; however, the in vivo effect of plasmin on FVIII in humans during the course of rt-PA induced thrombolysis has not yet been characterized. Here we showed that FVIII activity drops significantly immediately after thrombolysis as compared to levels measured on admission of patients. However, as the vast majority of patients had elevated FVIII levels on admission, this reduction is most probably due to plasmin-mediated degradation, and did not reach a level that would suggest a potential risk for intracerebral hemorrhage. In fact, FVIII levels measured at any point in time in this study were not associated with bleeding complications, which is in line with the results of studies in animal models. As opposed to FVIII activity, VWF antigen levels showed a rising tendency during the course of thrombolysis in our study. This, in theory might be due to two reasons. The first apparent reason is VWF degradation by plasmin, which has been shown before in vitro. As the test we used for measuring VWF antigen levels contains polyclonal antibody against VWF, the degradation of the protein leads to an increased antigen level. In an early paper describing the time course of certain hemostasis factors in a few patients (n= 7) with AMI treated by rt-PA induced thrombolysis, it was shown that thrombolysis treatment resulted in the elevation of VWF antigen levels, most probably due to the proteolysis of VWF multimers. The degradation of VWF multimers has been speculated to be a potential causative factor for hemorrhagic complications in AMI patients treated with thrombolysis. In our study, VWF antigen levels were found to be significantly higher at 24 h post-lysis in patients with SICH as compared to the rest of the cohort, but due to the relatively low number of patients with SICH in this population (n= 6), this association should be confirmed by other studies. As for the second reason for the elevation of VWF antigen levels post-lysis, it is plausible that the increase is due to endothelial damage caused by ischemic damage. Studies in AMI patients suggested that thrombolysis induced by streptokinase is associated with an increase of VWF antigen levels due to endothelial damage as a result of oxidative stress caused by the thrombolytic agent. Interestingly, in our study, VWF antigen levels showed an increase after thrombolysis only in patients with more severe stroke (NIHSS 6–16 and

NIHSS >16 on admission), while in the group of patients with less severe stroke (NIHSS 0–5) this elevation was not seen, suggesting that at least in part endothelial dysfunction is likely to contribute to this finding.

Many studies have investigated the association between FVIII and/or VWF levels and the risk of cardiovascular or cerebrovascular events. Despite few conflicting results, it has been well established that elevated FVIII and/or VWF levels predispose patients to AIS. In line with our findings, most studies revealed that in the majority of tested patients with AIS, high FVIII and VWF levels were found; moreover, baseline stroke severity as measured by the NIHSS score was associated with elevated FVIII and/or VWF levels. Furthermore, beyond these previously known results, here we describe a strong association between elevated FVIII/VWF levels during the course of thrombolysis and the ASPECT score in patients as assessed 24 h post-lysis. The only non-significant association in this respect was FVIII activity tested immediately after lysis, which was most probably due to plasmin-mediated degradation of the protein. The finding that the ASPECT score the day after stroke shows a strong association with the tested hemostasis parameters is of considerable interest, as it indicates a link between the investigated factors and stroke severity as verified by not only the NIHSS functional score but by imaging analysis as well. Similar findings on the relation of any hemostasis factors and the results of such imaging analysis is practically lacking in the literature as of yet.

Limited evidence is available on the possible association of FVIII/VWF levels with the etiology of stroke; moreover, reports are often discordant in this respect. Here, we could not find any association of FVIII/VWF levels with the subtype of stroke as classified by the TOAST criteria.

While the association between VWF levels and thrombolysis outcome following AMI has been studied before, surprisingly, similar data regarding ischemic stroke are much more limited. Here we show that a FVIII activity and VWF antigen level above the upper limit of the reference interval, as measured immediately after or 24 h post-lysis, confer a significant, independent risk for poor functional outcomes at 90 days post-event.

Our results indicate that both factors could be useful biomarkers having significant prognostic values on long-term outcomes, which might help with patient selection requiring alternative treatment post-lysis. At the same time, at least according to results of this cohort, pre-treatment FVIII and VWF levels were not indicative of thrombolysis

outcomes.

Although here we propose that our results have prognostic value in the studied patient cohort, nevertheless, we consider the relevance of our findings as potentially useful descriptive data which might provide basis for future research, while its clinical relevance remains to be fully elucidated. In the era of mechanical thrombectomy, the management of AIS faces new types of decision-making questions in the clinical practice. Useful biomarkers with predictive values regarding poor outcomes might be incorporated into algorithms designed to select candidates for alternative approaches rather than rt-PA alone, e.g. mechanical thrombectomy or other pharmacological approaches. Moreover, studies on VWF in particular might prove even more useful in the future, as preclinical and clinical studies on inhibitors of VWF are promising and show a safe antithrombotic potential. When used in combination with t-PA, VWF antagonists were able to prevent ongoing microvascular thrombus formation reducing stroke progression. These findings are particularly interesting in the light of our observations showing that VWF median and IQR values were constantly above the upper limit of the reference interval in the studied AIS population. Although drugs that target the inhibition of the action of VWF have not yet reached approval for the market, studies on FVIII/VWF levels during stroke and thrombolysis might provide useful descriptive data to understand the pathophysiology relevant to the potential clinical application of these inhibitors in humans in the future.

Limitations

Our study has limitations. The sample size is limited, but in the light of other studies on AIS patients treated by thrombolysis it is considered representative. Due to the limited number of patients with SICH and with poor outcomes in this cohort, despite the significant associations found, results presented here need to be verified by larger studies. We did not investigate AIS patients who were not suitable for rt-PA therapy. In theory, measuring FVIII/VWF levels of patients receiving and not receiving rt-PA and comparing the results with outcomes might be useful. However, due to the important baseline differences between the two groups (e.g. the group not receiving rt-PA might be highly heterogeneous regarding time window from symptom onset, baseline coagulation screening tests, effective anticoagulation, age, etc.) which may significantly affect the results, such comparison might fail to support relevant conclusions.

The lack of advanced neuro-imaging (e.g. perfusion and collateral circulation imaging)

limits the application of this study.

Finally, as the study was designed to find potential biomarkers with predictive values for long-term outcomes following thrombolysis, we did not perform any functional characterization studies on the plasmin-mediated effect of FVIII and VWF proteins. In case of FVIII activity, we assumed based on previous studies, that the reduction in activity levels as detected immediately after thrombolysis is due to plasmin-mediated degradation and did not perform any biochemical tests to prove this hypothesis. In case of VWF, functional activity tests, including ristocetin induced activity tests, collagen-binding assay, multimer testing, etc. were not measured in the patient population. Future studies are required to elucidate whether the qualitative changes of both proteins following stroke and thrombolysis would have any pathophysiological relevance and prognostic value.

Conclusion

Here we report the changes in FVIII activity levels and VWF antigen levels during the course of thrombolysis in a cohort of consecutive AIS patients. Elevated FVIII activity and VWF antigen levels immediately after lysis and 24 h post-therapy were shown to have independent prognostic values regarding poor functional outcomes at 90 days.

Summary of discussion

Cardiovascular and cerebrovascular diseases are among the leading causes of death in developed countries. Although these groups of diseases have been intensively researched because of their public health significance, better understanding of their pathomechanism and identifying biomarkers, predicting the risk of developing cardio- and cerebrovascular diseases and/or having prognostic value, remain a challenge.

One of the most serious complication of atrial fibrillation is cardioembolic ischemic stroke. Cardioembolic stroke occurs in approximately 21-37% cases of ischemic stroke. According to literature data, studying VWF in patients with atrial fibrillation may be a useful biomarker for cardioembolic ischemic stroke and may complement the currently used clinical scales using only anamnestic data. Our studies confirm the presence of increased VWF and associated elevated FVIII levels in patients with atrial fibrillation compared to non-atrial fibrillation controls. Our results also highlighted that the rate of increase is similar in intracardial and peripheral blood. Based on our results, FVIII and VWF may be useful biomarkers after the thrombolysis therapy of ischemic stroke, predicting post-thrombolysis severity and long-term outcome. Examination of the two parameters can help to better understand the causes of therapeutic failure, and the knowledge gained can provide a starting point in the future to develop alternative therapeutic approaches. At the same time, it should be emphasized that further investigation of the changes in the hemostasis parameters we have studied in both atrial fibrillation and ischemic stroke, and the results discussed in the thesis need to be studied with a larger population.

Summarizing our results, elevated FVIII and VWF levels may indicate thromboembolic risk associated with atrial fibrillation, while elevated FVIII and VWF levels after thrombolytic therapy in ischemic stroke are independent predictors of adverse long-term outcomes.

The candidate's own results and new findings

- By examining several markers of the hemostasis and fibrinolytic system in the intracardiac and peripheral blood samples of AF patients and non-AF controls, FVIII activity and VWF antigen levels were found to be significantly increased in AF patients in intracardiac and peripheral blood samples as well. Good correlation between FVIII activity and VWF levels suggests that elevated levels may be due to endothelial damage.
- Blood samples obtained from the left atrial appendage were not associated with increased prothrombotic differences in AF patients and non-AF controls as compared to blood samples obtained from the left atrium.
- Among the examined hemostasis or fibrinolysis parameters, local (intracardial), atrial fibrillation-specific differences were not detected. However, local (intracardial) non-atrial fibrillation-specific differences were observed in case of several parameters. We found that TAT complex, FM, PAP complex, D-dimer levels were significantly higher in the left atrium blood samples in both AF and non-AF groups. The elevated levels of these parameters during catheterisation indicated non-atrial fibrillation-specific transient thrombotic risk in both patient cohorts.
- By investigating blood samples of patients undergoing intravenous thrombolytic therapy following acute ischemic stroke prior to thrombolysis, immediately after thrombolysis and 24 hours after thrombolysis, we established that the VWF antigen level medians and the lower limit of the interquartile range were above the reference range in the tested patient cohort. The median FVIII activity prior to thrombolysis also exceeded the upper limit of the reference range but showed a significant reduction immediately after thrombolysis and increased again 24 h after thrombolysis.
- We observed that VWF antigen levels were significantly higher in patients with more severe stroke (NIHSS > 16 and NIHSS 6-16) as compared to patients with mild stroke (NIHSS <6) in blood samples taken prior to thrombolysis. In patients with more severe stroke, VWF antigen levels gradually and significantly increased after thrombolysis. Elevated VWF antigen levels as observed 24 hours

after thrombolysis showed significant correlation with the occurrence of symptomatic intracranial bleeding in the studied population.

- The relationship between more severe acute ischemic stroke and elevated VWF antigen levels was also supported by the observation that worse ASPECT score in the control radiological examination 24 h post-event was associated with significantly higher VWF antigen levels at all sampling occasions. FVIII as measured immediately after thrombolysis did not have significant correlation with an unfavorable ASPECT score determined at 24 h post-event, but there was a significant correlation between unfavorable ASPECT scores at 24 h post-event and elevated FVIII activity levels as measured in samples prior to thrombolysis and 24 h after thrombolysis.
- We observed good correlation between FVIII activity and VWF antigen levels ($r=0.748$, $p < 0.001$) in blood samples prior to thrombolysis. However, there was no significant correlation immediately after thrombolysis, which is surmized to be due to plasmin-mediated FVIII degradation.
- Using a multivariate logistic regression model, we demonstrated that elevated FVIII and VWF antigen levels detected immediately after and 24 hours after thrombolysis were independent risk factors of unfavorable long-term functional outcomes (mRS 90 days later thrombolysis ≥ 3) (immediately after thrombolysis FVIII: OR: 7.1, 95% CI: 1.8-21.7, $p= 0.003$, 24 hours after lysis: FVIII OR: 4.7, 95% CI: 1.4-15.4, $p= 0.011$, VWF: OR: 19.0, 95% CI: 1.9-187.0, $p= 0.012$).

Authors contribution

Intracardiac Hemostasis and Fibrinolysis Parameters in Patients with Atrial Fibrillation.

Csiba L, Csanádi Z, Muszbek L, Bereczky Z and Bagoly Z designed the study. Tóth NK, Csanádi Z, Kiss A, Hajas O, Nagy-Baló E, Kovács KB, and Sarkady F were involved in sample collection. Csanádi Z, Kiss A, Hajas O, Nagy-Baló E, Kovács KB were involved in clinical data preparation. Tóth NK performed the laboratory measurements. Tóth NK and Bagoly Z analyzed the data, designed and performed the statistical analysis, and wrote the paper.

Elevated Factor VIII and von Willebrand Factor Levels Predict Unfavorable Outcome in Stroke Patients Treated with Intravenous Thrombolysis.

Csiba L and Bagoly Z designed the study. Szekely EG, Czuriga-Kovács KR, Sarkady F, Berényi E, Láncki LI, Fekete K, and Fekete I were involved in sample collection and source data preparation. Tóth NK and Nagy O performed the laboratory measurements. Tóth NK and Bagoly Z analyzed the data, designed and performed the statistical analysis, and wrote the paper.

Summary

Introduction. Cardiovascular and cerebrovascular diseases, as the leading causes of mortality and long-term morbidity are intensively investigated disorders. Still, today the understanding of their pathomechanism as well as the identification of biomarkers as potential risk factors and/or prognostic markers remain a challenge.

Patients and methods. We have carried out two observational clinical studies in order to investigate the levels of certain hemostasis and fibrinolytic factors, particularly factor VIII (FVIII) and von Willebrand factor (VWF) in patients with atrial fibrillation and acute ischemic stroke. The first patient group consisted of 24 patients with atrial fibrillation and 14 patients with other supraventricular tachycardia (controls) undergoing transcatheter radiofrequency ablation. Blood samples were drawn from the femoral vein, left atrium and left atrial appendage before the ablation procedure. FVIII activity, VWF antigen level, fibrinogen, factor XIII, α_2 plasmin inhibitor activity, thrombin-antithrombin (TAT) complex, quantitative fibrin monomer (FM), plasminogen, plasmin- α_2 antiplasmin (PAP) complex, PAI-1 activity, and D-dimer were measured from all samples. The other study population included 131 consecutive acute ischemic stroke patients who underwent i.v. thrombolysis with recombinant tissue plasminogen activator (rt-PA). Blood samples were taken on admission, 1 and 24 h after rt-PA administration to measure FVIII activity and VWF antigen levels. Results were compared to stroke severity and to short- and long-term clinical outcomes.

Results. In atrial fibrillation patients, FVIII activity and VWF antigen levels were significantly elevated in intracardiac and peripheral blood samples as compared to controls. TAT complex, FM, PAP complex and D-dimer levels were significantly elevated in the intracardiac samples of both groups, indicating a temporary thrombotic risk associated with the catheterization procedure. When investigating ischemic stroke patients, significantly elevated VWF levels were detected on admission in case of more severe stroke. In a binary backward logistic regression analysis elevated FVIII and VWF levels after thrombolysis were independently associated with poor long-term functional outcomes (immediately after thrombolysis FVIII: OR:7.1, 95% CI: 1.7-28.4, $p=0.006$; VWF: OR: 6.31, 95% CI: 1.8-21.7, $p=0.003$).

Conclusions. Elevated FVIII and VWF levels might predict increased thromboembolic risk in atrial fibrillation patients, while in case of ischemic stroke patients, elevated FVIII and VWF levels after thrombolysis are independent predictors of poor long-term functional outcomes.

Publication list



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List of publications related to the dissertation

1. **Tóth, N. K.**, Székely, E. G., Czuriga-Kovács, K. R., Sarkady, F., Nagy, O., Láncki, L., Berényi, E., Fekete, K., Fekete, I., Csiba, L., Bagoly, Z.: Elevated factor VIII and von Willebrand factor levels predict unfavorable outcome in stroke patients treated with intravenous thrombolysis. *Front. Neurol.* 8, 1-10, 2018.
DOI: <http://dx.doi.org/10.3389/fneur.2017.00721>
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2. **Tóth, N. K.**, Csanádi, Z., Hajas, O., Kiss, A., Nagy-Baló, E., Kovács, K. B., Sarkady, F., Muszbek, L., Bereczky, Z., Csiba, L., Bagoly, Z.: Intracardiac hemostasis and fibrinolysis parameters in patients with atrial fibrillation. *Biomed Res. Int.* 2017 (3678017), 1-10, 2017.
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List of other publications

3. Székely, E. G., Czuriga-Kovács, K. R., Bereczky, Z., Katona, É., Mezei, Z. A., Nagy, A., **Tóth, N. K.**, Berényi, E., Muszbek, L., Csiba, L., Bagoly, Z.: Low factor XIII levels after intravenous thrombolysis predict short-term mortality in ischemic stroke patients. *Sci. Rep.* 8 (1), 1-9, 2018.
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