

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

**Prognostic value of thrombin generation in different
clinical pathologies**

by Linda Lóczi

Supervisor: Dr. Zsuzsa Bagoly



**UNIVERSITY OF DEBRECEN
KÁLMÁN LAKI DOCTORAL SCHOOL
DEBRECEN
2026**

PROGNOSTIC VALUE OF THROMBIN GENERATION IN DIFFERENT CLINICAL PATHOLOGIES

By **Linda Lóczi**, Clinical Laboratory Scientist

Supervisor: **Zsuzsa Bagoly**, MD, PhD, DSc

Kálmán Laki Doctoral School, University of Debrecen

Head of the **Defense Committee**: Prof. József Balla, MD, PhD, DSc, MHAS

Reviewers: Boglárka Brúgós, MD, PhD
Imre Földesi, PharmD, PhD

Members of the Defense Committee: Renáta Hudák, MD, PhD
Barbara Réger, PhD

The PhD Defense takes place at the Lecture Hall of Building “A”, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, the 8th of June, 2026, 1:00 PM.

INTRODUCTION

The identification of both haemorrhagic and thrombotic risk is a key issue for clinicians in the daily care of patients with a wide range of conditions. The haemostasis tests currently used in routine diagnostics do not provide a comprehensive assessment of an individual's coagulation profile and haemostatic balance.

Thrombin is a key coagulation enzyme essential for maintaining normal haemostatic balance. Thrombin generation assays (TGA) provide an innovative approach to the study of haemostasis by allowing the full kinetics of thrombin generation to be monitored. A unique feature of these assays is their ability to assess the combined effect of pro- and anticoagulant processes in the blood, which can help clinicians to assess increased thrombotic or haemorrhagic risk. As such, TGA has potential applications in a number of areas, including the diagnosis of inherited and acquired coagulation disorders, the assessment of thrombosis risk and the evaluation of the efficacy of various pharmaceutical therapies. For coagulation screening tests used in current haemostasis diagnostics (prothrombin time PT, activated partial thromboplastin time: APTT, and thrombin time: TT), the endpoint of the measurement is the onset of clot formation, i.e. the appearance of the fibrin filament, which is influenced by less than 5% of the total thrombin formed. As more than 95% of thrombin is formed after clot formation, coagulation screening tests do not provide adequate information on the amount of thrombin formed. TGA measures not only the amount of thrombin generated over a given time interval, but also the kinetics of generation. An additional advantage of the test is that, unlike conventional coagulation screening tests, it measures not only the initiation phase of coagulation, but also the amplification and propagation phases, and includes the effects of the natural coagulation inhibitor (antithrombin) and its inactivators (protein C and cofactor protein S). However, the standardisation of the method and the definition of reference ranges and cut-off values for clinical decision making are essential for the introduction of the test into clinical practice.

Over the past decades, TGA has been shown to provide information on the haemostasis balance in a number of clinical conditions: it can be useful in the diagnosis of certain coagulopathies, in the clinical assessment of breast cancer recurrence, and in the monitoring of certain anticoagulant therapies. In haemophilia, test results can help assess the risk of haemorrhagic complications and monitor treatment with different factor concentrates. Acceptance of the TGA in routine diagnostic/clinical practice may be facilitated by future experience with the diagnostic and prognostic utility of the test in different patient populations.

In the first part of the research on which this dissertation is based, we investigated the prognostic role of TGA in stroke patients. Since TGA can detect both hypo- and hypercoagulable states, we hypothesised that the test would be useful in predicting the outcome of haemorrhagic stroke. In the second half, we investigated whether post-transplant anti-HLA donor-specific antibodies in renal transplant patients were associated with hypercoagulability detected by TGA. We hypothesised that TGA-detected hypercoagulability, if persistent, may contribute to the pathomechanism of antibody-mediated graft rejection.

REVIEW OF LITERATURE

The haemostasis system

Haemostasis is a highly sophisticated physiological defence mechanism against vascular injury. The function of this system is both to contribute to the maintenance of blood fluidity in the vasculature through natural anticoagulant mechanisms and, in the event of endothelial injury, to initiate a coagulation process involving the activation of platelets and coagulation factors, clot formation and thus minimisation of blood loss. Maintaining the delicate haemostatic balance is necessary to ensure that the body responds appropriately to injury while maintaining adequate blood supply to organs and tissues.

In the early 1900s, revolutionary discoveries were made in the study of haemostasis and coagulation. By the late 1950s, all the coagulation factors had been identified. The cascade-like activation system of coagulation was first described in 1964 by MacFarlane and colleagues and by Davie and Ratnoff. The waterfall-like activation process was divided into two stages under *in vitro* conditions: intrinsic and extrinsic pathways, which continued along the common pathway.

The delicate balance of haemostasis is based on the coordinated action of three systems: the humoral system, which involves the process of coagulation through coagulation factors and natural anticoagulant factors; the cellular system, which is based on platelet adhesion, aggregation and secretion; and the vascular system, which activates the extrinsic pathway through vasoconstriction and the release of subendothelial procoagulant substances.

The previously known cascade system theory has now been replaced by a cell-based model of haemostasis, described in 2001. According to this model, the coagulation cascade can be divided into three phases: initiation, amplification and propagation.

In the initiation phase, activated coagulation proteins are produced on cells expressing tissue factor. In the amplification phase, platelets and cofactors are activated, leading to significant thrombin generation. Activated platelets secrete the contents of their granules (e.g. adenosine diphosphate: ADP is released), thereby promoting coagulation and further platelet aggregation. During the propagation phase, large amounts of thrombin and fibrin clot are formed on the surface of activated platelets. Thrombin activates Factor XIII (FXIII), which stabilises the clot by cross-linking fibrin chains and cross-linking α 2-plasmin inhibitor (α 2-PI), a central inhibitor of fibrinolysis, into the clot.

At the onset of blood clotting, von Willebrand factor (VWF) binds to subendothelial matrix proteins to promote rapid platelet adhesion. In the circulation, VWF forms a complex with

factor VIII (FVIII) by non-covalent binding. Through this binding, VWF stabilises the structure of FVIII, prolonging its half-life, and also regulates its activity, preventing activation by activated FX (FXa) and inactivation by activated protein C. VWF is stored in subcellular organelles of endothelial cells, the Weibel-Palade bodies, and is released in response to various activation stimuli. Elevated VWF levels are a sensitive marker of endothelial damage, in addition to other biomarkers (e.g. soluble P-selectin: sPsel, soluble E-selectin: sEsel, soluble VCAM-1, endothelial cell-derived microparticle assay, etc.). It is important to note that both VWF and FVIII are positive acute phase proteins and their levels can increase by up to a quarter in inflammatory processes.

The fibrinolytic system is responsible for the proper breakdown of the formed fibrin clot. The fibrinolytic system is not the subject of this paper and will not be discussed in detail. The central protease in fibrinolysis is plasmin, which is generated from plasminogen by plasminogen activators. Tissue-type plasminogen activator (t-PA) plays the major role in the activation of plasminogen. t-PA is a potent activator and its recombinant form is therefore used as a drug to dissolve thrombi. Fibrinolysis is physiologically a very slow process (taking hours or days), so the use of t-PA is common in fibrinolysis assays (e.g. clot lysis assay).

Compact clots with thinner fibrin fibres break down more slowly than looser clots with thicker fibrin fibres. At high thrombin concentrations, a denser, more branched but thinner fibrin clot is observed, whereas at low thrombin concentrations, a looser clot with thicker fibrin fibres is formed, which is associated with an increased risk of bleeding.

Historical background and principle of the thrombin generation assay

Thrombin is the key clotting enzyme and its generation is the most important step in coagulation. The thrombin generation assay (TGA) is a global test for coagulation that measures the amount of thrombin generated under in vitro conditions in response to a thromboplastin-containing reagent, monitoring the activation kinetics of thrombin generation over a defined period of time.

The first thrombin generation method was developed by Macfarlane and Biggs and published in 1953. The test was further developed in the 1990s by Coenraad Hemker, who first used a chromogenic substrate, and then described a method based on the use of a fluorescent substrate, which is still used today. He and his team then developed the CAT (calibrated automated thrombogram) assay, which allowed continuous monitoring of thrombin activity in a plasma sample in parallel with a calibration measurement. The current form of the TGA was finalised in 2008, when its correct laboratory use and the determination and validation of the various parameters were described.

The classic coagulation screening tests, PT, APTT and TT, are the most widely used tests for coagulation abnormalities worldwide. However, these tests do not reflect the full physiological process of clotting. Screening tests only provide information on the initial steps of coagulation and can only determine the clotting time, during which only a small fraction of the total amount of thrombin produced is generated. The TGA, on the other hand, reveals much more about the coagulation cascade, including the effect of the natural coagulation inhibitor (antithrombin) and its inactivators (protein C, cofactor protein S). Literature suggests that, unlike conventional coagulation screening tests, TGA results detect not only hypocoagulability but also prothrombotic, thrombotic states indicative of hypercoagulability, providing information on the degree of increased thrombotic risk.

CAT assay and TGA determination

The principle of the test is that the addition of thromboplastin to the plasma leads to the formation of thrombin, which can cleave the fluorescent substrate. The cleavage results in a fluorescent product that is readily detectable at specific wavelengths. The intensity and kinetics of the signal generated will be proportional to the thrombin formation, so that the process of thrombin generation can be followed for one hour by detecting the fluorescent signal.

The main parameters of the test are: lag time (minutes), which is the time from the start of the test to the start of thrombin generation; ETP (nM*min) is the endogenous thrombin potential or

area under the curve; peak thrombin (nM) is the maximum thrombin activity; time to peak (minutes) is the time required to reach maximum thrombin generation. Their names are used in English, as there is no generally accepted Hungarian translation.

Compared to previous TGAs, the CAT assay is a modification of the method with the addition of calibration. The calibration means that the colour/turbidity of the plasma (haemolysis, icterus, lipemia) does not affect the determination, making the method suitable for measuring not only platelet-free plasma but also platelet-rich plasma and whole blood. It has the advantage of eliminating the so-called "inner filter effect", correcting for variations due to substrate depletion and eliminating the " α_2 -Macroglobulin effect". The term "inner filter effect" refers to the fact that fluorescent molecules in the sample absorb some of the fluorescent light from the other cleaved molecules, so that the fluorescence intensity will not be proportional to the concentration of fluorescent molecules produced, and may give a result falsely lower than the amount of thrombin actually formed. The elimination of the " α_2 -macroglobulin effect" is another important advantage of the CAT assay. Antithrombin, a natural inhibitor of thrombin, inactivates the active enzyme by binding to the active site of thrombin. In addition to antithrombin, α_2 -macroglobulin can also inhibit thrombin by binding to another binding site (exosite) of thrombin. This renders the thrombin inactive, but it will still be able to cleave the small fluorescent molecules used in the test, resulting in a falsely higher generation of thrombin.

Reagents, application, limitations

TGA is not yet a routine laboratory test, but research shows it to be very promising in indicating hypo- and hypercoagulable states and pre-thrombotic states. There are several limitations to the clinical use of TGA. The test is not yet standardised, and different centres perform it in different ways. It is particularly sensitive to certain pre-analytical factors such as blood collection conditions, blood/citrate ratio, centrifugation, sample storage, etc. Depending on the manufacturer, the concentrations of tissue factor and phospholipids in the reagents marketed may vary. Another limiting factor is that the values obtained using different instruments and reagents are highly variable, so there is no official CLSI guideline reference range for the test parameters. The variation between laboratories could be reduced by standardising both the reagents used and the conditions under which they are used, and the use of reference plasma in future determinations could be a useful aid. Another advantage is that the test is currently time consuming and relatively expensive.

Thrombin generation kits are distributed by Diagnostica Stago (Asnières-sur-Seine, France) and Technoclone (Vienna, Austria). Stago offers its reagents for the Fluoroskan Ascent (FL) fluorimeter, while Technoclone recommends that its product is used on the Ceveron automated system. Recently, automated TGA based on the Diagnostica Stago CAT system (ST Genesia) has become available, eliminating the potential for error from manual measurement and aiming to enter the routine diagnostic market with specialised reagent kits (STG-ThromboScreen, STG-BleedScreen, STG-DrugScreen). TGA manufactured by Siemens can be used on the BCS-XP coagulometer. It should be emphasised that the commercially available thrombin generation reagents also have different tissue factor and phospholipid concentrations depending on the type of test sample (platelet-poor plasma vs. platelet-rich plasma). Platelet-poor plasma has a higher tissue factor and phospholipid content (e.g. Diagnostica Stago PPP reagent tissue factor: 5 pM, phospholipid: 4 μ M), whereas in platelet-rich plasma samples the reagent contains only tissue factor in low concentrations, the phospholipid surface is provided by platelets (e.g. PRP reagent tissue factor: 1 pM, no phospholipid). If the patient is on an anticoagulant (e.g. heparin therapy), a reagent with high tissue factor and phospholipid concentration can be used (e.g. PPP High reagent, tissue factor: 20 pM, phospholipid: 4 μ M), while in patients with haemophilia or other factor deficiencies, a reagent with low tissue factor concentration may be used (PPP Low reagent tissue factor: 1 pM, phospholipid: 4 μ M). The so called MP reagent (no tissue factor, phospholipid: 4 μ M) is recommended to study the coagulation effect of microparticles in PPP. Although the routine diagnostic use of TGA is not yet widespread, the test may be useful for a number of clinical conditions in the future to aid clinical decision-making. Literature data suggest that ETP and peak thrombin parameters, which are the most clinically relevant determinants, are associated with conditions that result in increased thrombotic risk. Studies have shown that TGA may be useful in pathologies with elevated arterial or venous thrombosis risk, e.g. diabetes mellitus, cancer, hereditary thrombophilias (e.g. antithrombin, protein C and protein S deficiency, FV Leiden mutation). The multiple reagents available make it suitable for monitoring the effect of anticoagulant therapy. One of the most promising future applications of the test is to assess the haemorrhagic risk of haemophilia patients and to monitor the therapy used.

Types of stroke, main clinical features of cerebral haemorrhage

A stroke is a rapidly developing group of symptoms caused by impaired blood supply to the brain, which persists for at least 24 hours and has no other cause than a lesion in the cerebral vasculature. The pathomechanism is ischaemic or haemorrhagic, depending on whether the disturbance is caused by an obstruction or haemorrhage. The symptoms depend on the type of stroke and the location and extent of its occurrence. In Hungary, there are 45-50 000 new stroke cases each year, of which about 80% are ischaemic in origin. Ischaemic stroke is caused by the blockage of cerebral blood vessels, which may be due to small or large artery thrombosis, embolism or some haemodynamic disturbance. The remaining 20% of strokes are haemorrhagic: in these case, a cerebral vessel ruptures, leading to bleeding into the surrounding brain tissue, putting pressure on the affected region. The majority of haemorrhagic strokes (about 15% of all strokes) are intracerebral haemorrhage (ICH), the remainder are subarachnoid haemorrhage.

Although the incidence of ICH is lower than that of ischaemic stroke, the mortality rate is much higher: 40% in the first month and 54% in the first year.

The aetiology of ICH is complex. Hypertension is one of the most common causes of ICH, accounting for 30-60% of haemorrhages. Persistently elevated blood pressure can lead to weakening of the walls of small (or less commonly large) blood vessels. Various vascular diseases (involving small and large arteries, veins), arteriovenous malformations and coagulation disorders may also be involved in the background of bleeding, as well as the increasing use of anticoagulant and antiplatelet therapy. It is important to emphasise that the development of ICH is a complex process of mechanisms that may be influenced by several factors simultaneously, but in about 5-20% of cases the underlying cause cannot be clearly identified. Risk factors include diabetes mellitus, smoking, age over 50, alcohol and drug use. Symptoms of ICH can develop suddenly and include headache, dizziness, nausea and vomiting, confusion, weakness, loss of feeling on one side of the body, speech problems, coordination problems, seizures. Most often there is a sudden onset of focal neurological deficit, determined by the site of the haemorrhage and the oedema that has developed. It is often accompanied by a decrease in the patient's level of consciousness, measured by the Glasgow coma scale (GCS). In the first few hours after an intracerebral haemorrhage, a steady deterioration in condition is observed in 15-20% of patients.

As neurological symptoms alone cannot clearly determine whether symptoms are caused by ischaemia or haemorrhage, brain imaging is essential to identify the type of stroke and assess

the extent of damage. Non-contrast CT scanning of the skull remains the gold standard imaging modality for the initial diagnosis of ICH because it is easily accessible and rapid. MRI scans with contrast can also be a useful imaging modality for clinicians to help detect old clots or previous lesions.

Mortality in non-traumatic ICH is higher compared to ischaemic stroke, but therapeutic options are limited and outcome is difficult to predict. Given the unfavourable outcome of ICH, timely medical intervention is key to treatment, as it avoids tragic outcomes, reduces the risk of permanent damage and improves survival. Once the diagnosis is made, the aim is to reduce the risk of haemorrhage and the extent of haematoma within the first 24-72 hours. The type and extent of treatment depends on the cause and location of the haemorrhage and the patient's condition. For ICH, immediate medical intervention within a short time window is of paramount importance, whereas for ischaemic stroke this should be within a 4.5- to 24-hour window from the onset of symptoms, depending on the subtype and the treatment modality. Given the unfavourable outcome of ICH, the emphasis is on conservative treatments such as blood pressure reduction, immobilisation and control of elevated intracranial pressure. Surgical intervention is only considered in certain cases, as its risk and efficacy are controversial, and studies are still ongoing to find less invasive therapies to improve prognosis.

Clinicians can use scales to assess the severity of symptoms and the patient's condition to make a diagnosis. Such neurological assessment scales include the GCS, the ICH score and the NIHSS, which can be determined shortly after a patient's admission to hospital.

The GCS was originally developed to determine the severity of coma or dysfunction following a traumatic brain injury, but it can be useful for any condition that leads to a disturbance of consciousness. Severity is assessed using eye opening, verbal and motor criteria. The GCS score at admission is a strong predictor of long-term outcome.

The ICH score is a widely accepted scale that estimates the risk of mortality by taking into account patients' demographic characteristics, functional clinical status and the results of brain imaging studies. A high ICH score is associated with worse outcome and higher mortality.

The NIHSS (National Institutes of Health Stroke Scale) system is used to assess stroke severity and has 11 components. The questionnaire assesses: state of consciousness, eye movements, facial asymmetry, motor function of arms and legs, speech and language, sensory perception, and independent activity. Scores range from 0 to 42 (for the most severe stroke). Higher scores may indicate a more severe neurological condition, i.e. a worse outcome.

However, status assessment scales alone are not sufficient to assess a patient's prognosis. The outcome of ICH can be highly variable, depending on the area of the brain that is affected.

Unfortunately, about 75% of patients are left with a loss of function that is associated with a loss of working capacity. Long-lasting symptoms often lead to disability and the majority of patients require ongoing care. The standard method for assessing long-term functional clinical outcome after acute stroke is the modified Rankin Scale (mRS).

After an acute stroke, certain complications may occur, such as pneumonia, aspiration, respiratory disturbances, pulmonary embolism and sepsis. Almost half of deaths are associated with complications after a stroke, mainly occurring during the first week in hospital. Patients with complications during hospitalisation have a higher mortality rate. The occurrence of venous thromboembolism (VTE) (deep vein thrombosis: DVT and/or pulmonary embolism) is a relatively common complication, occurring in about 2-7% of stroke patients, with a fourfold higher risk compared to patients with ischaemic stroke. The increased risk may be due to prolonged immobility following a major stroke, older age, infections, or hypercoagulability following haemorrhage, which is still less studied. The initiation of pharmacological prophylaxis in stroke patients may pose a serious dilemma in clinical practice, as there is currently insufficient evidence on the safety of low molecular weight heparin (LMWH) prophylaxis in the first 48 hours after the onset of ICH.

Kidney transplantation and antibody-mediated rejection (ABMR)

Chronic kidney disease

Acute kidney failure occurs when renal function deteriorates over a short period of time (a few hours or days) to the extent that more than 60% of the renal parenchyma is unable to do its job. Chronic kidney disease is characterised by structural and functional damage to the kidneys that persists over a long period of time (at least 3 months). The kidneys are no longer able to excrete toxic waste products into the urine, resulting in increased blood concentrations of toxic waste products, increased creatinine and urea concentrations, decreased glomerular filtration rate (GFR) and decreased urine volume.

Renal failure can be caused by a number of things: infection, diabetes, high blood pressure or even hereditary factors. The most common causes of chronic kidney disease include diabetes (36%), hypertension (30%), glomerulonephritis (12%), cystic kidney disease (3%) and interstitial nephritis (3%). Chronic kidney disease is a major public health problem, affecting 10-16% of the adult population worldwide. The majority of patients have a GFR <60mL/min and 1-2% have severe end-stage renal disease. Even in mild chronic kidney disease, the risk of developing cardiovascular or cerebrovascular disease is significantly increased. This is due to the rapid acceleration of atherosclerosis, which contributes significantly to the increased mortality associated with chronic renal failure. According to the National Kidney Foundation, patients with chronic kidney disease are at the highest risk of cardiovascular disease. Correct assessment of cardiovascular risk and reduction of risk factors is of utmost importance in this patient group, which can significantly reduce the development of complications through increased atherosclerosis. In addition to eGFR, proteinuria, which can be assessed by the albumin/creatinine or total protein/creatinine ratio determined from urine samples, plays an important role in the evolution of renal disease and in the estimation of cardiovascular risk. Timely treatment can help to prevent further progression of the disease and reduce some of the complications, but early detection of the disease is not easy, as the majority of patients are asymptomatic until the onset of end-stage renal disease.

Treatment of chronic renal failure: dialysis, transplantation

The treatment of chronic kidney failure focuses on treating the underlying disease, which may be supplemented by renal replacement therapies to support kidney function. For people with chronic kidney disease, renal replacement therapy (haemodialysis or peritoneal dialysis) or

kidney transplantation may be considered as a solution to replace kidney function. The optimal renal treatment depends on the underlying or comorbid conditions, the patient's general condition, age, available rehabilitation options and the patient's willingness to cooperate. The survival of transplanted patients is generally better than that of dialysis patients, so dialysis is not necessarily required before transplantation. Over the past 50 years, kidney transplantation has become a global practice for the treatment of chronic kidney disease. Today, organ transplantation is not only the most cost-effective treatment for end-stage renal disease, but can also ensure a longer and better quality of life. A transplanted kidney should be seen not only as a detoxifying organ that maintains salt and water balance and produces urine, but also as a hormone-producing organ that regulates haematopoiesis (hydroxylation of vitamin D, production of erythropoietin). The success of a kidney transplant depends on several factors. The operation usually lasts several hours and involves implanting the new kidney in the pelvic groove on the left or right side of the lower abdomen without removing the non-functioning kidney. The vessels of the new kidney are connected to the circulatory system and then the kidney is connected to the ureter and bladder. Transplantation may be done more than once in a single patient, depending on how long the previously transplanted kidney has been working properly. There are two types of organ that can be transplanted: from a brain-dead/deceased donor and from a living donor. However, the number of cadaveric kidneys is limited, so unfortunately the number of people waiting for a kidney is increasing and so is the waiting time. The demand for transplantable organs is increasing, with more and more patients requiring dialysis. According to the National Blood Service, the average waiting time for patients on the kidney transplant waiting list is currently 3.5 years. Living donor kidney transplantation is available in Hungary, but is much less common than deceased donor transplantation. Living donor kidney transplantation has several advantages (no waiting time, predictable surgery, possibility of transplantation before end stage, short cold ischaemia time) and its functional life is much longer than that of cadaveric kidneys. In the case of a living donor, the kidney is most often donated by a close relative, resulting in better histocompatibility.

A healthy kidney, or any other organ, can be transplanted if the recipient's blood group and immunological type are the best match for the donor (transplantation histocompatibility). Human leukocyte antigen (HLA) typing in transplantation is the testing of the immunological compatibility of the donor and the recipient, i.e. the search for a suitable donor organ for transplantation. The test involves the HLA typing of patients and potential donors and the detection of anti-HLA antibodies (donor specific antibodies: DSAs) in patients. If the recipient has had a previous transfusion, a previous kidney or other organ transplant, or is pregnant, the

likelihood of DSAs is higher. The higher the antigen match between the donor and the recipient, the greater the chance that the transplanted organ will successfully engraft and not be rejected. To prevent rejection, patients should receive combined immunosuppressive treatment (see later).

HLAs are special proteins the gene of which is located in the major histocompatibility complex (MHC) region of chromosome 6. The MHC system helps the body to distinguish foreign cells and tissues from its own, as cells recognised as foreign can trigger an immune response. The allogeneic response mediated by MHC molecules is the body's basic immunological defence mechanism based on the antigen recognition function of T lymphocytes. Their alloimmune response can lead to rejection of the transplanted cell/tissue (host-versus-graft reaction, rejection). HLA class I includes HLA-A, -B and -C genes, which are found on the surface of all nucleated cells and on platelets, while HLA class II includes HLA-DR, -DQ, -DP genes, which are found on antigen-presenting cells. In the case where the recipient produces antibodies that react with the HLA antigens of the donor, this results in an increased risk of rejection of the transplanted organ, a phenomenon known as antibody-mediated rejection (ABMR).

Donor-specific antibodies and antibody-mediated rejection

ABMR is one of the complications affecting long-term graft survival. ABMR is currently one of the major challenges in organ transplantation and is a common cause of graft loss and return to dialysis treatment in kidney transplant patients. The pathophysiology of ABMR is not yet fully understood, but a key role in its development is likely to be played by DSAs directed against HLA expressed on the endothelium of the graft and their presence greatly increases the likelihood of ABMR development. However, the presence of DSAs does not always indicate rejection, as 30-60% of kidney transplant recipients can have detectable levels of these antibodies without other clinical signs of rejection.

A central element in the pathophysiology of ABMR is the binding of anti-donor antibodies to graft endothelial cells, causing complement activation, inflammatory response, platelet activation, thereby activation of the coagulation cascade, and tissue damage.

Although DSAs are known to cause tissue damage in the graft endothelium, little is known about their effects on haemostasis. We can speculate that endothelial damage may involve activation of coagulation, but the number of studies on this is very limited. Circulatory disturbances at the microvasculature level in the context of ABMR lead to reduced renal function and further damage, ultimately leading to rejection.

Clear physical symptoms of rejection due to ABMR may not be evident in the early stages, but small changes in general well-being, such as unexplained fever or general weakness or pain in the graft area, hypertension, abnormalities in some laboratory parameters (elevated serum creatinine), reduced function of the transplanted organ: decreased urine output, fluid retention are worrying signs.

The diagnosis of ABMR is established by laboratory tests (DSA level measurement) and the Banff classification gold standard histopathology (renal biopsy) in addition to clinical symptoms. Today, the most widely used and sensitive method for the detection of DSAs is the so-called "single antigen beads" Luminex-SAB technique, which uses almost 200 HLA antigens bound to fluorescent beads to determine the alloantibody pool of an individual. As ABMR symptoms are "vague", it can only be clearly confirmed by histological examination. The purpose of a kidney biopsy is to assess the condition of the kidney, to check for rejection or other possible problems. However, the timing of the biopsy is a key issue, since despite its invasiveness, it cannot be replaced by any other laboratory method and cannot be repeated indefinitely, but if the test is performed too early, no abnormalities are confirmed, and if the sample is taken too late, irreversible lesions may be seen. According to the Banff classification, DSA serological testing against HLA (or other antigens) is required for the diagnosis of both acute and chronic ABMR, and C4d deposition in peritubular capillaries is also a specific feature (discussion of the categories of the Banff classification, the advantages and disadvantages of this classification are beyond the scope of this dissertation).

Several combinations of immunosuppressive treatments for renal rejection are known, and their individualised use is influenced by several factors (e.g. induction or maintenance treatment, disease aetiology, graft status). Immunosuppressive treatment is always personalised, and each centre will sub-categorise treatment combinations according to its own experience. A fundamental disadvantage of immunosuppressive therapy is that it can make patients more susceptible to various infections. The most commonly used maintenance immunosuppressive therapy is a triple combination of calcineurin inhibitors (e.g. tacrolimus, everolimus), corticosteroids and antiproliferative agents (e.g. mycophenolic acid: MPA). The treatment of ABMR is complex and wide-ranging, and in addition to modification of immunosuppressive therapy (alternative treatment and/or dose escalation), it may include plasmapheresis, intravenous immunoglobulin (IVIG) and even possible retransplantation.

The aim of patient follow-up is to monitor the improvement of the condition and to detect possible complications early, to determine the concentration of the drugs and thus to adjust the dose if necessary. Currently, there is no known laboratory parameter with a high predictive

value for the risk of ABMR. Despite the continuous development of new therapeutic approaches and transplantation procedures, ABMR remains a challenge in renal transplantation and intensive research is still ongoing to better elucidate the pathomechanism, improve early diagnosis and treatment.

AIMS

The aim of our study was to determine whether TGT parameters measured at admission in patients with ICH differ from those of healthy controls and whether they correlate with hematoma size and progression. We also assessed the potential of TGT as a predictive tool for short- and long-term outcomes in intracerebral hemorrhage.

In kidney transplant patients, we aimed to evaluate whether the presence of DSA is associated with endothelial damage and the development of a procoagulant state, and whether hypercoagulability can be detected using TGT. Furthermore, we analyzed whether TGT parameters correlate with the histologically confirmed presence of microvascular obstruction in DSA+ patients, thereby suggesting the prognostic value of TGT in the early detection of ABMR.

PATIENTS AND METHODS

Examination of patients with non-traumatic intracerebral haemorrhagic stroke

Our prospective, observational case-control study included consecutive patients with non-traumatic, CT-confirmed acute intracerebral haemorrhagic stroke in collaboration with the Neurology Stroke Center of the University of Debrecen. Patient recruitment started in June 2017 and was completed in September 2020. Inclusion criteria were: adult patients, CT-confirmed non-traumatic ICH, written informed consent (patient or relative). Exclusion criteria included cerebral aneurysm, arteriovenous malformation, traumatic intracerebral haemorrhage (epidural or subdural haemorrhage, cerebral contusion), malignancy, haemorrhaged cerebral metastases, severe liver and renal disease, known haemorrhagic diathesis and SARS-CoV-2 infection at hospital admission or follow-up. Anticoagulant therapy was also included as an exclusion criterion because previous studies have shown that all anticoagulants affect thrombin generation.

The time of onset of symptoms, basic anthropometric data (age, sex, weight, height), cerebrovascular risk factors, history of cerebrovascular and cardiovascular disease, and the presence of previous medication were recorded by clinical collaborators. The GCS score was used to assess level of consciousness. The NIHSS scoring system was used to assess stroke severity, which was assessed at admission and on day 7 after the event. Haematoma volume was estimated on non-contrast native CT scan at admission, and 14 days and 3 months after the event. The ICH score was used to estimate the risk of mortality. Patients were followed up for 3 months and mRS was also determined at admission (acute stage), on day 14 and day 90 to assess long-term outcome.

The study investigated the following outcomes: 1) long-term outcome at day 90 after the event (mRS 0-1 was considered a favourable outcome, while mRS 2-6 was considered unfavourable); 2) mortality at day 14 and 90.

As reference limits for TGA are not known, patients' results were compared with those of healthy controls. Healthy adult individuals who gave written informed consent were selected as controls. In their case, the exclusion criteria were: acute illness within the last 4 weeks, previous thrombosis, surgical intervention within the last 3 months, use of anticoagulants, malignancy, known bleeding disorder, autoimmune disease, chronic kidney or liver disease. Hypertension was allowed during inclusion.

Examination of kidney transplant recipients

In the observational case-control study, kidney transplant recipients were included in collaboration with the Division of Transplantation of the Department of Surgery of the University of Debrecen. Patient recruitment took place between 2019 and 2021. Inclusion criteria were age over 18 years, cadaver or living donor kidney transplantation and written informed consent. Individuals with a history of bacterial, fungal or viral infection, known haemorrhagic diathesis, anticoagulant treatment, or SARS-CoV-2 infection were excluded from the study. Retransplantation was not an exclusion criterion. All patients underwent SARS-CoV-2 antigen and/or PCR testing at inclusion and during follow-up. In case of a positive test result, the individual was excluded from the study.

The presence of DSAs in all patients was determined at the time of enrolment and at follow-up (see later), and patients were classified into case (DSA+) or control (DSA-) groups based on the results.

The patients' demographic and clinic data were recorded by clinical collaborators (age, sex, weight, height, date of transplant, medications including immunosuppressive treatment, cardiovascular risk factors, smoking, diabetes, etc.).

Patients were followed up and clinical data were recorded again after 6 months. For patients who underwent renal biopsy within 1 year of study inclusion, results were recorded according to the Banff classification and compared with TGA parameters and other haemostasis parameters studied.

Sample collection and laboratory measurements

Blood sampling

Native and anticoagulated (with 0.109M sodium citrate) peripheral blood samples were collected from the individuals included in our studies (Becton Dickinson, Franklin Lane, NJ). For ICH patients, blood was collected at hospital admission, prior to the start of treatment. Fasting samples were collected from healthy controls. For renal transplant patients, blood sampling and urinalysis were performed during outpatient clinic visits and 6 months after inclusion.

For all blood samples, routine laboratory tests such as electrolytes, glucose, liver and kidney function (serum creatinine, eGFR) tests, high sensitivity C-reactive protein (hsCRP) tests, and blood count were performed by standard laboratory methods at the Institute of Laboratory

Medicine, University of Debrecen (Roche Diagnostics, Mannheim, Germany and Sysmex Europe GmbH, Hamburg, Germany). In addition to blood tests, proteinuria and protein/creatinine ratio of the first urine sample in the morning were determined by routine laboratory methods to assess graft function in renal patients. In renal transplant patients, tacrolimus concentrations were determined from whole blood using a conventional immunochemical method (Abott Architect, Tacrolimus assay, Chicago, IL). Basic coagulation tests (PT, APTT, TT) and Clauss fibrinogen levels were determined on a BCS coagulometer for all patient groups using standard laboratory methods (Siemens Healthcare Diagnostic Products, Marburg, Germany) according to the manufacturer's instructions. Platelet-poor plasma (PPP) was obtained from the citrate-anticoagulated blood samples (centrifugation 1500 g, 20 min, room temperature) for further specific analyses. Previous preliminary experiments at our institute have demonstrated that plasma processed under these conditions will certainly be platelet and microparticle free. The resulting PPP was stored in aliquots at -80 °C until specific haemostasis studies were performed.

TGA, FVIII activity, VWF antigen levels were determined for both cohorts serving as the basis of the dissertation. In addition, for the ICH cohort, D-dimer levels were determined. In kidney transplant recipients, additional specific tests were determined: *in vitro* clot lysis test (CLA), sPsel and sEsel levels, complement levels, DSA presence, tacrolimus level measurement, histopathological examination of kidney biopsies. Patients recruited into the renal transplant cohort were followed up and after 6 months the above mentioned specific tests were repeated again.

The thrombin generation assay

The Thrombinoscope CAT test was used (Diagnostica Stago, Asnières-sur-Seine, France) following the manufacturer's instructions. Briefly, 20 µL of PPP-ReagentTM (containing 5 pM recombinant tissue factor and 4 µM phospholipid) was added to 80 µL of plasma and the mixture was incubated for 10 min in a U-shaped 96-well black polypropylene microplate (Greiner Bio-One). For all samples, our own calibrator (Thrombin CalibratorTM) was used to correct for fluorescence signal due to substrate depletion and to correct for plasma colour variability. To start thrombin generation, 20 µL of Flu-Ca-KitTM reagent (containing fluorescent substrate and Fluo-BufferTM containing CaCl₂) was used as a starting reagent, and duplicate determinations were performed for each sample. Measurements were performed on a Fluoroscanner Ascent FL fluorimeter (ThermoFisher Scientific, Waltham, MA, USA), TGA

parameters were determined using Thrombinoscope software (Diagnostica Stago). The following TGA parameters were used to evaluate the results: lag time (min), ETP (nM*min), peak thrombin (nM), time to peak (min).

Measurement of FVIII activity

FVIII activity was determined using a chromogenic method (Factor VIII Chromogenic kit, Siemens Healthcare Diagnostics, Marburg, Germany), which involves activation of FVIII in plasma by thrombin, followed by active FVIII catalyzing FX activation in the presence of activated FIX (FIXa), phospholipids and Ca^{2+} . FXa cleaves the specific substrate containing p-nitroanilide in the reagent. The initial rate of p-nitroaniline release, detected photometrically at 405 nm, is proportional to the activity of FX, and thus to the FVIII activity of the sample. The measurement was performed on a BCS coagulometer (Siemens Healthcare Diagnostic Products, Marburg, Germany). The reference range of FVIII activity is 60-168 %.

Measurement of VWF antigen level

The VWF antigen level was determined by immunoturbidimetric method (VWF Ag kit, Siemens Healthcare Diagnostics, Marburg, Germany). The VWF content of the plasma sample binds to specific anti-VWF antibodies in the reagent and induces aggregation of polystyrene particles, which leads to a change in the turbidity of the sample. The determination was performed on a BCS coagulometer. The reference range of VWF antigen level is 50-160 %.

D-dimer level measurement

D-dimer levels were quantified by immunoturbidimetry using monoclonal antibodies against D-dimer (Innovance® D-dimer), measured on a BCS coagulometer following the manufacturer's instructions (Siemens Healthcare Diagnostic Products, Marburg, Germany). The diagnostic limit for D-dimer is <0.5 mg/L.

***In vitro* clot lysis assay**

The assay conditions we used for *in vitro* clot lysis were based on methodologies from previous publications. The assay involved the formation of clots from plasma samples using recombinant tissue factor and phospholipid on a 96-well microplate, followed by the addition of tissue-type

plasminogen activator (rt-PA) to induce lysis, which was monitored in real time by turbidimetry. Each sample was analyzed by four parallel measurements. The plasma samples were thawed in a 37 °C water bath for 10 min before the experiment was performed. Recombinant human tissue factor and a 1000-fold dilution of phospholipid-containing reagent (Dade®Innovin®, Siemens, Marburg, Germany) and 100 ng/mL rt-PA (Alteplase, Boehringer Ingelheim, Ingelheim, Germany) were added to each sample in HEPES buffer (10 mM HEPES, 150 mM NaCl, 0.05% Tween 20, pH 7.4). Coagulation of the samples, and thus the coagulum lysis measurement itself, was initiated with HEPES buffer containing CaCl₂ injected by the device. Absorbance was detected at 37 °C, 340 nm using a TECAN Infinite m200 microtitre plate reader (TECAN Trading AG, Männedorf, Switzerland). The curves were evaluated using the online software Shiny App ClotlysisCL, with which the following parameters were calculated: maximum absorbance (OD), time to reach maximum absorbance (min), clot lysis times (CLT) determined at different time points: 10%CLT (min), 50%CLT (min), 90%CLT (min), and area under the curve (CLA AUC). The calculation of clot lysis times at different time points was based on the time points corresponding to 10%, 50% or 90% of the maximum turbidity, and in each case the time points between the respective 10%, 50% or 90% absorbance decrease time points after reaching the maximum turbidity.

Measurement of soluble P selectin and E selectin levels

Levels of sPsel and sEsel were determined by sandwich ELISA (Quantikine® ELISA, Bio-Techne, Abingdon, UK). sPsel reference limit: 37.9 ng/mL, sEsel reference range: 13.5-51.3 ng/mL.

Measurement of complement levels

Complement C3 and C4 levels were determined by nephelometry (Siemens BNII nephelometer, Munich, Germany) using Siemens AG reagents (Antiserum to Human C3c, C4) according to the manufacturer's instructions. The reference range of complement C3 is 0.9-1.8 g/L, the reference range of C4 is 0.1-0.4 g/L.

Measurement of donor specific antibodies

DSAs were detected by the Luminex® Single Antigen Bead assay (One Lambda Inc., Los Angeles, Canada) using a highly sensitive flow cytometric method as previously described. The

SAB technique consists of coating fluorophore impregnated beads with HLA class I (HLA-A, -B, -C) or class II (HLA-DR, -DQ, -DP) recombinant antigen. If the serum contains specific antibodies against HLA, they bind to the corresponding bead. The binding is detected by a specific secondary antibody. The fluorescent signals from each bead are detected by the Luminex system, which gives the results as mean fluorescence intensity (MFI). The threshold for DSA positivity is 500 MFI, at which point the patient's serum is shown to contain donor-specific anti-HLA antibodies.

Histopathological examinations

Of the selected individuals who were eligible and consented for the study, a kidney biopsy was performed and evaluated at one-year follow-up. Samples were pre-examined by hematoxylin and eosin (HE) staining, periodic acid-Schiff (PAS) and Masson's trichrome staining by light microscopy, and immunofluorescence (IgG, IgM, IgA, C3c, anti-fibrinogen, anti-collagen antibodies). Histological lesions were evaluated semi-quantitatively according to the Banff classification. The presence of microvascular obstruction was assessed by HE staining.

Ethics approval

Both studies were designed with the Declaration of Helsinki in mind. The studies were also approved by the University of Debrecen's Regional and Institutional Ethics Committee and the Scientific and Research Ethics Committee of the Health Science Council. Patients or their relatives and healthy volunteers were informed about their specific study and gave voluntary written informed consent to participate in it. For the ICH study, the ethics approval number is DE RKEB/IKEB 5349-2019, 16343-5/2017/ECIG, for the kidney transplant study, the ethics approval number is UD RKEB/IKEB H.5228-2019, H.0282-2021.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.0 software.

Data normality was tested using the Shapiro-Wilk test. If the data showed a normal distribution, the values were expressed as mean $\pm$ standard deviation (SD), in the case of a non-parametric distribution, the data were expressed as median and interquartile range (IQR).

Depending on normality, Student's t-test or Mann-Whitney U-test was used to compare two groups, paired t-test or Wilcoxon paired test was used for paired data. Categorical variables were compared using Fisher's exact test or χ^2 test, depending on the number of cases, using contingency tables. Pearson's or Spearman's correlation analysis was used to test the strength of the correlation between different continuous variables. ROC (receiver operating characteristics) analysis was performed to characterize the diagnostic performance of TGA. The optimal threshold of the test parameters was calculated using Youden's J index. Sensitivity, specificity, positive and negative predictive values were calculated using contingency tables and χ^2 test. For the ICH cohort, binary backward logistic regression models were used to examine whether each haemostasis factor was an independent predictor of specific outcomes (mortality and long-term functional outcome). The model included variables with significant differences based on univariate statistical analyses (Mann-Whitney U test, Fisher exact test, χ^2 test) and factors influencing outcomes based on literature data. The results of logistic regression were expressed as odds ratios (OR) and were characterized by 95% confidence intervals (CI). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Results of patients with non-traumatic intracerebral haemorrhagic stroke

Basic clinical data

In our first prospective, observational case-control study, we examined 87 consecutive patients with non-traumatic acute intracerebral haemorrhagic stroke and 164 healthy controls. One patient was diagnosed with SARS-CoV-2 infection on day 25 after the event and was excluded from the study. The presumptive cause of ICH in almost all patients was hypertension, as other major causes of ICH (as listed previously in the *Patients and methods* section) were excluded based on the exclusion criteria.

The average age of ICH patients was 67 ± 12 years, and 63% were male. The average age of healthy controls was 53 ± 9 years, and 34% were male. As expected, the most common cerebrovascular risk factor among patients was hypertension (97%). Among routine laboratory parameters, glucose, hsCRP, fibrinogen levels, white blood cell count and platelet count were significantly higher in ICH patients compared to controls (glucose median: 7.3; IQR: 5.8-9.8 mmol/L vs. 4.9; IQR: 4.6-6.0 mmol/L; $p < 0.0001$; CRP median: 2.5; IQR: 1.1-6.2 mg/L vs. 1.8; IQR: 0.8-3.4 mg/L; $p = 0.0031$; fibrinogen median: 3.8; IQR: 3.1-4.5 g/L vs. 2.4; IQR: 1.9-3.2 g/L; $p < 0.0001$; white blood cell count median: 8.5; IQR: 6.7-11.4 G/L vs. 6.9; IQR: 5.8-8.1 G/L; $p < 0.0001$; platelet count median: 226; IQR: 180-272 G/L vs. 249; IQR: 215-298 G/L; $p = 0.0039$). PT and APTT were significantly shorter in patients compared to the healthy control population (median PT: 8.1; IQR: 7.8-8.6 sec vs. 9.2; IQR: 8.3-9.9 sec; $p < 0.0001$; APTT median: 26.7; IQR: 24.6-29.2 sec vs. 28.4; IQR: 28.0-28.6 sec; $p = 0.0002$). D-dimer levels and FVIII activity were significantly higher in patients than in controls (median D-dimer: 0.63; IQR: 0.38-1.00 mg/L vs. 0.28; IQR: 0.18-0.38 mg/L; $p < 0.0001$; FVIII activity median: 228; IQR: 153-289% vs. 97; IQR: 83-123%; $p < 0.0001$).

Stroke characteristics and outcomes in the ICH cohort

The median NIHSS score on admission was 13 (IQR: 6-19). Mortality was 23% at day 14 and 41% at day 90 after the event. Looking at the mRS scores, only 20% of patients had a favourable outcome (mRS 0-1) for long-term functional outcome at the end of month 3. The median volume of haemorrhage at admission was 12.0 (IQR: 3.5-29.8) cm³. The median ICH score was 0 (IQR: 0-2). After the event, most patients received antihypertensive (89%), antacids and/or parenteral acid-suppressive therapy (94%). LMWH prophylaxis was used in 26 high-risk

patients (30%); 3 months after the event, all of these patients had at least mild residual symptoms (mRS 2-5) or had died (mRS 6). No antiplatelet agents were used during follow-up in the selected individuals. During the 90-day follow-up, four thrombotic events occurred in the study cohort: 1 deep vein thrombosis and 3 pulmonary embolisms.

Thrombin generation in patients and controls

When TGA parameters were evaluated in ICH patients and healthy controls, we found that peak thrombin was significantly higher in patients compared to controls (397.2 ± 93.9 nM vs 306.3 ± 85.3 nM; $p < 0.0001$). The time to peak parameter was significantly shorter in patients (median in patients: 5.5; IQR: 5.0-6.0 min vs. median in controls: 6.5; IQR: 5.6-7.8 min; $p < 0.0001$). Lag time and ETP did not differ between the two groups.

Lag time, ETP and time to peak parameters showed weak significant positive correlation with CRP in ICH patients and healthy controls (ETP: patients: $r = 0.2473$; 95% CI: 0.0323-0.4404; $p = 0.0209$ and controls: $r = 0.3610$; 95% CI: 0.2091-0.4960; $p < 0.0001$). Similar significant positive correlation was observed between TGA parameters and fibrinogen levels in patients (ETP: $r = 0.3520$; 95% CI: 0.1398-0.5333; $p = 0.0012$). In ICH patients, peak thrombin showed a weak significant positive correlation with white blood cell count ($r = 0.2385$; 95% CI: 0.0293-0.4277; $p = 0.0261$) and a significant negative correlation with APTT was observed ($r = -0.4277$; 95% CI: -0.5856-0.2385; $p < 0.0001$). D-dimer and FVIII activity did not show significant correlation between some TGA parameters measured in patients and controls.

Assessing of stroke severity and outcomes

The severity of stroke and short-term functional outcome showed no significant association with TGA parameters. In terms of long-term functional outcome, unfavourable outcome (mRS 2-6) was associated with significantly higher BMI and D-dimer level, higher NIHSS at admission, higher intracerebral bleeding volume (all three time points), and higher ICH score (BMI: mRS 0-1 median: 24.04; IQR: 21.78-28.25 kg/m² vs. mRS 2-6 median: 27.31; IQR: 24.93-31.60 kg/m²; $p = 0.0090$; D-dimer: mRS 0-1 median: 0.36; IQR: 0.28-0.58 mg/L vs. mRS 2-6 median: 0.72; IQR: 0.48-1.30 mg/L; $p = 0.0013$; NIHSS: mRS 0-1 median: 4; IQR: 4-6 vs. mRS 2-6 median: 16; IQR: 11-21; $p < 0.0001$; bleeding volume at admission: mRS 0-1 median: 2.3; IQR: 1.4-6.2 cm³ vs. mRS 2-6 median: 17.0; IQR: 6.5-37.1 cm³; $p < 0.0001$; Day 14 hemorrhage volume: mRS 0-1 median: 3.1; IQR: 1.4-4.8 cm³ vs. mRS 2-6 median: 19.0; IQR:

3.9-29.5 cm³; $p=0.0008$; Day 90 hemorrhage volume: mRS 0-1 median: 0; IQR: 0 cm³ vs. mRS 2-6 median: 1.0; IQR: 0-6.5 cm³; $p=0.0120$; ICH score: mRS 0-1 median: 0; IQR: 0-1 vs. mRS 2-6 median: 2; IQR: 1-3; $p=0.0005$). In patients with worse long-term outcome, the peak thrombin parameter was significantly elevated compared to patients with favourable long-term functional outcome (mRS 0-1) (mRS 2-6 median: 402.5 (IQR: 344.8-473.8) vs. mRS 0-1: 326.4 (294.2-416.1) nM, $p=0.0096$).

To investigate the prognostic value of TGA, ROC analysis was performed to determine the diagnostic efficiency of the peak thrombin parameter and its optimal threshold. Based on a statistically optimal peak thrombin threshold (339.1 nM) for long-term functional outcome, the sensitivity of this parameter was 80.8% and its specificity 64.7% for determining mRS 2-6 outcomes. Using this threshold, unfavourable outcome could be excluded with a high negative predictive value (NPV: 0.902; 95% CI: 0.802-0.954; PPV: 0.458; 95% CI: 0.279-0.649). In a binary regression model including age, sex, BMI, smoking habit, NIHSS at admission, D-dimer (>0.5 mg/L) and peak thrombin parameter (>339.1 nM), only NIHSS and peak thrombin remained in the model as independent predictors of adverse functional outcome (OR: 1.458; 95% CI: 1.181-1.801; $p<0.001$ and OR: 48.292; 95% CI: 4.667-499.720; $p=0.001$).

Association between TGA parameters and intracerebral haemorrhage volume

In patients with ICH, lag time and time to peak parameters showed a weak significant negative correlation with the amount of intracerebral haemorrhage on admission ($r=-0.2603$; 95% CI: -0.4642 – -0.0303; $p=0.0231$ and $r=-0.3698$; 95% CI: -0.5542 – -0.1509; $p=0.0010$). During patient follow-up, estimated bleeding volume at day 90 showed a significant positive correlation with ETP and peak thrombin parameters ($r=0.3838$; 95% CI: 0.0161-0.6600; $p=0.0363$ and $r=0.5383$; 95% CI: 0.2103-0.7574; $p=0.0021$, respectively). Although the primary objective of the study was not to investigate the association between possible VTE events and TGA parameters, it should be noted that the peak thrombin parameter measured at admission was above the calculated threshold in all patients who had a thrombotic event during follow-up. Mortality was not associated with TGA parameters at any of the time points studied.

Association between FVIII activity and D-dimer levels and intracerebral haemorrhage volume

FVIII activity showed no correlation with haemorrhage volume, while D-dimer levels showed significant correlation only with the estimated volume of intracerebral haemorrhage on day 14

($r=0.3504$; 95%CI: 0.0860-0.5689; $p=0.0087$). Mortality did not show any correlation with FVIII and D-dimer parameters at any time point.

Results of the examination of kidney transplant patients

Basic clinical data

The study included 43 kidney transplant recipients. Twenty-seven DSA+ cases and 16 DSA– controls formed the study groups. Examining the clinical parameters defined at inclusion, the two groups did not differ significantly in terms of basic demographic data, routine laboratory parameters and immunosuppressive treatment used. The mean age was 45.7 ± 10.9 years in the DSA– group and 46.4 ± 12.6 years in the DSA+ group. The mean time since transplantation was 19 ± 12 months in the DSA– group and 27 ± 19 months in the DSA+ group. The majority (82%) of DSA+ patients had anti-HLA class II antibodies. Hypertension was the most common cardiovascular risk factor among the patients studied (81%). Graft function (e.g. creatinine level, eGFR, urinary protein/creatinine ratio) did not differ significantly between the two groups. The immunosuppressive therapy used in the majority of patients (30/43; 70%) was a combination of tacrolimus, glucocorticoid and MPA.

Soluble markers of endothelial damage, TGA and CLA in DSA+ and DSA– kidney transplant recipients

Fibrinogen levels measured at the first sampling were significantly higher in the DSA+ group compared to the DSA– group (median: 3.6; IQR: 3.4-4.3 and 3.1; IQR: 2.7-3.5 g/L; $p=0.0081$). VWF antigen and sPsel levels were above the upper limit of the reference range in the majority of patients, but there was no significant difference between groups. VWF antigen levels showed a significant correlation with sPsel levels ($r=0.3788$; 95% CI: 0.0794-0.6155; $p=0.0122$). FVIII activity and sEsel levels did not differ between study groups. Among the TGA parameters determined from the first samples, ETP was significantly higher in the DSA+ group compared to DSA– patients (median: 1666; IQR: 1438-2012 and 1230; IQR: 1097-1659 nM*min; $p=0.0019$). The peak thrombin parameter, similar to ETP, was also significantly higher in DSA+ patients (median: 377.4; IQR: 342.9-404.5 and 328.9; IQR: 256.5-386.3 nM; $p=0.0203$), while there was no significant difference between groups for TGA time parameters. CLA parameters, including 50%CLT, did not differ between DSA– and DSA+ groups. At the first blood sampling, C3 and C4 complement levels did not differ between the groups studied. According to our results, C3 hypocomplementemia was not dependent on the presence of DSAs (DSA+ group: 4/27; 15% vs. DSA– group: 3/16; 19%; $p=0.9999$). The type of graft, i.e. whether it was from a living or cadaver donor, or the time until the development of DSAs had no effect on the parameters we tested.

Association between TGA parameters, DSA positivity and tacrolimus levels

In our study, ETP showed a significant positive correlation with the level of anti-HLA-II class DSAs (expressed as MFI) at the first sampling ($r=0.5494$; 95%CI: 0.1534-0.7934; $p=0.0081$). Of all other laboratory parameters tested, only haemoglobin concentration showed a significant correlation with the degree of DSA positivity at inclusion ($r= -0.4714$; 95% CI: -0.7508 – -0.0488; $p=0.0081$). TGA lag time ($r= -0.3667$; 95% CI: -0.6101 – -0.0645; $p=0.0192$), ETP ($r= -0.3909$; 95% CI: -0.6262 – -0.0904; $p=0.0128$) and peak thrombin parameters ($r= -0.3123$; 95% CI: -0.5685 – -0.0008; $p=0.0498$), as well as VWF antigen ($r= -0.3748$; 95% CI: -0.6144 – -0.0713; $p=0.0173$) and C4 complement levels ($r= -0.4247$; 95% CI: -0.6502 – -0.1304; $p=0.0063$) showed significant negative correlation with tacrolimus levels in the cohort studied.

Soluble markers of endothelial damage, TGA and CLA parameters during follow-up

Six months after enrolment, *de novo* DSAs appeared in 3 DSA– patients, while 5 DSA+ patients, the MFI values of anti-HLA antibodies fell below the threshold value (500 MFI). One patient dropped out of the second phase of the study due to participation in an interventional study. Accordingly, 18 DSA– and 24 DSA+ patients were studied 6 months later. Patients in the DSA+ group had significantly higher BMI compared to the DSA– group (mean: 25.1 ± 4.2 vs. 28.6 ± 5.5 ; $p=0.0302$). Time since transplantation was significantly longer in patients with DSA (in DSA+ patients: mean: 34.6 ± 16.9 vs. in DSA– patients mean: 23.9 ± 11.7 ; $p=0.0481$). Albumin levels were significantly lower in DSA+ patients (mean: 47 ± 3 vs. 44 ± 4 ; $p=0.0237$), while CRP was significantly higher compared to the DSA– group (median: 1.4; IQR: 0.6-2.2 and 3.1; IQR: 0.9-7.7; $p=0.0266$). Laboratory parameters reflecting graft function did not differ significantly between the two groups during follow-up either.

During follow-up, fibrinogen, VWF antigen levels, FVIII activity, sPsel and sEsel levels did not differ between groups. Similar to the results of the first blood sampling, VWF antigen levels were above the upper limit of the reference range in the majority of patients. ETP was again significantly elevated in the DSA+ group compared to the DSA– group 6 months after enrolment (median: 1701; IQR: 1382-1940 and 1458; 1221-1660; $p=0.0381$). During follow-up, the time parameters of peak thrombin and TGA (lag time and time to peak), similar to the first blood sampling, showed no difference between study groups. Consistent with the results of the first blood collection, 50%CLT and C3 and C4 levels at the second blood draw showed

no difference between DSA– and DSA+ patients. The incidence of C3 hypocomplementemia did not change during follow-up.

The *de novo* appearance of DSAs or decreased DSA positivity during follow-up in some patients did not show a significant correlation with changes in VWF antigen levels. It should be noted, however, that in patients who did not develop DSAs during follow-up (43%), VWF antigen levels were below the upper limit of the reference range. In contrast, only 29% of DSA+ patients had VWF antigen levels within the reference range. All three individuals with *de novo* DSA positivity had follow-up VWF antigen levels above the upper limit of the reference range, while VWF antigen levels did not show significant changes in patients with DSA positivity below the reference range. Interestingly, FVIII activity increased significantly in DSA– patients during follow-up compared to the first blood draw ($p=0.0200$) and a similar but non-significant trend was observed in DSA+ patients ($p=0.0938$). In both subgroups, 4 patients had FVIII activity levels above the upper limit of the reference range at 6 months after enrolment. In all three *de novo* DSA+ individuals, FVIII activity increased during follow-up, but no clear trend in the change in FVIII levels during follow-up was observed with decreasing DSA levels. The ETP parameter did not show significant changes in the results of the 6-month follow-up samples in any of the subgroups studied. Peak thrombin showed parallel results with ETP in each subgroup, with no significant change in peak thrombin values between the first and second sampling.

Association between TGA parameters, DSA positivity and tacrolimus levels during follow-up

In DSA+ patients, similar to the first sampling results, 6 months later, thrombin generation rates (ETP and peak thrombin) showed a significant positive correlation with DSA positivity (ETP: $r=0.5474$; 95% CI: 0.1097-0.0873; $p=0.0153$; peak thrombin: $r=0.6404$; 95% CI: 0.2489-0.8520; $p=0.0031$). Peak thrombin ($r= -0.3191$; 95% CI: -0.5770 – -0.0032; $p=0.0420$), FVIII activity ($r= -0.4034$; 95% CI: -0.6408 – -0.0957; $p=0.0098$) and fibrinogen levels ($r= -0.3255$; 95% CI: -0.5799 – -0.0011; $p=0.0448$) showed significant negative association with tacrolimus levels during follow-up. However, the degree of DSA positivity did not correlate with tacrolimus levels at any study time point. Time since transplantation did not show any association with the specific haemostasis parameters studied. VWF antigen and FVIII activity values showed a significant positive correlation at both study time points ($r=0.5876$; 95% CI:

0.3408-0.7587; $p < 0.0001$ and $r = 0.5919$; 95% CI: 0.3429-0.7631; $p < 0.0001$), with no significant difference between DSA– and DSA+ groups.

Association between some TGA parameters and microvascular obstruction in renal biopsies

Histological samples from kidney biopsies were evaluated according to the Banff classification. Biopsy sampling was performed in 15 patients (2 DSA– patients and 13 DSA+ patients) within one year of patient selection. Examination of HE stained histological sections confirmed microvascular obstruction in 10 DSA+ patients. In their cases, ETP was significantly higher compared to patients without histological evidence of microvascular obstruction (median: 1898; IQR: 1573-2165 vs. 1453; IQR: 1306-1656 nM*min; $p = 0.0425$). It should be emphasized that the levels of other haemostasis or complement parameters determined during the study were not associated with the presence of microvascular obstruction in this cohort.

Association between inflammation and the TGA parameters in DSA+ patients

In DSA+ patients, the TGA ETP parameter at the first sampling showed a significant positive correlation with CRP ($r = 0.4344$; 95% CI: 0.0534-0.7050; $p = 0.0236$), fibrinogen ($r = 0.4062$; 95% CI: 0.0008-0.6969; $p = 0.0439$), levels of all laboratory parameters tested. Six months later, the peak thrombin parameter measured in DSA+ patients was associated with fibrinogen ($r = 0.4640$; 95% CI: 0.0746-0.7307; $p = 0.0224$), VWF antigen ($r = 0.4701$; 95% CI: 0.0920-0.7297; $p = 0.0177$) and FVIII activity ($r = 0.5894$; 95% CI: 0.2533-0.7986; $p = 0.0019$), but similar correlations were not observed in DSA– patients at any time point. Based on the correlations seen between thrombin generation rates and acute phase proteins, these results suggest a relationship between inflammation and thrombin generation in the DSA+ patient group.

DISCUSSION

The TGA is a novel global haemostasis test with untapped potential for clinical practice in several areas. Since its original description, TGA has been used primarily as a research tool to investigate hyper- or hypocoagulability in conditions where the degree or direction of the shift in the haemostasis balance was not fully understood. However, the ability to detect hypo- and hypercoagulable states in a single measurement using TGA can be used not only for research purposes but also to aid clinical decision making in a number of pathologies. In particular, it can be of great help in the diagnosis of hypercoagulable states, as the test results correlate better with pre-thrombotic and thrombotic states than conventional coagulation screening tests.

ICH is a condition with high mortality and morbidity, requiring urgent care, so rapid diagnosis and prompt treatment are of paramount importance. Surprisingly, there are few studies available on this topic, despite the fact that laboratory tests with adequate predictive value could be useful in personalising the management of patients with ICH. Our study was one of the first to investigate the extent of thrombin generation in a cohort of patients with relatively large ICH, and showed that the extent of thrombin generation is associated with the development of haematoma and may be a promising test for predicting the outcome of ICH. At-admission TGA parameters (ETP and peak thrombin) of ICH patients showed good correlation with the estimated haematoma volume at day 90 post-event, a predictive marker of outcome. Our results showed that elevated peak thrombin was associated with adverse long-term functional outcome in patients and was a significant independent predictor of poor long-term outcome. Although one might legitimately expect hypocoagulability in patients with ICH, in contrast to the measured hypercoagulability, these results are consistent with previous literature showing that elevated D-dimer levels are associated with adverse outcome in ICH patients, indicating more extensive coagulation activation and clot degradation. Our results suggest that TGA may be even more useful than D-dimer in predicting the prognosis of ICH, since in logistic regression analysis including all relevant variables (including D-dimer), only peak thrombin and NIHSS remained in the model as independent predictors of poor outcome. The results of TGA in the ICH cohort studied clearly suggest that the balance of haemostasis in stroke patients is shifted towards hypercoagulability. When comparing thrombin generation results of patients and healthy controls, peak thrombin was found to be significantly higher in patients. This difference, based on an analysis of the relationship between some basic laboratory parameters and some parameters of TGA, could be explained by the presence of an increased inflammatory state in ICH patients. Since hypertension is suspected as the primary cause of ICH in the cohort we

studied, it is conceivable that the inflammation induced by the underlying accelerated atherosclerosis may be the trigger for hypercoagulability in patients. Although fibrinogen, as an acute phase protein, was associated with some parameters of TGA, its levels in patients were within the reference range and did not show significant differences between patient groups with different outcomes. Interestingly, PT and APTT showed a significant negative correlation with the peak thrombin parameter in the ICH cohort, suggesting that factors causing shorter coagulation times may be associated with higher levels of thrombin formation, i.e. higher peak thrombin values. Although the activity of FVIII, also an acute phase protein, was significantly higher in ICH patients compared to controls, FVIII levels did not show a correlation with specific TGA parameters in this cohort. Our results raise the need for further studies to elucidate the mechanisms (particularly with regard to inflammatory processes) that may contribute to the higher levels of thrombin generation we observed in ICH patients.

Since the MEGA study, it has been known from the literature that among many conditions requiring inpatient care (e.g. acute myocardial infarction, ischaemic stroke, rheumatoid arthritis, heart failure, renal failure, etc.), the risk of VTE in patients with haemorrhagic stroke is the highest, even after adjustment for sex and age. Prospective observational studies have clearly shown that, after adjustment for some influencing factors, patients with haemorrhagic stroke have a significantly higher risk of hospital-acquired VTE than patients with ischaemic stroke. In our study cohort, we recorded 4 VTEs during the 3-month follow-up period, despite the use of anticoagulant therapy in all patients considered clinically high-risk. Since the exact pathophysiology of the increased risk of VTE in ICH patients is not yet fully understood, our research findings may provide additional data to better understand the hypercoagulability in haemorrhagic stroke. Although TGA seems promising for assessing the extent of hypercoagulability in patients, further studies are needed to confirm whether TGA can help to better assess the risk of VTE in ICH patients and to individualize antithrombotic treatment.

TGA has previously shown promise in identifying haemostasis imbalances in a number of pathologies. Interestingly, in the cohort we studied, the evolution of intracerebral haematoma showed a positive correlation with the rate of thrombin formation. This information may be important for the future, as our understanding of the factors that induce/trigger haematoma growth or shrinkage is still in its infancy, but may also be of relevance for the design of new types of therapeutic approaches in the future.

In our second prospective observational study, we have shown for the first time that thrombin generation is increased in kidney transplant recipients with DSA compared to DSA- patients. To date, ABMR is the most common cause of allograft loss after renal transplantation (68).

Although the exact pathomechanism underlying ABMR is not yet fully understood, the literature suggests that the emergence of DSAs causes graft damage over time. Our results suggest that hypercoagulability may play a role in the pathological processes leading to loss of graft function. To date, only a few papers have been published in the literature investigating specific haemostasis markers in renal transplant patients, and to our knowledge, the extent of thrombin generation has not been previously investigated in this specific patient population.

Of note, the ETP parameter in the DSA+ group was significantly elevated compared to the DSA- group at both study time points (at inclusion and 6 months later), suggesting persistent hypercoagulability in patients with DSA. Although the increased risk of humoral rejection has been supported by several studies in patients with anti-HLA class II DSA, the exact pathomechanism is still unknown. In our cohort, anti-HLA class II antibody levels showed a significant positive correlation with ETP at both sampling times, suggesting a direct correlation between the presence of antibodies and the degree of pre-thrombotic status. It is questionable, however, whether the hypercoagulable state seen in DSA+ patients potentially plays a role in the pathomechanism of ABMR. In our study, a possible causal relationship between increased thrombin generation and graft rejection was suggested by the significantly higher ETP found in patients with signs of microvascular occlusion on renal biopsy compared to patients with graft histology without microvascular lesions. Although the number of patients with *de novo* DSAs in our study was too small (n=3) to draw firm conclusions about the thrombin generation enhancing effect of antibodies, it should be noted that ETP showed a slight increase after *de novo* appearance of DSAs in all three patients, which may also suggest a direct effect. Our study also revealed a correlation between hypercoagulation and graft function: the evolution of renal graft function (serum creatinine, eGFR at 6-month follow-up) showed a significant correlation with ETP and peak thrombin in DSA+ patients, and these correlations were not present in the group of patients without DSA. To confirm our observations, prospective studies in larger patient populations or translational model studies to confirm causal relationships are needed in the future.

Our studies showed that tacrolimus levels were negatively correlated with some parameters of TGA. Interestingly, we did not observe a direct correlation between tacrolimus levels and anti-HLA class II DSA positivity rates, but acute phase proteins (e.g. FVIII/VWF, fibrinogen, complement C4) showed a significant negative correlation with tacrolimus trough levels in patients. Our results suggest that TGA may also be significantly dependent on the degree of inflammation and immunosuppression, and suggest an indirect relationship between inflammation and TGA, suggesting that the degree of immunosuppression may be related to

the pathomechanism leading to hypercoagulability in kidney transplant recipients. Recently, it has been shown that proinflammatory cytokine levels are significantly elevated in renal transplant patients with DSA, and cause worse graft survival even in the absence of early signs of histological lesions. Van Loon and colleagues have shown a significant correlation between proinflammatory cytokines and the presence of DSAs, with their observations suggesting that DSAs induce immune activation that is not always detectable by histological examination. The authors described that the secretion of cytokines from endothelial cells, NK cells and monocytes is also mediated by DSAs. The association between DSAs, immune activation and activation of the haemostasis system during ABMR is an intriguing one, with useful potential for future treatment of patients.

Our results showed that the clot lysis test did not differ between the two groups, suggesting that in DSA+ patients only the balance of coagulation is upset, not the fibrinolysis. Our observations suggest a hitherto unknown role of the balance between hypercoagulation and prothrombotic state in the pathomechanism of ABMR, which calls for further investigation. The significance of these findings is twofold: on the one hand, TGA may help to determine the timing of renal biopsy and possible modification of immunosuppressive therapy, and on the other hand, the possible role of hypercoagulation in the pathomechanism of humoral rejection may suggest that prophylactic anticoagulation may be useful in cases of increased coagulation activation. The results of our study suggest that TGA may be useful to identify hypercoagulation in patients with DSAs, but more prospective studies are needed to confirm this, investigating the relationship between increased TG, hypercoagulation and the causes of graft rejection.

It should be noted that during the 6-month follow-up, there was no significant change in TGA, VWF, sEsel and sPsel levels in the subgroups studied with or without DSAs, indicating that the changes in haemostasis we observed were not transient in kidney transplant recipients.

We hypothesized that there would be a significant difference between the VWF antigen levels of DSA+ and DSA- patients, but this association was not confirmed in this cohort. In the majority of individuals included, VWF antigen levels were above the upper limit of the reference range, which was not dependent on the presence of DSAs. It has previously been reported that anti-HLA DSAs cause VWF secretion, which can lead to vascular inflammation and myocardial thrombosis, and it has also been described, similar to our results, that tacrolimus treatment reduced VWF antigen levels in patients. Thus, our previously published results are consistent with our findings, which suggest that VWF antigen levels are likely regulated by the degree of inflammation or immunosuppression in this patient group. However, in our cohort, in contrast to what has been previously described, patients without DSA also had a relatively

high proportion of elevated VWF antigen levels, a phenomenon that requires further investigation to explain. Literature suggests that elevated VWF antigen levels are a useful marker of graft vascular injury, but the exact source of secreted VWF has not been identified. Interestingly, sEsel, a marker of endothelial damage, was not elevated in the cohort we studied, but sPsel, a marker of endothelial injury and platelet activation, was elevated in the majority of patients, regardless of the presence of DSAs. This may be explained by the fact that increased VWF antigen levels may not only directly reflect the extent of endothelial damage, but also platelet activation, similar to sPsel. The significant correlation observed between sPsel and VWF antigen levels in this cohort based on the first blood sampling results, and between FVIII and VWF antigen levels in this cohort, may also suggest that elevated VWF antigen levels may be partly platelet-derived and not exclusively attributable to endothelial damage. However, the lack of correlation between sPsel and TGA parameters suggests that platelet hyperreactivity is not related to increased thrombin generation in the cohort studied, independent of the presence of DSAs. There may be reason to further suggest that measurement of VWF antigen levels may be more useful than determination of other acute phase proteins or inflammatory markers (e.g. CRP) in kidney transplant recipients, as elevated VWF antigen levels may be indicative of pre-existing vascular disease.

Despite the crucial role of DSAs in graft rejection, early detection of the ABMR process remains a challenge in clinical practice. In the search for markers and therapeutic targets of acute rejection, haemostasis testing may be important to prevent the development of myocardial thrombosis, ischaemia and reduced microcirculation.

The pathomechanism of microvascular obstruction may involve activation of not only the coagulation system but also the complement system as part of the response to DSAs. Several studies have investigated the role of complement-activating DSAs in the outcome of transplanted organs. The role of the complement system in the pathomechanism of allograft injury is now increasingly recognised. *In vitro* studies suggest that there are several links between the coagulation and complement systems (e.g. thrombin, among others, can cleave complement factor C3), however, the *in vivo* relevance of these processes is poorly understood. To investigate the potential thrombo-inflammatory dialogue between the complement system and coagulation, we measured the levels of C3 and C4 complement in the cohort. To our surprise, C3 and C4 levels and the degree of C3 hypocomplementemia did not differ in DSA+ and DSA- patients, neither at enrolment nor at follow-up. Interestingly, however, a significant positive correlation was observed between C3 and C4 levels and some TGA parameters, independent of the presence or absence of DSAs. Similar to the other acute phase proteins

examined in the cohort, C4 levels showed a significant negative correlation with tacrolimus levels. These results suggest that the haemostasis abnormalities described above, including increased thrombin generation or hypercoagulation, are not necessarily associated with C3 activation. As several new therapeutic options with novel mechanisms of action to prevent graft rejection, including therapeutic options involving the complement system, are currently emerging, it may be worthwhile to focus future research on the individual players of the thrombo-inflammatory axis, to better understand the individual roles of various pathways (coagulation activation, complement activation) underlying graft rejection in specific clinical conditions.

In evaluating the results of both studies, it should be emphasised that the current limitations in the use of TGA in clinical practice are that the test has yet to be standardised, which would allow inter-laboratory comparisons and the development of uniform reference ranges. It is important to note, however, that several haemostasis laboratory tests that are not standardised and do not have a single reference range (e.g. APTT) have become widespread in routine diagnostic practice worldwide. The number of publications in the literature currently available that investigate haemostasis balance in the pathologies we are studying (ICH, kidney transplant recipients) using specific coagulation tests is very limited. Our studies are therefore mainly hypothesis generating studies, aiming to gain insight into whether TGA is associated with adverse outcomes in ICH patients and with the presence of DSAs in renal transplant patients. Further long-term follow-up studies are needed to verify our results and optimise the study conditions, as well as translational experiments are required to confirm certain causal relationships.

Conclusion

In patients with ICH, we detected elevated levels of thrombin generation compared to healthy controls, which is presumably explained by the higher inflammatory parameters seen. Our results suggest that the TGA peak thrombin parameter measured at admission in patients with ICH may be useful in predicting the outcome of intracerebral haemorrhage.

In kidney transplant recipients, we found significantly increased levels of thrombin generation in DSA+ patients compared to DSA- patients. The degree of anti-HLA-II class DSA positivity showed a positive correlation with the TGA ETP parameter at patient enrolment and 6 months later, which may suggest that hypercoagulability is not transient in DSA+ patients. Tacrolimus levels were negatively correlated with some parameters of TGA and with VWF antigen and

FVIII levels. Our results suggest that the hypercoagulability associated with the presence of DSAs may contribute to the pathomechanism of ABMR through microvascular obstruction, which requires further studies to confirm.

Limitations

As with any clinical trial, the limitations of the studies must be taken into account when assessing their results. A limiting factor in interpreting the results of our study of ICH patients was the limited number of individuals included in the study. It should be noted, however, that compared to studies available in the literature on non-traumatic ICH patients that have examined specific haemostasis markers in patient samples at admission, our study has included the largest number of patients to date. The results presented here certainly need to be confirmed by further studies involving a larger number of patients. The study was performed in a single centre, which obviously contributed to the relatively small number of cases, but also to uniform patient care and sample management, and had the additional advantage that, due to the relatively narrow geographic region, only one patient was lost to follow-up during the 3-month follow-up. As there is currently no manufacturer-defined reference range for TGA, the TGA parameters of healthy control individuals were used as reference in this study. The age and sex of patients and controls differed significantly, which partly limited the direct comparison between patient and control group results. It should also be noted that our study was not designed to explore whether TGA or other haemostasis parameters could be helpful in assessing VTE risk or in the use of individualized anticoagulation therapy in ICH patients. Further clinical trials specifically designed to answer these questions are needed to address these hypotheses.

A limitation of our study of kidney transplant recipients is that the patient selection was also based in a single centre, which limited the number of cases, but there were also advantages in this patient group due to the uniform sample management and patient care. Unfortunately, the selection of patients for the study had to be stopped after the second wave of COVID-19, as it became impossible to include patients who were not affected by COVID-19 in some way (COVID-19 infection/post COVID-19 status or unvaccinated individuals), which could potentially affect the assessment of basic haemostasis parameters or TGA results. In our observations, the number of patients with *de novo* DSA was low, and therefore conclusions regarding the direct effect of the emergence of DSAs need to be confirmed by examining larger prospective cohorts. In our study, we did not investigate the effect of autoantibodies other than anti-HLA DSA (anti-endothelial cell antibodies, anti-MICA antibodies). The limited number of

cases prevented us from drawing firm conclusions about the relationship between hypercoagulation and changes in graft function and the development of ABMR. The aim of this study was not to determine whether TGA or other haemostasis tests could help in the personalised management of renal transplant patients or influence histological changes associated with graft rejection, and targeted studies are needed to test these hypotheses in the future.

SUMMARY

Thrombin generation assay (TGA) is a global haemostasis test indicating both hypo- and hypercoagulability and may hold promise for assessing haemostatic balance in various clinical conditions in the future. Its routine diagnostic use has several limitations, including the lack of standardisation and limited data on the diagnostic or predictive value of the test in certain pathologies. Our aim was to investigate whether TGA could be a useful test for predicting short- or long-term outcome in patients with intracerebral haemorrhage (ICH). In kidney transplant recipients, we hypothesized that the presence of anti-HLA donor specific antibodies (DSAs) would cause endothelial injury and that the resulting hypercoagulability could be detected by TGA, helping to detect antibody-mediated remodeling (ABMR) early. Two prospective observational studies were performed, including 87 ICH patients and 164 healthy controls, and 27 DSA+ and 16 DSA– renal transplant patients. TGA was performed at inclusion and after 6 months in kidney transplant recipients, determining lag time, peak thrombin, endogenous thrombin potential (ETP) and time to peak parameters. Short- and long-term outcomes of ICH patients were determined at day 14 and at the end of month 3 using NIHSS and modified Rankin scales (mRS), respectively. After 6 months, immunological and specific haemostasis tests (von Willebrand factor: VWF, FVIII activity, soluble E and P selectin, clot lysis test, complement C3, C4) reflecting graft status were repeated. Peak thrombin was significantly higher in ICH patients compared to controls and in patients with unfavourable outcome (mRS 2-6). With the statistically optimal threshold (339.1 nM), the sensitivity and specificity of peak thrombin for predicting mRS 2-6 outcome were 80.8 and 64.7%, respectively. In a logistic regression model, NIHSS and peak thrombin were shown to be independent predictors of adverse outcome. ETP was significantly higher in DSA+ kidney transplant recipients compared with the DSA– group, which persisted 6 months later. DSA levels were positively correlated with ETP, while tacrolimus levels were negatively correlated with ETP and VWF/FVIII levels, respectively.

CONCLUSION: Increased thrombin generation was detected in ICH patients compared to healthy controls. Our results suggest that TGA measured at patient admission may be useful in predicting the outcome of ICH. In DSA+ kidney transplant recipients, we detected elevated levels of thrombin generation compared to DSA– patients. Our results suggest that the hypercoagulability associated with the presence of DSAs may contribute to the pathomechanism of ABMR through microvascular obstruction, which requires further studies to confirm.

New results and findings of the thesis

- We detected elevated levels of thrombin generation in patients with ICH compared to healthy controls, which is presumably explained by the higher inflammatory parameters seen in patients. TGA lag time, ETP and time to peak parameters showed weak significant positive correlations with CRP and fibrinogen levels in both ICH patients and healthy controls.
- In terms of long-term functional outcome of ICH, an unfavourable outcome (mRS 2-6) was associated with significantly higher D-dimer levels, higher NIHSS at admission, higher haemorrhage volume (at all three study time points) and higher ICH score.
- Patients with poor long-term outcome had a significantly elevated peak thrombin parameter compared to patients with a favourable long-term functional outcome (mRS 0-1).
- We have shown that using the statistically optimal peak thrombin threshold (339.1 nM), an unfavorable outcome in ICH can be ruled out with a high negative predictive value.
- Based on our findings, the NIHSS score at admission and peak thrombin levels above the threshold are independent predictors of poor functional outcome.
- In ICH patients, the time parameters of TGA showed a weak significant negative correlation with the volume of intracerebral haemorrhage on admission. During follow-up of patients at day 90, estimated haemorrhage volume showed a significant positive correlation with ETP and peak thrombin parameters.

- We found significantly increased levels of thrombin generation in DSA+ kidney transplant recipients compared to DSA– patients at enrolment. This association persisted 6 months after enrolment.
- The degree of anti-HLA-II class DSA positivity showed a positive correlation with the TGA ETP parameter at patient enrolment and 6 months later, which may suggest that hypercoagulability is not transient in DSA+ patients.
- Tacrolimus levels were negatively correlated with some TGA parameters, VWF antigen, FVIII activity and complement C4 levels in the kidney transplant recipient cohort.
- Histological examination of kidney biopsy samples from DSA+ patients confirmed the presence of microvascular obstruction. Their ETP was significantly higher compared to patients without histological evidence of microvascular obstruction. Our results suggest that the hypercoagulability associated with the presence of DSAs may contribute to the pathomechanism of ABMR through microvascular obstruction.

- In DSA+ patients, TGA ETP at first sampling showed a significant positive correlation with CRP and fibrinogen levels. Six months later, the peak thrombin parameter measured in DSA+ patients showed a significant positive correlation with fibrinogen, VWF antigen and FVIII activity. Based on the correlations seen between the rate of thrombin generation and these acute phase proteins, the results suggest a relationship between inflammation and thrombin generation in the DSA+ patient group.

APPENDIX



UNIVERSITY of
DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN

H-4002 Egyetem tér 1, Debrecen

Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number:
Subject:

DEENK/44/2026.PL
PhD Publication List

Candidate: Linda Lóczi

Doctoral School: Kálmán Laki Doctoral School

List of publications related to the dissertation

1. **Lóczi, L.**, P. Szabó, R., Orbán-Kálmándi, R. A., Hodossy-Takács, R., Szilvási, A., Szalai, Z., Nagy, G., Antal-Szalmás, P., Nemes, B. Á., Bagoly, Z.: Increased thrombin generation in kidney transplant recipients with donor-specific antibodies directed against human leukocyte antigens.
Front. Immunol. 15, 1-14, 2024.
DOI: <http://doi.org/10.3389/fimmu.2024.1407407>
IF: 5.9
2. **Lóczi, L.**, Orbán-Kálmándi, R. A., Árokszállási, T., Fekete, I., Fekete, K., Héja, M., Tóth, J., Csiba, L., Bagoly, Z.: Thrombin generation as a predictor of outcomes in patients with non-traumatic intracerebral hemorrhage.
Front. Neurol. 13, 1-12, 2022.
DOI: <http://dx.doi.org/10.3389/fneur.2022.912664>
IF: 3.4

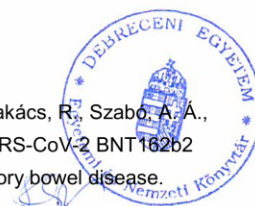
List of other publications

3. Szegedi, I., Bomberák, D., Éles, Z., **Lóczi, L.**, Bagoly, Z.: Cardiovascular disease and microbiome: focus on ischemic stroke.
Pol. Arch. Intern. Med. 135 (7-8), 1-10, 2025.
DOI: <http://dx.doi.org/10.20452/pamw.17088>
IF: 4.7 (2024)
4. Bagoly, Z., Tóth, E. L., Orbán-Kálmándi, R. A., **Lóczi, L.**, Deli, T., Török, O., Kozma, B., Baráth, S., Singh, P., Hevessy, Z., Tóth, J., Katona, É., Molnár, S., Krasznai, Z. T.: Complex evaluation of coagulation, fibrinolysis, and inflammatory cytokines in SARS-CoV-2 infected pregnant women: a prospective, case-control study.
Front. Immunol. 16, 1-18, 2025.
DOI: <http://dx.doi.org/10.3389/fimmu.2025.1556878>
IF: 5.9 (2024)





5. Bagoly, Z., Jouppila, A., Orbán-Kálmándi, R. A., **Lóczy, L.**, Bomberák, D., Kádár, Z. A., Deák, Á., Mátrai, Á. A., Bekéné Debreceni, I., Kappelmayer, J., Németh, N., Lassila, R.: Escalating doses of intravenous APAC demonstrate anticoagulant effect in pigs.
Thromb J. 23 (1), 1-12, 2025.
DOI: <http://dx.doi.org/10.1186/s12959-025-00742-8>
IF: 2.2 (2024)
6. Tóth, E. L., Orbán-Kálmándi, R. A., Bagoly, Z., **Lóczy, L.**, Deli, T., Török, O., Deliné Molnár, S., Baráth, S., Singh, P., Hevessy, Z., Katona, É., Fagyas, M., Szabó, A. Á., Molnár, S., Krasznai, Z. T.: Case report: Complex evaluation of coagulation, fibrinolysis and inflammatory cytokines in a SARS-CoV-2 infected pregnant woman with fetal loss.
Front. Immunol. 15, 1-10, 2024.
DOI: <http://dx.doi.org/10.3389/fimmu.2024.1329236>
IF: 5.9
7. Hudák, L., Kovács, K. B., Bagoly, Z., Szegedi, I., Bencs, V., **Lóczy, L.**, Orbán-Kálmándi, R. A., Péter-Pakó, H., Fülesdi, Z., Busi, B., Nagy, A. C., Perjési-Kiss, B., Oláh, L., Csiba, L.: Clinicopathological Observations in Acute Stroke Patients Treated with Intravenous Thrombolysis.
J. Clin. Med. 13 (19), 1-11, 2024.
DOI: <http://dx.doi.org/10.3390/jcm13196012>
IF: 2.9
8. Mátrai, Á. A., Varga, Á., Baráth, B., Ványolos, E., Orbán-Kálmándi, R. A., **Lóczy, L.**, Bagoly, Z., Jouppila, A., Lassila, R., Németh, N., Deák, Á.: Heparin-like effect of a dual antiplatelet and anticoagulant (APAC) agent on red blood cell deformability and aggregation in an experimental model.
J Thromb Thrombolysis. 57 (8), 1329-1338, 2024.
DOI: <http://dx.doi.org/10.1007/s11239-024-03040-8>
IF: 2.2
9. Ghansah, H., Orbán-Kálmándi, R. A., Bekéné Debreceni, I., Katona, É., Rejtő, L., Váróczy, L., **Lóczy, L.**, Laat, B. d., Huskens, D., Kappelmayer, J., Bagoly, Z.: Low factor XIII levels and altered fibrinolysis in patients with multiple myeloma.
Thromb. Res. 234, 12-20, 2024.
DOI: <http://dx.doi.org/10.1016/j.thromres.2023.12.004>
IF: 3.4
10. Stercel, V., **Lóczy, L.**, Kadenczki, O., Nemes, É., Nagy, B. J., Hodossy-Takács, R., Szabó, A. Á., Fagyas, M., Kappelmayer, J., Szabó, T., Bagoly, Z.: Effect of anti-SARS-CoV-2 BNT162b2 mRNA vaccination on thrombin generation in children with inflammatory bowel disease.
Front. Immunol. 14, 1-12, 2023.
DOI: <http://dx.doi.org/10.3389/fimmu.2023.1257072>
IF: 5.7





11. Ghansah, H., Bekéné Debreceni, I., Váróczy, L., Rejtő, L., **Lóczy, L.**, Bagoly, Z., Kappelmayer, J.:
Patients with multiple myeloma and monoclonal gammopathy of undetermined significance
have variably increased thrombin generation and different sensitivity to the anticoagulant
effect of activated protein C.
Thromb. Res. 223, 44-52, 2023.
DOI: <http://dx.doi.org/10.1016/j.thromres.2023.01.010>
IF: 3.7
12. Székely, E. G., Orbán-Kálmándi, R. A., Szegedi, I., Katona, É., Baráth, B., Czuriga-Kovács, K. R.,
Lóczy, L., Molnárné Vasas, N., Fekete, I., Fekete, K., Berényi, E., Oláh, L., Csiba, L., Bagoly,
Z.: Low [alfa]2-Plasmin Inhibitor Antigen Levels on Admission Are Associated With More
Severe Stroke and Unfavorable Outcomes in Acute Ischemic Stroke Patients Treated With
Intravenous Thrombolysis.
Front. Cardiovasc. Med. 9, 1-14, 2022.
DOI: <http://dx.doi.org/10.3389/fcvm.2022.901286>
IF: 3.6
13. **Lóczy, L.**, Kappelmayer, J., Tarr, T., Bagoly, Z.: Antiphospholipid syndrome and the risk of
myocardial infarction: current evidence and uncertainties.
Kardiol. Pol. 78 (1), 6-14, 2020.
DOI: <http://dx.doi.org/10.33963/KP.15090>
IF: 3.108

Total IF of journals (all publications): 52,608

Total IF of journals (publications related to the dissertation): 9,3

The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the
basis of the Journal Citation Report (Impact Factor) database.

04 February, 2026



ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr Zsuzsa Bagoly, for the support and faith she has shown in me throughout my PhD studies, for providing me with valuable professional advice and encouragement throughout my work since I was a TDK student. Thank you for being by my side even in the final stages.

I would like to thank Dr. Zsuzsanna Bereczky, Head of the Department of Clinical Laboratory Research of DE KK, and Prof. László Muszbek for providing me with the opportunity to carry out my research work at the institute.

I would also like to thank Prof. László Csiba for providing the blood samples and clinical results of the patients for our research, and all the staff of the Neurology Department who helped us in some way.

I would like to thank Dr. Réka P. Szabó and Dr. Balázs Nemes for providing us with clinical data and blood samples for our studies and for their professional advice during the collaboration.

I would like to thank all the co-authors who contributed their professional and practical knowledge to the preparation of the publications on which this dissertation is based.

I would like to thank Prof. János Kappelmayer, former director of the Institute of Laboratory Medicine, for providing us with the instruments used for our measurements. In addition, I would like to thank all the staff of the DE KK Department of Clinical Laboratory Research and the Institute of Laboratory Medicine who provided me with useful professional advice during the course of my research.

I am deeply grateful to my family, who supported me in every possible way throughout my studies and did everything they could to make this thesis possible.

The studies and the publications were made possible by the following grants: OTKA K120042, FK128582, GINOP-2.3.2-15-2016-00043, HUN-REN-DE Cerebrovascular Research Group, ÚNKP-19-3-DE-I-108, ÚNKP-23-4-I-DE-239, MTA-DE Lendület Haemostasis and Stroke Working Group.