FINAL REPORT
NKFI K120042 PROJECT
In vivo and in vitro investigation of hemostasis in acute stroke patients treated with iv. thrombolysis and mechanical thrombectomy

In the NKFI K120042 project, all activities were proceeding as originally planned in the Workplan. Inclusion of patients in the clinical study reached a number that was beyond our expectations. We were able to enroll n=539 acute ischemic stroke patients treated with iv. thrombolysis in the study, that highly exceeded the planned final patient number of n=300. At the end of the 4th year, 56 thrombi were collected from patients undergoing mechanical thrombectomy, which was also beyond our original expectations (n=30 thrombi). The large number of enrolled patients made it possible to perform laboratory measurements, statistical analysis and to publish results in well-recognized international journals. Despite the planned proceeding of the research project, a one-year prolongation was asked due to the COVID-19 pandemic, which caused some delay in the publication process of a few papers. As a result of this prolongation, the research group also had the opportunity to widen the scope of research to COVID-19 associated stroke and thrombolysis and publish results related to this phenomena. Thus, the project had a very successful last period of publishing results and until this report, a total of 19 papers were published based on the project, NKFI support was acknowledged in all papers, total IF: 60.818. The project provided the basis of 4 PhD thesis works (NKFI support acknowledged in all 4 PhD dissertations). Abstracts of the research group were presented as oral presentations at a number of international and national congresses during the projects period. The works received a number of achievements on these occasions (European Congress on Thrombosis and Hemostasis, 2019: Top 8 abstract of Congress; International Society on Thrombosis and Hemostasis (ISTH); 2020 „Daily Highlight of Congress“).

Detailed report of the accomplished work according to the Workplan.
I. Characteristics of enrolled patients with acute ischemic stroke (AIS) and thrombolysis/ thrombectomy. Biobanking, clinical data collection and laboratory measurements. All patients were enrolled at the Department of Neurology, University of Debrecen. Baseline demographic characteristics of the patient cohort (n=539 patients): age:median:68 (IQR: 60-76) years, male: 328/539 (60.9%); NIHSS:median: 7 (IQR:4-11); time-to-treatment:145 min (IQR:110-188). A total of 56 thrombi retracted by mechanical thrombectomy has been stored in our biobank, which is beyond our expectations. In all cases when a patient was undergoing mechanical thrombectomy a radiologist specialized in neuro-intervention was involved and a Solitaire stent retriever was used.

II. Laboratory measurements including ex vivo hemostasis measurements:
All laboratory measurements were carried out at the University of Debrecen, Division of Clinical Laboratory Sciences. Peripheral venous blood samples were collected from all patients immediately before thrombolysis and 24 hours after. The following laboratory parameters were studied from the blood samples: complete quantitative blood count, liver function parameters, kidney function tests and hsCRP. Screening tests of coagulation (PT, APTT, TT), quantitative fibrin monomer test, functional fibrinogen assay, D-dimer levels, PFA-100 closure times, thrombin generation assay, a list of specific hemostasis assays: FXIII, FVIII activity, VWF antigen levels, plasminogen, α2-plasmin inhibitor (α2-PI) activity and antigen levels were studied

from various subsets of patient samples. Moreover, the following ex vivo experiments were also executed from the blood samples: α2PI incorporation test and clot lysis, including a modified clot lysis where DNA and histones were also added to the plasma samples to imitate neutrophil extracellular trap (NET) formation.

III. Evaluation of extracted thrombi
Histopathological investigations have been performed on the extracted thrombi in the 2nd year of the study. Structural characteristics of the clots were studied and the localization of white blood cells, fibrin and lytic areas were identified. Thrombus size using the clot burden score (CBS) and a range of fibrinolytic proteins from parallel plasma samples were analyzed in a case-control subset of the study involving 200 patients.

IV. Statistical analysis.
Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS 22.0, Chicago, IL) with the following major statistical methods: Spearman's bivariant correlation, multivariate logistic regression analysis, Student's t test/Mann-Whitney U test, ANOVA/Kruskal-Wallis analysis, contingency tables, χ^2 test.

V. Summary of most important findings. Translational and clinical implications.

1/ Ex vivo experiments on specific fibrinolytic factors, the incorporation of α2 PI, FXIII levels, clot lysis assays and thrombolysis outcome
In the frame of the study, a comprehensive set of fibrinolytic markers were studied from the blood samples and correlated with outcomes, moreover, new assays were introduced by our research group. Fibrinogen, FXIII, a newly developed assay of α2PI incorporation, in vitro clot-lysis, soluble fibroblast activation protein and α2PI p.Arg6Trp polymorphism were measured from samples of AIS patients obtained before thrombolysis and of healthy controls. In healthy controls and in the subset of patients with good outcomes the extent of α2PI incorporation did not differ significantly (49.4 ± 4.6% vs. 47.4 ± 6.7%, p = 1.000). In patients suffering post-lysis intracranial hemorrhage, α2PI incorporation was significantly lower (37.3 ± 14.0%) as compared to controls and to those with good outcomes (p=0.004 and p=0.028, respectively). A manuscript was published based on the results (Bagoly Z et al Biomolecules, 2021;11: 347. IF: 4.879). Results provided the basis of a PhD dissertation (Baráth B, 2021).
Logistic regression analysis showed that a low FXIII level 24h post-lysis is an independent predictor of short-term mortality (<14 days), while major FXIII-A or FXIII-B polymorphisms do not influence thrombolysis outcomes. Results on the association of FXIII levels with stroke thrombolysis outcome were published in 2018 in the well-recognized international journal of Scientific Reports (Székely EG et al. Sci Rep 2018;8:7662. IF:4.122).
Cell-free DNA (cfDNA), clot lysis assay (CLA) and newly developed CLA assay supplemented with cfDNA and histones (mCLA) were carried out from the blood sample of AIS patients obtained before thrombolysis. Logistic regression analysis proved that 50% clot lysis time is a predictor of short-term therapy failure, while the AUC parameter predicts ICH occurrence. Results were published in Scientific Reports in 2021 (Orban-Kalmandi et al, Sci Rep. 2021;11(1):12713, IF: 4.379) Results provided the basis of a PhD dissertation (Orbán-Kálmándi R, 2021).

2/ The role of PAI-1 in predicting AIS thrombolysis outcomes

PAI-1 activity and antigen levels were measured from the blood samples of AIS patients on admission and after thrombolysis and the PAI-1 4G/5G polymorphism was determined. PAI-1 activity levels dropped transiently after thrombolysis, while PAI-1 antigen levels remained unchanged. Logistic regression analysis including age, sex, NIHSS on admission, BMI, history of arterial hypertension and hyperlipidaemia conferred a significant, independent risk for developing intracranial haemorrhage in the presence of 5G/5G genotype (OR:4.75, 95%CI:1.18-19.06). Results were published in 2019. (Szegedi I et al. Ann Clin Trans Neurol. 2019; 6: 2240-2250. IF: 3.660) The published results, together with findings on clot burden (see later) provided the basis of a PhD thesis (Szegedi I, 2021).

3/ Thrombin generation (TG) and endothel activation parameters (VWF, FVIII) as predictors of the outcome of thrombolysis

In a subset of samples TG was assessed from blood samples obtained before the administration of the thrombolytic agent. Endogenous Thrombin Potential (ETP) and Peak Thrombin were significantly lower in patients with cardioembolic IS. Symptomatic intracranial hemorrhage (SICH) was significantly associated with low ETP and Peak Thrombin levels. A multiple logistic regression model revealed that an ETP result in the lower quartile is an independent predictor of mortality within the first two weeks (OR: 6.03; 95%CI: 1.2-30.16, $p<0.05$) and three months after the event (OR: 5.28; 95%CI: 1.27-21.86, $p<0.05$). Results were presented as oral presentation at the European Stroke Congress (2017 May, Prague) and results were published in 2017 (Hudák et al, Plos One, 2017, 12:e0180477, IF: 2.806).

VWF levels and FVIII levels have been shown to be associated with endothelial damage. Elevated VWF antigen and FVIII activity levels were associated with worse 24h post-lysis ASPECT scores. Elevated FVIII/VWF levels post-lysis were independently associated with poor functional outcomes (mRS>3) at 90 days. Findings were presented at the European Stroke Congress in 2018. A manuscript was published based on the results (Tóth NK, Front Neurol 2018; 8:e721. IF: 3.508). Results provided the basis of a PhD thesis (Tóth NK, 2019).

Based on our studies including the measurement of a wide range of fibrinolytic and hemostasis factors, our main conclusion is that in patients with higher clot burden and increased consumption of fibrinolytic inhibitor (particularly $\alpha 2$ PI) and in the presence of the PAI-1 5G allele (which is associated with reduced levels of the inhibitor), the risk of hemorrhagic transformation is increased. Poor outcomes were associated with increased endothelial damage (elevated VWF levels and FVIII levels), increased consumption of FXIII and low TG. The summary of our experience including a comprehensive review on hemostasis and fibrinolytic markers predicting the outcome of AIS treatment was published in 2019, the paper has received a high number of citations (28) in this short period of time (Bagoly et al, Front Neurol 2019; 10:513, IF: 2.635)

4/ Hemostasis and fibrinolytic markers and the risk of stroke in patients with atrial fibrillation (AF). A chance to prevent cryptogenic stroke.

A wide range of fibrinolytic markers were measured in patients with AF and the risk of stroke in patients with AF undergoing ablation with different techniques and different anticoagulation strategies were studied in a smaller arm of the study. The presence of endothelial damage was found in AF patients as compared to controls. Our results

proved that uninterrupted dabigatran administration provides greater inhibition against intracardiac activation of hemostasis and thus reduces the risk of stroke as compared to vitamin K antagonists during cryoballoon catheter ablation of atrial fibrillation. Three papers were published based on our results (Tóth et NK al Biomed Res Int, 2017; 3678017, IF: 2.476, Bagoly Z et al J Clin Med. 2020; 9: 1-13. IF: 4.241 and Hajas O et al Cardiol Res Pract. 2020; 2020:e1570483. IF: 1.866). A review paper on potential biological markers of AF that could be helpful to prevent cryptogenic stroke were published in 2017 (Szegedi et al, Biomed Res Int 2017; 3678017. IF: 2.476).

5/ Stroke as a potential complication of COVID-19-associated coagulopathy

Given the situation caused by the pandemic, a narrative and systematic review of the literature on COVID-19 associated coagulopathy and stroke was performed by our group and published in 2020 (Szegedi et al J Clin Med. 2020, 10:9, IF: 4.241). Our findings showed that AIS is the most frequent type of stroke occurring in infected patients. In most cases, stroke was severe (median NIHSS:16) and most of the patients had one or more vascular risk factors. Laboratory findings in AIS patients were consistent with COVID-19-associated coagulopathy, and elevated D-dimer levels were the most common finding. The outcome was unfavorable in most cases, as a large proportion of the reported patients died or remained bedridden.

6/ Clot burden and thrombolysis outcome

Thrombus size calculated as the clot burden score (CBS), and a wide range of fibrinolytic factors were assessed in 200 anterior circulation AIS patients receiving intravenous thrombolysis treatment without thrombectomy: 100 AIS patients with large vessel occlusion (LVO) (CBS 0-9) and 100 age- and sex-matched AIS patients without LVO (CBS 10). In a univariate analysis, significant protective effect of the FXIII Leu34 allele against developing larger clots (CBS 0-9) could be demonstrated (OR:0.519; 95%CI:0.298-0.922, $p = 0.0227$). Multivariate regression analysis revealed that CBS is an independent predictor of short- and long-term functional outcomes, while such effect of the studied hemostasis parameters could not be demonstrated. A manuscript was prepared from the results was accepted in 2021 (Szegedi I et al PLoS One. 2021 7;16(7):e0254253. IF: 3.240)

7/ The effect of alcohol intoxication on stroke treatment

It has come to our attention that a significant number of AIS patients in our cohort were under the influence of alcohol. Literature on the treatment of AIS and particularly thrombolysis under the influence of alcohol is scarce. Our findings were published in the format of case series (Árokszállási T BMC Neurol. 2019; 29;19:14. IF: 2.233).

8/ The role of hypertension and its treatment on cognitive performance

As expected, a large percentage (72.5%) of our cohort with AIS had hypertension, in fact, this is by far the most common cerebrovascular risk factor in the studied cohort. In an arm of the study, we analyzed the influence of hypertension and its treatment with angiotensin-converting enzyme inhibition on the reversibility of alterations in arterial wall and cognitive performance associated with early hypertension. We found that hypertension-induced subclinical vascular and cognitive changes are reversible.

Two original papers were published on this topic. (Czuriga-Kovács KR et al J Clin Hypertens (Greenwich). 2019 ;21:658-667. IF: 2.444 and Csikai E. Medicine (Baltimore). 2019;98:e16966. IF: 1.870)