Final report

K128582 project:
Factor XIII, thrombin generation and fibrinolysis in patients with chronic inflammatory disorders
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Summary of project activities:

In the frame of the FK128582 project, all activities were carried out as originally planned in the Workplan. In addition, collaborative studies on factor XIII (FXIII), thrombin generation and fibrinolysis, closely related to the topic/methodology used in the grant, were also completed and published (see later). Enrollment of patients in both arms of the study was fully completed by the last year of the study. As the COVID-19 pandemic affected the enrollment of patients during the 2nd and 3rd year of the project, a 1-year extension was requested. Due to the pandemic, we had to face new, unexpected challenges: at first, the difficulties of patient enrollment (due to quarantine, temporary shut-down of outpatient ambulatory care units), while in the later phase of the project, the infection/post-infection/vaccination status of patients and controls had to be considered in the analyses, which potentially influenced hemostasis results. We managed to turn these challenges into an opportunity to study the effect of COVID-19 vaccination on hemostasis activation in the inflammatory bowel disease (IBD) patient population, and results of this arm of the study were published in a highly-ranked journal in 2023. In the first 2 years of the study, a series of new laboratory methods on fibrinolysis and thrombin generation were set up in the laboratory. As the effect of COVID-19 on hemostasis was not known at the time, at this early stage of the project, methods were tested on patient cohorts with arterial or venous thrombotic events of the pre-pandemic era, unaffected by COVID-19 disease or vaccination. In 2021-23, the IBD arm of the study was extended with the investigation of children with IBD. By the last year of the study, patient enrollment was completed and all measurements of the Workplan were carried out in full from the stored samples. Until this report, 21 papers were published on behalf of the research group in highly-rated international journals (NKFI support acknowledged in all papers, total IF: 100.279; the PI first or corresponding author in 16/21). Results of the project were presented at 10 congresses (NKFI support acknowledged in all published abstracts). A novel fibrinolysis assay that was set up in the beginning of the project provided the backbone of a PhD dissertation (Rita Orbán-Kálmándi, 2022, supervisor: Zsuzsa Bagoly). International collaborations with leading experts of the field were achieved and results were published (see later). As a direct result of the grant, in the last year of the project, the PI submitted her doctoral thesis of the Hungarian Academy of Sciences, which is currently under evaluation. Potential economic exploitation of the results has also begun, primarily by the research and development of novel fibrinolytic assays. During the last phase of the project, a research and development collaboration was signed between our research group and Aplagon Oy (Helsinki, Finland) on the pre-clinical studies of a novel antithrombotic drug with anti-inflammatory effects (APAC).

Detailed report of the project according to the Workplan

I. Establishment and verification of laboratory methods used in the project. Development of new fibrinolytic methods and their verification using stored plasma samples.

An important part of the project was the development and validation of new fibrinolytic assays. A new method for the measurement of α2 plasmin inhibitor (α2PI) incorporation into fibrin clots was finalized

in the second year of the project. A new, modified clot lysis assay, in which cell free DNA and histones are added to the sample solutions to mimic the presence of neutrophil extracellular traps, was also finalized and tested on stored clinical samples during the second year of the project. The new methods were verified and published in three studies in 2021 (Bagoly Z et al Biomolecules, 2021; Orbán-Kálmándi et al Front Neurol 2021; Orbán-Kálmándi et al et al Sci Rep 2021) and in another study published in 2022 (Szekely at al Front Cardiovasc Med 2022) and provided the basis of the PhD thesis of Dr. Orbán-Kálmándi (2022).

II. Enrollment of patients.

A. SLE/APS cohort.
In this arm of the study, 140 patients with SLE/APS and 140 controls matched for age, sex and smoking status were enrolled. Demographics and vascular risk factors were obtained from all participants. Disease activity, medication, history of thrombotic events/pregnancy complications were registered. Measurements of whole blood count, clinical chemistry, specific hemostasis tests, autoantibodies, and complement levels were carried out from stored blood samples. FXIII activity, FXIII-A_2B_2 and FXIII-B antigen levels, major FXIII polymorphisms, von Willebrand factor (VWF) antigen levels, factor VIII (FVIII) activity, thrombin generation (TG) were also measured.

B. IBD cohort.
Due to the COVID-19 pandemic, the number of enrolled IBD patients in the 2nd year of the project was somewhat below our original expectations, but by the end of the project, more IBD patients and controls were enrolled as originally planned (131 IBD patients: 79 adults and 52 children between the age of 12-18 and 217 age and sex-matched controls: 150 adults and 67 children). All IBD patients were followed and by the end of the project, the number of blood samples reached 2-5/patients. Routine chemistry, hemostasis, hematology tests, TG, and fibrinolysis assays were carried out from all stored samples. Macrophage-secreted enzymes, including angiotensin-converting enzyme (ACE) and chitotriosidase enzyme (CTO) activities were also tested. Complex statistical analysis was performed using Statistical Package for the Social Sciences (SPSS 22.0, Chicago, IL).

III. Results.

A. SLE/APS cohort.

FXIII levels and FXIII polymorphisms in patients with SLE and APS
Results. FXIII activity and FXIII-A_2B_2 antigen levels were significantly elevated in APS patients as compared to controls. In SLE patients, regardless of the presence of APS, FXIII-B levels were significantly elevated. FXIII-A_2B_2 and FXIII-B levels significantly correlated with C4 complement levels. A FXIII level in the upper tertile conferred a significant risk for arterial but not venous thrombotic events in SLE patients (OR: 4.43; 95% CI: 1.48-13.18, p=0.008). FXIII-B His95Arg polymorphism was found to be associated with a risk for venous thrombosis, while FXIII-B intron K polymorphism conferred a protective effect. After adjustments, only the effect of FXIII intron K polymorphism remained significant in the model (OR: 0.19; 95% CI: 0.04-0.79, p=0.022). Results were presented at two international meetings and abstracts were published. A manuscript was written based on the results and is currently under review.

Association between FXIII-A Val34Leu, fibrinogen levels (inflammation) and thrombus size
Results. Based on the above-described finding of elevated FXIII level and the risk of arterial thrombotic events, further *in vitro* and *in vivo* studies were pursued. Effects of FXIII-A Val34Leu and fibrinogen

levels on whole blood clots were studied *in vitro* in healthy controls and *in vivo* in patients with acute atherothrombotic events (acute ischemic stroke). In the collaborative study with the group of Prof. Wolberg at the University of North Carolina at Chapel Hill, we were able to demonstrate that the presence of FXIII-A Leu34 allele reduces the mass of *in vitro* formed whole blood clots, which effect mainly prevails at high fibrinogen concentrations (e.g. inflammation). FXIII-A Leu34 carrier status was associated with smaller thrombus burden *in vivo* in patients with AIS, which was consistent with the *in vitro* described whole blood clot mass reducing effects of the allele. Results were published in two well-rated journals (Kattula et al, J Thromb Haemost, 2020 and Szegedi et al, Plos One, 2021). Novel biochemical aspects of FXIII were published in J Thromb Haemost (Bagoly Z et al, 2019).
In another collaborative study with the group of Prof. Undas at the Jagiellonian University (Krakow, Poland), a renowned center on APS, we were able to demonstrate the effect of elevated FXIII in patients with venous thrombotic events (pulmonary embolism) and its effect on the fibrin clot phenotype. The paper was published in 2020 (Zabczyk M, J Physiol Pharmacol). As a follow-up of this study, the prothrombotic fibrin clot phenotype was studied by a large international collaborative study in which our research group participated (Natorska J et al, Thromb Haemost, 2023).
TG was also performed in patients with SLE and APS, but we had to conclude that the measurement is most likely affected by the presence of antiphospholipid antibodies. Results were presented as a poster at one international meeting, but this line of research was not pursued further.

VWF and FVIII levels in patients with SLE and APS
Results. VWF antigen levels and FVIII activity were significantly elevated in SLE and APS patients (primary or secondary) as compared to controls. In asymptomatic subjects with circulating aPL antibodies, VWF antigen levels and FVIII activity were within the reference range. Among SLE patients, VWF and FVIII levels were significantly elevated in those with a history of atherothrombosis. A VWF antigen level or a FVIII level in the upper tertile conferred a significant risk for arterial but not venous thrombotic events or pregnancy morbidity in SLE patients (VWF: OR: 29.6; 95% CI:5.12-170.7, $p<0.001$; FVIII: OR: 15.07; 95% CI:3.08-73.6, $p=0.001$).
Conclusions. Elevated VWF and FVIII levels are associated with increased atherothrombotic risk in SLE patients. A manuscript was written and is currently under review based on the results. Results were presented at IFCC Worldlab Congress (Tóth NK et al, IFCC 2022).
In the past few years it has been shown that in patients with APS, fewer arterial thrombotic events occur when treated with vitamin K antagonists (VKAs) as compared to direct oral anticoagulants (DOACs). On the contrary, in patients with nonvalvular atrial fibrillation (AF), DOACs seem to provide more effective inhibition against hemostasis activation as compared with VKAs. This hypothesis was proved by our group using the wide range of methodology introduced early in the project. In patients with AF, increased FVIII and VWF levels suggested permanent endothelial activation and the consequent hemostasis activation was more effectively inhibited by dabigatran as compared with VKA. Results were published in 2020 (Bagoly et al, J Clin Med).

Complex evaluation of thrombin generation, coagulation, fibrinolysis and inflammatory cytokines in a SARS-CoV-2 infected pregnant woman with pregnancy loss
Pregnancy loss and obstetric complications are among the clinical criteria of APS, but detailed demonstration of COVID-19-associated coagulopathy in a pregnancy with stillbirth hasn't been described so far. A comprehensive evaluation of coagulation, fibrinolysis and inflammatory cytokines using the LegendPlex flow cytometric method was carried out in a SARS-CoV-2 infected pregnant woman with pregnancy loss at 28th week of gestation. As compared to healthy age- and gestational age-matched pregnant controls, increased D-dimer, low FVIII activity, low FXIII level, marked hypocoagulability as demonstrated by TG, shortened clot lysis and decreased levels of fibrinolytic proteins were observed in the patient. These alterations most likely have contributed to the increased

bleeding observed during labour and in the early postpartum period. A manuscript was written and accepted for publication in the highly-ranked journal of Front Immunol (Tóth et al, 2024).

Chronic inflammatory diseases, APS, and the risk of thrombotic diseases (myocardial infarction, stroke or venous thromboembolism)
Results from the first half of the project pointed towards novel evidence on the risk of atherothrombotic diseases (myocardial infarction and stroke) in APS, chronic inflammation and thrombophilia. At the beginning of the project, therefore, we summarized our insights on this topic in three review papers (Lóczi et al, Kardiol Pol, 2020, Bagoly et al, Front Neurol, 2019, Bereczky et al, Kardiol Pol, 2019). Another narrative and systematic review paper was published during the pandemic on specific aspects related to COVID-19-associated coagulopathy (Szegedi et al, J Clin Med, 2020) and on the impact of risk interactions in venous thromboembolism (Bagoly Z, Pol Arch Intern Med, 2022).
As an economic exploitation related to this part of the study, a research and development contract was signed with Aplagon Oy (Helsinki, Finland), a biopharmaceutical company developing first-in-class therapeutics for thromboinflammatory diseases. Under the terms of this collaborative research and development, we are carrying out pre-clinical studies on a novel drug (APAC) that uniquely targets atherosclerotic sites, providing local, long-term antithrombotic and anti-inflammatory effect.

B. IBD cohort.

Thrombin generation in IBD patients
Results. As compared to healthy controls, in IBD patients lagtime and time-to-peak parameters were significantly prolonged ($p<0.0001$). ETP was significantly higher in adult IBD patients vs. controls (1896±39 vs. 1468±35 nM*min, $p<0.0001$). Peak thrombin was also significantly increased in IBD patients as compared to controls (377±98 vs. 285±91 nM, $p=0.0001$). This effect was not observed in children. ETP and peak thrombin were elevated with disease activity rise, indicating an association between hypercoagulability and disease progression. UC patients with higher pMayo index showed significantly increased ETP (pMayo 0-1: 1837±170 vs. pMayo 2-3: 2287±447 nM*min, $p=0.0475$).
Conclusion. TG is markedly elevated in adult IBD patients but not in children. In UC patients TG is associated with disease activity. In 2021, the presentation of preliminary results by a graduate student member of the research team at the National Student Research Meeting was awarded 1st prize. Results were presented at IFCC Worldlab Congress in 2022. A manuscript was prepared from the results and is currently under review in Eur J Clin Invest.

The effect of SARS-CoV-2 mRNA vaccination on TG in children with IBD
Results. Thirty-eight children with IBD (CD:18, UC: 20) aged 12-18 years and 62 healthy age-matched children were enrolled. Blood was collected before and 2-4 weeks after the second dose of BNT162b2 (Pfizer-BioNTech) vaccine dose. Whole blood count, fibrinogen, TG, inflammatory markers (CRP, ferritin), anti-SARS-CoV-2 antibody levels were investigated. Detailed clinical parameters including post-vaccination symptoms, COVID-19 history, disease activity scores (PUCAI, PCDAI) were registered. Baseline TG parameters did not differ between patients and controls. ETP showed a significant positive correlation with markers of inflammation and with PCDAI. Inflammatory parameters and TG did not increase in patients and controls post-vaccination. Vaccination significantly increased antibody levels in all investigated groups, but those receiving TNFα inhibitor therapy presented significantly lower SARS-CoV-2 S IgG/IgM levels. Our results support the safety of SARS-CoV-2 mRNA vaccination in children with IBD, highlighting observations of lower antibody titers in immunosuppressed children.
Results were presented at two international meetings, and a manuscript was written and accepted in the highly rated Front Immunol (Q1, IF: 7.300) based on the results (Stercel V et al, 2023).

Macrophage-secreted enzymes as potentially useful markers in the management of IBD. Results. Serum ACE and CTO activities were measured in 70 patients with IBD. Oral steroid therapy significantly decreased serum ACE activity among patients not taking ACEI (5.26 [4.70-7.73] U/L, n=12 vs. 8.96 [7.38-10.37] U/L, n=47; $p<0.0005$, respectively). ACE activity showed significant correlation with BMI ($r=0.3126$; $p<0.05$), while CTO activity with age ($r=0.3371$; $p<0.05$). Serum ACE and CTO activities were significantly higher in smokers with CD (11.29 [10.00-13.30] U/L; 667.10 [550.90-902.90] mU/L vs. 8.50 [6.63-10.14] U/L; 320.60 [192.00-693.80] mU/L; $p<0.05$, respectively). Conclusions: serum ACE and CTO activities may be useful biomarkers in IBD. ACE activity is suitable for monitoring the effect of steroid and ACEI therapy in clinical practice. Elevation of these biomarkers reflects the effect of smoking on macrophage-mediated inflammation in CD. Results were presented at Worldlab-Euromedlab Congress 2023 and a manuscript is currently under preparation based on the results.

Fibrinolysis and IBD

The clot lysis assay was carried out in the IBD cohort (79 adults and 52 children between the age of 12-18) and in 217 age and sex-matched controls. The test was found to be insensitive to detect alterations of fibrinolysis in IBD, therefore this line of research was not pursued. As an economic exploitation of the project, a product development collaboration was initiated with Technoclone, Austria, world-leading producer of diagnostics tests, equipments and research products in the area of fibrinolysis. Results and samples of the healthy control and diseased cohort are currently being used for the development of more sensitive assays.

FXIII, thrombin generation and fibrinolysis in patients with chronic inflammation and plasma cell disorders

Chronic inflammation is a common complication of most plasma cell disorders (PCDs) and represents a significant cause of morbidity and mortality in this population. Using the wide-range of methodology developed during the project, significant alterations in the balance of hemostasis and fibrinolysis was demonstrated by our research group in patients with PCD. FXIII activity, mixing studies, FXIII-A_2B_2 antigen, total FXIII-B antigen were measured from 17 untreated monoclonal gammopathy of undetermined significance (MGUS) patients, 33 untreated myeloma multiplex (MM) patients, and 30 age and sex-matched controls. Additionally, quantitative fibrin monomer (FM) test, thrombin-antithrombin assay, α2-PI activity, plasmin-α2-antiplasmin (PAP) complex, D-dimer, plasmin generation assay, clot lysis assay, ClotPro-TPA test, FVIII, VWF levels and TG assay was performed with or without the addition of activated protein C (APC).

Conclusions: Low FXIII levels due to consumption were observed in MM patients at diagnosis. Hypercoagulability and ongoing fibrinolysis were detected in MM and MGUS, indicating that a disturbed hemostasis balance is already present in the latter benign condition. Hypercoagulability was observed in MM and even in MGUS cases with very low monoclonal protein concentration. The findings have importance for the clinical practice indicating the necessity of specific hemostasis monitoring during the management of MM and MGUS.

Results on TG and on FXIII and fibrinolysis were well-received by the international scientific community and we were able to quickly publish the results in two papers in the higly-rated journal of Thromb Res (IF: 7.5, Q1). In both papers, the PI is corresponding /shared last author (Ghansah et al, 2023). The plasmin generation assay published in the paper is a novel assay for which the substrate was designed by the Synapse Research Institute, the Netherlands (inventor and patent holder of the thrombin generation assay used throughout the project). Collaborators from Synapse were authors on the paper and future collaborations are expected and awaited on the plasmin generation assay in the future.