NKFIH 129528 “Structural and functional aspects of fibrin and NET interactions”
FINAL REPORT

The results of the funded two-year project were published in 8 full-length papers in peer-reviewed journals with a total impact factor of 29.9 (2 D1, 3 Q1, 3 Q2). In addition, 5 abstracts were presented as talks or posters at two international scientific congresses organised by the International Society on Thrombosis and Haemostasis. The impact of the performed research is reflected in the number of independent citations (51) received in the first year after publication of the papers reporting results of the completed project. One of the papers (**#3**) was listed as a highly cited paper by the Clarivate Web of Science; as of September/October 2020 it received enough citations to place it in the top 1% of the academic field of Clinical Medicine based on a highly cited threshold for the field and publication year.

The research activities in the 1st year of the project were completed in full agreement with the workplan and the formulated objectives, whereas in the 2nd year of the project we were delayed by the COVID-19 lockdown in March-June, 2020, which was reflected in the delay of the publications too, but the work continued in line with the formulated objectives. We requested extension of the project and within the approved 3-month extension period by the end of the second year of the project all undertakings were completed (one publication related to objective #2 is pending, but the results were presented as a talk at the virtual ISTH congress 2020 (**abstract #4**, which has already received 1 citation).

In line with **objective #1** of the project we investigated the neutrophil extracellular traps (NETs)-related structural features of thrombi retrieved from different arterial localizations and their interrelations with routinely available clinical data and reported the results in **paper #2**. The ultrastructure and cellular composition of thrombi has a profound effect on the outcome of acute ischemic stroke (AIS), coronary (CAD) and peripheral artery disease (PAD). Thrombi extracted from AIS (n=78), CAD (n=66) or PAD (n=64) patients were processed for scanning electron microscopy, (immune)stained for fibrin, citrullinated histone H3 (cH3) and extracellular DNA. Fibrin fiber diameter, cellular components, DNA and cH3 were measured and analyzed in relation to clinical parameters. We found that DNA was least present in AIS thrombi showing a 2.5-fold lower DNA/fibrin ratio than PAD, whereas cH3 antigen was unvaryingly present at all locations. The NET content of thrombi correlated parabolically with systemic inflammatory markers and positively with patients’ age. The median platelet content was lower in PAD (2.2%) than in either AIS (3.9%) or CAD (3.1%) and thrombi from smokers contained less platelets than non-smokers. Fibrin fibers were significantly thicker in male patients with CAD (median fiber diameter 76.3 nm) compared to AIS (64.1 nm) or PAD (62.1 nm) and their diameter correlated parabolically with systemic inflammatory markers. We concluded that the observed NET-related variations in thrombus structure shed light on novel determinants of thrombus stability that eventually affect both the spontaneous progress and therapeutic outcome of ischemic arterial diseases.

When we evaluated the correlations between quantitative characteristics of the thrombus structure, in many subgroups of patients emerging from their clinical features the sample size was rather limited. In such cases, the adequacy of the regression model benefits from a preliminary screening of the input sample for non-informative, misleading and/or erroneous data points, known as outliers. In fact, the proper identification and rejection of outliers contributes to the quality of the regression model a lot more than the size of the input sample. Therefore, we developed a novel approach to reject such outliers in fuzzy datasets and thus improve the quality of the sample that is superior to a procedure that has insufficient rejection of outliers (thus aiming to maintain somewhat larger sample size). We published this approach in a high-impact journal in the field of artificial intelligence (**paper #7**). We divided the outlier detection procedure into cycles and each cycle consisted of two phases. In Phase 1 we applied a leave-one-out procedure for each non-outlier in the data set. In Phase 2, all previously declared outliers were subjected to a multiple testing procedure controlling the false discovery rate, and the non-confirmed outliers could return to the data set. Finally, we constructed a regression model over the resulting set of non-outliers. In that way we

ensured that the identified correlations between the composition and structure of thrombi were based on reliable and high-quality regression models.

In addition to the disease states discussed above, thrombosis is a frequent complication in malignancy. We addressed the contribution of NETs to cancer-associated thrombosis in a study reported in **paper #3**. Pancreatic cancer is associated with high incidence risk of venous thromboembolism (VTE). A recent study showed that increased plasma levels of the NET biomarker, citrullinated histone H3 (H3Cit), are associated with VTE in pancreatic and lung cancer but not in other types of cancer. In the study reported in **paper #3**, we examined the contribution of neutrophils and NETs to venous thrombosis in nude mice bearing human pancreatic tumours. We found that tumour-bearing mice had increased circulating neutrophil counts and granulocyte-colony stimulating factor, neutrophil elastase, H3Cit and cell-free DNA compared with controls. In addition, thrombi from tumor-bearing mice contained increased neutrophils, as well as higher levels of H3Cit and extracellular DNA. Thrombi from tumour-bearing mice also had thinner fibrin fibers consistent with increased thrombin generation. Importantly, neutrophil depletion and administration of DNase I reduced the thrombus size in tumour-bearing but not control mice. We concluded that neutrophils and NETs enhance venous thrombus formation in mice bearing human pancreatic tumours.

Our earlier studies have shown that in thrombi the polyanionic DNA confers mechanical and lytic resistance to fibrin and heparins interfere with the effects of NET components. Heparins are polyanions used not only as therapeutic agents, but they are also released by mast cells at entry sites of pathogens. Related also to **objective #1**, we continued the investigation of alternative polyanions. Platelets and microorganisms release a different type of polyanions (polyphosphates) of various size (in the range 60-1000 phosphate monomers). Within the framework of the current project we evaluated if the stability of fibrin is influenced by the type of polyanion, its molecular size or relative electric charge and reported the results in **paper #4**. Fibrin structure was approached with scanning electron microscopy (SEM) and pressure-driven permeation. An oscillation rheometer was used to investigate viscoelastic properties. Kinetic turbidimetric assays for the generation and dissolution of composite fibrin clots containing unfractionated heparin (UFH), and its partially or fully desulfated derivatives, as well as low molecular-weight heparin (LMWH), pentasaccharide (S5), and polyphosphates composed of 45 (P45), 100 (P100) or 700 (P700) monomers at average. The smaller polyanions P45, P100, LMWH, and S5 accelerated, whereas P700 and UFH retarded clot formation. All polyanions altered the fibrin structure: SEM and clot permeation showed thicker fibers with smaller (LMWH, S5, P700) or larger (UFH, P100) pores. All polyanions stabilized the clots mechanically, but the smaller P45, P100 and LMWH decreased the deformability of fibrin, whereas the large UFH and P700 increased the maximal bearable deformation of clots. Despite the size-dependent structural changes, all heparins caused a 10-15% prolongation of lysis-times with plasmin, and UFH-effects depended on sulfation patterns. The 20-35% prolongation of lysis-times caused by all polyphosphates was a kringle-dependent phenomenon, and was dampened in the presence of 6-aminohexanoate blocking the lysine-binding sites of plasmin. We concluded that polyanions of different chemical structure stabilize fibrin clots via size-dependent modulation of fibrin structure and kringle-dependent inhibition of plasmin-mediated fibrinolysis.

A logical continuation of this line of investigation was the work on the interference of heparins with the effects of NET components (histones and DNA) on fibrin structure and fibrinolysis. The results of this work were summarized in **paper #8**. In this study we characterized the combined effects of NET-components (DNA and histones) and polyanions (heparin derivatives and polyphosphates) on fibrin clot structure, mechanical properties and lytic susceptibility. Scanning electron microscopy, pressure-driven permeation, turbidimetry, oscillation rheometry were used for the characterization of the structure, viscoelasticity and kinetics of formation and lysis of fibrin and plasma clots containing histones+/-DNA in combinations with unfractionated heparin, its desulfated derivatives, low molecular-weight heparin (LMWH), pentasaccharide and polyphosphates of different sizes. Histones and DNA inhibited fibrin lysis by plasmin, but this behavior was not neutralized by negatively charged heparins or short polyphosphates. Rather, fibrin lysis was further inhibited by added polyanions. Histones inhibited plasma clot lysis by tissue plasminogen activator and the response to added heparin was size dependent. Unfractionated heparin, LMWH and pentasaccharide

had no effect, exacerbated or reversed histone inhibition, respectively. Histones increased the mechanical strength of fibrin which was exacerbated by smaller heparin and polyphosphate molecules. Histones increased fibrin diameter and pore size of fibrin clots and this effect was neutralized by all heparin variants but enhanced by polyphosphates. Despite their common polyanionic character, heparins and polyphosphates exert distinct effects on fibrin mechanical and fibrinolytic stability. Anti-fibrinolytic effects of histones were more often enhanced by polyanions not counteracted. We concluded that careful selection of anti-histone strategies is required if they are to be combined with thrombolytic therapy.

We could successfully complete the work related to **objective #3** and published the results in **paper #5**, in which we characterized the formation, structure, mechanical properties and lysis of fibrin clots generated by one of the virulence factors of Staphylococcus aureus. S. aureus causes localized infections or invasive diseases (abscesses or endocarditis). One of its virulence factors is staphylocoagulase (SCG), which binds prothrombin to form a complex with thrombin-like proteolytic activity and leads to uncontrolled fibrin generation at sites of bacterial inoculation. In the reported study recombinant SCG was expressed in Escherichia coli, purified and the amidolytic activity of its complexes with human prothrombin (SCG-PT) and thrombin (SCG-T) was determined using human thrombin as a reference. Fibrin clots were prepared from purified fibrinogen and human plasma using thrombin, SCG-PT or SCG-T as a coagulase. The kinetics of clot formation and lysis by tissue-type plasminogen activator (tPA) were monitored with turbidimetric assays. Fibrin ultrastructure was examined with scanning electron microscopy and small-angle X-ray scattering (SAXS). Fibrin clot porosity was characterized with fluid permeation assays, whereas the viscoelastic properties and mechanical stability were evaluated with oscillation rheometry. Compared to thrombin, the amidolytic and clotting activity of SCG-PT was 1.6 to 2.5-fold lower on a molar basis. SCG-T had equivalent amidolytic, but reduced clotting activity both on pure fibrinogen (1.6-fold), and in plasma (1.3-fold). The SCG-PT and SCG-T generated fibrin with thicker fibers (10 to 60 % increase in median diameter) than thrombin due to increased number of fibrin protofibrils per fiber cross-section. According to the fluid permeability of the clots SCG-PT and SCG-T promoted the formation of more porous structures. The shear stress resistance in the pure fibrin and plasma clots generated by SCG-PT was significantly lower than in the thrombin clots (243.8±22.0 Pa shear stress was sufficient for disassembly of SCG-PT fibrin versus 937.3±65.6 Pa in thrombin clots). The tPA-mediated lysis of both pure fibrin and plasma clots produced by SCG-PT or SCG-T was accelerated compared to thrombin, resulting in up to a 2.1-fold increase in tPA potency. Our results indicate that SCG generates a thrombus scaffold with a structure characterized by impaired mechanical stability and increased lytic susceptibility. This proneness to clot disintegration could have implications in the septic embolism from endocardial bacterial vegetation observed in S. aureus infections.

We could finish the experimental work related to **objective #2**, the partial results of which were reported in an oral presentation at the XXVIIIth Congress of the International Society on Thrombosis and Haemostasis held online only because of the COVID-19 pandemic (**congress abstract #4**) and the presenting author was awarded the Eberhard F. Mammen Award for best presentation by a young investigator. With this work we examined our hypothesis that citrullinated fibrin(ogen) is present in venous thrombi, altering the structural, mechanical and fibrinolytic properties of the clots. Citrullination, the conversion of peptidyl-arginine into peptidyl-citrulline is catalyzed by PAD (peptidyl-arginyl-deiminase) enzymes. Fibrin, the main scaffold of thrombi is susceptible to PAD2/4 released during NETosis by neutrophils, which are abundant in pathological thrombi. However, no study has investigated the presence and significance of citrullinated fibrin(ogen) in thrombi formed in vivo. We performed both in vivo and in vitro experiments. Thrombi were induced in a mouse inferior vena cava stenosis model. Citrullinated fibrin(ogen) was detected with a specifically designed Western blot-based protocol. In vitro citrullination of fibrinogen was performed with purified PAD4. The structure and biomechanical properties of fibrin formed from citrullinated fibrinogen were examined with a broad range of methods (scanning-electron/laser-scanning/atomic-force microscopy-SEM/LSM/AFM, rheometry, turbidimetry, permeability measurements, small-angle X-ray scattering-SAXS, nano-thromboelastography). Citrullinated fibrinogen was abundant in thrombi formed 1-48 h post ligation, but not in concurrent plasma samples. In pure fibrin clots, SEM confirmed a 34% decrease in median

fiber diameters after extensive citrullination, while the width/length ratios and fractal geometry of fibers remained preserved according to AFM and SAXS. LSM showed increased fiber density and accordingly clot permeability declined by >50% even after milder citrullination. Mechanical stability of citrullinated clots was compromised as evidenced by a 50% decrease in maximal cantilever pulling force in nano-thromboelastography and a 30% decrease in the critical shear stress needed to disassemble clots in rheometry. Both plasmin- and tPA-mediated fibrinolysis were hindered in pure fibrin and plasma clots containing citrullinated fibrinogen. With this work for the first time, we provided evidence for the presence of citrullinated fibrin(ogen) in venous thrombi. Furthermore, we showed that citrullination contributes to a clot structure that is lysis-resistant because of higher compactness and lower porosity, but mechanically vulnerable and consequently more prone to embolization.

In order to disseminate our research results and to place the current research in the context of the state-of-the-art we published an open-access review paper (**paper #1**) on the role of NETs in thrombosis. We summarized the evidence accumulated in the past 10 years that NETs and fibrin form a composite network within thrombi, as well as the variety of molecular pathways in the NET-fibrin interactions. Besides discussing the effects of various NET components on hemostasis, our review took a closer look at the interaction of these individual effects, with novel perspectives on how the NET and fibrin networks stabilize each other, thus justifying the lines of investigation in the current project. Similarities and molecular connections were also outlined between the processes responsible for the degradation (fibrinolysis and NET lysis) as well as elimination of these networks. In addition, the complex relationship of pathogens with the NET-fibrin network was discussed, with a particular focus on the role of peptidyl-arginyl deiminases (PADs) in NET formation as well as in pathogen intrusion, where PADs act as a virulence factor expressed by bacteria – an aspect that is currently underestimated in the domain of NET research, but formed **objective #3** in the workplan of the current project. The work performed in relation to **objective #3** prompted us to initiate the publication of a collection of papers on the advances in our understanding of the links between fibrinolysis and immunity, within the framework of which **paper #6** was published. Although not an original research article, this editorial is important to raise the awareness of the professional community to the novel aspects of the role of fibrinolysis in inflammation and energize networking of professionals of two independent fields (hemostasis and immunology).

The support from NKIFH definitely contributed to the sustainability of the human resources at the hosting research group, the development of their professional career and international networking. The funded research allowed two PhD students (Farkas Veronika és Farkas Ádám) to meet the research requirements for a PhD degree. The NKFIH funding contributed to the continuing collaboration with the research teams of Drs Colin Longstaff and Craig Thelwell (National Institute for Biological Standards and Control, South Mimms, UK) and Prof Kiril Tenekedjiev (University of Tasmania, Launceston, Australia) exemplified by co-authored publications (**papers #3,4,5,7**). New collaborations were also established: with the research team of Nigel Mackman (University of North Carolina at Chapel Hill, Chapel Hill, NC, USA) (**paper#3**) and Robert Medcalf (Monash University, Melbourne, Australia) (**paper#6**) The international visibility of the research supported by NKFIH was promoted by the active networking with international professional societies. During the project the PI was repeatedly elected to act as a Co-Chair of the Fibrinolysis Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis and as a Council Member of the International Society for Fibrinolysis and Proteolysis. Furthermore the PI acted as an editor of *BioMed Research International* (a Q1 journal in the Scopus ranking for Biochemistry, Genetics and Molecular Biology), as an Editorial Board Member of the *Journal of Thrombosis and Haemostasis* (a D1 journal in Hematology) and of *Thrombosis Research* (a Q2 journal in Hematology).

Peer-reviewed papers published with the funding support from NKFIH

1. Varjú I; **Kolev K**. Networks that stop the flow: a fresh look at fibrin and neutrophil extracellular traps. *Thromb Res* 2019; 175: 182:1-11

IF2.869 (Q2 Hematology; 11 independent citations)

2. Farkas ÁZ; Farkas VJ; Gubucz I; Szabó L; Bálint K; Tenekedjiev K; Nagy AI; Sótonyi P; Hidi L; Nagy Z; Szikora I; Merkely B; **Kolev K**. Neutrophil extracellular traps in thrombi retrieved during interventional treatment of ischemic arterial diseases. *Thromb Res* 2019; 175: 46-52

IF2.869 (Q2 Hematology; 13 independent citations)

3. Hisada Y; Grover SP; Maqsood A; Houston R; Ay C; Denis F; Noubouossie DF; Cooley BC; Wallén H; Key NS; Thålin C; Farkas ÁZ; Farkas VJ; Tenekedjiev K; **Kolev K**; Mackman N. Neutrophils and neutrophil extracellular traps enhance venous thrombosis in mice bearing human pancreatic tumors. *Haematologica* 2020; 105(1):218-225

IF7.116 (D1 Hematology; 23 independent citations)

4. Komorowicz E; Balázs N; Tanka-Salamon A; Varga Z; Szabó L; Bóta A; Longstaff C; **Kolev K**. Biorelevant polyanions stabilize fibrin against mechanical and proteolytic decomposition: Effects of polymer size and electric charge. *JOURNAL OF THE MECHANICAL BEHAVIOR OF BIOMEDICAL MATERIALS* 2020; 102 Paper: 103459

IF3.372 (Q1 Mechanics of Materials; 2 independent citations)

5. Farkas ÁZ; Farkas VJ; Szabó L; Wacha A; Bóta A; Csehi L; **Kolev K**; Thelwell C. Structure, Mechanical, and Lytic Stability of Fibrin and Plasma Coagulum Generated by Staphylocoagulase From Staphylococcus aureus. *FRONTIERS IN IMMUNOLOGY* 2019: 10: 2967

IF5.085 (Q1 Immunology; 1 independent citation)

6. **Kolev K**; Medcalf RL. Editorial: Fibrinolysis in Immunity. *FRONTIERS IN IMMUNOLOGY* 2020: 11: 582
7. Nikolova N; Rodríguez RM; Symes M; Toneva D; **Kolev K**; Tenekedjiev K. Outlier detection algorithms over fuzzy data with weighted least squares. *International Journal of Fuzzy Systems* 2021; 23: in press

IF4.406 (Q2 Artificial Intelligence)

8. Komorowicz E; Balázs N; Tanka-Salamon A; Varga Z; Szabó L; Bóta A; Longstaff C; **Kolev K**. Size- and charge-dependent modulation of the lytic susceptibility and mechanical stability of fibrin-histone clots by heparin and polyphosphate variants. *J Thromb Haemost* 2021; 19: in press

IF4.157 (D1 Medicine (miscellaneous))

Congress abstracts

1. Farkas ÁZ; Farkas VJ; Szabó L; Csehi LM; **Kolev K** ; Thelwell C. Structure, Mechanical and Lytic Stability of Fibrin and Plasma Coagulum Generated by Staphylocoagulase from Staphylococcus aureus. Res Pract Thromb Haemost. 2019; 3 (Suppl. 1): 457. wileyonlinelibrary.com/journal/rth2 https://doi.org/10.1002/rth2.12229

2. **Kolev K**; Longstaff C ; Balázs N; Tanka-Salamon A; Varga Z; Szabó L; Bóta A; Komorowicz E. Biorelevant Polyanions Modulate the Structure and Stability of Fibrin in a Size- , and Charge-dependent Manner. Res Pract Thromb Haemost. 2019; 3(Suppl.1): 217. wileyonlinelibrary.com/journal/rth2 https://doi.org/10.1002/rth2.12229

3. Hisada Y; Grover S; Maqsood A; Houston R; Ay C; Noubouossie D; Cooley B; Wallén H; Key N; Thålin C; Farkas AZ; Farkas VJ; Tenekedjiev K; **Kolev K**; Mackman N. Role of Neutrophils and Neutrophil Extracellular Traps in Mice Bearing Human Pancreatic Tumors. Res Pract Thromb Haemost. 2019; 3(Suppl.1): 189. wileyonlinelibrary.com/journal/rth2 https://doi.org/10.1002/rth2.12227

4. Varju I, Sorvillo N, Cherpokova D, Farkas V, Farkas A, Komorowicz E, Feller T, Kiss B, Kellermayer M, Szabo L, Wacha A, Bota A, Longstaff C, Wagner D, **Kolev K**. Fibrinogen Is Citrullinated in Venous Thrombi and Forms Fragile Clots with Increased Resistance to Lysis, Research and Practice in Thrombosis and Haemostasis 4: (S1) pp. 5-6., 2020

(1 independent citation)

5. Lovas M, Tanka-Salamon A, Szabó L, **Kolev K**. Polyphosphate Nanoparticles Are More Potent Stabilizers of Fibrin than Linear Polyphosphates and Histones Enhance their Effect, Research and Practice in Thrombosis and Haemostasis 4: (S1) pp. 364-365., 2020