Final Report on NKFIH K132623 project entitled “Development of treatment-specific diagnostic assays for personalized cardiovascular medicine”

We trust that we managed to achieve all the goals of the proposed research. In particular, we developed a method to measure the clinical efficacy of factor Xa inhibitor drugs, a challenging task with clinical impact. We provided a proof-of-concept that the developed method can be used for personalized medicine and submitted a patent application to further develop this method. We also established the basis of a similar approach for thrombin inhibitors. Our research aiming at the angiotensin converting enzyme 2 (ACE2) gained a significant interest concerning the unforeseen COVID-19 appearing after the start of our research. Furthermore, COVID-19 motivated us to use the developed methods, collected tissue samples for COVID-19 research, providing important details on human ACE2 expression, identifying circulating ACE2 as a biomarker for COVID-19 mortality and finally, identification of autoimmunity as a potential pathomechanistic step in acute COVID-19, as well as in Post-COVID symptoms.

Here in the formal report, we went through the major goals of the original proposal as listed in the workplan. It included the following major goals:

1. Determining the effectiveness of X-factor coagulation inhibitors and developing a personalized treatment method.
2. Developing a method suitable for measuring thrombin activity.
3. Developing a method suitable for measuring HMG-CoA reductase enzyme inhibition.
4. Investigating ACE2 with monoclonal antibodies, measuring activity using the ELISA method.
5. Assessing the applicability of methods developed for measuring the effectiveness of anticoagulant treatments in clinical studies.
6. In-depth examination of serum ACE2 in various clinical samples.
7. Preparing a patent application alongside publications.

Detailed research summary:

1. Determining the effectiveness of X-factor coagulation inhibitors and developing a personalized treatment method.

During the support period, we undertook a comprehensive research initiative aimed at developing a novel dilution-based assay for measuring Factor X activity and inhibition in biological samples. This work was motivated by the increasing clinical reliance on direct oral anticoagulants (DOACs), particularly those targeting Factor Xa, such as apixaban, rivaroxaban, and edoxaban. Despite their advantages over traditional anticoagulants, the lack of reliable and efficient methods to assess their efficacy has posed significant challenges in clinical settings. Our research aimed to fill this critical gap, ultimately enhancing patient safety and treatment outcomes.

Key Findings and Achievements:

Development of a Kinetic Factor Xa Activity Assay:

We successfully designed a chromogenic kinetic assay that allows for the direct measurement of Factor Xa activity in human plasma. This assay is characterized by a two-step process: first, we activated Factor X in plasma samples using Russell's Viper Venom, which catalyzes the conversion of Factor X to its active form, Factor Xa. Subsequently, we measured the activity of Factor Xa through a specific chromogenic substrate (Z-D-Arg-Gly-Arg-pNA). Our findings indicated that this assay is capable of detecting Factor Xa activity with a high degree of sensitivity, making it suitable for clinical applications.

Investigation of Dilution-Based Methodology:

One of the most significant discoveries from our research was the impact of sample dilution on apparent Factor Xa activity. We conducted a series of experiments where plasma samples were diluted in a range from 4-fold to 1024-fold. Our results demonstrated that lower dilutions resulted in decreased Factor Xa activity due to the presence of endogenous inhibitors. Note the increasing level of inhibition in parallel with increasing drug concentrations in Fig. 1.

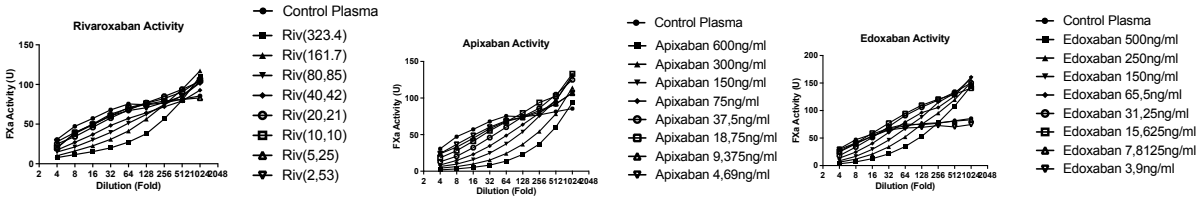

Figure 1: *Effects of factor Xa inhibitors on the enzyme activity at different dilutions.*

2. Developing a method suitable for measuring thrombin activity.

Our work on the efficacy of thrombin inhibitors is not as developed as it is in the case of the X-factor inhibitors. On the other hand, we tested the applicability of a similar method to that for factor-X inhibitors. We have got very promising results (Fig. 2). The different concentrations of the Thrombin inhibitor argatroban evoked different levels of inhibition of the enzyme activity, suggesting a good resolution of the assay. These findings suggest that a similar method can be established for thrombin than that for factor Xa inhibitors.

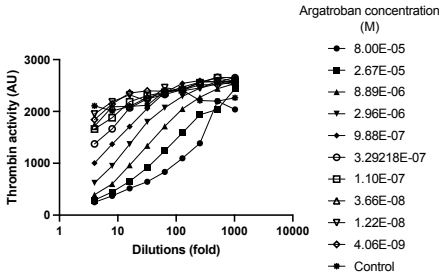

Figure 2: *Effect of argatroban on thrombin activity at different dilutions*

3. Developing a method suitable for measuring HMG-CoA reductase enzyme inhibition.

We have got suboptimal results on this goal. We set up the recombinant expression of the HMG-CoA reductase in an insect system. The recombinant enzyme was active, suitable for the determinations sought in the original proposal. However, the plasma concentrations of the statins were insufficient to be detected by the method applied above. Since the recombinant enzyme was inhibited with the expected IC_{50} values by the medical drug inhibitors, the most probable reason is that the free plasma concentration of statins is only a small percentage of the full concentration. Alternatively, statins are enriched in their target tissue (liver), therefore, they can be effective in the tissue, without having high enough concentration in the blood to be measured.

4. Investigating ACE2 with monoclonal antibodies, measuring activity using the ELISA method.

We made a significant step forward with this goal. We set up the measurement of ACE2 concentration (besides to the activity) in a variety of human (and animal) tissues. We managed to create a fairly detailed expression pattern (supported by activity) for the enzyme. These results will be detailed later in the report (see Section 6 for details).

5. Assessing the applicability of methods developed for measuring the effectiveness of anticoagulant treatments in clinical studies.

Clinical Validation of the Assay:

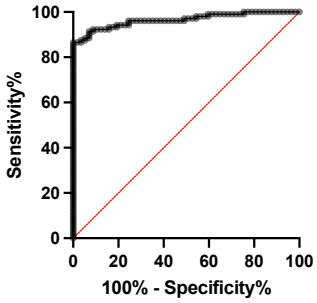

***Figure 3**: ROC analysis of identifying factor Xa inhibitor taking patients*

We conducted a clinical study involving more than 500 patients, divided into groups based on their treatment status: those receiving Factor X inhibitors and control subjects not on any anticoagulant therapy. In a particularly important sub-study, blood samples were collected at two time points: immediately before the administration of Factor X inhibitors (pre-drug) and three hours after taking the medication (post-drug). Our method significantly improved our ability to differentiate between patients on Factor X inhibitors and those not receiving treatment. Notably, we achieved a sensitivity of 87% and a specificity of 100% in identifying patients taking Factor X inhibitory medications, demonstrating the assay's efficacy as a diagnostic tool (Fig. 3).

Comparison with Traditional Assays:

We rigorously compared our dilution-based assay with traditional coagulation tests, such as activated partial thromboplastin time (aPTT) and prothrombin time (PT). Our findings revealed that these conventional assays lack the sensitivity required to detect low drug concentrations effectively, particularly below 100 ng/mL. In contrast, our dilution-based assay demonstrated the ability to detect Factor X inhibition at concentrations as low as 5 ng/mL for rivaroxaban and apixaban, and 15 ng/mL for edoxaban. This enhanced sensitivity positions our assay as a superior alternative to existing methods for assessing anticoagulant efficacy.

Our novel assay also proved invaluable in assessing the pharmacokinetics and pharmacodynamics of Factor X inhibitors.

We observed substantial variability in the efficacy of these drugs among patients, with some individuals exhibiting diminished effects at peak plasma concentrations. Most patients showed significant inhibition of Factor Xa activity at three hours post-drug administration, with a declining efficacy during the day (as expected, Fig. 4A). However, some patients demonstrated little to no change in activity during the day, indicating potential issues with over-dosing or diminished metabolism-excretion (Fig. 4B). In another

set of patients factor Xa inhibitory medication was without apparent effects (Fig. 4C) suggesting under-dosing, or diminished absorption. This variability underscores the importance of personalized treatment strategies, as our assay can be employed to tailor dosages based on individual patient responses to medication.

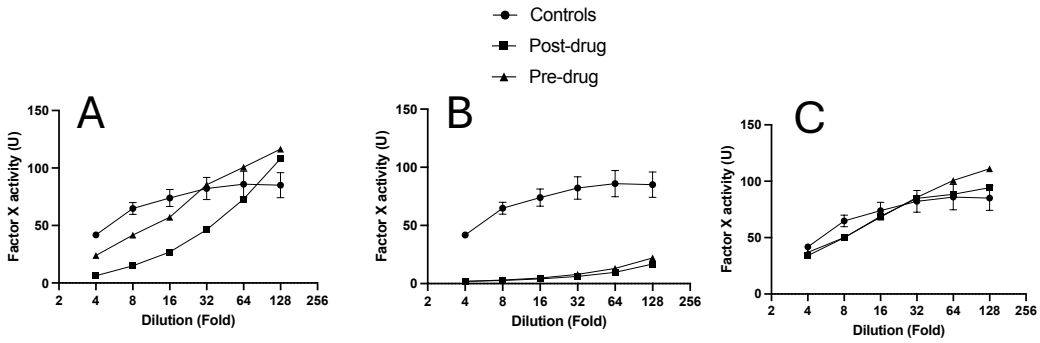

Figure 4: *Differences in factor Xa inhibitory drug effectiveness in clinical conditions*

Implications for Personalized Medicine:

The results of our research emphasize the critical role of personalized medicine in the management of anticoagulant therapy. By providing a reliable and efficient method to measure Factor Xa activity and inhibition, we can better tailor treatment plans to individual patient needs. Our assay enables healthcare providers to make informed decisions regarding dosage adjustments, particularly in patients with renal impairment, advanced age, or other factors that may affect drug metabolism. This personalized approach has the potential to improve patient outcomes and reduce the risk of adverse events associated with anticoagulant therapy.

Future Directions and Applications:

Looking ahead, we envision several applications for our dilution-based Factor Xa activity assay. The method can be integrated into routine clinical practice to monitor patients on Factor Xa inhibitors, allowing for timely adjustments to therapy based on individual responses. Additionally, the assay may be used in research settings to further investigate the mechanisms of action of Factor Xa inhibitors and their interactions with various physiological factors. Our findings also suggest that the assay could serve as a valuable tool in the identification of patients at risk for Factor Xa-related hemophilia, contributing to the broader field of coagulation research.

In conclusion, our research has led to the successful development of a novel dilution-based Factor Xa activity assay that addresses significant gaps in current methodologies for assessing anticoagulant efficacy. The findings from our studies pave the way for enhanced

patient management in anticoagulation therapy and contribute to the advancement of personalized medicine in this critical area of healthcare.

6. In-depth examination of serum ACE2 in various clinical samples.

The research proposal was submitted in early 2018. At the end of the first year, COVID-19 appeared, presenting an unprecedented health situation. We already had a research direction in line with COVID-19, since the cellular receptor (essential for cellular invasion) for SARS-CoV-2 is the ACE2. Motivated by COVID-19, we extended our proposed research efforts for in-depth clinical characterization of ACE2. We were lucky to develop the methods and techniques in the first year of the project, suitable for the timely research in the field, as part of our planned efforts in this application (see Section 4). Regarding the clinical articles published, we only want to showcase some of the most relevant ones, submitted from our laboratory.

Elevated ACE2 Activity in Aortic Stenosis Patients and Implications for COVID-19 Severity (GeroScience (2021) 43:19–29 https://doi.org/10.1007/s11357-020-00300-2)

The COVID-19 pandemic has highlighted the vulnerability of elderly patients, particularly those with pre-existing cardiovascular conditions. The angiotensin-converting enzyme 2 (ACE2) serves as the receptor for SARS-CoV-2, linking cardiovascular diseases to increased susceptibility to the virus. Our study investigated circulating ACE2 levels in patients with severe aortic stenosis (AS) and explored the implications of these levels for COVID-19 severity.

A total of 111 patients with severe aortic (AS) stenosis were enrolled and their serum ACE2 activity was measured. These patients were compared with 540 hypertensive individuals and 46 healthy controls. Various echocardiographic assessments and laboratory tests were conducted to evaluate the relationship between ACE2 activity and clinical parameters.

Major findings:

1. Patients with severe AS exhibited approximately four times higher circulating ACE2 activity (88.3 ± 61.6 mU/L) compared to hypertensive patients (20.6 ± 13.4 mU/L) and healthy controls (16.1 ± 7.4 mU/L).

2. The elevated ACE2 levels in AS patients were correlated with age, pulmonary pressure, and left ventricular dimensions, but not directly with systolic or diastolic blood pressure.
3. Notably, ACE2 activity did not significantly differ among patients treated with various RAAS inhibitors (ACEi, ARB) compared to those not on these medications.

Implications:

Elderly patients with severe aortic stenosis have significantly elevated serum ACE2 activity, which may reflect increased susceptibility to SARS-CoV-2 infections. This elevation could be attributed to factors such as age, pulmonary congestion, and cardiovascular dysfunction rather than hypertension alone. These findings suggested that circulating ACE2 activity may serve as a potential biomarker for assessing COVID-19 severity in patients with cardiovascular diseases. Understanding the relationship between ACE2 levels and COVID-19 severity in patients with cardiovascular conditions can direct clinical strategies and risk assessments for this vulnerable population.

Changes in ACE2 Levels in Cardiovascular Patients and Implications for COVID-19 (GeroScience https://doi.org/10.1007/s11357-021-00467-2)

Background:

The study investigated the role of angiotensin-converting enzyme 2 (ACE2) as a cellular receptor for SARS-CoV-2 and its modulation by common cardiovascular comorbidities. Given the higher mortality rates associated with COVID-19 in patients with cardiovascular diseases, understanding the changes in ACE2 levels could provide valuable insights for patient stratification and management.

Key Findings:

1. **Increased ACE2 Levels in Cardiovascular Patients:**

Serum ACE2 activity was significantly elevated in patients with hypertension (132% increase) and even more so in those with end-stage heart failure (689% increase). This suggests a strong correlation between ACE2 levels and the severity of cardiovascular disease.

2. **Demographic Influences:**

Factors such as male sex, obesity, and advanced age were associated with higher ACE2 levels, indicating that these demographics may be at greater risk for severe COVID-19 outcomes.

3. **Lung Cancer Impact:**

Patients with primary lung cancer exhibited higher circulating ACE2 activity, although (healthy) lung tissue ACE2 levels were unaffected by the cancer, suggesting a potential tumor-related secretion of ACE2.

4. **RAAS Inhibitors:**

The study found that the use of renin-angiotensin-aldosterone system (RAAS) inhibitors did not significantly affect circulating or tissue ACE2 levels, countering some concerns regarding the use of these medications during the COVID-19 pandemic.

5. **Correlations and Predictive Value:**

A strong negative correlation was observed between serum ACE2 activity and left ventricular ejection fraction, highlighting the potential of circulating ACE2 as a biomarker for cardiovascular disease severity and COVID-19 mortality risk.

Implications for Patient Management:

The findings underscored the importance of monitoring ACE2 levels in cardiovascular patients, particularly those at risk for severe COVID-19. Elevated ACE2 levels may serve as a biomarker for identifying patients who could benefit from more intensive monitoring and intervention strategies. Note, in a follow-up article, we proved that circulating ACE2 levels are indeed one of the most sensitive biomarkers to predict COVID-19 mortality. This work (International Journal of Infectious Diseases (2022), 115 8-16, https://doi.org/10.1016/j.ijid.2021.11.028.) has received 97 citations to this day, suggesting some significant interest and recognition.

7. Preparing a patent application alongside publications.

We managed to achieve this goal. The developed factor Xa efficacy measurement method has been submitted to the Hungarian Patent Office. Title: “Dilution-based Factor X activity assay”, Submission identifier: P2500013, Owner: University of Debrecen.

8. Miscellaneous activities

The appearance of COVID-19 motivated all of us to contribute to the better understanding of the disease. According to this need, we also performed some assays, from which I would like to highlight a singular direction with a potential long-term impact. These activities were not planned in the original (pre-COVID-19) research plan, but still, we are proud to reveal this facet of the viral infection. However, these findings are related to the project by using the methods, tissue samples and background knowledge accumulated during the implementation of the proposed research directions.

Anti-Cardiac Autoantibodies in Severe COVID-19 Patients (GeroScience https://doi.org/10.1007/s11357-022-00649-6)

Here we investigated the presence of anti-cardiac autoantibodies in patients hospitalized with severe COVID-19 in a Western blot-based approach, using the tissue samples collected for the other projects in the proposed research. Our study included 104 COVID-19 patients, alongside control groups of heart failure patients with dilated cardiomyopathy and those with severe aortic stenosis. We found that 68% of the severe COVID-19 patients developed anti-cardiac autoantibodies, with 39% exhibiting IgG and 51% showing IgM isotypes. Notably, 38% of patients targeted multiple cardiac antigens, indicating a broad autoimmune response.

Our findings suggest that severe COVID-19 is associated with the production of novel anti-cardiac autoantibodies, particularly IgM, and the reactivation of resident IgG autoantibodies. Despite these findings, the presence of these autoantibodies did not correlate with clinical outcomes or mortality rates, indicating that while autoimmunity may complicate recovery, it does not directly dictate the severity of the disease.

This research underscores the importance of understanding the autoimmune responses triggered by COVID-19, which may contribute to long-term complications such as myocarditis and dilated cardiomyopathy in survivors. Our work supports ongoing efforts to monitor and address the multifaceted impacts of COVID-19, reinforcing the need for comprehensive care strategies for affected individuals.

Research on anti-pulmonary autoantibodies in severe COVID-19 patients (GeroScience (2024) 46:1561–1574 https://doi.org/10.1007/s11357-023-00887-2)

In this retrospective study involving 104 patients with severe COVID-19, we investigated the presence of autoantibodies targeting lung tissue. Our findings revealed that more than 53% of these patients developed autoantibodies, with a predominant occurrence of the IgM class. This suggests that these antibodies were likely produced in response to the acute infection with SARS-CoV-2.

We observed a strong correlation between the presence of anti-pulmonary autoantibodies and adverse clinical outcomes. Specifically, patients with these autoantibodies exhibited worse pulmonary function, as indicated by the Horowitz index, and higher levels of multiorgan failure, measured by the SOFA score. Notably, patients categorized as "multi-producers," those with three or more distinct autoantibody clones, were older and had significantly poorer clinical outcomes, including increased mortality rates.

Among the identified autoantibodies, one specific antibody targeting a pulmonary protein of approximately 50 kDa was particularly associated with adverse outcomes. This finding raises the possibility of molecular mimicry, where the immune response to the virus may inadvertently target similar host proteins, leading to tissue damage and exacerbating the patient's condition.

The implications of our study are substantial. The development of lung-specific autoantibodies during severe COVID-19 may not only serve as biomarkers for disease severity but could also play a role in the long-term complications observed in post-COVID syndrome, commonly referred to as long COVID. The identification of these autoantibodies opens avenues for further research into their role in chronic symptoms experienced by patients after recovery from acute COVID-19.

Furthermore, our research emphasizes the potential of employing tissue proteome-wide tests to detect autoimmunity in post-COVID patients. By identifying specific autoantibodies, clinicians may better understand the underlying causes of persistent symptoms and tailor therapeutic interventions accordingly. This could lead to more effective management strategies for patients suffering from long-term effects of COVID-19, ultimately improving their quality of life.

As a matter of fact, we continued these research directions, and found significant long-term dysregulation of the immune system in post-COVID states. These suggest that

autoimmunity and humoral immune system dysregulation may not only contribute to the severity of the acute state of COVID-19, but also to the long term outcome.

Debrecen, 2025. 03. 07.

Sincerely,

Attila Tóth, PhD

Principal investigator