26TH CONGRESS OF THE INTERNATIONAL SOCIETY FOR FIBRINOLYSIS AND PROTEOLYSIS

PLASMINOGEN ACTIVATION AND FIBRINOLYSIS WORKSHOP OF THE HUNGARIAN SOCIETY OF THROMBOSIS AND HAEMOSTASIS

OCTOBER 11–14, 2023, BUDAPEST, HUNGARY

VENUE:
CENTRAL BUILDING OF THE HUNGARIAN ACADEMY OF SCIENCES
1051 BUDAPEST, SZÉCHENYI ISTVÁN TÉR 9

ISFP

SCIENTIFIC PROGRAMME, ABSTRACTS

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WELCOME FROM THE ORGANIZERS

Dear Colleagues,

It is a great pleasure and honour for me to invite you to participate in the joint meeting of the International Society for Fibrinolysis and Proteolysis, the International Workshop on Molecular and Cellular Biology of Plasminogen Activation and the Hungarian Society of Thrombosis and Haemostasis, which will take place from October 11–14, 2023 in Budapest, the beautiful capital of Hungary.

The first international conference on fibrinolysis was organized in 1972 and since then the biennial meetings have contributed largely to establish and continuously expand the international network of clinical and basic researchers who are interested in studying the physiological and pathological aspects of fibrinolysis, plasminogen activation, and extracellular proteolysis. We hope that the 2023 meeting in Budapest will continue these long-lasting traditions and will facilitate the exchange of scientific ideas and discoveries between interested scientists with a mixture of invited state-of-the-art talks and presentations of original research results. As a co-organizer, the Hungarian Society of Thrombosis and Haemostasis strongly encourages the participation of researchers from the region of Central and Eastern Europe to promote their integration in the world-wide scientific network in this exciting research field.

I hope you will join us in Budapest to experience some Hungarian hospitality.

Professor Krasimir Kolev

President of the congress

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The Hungarian Center of Excellence for Molecular Medicine (HCEMM) is a distributed Institute, whose scientists develop advanced diagnostics and treatment options in support of healthy ageing. Currently, the HCEMM Program is funded by an H2020 Teaming Grant, where Semmelweis University, the University of Szeged and the Biological Research Centre in Szeged cooperate with their advanced partner EMBL (with headquarters in Heidelberg). The support of the Hungarian government is also essential for the operation of the HCEMM, primarily through the Thematic Excellence Programme and the National Laboratories Programme. HCEMM works at the interface of academic and industrial research on topics related to Translational Medicine. The goal is to improve the quality of life for an ageing Hungarian population, while at the same time lowering the cost of health care provision through novel applications in the field of Molecular Medicine. The coordination of the various activities is managed by HCEMM Nonprofit Kft., with headquarters in Szeged, Hungary.

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This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 739593.

GENERAL INFORMATION

WIFI ACCESS AT THE VENUE:

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OPENING AND CLOSING REMARKS:

Presidential greetings: 11 October 2023, $10^{00} – 10^{15}$
Closing lunch: 14 October 2023, $12^{00} – 13^{30}$

REGISTRATION:

Wednesday,	11 October 2023	$08^{30} – 17^{00}$
Thursday,	12 October 2023	$08^{00} – 18^{00}$
Friday,	13 October 2023	$08^{00} – 16^{30}$
Saturday,	14 October 2023	$09^{30} – 12^{00}$

REGISTRATION FEES ON-SITE:

Non-member:	800 €
ISFP or HSTH (MTHT) member*:	600 €
Early-career** non-member:	600 €
Early-career** ISFP or HSTH (MTHT) member*:	500 €

**Members in good standing for 2023 are eligible for this category.*

***Resident, graduate student, postdoc (up to 5 years after PhD): a proof of status required.*

A limited number of daily tickets will be available on-site at 200 €/day. All fees include the VAT.

The registration fee includes: Access to Scientific Sessions, Poster Sessions, Opening Reception (Oct. 11, 6:30 PM), Networking Meeting (Oct. 12, 6:30 PM), Lunch on October 11th, 12th, 13th, 14th, Coffee Breaks on each day of the Congress.

Registration does NOT include access to the Gala Dinner (Oct. 13th, 7 PM), for which additional charge of 60 € applies.

Please wear the name badge you received at registration at all times during the event.

SOCIAL PROGRAMME:

Friday, October 13, 2023 $16^{30} – 18^{30}$ Visit to the Houses of Parliament

Friday, October 13, 2023 $19^{00} – 22^{00}$ Gala Dinner (Gerbeaud restaurant, 1051 Budapest, Vörösmarty tér 7–8), the band Búgócsiga will perform. No reservations for the dinner can be accepted on the spot.

You can visit the social programmes by pre-registration and by showing the QR code on your name badge.

ACCREDITATION:

Accreditation in the Hungarian Continuous Medical Education system (OFTEX): The "26th Congress of the International Society for Fibrinolysis and Proteolysis/ Plasminogen Activation and Fibrinolysis Workshop of the Hungarian Society of Thrombosis and Haemostasis" is accredited as an optional training course with code number 86770. The course is worth 18 credits, which are credited as optional points towards the qualifications listed on the OFTEX portals. Compliant by Medtech Europe Conference Vetting System

TECHNICAL INFORMATION – ORAL SESSIONS:

11 October 2023 – Reading room 1st floor
12–14 October 2023 – Grand Lecture Hall, 2nd floor
Presentations will be presented using a projector. Please give your presentation material in the form of a PowerPoint file, preferably on a USB flash drive, to the technical staff in the lecture hall at least 1 hour before the start of the session, but no later than the break before the start of the session.

TECHNICAL INFORMATION – POSTER SESSIONS:

Krudy Hall, Ground floor
The e-posters will be available for viewing all day at the Hall. The authors will be available in the indicated section for discussion and questions alongside their e-posters.

NON-MODERATED POSTER SESSIONS:

Thursday,	12 October 2023	13 15 – 13 45
Friday,	13 October 2023	11 45 – 12 15

LIABILITY AND OTHER INSURANCE:

The published conference registration and other fees do not include accident, sickness, baggage and liability insurance. In the event of accident, illness or damage, the organisers cannot provide any liability or compensation.

INFORMATION ON THE INTERNET:

https://regio10.hu/ISFP2023

GENERAL INFORMATION:

RÉGIÓ 10

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SCHEDULE AT A GLANCE

WEDNESDAY, OCTOBER 11

10 00 – 10 15	Greeting from the organizers
10 15 – 11 55	ORAL SESSION: Inflammation-related proteolysis. Interplay of plasminogen activation and the complement system
12 00 – 13 00	Lunch break
13 00 – 14 30	ORAL SESSION: Plasminogen and tranexamic acid in different clinical settings
14 50 – 15 30	Coffee break
15 30 – 17 05	ORAL SESSION: Fibrin and fibrinolysis
17 10 – 18 00	ISFP Council meeting
18 00 – 19 30	Opening reception

THURSDAY, OCTOBER 12

08 30 – 10 05	ORAL SESSION: Fibrin structure and function in physiology and pathology
10 05 – 10 45	Coffee break
10 45 – 12 20	ORAL SESSION: Fibrinolysis at the interface of immunity and infection
12 20 – 13 15	Lunch break
13 15 – 13 45	POSTER SESSION
14 00 – 15 35	ORAL SESSION: Carboxypeptidases and fibrinolysis
15 35 – 16 15	Coffee break
16 15 – 17 50	ORAL SESSION: Fibrinolysis in animal models
18 00 – 19 30	Networking Meeting

FRIDAY, OCTOBER 13

08 30 – 10 10	ORAL SESSION: Désiré Collen Young Investigator Awards competition
10 10 – 10 45	Coffee break
10 45 – 11 30	PLENARY LECTURE by the recipient of the 2023 ISFP prize
11 45 – 12 15	POSTER SECTION
12 15 – 13 30	Lunch break
13 30 – 15 05	ORAL SESSION: Hypofibrinolysis
15 30 – 16 30	ISFP general assembly
16 30 – 18 30	Social program
19 00 – 22 00	Gala dinner

SATURDAY, OCTOBER 14

10 00 – 12 00	ORAL SESSION: Thrombolytic therapy
12 00 – 13 30	Closing lunch

SCIENTIFIC PROGRAMME

WEDNESDAY, OCTOBER 11

10 00 – 10 15 **GREETING FROM THE ORGANIZERS**

10 15 – 11 55 **ORAL SESSION: INFLAMMATION-RELATED PROTEOLYSIS. INTERPLAY OF PLASMINOGEN ACTIVATION AND THE COMPLEMENT SYSTEM**

Location: (Reading room, 1st floor)

Chairs: *Evi Stavrou* and *Zoltán Prohászka*

10 15 – 10 50 *Evi Stavrou* (Case Western Reserve University School of Medicine, Cleveland, OH, US): Structural insights and targeted abrogation of the Factor XII – uPAR axis in wound healing

10 50 – 11 25 *Zoltán Prohászka* (Semmelweis University, Budapest, Hungary): Interplay of complement and haemostatic proteases in different clinical settings

11 25 – 11 45 *Iolanda Camerino* (Institute of Genetics and Biophysics, National Research Council, Naples, Italy): Modulation of glioblastoma-astrocytic cells interactions by urokinase-derived uPAcyclin decapeptide and relative variants

12 00 – 13 00 **Lunch break**

13 00 – 14 30 **ORAL SESSION: PLASMINOGEN AND TRANEXAMIC ACID IN DIFFERENT CLINICAL SETTINGS**

Location: (Reading room, 1st floor)

Chairs: *Ruby Law* and *Ian Roberts*

13 00 – 13 35 *Ruby Law* (Monash University, Melbourne, Australia): A critical role of plasminogen in infectious colitis

13 35 – 14 10 *Ian Roberts* (London School of Hygiene & Tropical Medicine, London, UK): The story of tranexamic acid: Clinical trials in acute severe bleeding

14 10 – 14 30 *Kristen A. Brown* (University of Colorado, Denver, Colorado, USA): Physiologic correction of tranexamic reversible fibrinolysis occurs in the majority of normothermic regional perfusion donors within one hour of circulatory arrest

14 30 – 14 50 *Kata Kálmán* (Semmelweis University, Budapest, Hungary): Monocyte-dependent antithrombotic effect of tranexamic acid

14 50 – 15 30 Coffee break

15 30 – 17 05 ORAL SESSION: FIBRIN AND FIBRINOLYSIS

Location: (Reading room, 1st floor)

Chairs: *Alisa Wolberg* and *Zsuzsanna Bereczky*

15 30 – 16 05 *Alisa Wolberg* (University of North Carolina, Chapel Hill, North Carolina, USA): Assessing plasmin generation in health and disease

16 05-16 25 *Cédric Duval* (University of Leeds, Leeds, United Kingdom): Thrombolysis is increased by EkoSonic Endovascular System via reduced fibrin fibre thickness and increased clot permeability

16 25 – 16 45 *Stephanie A. Smith* (University of Michigan, Ann Arbor, Mi, USA): Neutrophil serine proteases cleave fibrinogen to alter fibrin clot structure

16 45 – 17 05 *Timea Feller* (University of Leeds, Leeds, UK): The fibrin αC-region acts as a biomechanical “security latch” at high strains, but does not impact behaviour at low strain: implications for a new model of fibrin intrafibrillar structure

17 10 – 18 00 ISFP COUNCIL MEETING

18 00 – 19 30 Opening reception

THURSDAY, OCTOBER 12

8 30 – 10 05 **ORAL SESSION: FIBRIN STRUCTURE AND FUNCTION IN PHYSIOLOGY AND PATHOLOGY**

Location: (Grand Lecture Hall, 2nd floor)

Chairs: *Christopher D. Barrett* and *Nicola Mutch*

8 30 – 9 05 *Christopher D. Barrett* (University of Nebraska Medical Center, Omaha, Nebraska, USA): Fibrinolysis and thrombolytic therapy in COVID-19 respiratory failure

9 05 – 9 25 *Volodymyr Chernyshenko* (Palladin Institute of biochemistry of NAS of Ukraine, Kyiv, Ukraine): Limited proteolysis reveals fibrinogen structure

9 25 – 9 45 *János Kappelmayer* (University of Debrecen, Debrecen, Hungary): Low factor XIII levels and altered fibrinolysis in patients with multiple myeloma

9 45 – 10 05 *Ali Aftabjahani* (McMaster University, Hamilton, Canada): Fibrinogen αC domain is required for fibrinolysis up-regulation on thrombin-activated platelets

10 05 – 10 45 **Coffee break**

10 45 – 12 20 **ORAL SESSION: FIBRINOLYSIS AT THE INTERFACE OF IMMUNITY AND INFECTION**

Location: (Grand Lecture Hall, 2nd floor)

Chairs: *Kimberly Martinod* and *Paul Kim*

10 45 – 11 20 *Kimberly Martinod* (KU Leuven, Leuven, Belgium): Pathogen-driven interplay of NETs and fibrin in the virulence of Staphylococcus aureus

11 20 – 11 40 *Anna Tanka-Salamon* (Semmelweis University, Budapest, Hungary): Quantitative determination of neutrophil extracellular trap components in the systemic circulation of patients with arterial thrombosis and malignancy

11 40 – 12 00 *Sara Zalghout* (KU Leuven, Leuven, Belgium): Assessing the impact of staphyloccocal coagulases on in vitro NETs release and fibrinolysis in infective endocarditis

12 00 – 12 20 *Erzsébet Komorowicz* (Semmelweis University, Budapest, Hungary): Lytic and mechanical properties of fibrin formed by staphylocoagulase and co-polymerized with DNA and histones

12 20 – 13 15 Lunch break

13 15 – 13 45 POSTER SESSION
Location: (Krudy Hall, Ground floor)

- *Tamás Árokszállási* (University of Debrecen, Debrecen, Hungary): Acute alcohol consumption improves the outcome of intravenous thrombolysis treatment in acute ischemic stroke patients
- *Rita Orbán-Kálmándi* (University of Debrecen, Debrecen, Hungary): Spontaneous delivery without post-partum complications in a patient with congenital dysfibrinogenemia and IVF (donor embryo transfer) pregnancy
- *Désirée Coen Herak* (University of Zagreb, Zagreb, Croatia): Determinants of enhanced fibrinolysis in patients with hemophilia A and severe bleeding phenotype
- *Orr Zaacks* (Monash University, Melbourne, Australia): Setting the stage to assess the efficacy of DNase-1 as an adjunctive treatment for thrombolysis
- *Éva Katona* (University of Debrecen, Debrecen, Hungary): Incorporation of α2-plasmin inhibitor into plasma clots of patients with venous thrombosis

14 00 – 15 35 **ORAL SESSION: CARBOXYPEPTIDASES AND FIBRINOLYSIS**

Location: (Grand Lecture Hall, 2nd floor)

Chairs: *Laurent Mosnier* and *Patrizia Stoppelli*

14 00 – 14 35 *Laurent Mosnier* (The Scripps Research Institute, La Jolla, CA, USA): **TAFI and hemophilia**

14 35 – 14 55 *John Morser* (Stanford University School of Medicine, Stanford, California, USA): **Basic carboxypeptidases in plasma (CPB2 and CPN) and E. coli infection**

14 55 – 15 15 *Zikou Liu* (Australian Centre for Blood Diseases, Melbourne, Australia): **A novel plasma clot-based assay for assessing fibrinolysis: the Fluoro-Halo Lysis Assay (FHLA)**

15 15 – 15 35 *Craig Thelwell* (Medicines and Healthcare Products Regulatory Agency, Potters Bar, UK): **Development of the WHO 1st International Standard for TAFI, Plasma**

15 35 – 16 15 **Coffee break**

16 15 – 17 50 **ORAL SESSION: FIBRINOLYSIS IN ANIMAL MODELS**

Location: (Grand Lecture Hall, 2nd floor)

Chairs: *Matthew Flick* and *Roger Lijnen*

16 15 – 16 50 *Matthew Flick* (University of North Carolina, Chapel Hill, North Carolina,, USA): **The plasminogen/fibrinogen axis in the pathogenesis of obesity and metabolic syndrome**

16 50 – 17 10 *Maya Rodriguez* (Versiti Blood Research Institute of Wisconsin, Milwaukee, WI, USA): **Reduced liver tPA and higher plasma LDL particles in germ-free mice**

17 10 – 17 30 *Anton Ilich* (University of North Carolina, Chapel Hill, North Carolina, USA): **Measuring fibrinolysis in animals: adaptation and validation of two fibrinolysis assays**

17 30 – 17 50 *Michael B. Boffa* (The University of Western Ontario, London, Canada): **Prothrombotic effects of lipoprotein(a) in vitro and in a transgenic mouse model**

18 00 – 19 30 **NETWORKING MEETING**

FRIDAY, OCTOBER 13

8 30 – 10 10 **ORAL SESSION: DÉSIRÉ COLLEN YOUNG INVESTIGATOR AWARDS COMPETITION**

Location: (Grand Lecture Hall, 2nd floor)

Chair: *Ton Lisman*

8 30 – 8 50 *Anna Párkányi* (Semmelweis University, Budapest, Hungary): Viscoelastometry guided thrombolysis for pulmonary embolism – new protocol for the safety and efficiency – Preliminary results

8 50 – 9 10 *Claire S. Whyte* (University of Aberdeen, Aberdeen, UK): Plg-RKT localises plasminogen within thrombi

9 10 – 9 30 *Gael B Morrow* (University of Oxford, Oxford, UK): A novel method to quantify fibrin-fibrin and fibrin-α2AP cross-links in trauma patients; results from a sub-study of the FEISTY trial

9 30 – 9 50 *Charithani Keragala* (Monash University, Melbourne, Australia): A marked hyperfibrinolytic state explains the high D-dimer levels in patients with Vaccine-induced immune thrombotic thrombocytopenia (VITT): Evidence for the presence of a plasma cofactor that potentiates tPA-mediated plasminogen activation

9 50 – 10 10 *Mark Castleberry* (Versiti Blood Research Institute, Milwaukee, USA): Hepatocytes as a cellular origin of Serpine1 mRNA that contributes to platelet PAI-1 synthesis through apoB-lipoproteins

10 10 – 10 45 **Coffee break**

10 45 – 11 30 **PLENARY LECTURE BY THE RECIPIENT OF THE 2023 ISFP PRIZE**

Location: (Grand Lecture Hall, 2nd floor)

Chair: *Robert Medcalf*

Joost Meijers (Amsterdam UMC, Amsterdam, The Netherlands): **Factor XI: procoagulant, antifibrinolytic and a target for antithrombotic therapy**

11 45 – 12 15 **POSTER SECTION**

Location: (Krudy Hall, Ground floor)

- *Linda Lóczi* (University of Debrecen, Debrecen, Hungary): **The balance of hemostasis and fibrinolysis in patients with antibody-mediated rejection (ABMR) after kidney transplantation**
- *Rita Orbán-Kálmándi* (University of Debrecen, Debrecen, Hungary): **Alterations of fibrinolysis in SARS-CoV-2 infected pregnant women: a prospective, case-control study**
- *Rita Marchi* (University Hospitals of Geneva University of Geneva, Geneva, Switzerland): **The use of the Lysis Timer (Hyphen) in patients with bleeding disorders of unknown cause**
- *Timea Feller* (University of Leeds, Leeds, UK): Magnetic microrheology: **A tool to investigate local network mechanics**
- *Kateryna Baidakova* (Palladin Institute of biochemistry of NAS of Ukraine, Kyiv, Ukraine): **Fibrinogenases from the animal venoms in the study of fibrinogen structure and functions**

12 15 – 13 30 **Lunch break**

13 30 – 15 05 **ORAL SESSION: HYPOFIBRINOLYSIS**

Location: (Grand Lecture Hall, 2nd floor)

Chairs: *Hunter Moore* and *Helen Philippou*

13 30 – 14 05 *Hunter Moore* (University of Colorado, Denver, Colorado, USA): **The clinical relevance of differentiating hypofibrinolysis from fibrinolysis shutdown**

14 05 – 14 25 *Anders E. Aneman* (Liverpool Hospital, South Western Sydney Local Health District; Liverpool, Australia): **Fibrinolysis normalisation following cardiac bypass surgery: a prospective, observational study of recovery timeline and clinical associations**

14 25 – 14 45 *Micaella R. Zubkov* (University of Colorado, Denver, Colorado, USA): **Hypofibrinolysis is associated with portal vein thrombosis during liver transplantation**

14 45 – 15 05 *Christopher D. Barrett* (University of Nebraska Medical Center, Omaha, Nebraska, USA): **Plasminogen deficiency of pleural fluid in pleural space infections due to neutrophil inflammatory protease degradation: A cause of intrapleural lytic failure?**

15 30 – 16 30 ISFP GENERAL ASSEMBLY

16 30 – 18 30 Social program

Visit to the Houses of Parliament

Online registration for tickets required

19 00 – 22 00 Gala dinner

(Gerbeaud restaurant, 1051 Budapest, Vörösmarty tér 7–8.)

Online registration for tickets required

SATURDAY, OCTOBER 14

10 00 – 12 00 **ORAL SESSION: THROMBOLYTIC THERAPY**

Location: (Grand Lecture Hall, 2nd floor)

Chairs: *Zsuzsa Bagoly* and *Michal Zabczyk*

10 00 – 10 35 *Zsuzsa Bagoly* (University of Debrecen, Debrecen, Hungary): **Markers of fibrinolysis predicting the outcome of thrombolysis treatment in acute ischemic stroke**

10 35 – 11 10 *Michal Zabczyk* (Jagiellonian University Medical College, Krakow, Poland): **Altered fibrin clot structure and function in different clinical conditions**

11 10 – 11 30 *Dominik F. Draxler* (University of Bern, Bern, Switzerland): **The individual fibrinolytic capacity correlates with efficacy of ultrasound-assisted catheter-directed thrombolysis in patients with acute pulmonary embolism**

11 30 – 11 50 *István Szegedi* (University of Debrecen, Debrecen, Hungary): **Rotational thromboelastometry (ROTEM) measurements for the prediction of thrombolysis safety in acute ischemic stroke patients**

12 00 – 13 30 **Closing lunch**

Structural Insights and Targeted Abrogation of the FXII-uPAR axis in Wound Healing

Dillon Bohinc [1], Kara L. Bane [1], Maria de la Fuente [2], Shruti Raghunathan [3], Marvin T. Nieman [2], Coen Maas [4], Thomas Renne [5,6], Anirban Sen Gupta [3], Evi X. Stavrou [1,7]

[1] Department of Medicine, Hematology and Oncology Division, Cleveland, OH, USA; [2] Department of Pharmacology, CWRU School of Medicine, Cleveland, OH, USA; [3] Department of Biomedical Engineering Case Western Reserve University (CWRU) Cleveland, OH, USA; [4] Department of CDL Research, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands; [5] Institute for Clinical Chemistry and Laboratory Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [6] Irish Centre for Vascular Biology, School of Pharmacy and Biomolecular Sciences, Royal College of Surgeons in Ireland, Dublin, Ireland; [7] Medicine Service, Section of Hematology-Oncology, Louis Stokes Veterans Administration Medical Center, Cleveland, OH, USA

Background: We previously identified the FXII-uPAR signaling axis in neutrophils and found that it upregulates neutrophil functions. In sterile inflammation, the axis can be targeted to improve wound healing. Current strategies primarily focus on inhibition of FXII protease functions. Similarly, uPAR inhibition with antibodies can impair access of other ligands and adversely impact tissue proteolysis and fibrinolysis. To circumvent these issues, we propose to selectively abrogate the FXII-uPAR interaction. However, the precise binding sites between FXII and uPAR are not known.

Aims: To elucidate the binding interface between FXII and uPAR and create therapeutics that abrogate FXII-uPAR mediated proinflammatory effects.

Methods: We used hydroxyl radical footprinting (HRF), site-directed mutagenesis, and neutrophil functional assays to uncover the binding sites between FXII and uPAR and developed novel inhibitory peptides that selectively abrogate FXII-uPAR complex formation.

Results: The schema we pursued in solving the FXII-uPAR interface is detailed in **Figure 1A.** FXII-uPAR HRF revealed three distinct regions on FXII that contained highly protected amino acid residues **(Figure 1B).** Similar studies mapped the FXII binding sites on uPAR **(Figure 1C).** FXII amino acid residues involved in uPAR binding were replaced with alanine through site-directed mutagenesis and FXII variants were tested for their ability to bind uPAR using microscale thermophoresis. These studies provided the details for the development of FXII-derived peptide antagonists that interfered with FXII binding to uPAR **(Figure 2A)**, FXII-mediated ROS generation and NET formation in healthy human neutrophils, alone or in combination. In a murine model of diabetes, combination of FXII-uPAR inhibitory peptides, delivered within neutrophil-targeted nanoparticles, led to increased rate of wound closure **(Figure 2B)** and significantly reduced the expression of proinflammatory mediators.

Conclusions: Here we uncover the FXII-uPAR binding interface and present the design and testing of small molecule inhibitors that prevent FXII and uPAR effects in models of inflammation ex vivo and *in vivo.*

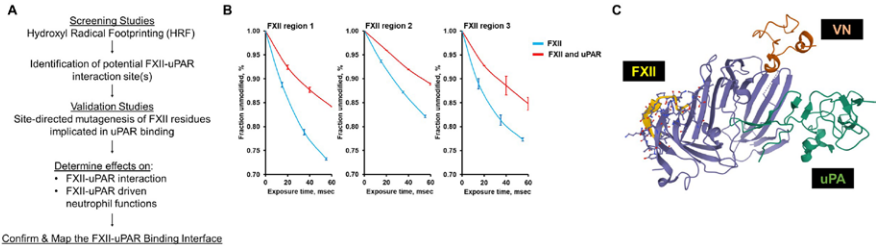
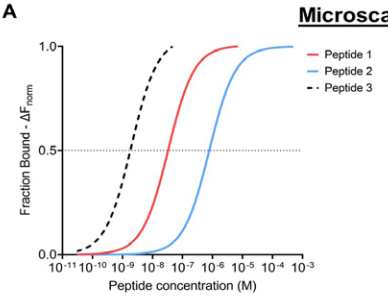


Fig 1. (A) Experimental workflow used in solving the FXII-uPAR binding interface. **(B)** Hydroxyl radical footprinting generated dose-response curves from three regions of FXII containing amino acid residues with the highest protection rates compared to the free protein. (**C**) Mapping of FXII-binding sites on uPAR are distinct from urokinase (uPA) and vitronectin (VN) binding regions.



FXII-derived peptide	Affinity for FXII (K_d, nM)
Peptide 1	33.45 ± 42.76
Peptide 2	781.8 ± 2601
Peptide 3	1.989 ± 2.466

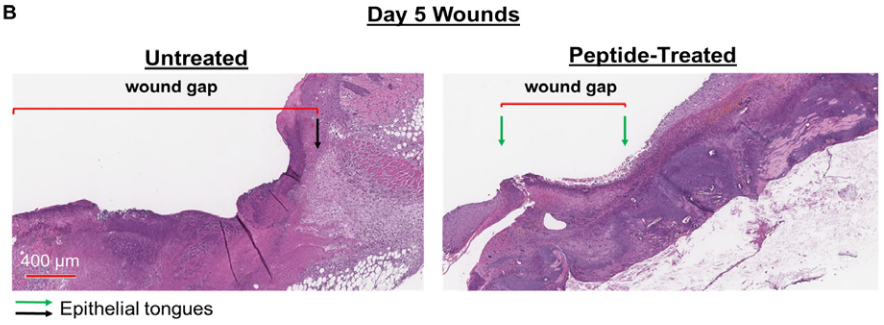


Fig 2. (A) Based on structural studies, three FXII-derived peptides were designed and tested for their ability to interfere with the FXII-uPAR interaction. **(B)** In vivo wound healing studies with FXII-uPAR inhibitory peptides were packaged in neutrophil-targeted nanoparticles for local delivery at wound sites. Day 5 histologic analysis showed that faster re-epithelialization rates in peptide-treated wounds compared to untreated wounds.

Interplay of complement and haemostatic proteases in different clinical settings

Zoltán Prohászka

Department of Internal Medicine and Hematology, Füst György Complement Diagnostic Laboratory, Semmelweis University, Budapest, Hungary

The humoral part of the intravascular innate immune system (IIIS) consists of the complex blood cascade system (the complement, the coagulation, the kallikrein/kinin [contact system], and the fibrinolytic systems) and individual proteins, mainly pattern recognition molecules and regulators belonging to the serine protease inhibitor family. Due to the common activators, to the shared scaffold surface, i.e. the endothelial cells and platelets, and to the similar regulator mechanisms, the interplay between complement and hemostasis is rich and abundant.

The current presentation will utilize illustrative case histories to highlight key aspects of the pathophisology of the IIIS, especially how severe infections and inflammation may lead immunothrombosis and multisystemic inflammation. Transplant-associated thrombotic microangiopathy (TA-TMA), severe COVID-19, and multisystemic inflammatory syndrome in children (MIS-C) will be discussed, together with observation data obtained in clinical case series studies. Our results point towards and important role of C1-inhibitor, the multi-functional serine protease inhibitor, in these diseases. Our novel methods that measure protease-inhibitor complex levels in these conditions seem to provide important observational data to support further research questions in this area.

Modulation of glioblastoma-astrocytic cells interactions by urokinase-derived uPAcyclin decapeptide and relative variants

Iolanda Camerino [1,2], Paola Franco [1], Amelia Cimmino [1], Francesca Gervaso [3], Alfonso Carotenuto [4], Luca Colucci D'Amato [2], Maria Patrizia Stoppelli [1]

[1] Institute of Genetics and Biophysics (IGB-ABT), National Research Council, Naples, Italy; [2] Dept of Environmental, Biological and Pharmaceutical Sciences and Technologies, University "L. Vanvitelli", Caserta, Italy; [3] Institute of Nanotechnology, National research Council, Lecce, Italy; [4] Dept of Pharmaceutical Sciences, University "Federico II", Naples, Italy

Background: Intensive studies on solid tumors, including Glioblastoma multiforme (GBM), that is the most devastating and widespread primary central nervous system tumor, have described a surrounding tumor microenvironment (TME), including different cell types (astrocytes, macrophages, endothelial cells). Emerging evidence shows that GBM cells progressively activate the surrounding astrocytes, enabling them to promote tumor survival, migration and invasion in the healthy tissue.

Aims: To characterize and modulate the supporting activity by astrocytic cells, human co-cultures of U87-MG and U251-MG GBM with SVG-A immortalized astrocytic cells were analyzed. Inhibition

of astrocytic pro-migratory activity was accomplished by the aid of novel mono- and di-substituted uPAcyclin decapeptides variants to identify therapeutically interesting variants.

Methods: GBM and astrocytic cell sensitivity to inhibition was tested by several migration and invasion cell-based assays and by intracellular molecular readouts. Vasculogenic assays in 3D-matrices were monitored by imaging and computational methodologies.

Results: Exposure of U87-MG and U251-MG GBM or SVG-A astrocytic human cells to nanomolar concentrations of uPAcyclin, binding to the αV integrin receptor, inhibits directional migration, matrix invasion and vasculogenic mimicry, without interfering with cell proliferation and survival. If U87-MG cells are exposed to the conditioned medium of SVG-A astrocytic cells, GBM cell migration is enhanced, unless SVG-A are pre-treated with nanomolar concentrations of uPAcyclin. Synthesis of Ala-substituted novel cyclopeptides included the uPAcyclin-Ala$^{2/5}$ and uPAcyclin-Ala^{8} decapeptides, endowed with a tenfold greater inhibitory potential of migration, invasion and vasculogenic mimicry of GBM cells, compared to uPAcyclin. In contrast, the uPAcyclin-Ala^{1} peptide is poorly inhibitory, although it shows a high affinity for the integrin target.

Conclusions: These studies shed light on the tumor-stroma interactions in GBM and indicate the functional relevance of single aminoacidic residues in uPAcyclin to identify therapeutically promising candidates for anti-GBM therapies.

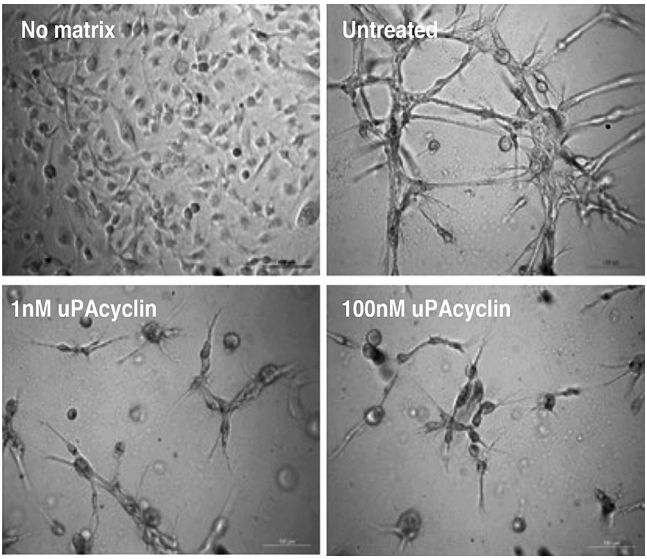


Figure 1. *Formation and inhibition of Vasculogenic Mimicry by U251-MG GBM cells*

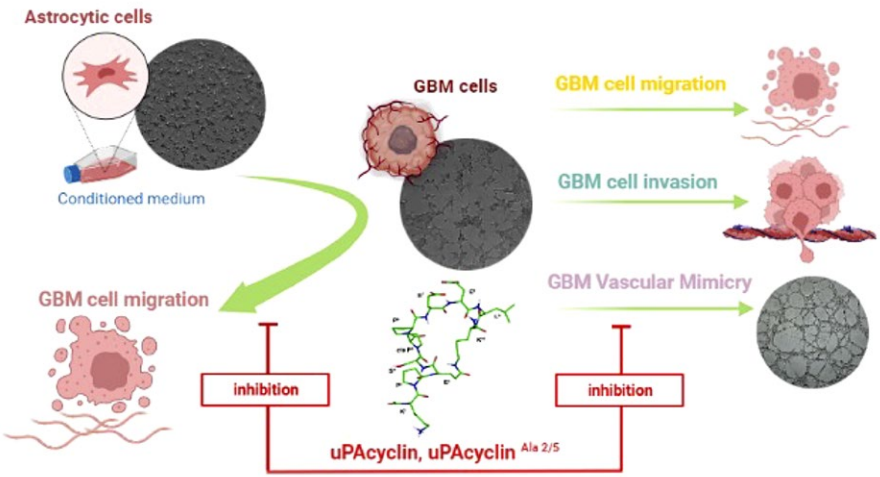


Figure 2. *The multiple activities of uPAcyclin in the GBM-astrocytic system*

A critical role of Plasminogen in infectious colitis

Ruby H.P. Law

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Viral, parasitic, or bacterial infections cause infectious colitis; *Salmonella, E. coli,* and *Clostridioides difficile* (previously known as *Clostridium difficile*) are the most common causes of this condition. These pathogens can cause inflammation and damage to the mucous membrane in the gastrointestinal tract. Among these infections, *C difficile* infection is a significant concern; it has caused three million infections and 14,000 deaths in the United States alone.

C. difficile is spread when spores are ingested. These spores are resistant to drying, heat, and disinfectants and can remain in the environment, especially in areas with infected individuals. They can also cause disease relapse, which may happen to 30% of patients.

Our work showed that human plasminogen could bind to and break down the *C. difficile* spore coat. This process facilitates spore germination, contributing to increased mobility and mortality in animal models of the disease.

We will discuss how plasminogen contributed to the spread of *C. difficile* infection and examine the effects of inhibiting plasminogen activation and plasmin activity on infection.

Physiologic Correction of Tranexamic Reversible Fibrinolysis Occurs in the Majority of Normothermic Regional Perfusion Donors within One Hour of Circulatory Arrest

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Background: Normothermic regional perfusion (NRP) is a strategy to increase organs available for transplantation after circulatory death. Following declaration of death, cannulation occurs with an oxygenating perfusion circuit to resuscitate organs. Donors receive heparin and red blood cell transfusions, allowing for real time assessment of the fibrinolytic system post circulatory death and during reperfusion. Serial coagulation assessment of NRP donors may provide insight into this process.

Aims: Determine the frequency of tranexamic acid (TXA) reversible systemic fibrinolytic activation after circulatory death and percentage of donors that spontaneously recover. Secondarily, to identify if fibrinolytic phenotype is associated with organ utilization rate.

Methods: NRP donors had blood assayed with the portable Quantra® device with QStat® cartridge. Samples were collected at initiation of perfusion, 60 minutes, and perfusion completion. Fibrinolysis was assessed using Clot Stability to Lysis (CSL), a parameter quantifying clot strength with and without TXA. The reference range is 93-100%. Donors were stratified by fibrinolytic phenotype at perfusion initiation, where CSL of 100% was hypofibrinolytic, 99-93% was physiologic, and < 93% was hyperfibrinolytic. Values >93% with subsequent blood draws were considered recovered.

Results: Nineteen donors from 12 hospitals were included for analysis; ages 18-67, 53% of livers were transplanted. Initial hyperfibrinolytic response was generated by 47% of the cohort. Of those, 75% recovered, the majority (50%) within the first hour. Organ utilization was highest in patients with physiologic CSL (Figure), followed by hyperfibrinolysis, with fewer hypofibrinolytic donors utilized. One donor who failed to correct fibrinolysis was declined.

Conclusion: Hyperfibrinolysis can be identified by a portable Quantra® device. This occurs in nearly half of NRP donors and most corrected by perfusion completion. These data support the diminished effects of TXA in reversing fibrinolytic activity as time progresses from circulatory arrest. Assessment of fibrinolysis phenotype at initiation of donor NRP may play a role in determining organ usability.

Monocyte-dependent antithrombotic effect of tranexamic acid

K. Kalman [1,2], *A. Raska* [1,2,3], *K. Balint* [1], *P. Csikos* [1], *K. Kolev* [1], *N. Wohner* [1,2,4]

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Background: Tranexamic acid (TXA) is a commonly used antifibrinolytic drug exerting its effect by inhibiting plasminogen activation and plasmin. Numerous trials showed that administration of TXA reduces bleeding, meanwhile provided no evidence on increasing thrombotic side effects, making its use in management of acute bleeding particularly appealing.

Aims: We studied effects of TXA on thrombus formation *in vivo* and *in vitro.*

Methods: Thrombus was induced with inferior vena cava (IVC) stenosis in mice. Mice received TXA injection after operation, they were sacrificed and thrombi harvested. Immediately before thrombus retrieval blood samples were taken. Plasma was prepared to analyze von Willebrand factor antigen (VWF:Ag) and monocyte chemoattractant protein-1 (MCP-1) levels via ELISA. Thrombin generation was measured in whole murine blood. White blood cells (WBCs) were harvested from murine bone marrow and incubated with plasminogen (+/- TXA, +/- N-Formylmethionine-leucyl-phenylalanine(fMLP) then added to fibrinogen and clotted with thrombin, subsequently plasminogen activation was measured.

Results: Odds for thrombus formation was 3.66 in untreated WT mice and 0.375 in TXA-treated animals. Plasma MCP-1 was 157.3±46.6 pg/mL in IVC-control and 29.8±9.3 pg/mL in TXA-treated mice (mean±SD, n=15), whereas baseline MCP-1 level was 22.1±5.6 pg/mL in non-stenotic mice. IVC stenosis did not result in any significant difference in plasma VWF:Ag levels (97.6±36.1% vs.120.7±18.6%). Thrombin generation was significantly faster in IVC stenotic mice compared to non-stenotic mice. This effect was partially reversed by TXA (time to peak values (TTP), $p<0.01$). Plasminogen activation was significantly increased on the surface of activated WBCs compared to non-activated cells, TXA treatment resulted in significant inhibition of plasminogen activation (slopes of F values plotted against time-squared $y=0.009911*x+11898$ vs. $y=0.002755*x+13183$, $p<0.001$).

Conclusion: TXA attenuates the formation of IVC stenosis-thrombi in mice. Our results provide clues for mechanistic understanding of the data from meta-analyses of clinical trials on thrombotic complications associated with TXA therapy.

Thrombolysis is increased by EkoSonic Endovascular System via reduced fibrin fibre thickness and increased clot permeability

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Background: The EkoSonic Endovascular System (EKOS) simultaneously delivers tPA and ultrasound (US) and is used for the treatment of venous thromboembolism (VTE). Despite studies supporting the use of EKOS to treat VTE, the mechanisms underlying acceleration of thrombolysis by EKOS are not fully understood.

AIMS: Quantify the *ex-vivo* effects of EKOS on tPA-mediated fibrinolysis, and on fibrin clot formation and structure.

Methods: Clots were formed from normal pooled plasma. For turbidity and lysis (+tPA) experiments, clots were formed in cuvettes containing EKOS catheters and optical density was measured at 340 nm. For scanning electron microscopy (SEM), clots were formed in open-ended tubes, with the EKOS catheter placed centrally. After ultrasound application, clots were immediately fixed and processed for SEM to analyse fibrin fibre thickness. For permeation experiments, clots were formed in cuvettes (with 4 x 1.5 mm holes drilled at the bottom) containing EKOS catheters, and after washing, the volume of liquid flowing through the clot was measured at regular interval. For all experiments, US were applied at 0, 8, 15, 30, or 47 Watts, with coolant.

Results: Application of US at 0, 9 and 15 W did not alter clot formation or lysis. However, lysis rates were significantly reduced at 30 W (0.8-fold) and 47 W (0.6-fold) US. Clot formation was unaffected by the application of US, but already formed clots exhibited a significant reduction in δOD (0.7-fold for 30 W, and 0.6-fold for 47 W) during US application, before returning to normal. SEM analysis showed that the application of 47 W US led to a 34% reduction in fibre thickness compared to no US. Average clot pore size was also increased by 26% when 47 W US was applied.

Conclusions: Our study indicates that EKOS promotes thrombolysis by thinning fibrin fibres and increasing clot permeability. These findings provide a structural underpinning for the mechanisms by which US accelerates fibrinolysis.

Neutrophil serine proteases cleave fibrinogen to alter fibrin clot structure

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Background: Growing evidence implicates neutrophils in the pathophysiology of thrombosis. With inflammation, neutrophils are recruited to injury sites, where they release neutrophil serine proteases (NSPs) from their granules such as cathepsin G (CatG), proteinase 3 (PR3), and elastase. We previously

observed cleavage of the fibrinogen αC-domain by CatG, but consequences for clot structure have not been described. The αC-domain, which plays important roles in fiber growth, mechanical stability, and susceptibility to fibrinolysis, is particularly susceptible to proteolysis due to its unstructured nature.

Aims: Evaluate the consequences of fibrinogen cleavage by NSPs on clot structure and properties.

Methods: Purified human fibrinogen (some including Alexa-Fluor488 labelled protein) was incubated with purified neutrophil CatG, PR3 or vehicle. Timed aliquots were subjected to SDS-PAGE, western blot, and N-terminal sequencing. In some experiments, proteolytic fragments were removed using concanavalin A affinity chromatography. Intact fibrinogen, PR3-cleaved fibrinogen (PR3Fg), CatG-cleaved fibrinogen (CatGFg), and CatG-cleaved fibrinogen with fragments removed were clotted with thrombin. Polymerization was monitored by turbidity and fibers imaged using laser-scanning confocal microscopy and scanning electron microscopy (SEM).

Results: Both CatG and PR3 remove the αC-domain. Thrombin-mediated polymerization of PR3Fg and CatGFg is faster than observed for control fibrinogen, with markedly more turbid clots. CGFg clots contained markedly fewer fibers on confocal microscopy, and the fibers were thinner when visualized with SEM. Removal of the released αC-domain fragments from the fibrinogen normalized the structure of the clot formed.

Conclusions: Truncation of the αC-domain by either NSP results in marked changes in clot structure. In the presence of these proteolytic fragments, fibrin polymerization is markedly impaired. Release of NSPs from activated neutrophils can modulate fibrin formation and structure, with potential consequences for immunothrombosis.

The fibrin αC-region acts as a biomechanical "security latch" at high strains, but does not impact behaviour at low strain: implications for a new model of fibrin intrafibrillar structure.

Tímea Feller [1], Helen R. McPherson [1], Simon D. A. Connell [2], Robert A.S. Ariëns [1]

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Background: High extensibility and relatively low stiffness of fibrin fibres are both essential properties to fulfil their mechanical role as an effective scaffold of blood clots. However, the structural underpinning of some of these remarkable biomechanical properties of fibrin are unknown. We hypothesise an important structural role for the fibrin αC-region in these characteristics.

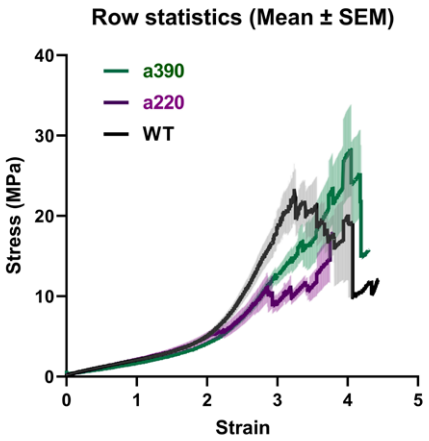
Aims: To elucidate the role of the αC-region in fibrin fibre biomechanical properties.

Methods: Either the αC-domain (a390) or the complete αC-region (a220) was truncated in recombinant fibrinogen variants. Clots were made with wild type (WT), a390 and a220 fibrinogens

on a striated surface. Individual fibrin fibres were stretched using fluorescent microscopy-combined lateral force sensing atomic force microscopy and the measured stress-strain behaviour was analysed.

Results: At strains<~1.5, fibre stiffness was similar in all variants (p>0.9999, figure) indicating that low-strain response is independent of the αC-region. At strains~1.8-2.8, fibre stiffness was somewhat lower in a390 and a220 clots compared with WT, suggesting some load bearing function of the globular αC-domain at these relatively higher strains. The fibre rupture strain was only decreased when the complete αC-region (p=0.007) but not αC-domain alone (p=0.8384) was truncated, showing a role of the flexible αC-region in fibre integrity. Rupture stress and fibre toughness decreased gradually with increasing removal of the αC-region.

Conclusion: Even though absence of the αC-region leads to apparent differences in network structure (McPherson Elife 2021), it does not lead to major differences in the biomechanical behaviour of individual fibrin fibres. Thus, other structural elements are load-bearing at low strain. We propose a novel model where the protofibril backbone is the main load-bearing element at low strain, yet branching of protofibrils enables their selective loading when the fibres are stretched. The αC-region acts as a security latch at very high strains thus contributing to fibrin's high extensibility.



Limited proteolysis reveals fibrinogen structure

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Palladin Institute of biochemistry of NAS of Ukraine, Kyiv

UDC 618.2: 577.29

Background: Proteolytic sites of macromolecules are located in the unstructured fragments or within the loops. So, information of the most probable proteolytic sites and the change of proteolysis pathway during molecular transformation can provide new information about structure of proteins.

Aim: In this work we focused on the new proteolytic sites within fibrinogen molecule to predict the structure of regions of the molecules where these sites are located.

Methods. Hydrolysis of human fibrinogen by different proteases from snake venoms, cultural liquids of bacteria or fungi was studied using SDS-PAGE (with densitometry using TotalLab TL100), Western-Blotting and MALDI-TOF. Molecular modeling was performed using I-TASSER,

Results: Analysis of hydrolysis of fibrinogen molecule allowed us to find two zones that are most exposed to proteolysis within αC-region neighboring to Aα504-505 and Aα413-414. We proposed 3D model of this structurally labile region of the molecule with the loops in these zones.

Another peptide bond that can be easily digested by proteases of different origin was Bβ42-43. The presence of a loop in this zone was assumed as the criteria for the selection of most appropriate model of 3D-structure of BβN-domain of fibrinogen.

Most remarkably protease from the venom of Echis multisquamatis changed its selectivity during fibrinogen conversion to fibrin and its polymerization. In particular the enzyme cleaved Bβ42-43 peptide bond in fibrinogen and monomeric fibrin, but not in polymerized fibrin. After fibrin polymerization studied enzyme started to cleave αC-regions. It indicated that BβN-domains are hidden in polymerized fibrin fibrils and also confirmed the formation of αC-polymers on the surface of fibrin fibrils.

Conclusions: Limited proteolysis of fibrinogen by specific proteases was assumed as a useful approach for the revealing of the structure of the fibrinogen molecule in solution.

Low factor XIII levels and altered fibrinolysis in patients with multiple myeloma

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* *Equal last authors*

Background: Acquired factor FXIII (FXIII) deficiency can be immune- or non-immune mediated and may cause severe bleeding symptoms. The incidence of acquired FXIII deficiency and its etiology in patients with multiple myeloma (MM) are poorly understood.

Aims: To assess FXIII levels and the balance of fibrinolysis in newly diagnosed, untreated MM and monoclonal gammopathy of undetermined significance (MGUS) patients.

Methods: FXIII activity, mixing studies, FXIII-A2B2 antigen, total FXIII-B antigen were measured in platelet-poor plasma from 17 untreated MM patients, 33 untreated MGUS patients, and 30 age and sex-matched healthy controls. Besides routine laboratory measurements, the balance of coagulation and fibrinolysis was evaluated using quantitative fibrin monomer (FM) test, thrombin-antithrombin assay, α2-antiplasmin activity, plasmin-α2-antiplasmin (PAP) complex, D-dimer, plasmin generation (PG) assay, clot lysis assay, and ClotPro-TPA test.

Results: FXIII-A_2B_2 levels were significantly lower in MM patients compared to controls [median (IQR):14.6 (11.2-19.4) vs. 21.8 (17.1-26.4) mg/L, p=0.0015], whereas total FXIII-B did not differ between groups. Decrease in FXIII activity was parallel to the decrease in FXIII-A_2B_2. An immune-mediated inhibitory mechanism was ruled out. Free/total FXIII-B was significantly higher in MM patients compared to MGUS and healthy controls, suggesting an etiology of consumption. In MM and MGUS patients, FM, D-dimer and PAP complex were significantly elevated compared to controls, indicating hypercoagulability and enhanced fibrinolysis.

Conclusions: Low FXIII levels due to consumption were observed in MM patients at diagnosis. Hypercoagulability and enhanced fibrinolysis were detected in MM and MGUS, indicating that a disturbed hemostasis balance is already present in the latter benign condition.

Fibrinogen αC domain is required for fibrinolysis up-regulation on thrombin-activated platelets

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Background: Platelets play a crucial role in coagulation; however, their involvement in fibrinolysis remains insufficently understood. Our recent findings revealed enhanced binding and activation of plasminogen by tissue-type plasminogen activator (tPA) on thrombin-activated platelets. This activation was likely dependent on generation of a novel C-terminal lysine residue, which is located at position 556 of fibrinogen alpha chain within the αC domain. To further validate the involvement of the αC domain of fibrinogen in regulating fibrinolysis on activated platelets, we used *Fga* ^{270} mice expressing a variant of fibrinogen with the alpha chain truncated after amino acid residue 270. We quantified plasminogen binding to platelets isolated from *Fga* ^{270}, wild-type (WT), and fibrinogen-deficient (*Fga* $^{-/-}$) mice using flow cytometry with or without activated thrombin-activatable fibrinolysis inhibitor (TAFIa).

Aims: To determine the role of the αC domain of fibrinogen in regulating platelet-dependent fibrinolysis.

Methods: Platelets were isolated from WT, *Fga* $^{-/-}$, and *Fga* ^{270} mice. Platelets were activated with thrombin (38 units/mL) for 10 minutes at 25°C. Some platelets were subsequently treated with TAFIa (18.5 nM) for 10 minutes at 25°C. To determine lysine dependence of plasminogen binding to the platelets, reaction mixtures were incubated with epsilon-aminocaproic acid (εACA) (20 mM) for 10 minutes at 25°C. These pre-treated platelets were incubated with a plasminogen derivative labeled with 5-iodacetamidofluorescein (5IAF-Pg), fixed, and analyzed by flow cytometry.

Results: *Fga* $^{-/-}$ and *Fga* ^{270} platelets exhibited significantly reduced plasminogen binding compared to WT platelets **(Fig. 1)**. TAFIa decreased plasminogen binding in activated WT platelets but had no impact on *Fga* $^{-/-}$ or *Fga* ^{270} platelets. εACA diminished plasminogen binding in all cases.

Conclusion: Our data confirms that the αC domain of fibrinogen is mediates enhanced direct binding of plasminogen to thrombin-activated platelets.

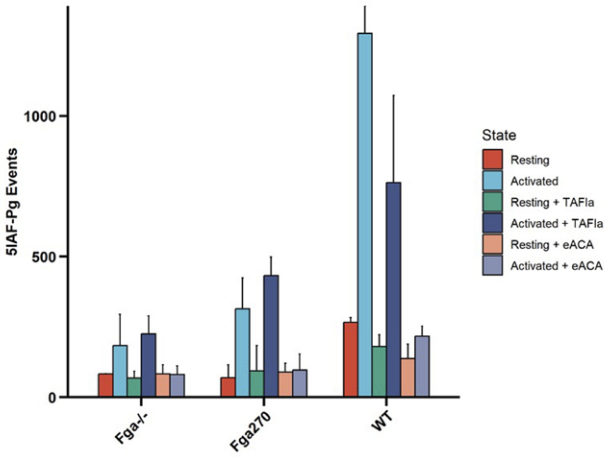

Figure 1: *Binding of plasminogen on activated platelets from WT, Fga $^{-/-}$ and Fga ^{270} mice in the presence or absence of TAFIa or εACA. Binding is shown as 5IAF-Pg Events determined by flow cytometry.*

Pathogen-driven interplay of NETs and fibrin in the virulence of *Staphylococcus aureus*

Kimberly Martinod

KU Leuven, Belgium

Dr. Martinod will discuss the contribution of neutrophil extracellular traps (NETs) to thrombosis. NETs can play a pathological role in human disease, having been shown to contribute to the detrimental host response in sepsis and in thrombus formation in both arterial and venous thrombosis. Citrullinated histones, generated during NET formation by the enzyme peptidylarginine deaminase 4 (PAD4) serve as useful biomarkers of ongoing NET formation. Studies in animal models showed a protective effect of PAD4-deficiency in conditions of venous thrombosis, MPN-driven thrombosis, as well as more chronic settings including cardiac fibrosis. We investigated the contribution of NETs in a mouse model of infective endocarditis (IE) driven by *Staphylococcus aureus.* Through neutrophil depletion experiments we found a crucial role of neutrophils in preventing IE development. Neither genetic prevention of NET formation nor deficiency in staphylococcal nucleases, indicating that key neutrophil effector functions were not from NETs. IE vegetations were surrounded by prominent areas of neutrophilic and bacterial infiltration around the aortic wall, with accompanying tissue damage. Use of a staphylocoagulase-null strain of *S. aureus* resulted in similar IE incidence but abrogated tissue damage and leukocyte recruitment around the vessel. Of note, altered thrombus composition with staphylocoagulase-deficiency was seen with more neutrophils and NETs within thrombi as opposed

to surrounding the vessel wall. This was lost in PAD4-deficient animals, indicating that NETs may be constraining infection only when fibrin is not present to hinder neutrophil access to the IE thrombus. This provides insight into potential therapeutic applications involving inhibiting NETs, and raises new questions about the contribution of staphylocoagulases in immunothrombosis.

Quantitative determination of neutrophil extracellular trap components in the systemic circulation of patients with arterial thrombosis and malignancy

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Background: Neutrophil extracellular traps (NETs) are formed in tumor tissues, arterial and venous thrombi and influence the outcome of these pathologies. The main components of NETs are DNA and attached histones, in which the arginine residues are partially converted to citrulline.

Aims: 1) To develop methods for NET-marker determination in plasma samples. 2) To quantify systemic NET-markers in arterial thrombosis (acute myocardial infarction and peripheral arterial thrombosis) with (Tu-AMI;Tu-PAT) or without (AMI;PAT) accompanying malignant tumor. 3) To investigate the lysability of plasma clots in relation to their NET-marker content.

Methods: Citrullinated H3 histone ($H3_{cit}$), double-stranded DNA (dsDNA), $H3_{cit}$-DNA complex and G-CSF plasma levels of 58 (32 AMI, 3 Tu-AMI, 14 PAT, 9 Tu-PAT) patients and 15 healthy volunteers were determined by ELISA, and Invitrogen Quant-iT Picogreen DNA reagent. The kinetics of formation and lysis of plasma clots from PAT patients was investigated by turbidimetry.

Results: The median plasma levels of the following NET markers were elevated: dsDNA 145% in AMI [130;160], 138% in Tu-AMI [131;144], 141% in PAT [135;164] and 147% [137;173] in Tu-PAT patients above their levels in healthy subjects (median[bottom;top quartile]). The H3cit levels increased in AMI (244%[128;360]) and Tu-PAT (263%[136;402]). H3cit-DNS was elevated in AMI (from zero to 3.6[0.3;7.8] ng/ml) and PAT (2.2[0;8.6] ng/ml) patients. The clotting time of recalcified plasma was reduced in all PAT patients (54%[47; 59]), while lysis of plasma clots by tissue type plasminogen activator was slowed down (lysis time increased up to 177%[125;184] in Tu-PAT patients). G-CSF levels did not increase in any patient group.

Conclusion: Only the dsDNA increased in all examined thrombotic states. The most specific NET-biomarker, the $H3_{cit}$-DNA complex was elevated in non-tumor patients. Increased stability of plasma clots containing histone and DNA implies that the elevated NET-components are not only biomarkers, but may act as pathogenic factors contributing to a systemic prothrombotic state.

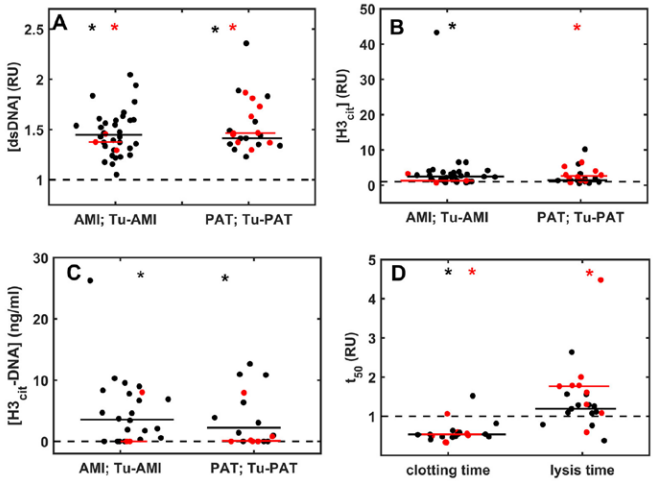

Figure Systemic NET markers in blood plasma and clotting/lysis kinetics of plasma clots

NET marker levels (A: dsDNA; B: citrullinated H3 histone (H3cit); C: H3cit-DNA complex) in plasma samples of patients with acute myocardial infarction without tumor co-morbidity (AMI, n=32) or with accompanying tumor (Tu-AMI, n=3), peripheral arterial thrombosis without tumor (PAT, n=14) or with accompanying tumor (Tu-PAT, n=9) and 15 healthy volunteers were measured. Clotting time and tissue type plasminogen activator induced lysis time of recalcified plasma from PAT, Tu-PAT patients and healthy volunteers were measured by turbidimetry (D). Black symbols indicate samples of patients without tumor, red symbols show tumor patients. Solid lines show median values, and asterisks of the same colour indicate difference significant at $p<0.05$ according to Kolmogorov-Smirnov test in comparison to the healthy control (mean shown in dashed lines).

Assessing the impact of staphyloccocal coagulases on in vitro NETs release and fibrinolysis in infective endocarditis

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Staphylococcus aureus (S. aureus) is the predominant causative organism of infective endocarditis and also a potent inducer of neutrophil extracellular traps (NETs). Multiple *S. aureus* virulence factors circumvent neutrophil-mediated defense mechanisms, including NET triggering and digestion. Others also target the coagulation system, in particular the staphylococcal coagulases [coagulase (Coa) and von Willebrand factor binding protein (vWbp)] which are involved in the generation of a fibrin shield around *S. aureus* that protect it from phagocytosis. These two virulence factors prevent NETs from

constraining the bacterial infection in our IE mouse model by restricting neutrophil access to the vegetation through the surrounding fibrin layer. As coagulases form staphylothrombin complexes that behave differently from endogenous thrombin, we hypothesized that staphylococcal coagulases could impact fibrinolysis or neutrophil-fibrin interactions in IE. Whether Coa and vWbp affect these interactions or directly alter neutrophil defense mechanisms (e.g. NET generation) (or both) remains obscure. Herein, we investigated the impact of *S. aureus* coagulases on NET release *in vitro.* Neutrophils were isolated from healthy donors and incubated with a WT laboratory strain of *S. aureus* (USA300) or the coagulase deficient strain (USA300 ΔcoaΔvwb). NET formation was quantified by measuring citrullinated histone ($H3_{Cit}$)-DNA complexes in cell culture supernatants. Both strains were able to induce NETs however, NETs release was significantly reduced by around 2-fold upon neutrophil stimulation with USA300 ΔcoaΔvwb compared to its WT counterpart **(Figure).** Similarly, the coagulase-negative staphylococcal strain *S. epidermidis* was incapable of releasing NETs, even together with platelets. Interestingly, this was not the case with eosinophils. Our results suggest that staphylococcal coagulases are critical for NET release and may indicate possible role(s) for coagulases in infectious context beyond their role in homeostasis. We are currently investigating the impact of these coagulases on fibrinolysis regulation in the IE model and their effect on neutrophil bactericidal activity.

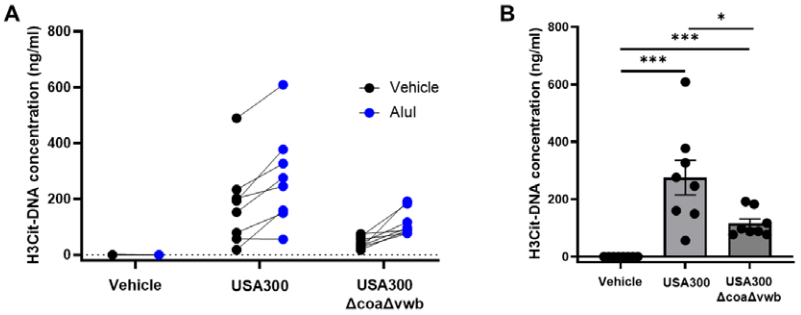

Figure. NET (neutrophil extracellular trap) release is diminished upon neutrophil stimulation by a *S.aureus* laboratory strain deficient in coagulases. In an *in vitro* NET release assay, isolated neutrophils (75 000 cells per well) from healthy volunteers were incubated with vehicle, *S. aureus* USA300 or *S. aureus* USA300 deficient in coagulases (Coa and vWbp) (multiplicity of infection [MOI] 100) for 3 hours, after which released NETs were digested by AluI (4U/ml) and measured in cell culture supernatants for H3Cit (citrullinated histone H3)-DNA complexes. **A,** H3Cit-DNA complex concentration in supernatants of paired digested (AluI, blue) vs nondigested (vehicle, black) samples (NETs or vehicle), n=8 . **B.** H3Cit-DNA concentration in AluI digested samples with corresponding mean, n=8. Each dot represents values from a single donor. Significance was determined by Mann-Whitney tests.

Lytic and mechanical properties of fibrin formed by staphylocoagulase and co-polymerized with DNA and histones

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Background: Staphylocoagulase (SCG) is a virulence factor of Staphylococcus aureus, one of the most lethal pathogens of our times. The complex of SCG with prothrombin (SCG/ProT) can clot fibrinogen, and SCG/ProT-induced fibrin and plasma clots have been described to show decreased mechanical and lytic resistance, which may contribute to septic emboli from infected cardiac vegetations. At infection sites, neutrophils can release DNA and histones, as parts of neutrophil extracellular traps (NETs), which in turn favor thrombosis, inhibit fibrinolysis and strengthen clot structure.

Aims: To characterize the combined effects of major NET-components (DNA, histone H1 and H3) on SCG/ProT-induced clot structure, mechanical and lytic stability.

Methods: Recombinant SCG was used to clot purified fibrinogen and plasma. The kinetics of formation and lysis of fibrin and plasma clots containing H1 or core histones+/-DNA were followed by turbidimetry. Fibrin structure and mechanical stability were characterized with scanning electron microscopy (fibrin fiber diameter), pressure-driven permeation (network porosity), and oscillation rheometry (viscoelastic parameters).

Results: Histones and DNA favored the formation of thicker fibrin fibers and a more heterogeneous clot structure including high porosity with H1 histone, whereas low porosity with core histones and DNA. As opposed to previous observations with thrombin-induced clots, SCG/ProT-induced fibrin was not mechanically stabilized by histones. Similar to thrombin-induced clots, the DNA-histone complexes prolonged fibrinolysis with tissue-type plasminogen activator (up to 2-fold). The anti-fibrinolytic effect of the DNA and DNA-H3 complex was observed in plasma clots too. Heparin (low molecular weight) accelerated the lysis of SCG/ProT-clots from plasma, even if DNA and histones were also present.

Conclusions: In the interplay of NETs and fibrin formed by SCG, DNA and histones promote structural heterogeneity in the clots, and fail to stabilize them against mechanical stress. The DNA-histone complexes render the SCG-fibrin more resistant to lysis and thereby less prone to embolization.

Acute alcohol consumption improves the outcome of intravenous thrombolysis treatment in acute ischemic stroke patients

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Background: Intravenous thrombolysis (IVT) by rt-PA improves the outcome in acute ischemic stroke (AIS), however, its therapeutic success is limited. Ethanol was shown to potentiate the effect of rt-PA in experimental ischemia models, moreover, human pilot trials proved safe administration of alcohol in AIS treated with IVT.

Aims: We aimed to investigate the effect of acute alcohol consumption on IVT outcomes.

Methods: A retrospective, cohort study of AIS patients treated with IVT was conducted based on the thrombolysis registry and medical electronic records of two large Hungarian stroke centres. AIS patients with detectable blood alcohol content during IVT were included as cases (alcohol group; n=60). For each case 3 control subjects who underwent IVT, but denied drinking alcohol were matched in terms of age, sex, affected brain area and stroke severity. Short- and long-term outcomes were determined using NIHSS at 7 days and modified Rankin scale (mRS) at 90 days, respectively.

Results: NIHSS at 24, 72 hours and 7 days after IVT were significantly lower in the alcohol vs. control group. Favorable long-term outcomes (mRS 0-2) were significantly more common in the alcohol group compared with controls (90% vs. 63%, $p<0.001$). Rates of hemorrhagic transformation did not differ between groups. Multiple logistic regression models identified acute alcohol consumption as significant protective factor against unfavorable short-term (OR:0.101, 95%CI: 0.040-0.255, $p<0.0001$) and long-term outcomes (OR:0.182, 95%CI: 0.062-0.535, $p=0.002$). In patients with blood ethanol levels >0.2%, significantly lower NIHSS scores were observed at 72 hours and 7 days after IVT as compared to those with admission ethanol levels of 0.01–0.2%.

Conclusion: Our study provides evidence that acute alcohol consumption significantly improves short- and long-term outcomes of IVT, without influencing safety. A randomized, controlled trial is warranted to investigate the effect of ethanol on IVT outcomes.

Spontaneous delivery without post-partum complications in a patient with congenital dysfibrinogenemia and IVF (donor embryo transfer) pregnancy

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Introduction: Congenital dysfibrinogenemia (CD) is a qualitative fibrinogen disorder characterized by normal fibrinogen antigen but low functional activity. The management of CD pregnancies is challenging, as bleeding or thrombotic risks and pregnancy-related complications are difficult to estimate.

Aim: Fibrinogen levels and fibrinolysis tests were investigated throughout the donor embryo transfer pregnancy of a woman with CD.

Patient and methods: A 33-year-old woman with CD (FGA heterozygous c.104G>A, p.Arg35His), a history of five unsuccessful assisted reproduction attempts and endometriosis underwent frozen donor embryo transfer. Before pregnancy, the patient was asymptomatic, with low functional fibrinogen levels (Clauss:0.6-0.7 g/L, antigen:2.4-3.4 g/L), other coagulopathy or thrombophilia were excluded. Besides coagulation screening tests, reptilase test, fibrinogen (Clauss and antigen), D-dimer, FVIII, plasminogen, α2-plasmin inhibitor, FXIII, plasminogen activator inhibitor-1 (PAI-1) activities, in vitro clot-lysis, ROTEM and ClotPro-tests were measured every 3 weeks. Pregnancy was closely monitored, detailed clinical parameters of pregnancy, labor, and post-partum period were registered.

Results: Fibrinogen levels increased towards the end of third trimester (Clauss:1.1-1.6 g/L; antigen:4.0-4.8 g/L). Reptilase time was prolonged throughout the pregnancy. D-dimer, FVIII and PAI-1 activity showed physiological increase, while FXIII activity decreased towards the end of third trimester. ClotPro TPA-test clotting time and maximum clot firmness was abnormal in the first two trimesters, but normalized at term. Besides few occasions of spotting in the first trimester, no bleeding or thrombotic complications occurred during pregnancy and postpartum. The patient did not receive prophylaxis or anticoagulation and delivered a healthy child per vias naturales at term (39 gw).

Conclusions: In pregnancies with CD, fibrinogen (Clauss/antigen) monitoring is suggested, and history is an important guide for management. Due to the physiological increase of fibrinogen during pregnancy, depending on CD type, the patient may be spared from unnecessary prophylaxis before childbirth and if no obstetrical contraindications arise, vaginal delivery should be encouraged.

Determinants of enhanced fibrinolysis in patients with hemophilia A and severe bleeding phenotype

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Background: Enhanced fibrinolysis leading to premature hemostatic clot lysis has been considered as an important mechanism contributing to the bleeding phenotype in hemophilia A (HA). However, factors associated with hyperfibrinolysis have not been completely determined.

Aims: The study aimed to investigate determinants contributing to enhanced fibrinolysis in HA patients.

Methods: The study group comprised of 27 healthy male controls and 62 (30 severe, 32 non-severe) HA patients, further classified according to bleeding phenotype using a modified scoring method (Humphries et al, Haemophilia 2017;23:e380-e382) that included age at first joint bleed, number of target joints and number of joint/muscle bleeds per year. Severe bleeding phenotype (bleeding score ≥5) was identified in 35/62, whereas 27/62 patients had mild bleeding phenotype (bleeding score ≤4). The investigation included the measurement of overall fibrinolysis potential (OFP), clot lysis time and fibrin clot permeability (Antovic, Semin Thromb Hemost 2010;36:772-9); activities of FVIII (one-stage method), FXIII (Berichrom FXIII, Siemens) and thrombin-activatable fibrinolysis inhibitor (TAFI, Stachrom TAFI, Diagnostica Stago); activated and inactivated TAFI (TAFIa/ai) antigen concentrations (Asserachrom TAFIa/TAFIai, Diagnostica Stago).

Results: Statistically significant difference for all parameters, except TAFI, were obtained between severe, non-severe HA patients and controls, as well as between HA patients with severe, mild bleeding phenotype and controls (Table 1). Only OFP was significantly different between patients according to bleeding phenotype (P=0.013), being higher in patients with severe bleeding phenotype (medians: 97.1% vs. 70.0%). The ROC analysis performed for discrimination of bleeding phenotype according to OFP demonstrated AUC=0.727 (95% CI: 0.564-0.856, P=0.008), sensitivity=77.8% and specificity=72.7% at a cut-off value of 84.0%.

Conclusions: Altogether, investigated fibrinolytic parameters, except TAFI, indicated enhanced fibrinolysis in severe compared to non-severe HA patients and healthy controls. Similar results obtained in bleeding phenotype groups confirmed the contribution of enhanced fibrinolysis to disease severity, revealing OFP as the best determinant of severe bleeding phenotype.

Table 1. Results of investigated parameters expressed as median and ranges in analyzed study group

Group	N	OFP (%)	CLT (min)	FCP (Ks)	FXIII (%)	TAFI (%)	TAFIa/ai (µg/L)
Severe HA patients	30	98.8 (45.5-100.0)	8.2 (3.3-21.3)	9.1 (3.1-28.0)	87.7 (54.2-145.4)	105.0 (53.7-131.0)	22.7 (16.2-40.8)
Non-severe HA patients	32	71.8 (40.9-100.0)	10.8 (6.2-20.1)	6.5 (3.1-10.2)	96.5 (48.4-156.0)	112.1 (81.7-196.6)	22.8 (10.5-30.1)
Healthy male controls	27	54.5 (23.9-100.0)	16.0 (9.2-22.0)	3.8 (1.0-17.1)	103.2 (63.4-136.1)	110.7 (88.2-187.6)	27.5 (17.9-47.8)
P (Kruskal Wallis)		**<0.001**	**<0.001**	**<0.001**	**0.006**	0.073	**0.003**
HA patients with severe bleeding phenotype	35	97.1 (44.7-100.0)	9.3 (3.3-21.9)	8.6 (3.1-28.0)	89.1 (53.2-145.4)	106.4 (53.7-162.5)	21.4 (16.2-35.7)
HA patients with mild bleeding phenotype	27	70.0 (40.9-100.0)	10.7 (3.5-19.0)	6.5 (3.1-12.3)	96.3 (48.4-156.0)	111.4 (80.9-196.6)	23.9 (10.5-40.8)
Healthy male controls	27	54.5 (23.9-100.0)	16.0 (9.2-22.0)	3.8 (1.0-17.1)	103.2 (63.4-136.1)	110.7 (88.2-187.6)	27.5 (17.9-47.8)
P (Kruskal Wallis)		**<0.001**	**<0.001**	**<0.001**	**0.021**	0.237	**0.002**

OFP: overall hemostasis potential; CLT: clot lysis time; FCP: fibrin clot permeability; TAFI: thrombin-activatable fibrinolysis inhibitor activity; TAFIa/ai: activated/inactivated thrombin-activatable fibrinolysis inhibitor antigen; HA: hemophilia A

Setting the stage to assess the efficacy of DNase-1 as an adjunctive treatment for thrombolysis

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Background: Neutrophil Extracellular Traps (NETs), which contain networks of cell-free DNA (CFDNA), are present within clots formed in patients with acute ischemic stroke (AIS) and contribute towards thrombolytic resistance. DNase-I is an endogenous endonuclease that is currently an FDA-approved treatment for patients with cystic fibrosis. We are investigating if DNase-1 could be an effective adjunctive treatment to improve the thrombolysis in patients with AIS.

Aims: To investigate the effect of DNase-I on thrombolysis of DNA containing fibrin clots.

Methods: DNA gel electrophoresis was used to determine the optimal concentration of DNase-I and investigate its activity in the presence of the thrombolytic agent tissue-type plasminogen activator (tPA) or its close variant tenecteplase (TNK). DNA gel electrophoresis, amidolytic assays (S2251) and a novel Fluoro-Halo Lysis Assay (FHLA) that uses fluorescently labelled fibrinogen incorporated in a plasma clot were used to determine whether there was any interaction between DNase-1 and tPA/TNK on plasmin generation and fibrinolysis in plasma clots.

Results: 40 U/mL DNase-1 efficiently digested 250 µg/mL calf thymus DNA in the plasma clot within 15 min. This activity was not interfered by the addition tPA or TNK (100 nM). Similarly, the fibrinolytic activity of tPA and TNK was not impacted by DNase-1 indicating that there is no interaction between the endonuclease and the plasminogen activators. Preliminary studies indicate that high concentration of DNA (250µg/ml) in plasma clots exerted partial inhibition (~10%) of both

tPA/TNK-induced fibrinolysis shown by FHLA but not when assessed using the S2251 amidolytic assay. The effect of DNase-1 in combination with tPA/TNK is still on-going.

Conclusion: The activity of DNase-1, tPA/TNK can independently function when added in combination. The presence of DNA inhibited the fibrinolytic, but not plasmin generating capacity of both tPA and TNK. The potentiating effect of DNase-1 on thrombolysis of DNA-containing plasma clots is underway.

Incorporation of α2-Plasmin Inhibitor into Plasma Clots of Patients with Venous Thrombosis

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Background: Alpha2-plasmin inhibitor (A2PI) is cross-linked to fibrin by activated factor XIII (FXIIIa). It has been shown that only cross-linked A2PI can efficiently inhibit fibrinolysis. Around 35% of secreted A2PI (PB-A2PI) cleaved in the circulation C-terminally and thus lacks the plasminogen-binding site (NPB-A2PI); it remains an active plasmin inhibitor, but reacts more slowly with plasmin. FXIIIa primarily cross-link PB-A2PI to fibrin. A2PI plasma levels have been shown to be elevated in venous thromboembolism (VTE).

Aim: To investigate the incorporation of different A2PI forms into fibrin clots of controls and patients with VTE.

Methods: Citrated plasma samples from 86 healthy controls and 84 VTE patients were clotted by thrombin and calcium. Total-A2PI and PB-A2PI levels were measured by ELISAs from the original plasma and the extruded serum samples. NPB-A2PI levels were calculated from total- and PB-A2PI values. Plasma FXIII was measured by ELISA and fibrinogen by the Clauss method. Statistical analysis was performed using the SPSS 26.0 software. Study was approved by the National Ethics Committee and informed consent was obtained from all participants.

Results: Plasma total-A2PI and NPB-A2PI levels (mean±SD) were significantly elevated in VTE patients (69±9 vs. 64±8 mg/L and 30±8 vs. 21±8 mg/L, respectively; $p<0.001$), while PB-A2PI levels were decreased (39±5 vs. 44±5 mg/L; $p<0.001$). The incorporation of NPB-A2PI did not differ significantly between controls and patients (13±4 vs. 15±7 mg/L; $p=0.099$). Although FXIII and fibrinogen levels were significantly higher in the VTE group, the amount of incorporated PB-A2PI was significantly lower (15±4 vs. 19±4 mg/L; $p<0.001$ and 39±8 vs. 44±6 % of plasma value; $p<0.001$).

Conclusion: Although the incorporation of NPB-A2PI is independent of FXIII levels, the elevation of NPB-A2PI seems to modify the covalent incorporation of PB-A2PI by FXIIIa that may have an effect on clot lysis and it should be further investigated.

Basic carboxypeptidases in plasma (CPB2 and CPN) and *E. coli* infection

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Background: There are two basic carboxypeptidases in plasma, procarboxypeptidase B2 (proCPB2; TAFI) and carboxypeptidase N (CPN). CPN is constitutively active while proCPB2 is activated to CPB2 (TAFIa) by thrombin or plasmin in the presence of thrombomodulin (TM) or glycosaminoglycans respectively. The complement anaphylatoxins, C3a and C5a, are substrates for both enzymes with the resultant products, C3adesArg and C5adesArg, being inactive. Previously we have investigated the role of CPB2 and CPN in models of vascular leak, hemolytic uremic syndrome and cobra venom factor challenge, showing that these enzymes have overlapping but non-redundant roles.

Aim: To determine the roles of CPB2 and CPN in *E. coli* infection.

Methods: E. coli was administered ip to wild type (WT), CPB2 deficient ($Cpb2^{-/-}$), CPN deficient ($Cpn^{-/-}$) and double deficient ($Cpb2^{-/-}/Cpn^{-/-}$) mice. The health of the mice using the Schrum score and their survival was followed. In some experiments mice were sacrificed at 18 hours and plasma collected for determination of CBC and markers for liver and kidney damage.

Results: $Cpb2^{-/-}$ mice had a longer median survival than WT mice while $Cpn^{-/-}$ mice had a shorter median survival time than WT with $Cpb2^{-/-}/Cpn^{-/-}$ mice having a similar median survival time to WT. The Schrum health score showed that the $Cpn^{-/-}$ mice were worse than the other groups of mice. In $Cpn^{-/-}$ mice, leukopenia was observed with a reduction in lymphocyte, monocyte and neutrophil count and in both $Cpb2^{-/-}$ and $Cpb2^{-/-}/Cpn^{-/-}$ mice an increase in liver markers (ALT, AST) was found.

Conclusions: Failure to inactivate anaphylatoxins in CPN deficiency leads to a worsening of *E. coli* infection while CPB2 deficiency improves outcomes with mice missing both enzymes having similar outcomes to WT. These results support the concept that CPN is key for inactivation of systemic C5a, whereas CPB2 functions as an on-demand supplementary anaphylatoxin inhibitor in inactivating excessive C3a and C5a formed locally.

A novel plasma clot-based assay for assessing fibrinolysis: the Fluoro-Halo Lysis Assay (FHLA)

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Background: *Ex vivo* assessment of plasminogen activator (PA) – induced fibrinolysis is crucial for guiding various clinical scenarios. Widely used methods include clot lysis assay that rely on instant formed clots where the rigidity and structural aspects of established clots are neglected, and chromogenic amidolytic assay that measures plasmin activity but not fibrinolysis *per se*. Here we present an advanced method focusing on the direct evaluation of fibrinolysis on established plasma clots.

Aims: To evaluate the feasibility and efficacy of the fluoro-halo lysis assay (FHLA) in assessing PA-induced fibrinolysis of plasma clots with and without tranexamic acid (TXA).

Methods: Human plasma was mixed with Alexa-488 labelled fibrinogen, with or without TXA, and clotted by thrombin in a halo shape on 96-well flat bottom plates. The fluorescence signal from fluorescent fibrin degradation product (FDP) in the centre area of the halo clots was recorded every minute for 1 hour after PA (alteplase or tenecteplase) addition. A fluoro-halo lysis index (FHLI) was then calculated to assess fibrinolytic capability and to compare the inhibition effects of TXA. Reaction mixtures were analysed by SDS-PAGE to detect the transformation of fluorescent components by time course.

Results: SDS-PAGE shows that Alexa-488 labelled fibrinogen was converted into insoluble network by thrombin and transformed into fluorescent FDP during fibrinolysis. Validation experiments accurately modelled fibrinolytic capability of alteplase or tenecteplase (1–100 nM). The FHLA offers same sensitivity of assessing TXA inhibition (0.4–100 µg/mL), with 98% cost reduction compared to the chromogenic amidolytic assay.

Conclusion: FHLA represents an advanced method for assessing fibrinolysis by directly measuring the destruction of *ex vivo* established fibrin clots. It also offers high throughput, high sensitivity, and cost-effectiveness. It is a valuable tool for future studies related to fibrinolysis and thrombolysis.

Development of the WHO 1st International Standard for TAFI, Plasma

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Background: The dysregulation of TAFI has been linked to various pathological conditions including thrombosis, bleeding disorders, and inflammatory diseases, and may therefore be important for the diagnosis, prognosis, and monitoring of these conditions. Variability in TAFI measurements among laboratories makes it difficult to compare results and establish accurate diagnostic thresholds. An International Standard (IS) for TAFI is required to harmonize the global measurement of TAFI in plasma.

Aims: To value-assign a candidate WHO IS for TAFI, plasma for TAFI activity and TAFI antigen in International Units (IU), and TAFI antigen in SI units (µg).

Methods: Commercial and local methods were used to measure TAFI activity and antigen in the study samples provided: candidate IS (A) normal plasma control (B) low TAFI control (C) high TAFI control (D) in addition to local fresh/frozen plasma pools (L) and local/kit calibrators (S) provided by the participants. The data was analysed centrally using a parallel line bioassay model and overall combined robust geometric means were calculated. TAFI was calculated in SI units using multi-dimensional liquid chromatography and triple quadrupole tandem mass spectrometry with stable isotope-labelled (SIL) TAFI. Traceability was by reference to calibrators of TAFI-depleted plasma spiked with a defined amount of purified TAFI (value assigned by amino acid analysis).

Results: A summary of the TAFI potency values calculated in the study is shown in **Table 1.** When the study data was reanalysed relative to the candidate IS (A) inter-laboratory variability was reduced when compared to the results relative to local or commercial calibrators, and the results were consistent with the values assigned independently by quantitative mass spectrometry.

Conclusion: Sample A (17/200) is suitable for establishment as the WHO 1st International Standard for TAFI, Plasma with assigned values for TAFI activity of 0.87 IU and for TAFI antigen of 0.92 IU and 7.43 (7.05-7.82) µg per ampoule.

Table 1. A summary of estimates for TAFI activity and antigen for Sample A (relative to Sample L with a nominal potency of 1.0 unit/ml) and for Samples B, C and D relative to Sample A. Potency estimates from the collaborative study were calculated from the robust geometric mean (GM) results of all valid assays together with the 95% confidence intervals (95% CI). The TAFI antigen values based on quantitative mass spectrometry are provided with the calculated coefficient of variation (CV%) values.

	TAFI activity		TAFI antigen (units)		TAFI antigen (µg) – collaborative study data		TAFI antigen (µg) – quantitative MS	
Sample	Robust GM (95% CI)	n	Robust GM (95% CI)	n	Robust GM (95% CI)	n	Mean (CV)	n
A	0.87 (0.84 – 0.89)	9	0.92 (0.87 – 0.99)	9	-		7.43 (4.5%)	3
B	0.94 (0.92 – 0.97)	8	1.00 (0.79 – 1.04)	6	8.08 (7.79 – 8.39)	6	7.88 (2.3%)	2
C	0.35 (0.30 – 0.40)	8	0.36 (0.28 – 0.48)	7	2.92 (2.21 – 3.86)	7	3.06 (18.0%)	3
D	0.60 (0.59 – 0.61)	9	0.73 (0.67 – 0.79)	7	5.83 (5.45 – 6.23)	7	6.07 (4.4%)	3

Reduced liver tPA and higher plasma LDL particles in germ-free mice

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Background: SARS-Cov-2 or rhinovirus infection have a high prevalence of adverse blood clotting and impaired fibrinolysis. Fibrinolysis is initiated by tPA converting plasminogen to plasmin, whereas PAI-1 inhibits tPA. Low density lipoprotein (LDL) is a carrier of microbial small RNA (msRNA) and transport RNA to immune cells. RNA viruses often hijack the lipid metabolism system to sustain their infection. However, the mechanism between fibrinolysis, lipoprotein, and immune response is unclear.

Aims: We are using germ-free mice as a model lacking immunological stimulus to investigate changes in fibrinolysis-related proteins and lipoproteins.

Methods: Germ-free mice are born, housed, bred, and euthanized in an axenic containment. This environment reduces immunological stimuli from infection. Germ-free mice were age, weight, gender and genetic background matched to the control mice raised in a non-germ-free environment.

Results: Comparing to the control mice, germ-free mice have a 2.16-fold higher tPA mRNA levels in the livers ($p<0.001$), without any difference in the PAI-1 mRNA levels. Low density lipoprotein receptor (LDLR) is the major receptor that clears LDL particles from the circulation through hepatocytes. LDL receptor-related protein 1 (LRP1) is required for fatty acids-induced tPA expression in hepatocytes, and LDLR facilitates LRP1-mediated endocytosis. Germ-free mice have lower liver LDLR levels in both mRNA and protein levels than their controls. Apolipoprotein B is the scaffold protein that holds the structure of LDL particles, which carry cholesterol, triglycerides and other proteins and RNAs in the circulation. Germ-free mice have lower plasma apoB and total cholesterol levels compare to their controls. Separating lipoprotein fractions by size-exclusion fast protein liquid chromatography (FPLC) revealed the LDL fractions in germ-free have higher cholesterol.

Conclusion: Germ-free mice have higher tPA mRNA levels in the livers and higher LDL particles in circulation and lower LDLR in the livers, indicating a slower LDL clearance rate.

Measuring fibrinolysis in animals: adaptation and validation of two fibrinolysis assay

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Global assays designed to evaluate fibrinolysis have provided insights into clot formation and resolution in humans. However, adaptation and validation of these assays for animal models remain limited. In light of this, we explored the use of two global tests of fibrinolysis, namely the plasma euglobulin clot lysis time (ECLT) and the whole blood halo assay, for use in animal samples. We performed the ECLT assay using a 96-well format and automatic readout on murine and canine plasma. Additionally, we adapted the halo assay, originally intended for human whole blood, in murine and canine samples.

Blood from mice and dogs was collected into tri-sodium citrate in line with ethical guidelines. The ECLT assay was performed following our previously published protocol (Ilich et al., *RPTH*, 2019). Our

findings demonstrated pronounced inter-species differences in mean ECLT values (human vs. murine vs. canine, [hours±SEM] 16.8±6.6 vs. 1.1±0.6 vs. 2.6+-0.9, Kruskal–Wallis test p<0.0001, Figure 1).

The halo assay (Bonnard et al., Sci Rep, 2017), which assesses fibrinolysis in whole blood, was performed with modifications, including the addition of human plasminogen to the sample, reduced sample volumes, and extending readout to 12 hours. The halo assay revealed no substantial differences between species but showed the capability to demonstrate hypofibrinolysis in a murine model of sickle cell disease (SS) (Figure 2A) and hyperfibrinolysis in Factor XIII deficient (FXIII-/-) mice (Figure 2B). Antifibrinolytic agents such as tranexamic acid (TXA) were detected with similar sensitivity in animal and human samples (data not shown). A trend towards hyperfibrinolysis was observed in hemophilic dogs (n=3) compared to healthy controls (n=3) (Figure 2C).

We conclude that plasma and whole blood global fibrinolysis assays may be adapted to animal models, thereby providing valuable tools for future hemostasis research.

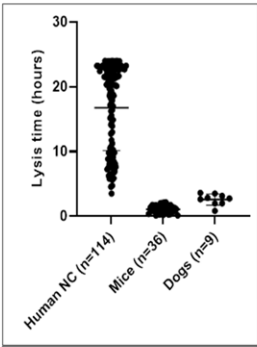

Figure 1. ECLT in normal human controls (NC), murine, and canine plasma, presented as time to 50% lysis (in hours), mean+-SEM.

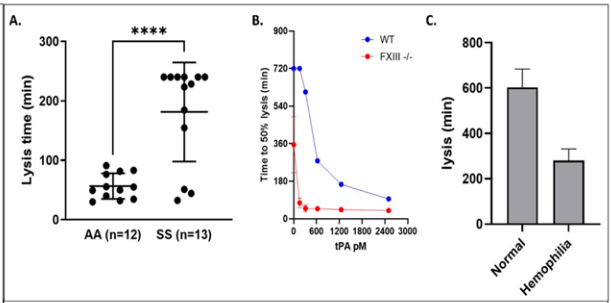

Figure 2. Modified halo assay in murine and canine models, presented as time to 50% lysis (in minutes). A – Sickle cell disease murine model; lysis time values between SS (mice with human hemoglobin S) and AA (mice with human hemoglobin A), challenged with 2.4 nM human tPA, **** - p<0.0001. B – FXIII deficient mice; lysis time values are plotted against various human tPA concentrations, WT - wild type. C – hemophilia A dogs vs. normal dogs, human tPA 1.2 nM, mean+-SEM, n=3, non-significant.

Prothrombotic effects of lipoprotein(a) in vitro and in a transgenic mouse model

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Background: Elevated levels of lipoprotein(a) (Lp(a)) are an independent and likely causal risk factor for atherothrombotic events. However, the impact of Lp(a) on coagulation and fibrinolysis remains controversial.

Aims: Evaluate the effects of Lp(a) on coagulation, fibrinolysis, thrombosis and thrombolysis *in vitro* and in a novel transgenic mouse expressing human Lp(a).

Methods: *(i)* Turbidometric clot lysis assays were performed using Lp(a)-deficient human plasma in the presence and absence of plasma-derived Lp(a). *(ii)* Lp(a)-deficient human blood containing or lacking added Lp(a) was rotated in a Chandler loop. Thrombi were added to autologous plasma containing tPA in the presence or absence of Lp(a); lysis was measured by release of fluorescent fibrin degradation products. *(iii)* Ten-week-old transgenic mice expressing human apo(a) and apoB100 (i.e., Lp(a)) and littermate controls expressing only human apoB100 were anesthetized and intravenously injected with fluorescent antibodies against platelets and fibrin. After laser injury of mesenteric vessels, intravital video microscopy of the resultant thrombi was performed to quantify platelet and fibrin volumes.

Results: *(i)* In plasma clot lysis assays, 250 nmol/L Lp(a) accelerated thromboplastin-initiated clot formation by 25% ($p<0.01$) but had no effect on lysis time. *(ii)* In the Chandler loop studies, inclusion of 250 nmol/L Lp(a) in the blood significantly decreased clot lysis (up to 15% reduction versus control; $p<0.05$), whereas Lp(a) added to the autologous plasma after thrombus formation had no effect. *(iii)* Following laser injury, thrombi in male Lp(a) mice ($n=6$) had significantly larger platelet (up to 49%; $p<0.01$) and fibrin (up to 54%; $p<0.05$) volumes than controls ($n=6$).

Conclusions: We have demonstrated prothrombotic effects of Lp(a) *in vitro* and, for the first time, *in vivo* in an animal model. Any attenuation of fibrinolysis by Lp(a) is likely attributable to effects on thrombus structure, rather than inhibition of tPA-mediated plasminogen activation.

Viscoelastometry guided thrombolysis for pulmonary embolism – new protocol for the safety and efficiency – Preliminary results

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Background: Thrombolytic therapy is only recommended in high-risk pulmonary embolism (PE) due to the bleeding risk. According to the literature, the risk of major haemorrhage is 13% among patients receiving rtPA treatment.

Aims: We aimed to increase the safety and efficacy of thrombolysis by using the 3rd generation viscoelastic tests.

Methods: Adult patients diagnosed with PE between December 2021 and May 2023 were enrolled in our randomized interventional study, performed at Semmelweis University Department of Anaesthesiology and Intensive Therapy.

In the viscoelastometry-guided group (VGG) viscoelastometric tests were performed hourly during thrombolysis. Echocardiography was performed every two hours to assess the right ventricular (RV) function. The dose of alteplase was modified based on the results.

In the control group (CG), the standard thrombolysis protocol (100 mg/2h alteplase) was performed, regardless of the viscoelastic measurement results.

Results: A total of 24 patients were eligible. 8 were excluded due to lack of consent, active bleeding or intermediate-low risk. 6 patients were randomized to the CG, while 10 patients were randomized to the VGG. The length of the thrombolysis was longer in the VGG compared to the CG (8.60 ± 1.76 h vs. 2 h), however, the cumulative rtPA dose was smaller (36.70 ± 11.48 mg vs. 100 mg). In the CG only 4 patients showed early (<24h) RV function improvement, in two cases, the RV dysfunction persisted despite the lysis. In contrast, in the VGG group RV dysfunction was dissolved in all cases, except for one patient with known chronic thromboembolic pulmonary hypertension. Severe, life-threatening bleeding occurred in two cases in the CG and one intracranial hemorrhage event occurred in the VGG.

Conclusions: The viscoelastometry-guided prolonged thrombolysis applied by our group seems to be safer and more efficient compared to the widely used protocol.

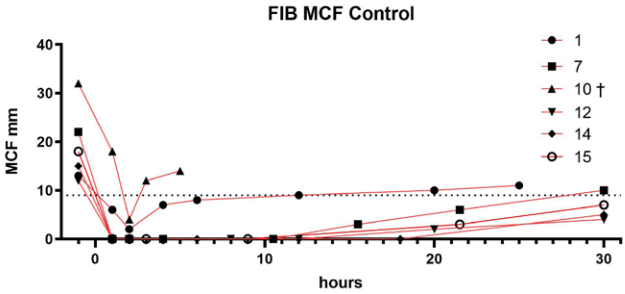

Figure 1. Thrombolysis performed according to the European Society of Cardiology guideline:100 mg alteplase in two hours. Alteplase administration was started at "0" hour. Four out of six patients showed prolonged (> 20 h) low level of maximal clot firmness (MCF) due to hypofibrinogenaemia after thrombolysis.
"•••••" indicates the lower limit of the normal range of maximal clot firmness on the FIB test (MCF>9 mm).

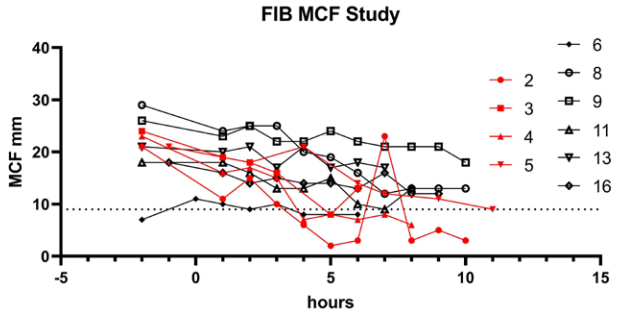

Figure 2. Viscoelastometry guided thrombolysis. Alteplase administration was started at "0" hour. The initial dosage was reduced from the patient "6" (50 mcg/kg*h (BW) or 2 mg/m^2*h (BSA)).
"•••••" indicates the lower limit of the normal range of maximal clot firmness on the FIB test (MCF>9 mm).

Plg-RKT localises plasminogen within thrombi

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Background: Binding of both exogenous and platelet-derived plasminogen is enhanced on the surface of activated platelets promoting plasmin generation. Plg-R_{KT} is a lysine-dependent transmembrane plasminogen receptor partially responsible for this binding.

Aim: To define the importance of Plg-R_{KT} in localising plasminogen within the thrombus microenvironment in ex vivo and *in vivo* models.

Method: Thrombi were formed in an *ex vivo* microfluidic model over collagen and tissue factor at 250 – 1000 s^{-1} using whole blood from wild type (WT) or Plg-$R_{KT}^{-/-}$ deficient mice. Dylight633 (DL633)-labelled plasminogen and AlexaFluor488-fibrinogen were incorporated ± tissue plasminogen activator (tPA). Thrombus formation was imaged using an EVOS fluorescence microscope. Thrombosis was induced in the carotid artery using 30% FeCl3 with prior infusion of AlexaFluor647-labelled fibrinogen and DyLight488-GP1bβ antibody to label platelets. After 5 min tPA was infused and real-time thrombus formation monitored for 30 min using a Zeiss Examiner upright intravital microscope equipped with spinning disk confocal.

Results: Plasminogen accumulation in thrombi formed ex vivo was attenuated in Plg-$R_{KT}^{-/-}$ deficient mice compared to WT mice. This effect was more apparent at low shear rate (250 s^{-1}). Upon inclusion of tPA, fibrin persistence was prolonged in Plg-$R_{KT}^{-/-}$ mice compared to WT. In the $FeCl_3$ induced injury model initial platelet accumulation within thrombi was significantly faster. Following infusion of tPA, fibrin(ogen) accumulation in Plg-$R_{KT}^{-/-}$ mice was significantly increased compared to WT. This indicates that in Plg-$R_{KT}^{-/-}$ mice the fibrinolytic potential was reduced tipping the balance in favour of fibrin formation.

Conclusion: Under physiological flow conditions, plasminogen is incorporated into developing thrombi. Plg-R_{KT} enhances plasminogen retention within the thrombus and limits thrombus persistence. A novel *in vivo* thrombolysis model reveals that Plg-R_{KT} influences initial platelet accumulation and fibrin deposition. These data demonstrate the functional importance of Plg-R_{KT} in localising plasminogen within thrombi influencing the thrombus architecture *in vivo.*

A novel method to quantify fibrin-fibrin and fibrin-α2AP cross-links in trauma patients; results from a sub-study of the FEISTY trial

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Background: Traumatic haemorrhage accounts for 4.9 million deaths every year. Due to widespread use of the anti-fibrinolytic agent, tranexamic acid (TXA), it is difficult to measure fibrinolytic resistance in trauma patients using conventional laboratory assays. At the final stage of coagulation, Factor XIIIa (FXIIIa) catalyses the formation of fibrin-fibrin and fibrin-α_2-antiplasmin (α_2AP) cross-links to increase clot stability. Developing a method to quantify fibrin-fibrin and fibrin-α_2AP cross-links overcomes the challenges posed by TXA on determining fibrinolytic resistance.

Aims: To develop a novel method to quantify fibrinolytic resistance and subsequent management in severely injured patients enrolled to the Fibrinogen Early in Severe Trauma Study.

Methods: Fibrinogen gamma chain (FGG-FGG), fibrinogen alpha chain (FGA-FGA) and FGA-α_2AP cross-links were quantified using ultra-performance liquid chromatography on a Fusion Lumos orbitrap in paired plasma samples pre- and post-fibrinogen replacement. Differences in the number of cross-links in trauma patients who received cryoprecipitate or fibrinogen concentrate (Fg-C) were analysed.

Results: There was a 1.8-fold increase in FGG-FGG, 2.2-fold increase in FGA-FGA and 1.4-fold increase in FGA-α_2AP cross-links post cryoprecipitate transfusion ($p < 0.0001$). Following Fg-C transfusion, there was no change to the number of cross-links. There was a significant increase in FXIII post-cryoprecipitate transfusion (1.3-fold; $p < 0.05$), whereas a significant decrease was observed in the Fg-C cohort (0.8-fold; $p < 0.05$). Furthermore, a strong positive correlation between the number of FGG-FGG cross-links and FXIII plasma level was observed in the cryoprecipitate, but not the Fg-C, cohort ($r^2 = 0.8$; $p < 0.05$). The number of cross-links was also positively correlated with the rate of thrombin generation ($p < 0.05$).

Conclusion: We have developed a novel method that overcomes the action of TXA to quantify fibrin cross-links in trauma patients. Using this novel approach we have shown that cryoprecipitate increases fibrin-fibrin and fibrin- α_2AP cross-linking when compared to Fg-C, which may impact the clot's susceptibility to fibrinolysis.

A marked hyperfibrinolytic state explains the high D-dimer levels in patients with Vaccine-induced immune thrombotic thrombocytopenia (VITT): Evidence for the presence of a plasma cofactor that potentiates tPA-mediated plasminogen activation

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Background: Vaccine-induced immune thrombotic thrombocytopenia (VITT) is a rare complication of adenovirus vector-based COVID-19 vaccines. VITT is associated with markedly raised levels of D-dimer yet how VITT modulates the fibrinolytic system is unknown.

Aims: We compared changes in fibrinolytic activity in plasma from patients with VITT, patients diagnosed with venous thromboembolism post-vaccination but without VITT (VTE-no VITT), and healthy vaccinated controls. A proteomic study of various immune markers in plasma was also included.

Methods: Plasma levels of plasmin-antiplasmin (PAP) complexes, plasminogen, and alpha2-antiplasmin (A2AP) from 10 patients with VITT, 10 patients with VTE-no VITT, and 14 healthy vaccinated controls were evaluated by ELISA and/or Western blotting. Fibrinolytic capacity was evaluated by quantitating PAP levels at baseline and after *ex vivo* plasma stimulation with 50 nM tissue-type plasminogen activator (tPA) or urokinase (uPA) for 5 minutes. Proteomic analyses was performed by Proximity Extension Assay screening of 192 markers of inflammation.

Results: Baseline PAP complex levels in control and VTE-no VITT individuals were similar but were ~7-fold higher in plasma from VITT patients ($p<0.0001$). VITT samples also revealed consumption of A2AP and fibrinogenolysis consistent with a hyperfibrinolytic state. Of interest, VITT plasma produced significantly higher PAP levels after *ex vivo* treatment with tPA, but not uPA compared to the other groups, suggesting the presence of a tPA-specific cofactor. Addition of *ex-vivo* D-dimer to VTE-no VITT plasma failed to potentiate tPA-induced PAP levels. Proteomic analyses has recently been obtained and preliminary analysis has revealed marked increases in expression pattern of some key mediators in the VITT cohort, including IL-10 and P-selectin.

Conclusion: A marked hyperfibrinolytic state occurs in patients with VITT and is associated with an unidentified plasma cofactor, unrelated to D-dimer, that potentiates tPA-mediated plasminogen activation. This finding explains the high D-dimer levels in patients diagnosed with VITT.

Hepatocytes as a cellular origin of Serpine1 mRNA that contributes to platelet PAI-1 synthesis through apoB-lipoproteins

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Background: Plasminogen activator inhibitor-1 (PAI-1) is a circulating serpin inhibitor that plays a critical role in hemostasis through its ability to inhibit the fibrinolytic activity of tissue plasminogen activator. Circulating PAI-1 protein is primarily found in platelets; anucleate cells produced by their megakaryocyte precursors. Although platelets cannot synthesize mRNA *de novo*, they possess the ability to sustain protein expression for up to ten days utilizing stored mRNA transcripts. I have previously published multiple studies that demonstrate that apoB-lipoproteins carry endogenous RNAs, mediating a dynamic intercellular RNA transport network between disparate cell types. Most apoB-lipoproteins are made in hepatocytes, where *Serpine1* (gene for PAI-1) mRNA transcript is also expressed. Hepatocyte-originated RNAs have showed to be transported by lipoproteins and regulate protein synthesis in recipient cells.

Aims: We are investigating the cellular sources of platelet PAI-1 protein and *Serpine1* mRNA transcripts, and evaluating the consequences of deleting *Serpine1* in megakaryocytes or hepatocytes on platelet PAI-1 synthesis, fibrinolysis and thrombosis.

Methods: We analyzed PAI-1 protein and *Serpine1* mRNA in the lipoprotein fractions and washed platelets from human blood donors, and from Hepatocyte-*Serpine1* knockout and control mice, generated by administering *Serpine1*$^{fl/fl}$ mice with an AAV8-TBG-Cre, or AAV8-TBG-GFP, respectively.

Results: We found that hepatocyte-derived apoB-lipoproteins carry abundant *Serpine1* mRNA, with the most in very-low-density lipoprotein (VLDL), which showed to increase PAI-1 mRNA intracellular levels. Western blot analyses indicate that apoB, the primary scaffold protein of VLDL, is found in washed platelets. Hepatocyte-*Serpine1* KO mice have lower circulating PAI-1 concentration by 70% than controls, indicating a contributory role of hepatocytes in platelet PAI-1 synthesis. Hepatocyte-*Serpine1* KO mice also have 50% lower intraplatelet PAI-1, 55% lower circulating *Serpine1* mRNA, lower VLDL secretion, and 20% lower plasma cholesterol.

Conclusion: We identified hepatocytes as a cellular origin of *Serpine1* mRNA that contributes to platelet PAI-1 synthesis.

The balance of hemostasis and fibrinolysis in patients with antibody-mediated rejection (ABMR) after kidney transplantation

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Background: Antibody mediated rejection (ABMR) is a common cause of graft loss. The development of *de novo* anti-HLA donor specific antibodies (DSAs) is associated with poor outcomes in kidney transplant recipients. It is surmised that an interaction between DSAs and the graft endothelium cause tissue injury, however, the exact underlying pathomechanism and optimal management of patients with DSAs remain undetermined.

Aims: We hypothesized that in kidney transplant recipients the presence of DSAs induce endothelial cell damage, leading to hypercoagulability and altered balance of fibrinolysis, contributing to ABMR.

Methods: In this observational cohort study, 21 kidney transplant recipients with DSAs (DSA+ group) and 20 age- and sex-matched transplant recipients without DSAs (DSA- group) were enrolled. Venous blood samples were obtained at baseline and the following measurements were carried out: routine laboratory tests including CRP, von Willebrand factor antigen (VWF), soluble E selectin (sEsel), thrombin generation assay, in vitro clot lysis assay (CLA). In order to correlate results with potential changes in DSA status over time, patients were followed and blood samples were taken again 6±1.5 months later.

Results: CRP, VWF and sESel levels did not differ between groups. As compared to DSA-patients, peak thrombin was significantly increased in the DSA+ group at baseline (median:349.6 [IQR:275.9-386.3] vs. 379.4 [IQR:342.7-436.6] nM, respectively, $p<0.028$). Similar results were found at follow-up. CLA parameters did not differ between DSA+ and DSA- groups at either blood sampling occasion. The extent of DSA positivity correlated positively with ETP, while tacrolimus levels negatively correlated with ETP and VWF antigen levels. CLA parameters did not correlate with DSA or tacrolimus levels.

Conclusions: In patients with DSAs, hypercoagulability was observed, potentially influencing the extent of tissue injury and ABMR. The balance of fibrinolysis, as detected by CLA, was not altered by the presence of DSAs in this patient cohort.

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Alterations of fibrinolysis in SARS-CoV-2 infected pregnant women: a prospective, case-control study

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Background and aim: Coronavirus disease-19 (COVID-19) is associated with disturbed coagulation and fibrinolysis balance. We aimed to investigate COVID-19-associated fibrinolysis alterations in third trimester pregnancies and their associations with the clinical course and post-partum hemostasis events.

Methods: In this observational case-control study, 100 women with acute COVID-19 infection at 24–40 gestational weeks (COVID-19+ group) and 95 healthy age and gestational week matched pregnant women (COVID-19- group) were enrolled. All women were outpatients with mild/no symptoms at admission. Acute infection was confirmed/ruled out using SARS-CoV-2 RT-PCR and/or antigen test. In addition to screening tests of coagulation, a comprehensive set of fibrinolysis markers including D-dimer, plasminogen activity, α2-plasmin inhibitor (α2PI) activity, FXIII activity and FXIII-A_2B_2 antigen, plasminogen activator inhibitor-1 (PAI-1) activity and antigen levels, in vitro clot-lysis were measured. Detailed clinical parameters of pregnancy, labor and post-partum period were registered.

Results: Clot-lysis times (CLT) were significantly shorter in the COVID-19+ group as compared to controls (50%CLT median [IQR]: 25 [21-42] min vs. 46 [41-58] min, respectively, p<0,001). A significant decrease in plasminogen activity was observed in the COVID-19+ group compared to control pregnancies (COVID-19+: 162 [IQR:143-190] % and COVID-19-: 174 [IQR:164-197] %, p= 0.002). In case of more severe COVID-19 (stage 2 disease), FXIII levels and plasminogen activity showed a significant decrease as compared to mild cases (stage 1 disease). Fibrinogen, D-dimer, PAI-1 activity and antigen levels did not differ between the groups. In the COVID-19+ group, postpartum hemorrhage (PPH) developed in 4 cases, associated with significantly reduced plasminogen and α2PI levels as compared to those without PPH. Thrombotic events did not occur in either group.

Conclusions: In this cohort, third trimester COVID-19+ pregnancies were associated with marked hemostasis alterations and hyperfibrinolysis. In patients with PPH, reduced plasminogen and α2PI levels were observed.

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The use of the Lysis Timer (Hyphen) in patients with bleeding disorders of unknown cause

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Background: Viscoelastic hemostatic assays are commonly used for the evaluation of fibrinolysis. More recently, the lysis timer, which assesses the global fibrinolytic capacity has been introduced with clinical setting purpose. A subset of patients with bleeding disorders of unknown cause (BDUC) tends to hyperfibrinolysis.

Aims: To compare the lysis time (LT) of a healthy control population with a group of BDUC patients.

Methods: Plasma was obtained from citrated blood at 3.2% sodium citrate by double centrifugation at 2500 g x 15 min at 20 °C. The lysis time (min) was measured in the Lysis Timer device (Hyphen Biomed) in a healthy blood donors (n = 145), and in BDUC patients (n = 15). The controls plasma (Hyphen) were also run. A mixture of tPA and silica (100 µL) was added to 100 µL of plasma, and incubated during 1 min at 37°C in the Lysis Timer. Then 100 µL of thrombin and calcium was added, and the progression of the reaction was measured until the complete dissolution of the plasma clot. The LT is measured from the primary derivatives of the light transmittance variation curve calculated by the software of the Lysis Timer. Samples were runned by duplicates. Fibrinolysis was also assessed by turbidity, and the time for 50% clot degradation (T50) was recorded.

Results: The LT was 41 (SD 6) and 34 (5) min in control and BDCU patients, respectively ($p<0.0001$). The controls provided by Hyphen: hyperfibrinolysis 15.3 (0.0) min, normal 37.9 (2.5) min, hypofibrinolysis 87.7 (6.4) min. The T50 from BDUC was 61 (14) min [controls: 66 (26)], and the Pearson correlation coefficient (r) between BDUC LT and T50 was 0.747.

Conclusions: The Lysis timer seems able to detect hyperfibrinoylsis in BDUC patients. An ongoing prospective study (NCT05713734) will assess the properties of the Lysis timer in a large cohort of patients with bleeding tendency.

Magnetic microrheology: A tool to investigate local network mechanics

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Background: Viscoelastic behaviour of blood clots is a complex result of the mechanical behaviour on multiple levels of organisation. It can be investigated at the fibre level with atomic force microscopy lateral fibre pulling or at the network level with methods measuring bulk viscoelastic behaviour like

rheology or thromboelastography. An important intermediate step between sub-micron single fibres and millimetre sized clots is the viscoelastic behaviour at the microscale.

Aims: To understand clot viscoelastic behaviour at the local network level using a novel home-built 2D magnetic microrheology system.

Methods: Paramagnetic microbeads of 4.5 µm in diameter were embedded in the clot by mixing them with the fibrinogen solution before initiating the clotting process with thrombin. We used both passive and active microrheology to characterise the mechanical behaviour of clots.

Results: For passive microrheology, a novel high frequency camera with frame rates up to 6000 Hz was used. The Brownian motion of the bead was tracked and used to calculate the viscous properties of the clot surrounding the bead. For active rheology, a magnetic force was applied to move the bead. From the resistance of the bead against the movement, and its time-dependant recovery, frequency-dependent viscoelastic properties (loss and storage moduli) of the clot were calculated. The size of the microbeads is approximately in the size-range of red blood cells (RBCs), thus by applying forces on them we can model conditions RBCs might experience under flow.

Summary/Conclusion: Simultaneous active and passive microrheology can be used to investigate the mechanical properties and behaviour of fibrin network at the microscale level revealing fundamental mechanistic detail. Together with the extremely low sample volume (~10–20 ul), magnetic microrheology is a powerful tool to provide complex viscoelastic characterisation of not only fibrin, but other soft hydrogels and biological polymers.

Fibrinogenases from the animal venoms in the study of fibrinogen structure and functions

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Background: Fibrinogen-specific proteases are prospective tools for study of the fibrinogen structure and functions during blood clotting. These enzymes also can be used for dissolving of blood clots.

Aim: Obtaining and characterization of fibrinogenases from snake and spider venoms and study of their action on fibrinogen molecule.

Methods: Crude venoms of *Bitis arietans, Gloydius halys, Vipera renardi, Vipera berus, Vipera lebetina, Echis multisquamatis* and *Brachypelma smithi* were fractionated using ion-exchange chromatography followed by size-exclusion chromatography using the FPLC system and analyzed by SDS-PAGE. Fibrinogen-specific proteases were detected using enzyme-electrophoresis. The specificity to

fibrinogen chains was determined by Western-Blot analysis using monoclonal antibodies targeted to Bβ- or Aα-chains of fibrinogen. Products of fibrinogen hydrolysis were detected by MALDI-TOF analysis.

Results: Purified fibrinogenases from the venoms of *B. arietans* and *E. multisquamatis* preferentially cleaved the N-terminal fragments of Bβ-chains of fibrinogen molecule. Fibrinogenases from other studied sources cleaved Aα-chains of fibrinogen molecule, hydrolyzing Bβ-chains after longer incubation. Interestingly enough most of enzymes were targeted to Bβ42-43 or Aα413-414.

Conclusions: Purification and characterization of fibrinogenases from studied venoms allowed obtaining a library of proteases targeted to different regions of fibrinogen molecule which provides an opportunity to conduct fundamental research and study of fibrin polymerization processes. All fibrinogenases must be assumed as potential fibrinolytic agents that could be tested in animal models of thrombosis.

Fibrinolysis normalisation following cardiac bypass surgery: a prospective, observational study of recovery timeline and clinical associations

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Background: Fibrinolysis, the natural process of blood clot breakdown, can be sensitively measured using rapid point-of-care viscoelastic testing with ClotPro® technology and the associated TPA-test®. Tranexamic acid is routinely given to mitigate hyperfibrinolysis during cardiac surgery requiring cardiopulmonary bypass, and to minimise post-operative bleeding. The postoperative kinetics of fibrinolysis normalisation and the clinical associations are undetermined.

Aims: To evaluate the kinetics of fibrinolysis following cardiac surgery with cardiopulmonary bypass and the correlations to organ function, risk scores and length of stay in the Intensive Care Unit (ICU-LOS).

Methods: Fibrinolysis was sequentially measured by the lysis time of the ClotPro TPA-test (TPA-LT). The associations between TPA-LT and biochemical markers of organ function, risk scores and ICU-LOS were determined using Spearman's correlation test, rho (r).

Results: Patient cohort characteristics are provided in Table 1. In 31 patients with a short ICU LOS (£ 4 days), fibrinolysis normalised more rapidly than in 29 patients requiring extended ICU aftercare, with a significant difference observed from the first postoperative day **(Figure 1).** In all patients, impaired fibrinolysis on Day 1 correlated with highest creatinine ($r=0.67$, $p<0.001$), lowest bicarbonate ($r=-0.37$, $p<0.01$), highest urea ($r=0.49$, $p<0.001$), Sequential Organ Failure Assessment (SOFA) score ($r=0.43$, $p<0.001$), Acute Physiology and Chronic Health Evaluation III (APACHE III) score ($r=0.48$, $p<0.001$), Australia and New Zealand ICU Risk of Death score ($r=0.58$, $p<0.05$), and ICU-LOS

(r=0.52, p<0.001). The pre-operative serum creatinine was not a significant mediator (p<0.18) of the association between TPA-LT and ICU LOS.

Conclusions: Delayed normalisation of fibrinolysis in postoperative cardiac surgery patients is associated with organ dysfunction and extended need for intensive care support. Early identification of impaired fibrinolysis may be used to target interventions in patients at risk for postoperative complications.

Table 1: Patient cohort characteristics

Variable	Median (Inter-Quartile Range) or percentages [%]
Age	66 (59-73)
Gender	Females 19 [32]
ICU LOS	85 (56-123)
Procedure:	
CABG alone	44 [73]
CABG + valve	8 [13]
Valve	5 [8]
Aorta	2 [3]

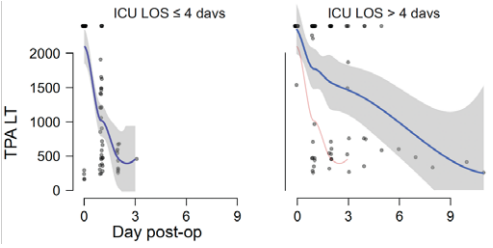

Figure 1: TPA LT (sec) in patients with a short (≤ 4 days) vs extended (>4 days) ICU LOS.

Hypofibrinolysis is Associated with Portal Vein Thrombosis During Liver Transplantation

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Background: Portal venous thrombus (PVT) at the time of liver transplant increases operative complexity and can lead to postoperative morbidity, and mortality. The pathophysiology of chronic PVT remains poorly understood, and failure of the fibrinolytic system is a potential unaddressed mechanism. We hypothesize that patients who fail to generate a fibrinolytic response (hypofibrinolysis) during the early phases of liver transplant are more likely to have PVT identified intraoperatively.

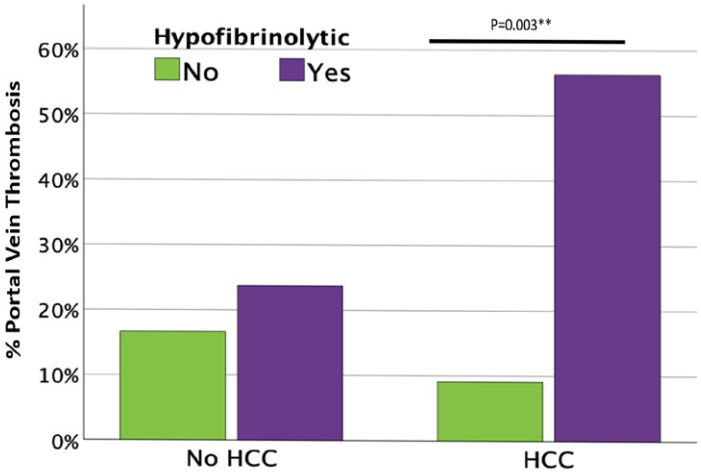
Aims: To identify whether PVT is associated with thrombelastography (TEG)-detected hypofibrinolysis during liver transplant surgery.

Methods: Patients from a prospective observational coagulation study were evaluated for the presence of PVT at the time of liver transplant by the operating surgeon. TEG was measured preoperatively, during the native hepatectomy and when the native liver was removed from the body. Hypofibrinolysis was defined as failure to generate a lysis at 30 minutes (LY30) >3% at any of these time points. The presence of hypofibrinolysis was also assessed in combination with hepatocellular carcinoma (HCC), another known PVT risk factor.

Results: 170 patients were included in the analysis. The median MELD was 23, 41% were female, and 21% had the diagnosis of HCC at the time of surgery. PVT was identified intraoperatively in 22% of

patients, which increased to 28% among patients with HCC. Hypofibrinolysis was identified in 41% of patients and was associated with a twofold increased rate of PVT (31% vs 15% p=0.015) compared to individuals without hypofibriolysis. Among patients with HCC, hypofibrinolysis was associated with an even higher rate of PVT 56% (vs 9% in patient with HCC without hypofibrinolysis; p=0.003) (Figure 1).

Conclusion: Hypofibrinolysis detected by TEG was associated with an increased rate of PVT, particularly in patients with HCC. Enhancing systemic fibrinolytic activity in cirrhotic patients with HCC may represent an unaddressed strategy to attenuate rates of PVT while the patient awaits definitive management with transplantation.



Plasminogen Deficiency of Pleural Fluid in Pleural Space Infections Due to Neutrophil Inflammatory Protease Degradation: A Cause of Intrapleural Lytic Failure?

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Background and Aims: Fibrin loculations in complex pleural space infections are frequently treated with tissue plasminogen activator (tPA) and DNAase via pleural catheter in an attempt to lyse loculations and drain the infection. However, this fails to resolve approximately 20% of cases and is also curious due to very high, repeat doses (6 over 3 days) of tPA required to get an effect relative to the small pleural space. Given that neutrophils are present in high levels in infected spaces, and that neutrophil proteases like elastase are known to degrade plasminogen, we hypothesized that plasminogen levels in infected pleural fluid are low due to neutrophil-mediated degradation and that plasminogen supplementation may be able to improve tPA-initiated lytic responses of infected pleural fluid.

Methods: Pleural fluid and plasma was collected from n=10 adult patients with pleural space infections with IRB approval prior to any intrapleural lytic therapy. Elastase activity was measured using an amidolytic assay, while plasminogen levels were measured via ELISA. Western blot analysis of pleural fluid, as well as plasminogen co-incubations with PMA-activated neutrophil supernatant and purified neutrophil elastase, were performed using antibody against Kringle domains 1-3. Turbidity assays were performed at 405nm wavelength to measure fibrin clot lysis by pleural fluid challenged with tPA (33pM and 330pM) without and with plasminogen supplementation.

Results: Infected pleural fluid had >4-fold higher elastase activity (p=0.02) and >3-fold lower plasminogen levels (p=0.04) compared to its respective plasma, while western blot analysis showed high levels of plasminogen degradation fragments consistent with neutrophil protease degradation. In 9 of 10 patients' pleural fluid there was no significant fibrinolytic response generated in response to tPA, while plasminogen supplementation rescued the fibrinolytic response in all patients' pleural fluid.

Conclusions: Plasminogen supplementation of the pleural space may improve intrapleural lytic efficiency and speed of disease resolution.

Markers of fibrinolysis predicting the outcome of thrombolysis treatment in acute ischemic stroke

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Currently, there are two proven reperfusion strategies for the opening of the occluded vessel in acute ischemic stroke (AIS) patients: intravenous thrombolysis using recombinant tissue-type plasminogen activator (rt-PA, alteplase) or tenecteplase, and mechanical thrombectomy. Both therapies must be delivered within a rapid time-frame in selected patients, moreover, mechanical thrombectomy is eligible in only a fraction of patients with large artery occlusion. Despite the unquestionable effectiveness of rt-PA as first-line treatment of AIS, successful reperfusion is achieved in only ~30–40% of patients, while ~6–8% of patients develop intracranial hemorrhage as side-effect. As of today, outcomes cannot be foreseen at the initiation of therapy and this remains one of the greatest challenges of AIS treatment. Studies on key players of fibrinolytic system during stroke thrombolysis provide a chance to understand thrombolysis failure and to develop better treatment options. On the other hand, if we can predict thrombolysis efficacy and particularly safety, that could be a game changer in improving acute stroke care. Due to the short time-frame before treatment, few studies are available on hemostasis tests predicting thrombolysis outcomes and several methodological issues are raised. Nevertheless, some assays show promising results and need to be further investigated and validated in large populations.

Altered fibrin clot structure and function in different clinical conditions

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Fibrinogen conversion into insoluble fibrin is the final stage of the coagulation cascade. Formation of more compact plasma fibrin clots displaying impaired susceptibility to fibrinolysis, so-called prothrombotic fibrin clot phenotype, represents a mechanism, which contributes to atherothrombosis as well as venous thrombosis. Growing evidence indicates that the prothrombotic fibrin clot phenotype can serve as a predictor of cardiovascular events in a long-term follow-up. Indeed, cardiovascular disease and venous thromboembolism (VTE), including prior myocardial infarction (AMI), ischemic stroke (AIS) or pulmonary embolism (PE), have been linked with prothrombotic fibrin clot phenotype. More compact fibrin meshwork characterized acute patients at the onset of AMI, AIS or PE, while hypofibrinolysis, particularly driven by higher plasminogen activator inhibitor type 1 (PAI-1) or thrombin activatable fibrinolysis inhibitor levels, has been identified as a persistent feature observed in patients following thrombotic events. Reduced clot susceptibility to lysis has also been observed in patients with deficiency of natural anticoagulants, including antithrombin or protein C. In patients with aortic stenosis hypofibrinolysis has been linked with PAI-1 overexpression in valvular interstitial cells and increased plasma PAI-1. Additionally, genetic variants of fibrinogen and fibrinogen post-translational modifications, a prothrombotic state, inflammation, increased oxidative stress, and the formation of neutrophil extracellular traps (NETs) contributed to the prothrombotic fibrin clot phenotype. Over 500 clot-bound proteins, including those unrelated to the coagulation system, have been identified within plasma fibrin clots and many of them were shown to modulate fibrin clot properties, even in acute state. This underlines how complex the process of clot formation is and how many mechanisms are engaged in its regulation. Traditional risk factors for cardiovascular disease, such as smoking, diabetes or hyperlipidemia, have also been linked with unfavorably modified fibrin clot properties. However, certain lifestyle changes and medications, particularly statins and anticoagulants, may help improve fibrin clot structure and function.

The individual fibrinolytic capacity correlates with efficacy of ultrasound-assisted catheter-directed thrombolysis in patients with acute pulmonary embolism

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Background: Acute pulmonary embolism (APE) is the third leading cause of cardiovascular mortality. Ultrasound-assisted catheter-directed thrombolysis (USAT) is nowadays available as an alternative reperfusion approach to systemic thrombolysis, and considered to provide an improved risk-benefit ratio. The lytic agent recombinant tissue-type plasminogen activator (rt-PA) activates the effector protease plasmin to induce fibrinolysis.

Aim: To characterize the individual fibrinolytic response in patients with APE, treated with USAT.

Methods: In a single-center cohort study, investigating the safety and effectiveness of treatment of USAT for intermediate-high or high-risk APE, pulmonary-arterial hemodynamic measurements were performed, and plasma samples obtained from 41 patients before treatment start, at 6h (during infusion of rt-PA), as well as at 24h after treatment start (post-lysis), between February 2021 and March 2023. Plasma levels of D-dimer and α2-antiplasmin were determined, and thromboelastography performed with the ROTEM system.

Results: At presentation, patients displayed elevated mean pulmonary artery pressures (PAPm 32.4 ± 7.5 mmHg), with a post-lysis reduction of 9.4 ± 8.3mmHg on average. During rt-PA infusion, a pro-fibrinolytic response was evident by a significant increase in D-dimer ($p<0.0001$), INTEM ML ($p<0.05$), as well as consumption of the endogenous plasmin inhibitor α2-antiplasmin ($p<0.0001$). In the 32 patients with an initial PAPm of >25 mmHg, the reduction of PAPm correlated positively with D-dimer levels ($r=0.3888$, $p<0.05$), and negatively with α2-antiplasmin levels at 6h ($r=-0.4307$, $p<0.05$), indicating that the individual fibrinolytic potential modulates treatment efficacy.

Conclusion: The extent of the fibrinolytic response during thrombolytic therapy correlates with efficacy of USAT in APE. Further analyses are currently underway to assess the capacity of various fibrinolytic parameters, evaluated before treatment start, to predict treatment efficacy.

Rotational thromboelastometry (ROTEM) measurements for the prediction of thrombolysis safety in acute ischemic stroke patients

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Background: Intravenous thrombolysis using recombinant tissue-type plasminogen activator (rt-PA) is an effective treatment of acute ischemic stroke (AIS), however, 6-8% of patients develop intracranial hemorrhage (ICH) as side-effect. As of today, the occurence of therapy-associated ICH cannot be foreseen at the initiation of thrombolysis.

Aims: We aimed to investigate whether the rotational thromboelastometry (ROTEM) measurement, a point-of-care test (POCT) to study the viscoelastic properties of clot-formation and lysis could predict the safety of thrombolysis, and whether the predictive value of the test can be improved by modifying the assay.

Methods: In this prospective observational study, blood samples of 114 AIS patients, all receiving i.v. rt-PA were taken before and immediately after thrombolysis. ROTEM was performed at both time points using citrated whole blood in a ROTEM SIGMA device and the Cartridge Complete kit. In order to mimick the in vivo effect of rt-PA, the test was also performed in the presence of 140 ng/mL rt-PA (mROTEM-t-PA) added directly to pre-thrombolysis blood samples. Stroke severity was determined by NIHSS on admission. Therapy-associated ICH was classified according to ECASSII. Long-term outcomes were defined at 3 months post-event by the modified Rankin Scale.

Results: In pre-thrombolysis blood samples, clot firmness showed strong correlation between ROTEM and mROTEM-tPA measurements (A5: $r=0.7564$, $p<0.0001$). In case of more severe stroke (NIHSS>15), higher clot firmness was observed in pre-thrombolysis samples. Clot lysis index of mROTEM-tPA showed strong correlation with post-thrombolysis ROTEM results (LI45: $r=0.7192$, $p<0.0001$). Using the mROTEM-tPA test before thrombolysis, a LI60>0% parameter excluded the occurrence of post-lysis ICH with 100% sensitivity and 45% specificity (100% negative predictive value, $p=0.038$) in this cohort.

Conclusions: Based on our results, the modification of ROTEM is necessary to predict thrombolysis outcomes. The mROTEM-tPA might be used to exclude post-lysis ICH using pre-thrombolysis samples with high negative predictive value.

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