SYSTEMS BIOLOGY AND SYSTEMS PHYSIOLOGY: REGULATION OF BIOLOGICAL NETWORKS

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Topic: Cell as a complex biological system: topical issues of regulation of cell division and metabolism

Disordered gears for the tubulin motors: emergent properties of unstructured protein domain ensembles

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Background: Cells use mechanical forces to support vital functions such as transfer of genetic material during cell division. One of the key points of intracellular force generation is kinetochore, a multi-protein complex linking centromeric regions of chromosomes with the microtubules in the mitotic spindle. Microtubule elongation and shortening produces mechanical force that moves chromosomes and provides a mechanical cue for the signaling cascade that regulates mitosis, the checkpoint. However, it is poorly understood how the microtubule-generated force is transmitted to its cargo.

Aims: Given the complexity of the kinetochore, which is built by over a hundred polypeptides present in multiple copies, we take a reductionist approach. Using in vitro reconstitution, we aim to recreate a force-coupler, protein complex that is able to capture microtubule-generated force, and to prevent microtubule ends from detaching.

Methods: We use single-molecule fluorescence microscopy to follow how recombinant protein complexes interact with the ends of dynamic microtubules. To recreate force-coupling attachments in vitro, we use optical tweezers, a method to manipulate micro-particles and measure piconewton-range forces. Using glass beads coated with recombinant protein complexes, we let dynamic microtubules pull on them, and interrogate the force at which the microtubule is stalled in its shortening, and the duration of this stall.

Results: We find that the most efficient force-couplers are made using disordered, positively charged protein domains with poor sequence conservation, but similar physicochemical properties. A number of kinetochore components from a range of evolutionary distinct organisms contain such sequences.

Conclusions: We hypothesise that most of the cellular processes that use microtubule dynamics as a source of energy rely of similarly disordered, positively charged sequences. Such processes likely include microtubule-chromosome interactions, membrane remodelling by dynamic microtubules in the context of microtubule-mitochondria and microtubule-ER contacts, and possibly interactions between microtubules and actin at the cell periphery.

Role of mechanical forces in regulation of filopodia dynamics and activation of Ca²⁺-dependent signaling

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Background: The process of cell migration process plays the central role in development and maintenance of multicellular organisms. Wounds healing, immune response to exogenous pathogens, embryonic tissues formation – these are just a few examples of a large number of
vital biological processes that rely on highly ordered collective cell migration, which is required for proper organism functioning. To guide their motion through extracellular matrix (ECM), cells produce dynamic membrane projections known as filopodia, which are responsible for mechanical and chemical sensing of the surrounding microenvironment as well as for formation of initial adhesion contacts with ECM or other cells. Numerous experimental studies suggest that abnormally high filopodia activity is a typical feature of aggressive cancer cells that results in their high motility, leading to formation of metastases in healthy tissues. Thus, understanding of molecular mechanisms responsible for the regulation of the filopodia dynamics and sensing of the surrounding environment may provide important insights into the cell migration as well as cancer development processes.

**Aims:** Recently, a number of proteins necessary for filopodia formation, growth and adhesion have been identified. However, mechanosensing molecular pathways that allow filopodia guide cell migration in response to both external and cell-generated forces remain unknown.

**Methods:** Statistical physics, polymer field theory, transfer-matrix calculations.

**Results:** To gain insights into the role of mechanical forces in regulation of the filopodia dynamics as well as in generation of intracellular signals that guide cell migration, we have developed an experimental approach based on optical tweezers allowing one to observe changes in the behaviour of filopodial protein complexes in response to precise and highly localized application of mechanical force. By using this experimental setup in combination with pharmacological inhibition and knockdown assays, we have identified several key components, such as myosin IIA and formins, contributing to regulation of filopodia adhesion and dynamic properties in a force-dependent manner. Furthermore, it has been found that stretching forces of tens of pN strongly promote Ca$^{2+}$ influx into filopodia through L-type Ca$^{2+}$ channels, causing persistent Ca$^{2+}$ oscillations, which result in activation of calpain protease involved in regulation of cell adhesion complexes.

**Conclusions:** Overall, our study suggests existence of an intricate interplay between several different types of force-generating and mechanosensing cytoskeletal and membrane protein complexes, whose collective action helps to guide cell migration in a force-dependent manner.

**Structural and computational insights into the mechanisms of microtubule assembly and force generation**

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**Background:** Microtubules (MTs) are essential cytoskeletal polymers in all eukaryotic cells. Thanks to their dynamic instability, MTs efficiently search and capture chromosomes in mitosis. Upon association with chromosome-bound kinetochore proteins, MT tips produce pulling and pushing forces that aid in the accurate segregation of sister chromatids. However, detailed mechanisms of MT dynamics and force generation remain poorly understood.

**Aims:** We aim to investigate the structures of growing and shortening MTs *in vitro* and *in vivo* and construct a comprehensive computational model of MT dynamics and force production.
Methods: To achieve this goal, we use a combination of cryoelectron tomography of MT ends and Brownian dynamics simulations.

Results: In contrast to most previous models, our descriptions of MT tips by electron tomography in vivo (six species) and in vitro (under several experimental conditions) found that the ends of these dynamic polymers display flaring protofilaments (PFs) in both growing and shrinking states. We construct and systematically analyze a Brownian dynamics model for MT dynamics and force production. We demonstrate that a description of MT assembly and disassembly with flared ends can be achieved under a range of conditions, using simple tubulin lateral interaction energy potentials. However, force generation experiments put constraints on model parameters, pointing to the presence of a high and steep activation energy barrier in the lateral tubulin interaction energy potential. When properly constrained, the model describes the development of large pulling forces by shortening MTs and considerable pushing forces by growing MTs.

Conclusions: Curved protofilament morphology at MTs ends has profound implications for MT dynamics, for mechanical force generation, and for the regulation of both processes by associated proteins. It also enables sustained interactions between kinetochores and growing MTs under assisting forces. A load-dependent acceleration of MT growth rates provides an explanation for the long-standing problem of synchronizing the assembly and disassembly of MTs connected to opposite spindle poles during metaphase chromosome oscillations.

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Development of in silico approach for investigating EB1 and microtubules interaction

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Background: Microtubules are highly dynamic tubulin polymers which form cytoskeleton with a help of other intracellular polymers and microtubule associated proteins. Stochastic switching between polymerization and depolymerization phases and back are named catastrophe and rescues, correspondingly. Along with other cellular filaments, they perform cellular housekeeping functions and accurately segregate the chromosomes during cell division. One of the most ubiquitous microtubule associated proteins is the end-binding protein 1 (EB1). Its remarkable feature is the ability to recognize growing microtubule ends and associate with them. EB1 can significantly affect the parameters of dynamic instability of the microtubules. The mechanism of such a specific recognition of the growing microtubule ends is under debate, and the mechanism of microtubule dynamics regulation is poorly investigated.

Aims: Development of a modeling approach to investigate EB1 interaction with a dynamic microtubule in order to establish the mechanism of specific localization of the protein near the microtubule growing end.

Methods: We simulated microtubule behavior in the presence of EB1 proteins using stochastic Monte-Carlo modeling. The method describes the evolution of the system as a time sequence of predetermined events. Our model is based on our experimental data on the structure of
polymerizing and depolymerizing microtubule ends. It allows us an explicit account for the conformational states of tubulin dimers. EB1-microtubule interaction simulation is performed by introducing additional events in the model. The data, which we used for model calibration, included experimentally measured microtubule dynamic instability parameters (polymerization and depolymerization rates, catastrophe and rescue frequencies) and recently established EB1 localization at the microtubule end revealed by precise in vitro fluorescent investigation.

Results: We described the experimental data for EB1 localization at the growing microtubule tips, based on the assumption that the affinity of EB1-microtubule interaction depends on the nucleotide state of the tubulin dimers. In agreement with the experimental data, in the model EB1 localization area becomes larger as the free tubulin concentration increases.

Conclusions: Nucleotide-dependent EB1-tubulin binding explains the specific ability of EB1 to track with the microtubule growing ends. The proposed model can be useful for further investigation of EB1 effects on microtubule dynamic instability parameters.

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Spatiotemporal dynamics of platelet GPVI signaling is tightly regulated by structural aspects of GPVI ligands

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Background: Platelets are non-nuclear cells, responsible for preservation of the circulatory system integrity. Upon blood vessel trauma platelets are activated by contact with disturbed endothelial cells and exposed sub-endothelial matrix, which consists mostly of collagen. This results in platelet plug formation that prevents the blood loss. It is believed that platelet activation in these circumstances is majorly mediated by platelet collagen receptor GPVI. Recently it was suggested that platelet GPVI is also responsible for maintaining thrombus stability by binding to fibrin/fibrinogen. In order to specify GPVI roles in human physiology, a systems biology analysis of the actions of different ligands on platelet activation through GPVI is required.

Aims: Analyze role of ligand structure in platelet GPVI signaling to determine the theoretical impact of dimerized GPVI ligation by fibrin/fibrinogen and compare to GPVI activation by collagen/CRP.

Methods: A computational systems biology model based on scheme of biochemical events during platelet activation by GPVI ligands was constructed and analyzed. The scheme of platelet GPVI signaling was based on the previously published data. Briefly, GPVI ligation results in GPVI multimerization and GPVI intracellular ITAM motif phosphorylation. This results in Syk kinase activation and LAT-signalosome formation, which becomes the center of the calcium and phosphoinositide signaling. Model was solved using LSODA implemented in Python 3.8. For model validation flow cytometry of Fura-RED loaded human platelets using BD FACS Canto II was performed.
**Results:** Systems biology analysis of the GPVI signaling pathway revealed that platelet activation via GPVI is significantly affected by the GPVI clustering pattern. Based on model fitting of the experimental data from Poulter et al. Blood, 2016 it was considered that GPVI clustering is ligand-dependent. Fibrin/fibrinogen binding was assumed to be clustering not more than 4 GPVI units, while immobilized collagen could induce higher order of GPVI clustering. Model predicted that fibrin/fibrinogen could not induce sufficient platelet activation, while collagen-induced activation was detectable at all physiological concentrations of collagen. This result remained valid at all physiological values of the unknown parameters.

**Conclusions:** Based on the theoretical predictions, GPVI activation by fibrin/fibrinogen is significantly less pronounced in comparison to activation by collagen.

**Erythrocytes-bioreactors and limitations of their efficiency**

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**Background:** Erythrocytes-bioreactors (EBRs) are erythrocytes with encapsulated enzymes that catalyze some biochemical reactions absent in normal RBC. EBRs may be useful in treatment of some diseases. EBRs for removing ethanol, methanol, ammonium, asparagine, and other compounds from the bloodstream were obtained *in vitro* and tested *in vivo* by different scientific groups. But, mechanisms that limit the efficiency of EBRs were still unclear.

**Aims:** Built-in metabolic pathways interact with the native metabolic system of RBC. This interaction may lead to different consequences from decrease of EBR’s efficiency to the risk of death of the cell (for instance, due to osmotic lysis). The aim of this work is to study possible restrictions of EBR’s efficiency by the case of ammonium-neutralizing (based on glutamate dehydrogenase and alanine aminotransferase reactions) and ethanol-neutralizing (based on alcohol dehydrogenase and acetaldehyde dehydrogenase reactions) using mathematical modelling of metabolic systems.

**Methods:** Mathematical models were systems of first-order ODE describing the rates of metabolites concentrations change. Reactions of built-in systems and reactions of some RBC metabolic pathways (mainly glycolysis and pentose phosphate pathway) were included to the models. Numeric solutions were obtained by Runge-Kutta methods in MATLAB.

**Results:** Two main factors that limit efficiency of ammonium-neutralizing EBR are pyruvate influx from external media and total rate of NADPH oxidation in the cell (by both embedded pathway and natural oxidative reactions). Maximum possible ammonium consumption rate is about 12 mmoles/h per liter of pure cells. This rate cannot be increased neither by increase of ammonium neutralizing enzymes activity nor by increasing of external pyruvate concentration because of losing of steady state in glycolysis when ammonium consumption rate is higher than 12 mM/h. In the case of ethanol-neutralizing EBRs increasing of ethanol-neutralizing enzymes activity leads to losing of steady state in glycolysis as well. Maximum possible ethanol oxidation rate is defined by the critical value (about 0.8) of NAD fraction in the nicotinamide dinucleotides pool.
Conditions in which the system exceeds this value are defined in their turn by pyruvate influx from external media because NADH is reduced in lactate dehydrogenase reaction that uses pyruvate as a substrate.

**Conclusions:** There are two groups of restrictions of EBR’s efficiency. The first group includes restrictions caused by low permeability of RBC membrane for substrates or products of target reactions. Restrictions of this type lead to low EBR efficiency. Restrictions of the second group are caused by interaction of target reactions with the cell metabolic system. Such interactions may lead to losing of steady state of metabolic system and death of the cell. Mechanisms of revealed limitations are universal and may be applied for analysis of EBRs with different built-in metabolic pathways.

**Numerical modelling of Ndc80 complex interaction with microtubules in an ultrafast force clamp assay**

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**Background:** The Ndc80 complex connects mitotic chromosomes to spindle microtubules (MTs) with Hec1 and Nuf2 domains. Macroscopic patterns of mitotic chromosome motion suggest that Ndc80-MT interaction is asymmetrical and force-dependent: it should provide fast motion to the minus-end of MTs and strong binding while moving to the plus-end. These properties can be studied with an ultrafast force-clamp spectroscopy assay (UFFC).

**Aims:** We investigated how energies of domain-MT interaction and mechanical properties of Ndc80 affect its motion in absence of or under force and estimated these parameters from experimental data.

**Methods:** We proposed a mechanical model of UFFC experimental system considering its elastic properties and viscous friction. Hec1 binding to MT was modeled with a periodic lattice of potential wells along MT and a 2nd potential well with barrier defined Nuf2-MT interaction. We assumed constant binding of Hec1 site, Nuf2 binding depended on Ndc80-MT angle constrained with torsion spring. Langevin dynamics simulations were used to numerically obtain UFFC system evolution and model free diffusion of Ndc80 on MT surface. We calculated force acting on the molecule given the force applied to the MT “dumbbell” in experiments and searched parameter space to best fit with the model experimentally obtained Ndc80 diffusion coefficient and force-velocity curves.

**Results:** Diffusion motion simulations require 6-7 kT deep wells for Hec1 and Nuf2 depending on barrier height and torsion stiffness of Ndc80. UFFC simulations reproduce experimentally observed asymmetrical motion to plus- and minus-ends for weak torsion springs and non-zero depth of the 2nd well. Model with hard torsion springs shows vanishing asymmetry in motion which plateaus as stiffness decreases. Speed of the dumbbell motion under given force to the minus end is primarily governed by the depth of Hec1 well, however motion to the plus end strongly depends on both site energies as well as barrier height. We found that diffusive motion and motion under force in experiments may be reproduced simultaneously in model with 7 kT Hec1 well, 6 kT well and 20% (1.4 kT) barrier for Nuf2 and 0.001 pN·µm torsion stiffness.

**Conclusions:** These findings suggest that biologically significant asymmetrical properties of Ndc80-MT gliding under force depend on both Hec1 and Nuf2 domains that exhibit similar...
binding to MT surface. Overall dynamics of such motion strongly depends on both mechanical properties of Ndc80 and binding energies of its domains.

**Accelerated death of Wiskott-Aldrich Syndrome megakaryocytes**

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**Background:** Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disorder affecting only hematopoietic cells and caused by mutation in WAS gene. WAS has wide clinical phenotype range with varying degree of such symptoms as microthrombocytopenia, eczema, combined immunodeficiency, autoimmunity and malignancies. Recent studies reported accelerated death of WAS platelets, T and B lymphocytes but there are few data for megakaryocyte (MK) status in WAS.

**Aims:** To study WAS MK morphology and viability before or during thrombopoietin receptor agonist romiplostim treatment.

**Methods:** All procedures were approved by Ethical Committee, donors and patients gave their written consent. Bone marrow aspirate was collected into EDTA-containing tubes. The mononuclear cell fraction was purified using Histopaque 1077 (Sigma), and placed on anti-CD61 antibody or monafram covered microarray for MK attachment. Confocal microscopy was employed for viability evaluation by phosphatidylserine (PS) externalization and/or mitochondrial membrane potential. Maturation stage and sizes of MK were determined by May-Grünwald-Giemsa staining and light microscopy.

**Results:** We examined samples of 14 WAS patients and 7 healthy donors. The fraction of dead MK with exposed PS varied in WAS patients from 0 to 83% (25±25% average±SD) and in healthy donors from 0 to 11% (2±4%). The PS+ MK fraction correlated with the disease severity Zhu score (Pearson C=0.73, p<0.01). MK with exposed PS were with collapsed mitochondria. Analysis of MK size and maturation stages distribution did not provide significant differences between healthy and WAS MK. Romiplostim treatment increased percentage of megakaryoblasts from 20±19% before treatment to 48±20% during treatment and decreased percentage of MK on maturation stage 3 from 36±22% before treatment to 18±16% during treatment. The increase in micromegakaryocyte fraction was only observed when patients had a mutation in the WH1 domain of WAS protein.

**Conclusions:** WAS megakaryocytes have increased tendency for programmed cell death that could contribute to thrombocytopenia.

**Permitted and restricted steps of human kinetochore assembly in mitotic cell extracts**

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**Background:** Assembly of kinetochore and its proper interaction with spindle microtubules are critical steps for accurate chromosome segregation. In human cells, assembly of outer kinetochore layers occurs during mitosis based on two major constitutive proteins CENP-C and CENP-T, which recruit main kinetochore microtubule binder Ndc80 complex and other kinetochore components. Hierarchical recruitment of kinetochore proteins from cytoplasmic pool is a highly regulated process, but the underlying steps are not well understood. Also, it is not clear how different kinetochore proteins contribute to interaction with spindle microtubules.

**Aims:** The ultimate goal of our research is to gain understanding of mechanisms driving kinetochore assembly and interaction with microtubules in mitosis.

**Methods:** Published work by others has highlighted strategies that were successful for kinetochore assembly reconstitutions in non-human systems. They include purification of preassembled kinetochores from cells, and assembly of kinetochore de novo building on inner kinetochore components. We applied analogues approaches for human cell system. First, we examined presence of pre-assembled kinetochore particles with microtubule binding activity in cell extracts prepared from mitotic HeLa cells. Second, we investigated kinetochore assembly de novo by analyzing the recruitment of GFP-fused kinetochore proteins from human mitotic cell extracts to the inner kinetochore components immobilized on the microbeads.

**Results:** By analyzing the recruitment of GFP-fused proteins from human mitotic cell extracts to inner kinetochore components, we reconstructed the interaction between CENP-C and CENP-A–containing nucleosomes. However, subsequent phospho-dependent binding of the Mis12 complex was less efficient, whereas recruitment of the Ndc80 complex was blocked. Recombinant CENP-T/W complex also failed to recruit native Ndc80 complexes, which we show partially results from the auto-inhibition of the full length Ndc80 complex. Consistently, the microtubule-binding activity of native kinetochore components, as well as those assembled using a combination of native and recombinant human proteins, was weaker than that of recombinant Ndc80 complex alone.

**Conclusions:** Our work suggests existence of the yet unidentified regulatory mechanisms that permit interactions between these proteins specifically at the assembling kinetochore. Such inhibitory mechanisms are likely to guard against spurious formation of kinetochores in the cytosol of mitotic human cells.
Topic: Functional regulation of human body systems: from theory to practice

Regulation of, and variation in, the mechanisms that lead to thrombus formation

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Background: Blood clot formation is an intricate and nonlinear process driven by the interplay of plasma coagulation factors (the coagulation cascade) and platelets. Clots form normally during hemostasis where they seal injured vessels to stop bleeding, but can form inappropriately leading to either serious bleeding or pathological clot formation (thrombosis).

Aims: Our ability to identify factors that alter clot formation and determine a donor’s susceptibility to medication would be enhanced by a better understanding of the mechanisms that regulate coagulation and platelet activation and how these vary in health and disease.

Methods: We present mathematical and computational models that help reveal some of the mechanisms that regulate and vary clot formation.

Results and Conclusions: The models and theoretical techniques have been developed alongside novel experimental approaches to produce quantified high-density data, some of which will also be reported alongside machine-learning approaches utilised to unravel variability in mechanisms seen in large cohorts of donors.

The relationship between thrombin generation parameters and their sensitivity to coagulation factors, inhibitors and platelets

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Background: Thrombin generation (TG) is a global assay of blood coagulation widely applied in research, diagnostics, and pharmacology. Several parameters are usually used for characterization of TG and clotting of plasma (fibrin generation, FG).

Aims: We studied the significance and interconnectedness of TG and FG parameters.

Methods: TG and FG in vitro and in silico experiments.

Results: Computer simulation showed that parameters can be divided into two groups within which they correlated strongly positively (R=0.67 to 0.99): (i) clot time, lag time and time to thrombin peak (R=0.89 to 0.99) and (ii) thrombin peak, maximal thrombin generation velocity, and endogenous thrombin potential (R=0.67 to 0.94). There was no correlation between parameters of different groups (R=−0.24 to −0.48). This was confirmed in TG experiments in coagulation factor (f) VII−, fX−, and fII− deficient plasmas titrated with normal plasma. Experimental correlations were between 0.94 and 0.97 for the first group, and 0.86 to 0.99 for the second; with little, if any, correlation between groups (R=−0.41 to -0.56). Sensitivity analysis of TG in silico showed that parameters of the first group were sensitive to reactions and
Concentrations of reactants involved in the initiation of coagulation, while parameters of the second group were sensitive to concentrations of fiI, antithrombin and platelets.

**Conclusions:** The mathematical model of TG developed in this work can be used for experimental planning and analysis in basic and applied research of coagulation. Six widely used assay parameters for TG may be redundant and can be reduced to two representing key regulatory components of coagulation system.

**Purinergic signaling in bone adaptation to mechanical forces**

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**Background:** Physical forces are vital for successful function of musculoskeletal, urinary, respiratory, and other organ systems; however, they can be destructive and require active adaptation. Mechanoadaptation includes a cascade of signals starting from intracellular calcium ([Ca2+]i) transients and release of ATP that acts via receptors of the purinergic (P2) family, which includes seven P2X ligand-gated ion channels and eight P2Y G-protein-coupled receptors.

**Aims:** We aimed to examine how ATP released from mechanically stimulated cells coordinates the responses of neighboring bone cells.

**Methods:** Single murine osteoblast was stimulated with a glass micropipette and the changes in [Ca2+]i in fura-2 loaded cells or luciferin fluorescence were recorded.

**Results:** Mechanical stimulation of a single murine osteoblast was found to result in a release of 70 ± 24 amole ATP, which stimulated calcium responses in neighboring cells. Osteoblasts contained ATP-rich vesicles that were released upon mechanical stimulation, however, pharmacological interventions that promoted vesicular exocytosis reduced ATP release, while inhibitors of vesicular release potentiated ATP release. In search of an alternative route of ATP release, we found that mechanical stresses induced reversible cell membrane injury in vitro and in vivo. Calcium/PKC-dependent vesicular exocytosis facilitated membrane repair, thus maintaining cell viability and reducing ATP release. To understand how purinergic signal conveys information about the mechanical stimulus to the neighboring bone cells, we developed a mathematical model describing injury-related ATP and ADP release, their extracellular diffusion and degradation, and purinergic responses of neighboring cells. We found that the total amount of ATP released, which depended on injury severity and repair dynamics, determined the overall number of responders and maximal distance from the injury at which purinergic responses were stimulated. Peak ATP concentrations allowed cells to discriminate between minor and severe injuries that led to the release of similar amounts of ATP due to different injury repair kinetics. ADP-mediated signaling became relevant in larger tissue-level injuries, at sites distant from the initial injury.

**Conclusions:** Thus, mechanotransductive purinergic signalling fields depended on the severity of the stimulus and dynamics of injury repair, which enabled neighbouring cells to evoke responses appropriate to their proximity to and the degree of the mechanical stimulation.
Platelets from children are moderately hypo-reactive compared to platelets from adults

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Background: In our previous studies we demonstrated that term newborns have lower platelet function compared to adults. However, studies on platelet function in older children are rare and their results are controversial.

Aims: We compared platelet function in children aged 1 to 18 years old to that of healthy adult volunteers.

Methods: Venous blood was collected into vacuum plastic tubes with sodium citrate, final concentration 3.8%. 20 µL of blood was diluted 1:20 in HEPES-buffered Tyrode buffer. For platelet activation, a mixture of thrombin receptor activating peptides SFLLRN (80 µM) and AYPGKF (80 µM), adenosine diphosphate (ADP, 10 µM) and 2.5 mM CaCl$_2$ was used. We determined forward scatter (FSC), side scatter (SSC), CD42b-PE, CD61-PE, CD62P-Alexa Fluor 647, PAC-1-FITC, annexin V-Alexa Fluor 647 binding and mepacrine release levels.

Results: Children were divided into 3 age groups: 1 to 5 years old (n=16), 6 to 10 years old (n=23) and 11 to 18 years old (n=32); the control was healthy adults aged 19 to 40 years old (n=49). Resting platelets of children from the youngest age group had 1.2-fold smaller FSC and SSC and less dense granules than adults. Procoagulant activity of platelets of children aged 1 to 5 and 6 to 10 years old was 2.5-fold higher compared to adults. No differences in PAC-1 binding, CD62p, CD42b and CD61 expression levels between children and adults were found. After activation platelets of children aged 1 to 5 years old had 1.2-fold smaller FSC in comparison with adults' platelets. No differences in SSC between children and adults were found. Though we found no differences in mepacrine fluorescence between children and adults, platelets of children from the youngest age group had lower dense granules release, which was reflected in 1.1-fold lower degranulation index and 1.2-fold lower dense granules secretion compared to adults' platelets. Children from the youngest age group had lower alpha-granules release, which was reflected in 1.2-fold lower P-selectin level in comparison with adults. We found no difference in PAC-1 binding level, CD42b and CD61 expression levels and procoagulant activity between children and adults in response to stimulation.

Conclusions: Platelets from children have smaller size, lower dense and alpha-granules secretion and are moderately hypo-reactive compared to platelets of adults. Pediatric platelets reach adults’ level of platelet function at the age of 5.

The influence of inflammation on multiple thrombus formation and blood circulation in the lungs during the coronavirus disease

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**Background:** In 2020, the world was suddenly overwhelmed by the epidemic provoked by the new coronavirus infection named “COVID-19”. Starting in China in winter 2019, it spread all over the world and got the status of pandemic several months later. All countries continue struggling with its medical and social consequences.

With the outstanding efforts of medical and research communities, the characteristics of this disease have been outlined but there are still many open questions, there is no effective treatment, and vaccination is expected only during the next year. The coronavirus disease will require further investigation in order to overcome its consequences and to prevent similar events in the future.

The main cause of mortality during the coronavirus disease is related to the multiple thrombus formation in the lung arteries. Vessel occlusion influences pulmonary blood circulation and reduces blood oxygenation, possibly leading to the failure of various body systems.

**Aims:** Investigation of clot formation in the lung arteries and of pulmonary blood circulation depending on the level of lung inflammation; evaluation of the blood oxygenation level and the critical lung damage.

**Methods:** Mathematical modelling based on the two-dimensional and quasi-one-dimensional approaches to blood circulation and clot growth, analytical investigations of these models, numerical simulations in both full (two-dimensional) and quasi-one-dimensional formulations.

**Results:** A mathematical model of clot growth in a network of blood arteries was developed and investigated analytically and numerically. Blood flow velocity, pressure, and flux distribution in the pulmonary tree were determined depending on the lung area affected by the inflammation.

**Conclusions:** Pulmonary blood circulation and oxygenation level are evaluated depending on the level of lung inflammation.

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**Membrane-dependent reactions can be supported by surface of Red Blood Cells**

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**Background:** Membrane-dependent reactions are necessary for the normal work of the coagulation cascade. Platelets and their microparticles are considered as the main physiological source of negatively charged membranes. Some studies showed that RBCs influence plasma coagulation. Different ways are proposed to explain this phenomenon. But there is still no answer to which of them is more important.
**Aims:** In this work we investigated the ability of RBCs to support membrane-dependent reactions on the example of fX activation via intrinsic tenase. Also we studied the binding of components of this complex with the surface of RBCs.

**Methods:** Written informed consent was given by all participants, and the study was approved by the institutional ethical committee. RBCs and platelets were isolated from whole blood by washing. Binding of FITC-labeled coagulation factors with the platelets and RBCs was study by flow cytometry. The concentration of activated FXa was determined from the rate of hydrolysis reaction of a specific substrate S2765.

**Results:** It was shown RBCs could take part in activation of FXa by intrinsic tenase. The amount of generated enzyme was proportional to the concentration of cells in the range of of 46,86-2000 (for RBCs) or 0,17-500 (for platelets) cells/ul, and the effect of 2x106 cells/ ul RBCs was similar to the of 0,25 x106 cells/ ul thrombin-activated platelets. It was shown coagulation factors predominantly interact with PS-positive platelets or RBCs.

**Conclusions:** These results indicate that RBCs can participate in coagulation by providing phospholipid membranes for the intrinsic fX activation and that their effect at physiological hematocrit can be comparable to that of platelets at their physiological concentration.

**Developments leading to a Factor XI enhanced model of blood coagulation**

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**Background:** Computational modelling of blood coagulation, namely computer simulations of a mathematical/mechanistic model, has increased in importance in the last two decades.

**Aims:** Computational modelling can test hypotheses prior to experimental verification, can predict the sensitivity of specific mechanisms of coagulation to various anticoagulant and anti-thrombotic drugs, and contribute to the development of safe and effective hemostatic agents and antithrombotic drugs.

**Methods:** In this talk, we present recent contributions in computational modelling of coagulation from our group that led to a mechanistic model which uses factor XI-driven enhancement to enter self-sustained thrombin production with initial zero activated enzymes.

**Results:** These include a) a review detailing consensus values of kinetic constants for each reaction, b) a mechanistic model which can aid clot-based assays, c) equations for fibrin polymerization which capture the lag time of clotting, and d) detailed analysis which identifies the need to enhance production of factors IXa and Xa via factor XI.

**Dissecting complex regulation of germinal centers by follicular regulatory T cells**

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**Background:** Follicular regulatory T cells (Tfr) play various roles in the control of B cell and antibody responses. Tfr repress foreign antigen-specific germinal center (GC) response at its peak, but at the same time promote GC B cell cycling and support affinity maturation. Tfr are
also involved in the control of autoreactive B cells and prevention of autoimmunity. The cellular and molecular mechanisms of the multifaceted Tfr-mediated regulation of B cells are poorly understood. In our previous study we showed that proinflammatory chemokine CCL3 produced by foreign Ag-specific GC B cells promotes their direct contacts and modest negative regulation by Tfrs at the peak of GC response. However, which subsets of GC B cells upregulate production of CCL3, the longer-term effects of CCL3-facilitated GC-Tfr cell encounters and the role of cognate GC-Tfr interactions on the GC response are not known.

**Aims:** 1) Determine the role of CCL3-mediated Tfr-GC B cell encounters in the long-term regulation of foreign Ag-specific GC response. 2) Determine whether cognate GC-Tfr cell interactions (when Tfr recognize MHCII/self-peptides on the surface of GC B cells via T-cell receptors, TCR) repress GC response in vivo.

**Methods:** Flow cytometry analysis, qPCR, cell sorting. Myc-GFP, FoxP3-DTR, Hy10 CFP, Hy10 CCL3-KO GFP, CD45.1, CD45.2, CCL3-KO (C56BL/6 background) mice were used in the study. Antigens used for immunization of mice: NP-KLH, SA-DEL, SA-NucPrs, DEL-OVA.

**Results:** By utilizing Myc-GFP transgenic mice, cell sorting and qPCR analysis we established that CCL3 is upregulated in the MYC+ GC B cells (the cells undergoing positive selection in GCs). We then found that B cell-intrinsic production CCL3 was important both for modest repression of foreign antigen-specific B cells at the peak of GC response, but also for their sustained participation in GCs, affinity maturation, memory and plasma cell development (by performing flow cytometry analysis of GC responses at various times after immunization in mixed CCL3+/+ CD45.1/CCL3−/− CD45.2 bone marrow chimeric mice and in adoptive transfer experiments). The observed effects were dependent on the presence of Tregs. In addition, by using mice immunized with streptavidin-DEL (SA-DEL) antigen and boosted with SA conjugated to nuclear proteins (Nuc-Prs), we found (by flow cytometry analysis) that acquisition of Nuc-Prs by SA-specific GC B cells leads to rapid expansion of Tfr cells, and Tfr-mediated suppression of GC and more specifically Nuc-Prs acquired GC B cells. The experiments where repeated 2-6 times with 3-7 mice per each time point. Students T-test or ANOVA statistical analysis was applied as appropriate.

**Conclusions:** Based on our results and previous findings, we suggest a novel model of GC check-point control by Tfr cells. GC B cells that undergo positive selection transiently upregulate CCL3 that promote their encounters with Tfr cells. Tfr cells then “examine” selected GC B cells for the surface expression of MHCII/nuclear peptides (self-peptides). Cognate recognition of self-peptides leads to Tfr cell expansion and negative control of GC B cells, while non-cognate interactions with Tfr promote GC B cell selection, sustained participation in the GCs and affinity maturation. Further experiments are underway to test the hypothesis outlined above.

**Interaction of the coagulation factors of the intrinsic tenase complex with the subpopulations of activated platelets**

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**Background:** One of the major membrane-dependent reactions of blood coagulation is activation factor X by intrinsic tenase complex. However, the interaction of the factors included in this complex with the subpopulations of activated platelets has not been studied yet.

**Aims:** To investigate the equilibrium and kinetics binding of factors IXa and X with subpopulations of activated platelets, as well as the effect of factor VIIIa on this binding. To determine the conditions for the competition of blood coagulation factors when binding to the platelets’ subpopulations.

**Methods:** Platelets were obtained from donor blood by gel filtration purification, and phospholipid vesicles were formed by extrusion. Native fIXa and fX was labeling with Alexa Fluor 647, commercial FITC-labeled antibodies were used for observation of recombinant fVIIIa (activated by thrombin). The suspension of incubated with labeled proteins platelets or vesicles was analyzed in a flow cytometer BD FACSCanto II (BD Bioscience, San Jose, CA, USA).

**Results:** The binding of factor IXa to the membrane of procoagulant platelets occurs through one low-affinity binding site, which is a negatively charged phosphatidylserine. In this case, the presence of factor VIII strongly increases the binding of factors IXa and X. Also, at physiological concentrations of factor IXa, its reciprocal cooperation is observed, including with the zymogen, but there was the competition effect with factor X. The factor IXa multimerization may be the cause of its cooperativity, on its basis a mathematical model of the interaction of a factor IXa with a membrane was written.

**Conclusions:** There were obtained the constants of binding of intrinsic tenase factors to the membrane of activated platelets (fIXa: 0.7 ± 0.1 mkM, fIXa in the presense of fVIIIa: 67 ± 16 nM, fX in the presence of fVIIIa: 109 ± 10 nM) and phospholipid vesicles (fIXa: 1.0 ± 0.1 mkM). The conditions of competition between coagulation factors were determined also (fIXa-fIXa cooperation in [0 – 400 nM], competition in [400 – 4200 nM], fIXa-fX cooperation [0 – 4200 nM], fIXa-fX competition [0 – 4200nM]).

**Quantitative Systems Pharmacology modeling and its use in the development of new drugs**

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**Background:** Quantitative Systems Pharmacology (QSP) modeling combines pharmacology, molecular/cellular biology, to dynamically predict physiological outcomes. This analysis type comprehensively integrates the knowledge of previous research of similar biological systems, pathology, and new experimental preclinical evidence/clinical study data. QSP models may be represented by the systems of ordinary differential equations describing the dynamical properties and interactions within the studied system [Hugo Geerts et al. J Pharmacokinet Pharmacodyn. 2013 Jun;40(3):257-65]. In current research QSP models are used to analyze dyslipidemia and its treatment with different drug modalities.

**Aims:** The objective of this research was to benchmark two anti-PCSK9 pharmacological modality classes: monoclonal antibodies (mAbs) and small interfering RNA (siRNA) in their potency to inhibit proprotein convertase subtilisin/kexin type 9 (PCSK9) thereby lowering low-density lipoprotein cholesterol (LDLc).
Methods: QSP model of PCSK9 inhibition model was generated to: 1) describe the dynamic interplay between LDLc and PCSK9; 2) perform quantitative assessment of two anti-PCSK9 drug modalities: monoclonal antibodies (mAb) and synthesis inhibitors (siRNA); 3) provide the analysis for multiple non-LDLc biomarkers.

Results: We demonstrated that LDLc decreased proportionally to PCSK9 reduction for both mAb and siRNA modalities. At marketed doses, however, treatment with mAbs resulted in an additional ~20% LDLc reduction compared with siRNA. We further used the model as an evaluation tool and determined that no quantitative differences were observed in other biomarkers, suggesting that the disruption of PCSK9 synthesis would provide no additional effects on lipoprotein-related biomarkers in the patient segment investigated [Victor Sokolov et al. J Lipid Res. 2019 Sep;60(9):1610-1621].

Conclusions: Model simulations indicated that siRNA therapies reach reduction in LDLc levels similar to the achieved with different marketed drug modalities (mAbs) given the current threshold of 80% PCSK9 inhibition by siRNA can be exceeded.

Combining computational modelling and machine learning to predict the specific response of patients to anticoagulant therapy in flow

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Background: Anticoagulant drugs prevent the incidence of prothrombic complications by inhibiting several pathways of the coagulation cascade. They also induce bleeding if the administered dose is excessive. Patients respond to each anticoagulant treatment differently because each individual patient has a specific composition of the plasma and specific kinetics of the coagulation biochemistry. Therefore, the fast and reliable prediction of the clotting response to anticoagulant therapy would be beneficial for the management of patients with prothrombotic risks.

Aims: We introduce a novel methodology that combine computational modelling and machine learning to predict the patients’ response to anticoagulant therapy under flow.

Methods: We use a previously developed model to fit the thrombin generation curves of real patients. Next, we extend the same model to simulate the spatio-temporal dynamics of thrombus growth for each patient under venous flow conditions. Then, we conduct numerical simulations to quantify the effects of anticoagulant drugs on clot growth and thrombin generation.

After the numerical analysis of the model, we use it to generate a database of virtual patients by varying three of the model most sensitive parameters in the physiological range. Then, we divide this cohort of virtual patients in different groups and we administer different treatment regimens for each group. Finally, we use the obtained database to train a support vector machine algorithm for the fast prediction of the clotting response of individual patients.

Results: We analyse the effects of different anticoagulant drugs on plasma coagulability and on clot growth dynamics. We present a quantification of the impact of anticoagulation on the
relative incidence of thrombotic and bleeding complications in a cohort of patients. The trained machine-learning algorithm yields an accuracy of over 95% and provides a prediction of the response of each patient instantaneously.

Conclusions: The proposed approach can be used as a tool for clinical decision-making and management of patients with prothrombotic disorders.

New serine protease inhibitors from Brassica Rapa and Beta Vulgaris

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Background: Nowadays thrombosis is characterized by high mortality and disability rate. Research has shown considerable impact of contact in pathologic clot formation. Factor XII which starts contact activation pathway belongs to the class of serine proteinase. And the most selective factor XII inhibitor (also trypsin inhibitor) was excreted from corn, that’s why we supposed that trypsin inhibitors from Beta vulgaris and Brassica rapa could inhibit contact activation pathway.

Aims: Excrete serine proteinase inhibitors from Beta vulgaris and Brassica rapa and examine its activity on trypsin and factor XII.

Methods: Proteins were extracted using 0.1% solution of sodium chloride, then the solution of proteins underwent temperature denaturation and filtration. For further purification we used affinity chromatography with trypsin. Analysis of proteins purity and molecular mass rating was performed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein concentration was measured using the bicinchoninic acid protein assay. Proteins inhibiting activity was evaluated by kinetic chromogenic assay.

Results: The mixture of proteins with molecular masses of 25 and 75 kDa in Beta vulgaris, 25 and 37 kDa in Brassica rapa, was extracted. Using Kinetic chromogenic assay, proteins were proved to be the inhibitors of trypsin.

Conclusions: Thus we generated a mixture of proteins having an inhibitory capacity towards trypsin.

Integral assays of hemostasis in patients with COVID-19

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Background: Blood coagulation abnormalities play a major role in the COVID-19 pathophysiology. However, specific details of hypercoagulation and anticoagulant treatment require investigation.

Aims: To characterize the hemostatic balance in COVID-19 patients on heparin.

Methods: Integral coagulation tests thrombodynamics (TD) and thromboelastography (TEG) were performed in 153 COVID-19 patients observed in a hospital setting. The study was approved by the Ethic Committee of the National Medical Research Center of Pediatric Hematology, Oncology and Immunology, and informed consent was obtained from all patients. All patients received medium-dose LMWHs depending on body weight according to routine hospital protocol for COVID-19 patients: enoxaparin (80-240 mg/day), dalteparin (5000-15000 units/day), or fondaparinux (5 mg/day). The patients on ECMO were on unfractioned heparin (500-1250 units/hour depending on weight and bleeding tendency).

Results: The patients before treatment (n=30) had extreme hypercoagulation by all integral assays (Fig. 1). The major parameters of TD (initial (Vi) and stationary (Vs) clot growth rates) exceeded the normal range in 83% and 76% of patients, respectively. The citrated native TEG parameters R, K, angle α and MA showed hypercoagulation in 24%, 17%, 48%, and 54% of these patients, respectively. The patients on treatment showed significant heparin response by Vs: 77% of TD measurements showed hypocoagulation, 15% were normal and 8% remained in hypercoagulation. The TEG parameters showed less response: angle α in citrated native TEG showed hypocoagulation in 24% of measurements only, while R and angle α for citrated kaolin TEG parameters in ECMO patients showed hypocoagulation in 63% and 54%, respectively. Since 8-17% of patients remained hypercoagulable on anticoagulant therapy, this may indicate that standard anticoagulation was insufficient in these patients.

Conclusions: Patients with COVID-19 have severe hypercoagulation, which remains in some patients after anticoagulation treatment, while others have significant hypocoagulation in these conditions. The data suggest critical issues of hemostasis balance in COVID-19 patients, and indicates potential importance of controlling their integral coagulation status.

Both dual-activation and apoptosis stimulation of platelets result in the necrotic procoagulant phenotype

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Background: Because both apoptotic and necrotic death pathways can lead to surface PS exposure and a procoagulant platelet surface, an accurate description of agonist-induced procoagulant platelets is required to differentiate these platelets from other potential procoagulant forms.
Aims: To compare apoptotic and necrotic platelets in terms of their ability to take part in membrane-dependent coagulation reactions, as well as the ability to adhere and aggregate and morphological appearance.

Methods: Platelets were isolated from whole blood by gel-filtration. Calcium ionophore A23187 (10 μM), thrombin (100 nM), CRP (20 μg/ml) and their mixture were used to necrotic platelets. ABT737 (1 μM) was used for apoptotic platelets. Non-activated platelets were used as a control.

Results: Both types of PS-positive platelets (apoptotic and necrotic) are characterized by a spherical shape, with non-uniformly distribution Annexin V. Annexin V is concentrated in a small area region (“cap”). However, apoptotic platelets, in contrast to necrotic ones, do not exhibit α-granular proteins on their membrane. Apoptotic and necrotic platelets do not differ in their ability to bind coagulation factors and take part in procoagulant reactions of blood coagulation. Both types of PS-positive platelets have not active integrines αIIbβIII on their membrane so worse than PS-negative platelets could aggregate and adhere to place of injure.

Conclusions: No significant differences of necrotic and apoptotic platelets were found in functional properties and morphological appearance.

Thrombosis and hemostasis in children with Acute Lymphoblastic Leukemia

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Background: Approximately 70% of children with ALL (Acute Lymphoblastic Leukemia) suffer from CIC (central venous catheter) thrombosis during the treatment. From 5 to 36% of this thrombosis have clinical manifestation. There is still no diagnostic keys for this problem.

Aims: To evaluate the state of hemostasis in children with ALL to identify the high-risk group for thrombosis.

Methods: 73 patients (45 boys, 28 girls, from 1 to 17 years) with ALL during Consolidation Phase of ALL-MB-2015 protocol (10 points of blood collection throught 18 weeks) were enrolled in this study. All thrombosis were detected by ultrasound. Clotting times (APTT, TT, PT), fibrinogen, antithrombin III (ATIII), thrombomodulin (TM) and D-dimer concentrations, Thromboelastography (TEG) and Thrombodynamics (TD) were performed before and during the treatment. For calculations the program OriginPro 8.0 (Microcal Software, Northampton, MA, USA) was used. The Pearson coefficient was used to assess the statistical significance (p).

Results: Clotting times (APTT, TT, PT) were in normal or hypocoagulation area (nearly 50% of points) caused by procoagulant factors decrease during L-asparaginase treatment: fibrinogen lowered in 72% of points. But clotting times were not sensitive to natural anticoagulants decrease confirmed by ATIII reduction in 41% of points. TEG parameters were normal (66%) or in hypocoagulation (34%) area during the treatment most caused of platelets and erythrocytes count failure. Thrombosis occurred in 40 patients (55%). TD with clot growth rate (Vs) was the only revealed hypercoagulation in 64% of points. But there were no differences (p>0.05) in TD between groups patients with and without thrombosis. We detected threefold increase of TM
levels in patients with thrombosis (p<0.001) compared to patients without thrombosis. Perhaps endothelial dysfunction also contributes to hypercoagulation beyond plasma clotting. We managed to identify the high-risk group for thrombosis: if the patient had hypercoagulation by TD and had impaired lysis function (normal D-dimes) then the risk of thrombosis is from 45 to 53% regardless of the ATIII decrease.

**Conclusions:** A possible mechanism for the development of thrombosis in ALL is a serious damage in endothelium and in lysis function. Hypercoagulation were detected in most patients by TD, but some with periodical increased D-dimers retain the lysis function while those with D-dimer only normal range had thrombosis complications. There was no thrombosis in patients with normal TD.

**Phosphatidylserine expression on platelets leads to additional and delayed thrombin generation**

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**Background:** Blood plasma coagulation is greatly affected by platelets, however, the impact of various stages of their activation on the process of thrombin generation have not been studied.

**Aims:** To investigate how platelets are involved in thrombin generation and fibrin clot formation.

**Methods:** We used fluorescence video microscopy to monitor fibrin formation and thrombin generation, confocal microscopy and flow cytometry to assess platelet activation during clotting. Thrombin generation test (TGT) was performed in the platelet-rich plasma (PRP, 40-200x10³ cells/μL) or platelet-free plasma (PFP) of healthy volunteers, plasma from a patient with gray platelet syndrome and a patient with afibrinogenemia. Coagulation was activated using 5 pM tissue factor.

**Results:** Thrombin generation curve in the PRP had two peaks. The time of the first peak did not depend on platelet concentration, coincided with the time of the only peak in the PFP, and followed plasma clotting. The time of the second peak decreased as the platelet concentration increased, leading to the confluence of the two peaks at the platelet concentration of 200x10³ cells/μL. The amplitudes of both peaks increased equally with increasing platelet concentration. In the presence of the platelet activation inhibitor prostaglandin E1 (10 μM), the second peak disappeared (the area under the curve decreased by a factor of 2), whereas in the presence of thrombin-like platelet activators 1 and 4 (200 and 550 μM, respectively), the second peak shifted to the first (with the retention of area under the curve). The addition of artificial lipid microvesicles (0.5-4 μM) led to the increase in the amplitude of the first peak and a shift of the second peak to the first. Microscopic and cytometric data showed that alpha granules were released completely before the formation of the first peak, while phosphatidylserine (PS) expression started after the formation of the first peak and coincided in time with the formation of the second peak. In the plasma of a patient with gray platelet syndrome (alpha granules deficiency), a two-peak generation of thrombin was also observed. The addition of 4 μM lipids to
the PFP after the thrombin peak formation resulted in a second peak; the addition of activated 1 nM activated factor V did not affect thrombin generation.

**Conclusions:** Thrombin generation in the presence of platelets occurs for a longer time than in the PFP due to the presence of two stages of generation. Depending on the initial conditions, these stages can proceed almost simultaneously or be spaced in time. The second stage of generation is associated with the release of PS and does not depend on the release of alpha granules. Moreover, the amount of phosphatidylserine bearing surface presented in plasma is sufficient for fibrin clot formation.

**Hereditary spherocytosis in children: hypercoagulation and risk of thrombosis**

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**Background:** Children with hereditary spherocytosis (HS) do not have the dramatically high risk of spontaneous thrombosis that is more likely in adults. The risk of thrombosis in children is nearly 5% but it is several times higher than in general children population. Risks increase in adolescence and after surgery.

**Aims:** To evaluate the state of hemostasis in children with HS during hemolytic crisis and in without.

**Methods:** Patients with HS during hemolytic crisis (28 patients, 18 boys and 10 girls, age 0.5 to 17 years) and patients with HS without hemolytic crisis (34 patients, 20 boys and 14 girls, age 1 to 17 years) were enrolled in this study. Clotting times (APTT, TT, PT), fibrinogen and D-dimer levels, Thromboelastography (TEG) and Thrombodynamics (TD) assays were used to assess the hemostasis state. For calculations the program OriginPro 8.0 (Microcal Software, Northampton, MA, USA) was used. The Mann-Whitney U-test was used to assess the statistical significance (p).

**Results:** APTT, TT and PT were not significantly different between the two groups (p>0.05). Fibrinogen levels were decreased in patients with HS in common compared to normal ranges (2.1 ± 0.4 mg/ml), but there were no significant differences between two groups (p>0.05). This may be caused by the well known consumption of fibrinogen during acute hemolysis. All TEG parameters (R, k, alpha and MA) revealed hypercoagulation in HS patients during hemolytic crisis compared to HS patients without crisis (for MA: 62±5 mm vs. 57±4 mm, p<0.05). Also TD parameters (Vi, Vs) revealed hypercoagulation in group with crisis by clot growth rates (for Vs: 31±4 μm/min vs. 26±3 μm/min, p<0.05). There were no significant differences between two groups (p>0.05) in D-dimer levels, but 4 children from group during crisis had the elevated D-dimers compared to 1 child in group without.

**Conclusions:** We revealed that exacerbation of hemolysis is directly related to the increased blood clotting in patients with HS. It is necessary to evaluate the hypercoagulation state with global hemostasis assays in children with HS for predicting thrombotic complication which can occur in postoperative period.
The computational analysis of the substrate delivery pathways for the enzymatic complexes of prothrombinase, intrinsic and extrinsic tenases

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**Background:** Phospholipid membrane is critically important for reactions of blood coagulation. Enzyme-cofactor complexes which catalyze those reactions are located on the membrane surface. The examples of such enzyme complexes are prothrombinase, intrinsic and extrinsic tenase. The former catalyzes the activation of prothrombin, while the latter two activate factor X. The reaction mechanisms of prothrombinase and tenases are similar. The complex of an enzyme (Factor VIIa, Factor IXa or Factor Xa) and a cofactor (tissue factor (TF), Factor VIIIa or Factor Va) is formed on the phospholipid surface and then it can catalyze the substrate hydrolysis. However, the functioning of these complexes is significantly complicated by the fact that their substrates are able to reversibly bind to the membrane and consequently they are distributed between the membrane and solution. The preferred substrate delivery pathways could depend on the reaction conditions and the mechanisms of such reactions are not clear.

**Aims:** Computational analysis of the substrate delivery pathways for the enzymatic complexes of prothrombinase, intrinsic and extrinsic tenase under different reaction conditions.

**Methods:** The computational models of (A) prothrombinase, (B) intrinsic tenase and (C) extrinsic tenase functioning on the phospholipid vesicle surface were constructed. The models were constructed as systems of ordinary differential equations. The models were integrated in Matlab and COPASI. The model parameters were tuned for them to accurately describe experimental data from (Hathcock et al. 2005, Stone et al. 2005, Panteleev et al. 2006).

**Results:** Model (A) predicted that in presence of high concentrations of enzyme (Xa) or cofactor (Va) prothrombin from solution is the preferred substrate for prothrombinase. On the other hand, in presence of high concentrations of phospholipid, the membrane-bound prothrombin was the preferred substrate. Similar results were obtained in the model (B): in presence of high concentrations of cofactor (VIIIa), the factor X from solution was the preferred substrate. In contrast to these results, the model (C) predicted that only in presence of high surface concentrations of the cofactor (TF) (more than 2.64×10⁻³ nmol/cm²), the solution-phase factor X is the preferred substrate. In other cases the membrane-bound factor X is preferred in presence of both high and low phospholipid concentrations.

**Conclusions:** For prothrombinase and intrinsic tenase the way of substrate delivery switches from the solution-dependent to the membrane-dependent with the rise of phospholipid concentration. In contrast, the extrinsic tenase binds preferentially the membrane-bound substrate in presence of all phospholipid concentrations. For all complexes solution-phase substrate is the preferential one in presence of high cofactor concentration.

Theoretical study of antibodies response in COVID-19

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Background: Development of antibody-based drugs and vaccines against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a global priority. The mechanism that controls long-lasting adaptive immune response is still unclear. In the last few months, more cases of reinfection with SARS-CoV 2 are registered in different countries.


Methods: A mathematical model based on laws of chemical kinetics was developed and analyzed. Briefly, virus particles interact with antigen-presenting sites (APS) and activate them. While activated, these sites are capable of stimulation of the B-cells proliferation and differentiation into memory B-cells and plasma cells. The latter are the main sources of antibodies against pathogens. These antibodies bind to virus particles and promote their effective elimination. The system of ODE equations was solved using LSODA method implemented in COPASI software (COPASI.org).

Results: Within the framework of this model, we could describe the viral load dynamics and the antibodies response in patients with COVID-19 (https://doi.org/10.1016/S1473-3099(20)30196-1, https://doi.org/10.1038/s41586-020-2196-x). System biology analysis revealed main parameters regulating viral concentration and kinetics of antibodies. It was found that the maximal activation rate of antigen-presenting sites determines the highest value of antibodies concentration, whereas plasma cell death rate explains fast decrease of antibody concentration in blood. When we set the antibodies concentration at the initial state to be non-zero, we could observe more efficient viral clearance compared to when the antibodies are initially absent.

Conclusions: Theoretically, the specific antibodies response is determined by the activation rate of the antigen presentation, and the rate of plasma cell death.

Modeling the viral load dynamics in coronavirus infection

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Background: COVID-19 is an infectious disease caused by SARS-CoV-2 virus. Mathematical modeling can shed light on the manifestation of such a phenomenon as antibody-dependent enhancement of coronavirus infection (ADE). The most common mechanism of ADE occurs when a non-neutralizing or poorly neutralizing antibody binds to a virus particle. The antibody-virus complex binds to the Fc-receptor of leukocytes and is absorbed by these cells. If the antibody is suboptimal, the virus can free itself from the antibody and cause adverse immune reactions.

Aims: The aim of this work is to study theoretically the viral load dynamics in SARS-CoV-2 infection.

Methods: A mathematical model based on the laws of chemical kinetics was developed. The model was fitted to the data of SARS-CoV-2 infection in patients (10.1016/j.mbs.2020.108438). In the model, virus particles could infect healthy pneumocytes and replicate in them. A few days
after the onset of symptoms, seroconversion occurred and virus-specific antibodies appeared. Antibody concentration was set as a step function, where the seroconversion day was taken from the same dataset as the viral load dynamics. Free and antibody-bound virus particles were engulfed by macrophages and eliminated from the body. To simulate ADE, we assumed that upon engulfing antigen-antibody complex macrophage could become infected with probability $\rho$. The system of ODEs was integrated using COPASI software (http://copasi.org/).

**Results:** The model described the viral load dynamics in patients with SARS-CoV-2. The analysis of the model revealed that the viral load dynamics is determined by pneumocyte infection rate, pneumocyte virus production rate and concentration of antibodies. The initial viral load did not influence its further dynamics. Additionally, it was found that for 6 out of 7 described patient data macrophage infection did not influence the viral load dynamics when parameter $\rho$ was less than 0.01. In a model patient, for which the medians of the parameters were taken, the infection of macrophages significantly influenced the dynamics of the concentration of viruses only in case when antibodies already existed before the infection, and the parameter $\rho$ was greater than 0.001.

**Conclusions:** Theoretically, the viral load dynamics is not influenced by macrophage infection in the absence of pre-existing antibodies.

**Deciphering platelet calcium signaling**

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**Background:** Human platelets have a unique feature: one relatively simple signal simultaneously controls different functional responses of the platelet depending on type and amount of an activator. Platelets exhibit series of calcium spikes upon activation, so we subjected platelet calcium profiles to a number of tests in order to understand the principles of signal encoding in them.

**Aims:** Our goal was to understand how a platelet interprets extracellular stimuli by a series of calcium spikes.

**Methods:** We obtained calcium profiles using TIRF microscopy and microfluidic systems that allowed us to control platelet activation. We analyzed calcium profiles for resting platelets, platelets activated with 10 μM ADP and non-fibrillar collagen type I. We used FFT to extract the possible fundamental frequency from a signal. Using thresholding to eliminate the noise, we performed feature extraction which resulted in distributions of peak amplitudes, widths, areas, numbers per signal and interspike intervals. Statistical differences between types of platelet activation were measured using Kolmogorov-Smirnov criterion. Also, we tested interspike intervals to be of Poissonian nature or not.

**Results:** Fourier analysis did not reveal a fundamental frequency of platelet calcium oscillations due to stochasticity of calcium spikes and Gibbs effect caused by peaks. We observed statistical differences between platelet calcium spikes’ features for all types of activation, with
interspike intervals which displayed 3 s shifts between non-activated and activated cells. Also, the large (more than 10 s) interspike intervals were observed only for resting platelets. Resting platelets exhibited 0.02 - 0.05 peaks per second, while in response to collagen or ADP platelets tend to 0.25 - 0.35 peaks per second. We showed that means and standard deviations of interspike intervals were in linear relation for all types of activation, and there was a minimum interspike interval of 1.7 ± 0.3 s predicted.

**Conclusions:** We showed that distributions of platelet calcium peak features were statistically different between each other upon activation, with interspike intervals and number of peaks per second giving the most dramatic results, indicating the importance of temporal properties of the signals. We showed that occurrence of platelet calcium peaks can be interpreted as a Poissonian process with a deterministic part that is constant between types of platelet activation. Fast Fourier Transform is not applicable for platelet calcium dynamics.

**Analysis of fibrin formation and thrombin generation dynamics under arterial blood flow conditions**

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**Background:** One of the urgent tasks in the field of thrombosis and hemostasis involves understanding the mechanisms governing blood coagulation under arterial flow rates. It is especially important to study the initial stages of blood clotting, since the possibility of forming a stable core of the further blood clot depends on the coagulation reactions taking place after the vascular injury. These reactions demonstrate threshold dependencies on several key parameters, including shear rate, surface density of tissue factor and the size of the damaged area of the vessel wall.

**Aims:** The goal of this work is experimental analysis of the spatiotemporal parameters of plasma coagulation on high-density tissue factor at arterial shear rates and further clarification of the mechanisms that regulate the observed processes.

**Methods:** Microfluidic parallel-plate flow chambers were used for the in vitro experiments. Vesicles containing tissue factor were localized on the activated plastic surface. The platelet-free plasma of healthy donors supplemented with fluorescent probes was perfused through this system at flow rate corresponding to arterial shear rate (1000 s⁻¹). Both thrombin activity and fibrin formation on the activator area were detected using fluorescence microscope in real time.

**Results:** Despite arterial flow rates and the absence of platelets, fibrin polymerization starts downstream from the beginning of the tissue-factor-containing region and visibly propagates in two directions: against the flow and away from the surface. Polymerization process starts soon after plasma contact with tissue factor (1-2 minutes). The fibrin growth has a tendency to stop after reaching approximately 10 μM height.

**Conclusions:** High density tissue factor is capable of initiating plasma coagulation in the absence of platelets even under arterial flows. However, the mechanisms limiting the propagation of fibrin polymerization are poorly understood.
DYNAMICS OF BLOOD CLOT FORMATION IN A MICROFLUIDIC MODEL OF THROMBOSIS

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Background: One of the major goals of the hemostasis system is to seal any penetration in blood vessels. To do this, it must be able to form blood clots rapidly, locally, limit their growth to avoid unnecessary occlusion and dissolve formed clots after the vessel wall is no longer damaged.

In modern in vivo experiments it was shown that thrombi have a heterogeneous structure. Similar data were also obtained from ex vivo experiments using artificial flow chambers. However, exact spatiotemporal parameters of thrombus and fibrin clot growth under blood flow conditions have not been determined in ex vivo models. In attempt to address this issue a new model of thrombosis was developed to study different properties of thrombus formation.

Aims: The main goal of this work was experimental analysis of simultaneous platelet aggregation and fibrin formation in a microfluidic model of thrombosis.

Methods: In order to achieve our goals we developed a microfluidic model of thrombosis - an ex vivo system in which human whole blood is perfused at venous shear rate of 200 1/s using a syringe pump through a channel over an “activation zone”, where type I collagen and high density tissue factor (TF) are immobilized.

To create this zone a new method of incubation and localization of proteins was developed. In this method, TF and collagen were applied in a shape of a straight stripe on a plastic surface so that their coverage areas coincided.

Experiments with whole recalcified human blood of healthy donors were imaged in real time on the upstream edge of the activation stripe using a confocal microscope in a z-stack mode, allowing to measure both platelet aggregate height and fibrin distribution at any given moment.

Results: Throughout the course of the research the microfluidic model of arterial thrombosis was developed and tested, which made it possible to study dynamics and physical qualities of thrombus formation induced by collagen and TF.

The analysis of thrombus formation dynamics made it possible to determine the parameters of platelet aggregate growth and fibrin formation in detail. The most important result of the study is the observation of both platelet aggregate and fibrin clot growth rate plateauing under constant flow conditions. Fibrin height on average constitutes (50% +/- 20%) of the aggregate height. In most of the performed experiments thrombus growth rate significantly decreases after 10 minutes of whole blood perfusion.

Conclusions: Obtained results made it possible to put forward a hypothesis that there is a pronounced influence of blood shear rate on dynamics of the thrombus formation. However, mechanisms limiting thrombus propagation in the developed model are yet to be fully determined.
Computational modelling of agonist-driven thrombus formation under arterial blood flow

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\textbf{Background:} Platelet accumulation at the site of vascular injury is regulated by soluble platelet agonists, markedly thrombin and ADP, which are in large responsible for the formation of thrombus core and shell, respectively. However, the interplay between thrombin generation, granule secretion and overall thrombus dynamics is poorly understood.

\textbf{Aims:} This work is aimed at the development of computational tools for detailed exploration of thrombus heterogeneity driven by spatial and temporal segregation of the major soluble platelet agonists.

\textbf{Methods:} Adopting a previously developed two-dimensional \textit{in silico} model focused on the thrombus shell formation, we revise it to introduce platelet agonists. Blood flow is simulated via computational fluid dynamics approach. In order to model soluble platelet activators, we apply Langevin dynamics to a large number of non-dimensional virtual particles. Taking advantage of the available data on platelet ADP-containing dense granule secretion kinetics, we model platelet degranulation as a stochastic thrombin-dependent process.

\textbf{Results:} The new model was able to qualitatively reproduce hierarchical structure of thrombus as observed \textit{in vivo}, as well as impaired thrombus formation observed under either thrombin or ADP inhibition.

\textbf{Conclusions:} The developed model describes the core-and shell organization of microvascular thrombus and links rapid thrombus growth with dense granule secretion process, in line with available experimental data.

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\section*{A new mathematical model of hemostasis in microvasculature for evaluation of local hemodynamic parameters}

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\textbf{Background:} Hemostasis in microvasculature represents a poorly understood process. Direct measurements of key hemodynamics parameters during hemostasis are experimentally
challenging, however, such information is crucial for understanding the exact mechanisms that drive this process.

**Aims:** The general aim of this work was to obtain the estimates of wall shear rates during hemostatic response to the injury of the microvascular tree.

**Methods:** To estimate the dynamics of shear rates we developed a new model of bleeding in microvasculature based on Tsiklidis’ model. This model may be applied to bleedings caused by small injuries like a cut on the finger. Model simulates bleeding in a symmetrical vascular tree, using Poiseuille equations and takes into account both vasoconstriction and thrombi formation. Model was validated using Sawada’s experiments, who studied bleeding in pig’s ear after incision.

**Results:** Model correctly estimates the bleeding times in small vessel and predicts extremely high shear (up to 40 000 1/s) during bleeding in arterioles. Interestingly, sensitivity analysis showed that bleeding dynamics and critical shear rates are very sensitive to the length of the growing hemostatic plug.

**Conclusions:** The developed model predicts extremely high shear rates and short bleeding times during hemostasis in microvasculature. The length of the growing hemostatic plug may significantly impact local hemodynamics and hence the dynamics of hemostasis.

**Conformational dynamics of von Willebrand factor in conditions of vessel stenosis**

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**Background:** Multimeric glycoprotein von Willebrand factor plays an important role in thrombus formation under extreme hydrodynamic conditions. However, despite active research, the exact mechanisms driving biomechanical thrombus formation at the sites of vessel stenosis are poorly understood.

**Aims:** The general aim of this study is elucidation of the mechanisms that initiate thrombus formation under conditions of vessel stenosis.

**Methods:** *In vitro* studies were performed using PDMS microfluidic chambers. Hirudinated human blood was perfused through rectangular channels coated with fibrinogen, with symmetric two-sided liner stenosis with 90% lumen reduction, as described in [1]. The observations were made in the region of maximum lumen reduction with fluorescent microscope. To study a possible role of von Willebrand factor, the coarse-grained molecular dynamics simulations of this molecule under the corresponding hemodynamic conditions were performed. Computational modeling of shear-induced diffusion of von Willebrand factor molecules under similar hydrodynamic conditions were used for vWF transport analysis.
Results: Experimental distribution of the positions of first platelet aggregates formed in the stenosed region of the chamber peaks at the distance of 300-600 μm from the beginning of the stenosis zone. Using in silico model it was shown that large vWF multimers moving on the certain height (10 μm) from the bottom of the channel in the area of maximum lumen reduction experience the most significant conformational changes - both maximum extension and inter-dimer extensional forces. Analysis of vWF transport to the chamber floor revealed that theoretical and experimental distributions are consistent in case when shear diffusion coefficient of VWF matches the value corresponding to platelets.

Conclusions: Our results reinforce the proposed mechanism responsible for initiation of platelet thrombus formation due to flow disturbance, described earlier [1]:
- unfolding and activation of vWF molecules due to elongational velocity gradient near stenosis entrance;
- shear-induced diffusion of flow-activated von Willebrand molecules to the vessel wall;
- interaction between platelets and adhered flow-activated von Willebrand molecules resulting in platelet adhesion, followed by platelet aggregation.

In vitro thrombosis model demonstrates two types of thrombus dynamics

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Background: Non-occlusive arterial thrombosis models in vivo demonstrate three-phase thrombus dynamics: rapid thrombus growth is followed by disaggregation and stabilization of thrombus size. However, such dynamics does not occur in vitro. In order to address this problem, we designed in vitro flow chamber, which better mimics hemodynamic conditions found in microvasculature.

Aims: The major goal of this work was to analyze the dynamics of thrombus formation on type I fibrillar collagen in a novel microfluidic model of arterial thrombosis.

Methods: Polydimethylsiloxane (PDMS) microfluidic flow chamber with square cross-section (50x50 μm) channel was attached to polycarbonate slide. Hirudinated whole human blood supplemented with fluorescent probes was pumped with a syringe pump into a primary channel of a microfluidic chamber with a shear rate of 1000 s⁻¹. The primary channel was further divided into two sub channels, the first leading to the region with immobilized type I fibrillar collagen and the second acting as a bypass. Channel bifurcation imitates in vivo conditions by creating a quasi-stationary pressure drop between the bifurcation start and end. Collagen was immobilized on a 100 μm - width stripe using microfluidics, with fibers aligned perpendicularly to the blood flow. Thrombus growth was visualized in real-time using epifluorescence microscope.

Results: Two types of thrombus growth dynamics have been observed in our in vitro system: intensive thrombus growth leading to channel occlusion or formation of non-occlusive thrombus with further disaggregation. The exact mechanisms responsible for the observed behavior are yet to be determined.
**Conclusions:** The developed microfluidic model of thrombus formation demonstrates both occlusive and non-occlusive thrombosis scenario, rendering it a valuable tool for future research and applications.

**Lattice-Boltzmann Intravascular Thrombolysis**

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**Background:** Ischemic stroke, and more generally, cardio-vascular diseases, are amongst the top issues in public health.

**Aims:** Ischemic stroke results from the occlusion of a brain vessel due to the formation of a thrombus. One way to remove this thrombus is to inject a chemical into the patient's bloodstream, which will breakdown the clot's fibrin fibers: this is called Intravascular Thrombolysis (IVT).

**Methods:** We present a novel 3D mesoscopic lattice-Boltzmann (LB) model, which simulates this process. The thrombus is a porous medium described with Walsh's Partially Bounce-Back (PBB) dynamic, and varies in time as lysis occurs, following Diamond et al.

**Results and Conclusions:** The model is able to reproduce the observed blood clots permeabilities, and to recover lysis times from literature.

**Anomalous transport, adhesion and aggregation properties of platelets**

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The transport of platelets in blood is commonly assumed to obey an advection-diffusion equation. Here we propose a disruptive view, by showing that the random part of their velocity is governed by a fat-tailed probability distribution, usually referred to as a Lévy flight. Although for small systems, it is hard to distinguish it from the generally accepted Brownian motion, for larger systems this effect is dramatic as the standard approach may underestimate the flux of platelets by several orders of magnitude, compromising in particular the validity of current platelet function tests. We will show experimental and numerical evidences of this anomalous diffusion, and also discuss the deposition properties of platelets in healthy and pathological situations.
Topic: Fundamental and applied aspects of cell diagnostics and therapy

Acute leukemia diagnosis using an anti-cluster-of-differentiation antibody microarray

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Background: Acute leukemia diagnosis is based on enumeration and lineage determination of immature hematopoietic cells (blasts) in bone marrow aspirate. This analysis is usually performed separately by morphology in films and by determination of lineage-specific cluster-of-differentiation (CD) molecules on the cell surface by flow cytometry. Earlier we have developed an anti-CD antibody microarray for leukocyte “panning” by their surface CD antigens and a method for the morphology analysis of microarray-captures cells. Here we show the diagnostic algorithm for microarray-based analysis of bone marrow aspirate mononuclear fraction for acute leukemia diagnosis.

Aims: The aim of the study was the development of a diagnostic algorithm based on the number of cells with blast morphology captured by the different antibody spots on the microarray.

Methods: The bone marrow aspirates from 196 pediatric patients with acute leukemia (130 patients with acute myeloid leukemia (AML) and 66 patients with acute lymphoblastic leukemia (ALL)) and 20 healthy donors (material obtained during preparation for haploidentical transplantation) were studied using the anti-CD antibody microarray containing 41 antibodies against lineage-specific CD markers and negative control. 41 patients with AML and 23 patients with ALL were used as a training cohort during the diagnostic algorithm development.

Results: The presence of more than 25% of cells with blast morphology among all anti-CD45RA-captured cells on the microarray is found to determine the patients with acute leukemia with 94% sensitivity and 97% specificity. We have suggested a scoring system for the blast lineage determination based on the presence or absence of cells with blast morphology captured by the antibodies against lineage-specific CD markers on the microarray. The myeloid score is increased by 1 for the presence of blast cells in the antibody spots against CD11c, CD13, CD33, CD64, CD117, CD41 and CD61 and by 0.5 for the presence of blast cells in the antibody spots against CD11b and CD15. The B-score is increased by 1 for the presence of blast cells in anti-CD10, CD19 and CD22 spots and the T-score is increased by 1 for the presence of blast cells in anti-CD1a, CD3 and CD5 spots and by 0.5 for anti-CD2 and anti-CD7 spots. The B-score of 2 and higher determines the patients with B-ALL with 97% sensitivity and 100% specificity and the myeloid score of 2 and higher determines the patients with AML with 96% sensitivity and 97% specificity. The T-ALL, however, could not be reliably determined using the combination of B, T and myeloid scores. Additional analysis is needed to verify the T-cell nature of the blasts for reliable T-ALL diagnosis on the microarray.

Conclusions: The anti-CD antibody microarray for bone marrow aspirate mononuclear cell panning and morphology analysis permits to diagnose the presence of acute leukemia and blast attribution to B-lymphoid or myeloid lineage with high sensitivity and specificity.
The Role of Dopamine, Calcium and alpha-Synuclein in Selective Vulnerability of Neurons in Parkinson’s Disease

Eugene Mosharov¹,²


Background: Parkinson’s disease (PD), the second most common neurodegenerative disorder, is marked by bradykinesia, resting tremor, muscular rigidity, and postural instability. Although multiple brain regions are affected in late-stage PD, two catecholaminergic neuronal populations degenerate early, before the onset of the motor symptoms - dopaminergic (DA) neurons of the substantia nigra (SN) pars compacta and noradrenergic (NE) neurons of the locus coeruleus (LC). Interestingly, dopaminergic neurons of the ventral tegmental area (VTA) that neighbors the SN are largely spared in PD.

Aims: To identify unifying and differential events that underly a cascade of neuropathological changes in SN and VTA neurons during PD-like neurodegeneration.

Methods: Optical, electrophysiological and electrochemical recordings from SN and VTA neurons in postnatal cultures and acute midbrain slices treated with a parkinsonian neurotoxin MPP+ and dopamine precursor L-DOPA.

Results: Significant metabolic differences were found between SN and VTA neurons both under rest and under stress. Our data support the multi-hit hypothesis of neurodegeneration where synergistic interactions between calcium, dopamine and a small presynaptic protein alpha-synuclein lead to imbalanced protein turnover and selective vulnerability of SN and LC neurons.

Conclusions: Decreasing the levels of any the toxicity mediators provides neuroprotection, offering multiple opportunities for targeted drug interventions aimed at modifying the course of PD.

Filtration-based method for isolation and cytological analysis of circulating tumor cells

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Background: Detection and analysis of circulating tumor cells (CTC) is a promising diagnostic method in oncology. However, the minute number of CTC in blood (few cells per milliliter) raises certain technological problems in their separation. One of the approaches to CTC separation is based upon their larger size and worse deformability compared to leucocytes and consists in filtration through filters with pores of micrometer diameters. As cancer diagnosis involve cytological investigation of tumor cells, clinically acceptable CTC-detecting method should permit cytological analysis of filtrated cells.
Aims: The main purpose of this work is to develop a method for CTC isolation based on peripheral blood filtration through a semi-transparent track-etched polymer membrane with pore diameters of 5-7 μm permitting further cytological analysis of separated tumor cells directly on the filter.

Methods: Track-etched PET membrane of 15-20 micrometer thickness, pore diameter 7 ± 0.5 micrometers, pore density 105/cm² and light transmission of 75% (courtesy of LNR JINR, Dubna), was placed into a sealed plastic cassette ensuring the flow of the filtrated cell suspension in the direction perpendicular to the membrane surface. Three cell lines: MCF7, T47D and MDA-MB-231 in phosphate buffer solution, pH 7.4, were used as a model system for CTC-containing blood. After the filtration the cassette was disassembled, the membrane with captured cells was placed onto the glass slide, dried and stained by May-Grunewald-Giemsa. The number of captured cells was determined cytologically by bright-field microscopy. The filtration capacity of the filter was determined as the ratio of the absolute number of the captured tumor cells found to the calculated number of tumor cells introduced into the suspension to be filtered.

Results: Cytologically the membrane-detained cells of all three cell lines after drying and staining directly on the membrane closely resemble the same cells in smears. The transparency of the membrane used was sufficient for cytological investigations of CTC by bright-field microscopy. The filtration capacity of filters for tumor cell lines in the buffer, according to the results of 66 experiments with the estimated number of tumor cells in the filtered suspension from 17 to 3500 pieces, was 55±17%. For suspensions containing tumor cell lines in diluted normal peripheral blood with the estimated number of tumor cells in the suspension from 28 to 420 cells, the filtration capacity according to the results of 30 experiments was 30±4%.

Conclusions: The proposed protocol for drying and staining of cells retained by the filters permits to conduct a cytological study of the CTC directly on the filter. The described method can be used for cytological examination of CTC in patients with stages I-IV carcinomas.

Impact of activator parameters on thrombus dynamics in the microfluidic model of thrombosis

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Background: In vitro microfluidic systems are widely used to simulate thrombus formation after vessel wall injury. However, there are significant differences in the dynamics of thrombus formation observed in vitro and in vivo, and many of the mechanisms regulating thrombus growth are still unknown.

Aims: The aim of this work is to analyze the dependence of thrombus formation dynamics initiated by type 1 collagen in a microfluidic system on the parameters of the activator, hemodynamic conditions, and the presence of aspirin.

Methods: Microfluidic flow chambers made of polydimethylsiloxane (PDMS) were used, having three channels of rectangular cross-section, 50 μm in height, 1000 μm in width and 1 cm long. Type 1 collagen was immobilized on the glass coverslip as a strip using a special flow chamber
(with 1 mm wide channel), after which another chamber was installed so that in the middle of each channel, a 1x1 mm area with collagen was obtained. Hirudinated human blood supplemented with fluorescent probes was perfused through the system and microscopic images were obtained in real time and further analyzed.

**Results:** Immobilization of collagen using microfluidic system results in oriented fibers. Analysis of thrombus formation parameters on different concentrations of collagen revealed a threshold value of concentration (0.1 mg/ml), at which the growth of thrombi begins. The orientation of collagen fibrils, as well as the shear rate of blood flow, significantly affects the dynamics of thrombus formation in this microfluidic model. The synthesis of thromboxane A2 plays a significant role in the observed stable growth of thrombi over long periods of time (5-20 minutes).

**Conclusions:** There is a threshold value of collagen concentration in case when microfluidic immobilization of collagen is used. Both fibril orientation and shear flow velocity impacts the dynamics of thrombus and the first layer of platelets formation in the considered model. Thromboxane A2 synthesis is crucial for the long-term stable growth of thrombi.

**How the Length of the Incubation Time Affected Platelet Activation in Platelet Function Assay**

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**Background:** Using the flow cytometry method in the platelet function diagnostics has several advantages, allows us to obtain more comprehensive information about the clue parameters of platelets describing their function activity. However, there is no unified protocol for that test, and the optimal conditions for the testing stay unclear.

**Aims:** Our study aimed to identify the alterations of the platelet activation parameters over time to assess the optimal test conditions.

**Methods:** Blood samples from three healthy adults collected in tubes with sodium citrate (final concentration 3,8%) were diluted by a modified Tyrode buffer. Platelets were stimulated by the addition of a mixture of the collagen-related peptide (1.25 μg/ml), PAR-1 activating peptide SFLLR-NH₂ (12.5 μM), and 2.5 mM CaCl₂. The samples incubation with activation mixture was performed for 1, 5, 10, 20, and 30 minutes after that was the incubation with immunofluorescent antibodies for 2 minutes. We determined forward scatter (FSC), side scatter (SSC), CD42b-PE, CD61-PE, CD62P-Alexa Fluor 647, PAC-1-FITC, annexin V-Alexa Fluor 647 binding, and mepacrine release levels. Assess was performed by the flow cytometry method.

**Results:** We established that most of the parameters (CD42b, CD61, CD62p, and the release of mepacrine from dense granules) reached saturation approximately in 1-3 minutes of activation, while the PAC-1 attained it in 10 minutes. The percent of procoagulant platelet continued to increase throughout all time. When the activation was performed for 30 minutes, blood clotted in all samples.

**Conclusions:** We determined that the optimal activation time frame for our test is 10 minutes.
Immunofluorescence Staining of Blood Smears for the Diagnostics of Platelet Disorders

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Background: The method of immunofluorescence staining of blood smears is the relatively new approach to identify the various platelet pathologies like a Bernard-Soulier, Gray platelet, Wiskott-Aldrich syndromes, MYH9 disorders, and others. While this method has many perspectives, it also demands the revision and standardization to integration into clinical practices.

Aims: Our study aimed to verify the reproducibility of the method and its applicability to the diagnostics of platelet pathologies.

Methods: Blood samples from healthy adults and children with established platelet disorders collected in tubes with sodium citrate (final concentration 3.8%) after sampling the blood smears were prepared, air-dried, and fixed in the acetone. For immunofluorescence the primary antibodies Myosin, LAMP 1 (H5G11), LAMP 2 (H4B4), VWF, P-Selectin (CD62P), CD63 (delta-granules), Ib/IX CD42a (FMC25), IIb/IIIa CD41 P2, β1-Tubulin (2.1.), α-Tubulin (RM113) and secondary antibodies ALEXAFluor 568, ALEXAFluor 488 were used. Blood smears were assessed by immunofluorescence microscopy.

Results: Three groups of patients with different platelet disorders (5 patients with MYH9 disorder, 5 patients with Wiskott-Aldrich syndrome, 1 patient with Bernard-Soulier syndrome) confirmed by the genetic analysis were assessed. For all patients, we were able to identify the deviations of platelet disorder markers associated with established diagnosis by immunofluorescence microscopy. The patient with Bernard-Soulier syndrome showed significantly reduced expression of GPIbIX that is the main marker of this disorder; for the patients with MYH9 the inclusion bodies in the granulocytes were detected, and the small platelet size with the aberrant β1-tubulin distribution let us confirm the diagnose of the patients with Wiskott-Aldrich syndrome.

Conclusions: We determined that the immunofluorescence staining of blood smears enables us to receive reproducible results and implement the diagnostic of platelet disorders.

Virtual erythrocyte as a testing ground for evaluating the effectiveness of systems biology methods

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The last decades have been characterized by the birth of a multitude of "-omics" -proteomics, metabolomics, lipidomics, etc., claiming the title of new sciences. These approaches have in
common an impressive ability to increase the amount of information about the certain cells by orders of magnitude. As in the situation with genomics, there is a feeling of completeness, that for example, we know all proteins, or all metabolic networks present in a given cell. This stimulated a large flow of work trying to develop formal methods of analysis that, using computers, would automatically allow us to obtain not only answers to the question "what is in a given cell?", but also to the question "how does it work?" Applying any theoretical approach, we get some "answers", which are often impossible to quickly check for correctness due to inconsistency and noisy experimental data. It seems to us that a good test of the effectiveness of new analytical approaches can be applied to complex virtual biological systems, the structure of which, by definition, is precisely known. In this talk we will present the results of such a "test" on the virtual human erythrocyte. The results so far do not look very encouraging.

Evaluation of platelet function and bleeding in children with immune thrombocytopenia

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Background: Immune thrombocytopenia (ITP) is believed to be associated with platelet function defects. However, their mechanisms are poorly understood, in particular with regard to differences between ITP phases, patient age, and therapy.

Aims: We investigated platelet function and bleeding in children with either persistent or chronic ITP, with or without romiplostim therapy.

Methods: The study included 151 children with ITP, of whom 56 had disease duration less than 12 months (grouped together as acute/persistent) and 95 were chronic. Samples of 57 healthy children were used as controls, while 5 patients with leukemia, 5 with aplastic anemia, 4 with MYH9-associated thrombocytopenia, and 7 with Wiskott-Aldrich syndrome were used as non-ITP thrombocytopenia controls.

Results: Whole blood flow cytometry revealed that platelets in both acute/persistent and chronic ITP were increased compared with healthy donors. They were also pre-activated by PAC1, CD62P, cytosolic calcium, and procoagulant platelets. This pattern was not observed in other childhood thrombocytopenias. Pre-activation by CD62P were higher in the bleeding group in the chronic ITP cohort only. Pre-activation by PAC1 and procoagulant platelets were significantly associated with bleeding in the acute/persistent patient cohort. Romiplostim treatment decreased size and pre-activation of the patient platelets, but not calcium.

Conclusions: Our data suggest that increased size, pre-activation, and cytosolic calcium are common for all ITP platelets, but their association with bleeding could depend on the disease phase.
The diagnosis of platelet defects using low-angle laser light diffraction method

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Background: A recently developed low-angle laser light diffraction method evaluates platelet aggregation in a diluted sample. This method requires a low sample volume, provides additional information about aggregate structure and platelet shape change and uses standardized buffer as a medium.

Aims: Validation of the low-angle laser light diffraction method for measurement of platelet aggregation.

Methods: We diluted platelets by modified HEPES buffer to 10,000 platelets/µl, stimulated by 800 nM ADP, and evaluated initial velocity and amplitude of aggregation at 1.5° and 12°. Healthy adults (n=32, 21-40 years old) and healthy children (n=20, 1-18 years old), pediatric patients with Glanzmann’s thrombasthenia (TG, n=2), hypo- and dysfibrinogenemia (n=1), as well as patients with bleedings (n=19) were included in the study.

Results: Reference ranges for healthy adults and children were determined. The parameters characterizing platelet aggregation and shape change between these control groups were not significantly different. Correlation between light scattering amplitude measured by light diffraction method and light transmission measured by LTA was moderate both in the group of healthy children and patients. There was no aggregation after the addition of 800 nM ADP for a patient with hypo- and dysfibrinogenemia, two patients with diagnosed Glanzmann’s thrombasthenia. These results correlate with the results of LTA. We found that the parameters of platelet aggregation and the shape change were independent of the blood / citrate ratio, the intake of a fat meal or physical activity before blood sampling.

Conclusions: To summarize, the effect of preanalytical variables on the research results was estimated; the method was validated on pediatric patients.

Flow cytometry for the study of platelet surface glycocalyx

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Background: Most of the platelet surface proteins are glycoproteins (GP), for instance, GPIba, GPIIb/IIa and GPVI. Platelets’ GPs are usually modified with complex carbohydrates like N-linked glycans (N-glycans) and O-linked glycans (O-glycans), which are “covered” with sialic acids. Normally desyalylation (sialic acid loss) occurs during platelet aging, and it leads to
platelet removal from circulation by hepatocytes. But some pathologies can make desyalylation occur more often due to the release of enzymes – neuraminidases, which can be viral, bacterial, or released from platelets, or the binding of autoantibodies, which leads to a decrease of platelet concentration in the circulation and can lead to severe bleeding.

**Aims:** The aim of this work was to develop a method of platelet glycocalyx study using FITC-labeled lectins by flow cytometry.

**Methods:** Venous blood was collected into citrate tubes. Platelet rich plasma (PRP) was obtained with centrifugation at 100g for 5 minutes. To obtain washed platelets two more centrifugations at 400g for 5 minutes are required. There were RCA-1 (binds with β-galactose) and SNA EBL (binds with sialic acids) lectins and flow cytometer NovoCyte3000 used.

**Results:** It was shown that lectin binding depends on lectin and platelet concentrations, e.g. increase in platelet concentration from 1*10^3 per microliter to 50*10^3 per microliter relults in reducing RCA-1 lectin binding, while lectin binding reaches saturation at 12.5 µg per microliter for RCA-1 and at 1-4 µg per microliter for SNA EBL and it does not depend on platelet concentrations for 1*10^3 -10*10^3 per microliter. What is more, it was shown that lectin binding reaches saturation after 30 minutes of incubation. Also it was shown that adding plasma reduces lectin binding 9 times and the reduction of binding is quite similar for 2, 5, 10 percent of plasma in solution and diluted PRP.

**Conclusions:** There were the optimum lectin and platelet concentrations and binding time for the study of platelet glycocalyx defined.

**Erythrocytes-bioreactors for removing ammonium from the blood**

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**Background:** Ammonium has serious neurological toxicity, so its long action and high levels are main risk factors during hyperammonemia, which is an acute or chronic life-threatening condition arising at liver pathologies, deficiencies in urea cycle enzymes, or acute gastrointestinal infections [1]. Erythrocytes (RBCs) with encapsulated enzymes removing ammonium from the bloodstream (ammocytes) can be considered as more promising drugs compare to currently used medications. This study was devoted to development of effective ammocytes.

**Aims:** Development of erythrocyte-bioreactors that efficiently remove ammonium from the bloodstream.

**Methods:** Mathematical modelling to select a more promising enzyme system for encapsulation into RBCs and experimental methods of drug encapsulation using various hypoosmotic effects (hypoosmotic lysis by dilution, dialysis in bags and flow dialysis) were used to obtain more efficient ammocytes. Glutamate dehydrogenase (GDH) from Proteus sp. was also considered as a more promising enzyme for the ammocytes production.
Results: A mathematical model of ammocytes was developed and used to study the limitations of the ammocytes efficacy [2]. It was shown that the low permeability of the RBC membrane for glutamate (GLU) and α-ketoglutarate (AKG) was the reason for the low efficacy of previously developed ammocytes based on glutamine synthetase (GS) [3,4] or GDH [5,6]. A new enzyme system based on GDH and alanine aminotransferase (AAT) was proposed, which had no limitation in the transport of the substrate (AKG) and product (GLU) of the built-in reactions, since they were consumed and produced in a cyclic mode. To increase the encapsulation efficiency of GDH, whose molecules are large and polymerize at concentrations in suspension above 0.1 mg/ml, various encapsulation methods were compared. Flow dialysis was shown to give the best percentage of GDH encapsulation. In addition, it was shown that the GDH from Proteus sp. did not polymerize at increasing concentrations in the medium. This enzyme was used instead of usually used bovine liver GDH. All this together allowed to increase the specific activity of GDH inside RBCs by more than 20 times.

Conclusions: Encapsulation of GDH and AAT into RBCs allowed to obtain a working enzyme system, capable to remove ammonium from the blood during a long period of time. The use of the most efficient enzymes’ encapsulation method and GDH from Proteus sp. allowed to increase the specific activity of GDH inside RBCs. Thus, the obtained ammocytes can be sufficiently effective for clinical use.


Statistical description of cell margination in blood flows and implications in microfluidics

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Background: Uderstanding diseases related to our circulatory system often incorporates the need to understand local blood flow conditions. In recent years the co-operation between experimental and simulated microfluidic evolved significantly, primarily due to the quickly growing possibilities on the computational side.
**Aims:** In the current talk a recent effort is presented which aims to provide a multi-scale description of cell migration inside blood flows.

**Methods:** For the detailed description of the cellular level the open-source HemoCell is applied. Since this model is computationally expensive, two multi-scale couplings are realised to allow to push the computations to larger spatial domains.

**Results:** The multi-scale model accuracy is compared to well-known experimental measurements. Furthermore, several implications are discussed which have significant impact on the typical assumption made in microfluidic experiments (e.g. to approximate the actual wall shear rate from the flow rate).

**Conclusions:** The current numerical simulations seem to have reached the maturity when they can be actively and effectively used as supplementary tools for designing or evaluation microfluidic experiments.