13th Meeting of the
Slovenian Biochemical Society
with International Participation

13. srečanje
Slovenskega biokemijskega društva
z mednarodno udeležbo

Book of Abstracts

24 - 27 September 2019

Dobrna, Slovenia

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Knjiga povzetkov

Dobrna, 24 - 27 September 2019

The 13th Meeting of the Slovenian Biochemical Society with International Participation is organised by the Slovenian Biochemical Society and the National Institute of Chemistry, Ljubljana, Slovenia.

13th Meeting of the Slovenian Biochemical Society with International Participation
13. srečanje Slovenskega biokemijskega društva z mednarodno udeležbo

Editor: Matic Kisovec
Technical Editors: Anja Golob-Urbanc, Gašper Šolinc
Published by: Slovenian Biochemical Society, Ljubljana
Reviewed by: Matic Legiša, Anja Golob-Urbanc, Gašper Šolinc
Cover Design by: Matic Kisovec (graphics from vecteezy, freepik, smashicons)
Organised by: National Institute of Chemistry, Ljubljana, Slovenia and the Slovenian Biochemical Society
Printed by: Tiskarna knjigoveznica Radovljica d.o.o., Radovljica

Circulation: 250

Complimentary publication

CIP - Kataložni zapis o publikaciji
Narodna in univerzitetna knjižnica, Ljubljana

577.1(082)

SLOVENIAN Biochemical Society. Meeting with International Participation (13 ; 2019 ; Dobrna)
Book of abstracts = Knjiga povzetkov / 13th Meeting of the Slovenian Biochemical Society with International Participation = 13. srečanje Slovenskega biokemijskega društva z mednarodno udeležbo, Dobrna, 24-27 September 2019 ; [organised by National Institute of Chemistry and the Slovenian Biochemical Society ; editor Matic Kisovec]. - Ljubljana : Slovenian Biochemical Society, 2019

ISBN 978-961-93879-8-6
1. Kisovec, Matic
COBISS.SI-ID 301584384

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Foreword

It is a great pleasure to welcome you at the 13th Meeting of Slovenian Biochemical Society with International Participation in Dobrna. The Meeting is organized by the Slovenian Biochemical Society together with the National Institute of Chemistry, Ljubljana, Slovenia.

Like previous meetings, this one aims at displaying most recent developments in biochemistry and related disciplines. The assortment of distinguished international and Slovenian speakers will present new discoveries in biochemistry, molecular biology, genomics, systems biology, cell biology, structural biology, synthetic biology, biotechnology etc. Furthermore, new technologies supporting various disciplines will be presented by the sponsors.

This meeting is all about communication and networking between world class scientists, established senior as well as enthusiastic young researchers. The meeting is a great opportunity for exchanging knowledge and experience as well as for establishing new scientific collaborations and connections with industrial partners.

We also invite you to participate at the round table discussion on *Integrity in science* with the lecturer from the FEBS Journal, Cambridge. We also warmly invite you to attend all social events.

We wish you all a productive and enjoyable meeting as well as a pleasant stay at Dobrna.

Sincerely,
Organizing Committee

Committees

Organising Committee

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Kristina Sepčić
Boris Turk

General Information

Conference Venue

Dobrna, The Ceremony Hall of the Zdraviliški Dom Spa, Dobrna 50, SI-3204 Dobrna, Slovenia

Registration and Information Desk

The registration desk will be located in the lobby of the Ceremony Hall of the Zdraviliški Dom Spa. The certificate of attendance will be issued at the registration desk.

Registration and Information Desk Opening Hours

Tuesday, 24 September 2019, 08:30–18:00
Wednesday, 25 September 2019, 08:00–18:00
Thursday, 26 September 2019, 08:00–16:00
Friday, 27 September 2019, 08:30–12:00

Name Badges

All participants are kindly requested to wear badges at all times during the conference.

Presentation Preview and Deposition

Speakers are kindly requested to deliver their presentations to the computer technician in the conference hall half an hour before the start of the session.

Poster Display

Poster session I will be held on Tuesday, 24 September 2019 from 18:00 to 21:00 in the lobby of the Ceremony Hall of the Zdraviliški Dom Spa.

Poster session II will be held on Wednesday, 25 September 2019 from 18:00 to 21:00 in the lobby of the Ceremony Hall of the Zdraviliški Dom Spa.

Poster presenters are kindly asked to mount their posters between 10:00 and 16:00 on Tuesday, 24 September 2019 and remove them by Friday, 27 September 2019 before 10:00.

Presenters should look-up the numbers assigned to their posters in the Book of abstracts and pin up their posters on the display boards with the corresponding number. Material for mounting the posters will be available at the venue. Presenters are responsible for setting and removing their posters.

Sponsor Exhibitions

All sponsors exhibiting at the symposium will have their desks ready in the lobby by Tuesday, 24 September 2019 at 12:00. All tables will be assigned in advance.

Social Programme

Social programme includes:

- "Welcome Dinner" on Tuesday, 24 September 2019 from 18:00 to 21:00,
- "Get Together Dinner" on Wednesday, 25 September 2019 from 18:00 to 21:00,
- "Conference Dinner" on Thursday, 26 September 2019 at 19:00.

Coffee Breaks and Lunch

All participants are invited to refreshments during the coffee breaks, which will be held in the lobby of the Ceremony Hall of the Zdraviliški Dom Spa. Lunch will be available for all registered participants, in the Dining room of the Hotel Vita.

Schedule Outline

Tuesday 24	Wednesday 25	Thursday 26	Friday 27
9:00-9:45 **Satellite meeting: ERA CoBiotech project MISSION**	8:30-10:30 **Molecular interactions and networking**	8:30-10:30 **Functional genomics and systems biology**	8:30-10:30 **Molecular basis of disease II**
9:45-10:15 **Coffee break**	10:30-11:00 **Coffee break**	10:30-11:00 **Coffee break**	10:45-11:15 **Coffee break**
10:15-12:00 **Satellite meeting: ERA CoBiotech project MISSION**	11:00-13:00 **Protein Structure and Function**	11:00-12:45 **Molecular basis of disease I**	11:15-12.00 **Closing EMBO lecture** EMBO excellence in life sciences
11:00 → **Registration**			
14:15-14:30 **Opening of 13th Meeting of the Slovenian Biochemical Society**	13:00-14:00 **Lunch**	12:45-14:00 **Lunch**	12:00-12:15 **Closing remarks**
14:30-16:30 **Synthetic biology**	14:00-16:00 **Biotechnology and bionanotechnology**	14:00-16:00 **Cell signalling and membranes**	
16:30-17:15 **Coffee break**	16:00-16:30 **Coffee break**	16:00-16:30 **Coffee break**	
17:15-18:00 **Opening FEBS lecture** FEBS	16:30-18:00 **Round table EMBO: Integrity in Science** EMBO excellence in life sciences	16:30-18:00 **SBD assembly**	
18:00-21:00 **Poster session I and Welcome dinner**	18:00-21:00 **Poster session II and Get together dinner**	19:00 → **Conference dinner**	

Schedule

Tuesday, 24 September 2019

7:20	Bus to Dobrna (from P&R Barje, Ljubljana)
9:00-12:00	Satellite meeting: ERA CoBiotech project MISSION
9:00-9:15	Coordinator **Andriy Luzhetskyy**, Saarland University, DE Welcome and introduction of ERA CoBiotech project MISSION
9:15-9:45	**Chengzhang Fu**, Helmholtz institute, DE Cloning of the griselimycin and myxopyronin biosynthetic gene clusters
9:45-10:15	Coffee break
10:15-10:45	**Hrvoje Petković**, University of Ljubljana, SI MISSION chassis for biosynthesis of diverse natural products
10:45-11:15	**Judith Becker**, Saarland University, DE Mission to morphology - bioprocess engineering for natural product formation in *Streptomyces*
11:15-11:45	**Lilya Horbal**, Saarland University, DE Transcriptional Cluster "refactoring" to access and expand nature's chemical diversity
11:45-12:00	**Conclusion: Hrvoje Petković,** University of Ljubljana, SI

8:30 →	Registration
14:15-14:30	Opening of 13th Meeting of the Slovenian Biochemical Society
14:30-16:15	Synthetic biology (chair: Roman Jerala)
14:30-15:00	**Bruno Correia**, Swiss Federal Institute of Technology Lausanne (EPFL), CH Computational design of synthetic proteins for biomedicine
15:00-15:30	**Tom F. A. de Greef**, Eindhoven University of Technology, NL Programmable DNA-based communication in populations of synthetic cells

15:30-15:45	**Duško Lainšček**, National Institute of Chemistry, SI CCExo: A new tool for the enhanced genome editing
15:45-16:00	**Neža Omersa**, National Institute of Chemistry, SI Protein logic gate operation on lipid vesicles
16:00-16:15	**Žiga Strmšek**, National Institute of Chemistry, SI Coiled-coil protein origami as the scaffold for presentation of functional protein domains
16:15-16:45	Coffee break
16:45-17:45	Opening FEBS lecture (chair: Marjetka Podobnik)
	**The FEBS National Lecturer** **Petra Schwille**, Max Planck Institute of Biochemistry, DE Bottom-up design of protein self-organization
18:00-21:00	Posters I and Welcome Dinner
21:00 →	Bus to Ljubljana

Wednesday, 25 September 2019

7:00	Bus to Dobrna (from P&R Barje, Ljubljana)
8:30-10:30	**Molecular interactions and networking** (chairs: Kristina Sepčić, Kristina Gruden)
8:30-9:00	**Oren Tzfadia**, Ghent Univeristy, BE MorphDB: Prioritizing genes for specialized metabolism pathways and gene ontology categories in plants
9:00-9:30	**Matej Butala**, University of Ljubljana, SI Viral small proteins that control the bacterial cell fate
9:30-9:45	**Anna Coll**, National Institute of Biology, SI The potato transcription factor StERF49 increases susceptibility to potato virus Y
9:45-10:00	**Kristina Sepčić**, University of Ljubljana, SI Aegerolysins - lipid-binding proteins with potential applications in biomedicine and biotechnology
10:00-10:15	**Matic Legiša**, National Institute of Chemistry, SI Combined inhibition of two cancer specific 6-phosphofructo-1-kinase isoforms prevents lactic acid excretion in tumorigenic cell lines
10:15-10:30	**Christine Strasser**, Carl Zeiss AG, CH LSM 9 Family with Airyscan 2
10:30-11:00	Coffee break
11:00-12:45	**Protein Structure and Function** (chairs: Jurij Lah, Brigita Lenarčič, Boris Turk)
11:00-11:30	**Jim Huntington**, University of Cambridge, UK The structural basis of antitrypsin deficiency
11:30-12:00	**Boris Turk**, Jožef Stefan Institute, SI Extracellular cysteine cathepsins as targets for diagnostics and therapy in cancer
12:00-12:15	**Andreja Kežar**, National Institute of Chemistry, SI Cryo-EM analysis of potato virus Y and virus-like particle reveals structural plasticity of the coat protein
12:15-12:30	**Jure Loboda**, Jožef Stefan Institute, SI Structural insight in peptidyl substrate binding to cysteine cathepsins
12:30-12:45	**Sara Drmota Prebil**, University of Ljubljana, SI Towards understanding the effect of calcium binding on the structure of human non-muscle α-actinin-1
12:45-14:00	Lunch in Hotel Vita
14:00-16:00	**Biotechnology and bionanotechnology** (chair: Hrvoje Petković)
14:00-14:30	**Andriy Luzhetskyy**, Saarland University, DE Actinobacteria biosynthetic potential: bridging *in silico* and *in vivo*

14:30-15:00	**Damjana Drobne**, University of Ljubljana, SI Importance of design in nanotechnology
15:00-15:15	**Urša Čerček**, Jožef Stefan Institute, SI Cold atmospheric plasma induces stress granule formation via EiF2-signalling
15:15-15:30	**Matic Kisovec**, National Institute of Chemistry, SI Quartz crystal microbalance as a bioprocess in-line sensor
15:30-15:45	**Aleš Berlec**, Jožef Stefan Institute, SI Development of nanofiber delivery system that enables high loading and long-term viability of probiotics
15:45-16:00	**Luka Jeromel**, Lek Pharmaceuticals, SI Using Knowledge for Downstream Process Design – Mechanistic Modelling
16:00-16:30	Coffee break
16:30-18:00	**Round table EMBO: Integrity in Science**
EMBO excellence in life sciences	**Paraminder Dhillon**, The FEBS Journal, UK Promoting research integrity and reproducibility through publication best practice
18:00-21:00	Posters II and Get Together Dinner
21:00	Bus to Ljubljana

Thursday, 26 September 2019

7:00	Bus to Dobrna (from P&R Barje, Ljubljana)
8:30-10:30	**Functional genomics and systems biology** (chair: Damjana Rozman, Uroš Potočnik)
8:30-9:00	**Adil Mardinoglu**, KTH - Royal Institute of Technology, SE The use of systems biology in treatment of liver diseases
9:00-9:30	**Martin Trapečar**, Massachusetts Institute of Technology, US Humans-on-a-chip and systems immunology for the studies of complex inflammatory diseases
9:30-9:45	**Maja Zagorščak**, National Institute of Biology, SI A clique-based method for improving motif scanning accuracy
9:45-10:00	**Nejc Nadižar**, University of Ljubljana, SI BiOpenBank: open source solution for management of biological samples and sample associated data in small biobanks
10:00-10:15	**Miha Milek**, Berlin Institute for Molecular Systems Biology, DE Investigating 2'-O-methylation writers in human RNA
10:15-10:30	**Uršula Prosenc Zmrzljak**, LABENA d.o.o., SI Minimal residual disease monitoring by droplet digital PCR in acute promyelocitic leukemia
10:30-11:00	Coffee break
11:00-12:45	**Molecular basis of disease I** (chair: Tamara Lah Turnšek, Mateja Manček Keber)
11:00-11:30	**Juergen Dittmer**, Martin Luther University Halle - Wittenberg, DE The impact on tumor stroma on drug response in breast cancer
11:30-12:00	**Peter Korošec**, University Clinic of Pulmonary and Allergic Diseases Golnik, SI Molecular mechanisms of anaphylaxis
12:00-12:15	**Ana Mitrović**, Jožef Stefan Institute, Slovenia Cysteine cathepsins B and X in cancer stem cells
12:15-12:30	**Vashendriya Hira**, National Institute of Biology, SI Microscopic imaging of similarities between stem cell niches in glioblastoma and bone marrow
12:30-12:45	**Mirko Cevec**, National Institute of Chemistry, SI Study of RNA G-quadruplex structures in the 5'-UTR of the human NRAS proto-oncogene
12:45-14:00	Lunch in Hotel Vita
14:00-16:00	**Cell signalling and membranes** (chair: Igor Križaj, Metka Lenassi)
14:00-14:30	**Guillaume van Niel**, French Institute of Health and Medical Research (Inserm), FR Live tracking of endogenous exosomes *in vivo*

14:30-15:00	**Toni Petan**, Jožef Stefan Institute, SI Lipid droplets: fatty managers of stress and inflammation
15:00-15:15	**Valentina Kubale**, University of Ljubljana, SI Internalization and signalling properties of BILF1 receptors encoded in Epstein-Barr virus and porcine lymphotropic herpesviruses
15:15-15:30	**Marija Holcar**, University of Ljubljana, SI Development of a method for extracellular vesicles isolation from human blood and evaluation of its clinical value in malignant mesothelioma
15:30-15:45	**Tamara Lah Turnšek**, National Institute of Biology, SI Chemokine CCL5 signalling is important for invasion of glioblastoma in its microenvironment
15:45-16:00	**Monica Sevillano**, Promega GmbH, DE HiBiT Technology - New tool to study protein biology and cellular responses
16:00-16:30	Coffee break
16:30-18:00	**SBD assembly**
19:00 →	Conference dinner
22:00	Bus to Ljubljana

Friday, 27 September 2019

7:30	Bus to Dobrna
9:00-10:45	**Molecular basis of disease II** (chair: Iva Hafner Bratkovič, Boris Rogelj)
9:00-9:30	**Pablo Pelegrin**, Biomedical Research Institute of Murcia (IMIB), ES Understanding the role of the NLRP3 inflammasome in sepsis
9:30-10:00	**Marjan Slak Rupnik**, Medical University of Vienna, AT Complexity and simplicity in beta cell collectives
10:00-10:15	**Marko Fonović**, Jožef Stefan Institute, SI Legumain as a modulator of immune response
10:15-10:30	**Mirjana Liović**, University of Ljubljana, SI iPS cells as disease models for epidermolysis bullosa
10:30-10:45	**Anja Pišlar**, University of Ljubljana, SI Cathepsin X as a potential therapeutic target for treatment of Parkinson's disease
10:45-11:15	Coffee break
11:15-12:00	**Closing EMBO lecture** (chair: Janko Kos)
EMBO excellence in life sciences	**Antonella Viola**, University of Padova, IT Mitochondria in inflammation and immunity
12:00-12:15	Closing remarks
12:30	Bus to Ljubljana

Lecture abstracts

L1

Computational Design of Functional Proteins for Biomedicine & Synthetic Biology

Bruno Correia

Institute of Bioengineering, Ecole Polytechnique Federal de Lausanne, Switzerland

Finely orchestrated protein activities are at the heart of the most fundamental cellular processes. The rational and structure-based design of novel functional proteins holds the promise to revolutionize many important aspects in biology, medicine and biotechnology. Computational protein design has led the way on rational protein engineering, however many of these designed proteins were solely focused on structural accuracy and completely impaired of function. At the methodological level, I will present a computational design strategy centred on the exploration of *de novo* protein topologies and the use of structural flexibility with the ultimate goal of designing functional proteins. This approach aims to solve a prevalent problem in computational design that relates to the lack of optimal design templates for the optimization of function. By expanding beyond the known protein structural space, our approaches represent new paradigms on the design of *de novo* functional proteins.

Beyond the novel approaches, developed in our group, for the *de novo* design of functional proteins I will also showcase important applications in the domains of vaccine design, T-cell based-therapies, biosensors and synthetic biology.

Programmable DNA-based Communication in Populations of Synthetic Cells

Tom F. A. de Greef

Instituut voor Complexe Moleculaire Systemen, Eindhoven University of Technology, Netherlands

The development of highly orthogonal molecular communication channels is a crucial step towards engineering artificial cell-scale systems. Here, we present a general and scalable platform entitled 'Biomolecular Implementation of Protocellular Communication' (BIO-PC) to engineer multichannel molecular communication networks between populations of non-lipid microcapsules. Our method leverages the modularity and scalability of enzyme-free DNA strand-displacement circuits to develop protocellular consortia that can sense, process and respond to DNA-based messages. We engineer a rich variety of biochemical communication devices capable of cascaded amplification, bidirectional communication, sender-receiver functions and distributed computational operations. Encapsulation of DNA strand-displacement circuits allows their use in concentrated serum where non-compartmentalized DNA circuits cannot operate. BIO-PC enables reliable execution of distributed DNA-based molecular programs in biologically relevant environments and opens new directions in DNA computing and minimal cell technology.

L3 – Opening lecture

Bottom-up design of protein self-organization

Petra Schwille, FEBS National Lecturer

Max Planck Institute of Biochemistry, Germany

Living systems employ self-organized protein pattern formation to regulate important life processes in space and time. Although pattern-forming protein networks have been identified in various pro- and eukaryotes, their systematic experimental characterization is challenging due to the complex environment of living cells. In turn, cell-free systems are ideally suited for this goal, as they offer defined molecular environments that can be precisely controlled and manipulated. We demonstrate the power of reconstitution approaches using the *E. coli* Min system, a model system for protein self-organisation based on the reversible and energy-dependent interaction of the ATPase MinD and its activating protein MinE with a lipid membrane. Patterns formed are dramatically dependent on protein features, such as membrane affinity and ATP turnover, and can be regulated and controlled in many ways, as will be demonstrated in my talk. Thus, a full *de novo* design of protein self-organization on desired spatial and temporal scales is within reach.

MorphDB: Prioritizing Genes for Specialized Metabolism Pathways and Gene Ontology Categories in Plants

Arthur Zwaenepoel[1,2,3], Tim Diels[1,2,3], David Amar[4], Thomas Van Parys[1,2,3], Ron Shamir[5,] Yves Van de Peer[1,2,3,5*] & Oren Tzfadia[1,2,3*]

[1]Department of Plant Biotechnology and Bioinformatics, Ghent University, Belgium
[2]VIB Center for Plant Systems Biology, Belgium
[3]Bioinformatics Institute Ghent, Ghent University, Belgium
[4]Stanford Center for Inherited Cardiovascular Disease, Stanford University, USA
[5]Blavatnik School of Computer Science, Tel-Aviv University, Israel
[6]Genomics Research Institute, University of Pretoria, South Africa

Recent times have seen an enormous growth of 'omics' data, of which high-throughput gene expression data are arguably the most important from a functional perspective. Despite huge improvements in computational techniques for the functional classification of gene sequences, common similarity-based methods often fall short of providing full and reliable functional information. Recently, the combination of comparative genomics with approaches in functional genomics has received considerable interest for gene function analysis, leveraging both gene expression based guilt-by-association methods and annotation efforts in closely related model organisms. Besides the identification of missing genes in pathways, these methods also typically enable the discovery of biological regulators (*i.e.* transcription factors or signaling genes). A previously built guilt-by-association method is MORPH, which was proven to be an efficient algorithm that performs particularly well in identifying and prioritizing missing genes in plant metabolic pathways. Here, we present MorphDB, a resource where MORPH-based candidate genes for large-scale functional annotations (Gene Ontology, MapMan bins) are integrated across multiple plant species. Besides a gene centric query utility, we present a comparative network approach that enables researchers to efficiently browse MORPH predictions across functional gene sets and species, facilitating efficient gene discovery and candidate gene prioritization. MorphDB is available at http://bioinformatics.psb.ugent.be/webtools/morphdb/ morphDB/index. We also provide a toolkit, named 'MORPH bulk' (https://github.com/arzwa/morph-bulk), for running MORPH in bulk mode on novel data sets, enabling researchers to apply MORPH to their own species of interest.

L5

Viral small proteins that control the bacterial cell fate

Nadine Fornelos[1], Anja Pavlin[2], Nathanael Caveney[3], Douglas F. Browning[4], Guillermo Caballero[3], Liza H. de Castro[3], Miha Bahun[2], Zdravko Podlesek[2], Vesna Hodnik[2,!], Margarita Salas[5], Natalie C. J. Strynadka[3], Matej Butala[2]

[1] The Broad Institute of MIT and Harvard, Cambridge, USA
[2]Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia
[3] Department of Biochemistry and Molecular Biology and the Centre for Blood Research, University of British Columbia, Canada
[4] Institute of Microbiology and Infection, School of Biosciences, University of Birmingham, UK
[5] Instituto de Biología Molecular 'Eladio Viñuela' (CSIC), Centro de Biología Molecular 'Severo Ochoa' (CSIC-Universidad Autónoma de Madrid), Spain
[!] Present address: Sandoz, Mengeš, Slovenia

Transcription in most bacteria is tightly regulated in order to facilitate bacterial adaptation to different environments, and transcription factors play a key role in this. The tectiviral bacteriophage GIL01 is known to infect *Bacillus thuringiensis*, an insect pathogen of use in agriculture. In *B. thuringiensis*, GIL01 is seen to establish a stabile, quescent residence within its host upon infection, switching to a lytic state when its host undergoes DNA damage. We show that the small phage-borne protein, gp6, activates of the lytic cycle. We determined that unlike most temperate phages, GIL01 lysogeny is not established by a dedicated phage repressor but rather by the host's regulator of the SOS response, LexA. LexA binds near the promoter directing the expression of genes involved in GIL01 replication and regulation, with LexA affinity for this region increased through interaction with a viral co-repressor protein gp7. gp7 directly interacts with LexA and increases repressor affinity for a non-canonical target site, which is crucial for the establisment of lysogenic state. We resolved the crystal structure of the 50-amino acid gp7 protein and according to SAXS data we generated a structural model of gp7 in complex with LexA which illustrates that gp7 positions LexA in a DNA bound conformation. Furthermore, we show that gp7 can interact with LexA from a human pathogen *Staphylococcus aureus*. This is of great relevance as LexA is increasingly associated with resistance phenotypes in response to antibiotics. Thus, gp7 is an attractive protein for the structure-based drug design to be used as antibiotic-coupled therapeutics.

1. Caveney NA, Pavlin A, Caballero G, Bahun M, Hodnik V, Castro L, Fornelos N, Butala M, Strynadka NCJ. Structural Insights into Bacteriophage GIL01 gp7 Inhibition of Host LexA Repressor. Structure. 2019 In press.
2. Fornelos N, Browning DF, Pavlin A, Podlesek Z, Hodnik V, Salas M, Butala M. Lytic gene expression in the temperate bacteriophage GIL01 is activated by a phage-encoded LexA homologue. Nucleic Acids Res. 2018 Oct 12;46(18):9432-9443.

The structural basis of antitrypsin deficiency

James A. Huntington

Cambridge Institute for Medical Research (CIMR), University of Cambridge, UK

Antitrypsin deficiency is a disease with multiple manifestations, including emphysema, COPD, liver cirrhosis and hepatocellular carcinoma. The underlying cause is the misfolding and accumulation of stable polymers of mutant α_1-antitrypsin (α1AT) in the endoplasmic reticulum of hepatocytes. Over 120 such mutations have been described, but the most prevalent in Northern Europe is the Z allele (~4% carriers), where a negatively charged glutamate residue is substituted for a positively charged lysine at position 342. Incidence of ZZ homozygocity is typically 1 in 2000, but can be as high as 1 in 500 in some countries (e.g. Latvia), and is associated with reduced levels of functional α1AT in the blood (~15% of normal). The reduced secretion of α1AT is the cause of lung disease, and the accumulation of polymers within hepatocytes is the cause of liver disease. It is well established that the Z mutation retards the folding of α1AT and leads to the accumulation of a polymerigenic intermediate. However, the nature of this intermediate and of the intermolecular linkage of Z α1AT polymers have remained a matter of conjecture. We recently solved a crystal structure of a Z-α1AT polymer purified from Pichia pastoris. The structure revealed that polymers are linked by a runaway C-terminal domain-swap, consistent with the position of many of the disease-causing mutations, recent folding data, and other structural studies. In addition, it provides important information into the nature of the polymerigenic intermediate, shows why the Z mutation retards folding, and provides the structural basis for the rational design of chemical chaperones to improve the secretion of Z-α1AT.

L7

Extracellular cysteine cathepsins as targets for diagnostics and therapy in cancer

Boris Turk[1,2]

[1]Department of Biochemistry and Molecular and Structural Biology, Jožef Stefan Institute, Slovenia
[2]Faculty of Chemistry and Chemical Technology, University of Ljubljana, Slovenia

Understanding the precise role of an individual protease in health and disease remains a major challenge. There are several ways how to address this issue, including the chemical biology approaches with small molecule inhibitors and activity-based probes, as well as by engineered macromolecules (e.g. DARPins). In addition to helping unravel the physiological role of a protease, these approaches offer a major potential for noninvasive optical imaging by monitoring protease activities in situ, i.e. on disease site. Moreover, they enable also validation of proteases as drug targets, in vivo validation of drug candidates and evaluation of the diagnostic potential of the target proteases. Among the proteases found to be tightly linked with inflammation-associated diseases, including many types of cancer, are also cysteine cathepsins that can be found extracellularly at the sites of inflammation due to their secretion from primarily infiltrated immune cels, such as macrophages. Furthermore, since they are heavily upregulated in a number of inflammation-associated diseases, they are therefore perfect targets for such approaches. There is increasing evidence that monitoring cathepsin activity in vivo may be applicable to diagnostic imaging, such as demonstrated primarily for cancer. Moreover, cathepsins can be also used as targets for targeted drug delivery approaches combined with diagnostics, thereby offering a theranostic potential.

Actinobacteria biosynthetic potential: bridging *in silico* and *in vivo*

Luzhetskyy Andriy[1,2]

[1]Department of Pharmaceutical Biotechnology, Saarland University, Germany
[2]Helmholtz-Institute for Pharmaceutical Research Saarland, Germany

Natural products have great importance for medicine, biotechnology and agriculture and remain in the research focus despite a growing challenge in the discovery of new compounds. *Actinomycetes* and representatives of genus *Streptomyces*, as one of the most prolific microbial sources of natural products, were objects of various extensive screening studies and lots of compounds readily produced under laboratory conditions were already isolated and characterized. However, numerous genome and transcriptome sequencing studies indicate that only small portion of biosynthetic clusters is active under laboratory conditions with the majority of clusters remaining silent. In this respect silent biosynthetic clusters seem to be promising source of new compounds making strategies for their high throughput activation highly welcomed.

To address the problem of disclosing entire biosynthetic potential of actinobacteria, we aimed to develop a panel of genetically optimized host stains for heterologous activation of silent clusters and expression of cryptic clusters driven by synthetic genetic controlling elements such as synthetic promoters, ribosome binding sites, terminators etc. We used several different parallel approaches to improve chassis strains and to confer it above mentioned super-host properties, with genome minimization, copy number increase of integrative plasmids and introduction of distinct rpsL mutations being the major ones. The constructed strains have been characterized by a lack of indigenous biological activity and biosynthetic clusters, high production levels of heterologous compounds and increased rates of silent clusters activation without detrimental effects on growth characteristics.

A number of cryptic gene clusters from S. albus subsp. chlorinus NRRL B-24108, Frankia alni ACN14a and Frankia sp. CcI3 have been expressed in the newly developed host panel leading to the isolation of several novel compounds. The biosynthetic mechanisms of these compounds as well as the properties of the improved super host strains will be discussed.

1. Myronovskyi M., Luzhetskyy A. (2016) Native and engineered promoters in natural product discovery. Nat Prod Rep. 33 (8): 1006-19.
2. Horbal, L., Luzhetskyy, A. (2016) Dual control system - A novel scaffolding architecture of an inducible regulatory device for the precise regulation of gene expression. Metab Eng. 37: 11-23.

L9

Importance of design in nanotechnology

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Nanotechnology is defined as manipulation of matter on an atomic, molecular, and supramolecular scale. Scientists doing nanotechnology refer to their activity as “design” (Loeve, 2018). Nanodesign generally starts with the basic laws of physics at the nanoscale, then addresses principles and rules of engineering and finally touches upon the “broader” environmental and societal issues. The approach to nanotechnology design is contradictory: it is supposed to be user-centric although *sensu stricto* there is no “user” of nanotechnology. Nano-components are always packaged into larger modules or systems so that users have no direct relationships with the nanomaterials embedded in the final product. This “invisible” technology is supposed to be cut off from all sensible experiences whereas design traditionally focuses on the shaping of the user’s experience. *There is one exception* to this: the fears around new technologies. New technologies often prompt strong emotional reactions *in particular with regard to safety*. Therefore, technology developments and safety awareness need to complement each other in order to achieve sustainability of technology development and promote a new safety culture in nanotechnologies.EU Commission *has invested* a remarkable amount of resources to support the research on safety of nanomaterials but the results of research do not serve well the urgent ethical and social implications of nanotechnology. Responding to the challenge of nanotechnology will require confronting “philosophical” questions about the sort of society we wish to create and the role that technology might play in creating it. In this view again design is inescapable, but this time social design. The lecture will emphasise the role design in any new technology.

1. Loeve, S. (2018). Design and Aesthetics in Nanotechnology. In Loeve, S. Guchet X., & Bensaude Vincent B. (eds.), *French Philosophy of Technology. Classical Readings and Contemporary Approaches*: Springer, 361-384.

Promoting research integrity and reproducibility through publication best practice

Paraminder Dhillon

The FEBS Journal, Cambridge, UK

Research rigor and reproducibility is fostered through the generation of manuscripts that adhere to best practice in scientific publishing. Many publishers and journals, including FEBS Press, are committed to promoting high standards in publication ethics by incorporating a series of detailed checks into their submission workflows and by providing guidance to authors on the adoption of ethical practices. In this talk, I will highlight the importance of adhering to guidelines on authorship, conflict of interest, data generation and image manipulation, methodology detail and other aspects of manuscript preparation, with examples of good and bad practice.

L11

The use of systems biology in treatment of liver diseases

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To develop novel strategies for prevention and treatment as well as to gain detailed insights about the underlying molecular mechanisms of liver diseases, it is vital to study the biological functions of liver and its interactions with other tissues and gut microbiota. Biological networks can provide a scaffold for studying biological pathways operating in the liver in connection with disease development in a systematic manner. In my presentation, I will present our recent work where biological networks have been employed to identify the reprogramming in liver physiology in response to NASH/NAFLD. I will further discuss how this mechanistic modelling approach can contribute to the discovery of biomarkers and identification of drug targets which may lead to design of targeted and effective treatment strategies.

Humans-on-a-chip and Systems Immunology for the studies of complex inflammatory diseases

Martin Trapečar[1], Emile Wogram[2], Devon Svoboda[2], Tenzin Lungjangwa[2], Jason Velazquez[1], Kirsten Schneider[1], Charles W. Wright[1], David Trumper[3,4], Rudolf Jaenisch[1,2], Linda G. Griffith[1,3,5]*

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The gut-liver axis is a highly connected system whose roles include processing of gut-derived products, regulation of metabolic homeostasis and stability of systemic immune function. Short-chain fatty acids (SCFA), which act as signalling molecules between the gut microbiota, the hosts metabolism and immune status, became recently of increased interest as potential immunometabolic mediators. Numerous studies showed SCFA to exert beneficial effects on the host however, contrary to this finding lies recent insight which shows SCFA to significantly enhance effector function of activated CD4 and CD8 T cell. Several animal and human trials found SCFA to either lack any therapeutic efficacy or to exacerbate inflammation. Conflicting results suggest the function of SCFA to be context and disease severity dependent, thus their role in T cell activation and metabolism under conditions of local and systemic inflammation in humans demands urgent clarification. Recently-developed human physiomimetic technology, in combination with systems immunology, offers new possibilities for illuminating mechanisms in multi-organ inflammatory diseases by modeling complex interactions with multiple interconnected organ specific microphysiological systems (MPS). We have created a new physiomimetic platform specifically designed to mimic the gut-liver-brain axis by connecting a gut MPS (primary human epithelium, dendritic cells and macrophages), a liver MPS (human hepatocytes and Kupffer cells), a brain MPS (iPS derived neurons and microglia) as well as circulating Treg/Th17 cells. Using a multiomics approach we were able to gain new insight in how SCFA affect acute inflammation such as during onset of IBD and chronic inflammation like Parkinson's disease (PD) and show their effect to be largely dependent on the metabolic state of receptive cells. Recreating hallmarks of human innate and adaptive immune mechanics of the gut-liver-brain axis highlights the unique potential of human physiomimetic technologies to fill in the gaps in understanding complex human diseases through mechanistic correlation of various multiomic observations and systems immunology to human clinical data.

L13

The impact of tumor stroma on drug response in breast cancer

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Tumor-residing stromal cells, like carcinoma-associated fibroblasts (CAFs), are in close contact with cancer cells and modify their behavior. For instance, in their presence, drug responses of cancer cells can be significantly altered. Certain cytokines or growth factors secreted by stromal cells may trigger changes in signaling pathway activities which may bestow cancer cells with higher apoptotic resistance. CAFs have been shown to protect breast cancer cells against anti-estrogens by upregulating the PI3K/AKT, the MAPK and/or the NFkB pathways. However, some of these effects are only seen in short-term treatments. Long-term treatments may lead to opposite effects and may provoke addiction to certain permanently activated proteins. Besides its influence on drug resistance, such permanent changes in protein activity may influence tumor progression and metastasis.

Molecular mechanisms of anaphylaxis

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Anaphylaxis is rapidly-progressing hypersensitivity reaction characterized by life-threatening airway, breathing, and/or circulatory problems following exposure to minute amounts of allergens (e.g. certain foods, single insect stings or medications), and that is usually associated with skin and mucosal changes. Thus, it can be considered as one of the most aberrant examples of an imbalance between the cost and benefit of an immune response. Immunopathogenesis and pathophysiology of anaphylaxis involve effector molecules like IgE antibodies and FcεRI receptor, potential effector cells (mast cells, basophils, monocytes/macrophages, neutrophils and platelets), and many potential mediators (histamine, PAF, CysLTs, tryptase, prostaglandins and cytokines/chemokines) which contribute (positively or negatively) to the clinical signs and symptoms. Insight in the molecular mechanisms of anaphylaxis was gained from analyses of animal models, including mice genetically deficient in the antibodies, or humanized for antibody receptors or effector cells. However, only limited data on molecular mechanisms of anaphylaxis from human subjects are currently available due to the life-threatening nature of this disorder. Therefore, we recently analysed global transcriptional changes (RNA-sequencing) in patients with anaphylaxis presenting at emergency department, in patients with anaphylaxis under the controlled setting of double-blind placebo-controlled food challenges to peanut (DBPCFC), and in mouse models of different severity of IgE-mediated food induced anaphylaxis. We showed that anaphylaxis is associated with extensive transcriptional alternations and activation of specific biological and molecular pathways.

L15

Live tracking of endogenous exosomes *in vivo*

Guillaume van Niel

Institute of Psychiatry and Neurosciences of Paris, INSERM, France

Extracellular vesicles (EVs) such as exosomes are released by a wide variety of cell types and found in all organism tested so far. Even though EVs have been implicated in many important physiological and pathological processes, our understanding of their relevance *in vivo*, remains poorly understood, mainly due to the lack of relevant model organisms that allow for accurate spatiotemporal assessment of EV biogenesis, transfer and fate at the single vesicle level.

We developed an animal model to study endogenous exosomes *in vivo* by expressing tetraspanin-pHluorin, fluorescent reporters for exosome secretion (Verweij *et al*., JCB 2018), in transparent zebrafish embryo in a cell type specific manner (Verweij *et al* Dev Cell 2019). Using a combination of in vivo light- and in situ electron microscopy techniques and proteomic *ex vivo* analysis, we explored the physiology of exosomes, including their biogenesis, transfer, uptake and fate. Our data reveal for the first time the release, journey, targets and the fate of endogenous exosomes *in vivo* and shed new light on the role of this intercellular communication.

Lipid droplets: fatty managers of stress and inflammation

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Lipid droplets (LDs) are cytosolic fat storage organelles that have long been regarded as inert fat depots. They are now emerging as dynamic organelles with roles extending well beyond lipid storage and energy metabolism. LDs are composed of a neutral lipid core containing triglycerides and sterol esters, covered by a monolayer of phospholipids and proteins. They bud from the endoplasmic reticulum membrane and are released into the cytosol, where they grow, fuse, shrink, and engage in contacts with other organelles to exchange proteins and lipids. They are broken down by lipolysis or by selective autophagic degradation, i.e. lipophagy. Their aberrant accumulation in different tissues has been associated with metabolic diseases, inflammation, immunity, neurodegeneration and cancer. However, we are only beginning to understand how and why LDs form and are broken down in response to various stimuli. Intriguingly, their biogenesis is induced in cells exposed to a variety of stressful conditions, including nutrient deprivation, oxidative stress and inflammation. Our recent studies in cancer cells have shown that LDs are necessary for the protection against nutrient and oxidative stress during starvation. We have found that LDs maintain membrane integrity and mitochondrial function by regulating the trafficking of fatty acids in stressed cells. They also act as antioxidant organelles by storing oxidation-prone polyunsaturated fatty acids in the form of inert triglycerides, thereby preventing oxidative stress, while simultaneously releasing fatty acids for mitochondrial energy production and redox balance. Our latest findings also identify LDs as platforms for the synthesis of prostaglandins, leukotrienes and other eicosanoids, which are potent inflammatory mediators that promote tumourigenesis. LDs thus integrate metabolic and signalling pathways to regulate stress response pathways and emerge as targets for novel therapeutic interventions in cancer and other diseases

L17

Understanding the role of the NLRP3 inflammasome in sepsis

Pablo Pelegrin

Biomedical Research Institute of Murcia, Spain

Sepsis characterises by a systemic inflammatory response that is followed by an immunosuppression of the host. Metabolic defects and mitochondrial failure are common in immunocompromised septic patients. The NLRP3 inflammasome is important for establishing an inflammatory response after activation by the purinergic P2X7 receptor. Here, we study a cohort of individuals with intra-abdominal origin sepsis and show that patient monocytes have impaired NLRP3 activation by the P2X7 receptor. Furthermore, most sepsis-related deaths are among patients whose NLRP3 activation is profoundly altered. In monocytes from septic patients, the P2X7 receptor is associated with mitochondrial dysfunction; moreover, activation of the P2X7 receptor results in mitochondrial damage, which in turn inhibits NLRP3 activation by HIF-1α. We also show that mortality increases in a mouse model of sepsis when the P2X7 receptor is activated in vivo. Our data reveal a molecular mechanism initiated by the P2X7 receptor that contributes to NLRP3 impairment during infection.

Complexity and simplicity in beta cell collectives

Marjan Slak Rupnik[1,2,3], Andraž Stožer[1], Dean Korošak[2]

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Pancreatic islets are composed of cell collectives sensing plasma nutrient levels and responding with an adequate release of hormones to distribute these nutrients among cells in the body to support their energy usage or energy storage as well as housekeeping and growth. Communication among the entities of such a collective has been found to involve both strong direct, short range interactions through gap junctions, and paracrine long-range signalling, but how exactly and on which temporal scale these interactions shape the emergent properties of these dynamic cell networks is still unknown. Here we borrow approaches from statistical physics to bridge the limitations of single cell biology and to describe the functional behaviour of islet cell collectives.

When stimulated with specific ligands, islet cell collectives respond with intermittent increases in cytosolic calcium concentration to drive regulated exocytosis of hormones. We used these recurrent transitions from silent to activated phase to assess pairwise correlations among all responsive cells in the islet and used the dynamical network approach to model various aspects of the cell collective behavior. We found strongly correlated states of cells in the active phase coexisting with mostly, but not exclusively, weak pairwise correlations widespread across the islet. Within an islet as a whole, these phase-dependent processes can replace each other within the timeframe of some tens of seconds to generate a statistical conservation law governing the hormonal release from individual cells.

The major conclusion of our work is that hormonal release in pancreatic islet is a collective dynamical process shaped by different forms of cell-cell interactions, operating on a temporal scale much faster than typical protein expression changes. Our results shed a new light regarding the nature of functional cellular heterogeneity within the islet in normal and diseased condition

L19 – Closing lecture

Mitochondria in Inflammation and Immunity

Antonella Viola[1,2]

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Mitochondria are dynamic organelles controlling multiple cell functions, including signalling, metabolism, and cell death. In addition, these organelles are engaged in the regulation of inflammatory responses through mechanisms involving generation of reactive oxygen species (ROS), release of damage-associated molecular patterns (DAMPs), Ca^{2+} buffering, and production of metabolites. These concepts will be discussed on the basis of the existing literature and novel, unpublished results.

Abstracts of short lectures

SL1

CCExo: A New Tool for the Enhanced Genome Editing

Duško Lainšček[1], Vida Forstnerič[1], Špela Malenšek[1], Roman Jerala[1,2]

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The CRISPR/Cas system is a potent tool which has revolutionized genome engineering and regulation of gene transcription in cells and organisms. This gene-editing tool consists of a guide RNA (gRNA), which targets the Cas9 endonuclease to the desired genomic site. Cas9 catalyzes the formation of double-strand DNA breaks that are then repaired by different cell mechanisms. Error-prone Non-homologous end joining occurs, resulting in random indel (insertion-deletion) mutations, which can lead to functional gene inactivation by either frameshift or deletions. Depending on the size (tens of base pairs) of indel mutations, higher rates of "knock-out" can be achieved. To achieve greater indel mutations, CRISPR system can be coexpressed in cells with DNA exonucleases, which cause increased recessions of DNA following DNA breaks. We show that joint action of the CRISPR system with different exonucleases significantly increases the percentage of indel mutations at various targeted genes. Of the different exonucleases tested, the *E.coli*-derived exonuclease III (EXOIII) exhibited the best performance in terms of indel formation. To further improve the rate of indel mutations, Cas9 and EXOIII were brought into the proximity via coiled-coil forming heterodimeric peptides (CCExo). This resulted in increased indel formation compared to the classical CRISPR/Cas system as well as more efficient than cointroduction of non-interacting and genetically fused Cas9-EXOIII. We performed a case study for the use of the CRISPR-EXO system as a potential anti-cancer therapeutic tool. The Philadelphia chromosome, which occurs in leukemic cancer cells, is the result of characteristic the reciprocal genome translocation t(9:22) and is responsible for higher proliferation of tumorous cells. Using the CCExo system, we achieved a higher degree of indel mutations at the translocation site, which resulted in greater killing of cancer cells, thus providing a useful potential anti-cancer therapeutic tool.

Protein logic gate operation on lipid vesicles

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Lipid vesicle-encapsulated cargo formulations are very useful delivery systems in molecular biology and therapeutics. They can be designed to sense environmental conditions for targeted delivery and release of desired vesicle contents. We designed a protein logic gate system based on pore-forming toxin that responds to pH, metalloprotease activity and reducing environment. In its OFF state, the system is inhibited by highly specific protein inhibitor and pH ≥ 6.5. Designed on a Boolean AND-OR logic, the gate is opened when pH drops below 6.5 in addition to either metalloprotease or reductant presence. Upon activation, extremely large and well-defined pores are formed in the lipid membrane, allowing for fast and accurate release of molecules up to 150 kDa to the desired place of action. The system is modular and allows for introduction of other enzyme recognition sites on the protein inhibitor or different chemistries used for conjugation of the inhibitor to the lipid membrane, which allow implementation of additional input signals.

SL3

Coiled-coil protein origami as the scaffold for presentation of functional protein domains

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Coiled-coil protein origami (CCPO) is a class of designed protein folds constructed from concatenated coiled-coils (CC). CCPOs are highly modular, rigid and robust scaffolds that have been used to construct several polyhedral protein cages. In order to determine the suitability of the system for future application development such as vaccines, targeted delivery vehicles, sensors, cell receptor inhibitors or activators, the strategies to present protein domains on the vertices of the scaffold was investigated. Here we show that folded protein domains can be incorporated into the selected vertices of the CCPOs by genetic fusion between the CC modules. Fluorescent protein domains have been successfully incorporated into a single and into up to all four vertices of a tetrahedral protein cage. This strategy enables precise addressing of each vertex with a selected protein domain and incorporation of functionality. Successful presentation of two different fluorescent proteins (GFP and RFP) on all four vertices at all possible ratios between RFP and GFP were experimentally confirmed by SEC-MALS and SEC-SAXS. Furthermore, all purified proteins exhibited the expected fluorescence. In order to confirm that enzymes retained their activity upon insertion into the CCPOs, luciferase was inserted into the vertices and up to three copies of enzyme with GFP on the fourth vertex were successfully incorporated into the scaffold. As an alternative strategy to modular incorporation of the desired proteins SpyCatcher/SpyTag system was incorporated. SpyCatcher is a protein that forms isopeptide bond with its partner – SpyTag. Three copies of SpyCatcher were incorporated into the vertices of tetrahedral CCPO where upon the addition of SpyTag-RFP the covalent complex was formed. The presented system for presentation of protein domains at the vertices of CCPOs represents a technology for diverse applications where the fixed orientation, distance and/or placement of different protein domains is required.

The potato transcription factor StERF49 increases susceptibility to potato virus Y

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Potato is the world's most widely grown tuber crop and potato virus Y (PVY) is one of the major potato pathogens causing severe crop losses worldwide. It is thus important to improve our understanding of potato-PVY interaction for more efficient crop breeding strategies. The analysis of transcriptomics data previously obtained in our group suggested that the ethylene response factor StERF49 is an important player in *Ny*-mediated hypersensitive response to PVY. To study the role of this gene in PVY infection, we generated stable transgenic potato lines where *StERF49* was knockdown (shERF49). The shERF49 lines presented significantly lower viral levels and slower symptom development compared with the corresponding non-silenced potato plants. Therefore, we concluded that StERF49 increases potato susceptibility to PVY infection. We performed differential gene expression analysis of PVY infected and mock inoculated leaves of shERF49 plants. Several gens involved in the protein degradation pathways seem to be regulated by StERF49 after PVY infection. On the other hand, StERF49 strongly accumulates in plant cell nucleus only when the virus is present. Considering these results we hypothesis that PVY manipulates the host defence mechanisms for its own benefit by preventing degradation of StERF49. We are further studying the complex network of plant signalling pathways associated to this response. Using *in silico* promoter analysis, Y1H and transactivation assays we identified two transcription factors, part of the ethylene signalling pathway, which activate the expression of *StERF49* gene. Our findings will uncover new molecular mechanisms involved in potato-PVY interaction.

SL5

Aegerolysins – lipid-binding proteins with potential applications in biomedicine and biotechnology

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Aegerolysin protein family (Pfam 06355, InterPro IPR009413) comprises low molecular (15-20 kDa), acidic, beta-structured proteins, found in several eukaryotic and bacterial taxa. Although they appear to be among major proteins secreted by the organisms that produce them, their functions and biological roles remain poorly understood.

The common feature of the aegerolysins is their ability to bind different lipids and lipid membranes. Some aegerolysins can target sphingomyelin/ cholesterol membrane nanodomains, while aegerolysins from the fungal genus *Pleurotus* preferentially bind to ceramide phosphoethanolamine (CPE), which is the major membrane sphingolipid in invertebrates (particularly insects). Moreover, the genomes of some aegerolysin-producing fungi have nucleotide sequences that encode proteins with membrane-attack complex/ perforin (MACPF) domain. In the presence of a protein with a MACPF domain, fungal aegerolysins can function as bi-component lytic complexes for target cell membranes.

Selected fluorescent fusion derivatives of fungal aegerolysins could be used as useful tools to track raft-like membrane nanodomains composed of sphingomyelin and cholesterol. Moreover, the selectivity of some aegerolysin-based cytolytic complexes for increased membrane sphingomyelin/ cholesterol contents can be exploited for selective killing of urothelial carcinoma cells. Finally, due to their specific interaction with CPE, some cytolytic complexes based on *Pleurotus*-derived aegerolysins could represent a novel promising class of biopesticides for controlling plant pests.

Combined inhibition of two cancer specific 6-phosphofructo-1-kinase isoforms prevents lactic acid excretion in tumorigenic cell lines

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Lactate generation in tumors has been correlated with poor clinical outcomes. It was shown to induce angiogenesis, migration of cancer cells and contributes to immune escape. In the fast growing cells with enhanced glycolysis, lactate formation is required for redox balancing where redundant NADH is re-oxidized by reducing pyruvate. We showed that modified 6-phosphofructo-1-kinase (PFK) a rate-limiting enzyme of glycolysis, might be the pivotal factor of deregulated flux in cancers. In several cancer cells only the shorter 45 or 70 kDa fragments, and no 85 kDa native enzymes were detected. Shorter fragments that were formed by proteolytic cleavage of the C-terminus, retained activities but became insensitive to feed-back inhibition.

In order to find a drug that would inhibit overactive PFKs, a large-scale target based virtual screening was performed by docking small-molecule ligands on two forms of ATP binding sites of PFKs (PDB 4xyj) by a supercomputer. One form was found in PFK-M (muscle) and PFK-P (platelet) and another in the PFK-L (liver type) isoenzyme. Forty compounds with the highest scores were selected out of 4.5 million and thirtyfour were chosen for testing. Two compounds from the 1st generation of screening inhibited recombinant PFK-M enzyme and also prevented lactate excretion by Jurkat cells. Additional three compounds (2nd generation) specifically decreased the activity of recombinant PFK-L isoform and reduced lactate formation by Colo 829, Caco2 and MDA-MB-231 cells. However, complete inhibition of lactate formation was observed only with the combined inhibition of the 1st and 2nd generation of inhibitors. Efficient inhibition of modified PFKs was confirmed by measuring the levels of glycolytic intermediates by mass spectrometer.

To conclude, by inhibiting modified cancer specific PFKs, enhanced glycolysis is reduced. As a consequence, no redundant NADH must be re-oxidated anymore therefore deleterious lactate generation is stopped in tumors.

SL7

Cryo-EM analysis of Potato virus Y and virus-like particle reveals structural plasticity of the coat protein

Andreja Kežar[1], Luka Kavčič[1], Martin Polák[2], Jiří Nováček[2], Ion Gutiérrez-Aguirre[3], Magda Tušek Žnidarič[3], Anna Coll[3], Katja Stare[3], Kristina Gruden[3], Maja Ravnikar[3], David Pahovnik[4], Ema Žagar[4], Franci Merzel[5], Gregor Anderluh[1], Marjetka Podobnik[1]

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PVY is among top 5 economically most important plant viruses. It infects potato, tobacco and other Solanaceae and can severely affect their yields. PVY flexible filamentous virion consists of thousands of coat protein (CP) copies assembled around the viral (+)ssRNA. Heterologous expression of CP in bacteria leads to production of filamentous RNA-free virus-like particles (VLPs).

We determined cryo-EM structure of PVY at 3.4 Å resolution and cryo-EM structure of VLP at 4.1 Å resolution. PVY virion shows a left-handed helical symmetry of CPs encapsidating the viral RNA, with 8.8 CP units per turn and diameter of 130 Å. On the other hand, in the absence of viral RNA, VLPs are built of stacked rings of 8 CP units and diameter of 125 Å. Each CP unit is built of three regions: structurally extended N- and C-terminal regions and a core region with defined secondary structure elements. Unique architectures of both types of filaments are enabled by flexible N- and C-terminal regions of CP, which can structurally adapt to available binding partners. The role of terminal regions in PVY and VLP filament assembly was confirmed by mutational analysis. We show that in VLP, the N-terminal region is essential for the filament assembly. Sequential deletion of this region eventually leads to formation of individual octameric rings or even soluble monomeric CP units. On the other hand, deletion of the C-terminal region does not affect filament assembly and results in formation of hollow VLP filaments. Moreover, we show that both terminal CP regions are essential for successful viral infection of host plant.

These near-atomic structures of PVY and VLP are important contributions to understanding of PVY assembly mechanism as well as of various steps of infection cycle. Moreover, knowing the well-defined structure of these filaments could accelerate the development of nanotechnology applications such as nanowires, nanotubes, biosensors, templates, etc. based on PVY and corresponding VLPs.

Structural insight in peptidyl substrate binding to cysteine cathepsins

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Cysteine cathepsins are lysosomal peptidases involved in numerous physiological and pathological processes, such as protein degradation, protein processing, antigen presentation, cancer and CNS disorders. We are trying to understand how these proteases select their endogenous substrates. Analysis of proteomic study of the cell lysate, which was treated with several cathepsins, suggested representative peptides for the study interactions between the peptidyl substrates ad cysteine cathepsins. Using structural analysis and mass spec, we made an attempt to gain insight in the differences between the binding and processing of short peptides and proteins using native and catalytic-site mutant human cathepsin V. Three generation of peptides were synthetized: The first generation peptides were designed to explore possible cooperative effects of amino acids binding into S1 and S2' subsites of the enzyme. However, the peptides did not bind to the protein structure as anticipated. The second generation peptides were hexapeptides selected from the proteomic data. In crystal structures we observed mainly shifted peptides from their expected binding, possibly due to interactions of their charged termini, and possibly a few cleaved fragments. The third generation of peptides are of various lengths with N- and C- termini protected with acetyl and amide groups. This analysis is ongoing. So far, we have shown that peptides bind differently to cathepsin V as expected from proteomic data, as their binding is clearly affected by complex active site interactions beyond single substrate amino acid – enzyme subsite affinity.

SL9

Towards understanding the effect of calcium binding on the structure of human non-muscle α-actinin-1

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α-actinin-1 is one of the Ca^{2+}-sensitive non-muscle cytoskeletal isoforms of the α-actinin, a member of the spectrin superfamily of F-actin-binding proteins. All isoforms of α-actinin are functional as antiparallel dimers and share a common basic structure (N-terminal ABD, flexible neck region, rod domain and C-terminal calmodulin-like domain (CaMD)). In non-muscle cells, Ca^{2+} binding to EF-hands within the CaMD of α-actinin-1, results in reduced actin bundling capacity of the α-actinin dimer. We hypothesise that the molecular basis of Ca^{2+} regulation of α-actinin-1 bundling activity lies in modulation of structural dynamics of functional domains ABD and CaMD.

In order to elucidate the impact of Ca^{2+} binding on structure and dynamics of isolated CaMD, we determined high-resolution NMR structures of CaMD in both apo and holo forms. Furthermore, by using ITC and MS, we showed that CaMD has only one Ca^{2+}-binding site, located in EF1-hand with K_d of ~100 μM.

Taking into account the fact that CaMD was studied out of the context of the full-length α-actinin-1 molecule, we successfully crystallized apo and holo forms of α-actinin-1 half-dimer (ACTN1-hd). Several diffraction datasets with the highest resolution of 3.5 Å were recorded. Structure determination is in progress and will give us insight into the most important intramolecular interactions. Additionally, using ITC we confirmed that there is one Ca^{2+}-binding site in EF1-hand of ACTN1-hd with the K_d of ~20 μM. This is in line with our prediction that the presence of other domains and/or target proteins can significantly affect Ca^{2+} binding.

Being aware that crystal structures typically do not provide insight into structural dynamics, we aim to more specifically address the question of the Ca^{2+} regulation of the intramolecular dynamics in the context of the whole molecule using solution NMR. We thus prepared segmentally labelled ACTN1-hd to uniquely show that ABD loses its flexibility upon Ca^{2+} binding.

Cold atmospheric plasma induces stress granule formation via EiF2-signalling

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Cold atmospheric plasma is an ionized gas that has shown great promise in regenerative medicine and oncology treatments, but with exact mechanisms underlying these effects remaining unknown. Besides physical effects (production of ultraviolet rays, heat and electromagnetic fields), in cell cultures chemical effects of plasma (production of reactive oxygen nitrogen species) pervade. So far many “late” cell responses as growth promotion or cancer cell apoptosis, to plasma treatment have been demonstrated, whereas the early response has not yet been addressed. The initial response of cells exposed to various stress stimuli (oxidative, osmotic, nutrient deprival), is a halt of protein translation accompanied by recruitment of over 140 proteins that intertwined with cytoplasmic RNAs form transient cytoplasmic structures - stress granules (SG). As no study so far addressed plasma effect on SG formation, we aimed to employ an atmospheric pressure plasma jet with argon gas flow, to treat the Flp-In SH-SY5Y cells stably expressing a SG constituent - mScarletG3BP1 protein, for the analysis of SG assembly dynamics. Two known cell stressors NaAsO2 and sorbitol were evaluated sidewise for early stress response and SG characteristics comparisons. The SG dependence on eIF2a signalling pathway in plasma exposed cells was evaluated by ISRIB inhibitor. We demonstrated that plasma induces SG formation in cells in flow/time dependent manner, with the SG characteristics resembling arsenite stress response. Plasma at flow rates exerting no toxic effects in cells, induced eIF2 signalling, but only to half the extent the Arsenite effect. This mirrored in pronounced ISRIB inhibition of plasma induced SG formation via eIF2 pathway. Our results provide insight into plasma induced cell signalling and SG assembly. Unravelling mechanisms and pathways of plasma response regulation may implicate on novel direct or adjuvant plasma therapies in oncology and regenerative medicine.

SL11

Quartz Crystal Microbalance as a bioprocess in-line sensor

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Protein A multicolumn continuous chromatography in downstream processing of recombinant immunoglobulins (IgGs) is a promising approach enabling significant improvement in resin utilization and overall process economic optimization. Integrated columns enable the capture of IgGs from flow through and wash cycle of the first column on the subsequent column in series.

The key control point when loading in a multicolumn system is determination of IgGs in the breakthrough from the column. This is important for the redirection of fluid flow from waste to loading the next column in the series. UV absorbance is a reliable and sensitive detection mode enabling estimation of total protein concentration in protein A column outflow. However, UV detection lacks specificity for IgGs and relies on assumption of constant contribution of host cell proteins to the total protein amount in the cell-free harvest. This is not always the case, especially in harvests from perfusion culture bioreactors. Quartz Crystal Microbalance (QCM) is a mass detector that enables specific detection of selected component in crude samples through immobilized ligands on the sensor surface. We prepared a QCM sensor surface using protein A as the ligand. We demonstrated sensors linear detection range of IgGs and ligand stability in long term repeated detection-regeneration cycles. Modular QCM sensor was integrated inline after UV detector of chromatographic system. Detection of IgGs in breakthrough fluid was specific and can even precede the detection by the UV detector. Using the same ligand in chromatography column and QCM sensor enabled concomitant column and sensor regeneration, suggesting ability for continuous and automated sensor performance in continuous protein A chromatography.

Development of nanofiber delivery system that enables high loading and long-term viability of probiotics

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The interest in probiotics has grown in recent years due to increased awareness of the importance of microbiota for human health. Electrospinning is an established technique to produce fibers with small diameter in the range of several nanometres to micrometres, often called nanofibers. Incorporation of probiotics in nanofibers allows for the concomitant drying of the bacteria and the preparation of solid dosage form, thereby offering considerable advantage over e.g. lyophilization. We have incorporated ten different species of lactobacilli in poly(ethylene oxide) nanofibers and characterized them using scanning electron microscopy. All bacteria retained considerable viability (1-2 logs decrease) that correlated with cell hydrophobicity. We have focused our efforts on the probiotic *Lactobacillus plantarum* ATCC 8014 by developing poly(ethylene oxide) and composite poly(ethylene oxide)/ lyoprotectant nanofibers. High loading was achieved for *L. plantarum* cells (up to 7.6×10^8 colony-forming unit /mg) that were either unmodified or expressing mCherry fluorescent protein. The initial concentration of *L. plantarum* electrospinning solution was reported as the most critical parameter for its high viability after electrospinning, whereas the applied electric voltage and relative humidity during electrospinning did not vitally impact upon *L. plantarum* viability. The presence of amorphous lyoprotectant (especially trehalose) in the nanofibers promoted *L. plantarum* survival due to lyoprotectant interactions with *L. plantarum* cells. *L. plantarum* cells in nanofibers were stable over 24 weeks at low temperature, thereby achieving stability comparable with that in lyophilizates. The poly(ethylene oxide) nanofibers released almost all of the *L. plantarum* cells over 30 min, which will be adequate for their local administration. Our integrated approach enabled the development of a promising local nanodelivery system for lactobacilli for topical or vaginal administration.

SL13

Using Knowledge for Downstream Process Design – Mechanistic Modelling Approach

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One of the key steps in the manufacturing of the biological entities is the purification of the cell broth. The broth is produced in bioreactors and contains, together with pharmacologically valuable ingredient, high amount of process and product related impurities. Development of downstream process follows two key objectives: efficiency and quality. Final product should be produced with high yields with optimized expenses, and repeatedly and robustly purified to the sufficient quality. Increasing demands on product quality result in higher complexity of the processes. In addition, in order to optimize speed to market time the downstream development starts in parallel with the development of the upstream process, which leads to frequent changes in the development material quality. In order be able to adopt to these changes quickly we are introducing the knowledge based approach to the Downstream Process Design.

Increasing demand for the high throughput downstream process development as well as the confinement of the limitations posed on the availability of the development material naturally lead to the *in-silico* development of the protein purification process. By application of mechanistic modelling in the initial steps of the development, we drastically decrease the number of required experiments as well as material consumption. Even for non-platform biological entities the initial acceptable (working) downstream process may be developed within 1 month. By gathering knowledge, refining the initially parameterized model, we build strong knowledge foundations, which is of great importance in later phases at process upscale or as a manufacturing support.

In this talk, we present how this concepts are integrated into every day development decisions. We are utilizing the mechanistic modelling tools developed by Rushd Khalaf. These tools are operated within the context of work packages (unit processes) that are easy to learn and use. The development starts on high throughput Tecan system, where we determine early model parameter estimates. By simulation, interesting experimental space is selected and larger scale confirmation experiments are conducted. Unnecessary experiments that do not increase system knowledge are avoided. Instead of aspiring to a perfectly parameterized model, the goal of this design tool is to refine the model parameter estimates with each new experiment until a satisfactory set of process parameters is found.

A Clique-Based Method for Improving Motif Scanning Accuracy

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Background: Motif scanning is a very common method in bioinformatics. Its objective is to detect motifs of sufficient similarity to the query, which is then used to determine family membership, structural or functional features or assignments. Considering a variety of uses, accuracy of motif scanning procedures is of great importance.

Results: We present a new approach for improving motif scanning accuracy based on analysis of in-between similarity. Given a set of motifs obtained from a scanning process, we construct an associated weighted graph. We also compute the expected weight of an edge in such a graph. It turns out that restricting results to the maximal clique in the graph, computed with respect to the expected weight, greatly improves accuracy of the scan. We tested the method on three motif-characterized protein families from five plant proteomes. The method was applied to three iterative motif scanners - PSI-BLAST, jackhmmer and IGLOSS - with very good results.

Conclusions: We present a method for improving protein motif scanning accuracy, successfully applied in several un-gapped situations. The method has wider implications, for general pattern recognition and feature extraction strategies, as long as one can determine the expected similarity between objects under consideration.

SL15

BiOpenBank: open source solution for management of biological samples and sample associated data in small biobanks

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With development and advancement of different technologies it is only natural that more samples and data from the experiments will be collected in research environments. This presents an obstacle with storage, tracking, management and analysis of physical samples as well as the data describing these samples and their analyses.

BiOpenBank presents our initiative to develop an open source information system for storage and management of biological samples. BiOpenBank is compliant with recent standards, regulations and good practices, such as ISO 20387:2018, GDPR and MIABIS 2.0. Core functionalities provide physical identification and tracking of samples through QR codes, which provide unique sample identifiers. Management and display of stored information is implemented with web-based user interface. New samples can be added to the system manually *via* web interface or through the import of standardized files. Records can be exported in standardized formats that can be further analyzed with other software tools.

BiOpenBank was designed as a flexible system and can be easily customized to the user demands. It combines user friendliness, low-maintenance and development with open-source platforms. This makes the system more accessible to scientific and research community. BiOpenBank has already provided a basis for different projects, such as diagnosis of Alzheimer's disease and hepatocellular carcinoma, and analysis of genetic predispositions in psychiatric disorders and familial erythrocytosis.

This work was partially supported by the projects "Establishment of open information system for the management of biological samples in the biobank repositories" and "Standardization of procedures for obtaining biological samples and information system for biobanks" co-financed by the Republic of Slovenia and the European Union under the European Social Fund.

Investigating 2’-O-methylation writers in human RNA

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RNA modifications are changes of the chemical composition of ribonucleotides and thus have the potential to influence gene expression. Recently, 2’-O-methylated nucleotides (Nm) have been shown to be present in mRNA. However, enzymes that catalyze the formation of 2’-O-methyl groups on mRNA are unknown. FtsJ RNA methyltransferase homolog 3 (FTSJ3) is a nucleolar RNA-binding protein that exhibits poly(A)+ RNA-binding activity. In this work we performed PAR-CLIP to identify *in vivo* RNA substrates of FTSJ3 in HEK293 cells. We found that FTSJ3 binds predominantly to GC-rich regions of pre-rRNA, rRNA, mRNA and pre-mRNA. We next used a 2’-O-methyl sequencing approach and identified a candidate FTSJ3-dependent site in 28S rRNA, which corresponds to the yeast 25S rRNA site (Um2921) methylated by the likely orthologue SPB1. Further experiments will assess the *in vitro* RNA 2’-O-methylation activity of FTSJ3 on rRNA and mRNA and its impact on the regulation of gene expression.

SL17

Cysteine cathepsins B and X in cancer stem cells

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Disease relapse and therapy resistance are currently the main challenges in cancer treatment. Failure in cancer treatment has been, among the phenotypically and functionally heterogenous population of cells with different degree of differentiation within tumors, mainly attributed to the subpopulation of cancer stem cells (CSCs). CSCs represent a small subset of cells with a tumorigenic potential that are resistant to the convenient antitumor therapies and can give rise to a disease relapse. Therefore, to improve the effectiveness of treatment and patient's survival, new drugs and approaches, affecting besides differentiated also CSC, are urgently needed. Due to overexpression and important role in various stages of development and progression of cancer, redundant lysosomal cysteine carboxypeptidases cathepsins B and X could serve as promising molecular targets to enhance CSCs directed therapy. Increased expression of cathepsins B and X is associated with less differentiated cell phenotype and they play important role in degradation of extracellular matrix, tumor invasion, migration, metastasis and angiogenesis. Therefore, in the present study we evaluated expression and activity of cathepsins B and X in cells with CSC phenotype (CD44+/CD24-), isolated from breast cancer cell lines, based on their ability to form tumorspheres *in vitro*. We demonstrated that in CSCs following tumorsphere formation protein levels and activity of cathepsins B and X were significantly higher compared to single adherent cells as shown by western blot, ELISA and enzyme kinetics activity assay. Additionally, treatment of cells with CSCs phenotype with cathepsin inhibitors resulted in decreased expression of CSCs markers as shown by western blot. Taken together, inhibition of cathepsins B and X in CSCs could serve as promising tool to perform CSC directed treatment in cancer that may overcome the limitations of current antitumor therapy.

Microscopic imaging of similarities between stem cell niches in glioblastoma and bone marrow

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Glioblastoma is the most aggressive primary brain tumor. Slowly-dividing and therapy-resistant glioblastoma stem cells (GSCs) reside in protective peri-arteriolar niches and are held responsible for glioblastoma recurrence. We have determined similarities between GSC niches and hematopoietic stem cell (HSC) niches in bone marrow in previous studies [1]. Acute myeloid leukemia (AML) cells hijack HSC niches and are transformed into therapy-resistant leukemic stem cells (LSCs). Current clinical trials are focussed on removal of LSCs out of HSC niches to differentiate and to become sensitized to chemotherapy [2]. In the present study, we elaborated further on these similarities by fluorescence immunohistochemistry and imaging of 15 biomarkers in paraffin sections of human glioblastoma and human bone marrow. We found all 15 biomarkers to be expressed both in hypoxic peri-arteriolar HSC niches in bone marrow and hypoxic peri-arteriolar GSC niches in glioblastoma (Fig). Our findings implicate that GSC niches are being formed in glioblastoma as a copy of HSC niches in bone marrow. These similarities between HSC niches and GSC niches enable the development of novel strategies to force GSCs out of their niches, in a similar manner as in AML, to induce GSC differentiation and proliferation to render them more sensitive to anti-glioblastoma therapies.

SL19

Study of RNA G-quadruplex structures in the 5'-UTR of the human NRAS proto-oncogene

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Naturally occurring RNA guanine quadruplex (G-quadruplex) sequences have a large diversity of loop lengths and arrangements, nevertheless, they usually adopt the parallel conformation in which all four strands are oriented in the same direction. RNA G-quadruplexes have been shown to be involved in the modulation of translation, the regulation of (alternative) splicing, and the subcellular transport of mRNAs, among other processes. They also seem to hold great promise in therapy, both as therapeutic targets as well as therapeutic agents themselves, and can act as tunable devices depending on cellular conditions.

We will show our results on RNA G-quadruplex structures in the *neuroblastoma RAS viral oncogene homologue* (*NRAS*) proto-oncogene using NMR, CD and UV spectroscopies. *NRAS* proto-oncogene encodes for protein called N-Ras, which is involved in regulating cell division through a process known as signal transduction. RNA G-quadruplex forming sequence is present in the 5'-UTR of *NRAS* mRNA and is known that stops translation. We prepared modified RNA G-quadruplexes with different G-tract and loop lengths in order to stabilize the two most probable structures.

Internalization and signalling properties of BILF1 receptors encoded in Epstein-Barr virus and porcine lymphotropic herpesviruses

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Several herpesviruses express viral G protein-coupled receptors (vGPCRs) homologous to host receptors, allowing viruses to establish a lifelong infection. The human Lymphocryptovirus, Epstein-Barr virus (EBV) is highly prevalent oncovirus, which establishes a lifelong infection in B cells. EBV encodes an orphan vGPCR (BILF1) with constitutive $G\alpha_i$ activity; linked to cell transformation. By downregulating surface expression of MHC class I molecules, BILF1 acts as important immunoevasin. Porcine Lymphocryptoviruses, porcine lymphotropic herpesviruses (PLHV 1-3) are widespread and closely related to EBV. They encode BILF1 receptor homologs. EBV and PLHV1 are both associated with post-transplant lymphoproliferative disorder (PTLD) causing similar clinical symptoms. Considering the similarities between PLHV1 and EBV, we hypothesize that pigs could be used as in vivo models for studying EBV infection in humans. With this project, we aim to characterize and compare EBV and PLHV-encoded BILF1 receptors in human HEK-293 and porcine PK-15 cells, focusing on receptor signaling, internalization properties and the impact on MHC class I downregulation. Co-localization with membrane marker showed that all studied BILF1 receptors mainly localizes at the cell surface. Higher surface expression was observed for EBV-BILF1 compared to porcine BILF1 in both cell lines, but it was more distinct in HEK-293 cells. Similar to EBV-BILF1, we confirmed constitutive internalization for porcine BILF1 receptors, with approximately 80% of PLHV1-2-BILF1 residing at the surface after 30 min. Using transcription factor assay in HEK-293 cells, EBV-BILF1 and PLHV-1-3 BILF1 showed constitutive activation of transcription factor NF-κB. Furthermore, we show that PLHV-1-3 BILF1 constitutively activated $G\alpha_i$ to a similar extent as EBV-BILF1. Based on similarities between BILF1 receptors on cellular level, we aim to further investigate their role in immune evasion with focus on MHC class I downregulation.

SL21

Development of a method for extracellular vesicles isolation from human blood and evaluation of its clinical value in malignant mesothelioma

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Introduction: Extracellular vesicles (EVs), phospholipid bilayer-enclosed particles, are abundantly released into body fluids. EVs' morphology and molecular cargo reflect (pato)physiological state of their cell-of-origin, making them ideal candidates for minimally invasive biomarkers. However, they are largely unexplored due to lack of established isolation methods from complex fluids. Here, we established a method for effective isolation of pure EVs from blood and used it to evaluate EV-miRNAs as biomarkers of treatment outcome in malignant mesothelioma (MM).

Methods: For method development, EVs were isolated from plasma of 10 healthy subjects, using density-based (ultracentrifugation on 20% sucrose cushion; sUC) and size-based (size exclusion chromatography; SEC) isolation methods. Size, concentration and purity of isolates were determined with NTA, AF4-MALS, TEM and miRNA levels with qPCR. For biomarker study, levels of miR-126-3p, miR-625-3p and miR-625-3p were analysed in sUC-EVs from serum of 20 MM patients (before and after treatment) and 10 healthy asbestos-exposed controls with qPCR. All subjects provided informed consent, NMEC approved the study.

Results: SEC generally led to a higher number but lower quality of isolated particles compared to sUC ($P<0.001$), due to contamination with lipoproteins and aggregates. sUC method was highly repeatable and resulted in purer EVs isolate with more miRNA cargo. NTA detected $3.46*10^9$ particles/mL (mean size 109 nm) and AF4-MALS $0.73*10^9$ particles/mL (R_{geom} 195 nm).

In the biomarker study, relative expression of EV-miR-126-3p was significantly higher in MM compared to controls ($P<0.001$). miR-625-3p increased significantly after treatment in patients with poor treatment outcome ($P=0.017$). miRNA expression at diagnosis was not associated with survival in MM.

Conclusion: sUC method led to higher yield and purity of isolated EVs and was used in the MM biomarker study. EV-miRNAs show potential as diagnostic biomarkers in MM.

Chemokine CCL5 signalling is important for invasion of glioblastoma in its microenvironment

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Background: Glioblastoma microenvironment consists of cancer cells, glioblastoma stem cells (GSCs), mesenchymal stem cells (MSCs) and infiltrating immune cells. The chemokine CCL5 /RANTES is inflammatory mediator, which interacts mostly, with the receptor CCR5 and thereby promotes intracellular interactions in the tumour and its microenvironment. CCL5/CCR5 signalling axis involves mainly PI3K/ Akt kinase signalling, affecting cell invasion. CCL5 is elevated in aggressive mesenchymal glioblastoma subtype and had an impact on shorter patients' survival.

Aim: To localise the expression levels of CCL5 and CCR5 in series of tissues, established differentiated glioblastoma cells and GSC lines using immunohistochemistry, immunocytochemistry and mRNA analyses from fresh patient tumour samples. We also aimed to confirm CCL5 effects on invasion of established glioblastoma cells derived from patient.

Results: CCL5 and CCR5 mRNA levels are highly expressed in glioblastoma tissues compared to low grade glioma and non-tumour brain. MSCs that reside in glioblastoma, found in association with GSCs, also use a variety if cytokines to mediate their intra-tumour communications, significantly affecting tumour behaviour. We found that CCL5 protein is expressed in MSCs, but not in GSCs. CCR5 is expressed in macrophages, differentiated GB cells, and in GSCs. CCR5 is the main co-receptor used by macrophage (M)-tropic strains of human immunodeficiency virus type 1 (HIV-1) and HIV-2, which are responsible for viral transmission. A CCR5 antagonist and FDA approved drug to treat HIV infection is Maraviroc. Maraviroc reduced the invasion through matrigel. Maraviroc also specifically and highly significantly ($P < 0.001$) inhibited chemotactic invasion of differentiated glioblastoma cells towards MSCs, proving that they also also secrete CCL5.

Conclusion: These results suggest that targeting CCL5/CCR5 axis is a promising adjuvant tool in the treatment of glioblastma.

SL23

Legumain as a modulator of immune response

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Legumain or asparaginyl endopeptidase (AEP) is a member of the CD clan of cysteine proteases and cleaves protein substrates exclusively after asparagine or (to a minor extent) after aspartic acid residues. Mammalian legumain is most abundantly expressed in immune cells, however its exact role in molecular mechanisms of immune response remains largely unknown, since only a few of its physiological substrates have been identified to date. In order to improve our understanding of legumain physiology, we performed a comprehensive proteomic analysis of tissue samples from legumain null mice. The analysis showed that legumain activity affects a distinct group of peroxidases and CLPs (chitinase-like proteins) which are crucial effectors of inflammation and immune response against pathogen infection. Peroxidases are expressed primarily by neutrophils where they catalyse the formation of hypochlorous acid which kills invading pathogens. Chitinase like proteins are also closely related with neutrophils since trigger their accumulation through the stimulation of cytokines. We have determined that legumain deficiency causes strong upregulation of peroxidase activity and that it affects proteolytic processing and localization of chitinase-like proteins in immune cells. This indicates that legumain deficiency might affect immune response by stimulating neutrophil formation. Here, we will present possible physiological implications of this finding.

iPS cells as disease models for epidermolysis bullosa

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Epidermolysis bullosa (EB) is a severely disfiguring and often lethal hereditary skin fragility disorder that is divided into three main types: simplex, junctional and dystrophic EB, which include over 30 clinical subtypes. The disease causing mutations affect at least 18 structural proteins lying within the epidermal-dermal junction of skin. Chronic skin blistering in patients with EB causes severe health complications including squamous cell carcinoma.

Up to now a variety of therapeutic approaches have been devised and tested, such as gene-, protein-, and cell-based therapies, yet most of these are expensive, very invasive and include risks for patients' health as well. However, new pharmacological approaches for treating this type of wounds could prove much more cost-efficient and less invasive by promoting faster wound healing and less scarring. As this requires testing a large number of molecules (drug repurposing, new substances), it is a difficult task to achieve without a good disease model for *in vitro* testing.

Induced pluripotent stem cell (iPSC) technology allows generating pluripotent stem cells from differentiated somatic cells. These iPSCs can in turn be used to produce pools of different cell types to build tissue mimicking 3D models.

We already generated iPSC cells from cells derived from a skin biopsy of a normal control individual (MLi003-A) and a patient (MLi002-A) with the simplex phenotype of EB (EBS). We also reprogramed fibroblasts from two recessive dystrophic EB and one dominant dystrophic EB patient, by using non-integrating Sendai virus vectors containing the four Yamanaka factors. At the same time, we tested differentiation protocols to obtain keratinocytes and fibroblasts.

The EB iPSC lines and the resulting 3D skin models will be used in ongoing and future projects on EB disease mechanisms, for screening libraries of compounds for systemic therapy and for testing novel therapy approaches.

SL25

Cathepsin X as a Potential Therapeutic Target for Treatment of Parkinson's Disease

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Neuroinflammation is closely implicated in the pathogenesis of neurodegenerative disorders, such as Parkinson's disease (PD), where the hallmark of neuroinflammation is activated microglia. Microglia-derived lysosomal cathepsins, including cathepsin X, are increasingly recognized as important mediators of the inflammation-induced neurodegeneration. Recent study revealed that up-regulated expression and activity of microglial cathepsin X as wells as increased release of cathepsin X after lipopolysaccharide (LPS) stimulation leads to microglia activation-mediated neurodegeneration. Cathepsin X inhibitor caused neuroprotection via its suppression of microglia activation. Moreover, the immunomodulatory role of cathepsin X has been also shown in microglial co-activation, where cathepsin X inhibition proved to diminish increased neuroinflammation by LPS and poly(IC) co-stimulation. Our recent study revealed that LPS also induced the expression and upregulated enzymatic activity of cathepsin X in brain regions observed in *in vivo* models of PD, with a preference cathepsin X upregulation in microglia cells and astrocytes in lesioned striatum. Taken together, these findings propose the potential function of microglial cathepsin X in inflammation-induced neurodegeneration. Knowing the involvement of cathepsin X in the neurodegenerative processes represent a step towards the development of new molecules for the treatment of neurodegenerative diseases.

Abstracts of satellite meeting lectures

SM1

Heterologous expression of the griselimycin and myxopyronin biosynthetic gene clusters

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Griselimycin (GM) and methylgriselimycin (MGM) are anti-tuberculosis (TB) cyclic depsipeptides via targeting the prokaryotic DNA polymerase sliding clamp DnaN.[1] In addition, GM has no cross-resistance with available anti-TB drugs which makes GM serve as an excellent starting point to combat drug-resistant TB. The complete 65 kb GM biosynthetic gene cluster (BGC)[2] has been cloned into a Bacterial artificial chromosome (BAC) plasmid. The resulting construct has been added with a site-specific integration gene cassette to facilitate the integration of GM BGC into the chromosome of heterologous expression hosts. The GM BGC has been successfully expressed in an engineered *S. lividans* strain to produce GMs. The engineering on the regulatory genes and transcription reconstitution to improve GMs production are ongoing.

Myxopyronins (MXNs) are α-pyrone antibiotics discovered from *Myxococcus fulvus* strains which inhibit the prokaryotic RNA polymerase (RNAP). The binding site of MXN is distant from targets of previously characterized RNAP inhibitors including rifamycins, which makes MXN a promising candidate to combat antibiotic resistance.[3] Previously, the myxopyronin BGC spanning 53 kb was cloned and successfully expressed in *Myxococcus xanthus* DK1622.[4] Currently, the construct harboring MXN BGC has been swapped with a BAC backbone and added with a site-specific integration gene cassette to facilitate the integration of it into the chromosome of streptomycete hosts to facilitate the potential industry production. The engineering on the regulatory genes and transcription reconstitution of MXN BGC to make it working in streptomycete strains are in process.

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Streptomyces rimosus, a powerful industrial strain for production of oxytetracycline; perspectives of this industrial chassis

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Streptomyces rimosus is used for industrial production of the medically and agriculturally important broad spectrum antibiotic oxytetracycline (1). It is one of the most efficient high-producing strains ever developed for industrial fermentation processes. In addition to this high efficiency, there are a number of properties of *S.rimosus* that make this industrial strain attractive as a cell factory for the production of diverse heterologous bioproducts: *S. rimosus* has a rapid growth rate, and as with *Escherichia coli*, it can reach high cell densities in a short period of time. This host strain does not form complex mycelia or pellets, but as for *E. coli*, it grows as relatively short fragments; and finally, *S. rimosus* shows high transformation efficiency and is genetically amenable. An additional advantage of S. rimosus is its 'industriability', with its robust and economically favourable medium, culturing and bioprocessing conditions, which have been developed and scaled up to industrial applications over the years. *S.rimosus* also has GRAS status (generally regarded as safe). Considering these favourable properties of *S. rimosus*, this host holds promise as a powerful platform that can accommodate a number of new approaches for drug discovery and process development of natural products.

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Mission to morphology - bioprocess engineering for natural product formation in *Streptomyces*

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Streptomyces is the most successful supplier of natural products for therapeutic use. About 70% of our antibiotics today originate from this soil bacterium. Two times, *Streptomyces* has been awarded the Noble Price: as producer for the antibiotic streptomycin (1952) and for the anti-infectant ivermectin (2015). Unfortunately, the level of the products of interest in native producers is typically far too low to support further development so that the enhancement of the natural biosynthetic potential is important. One of the peculiarities of *Streptomyces* is their complex morphology. In suspended culture, the microbe occurs in diverse shapes and forms individual cells, hyphae and even pellets. Previous studies with filamentous fungi have shown that morphology closely correlates with production [1-3]. An interesting way to tailor morphology is the use of micro particles, which are added to the culture.

Here, we demonstrate *Streptomyces* strains and micro particle based bioprocesses with a tailored cellular morphology and a significantly enhanced production performance. Moreover, we provide a system's view into the cellular response of the engineered microbe.

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Transcriptional Cluster “refactoring” to access and expand nature’s chemical diversity

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There are thousands of prokaryotic natural products (NPs) that have been once detected and never properly characterized. The supply of sufficient amounts of complex natural products for studying of pharmacological properties is a significant bottleneck in drug development. Such NPs comprise an alternative treasure trove of potentially new chemical scaffolds and biological activities awaiting discovery. The problem is that the secondary metabolite gene clusters coding for above-mentioned entities are silent or poorly expressed, thus need to be “refactored” in order to get access to chemical compounds they code for. Therefore, there is a need in methods that will allow quickly turning on or up biosynthetic pathways, and tuning their metabolic profile.

Herein, we report an alternative random rational strategy for the native and heterologous expression of biosynthetic gene clusters. The approach is based on creation of a library of clusters with random combination of promoters and selection for the one with high production of metabolite of interest. As a case study, we used gene clusters coding for bottromycin and pamamyicn – promising compounds that might be blue prints of new class of antibacterials. Using above-mentioned approach, we succeeded to optimize/tune the expression of genes in operons and created modified gene clusters that provide 20-80 times higher yield of bottromycins and pamamycins in comparison to the natural ones and thus allowed to identify several new molecules not described before. We anticipate that this generic strategy should facilitate the overproduction of known and discovery of new potentially active NPs in *Actinobacteria*.

Abstracts of talks by the sponsors

Minimal residual disease monitoring by droplet digital PCR in acute promyelocytic leukemia

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Acute promyleocitic leukemia (APL) is a hematological malignancy characterized by a specific chromosomal translocation t(15;17)(q24;q21), forming oncogenic PML-RARA fusion transcript. A major component of current induction therapy is all-trans retinoic acid (ATRA), which is commonly combined with other agents, like anthracycline or arsenic trioxide. Achievement of undetectable level of PML-RARA fusion transcript is required for complete remission, while persistence or re-emergence of PML-RARA fusion transcript presents particular risk for disease relapse. Thus, determination of minimal residual disease (MRD) after remission is of crucial importance for recognition of potential relapses. Real time quantitative PCR (qPCR) is the method generally used for monitoring MRD in APL. Requirement of controlled standards and standard curves for accurate detection of MRD can sometimes lead to variable quantification, especially when copy number of PML-RARA is near an assay's limit of detection. Fluctuating levels of MRD can be confusing and therefore introducing a more sensitive and accurate method like droplet digital PCR (ddPCR), allowing absolute quantification independently of standard curves, represent the future of molecular MRD monitoring. We followed 22 APL patients from Clinical Hospital Rijeka that are enrolled in MRD follow-up from 2014. We compared three methods for MRD detection: TIB MOLBIOL method that is qPCR based method used in routine diagnostics, a qPCR method with primers and probes designed and approved by "A Europe Against Cancer Program" working group and a ddPCR method with these same primers and probes as for qPCR. We can conclude that ddPCR allows reliable MRD detection, with clear and non-confusing results, but on the other hand is extremely sensitive, so every deviation from optimal assay performance is immediately detected. This allows diagnostic institutions to reliably determine patient status and initiate treatment on time, if needed.

SP2

HiBiT Technology - New Tool to study Protein Biology and Cellular Responses

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Intracellular signaling pathways are mediated by changes in protein abundance and post-translational modifications. A common approach for investigating signaling mechanisms and the effects induced by synthetic compounds is through overexpression of recombinant tagged proteins. Nevertheless, it is well known that overexpression can cause artificial effects. Regularly used protein tags include either autofluorescent proteins of high molecular weight or small epitope tags which require very tedious, antibody-based detection procedures. Here we describe a new method based on the combination of a new bioluminescent protein tagging system and CRISPR/Cas9. This new method enables the cloning-free generation of endogenous protein assays and allows the simple quantification of intra- and/or extracellular protein levels after experimental manipulation.

We developed a new protein tagging and detection method – the HiBiT system – which is based on a split version of NanoLuc luciferase. HiBiT is a 1.3 kDa peptide (11 amino acids) capable of producing bright and quantitative luminescence through high affinity complementation with an 18 kDa subunit derived from NanoLuc (LgBiT). The small tag size of HiBiT and the ability to measure protein expression at endogenous levels makes HiBiT well suited for CRISPR-mediated genome editing. We, therefore, established a straightforward, cloning-free and rapid CRISPR/Cas9 protocol for the specific insertion of HiBiT into the genome. The importance of low endogenous levels of expression in assay response is demonstrated.

Poster Abstracts

Poster Session I

PI 1

Design of split superantigen fusion proteins for cancer immunotherapy

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Several antibody-targeting cancer immunotherapies have been developed based on T cell activation at the target cells. One of the most potent activators of T cells are bacterial superantigens, which bind to class II major histocompatibility complex (MHC class II) on antigen presenting cells and activate T cells through T cell receptor. Strong T cell activation is also one of the main weaknesses of this strategy as it may lead to systemic T cell activation. To overcome the limitation of conventional antibody-superantigen fusion proteins, we have split a superantigen staphylococcal enterotoxin A (SEA) from *Staphylococcus aureus* into two fragments, individually inactive, until both fragments came into close proximity and reassemble into a biologically active form capable of activating T cell response. A screening method based on fusion between SEA and coiled-coil heterodimers was developed that enabled detection of functional split SEA designs. The split SEA design that demonstrated efficacy in fusion with coiled-coil dimer forming polypeptides, was fused to a single chain antibody specific for tumor antigen CD20. This design selectively activated T cells by split SEA-scFv fusion binding to target cells.

Triangular protein nanostructures self-assembled *in vivo*

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Conceptual and computational advances triggered an explosion of designed protein structures in the last decade. Recently we developed an inovative strategy to create *de novo* protein nanostructures, called coiled-coil protein origami (CCPO). CCPO are single-chain polyhedral structures constructed from concatenated coiled coil-forming building modules that can be of different sizes and properties. For testing new designed CCPO building modules, simpler polyhedra must be used that should maintain most features relevant to larger scaffolds. We show the design and characterization of nanoscale triangles based on a single polypeptide chain, composed of six concatenated coiled coil dimer-forming segments connected by flexible linker peptides. The polypeptides self-assembled in bacteria into nano-triangles in agreement with the design, and the shape of the polypeptides was determined with small angle X-ray scattering.

PI 3

Minimization of gas vesicle cluster

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Gas vesicles are hollow protein structures found in some photosynthetic microorganisms that provide buoyancy and thus allow them to float near the surface of water. Those structures are cylindrical in shape with cones on each end. Their size is from 100 to 2000 nm in length and 45 to 200 nm in width. They are hollow and filled with gas by diffusion. The different density makes them useful as contrast agents in MRI and ultrasound techniques. Genes that are required for their formation form a gene cluster which comprises 11 genes in the genome of *Bacillus megaterium*. The said cluster has been successfully expressed in *E. coli* and in a slightly modified version recently also in mammalian HEK293 cells. As the cluster is relatively long the expression in mammalian cells is low and only results in few gas vesicles, which are not sufficient for ultrasound techniques. Our goal was to identify which of the genes are essential for gas vesicle formation and to prepare a shortened construct for use in bacteria and possible use in mammalian cells. Gene knock-out variants of the cluster from *B. megaterium* were prepared and used for the transformation of *E. coli*. The formation of functional gas vesicles was monitored with flotation assay. Gas vesicles were then isolated, purified and evaluated using dynamic light scattering and transmission electron microscope. Our experiments determined four candidate genes (GvpR, GvpN, GvpT and GvpU) that are not essential for the functional gas vesicle production.

Nanobodies targeting coiled-coil protein origami cages for structure determination and introduction of delivery application

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The modular construction of coiled-coil protein origami (CCPO) cages provides the possibility to construct polyhedra with different CC modules at selected positions (1, 2). CCPO tetrahedral cage that self assembles from a single polypeptide chain and is composed of six different CC module pairs was used for the immunization for the production of nanobodies. Small-angle X-ray scattering analysis and crystallization studies with protein or its specific peptide pairs demonstrated formation of a complex comprising a single molecule of tetrahedron variant TET12SN and up to four nanobody molecules that are either on the inner or outer surface of the tetrahedron. We used binding pattern for nanobodies on homodimeric CC module APH, which is the main target for the generated nanobodies, for the transplantation of this binding site onto another CC module, while maintaining the CC orthogonality. Binding and crystallization studies of tetrahedrons with partially or completely inactivated binding pattern for nanobodies or transplantated binding site for nanobodies as well as other polyhedral variants with repeated CC modules specifically targeting single or multiple nanobodies to the selected sites within CCPO cages are in progress. The availability of nanobodies targeting different CC modules represents a unique opportunity to address defined sites within the CCPO cages and engineer new polyhedral cages with selected number and position of binding sites for nanobodies or their fusion with other functional domains, what will enable development of innovative applications of nanobodies, where designed CCPO polyhedra will be used for protein delivery through nanobody-cargo genetic fusions.

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PI 5

Sonogenetics - transcriptional regulation by ultrasound

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Methods for controlling and regulating cellular processes are of key importance in the basic research of cell biology and understanding of cellular mechanisms. Currently, the most advanced tools rely on light-responsive proteins which allow selective regulation upon stimulation with specific wavelength. Light signal can be easily regulated and it can be restricted to the selected location; however, its poor penetration and scattering require the use of invasive procedures to stimulate cells in deep tissues. In contrast, ultrasound is able to noninvasively penetrate into deep tissues with high spatiotemporal precision. The fact that ultrasound is widely used in medical and therapeutic applications presents an additional advantage in terms of safety.

Synthetic systems for regulation of gene expression are often developed based on known cell signaling pathways. While increase of the intracellular calcium concentration is mostly regulated by release from intracellular stores, activation of mechanosensitive Ca^{2+} channels on cell surface can be achieved by ultrasound waves and also leads to calcium influx. We present a novel synthetic system for regulation of target gene expression by ultrasound. Synthetic calcium-dependent transcription factors were designed based on regulatory domains of calcium-dependent transcription factor NFAT (nuclear factor of activated T cells). We successfully show that our system can be activated based on changes in intracellular calcium concentrations by ultrasound in mammalian cells and *in vivo*.

De novo designed parallel heterodimeric coiled coil peptide pairs with high affinity

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Coiled coil (CC) peptides represent attractive building blocks for assembling modular protein structures. Here we describe *de novo* design of three sets of orthogonal CC forming peptides with low K_d's. CC dimer formation is based on interactions between two alpha helices assuming a twisted super-coiled structure with a heptad repeat periodicity. Our group previously designed several orthogonal CC peptide pairs with K_d in the micromolar range. In order to extend the toolbox of CC-peptide we designed a set of tighter binders that would be more suitable for *in vivo* application. The original pairs were modulated by the number of asparagine residues along the hydrophobic seam. At the *b, c* and *f* position, alanine residues were placed in order to increase the stability of CC pairs. Pairing specificity was achieved based on the electrostatic complementarity between the *e* and *g* charged residue patterns. Furthermore, we tuned the stability of CCs by introducing a polar glutamine residue at the *f* position in all heptads or by introducing a second asparagine instead of isoleucine at the *a* position of the first or fourth heptad. The Asn at the *a* position in the fourth heptad reduced the thermal stability and prevented formation of homodimers. Formation of CC dimers *in vivo* was measured by reconstitution of split luciferase fused to orthogonal peptides Luciferase activity was proportional to the stability of the CCs. Peptide displacement of the reconstituted split luciferase could be controlled by the CC building blocks with the increased stability. The luciferase assay confirmed the traits of *in vitro* coiled coil characterization. The set of orthogonal peptides with different thermal stability of CC's extends the toolbox of peptides that can be used as regulators of enzymatic activity or transcription regulators.

PI 7

In vitro evolution as a tool to study the specificity of proteins for membrane lipids

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The interplay between proteins and cellular membranes is enabling the course of many crucial biological processes such as signaling, membrane trafficking and molecular mechanisms of attack and defense. Membrane targeting protein domains evolved to specifically recognize a distinct membrane component and bind to it. We are particularly interested in the molecular mechanism of specific recognition of cholesterol, the major lipid in mammalian cell membranes. We used a membrane binding domain of perfringolysin O, a bacterial toxin which binds exclusively to cholesterol containing biological and model membranes. For comprehensive characterization of the lipid-binding protein surface we developed an *in vitro* evolution approach using modified ribosome display combined with next generation sequencing. By preparing gene library of cholesterol-binding protein domain with random nucleotides at chosen positions and performing selection after *in vitro* translation, we measured the ability of hundreds of thousands of protein variants to bind to model membranes with different composition. The change in the frequency of each amino acid from input to selected library served as a measure of its function in binding. The most abundant motifs consist of amino acid residues, which are the same or chemically similar to the amino acids in the wild type toxin indicating that these amino acids are crucial for cholesterol recognition. Although the majority of abundant motifs were similar to the naturally occurring ones, we also identified cholesterol specific variants not present in nature and may use a different recognition mechanism. Our approach can be used as a tool to design new toxin-derived molecules for research, biotechnology, or medical treatments.

Tumour Spheroid Cell Model with Stem-like Properties

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Most currently available chemotherapeutic drugs and radiotherapy lack the ability to effectively kill cancer stem cells (CSCs) therefore, tumour resistance and recurrence eventually occur. Targeting the CSC population has thus emerged as a very promising concept and therapeutic option to eradicate tumours and prohibit resistance and recurrence. Since CSCs comprise only a small proportion of the tumour tissue and are thus hard to obtain in quantities sufficient for anti-CSC compounds testing, models capable of producing large enough amounts of CSC are needed.

Here, we have developed a head and neck squamous cell carcinoma (HNSCC) spheroid tumour model with stem-like properties. We plated commercially available FaDu cells on ultra-low attachment plates with culture media containing epithelial and basic fibroblast growth factors. After 11 days the cells from spheroids were analyzed for expression of stem cell biomarkers. The analyses confirmed enrichment of our spheroid model for CSC-like cells. Cells from spheroids had increased expression of CD44 and CD90 proteins compared to the adherent FaDu cells. Additionally, cells from spheroids showed increased mRNA expression of selected stem cell genes, CD73, CD133 and Nanog. Curcumin treatment of spheroids resulted in decreased “stemness” of the cells as shown by decreased expression of CD44 and CD90 proteins and mRNA expression of CD73, CD133 and Nanog. Thus, could be used as a positive control in screening for novel bioactive compounds with anti-CSC activity. Due to high reproducibility and low inter-experimental variability, the designed model is suitable for high throughput applications, such as screening for novel bioactive compounds with anti-CSC activity.

PI 9

Interacting partners of the human retrotransposon L1 ORF1p

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Long interspersed nuclear element 1 (L1) is the dominant and the only active autonomously replicating retrotransposon in human. Approximately 500,000 copies of full-length and truncated copies of L1 comprise as much as 17 % of the human genome. Due to its 'copy and paste' mechanism of replication, L1 has played an important role in genome evolution. L1 insertions have been associated with a number of Mendelian disorders, as well as some complex diseases, including cancer. Bicistronic L1 mRNA is translated into two proteins, ORF1p and ORF2p. The latter is expressed at significantly lower levels and possesses endonuclease and reverse transcriptase activity, while the precise function of the RNA-binding protein ORF1p in the process of the L1 retrotransposition has not yet been uncovered. ORF1p, ORF2p and L1 mRNA form ribonucleoproteins (RNPs), which further interact with additional host proteins. The aim of our study was to determine interacting partners of ORF1p to explain certain pathways of L1 retrotransposition. We characterised localisation of endogenous ORF1p in cell lines 2102Ep and nTERA2 as well as the localisation of GFP-, Flag- and myc-tagged ORF1p in cell lines HEK293T and HeLa by immunocytochemistry. ORF1p was predominantly localised within cytoplasmic clusters, rarely in nucleus, while in some cells, we also detected a diffuse cytoplasmic localisation. In sodium arsenite stressed 2102Ep cells, endogenous ORF1p partially reorganized into larger aggregates. In order to identify proteins in vicinity of the endogenous ORF1p in (un)stressed 2102Ep, we used a novel proximity-based labelling method called biotinylation by antibody recognition. The identified ORF1p interaction partners will enable further characterisation of ORF1p clusters and, possibly, of the role of the protein in L1 lifecycle.

The effect of listeriolysin O binding to cholesterol-rich membranes studied by solid-state NMR

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Listeriolysin O (LLO) is a cholesterol-dependent cytolysin (CDC), secreted from pathogen bacterium *Listeria monocytogenes*, that causes human and animal disease called listeriosis. The protein forms large pores on target membranes and in that way enables the bacteria to escape from host's phagosome. The process results in bacterial survival, its cytosolic replication, and subsequent cell-to-cell spread. Although it is known that LLO binds and forms pores on lipid membranes that are rich in cholesterol, its interactions with cholesterol and the effect on lipid membranes at molecular levels have not been fully explained yet. To look the process in more details we have prepared recombinant LLO and incubated it with different liquid-disordered or liquid-ordered cholesterol-rich lipid membrane systems. With ^{2}H, ^{31}P and ^{13}C solid-state NMR experiments we have observed changes in different lipid membranes upon the protein binding. We showed that LLO binding significantly affects the dynamics of phospholipid acyl chains in more liquid-disordered cholesterol-rich bilayers, whereas it does not affect the lipid bilayer organization. We have also provided the proof of direct interaction of LLO with membrane cholesterol by observing changed holesterol dynamics in the presence of the protein[1].

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PI 11

Lytic/ lysogenic switch of the *Bacillus thuringiensis* temperate bacteriophage GIL01.

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The GIL01 bacteriophage, is a tectiviral temperate phage, which infects the insect pathogen *Bacillus thuringiensis*. Unlike most temperate phages, GIL01 lysogeny is not established by a dedicated phage repressor but rather by the host's regulator of the SOS response, LexA. However, LexA is unable to maintain lysogeny unless the small phage-encoded protein gp7 is also present. Gp7 directly interacts with LexA to enhance its DNA binding to phage promoter. We obtained the crystal structure of the 50-amino acid gp7 protein and according to SAXS data we generated a structural model of gp7 in complex with LexA which illustrates that gp7 positions LexA in a DNA bound conformation. To determine how GIL01 establishes the lytic cycle, we examined the regulatory mechanisms at the lytic promoter. We show that lytic promoter is also repressed by LexA/gp7 complex and that the second phage-borne small protein, gp6, is the key activator of the lytic cycle. Surprisingly, gp6 is homologous to LexA itself and, thus, is a rare example of a LexA homologue directly activating transcription. We propose that the interplay between these two LexA family members, with opposing functions, ensures the timely expression of GIL01 phage late genes.

Phosphoproteomic analysis of legumain deficient mice.

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Legumain is an asparaginyl specific protease which belongs to the cysteine proteinase family. It is primarily located in the endolysosomal system, however, under specific physiological conditions it can also be translocated to the cytoplasm, nucleus or extracellular space. The primary structure of legumain is highly conserved among various species, which indicates its evolutionary and physiological importance. Interestingly, legumain null mice are viable, fertile and they exhibit no behavioural abnormalities. Their phenotype showed reduced body weight, abnormal kidney function and hyperinflammation. Very little is known about the molecular mechanisms that cause observed phenotypic changes, since no system wide studies on legumain deficient mice were reported yet. It has been shown that legumain deficiency significantly increased the levels of EGF receptor, which could have a profound impact on general cellular signalling. However, legumain activity could also affect other receptors and kinases. In our study, we performed a phosphoproteomic analysis of legumain knockout mice tissue samples in order to determine phosphorylation signaling pathways which are affected due to the legumain deficiency. This information will provide a better understanding of legumain physiology and its putative pathological roles.

PI 13

NLP proteins as pore-forming toxins

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Necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs) are produced by a wide variety of phytopathogenic microorganisms. They trigger leaf necrosis and immunity-associated responses in dicot plants. Based on structural similarity of NLPs to well-known pore-forming toxins actinoporins from sea anemones, and ability to induce leakage from plasma membrane vesicles, membrane disruption was proposed to be the underlying mechanism of their observed cytotoxicity. Our group recently reported that glycosylinositol phosphorylceramides (GIPC), the most abundant class of plant sphingolipids, are receptor molecules for NLP binding to plasma membranes. Furthermore, structural studies unveiled conformational changes upon binding of NLPs to GIPC sugars and in combination with biochemical and biophysical studies proposed a model of early steps of NLP membrane interaction. However, the exact mechanism of membrane disruption by the toxin action remains to be elucidated.

We are exploiting various model lipid systems to observe NLP action on membranes, composed of commercially available lipids and plant-isolated GIPC. Phytosterols are added to the lipid mixture to further mimic the composition of plant plasma membrane. Planar lipid bilayers are considered to be the most suitable approach to directly monitor permeabilizing activity of proteins through ionic current measurements, and giant unilamellar vesicles, conveniently followed with confocal microscopy, provide visual information after NLP – membrane interaction about changes in morphology and integrity of the vesicles and differential leakage of different-sized probes into the vesicles. Our study will contribute to understanding of the nature and mechanism of interaction of NLP proteins with lipid membranes, be it either via pore formation or some other membrane destabilizing process.

Mechanisms of action and interplay of three key virulence factors of Listeria monocytogenes

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Intracellular bacterium *Listeria monocytogenes* is a causal agent of listeriosis, a rare but potentially deadly disease. One of the crucial pathophysiological adaptations of the pathogen is the ability to escape primary and secondary vacuoles of the host cell, thus releasing the bacterium in its replicative niche. This process is facilitated by three key virulence factors: a cholesterol dependent pore-forming protein and cytolysin listeriolysin O (LLO), a broad-range phospholipase C (PC-PLC) and a phosphatidylinositol-specific phospholipase C (PI-PLC). Our aim is to study a molecular mechanism of action of these three proteins, and in particular their potential synergy in transmembrane pore formation by LLO, to better understand their role in in host cell membrane disruption. All three proteins are prepared recombinantly in *E. coli* and purified at a high level. An increased haemolytic rate on bovine erythrocytes by LLO with simultaneous addition of both phospholipases was observed. Pre-incubation of POPC/cholesterol lipid vesicles with PC-PLC seem to significantly increase LLO binding and the presence of PC-PLC seems to increase permeabilization of lipid vesicles by LLO, while PC-PLC does not cause any leakage on its own. PC-PLC thus enables pore formation by LLO in membranes with cholesterol content lower than usually required for LLO pore formation, thereby suggesting that PC-PLC may increase the availability of membrane cholesterol. We are currently aiming towards discovering the mechanisms and interplay between the three virulence factors by imaging protein-lipid interactions using various biochemical and biophysical techniques as well as approaches of structural biology.

PI 15

Role of the unique insertion sequence in subtilisin-like proteinase from hyperthermophile *Aeropyrum pernix* K1

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Pernisine is extracellular subtilisin-like proteinase from hyperthermophilic archaeon *Aeropyrum pernix*. Extraordinary stability at high temperatures and presence of denaturants makes pernisine suitable for industrial applications, because of its ability to degrade infective prion protein aggregates. Pernisine is homologous to the intensively studied Tk-subtilisin from *Thermococcus kodakarensis*, which contains three insertion sequences and six additional Ca^{2+} binding sites compared to mesophilic subtilisins. These insertion sequences, together with additional Ca^{2+} binding sites, contribute to folding and high thermostability of Tk-subtilisin. All Tk-subtilisin insertions and calcium binding sites are conserved in pernisine. However, pernisine contains an additional insertion sequence, absent in other subtilisin-like proteases characterized to date. Here we investigated the role of this unique insertion sequence in pernisine. This insertion consists of eight amino-acid residues and is located at the N-terminus of pernisine catalytic domain. The three residues of this insertion (D134, V135 and N136) form conserved calcium-binding motif DX[D/N]XDG, which constitutes an additional surface loop, as predicted in the 3D model of pernisine. Using spectrofluorimetry, circular dichroism and polyacrylamide gel electrophoresis, we have shown that compared to the wild-type pernisine, it's variant with deleted insertion sequence requires higher concentration of calcium ions to initiate conformational transition from unfolded to folded state of pernisine. Furthermore, pernisine lacking this insertion shows lower activity and thermal stability than its wild-type variant. Together, our results indicate that this novel insertion is an additional Ca^{2+} binding site, contributing to the adaptation of pernisine to the extreme environment of its host organism *A. pernix*.

A platform for protein production and crystal structure determination

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A platform for protein production and crystal structure determination involves several steps: molecular cloning, small-scale expression screen, large-scale protein production, protein purification, protein crystallization and finally protein structure determination.

We established a system of parallel cloning into a range of *E. coli* expression vectors containing different tags (His_6, GST, MBP, SUMO) and different *E. coli* host strains. Small-scale expression screen is performed in 96 deep-well format with auto-induction medium. This approach enables us to screen multiple constructs simultaneously and select the most promising ones for large-scale expression and protein production. When needed alternatives to prokaryotic expression system are used. We have the capacity and know-how for yeast (*Pichia pastoris*), insect cell and mammalian cell expression systems. For expression of multi-protein complexes we use MultiBac/MultiMam technology (Geneva Biotech).

For standard protein purification usually the multistep chromatography system ÄKTAxpress is used. As first two steps Ni affinity chromatography followed by size-exclusion chromatography are performed. When necessary, additional purification steps such as ion-exchange and affinity chromatography are introduced subsequently. Protein tags are removed before crystallization. We use mass spectrometry to confirm identity and molecular weight of expressed proteins. For protein characterization differential scanning fluorimetry (DSF) and solubility test are implemented.

In order to establish initial crystallization conditions commercial screens are applied. To ensure rapid set up of crystallization plates and their observation automated pipetting robot and imaging system, which records plates by predefined schedule, are used. Optimized crystals are tested on our in-house rotating anode (X-ray). Final high-quality diffraction data for crystal structure determination are collected at synchrotron source.

PI 17

The interaction of RahU protein with invertebrate lipids and its biological role

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Aegerolysin RahU of the opportunistic pathogen *Pseudomonas aeruginosa* is a bacterial protein belonging to the aegerolysin protein family (Pfam 06355), mainly composed of proteins originating from fungi and bacteria. The common property of fungal and bacterial aegerolysins is their ability to interact with membrane lipids. Biological function of aegerolysin RahU remains to be determined. It has been shown that RahU interacts with oxidized low-density lipoprotein, lysophosphatidylcholine and bacterial biosurfactants rhamnolipids. To elucidate the biological importance of its interaction with selected lipids, we successfully purified single alanine substitution RahU mutants in the *Escherichia coli* heterologous expression system. With cosedimentation assay and surface plasmon resonance spectrometry, we showed that RahU specifically binds ceramide phosphoethanolamine, the main sphingolipid in the membranes of invertebrate hosts, and identified crucial residue for this interaction. Therefore, this is the first report of a bacterial aegerolysin interaction with the aforementioned lipid. We have also shown the binding of RahU to the polar head of ceramid phosphoethanolamine, *O*-phosphorylethanolamine. The possibility of identifying the sensor for host lipids and manipulation of this process, offers us an opportunity to modulate the infection, caused by *Pseudomonas aeruginosa.* Therefore our results add to the growing knowledge on virulence potential of aegerolysin RahU and starting point for developing new antimicrobial agents.

Proteomic and biological characterisation of the venom from *Vipera ursinii ssp*., a karst viper from Croatia

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Croatian snake *Vipera ursinii* (*VuCro*) belongs to the karst viper group of the Orsini's vipers (*Vipera ursinii,* BONAPARTE 1835), which inhabit high mountain grasslands. *VuCro* bites are rare and medically less significant than are those of other Vipera species, causing only mild and negligible local symptoms that spontaneously resolve for a couple of days without need for medical treatment or antivenom therapy. However, *VuCro* is a highly threatened viper species that appears only in five isolated localities in Croatia and is under the European Council species' conservation program. In line with that, we report here for the first time the composition and biological activity of the *VuCro* venom, and compare it with the venom of *V. ammodytes ammodytes* (*Vaa*), the most venomous European snake of the greatest medical importance. Crude *VuCro* venom was separated by one- and two-dimensional gel electrophoresis, proteins were in-gel trypsinised and resulting peptides analysed by LC-ESI-MS/MS. We identified 24 proteins that belong to seven protein families, typical for viperid venoms: metalloproteinase (MP), serine protease, secreted phospholipase A_2 ($sPLA_2$), cysteine-rich secretory protein, snake C-type lectin-like protein (snaclec), serine protease inhibitor and venom nerve growth factor. When compared to much more complex *Vaa* venom [1], it lacks disintegrins and *L*-amino acid oxidases, and has a substantially lower amount of snaclecs. MPs are the most abundant components of *VuCro* venom and haemorrhagic potential of both *VuCro* and *Vaa* venoms, determined in rats, was of comparable magnitude. Nevertheless, *VuCro* venom was less lethally toxic in mice than *Vaa* venom, with 3.4 times higher LD_{50}. Interestingly, the pattern of mice dying indicated the presence of a strong neurotoxic component in the former, although proteomics and immunological studies revealed the lack of basic $sPLA_2$s that are the main neurotoxins of *Vaa* venom.

1. Leonardi et al., 2019, *J. Proteome Res.* 18, 2287−2309

PI 19

Can structure of annexin A11 explain its ALS-associated mutations?

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Annexin A11 (ANXA11) is a member of annexin family of calcium dependant phospholipid-binding proteins. Annexins have structurally conserved core and unique N-terminal domains with ANXA11 having the longest N-terminal domain of the family. Mutations in the annexin A11 gene (*ANXA11)* have been associated with amyotrophic lateral sclerosis (ALS), an incurable neurodegenerative disease of motor neurons. Therefore, we wanted to determine ANXA11 crystal structure to explain the functions of known ALS mutations. Structural predictions showed a high degree of intrinsic disorder in the proline and tyrosine rich N-terminal region of the protein indicating potential unsuccessful crystallisation. Therefore, we have expressed and isolated 3 ANXA11 constructs: wild type and 2 truncated forms at N-terminal site to get soluble and stable forms of ANXA11. Protein crystals were grown in Wizard solutions (Molecular Dimensions) and optimised for pH, salt and protein concentration. Diffraction data was collected at Elettra synchrotron in Trieste, Italy. Only truncated ANXA11, lacking most of the N-terminal part, successfully crystallised. Crystals diffracted to 2,2 Å and we solved the crystal structure using molecular replacement. Structure of ΔN-ANXA11 has conserved core domain of 4 homologous annexin repeats with slight differences in the middle part. Prediction of disordered N-terminal domain explains unsuccessful crystal growth of the wild type and of the partially truncated N-terminal ANXA11. N-terminal part is too dynamic to enable crystal formation. Further analysis, based on the crystal structure of ANXA11 is needed to explain the changes of mutated forms in protein interactions and their functions

Advanced isotopic labelling of MurD for protein-based NMR studies of ligand-MurD binding

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Muramyl ligase D (MurD) is a 47.7 kDa enzyme, belonging to the family of ATP-driven bacterial enzymes Mur ligases, which are involved in the biosynthesis of the bacterial peptidoglycan. Therefore, Mur ligases are an attractive target for the development of novel antibacterial agents.

An important aspect in the design of effective Mur ligases inhibitors appears to be the dynamic nature of ligand-MurD complexes, as revealed by our studies. We investigated the binding mode of MurD inhibitors with various molecular scaffolds in aqueous environment, using both ligand-based and protein-based NMR methods in combination with extensive molecular dynamic (MD) simulations.

In order to carry out the protein-based NMR studies of ligand-MurD binding we have prepared various types of isotopic labelled *Escherichia coli* MurD, including selectively $^{13}CH_3$ labelled MurD and perdeuterated ^{15}N labelled MurD.

With this aim, MurD was heterologously expressed in *E. coli* strain BL21 (DE3). We optimised the expression and isolation protocol to increase the yield and purity of the labelled protein. The crucial step towards a higher yield of perdeuterated MurD was the optimisation of the unfolding/refolding process at the end of the purification process, in order to exchange ^{2}H at the amide group into ^{1}H. In these conditions aggregation and misfolding of a protein should be minimised.

Our NMR studies of selectively $^{13}CH_3$ labelled MurD have provided a solid basis for the effective characterization of a site specific MurD ligand binding, as demonstrated here for the various types of MurD inhibitors. The addition of perdeuteration was crucial for the amide ^{1}H and ^{15}N backbone assignment of opened and closed MurD state, which is essential for ^{15}N relaxation studies of MurD domain motions in relation to ligand binding.

This work was supported by the Slovenian Research Agency (Grant numbers J1-8145 and P1-0010) and EN-FIST Centre of Excellence.

PI 21

ATP binding to the Nerve Growth Factor: a molecular switch for neurotrophins signaling?

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Nerve Growth Factor (NGF) is the prototype of the neurotrophins family and induces cell growth and differentiation in neuronal cell types. Despite its discovery in the '50s, many molecular and functional properties remain elusive.

Small endogenous NGF ligands with biological modulating effects are of increasing interest. Among these, ATP showed to mediate NGF neurotrophic activity through its receptors, although the molecular determinants of the binding mechanism are yet to be unraveled. We thus undertook a biophysical study on NGF/ATP binding.

We obtained ^{15}N- and $^{13}C^{15}N$-labeled recombinant human NGF (rhNGF), through the optimization of protocols previously established for mouse NGF (rmNGF). Interestingly, rhNGF and rmNGF differ in both their biochemical/biophysical and functional properties, despite their highly similar 3D structures. We performed 2D and 3D NMR experiments in order to complete the backbone and side chains resonance assignment of rhNGF.

Differential Scanning Fluorimetry has been exploited to investigate the binding effects of ATP and of a set of divalent ions on rhNGF. We showed by FT-IR that NGF retains its secondary structure also in the presence of ATP. We then moved to investigate the rhNGF/ATP binding mode, using protein-based solution NMR. We recorded 2D HSQC spectra following a titration with increasing amounts of ATP. We identified by NMR guided molecular docking the likely ATP binding site on rhNGF. Finally, we investigated by Isothermal Titration Calorimetry the thermodynamic signature of ATP on rhNGF. In order to suggest an effect of ATP binding on the NGF/receptors signaling pathways, we undertook a study on the NGF/receptors binding by means of SPR. The effect of different ions, Mg^{2+} and Zn^{2+}, was also investigated. The obtained results will be discussed in light of the role played by NGF and proNGF in neurotrophic mechanisms in health and disease.

Acknowledgements: ARRS Grant numbers J1-8145 and P1-0010. EN-FIST Centre of Excellence.

Bacterial surface-layer-protein assemblies at atomic scale

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S-layers are (glyco)protein coats displayed on the external surface of many bacterial and archaeal species. As the outermost layer, the S-layer is in direct contact with the environment and thus involved in e.g. adhesion to epithelial cells and intestinal components. S-layers however remain poorly understood, primarily due to lack of structural knowledge.

Here we report the structural characterization of the bacterial surface layer proteins SbsC from G*eobacillus stearothermophilus* and SlpA from *Lactobacillus acidophilus.* Several soluble fragments as well as full-length proteins self-assembling into 2D-crystals were produced. To elucidate the structure of the complete self-assembled S-layer, an integrative structural biology approach combining X-ray crystallography, mass spectrometry and electron microscopy has been applied. We determined the atomic structures of soluble fragments by X-ray crystallography. These individual domains were then fitted into the 3D-volume obtained by cryo-electron tomography and sub-tomogram averaging of 2D-crystals. To additionally characterize the binding of S-layer proteins to the bacterial cell wall, we performed isothermal titration calorimetry (ITC) experiments. The obtained results allow us to learn more about the cell wall attachment and self-assembly formation of s-layers on atomic scale.

Acknowledgments: This work has been supported by the Austrian Science Fund (FWF, project P29432).

PI 23

Evaluation of computational approaches for use of chemical crosslinking-based spatial restrains in modeling of homo-oligomeric complexes

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Many proteins self-associate to form oligomeric protein complexes known as homo-oligomers, which are often critically associated with their biological function. As part of their structural characterization, the path from subunit structure to the model of the oligomeric form is usually far from trivial. Here, researchers often rely on computational approaches, guided by low-resolution structural restrains, to provide a reliable structural model. As a source of restraints chemical crosslinking coupled with mass spectrometry (XL-MS) is commonly employed. However, the successful application of XL-MS to guide modeling of homo-oligomers is often hindered by the method's inherent inability to distinguish *intra*- and *inter*-subunit connections. To evaluate how different scoring approaches and different methods for inter-residue distance calculations influence the quality of modeling outcome we preformed a comprehensive computational analysis of available 3D structures of homo-oligomers. Here, we generated a list of all potential surface-exposed lysine-lysine crosslinks and evaluated them using different scoring functions. Particularly, we were interested in the difference between Solvent Accessible Surface distances (SASDs), which respect the impenetrability of the molecular surface, and Euclidean distances (EUCs), which are calculated as the direct distance between two residues. Our results show that using SASDs instead of more commonly used EUCs reduces the assignation ambiguity and thus delivers better modeling precision. Modeling performance can be further improved by considering ambiguous connections as *inter*-subunit only when the intra-subunit option exceeds the distance threshold, and by explicitly defining symmetry in the scoring function. Our findings provide guidelines for proper evaluation of chemical crosslinking-based spatial restrains in modeling of homo-oligomeric protein complexes.

Determination of kinetic parameters of paraoxonase 1 based on time-course data

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A century after Leonor Michaelis and Maud Menten founded enzyme kinetics, turnover number k_{cat} and Michaelis constant K_m remain the basic parameters which are considered for the quantification and comparison of various enzyme-catalyzed reactions. Unfortunately, these parameters are still widely determined in terms of initial-rate measurements at different substrate concentrations, and by plotting the results in a linearized form, usually as a Lineweaver-Burk diagram, from which the Michaelis constant K_m and limiting rate V_{max} are estimated.

We present here an alternative approach for determination of these parameters from progress curves by direct fitting of a reaction model on time-course data with the program Dynafit, which is free for academic use. The advantage of fitting progress curves over using initial rates is that the progress kinetics analysis is quicker, and is arguably more accurate, because the experimental setup requires fewer assays and analysis steps.

To demonstrate these advantages, we used paraoxonase 1 (PON1), a human plasma enzyme with lactonase activity. We measured curves of product concentration against time for the reaction of recombinant PON1 with the substrate dihydrocoumarin (DHC) at different concentrations and then evaluated kinetic parameters from fitting progress curves and from initial rates. We obtained similar results by both methods, but the confidence intervals we received for K_m via progress curves' analysis were almost an order of magnitude smaller than via initial-rate analysis. Since PON1 is a human enzyme involved in aging and several diseases, we propose that the abovementioned method should be considered for the determination of its kinetic parameters in future diagnostic tests.

PI 25

Substrate recognition and cleavage of *Staphylococcus aureus* GH73 family hydrolazes is accompanied by a large conformational change and domain movement

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The bacterial cell wall enables the cells to withstand osmotic pressure and thus it is essential for their survival in a large set of different habitats. Yet for cells to grow and divide, it has to be remodeled in a controlled manner. Cell wall is composed of glycan strands comprising alternating N-acetylglucosamine and N-acetylmuramic acid and cross-linked by peptides. Peptidoglycan hydrolases are enzymes involved in the cell wall remodeling. They are important for peptidoglycan maturation, turnover and recycling, as well as antibiotic resistance.

Glycoside hydrolases family 73 are N-acetylglucosaminidases. Their structures share a lysozyme-like fold with highly conserved core that consists of five or six helices with the catalytic site glutamate on top of the central helix. Crystal structures of two N-acetylglucosaminidases, SagB and AtlA-gl, revealed very similar fold to *S. aureus* N-acetylglucosaminidase AtlE, however with unexpected difference in their L- and R-domain packing and subsequently wide open active site cleft. Thereby they provide an unexpected insight into the substrate binding mechanism. By structural analysis and mutational studies, we demonstrated that the substrate must interact with active site residues on the left and right side of the active site cleft. This suggests, that in order to cleave the carbohydrate chain in peptidoglycan, these two enzymes bind to the relatively rigid substrate first and cleave it in a later step in which the two domains are brought together. Hence, a substantial domain rearrangement associated with conformational change has to occur for the reaction to transpire. This indicates that enzymes can adapt the shape beyond the range of lowering the energy barrier of the reaction intermediate and beyond the range of structural changes of the induced fit mechanism.

Fungal lectin KKP affects bacterial adhesion

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Antimicrobial resistance against commonly used antibiotics is a major threat to public health. An alternative approach to reduce antimicrobial resistance is focused on the search for new antimicrobial strategies that prevent bacterial adhesion. Adhesion of bacteria to an abiotic surface represents the first and key step in environmental persistence in form of biofilms. Since bacterial surface is covered in oligosaccharides and polysaccharides these glycans greatly influence the interactions of bacteria with any surface. Lectins are proteins that specifically and reversibly bind carbohydrates. They are ubiquitously present in all groups of organisms and represent key players of molecular recognition. Analogously to endogenous bacterial lectins or adhesins that contribute to bacterial adhesion, heterologous lectins can affect bacterial adherence by interacting with bacterial surface glycans and represent a possible way of preventing bacterial adhesion. Fungal lectins with their unique characteristics and high stability represent ideal candidates. In our study, we prepared a recombinant lectin KKP from the model mushroom *Coprinopsis cinerea.* We evaluated its effect on growth and adhesion of several pathogenic and commensal microorganisms. The overall effect of lectin KKP on growth and adhesion of microorganisms was very similar for the strains of the same species but varied significantly between different species, pointing towards very specific target recognition and binding. We confirmed that lectin KKP specifically binds to the cell wall proteins and extracellular proteins of *Lactobacillus* and *Campylobacter* strains. In summary, lectin KKP shows potential to be used as an alternative antimicrobial substance for prevention of *Campylobacter* adhesion and as a supplement to antibiotic therapy that preserves the growth and function of commensal bacteria.

PI 27

Visualization of oligomeric Cyt2Aa toxin from Bacillus thuringiensis on model membrane systems

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Bacillus thuringiensis (*Bt*) is a gram-positive bacterium, which produces insecticidal proteins during the sporulation phase. These insecticidal proteins are located in parasporal crystals consisting of two delta-endotoxin families, Crystal (Cry) and Cytolytic (Cyt) toxins. *In vitro*, Cyt toxins display cytolytic activity toward bacterial cells, a variety of insect as well as mammalian cells. They bind to cell membranes with unsaturated phospholipids such as phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin. Although *Bt* is being used as a bioinsecticide, the mechanism of the Cyt family is still not understood. Currently, there are two proposed models of Cyt membrane integrity disrupting: the pore-forming model, where multiple monomers come together and form an oligomeric pore in the lipid bilayer, and the detergent-like model, where monomers aggregate on the membrane surface until they reach a critical concentration; the membrane is then disrupted by a detergent-like activity. In an attempt to shed light on the mechanism of this potentially commercially important protein, we focused our research on oligomerization of Cyt2Aa on model membrane systems such as multilamellar lipid vesicles and planar lipid membranes and visualized the oligomers. When incubated with lipid vesicles the protein forms oligomers on the lipid bilayer. We visualized the oligomers with Native-PAGE and transmission electron microscopy (TEM). TEM revealed Cyt2Aa oligomers bound to the vesicles but we did not find any classical ring-shaped pores. At longer incubation times Cyt2Aa oligomers were also present in the area surrounding the vesicles. These oligomers retained the general shape and were of similar size. Interestingly Cyt2Aa forms similar oligomers in the presence of detergents alone even in the absence of lipid bilayers. Further detailed biophysical and structural studies will be needed to clarify the mechanism of Cyt2Aa's oligomerization and membrane disruption.

Insight into thyroid hormone formation

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Iodine is essential element in the synthesis of thyroid hormones. It is incorporated in T3/T4 hormones responsible for regulation of basal metabolism of almost any cell. T3 and T4 hormones are produced in the thyroid gland. Improper function of the T3/T4 hormone results in impaired metabolism that severely decrease the quality of life. More than 10% of population is affected by thyroid disorders. Nowadays, the common cause of thyroid hormone related disorders is impaired iodine metabolism that can result in hypothyroidism (reduced hormone production) or in hyperthyroidism (increased hormone production).

Despite the proteins involved in hormone synthesis have been identified, the exact molecular mechanism remains unclear. Precursor of hormones thyroglobulin is stored inside the lumen of thyroid follicular cells. There its tyrosine residues are iodinated by thyroid peroxidase and cross-linked. With the help of recombinantly produced thyroid peroxidase we established *in vitro* system for production of thyroid hormones from thyroglobulin. Our still ongoing analysis revealed iodination pattern and sites responsible for hormone formation.

PI 29

How is salicylic acid perceived in potato?

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Potato (*Solanum tuberosum*) is one of the most important crops of our time, produced, processed and consumed by countries all around the globe. At the same time it is exposed to a number of plant pathogens, causing high loss in yield and lower product quality. Plants respond to pathogen infections with a complex network of signalling cascades, which are orchestrated by hormones and ultimately affect the expression of defence genes. One of the main hormonal pathways in plant immunity is the salicylic acid pathway, which is also a key component of potato defence response to potato virus Y (PVY), the most dangerous virus infecting potato. The salicylic acid pathway has been extensively studied in *Arabidopsis thaliana*, where it regulates the expression of *PR-1* defence gene through NPR transcription cofactors and TGA transcription factors. Although this pathway has also been investigated in some economically important crops, the transcription regulation during potato PVY infection is mostly unknown and data on similar mechanisms in potato are scarce. We have selected three potato TGA transcription factors involved in potato defence response, investigated the chromosomal arrangement of their genes, classified them phylogenetically and analysed their protein sequences and domains *in silico*. We determined the localization of all three TGAs in plant cells and studied the protein-protein interactions among them, their interactions with potato NPR cofactors and proteins from other signalling pathways using the yeast two-hybrid assay. As we found several positive interactions between investigated proteins in yeast, we continued with their confirmation *in planta* with co-immunoprecipitation experiments, currently in progress. The results of our study provide new insights into salicylic acid mediated transcription regulation of defence genes and will help us to better understand the potato immune response on a molecular basis.

The role of thiopurine S-methyltransferase in biotransformation of selenium compounds

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Thiopurine S-methylatransferase (TPMT) is one of the most important deactivating enzymes of cytostatic and immunosuppressive thiopurine drugs. Its activity-affecting genetic polymorphisms have been successfully implemented in clinical practice as pharmacogenomic markers predicting the patient's response to thiopurines. Despite its remarkable participation in thiopurine metabolism, the endogenous role of this enzyme remains unknown.

To elucidate the biological background of TPMT, we studied its involvement in selenium metabolism. We were able to demonstrate *in vitro* binding of selenocysteine (Sec) to human recombinant TPMT (rTPMT) by using STD NMR spectroscopy and tryptophan fluorescence measurements We further found that after the incubation of Sec with rTPMT and the methyl donor, S-adenosylmethionine, a methylated entity, Se-methlyselenocysteine (MeSec) is enzymatically formed. To transfer this finding to biological level, we extended the experiments to lymphoblastoid cell lines (LCLs) derived from different consenting individuals. We genotyped cells for the most common genetic polymorphisms in the TPMT gene (TPMT*2, TPMT*3A, TPMT*3B and TPMT*3C) and measured their TPMT activity. Expectedly, we observed significant genotype-activity correlation, designating that LCLs carrying at least one variant allele have significantly decreased TPMT activity. When further evaluating the sensitivity of the cells to different selenium compounds, we found that the sensitivity of LCLs towards Sec negatively correlated with TPMT activity. Similarly, the cells with wild-type TPMT were less sensitive to sodium selenite compared to LCLs with heterozygous or double-variant genotype.

Our study revealed Sec as the first known biological compound acting as a substrate for TPMT. Whether TPMT is protecting cells against reactive selenium compounds or plays a role in selenium-mediated (anti-)oxidative reactions, remains to be answered in forthcoming studies.

PI 31

Characterization of the export system of C. difficile surface proteins

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In bacteria, the majority of exported proteins are involved in the maintenance of cell wall structure, nutrient acquisition and communication with the environment. Most of them are translocated to the bacterial surface using SecA systems. SecA proteins are ATPases that undergo a series of conformational changes during cycles of ATP binding and hydrolysis. These are required for sequential translocation of target proteins through the protein-conducting membrane channel. In bacterial pathogens, surface proteins are also in ideal position to interact with the host cell. This makes them, and the system that exports them, important virulence factors. *Clostridium difficile* (*C. difficile*) is a detrimental hospital pathogen causing hard-to-treat and often life-threatening intestinal infections worldwide. Its outermost surface is covered with the proteinaceous S-layer, composed of 29 proteins. The export system that specifically translocates these S-layer building blocks to the cell surface, is the SecA2 system. We solved the structure of *C. difficile* SecA2 using molecular replacement. It revealed a multidomain organization similar to its homologs. The ATPase part of the molecule is composed of two domains, NBD1 and 2, that form a nucleotide binding pocket. The preprotein-crosslinking domain recognises and interacts with the target protein destined for translocation. The helical scafold domain is the one that interacts with the transmembrane channel, and neighbours the helical wing domain. Finally, our analyses of evolutionary conservation revealed differences that suggest a unique adaptation of SecA2 proteins for a specific type of substrate. Our studies may provide the basis for development of *C. difficile*-specific therapies that interrupt the secretion pathway of its virulence factors.

Analysis of EpCAM's lateral oligomerization shows cis-dimerization is more robust than previously expected.

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Epithelial cell adhesion molecule (EpCAM) is a transmembrane glycoprotein expressed in most epithelial cells. As a signalling molecule and a regulator of cell-cell adhesion, it is involved in cell proliferation, differentiation, and migration. Because of elevated expression in carcinomas, EpCAM is also an important tool in cancer diagnostics and treatment.

EpCAM is subjected to regulated intramembrane proteolytic cleavage (RIP) - a process catalysed by sheddases, similar to the processing of the amyloid precursor protein. RIP is a key mechanism in EpCAM signalling that results in mitogen expression and cell cycle propagation. We have recently shown that EpCAM predominately exists in the *cis*-dimeric state that sterically prevents access to the α- and β-sheddases' cleavage sites, which are buried within the dimerization surface. For the cleavages to occur we inferred the *cis*-dimeric form of the extracellular domain must temporarily dissociate.

To investigate the relationship between the oligomeric state and the cleavage rate in greater detail we expressed mutants of soluble extracellular domains with hampered dimerization interface. Their inability to form *cis*-dimers was confirmed by SEC-RALS/LALS analysis. However, when these mutations were introduced into full-length EpCAM, mutants were still able to dimerize, as revealed by FLIM-FRET oligomerization analysis. Additional attempts to abolish dimerization by switching the transmembrane region were also unsuccessful. This shows EpCAM *cis*-dimers are much more robust than previously expected and opens new questions regarding the relationship between EpCAM oligomerization and its signalling function.

PI 33

Systems biology of oxytetracycline production in *Streptomyces rimosus*

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Oxytetracycline (OTC) is an aromatic polyketide with broad-spectrum antimicrobial activity. It is produced by *Streptomyces rimosus* during submerged fermentation on industrial scale (Petkovic et al., 2006; Pickens and Tang, 2010). Small-scale cultivation in a chemically defined medium enables us to specifically study molecular details of growth and OTC production. With this objective, two different strains: *S. rimosus* ATCC 10970 (R7), a wild-type soil isolate that naturally produces OTC and *S. rimosus* HP0508, an advanced OTC producer, were cultivated in a synthetic medium. Both strains efficiently utilized mannitol as a substrate for growth and OTC formation. In addition to this, cultivation under phosphate limitation supported production. Amongst the strains, *S. rimosus* HP0508 exhibited higher OTC production potential, yielding up to twenty times as much as the *S. rimosus* R7 strain. First metabolomics studies on cellular level, revealed similar pools of malonyl-CoA in both strains, which indicates sufficient availability of this important OTC building block. Further in depth multi-omics profiling is necessary to fully unravel differences in growth patterns and OTC production capacities. These analyses are foundation for future development of superior *S. rimosus* producer strains.

Multiplex Quantification of Six in EU Authorised Genetically Modified Soybean Lines Using Crystal Digital PCR

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The story of genetically modified organisms (GMOs) is a complex one, both from the research and legislative point of view. From their humble origins in 1992, when the first genetically modified crop was set to the market, GMOs have diversified and grown in number. The majority of countries around the globe have imposed legislation that helps regulate the use of GMOs, and a plethora of different detection and quantification methods have been developed to determine the presence and quantity of GMOs in the food and feed supply chains.

In the European Union (EU), legislation includes a labelling system for all food and feed products that contain, consist of, or are produced from authorised GMOs at a 0.9% per ingredient. DNA-based approaches are the most common for detection and quantification of GMOs, with quantitative real-time PCR (qPCR) being the gold standard. With the rise in number of authorised GMOs a strategy for faster quantification was needed. A screening qPCR approach where the most common GM elements are detected was proposed. This shortened the time of the analysis and reduced the costs. However, since the introduction of screening, GMOs have diversified, and many of the new lines do not contain any of the most common GM elements. Another way to enhance the time- and cost-efficiency is quantification of multiple targets in one reaction – multiplexing. Multiplex quantification by qPCR is practically impossible, and a new approach is needed yet again.

The aim of our study was to use new technologies to develop fast, sensitive and cost-efficient methods for multiplex quantification of GMOs. One of the most promising technologies for nucleic acid quantification is digital PCR. We have used Naica platform from Stilla Technologies to develop a crystal dPCR multiplex assay for fast and precise quantification of six in the EU authorised GM soybean lines that cannot be detected using the standard qPCR screening approach.

PI 35

Vitamin C: biochemistry and detection

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Vitamin C or L-ascorbic acid is a water-soluble essential nutrient for many animals and humans required for multiple biological functions. Ascorbic acid is a cofactor of several enzymes that participate in the hydroxylation of collagen molecules, including prolil-4-hydroxilase and lysyl-hydroxilase as well as for two alpha-ketoglutarate-requiring dioxygenase reactions in the pathway of carnitine biosynthesis. By preventing defects in the synthesis of collagen Vitamin C prevents the disease scurvy. Ascorbic acid also needed for normal metabolism of tyrosine and is one of the most important antioxidants and can reduce transition metals such as iron from ferric Fe^{3+} to ferrous Fe^{2+} ions. It also plays an important role in signalling in cells as well as gene expression. Vitamin C contributes to immune defence by supporting various cellular functions of both the innate and adaptive immune system. It accumulates in phagocytic cells, such as neutrophils, and can enhance chemotaxis, phagocytosis, generation of reactive oxygen species, and ultimately microbial killing. It is also needed for apoptosis and clearance of the spent neutrophils from sites of infection by macrophages. The role of vitamin C in lymphocytes is less clear, but it has been shown to enhance differentiation and proliferation of B- and T-cells. Infections significantly impact on vitamin C levels due to enhanced inflammation and metabolic requirements. Quantitative detection of ascorbic acid can be conducted various methods such as HPLC method, redox based titrations with iodine or 2,6-Dichlorophenolindophenol (DCPIP). In this latter method by stoichiometric titration DCPIP oxidises ascorbic acid, and the titration end point is detected by a noticeable colour change in the solution. Vitamin C is found in various fruits and vegetables and is also sold as a dietary supplement. The recommend level of daily intake for adults is 80 mg per day according to the European Commission Council on nutrition labelling.

Systems biology of rare omega fatty acid biosynthesis in *Yarrowia lipolytica*

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Omega-3 fatty acids are polyunsaturated fatty acids (PUFAs) of recognized value with benefits in improving cardiovascular health, immune function, mental health and infant cognitive development. Their major source, marine oily fish, is not sufficient to satisfy the increasing demand. Hence, sustainable production processes, which yield omega-3 fatty acids from renewable resources using metabolically engineered cells, emerge as promising alternatives. As highlighted by its species name, *Yarrowia lipolytica* is an oleaginous yeast, which is well-known for its capacity to metabolize and its high lipid content. Recombinant *Y. lipolytica* Po1h:pSynPfaPptAf4 overexpresses a myxobacterial biosynthetic cluster and successfully accumulates PUFAs during small-scale batch fermentation. Here, we unravelled molecular details of the production process using gene expression profiling. For analysis of the PUFA cluster expression profile, RNA samples were obtained from a glycerol-based production process sampled at different points over time. Quantitative real-time analysis indicated clear differences in specific *pfa* gene expression levels between growth and production phase as well as the influence of different nutrient limitations. The genes responsible for PUFA production were expressed already after the transition to the stationary phase, triggered by a limitation for phosphate. The products, however, were detectable only later, when also the initial glycerol consumption was completed. This suggests that the fatty acids of interest were synthesized from existing lipid pools through recycling processes rather than *de novo* from the initial carbon source. A more global analysis of the cellular program of the yeast promises better insights into the underlying metabolism and hopefully support rational strain improvement in the future.

PI 37

Structure-based engineering of flexuous filamentous virus-like particles for potential bionanotechnological applications

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Plant viruses represent an ideal starting point for fabricating structurally controllable nanoscale devices with novel functionalities. Viral particle can also be utilized in a non-infectious nucleic acid-free form, called a virus-like particle, which often retains similar symmetry, shape and size as the native virus. Several viruses and their respective VLPs are currently being developed for nanomedical applications, yet the vast majority of platforms under investigation are still of spherical nature.

In our group, we study Potato virus Y (PVY), which belongs to the *Potyvirus* genus of flexible filamentous viruses. Our recent near-atomic structures of the PVY virion and the corresponding VLP have put forth a solid foundation for further protein engineering of VLPs. By systematic deletion of the unstructured N- or C-terminal part of the coat protein (CP), both essential building blocks of VLP, we have produced recombinant protein nanoparticles of various shapes and sizes, such as hollow nanotubes and nanorings with distinct biophysical properties. High structural organization of the VLP as a template enables effective modularization of its solvent exposed filament surface in order to gain novel functionalities. By inserting reactive functional groups via CP mutagenesis, we have produced and characterized various modified VLPs with the possibility of biomolecules chemical conjugation on already assembled filament. Alternatively, we can obtain decorated VLPs via fusion protein construction with a chosen peptide from a heterologous source being presented on the surface of the filament, however the maximum capacity and requirements for successful filament assembly of such presentation platform has to be further examined. Tailoring of the PVY CP can thus result in diverse VLP properties and functionalities, with potential for applications in the field of nanotechnology and medicine as well as in materials science.

The effect of stabilized in basil extract silver nanoparticles on rats' liver fibrosis experimental model.

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Due to the increasing pace of nanotechnology development and the use of nanoparticles in various fields, it becomes inevitable human contact with this type of compounds. The question of studying their effects on the central organ of the detoxification system of the body, the liver, is becoming topical.

The aim of this work was to study the effect of stabilized in *O. araratum* extract silver NPs on an experimental model of Wistar albino rats liver fibrosis. Silver NPs were synthesized using 50% ethanol extract of *O.araratum*. Liver fibrosis of experimental animals was caused by carbon tetrachloride i/p injection. To screen the effects of silver NPs, biochemical parameters of experimental animals' plasma was carried out.

Scanning electron microscopy revealed that the synthesized nanoparticles had a spherical shape with a diameter of 11±5nm. It was found that the extract of *O.araratum* has lipid-lowering properties (total cholesterol -1.36±0.02 mmol/l, LDL -0.73±0.01 mmol/l, HDL-0.63±0.01 mmol/l), inhibits albumin synthesis (3.06±0.02 g/l), leads to an increase of total protein content (61.86±0.016 g/l), but at the same time increases the activity of AsT (7.66±0.012 U/l) compared to control animals 1.4 times. However, the combined effect with AgNPs normalizes these indicators (total cholesterol -2.06±0.001 mmol/l, LDL -1.48±0.024 mmol/l, HDL -0.58±0.013 mmol/l, AcT -2.94±0.003 U/l, albumin -13.06±0.031 g/l, total protein -22.68±0.02 g/l), which indicates the hepatoprotective properties of these nanoparticles. An additional study of AgNPs possible toxic properties revealed that they aren't toxic for both healthy mice and S-180 sarcoma mice.

It can be argued that silver NPs stabilized in *O.araratum* extract normalize biochemical parameters of Wistar albino rats' blood plasma in an experimental model of liver cirrhosis and they aren't toxic for mice in studied concentrations.

PI 39

Establishment of CRISPR/Cas9-mediated microRNA knock-out in potato

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To date, CRISPR/Cas9-mediated microRNA editing has not been established in potato yet. However, there is a growing evidence that small noncoding RNAs can be targeted by CRISPR/Cas9 system in plants. The novel gene-editing strategy is a challenge yet worth accepting, due to the compelling robustness, specificity, and stability for the modification of microRNA expression. In potato, CRISPR/Cas9 technology was mostly used in combination with Agrobacterium-mediated stable transformation. This could pose a problem, especially in case of time-consuming stable transformation with sgRNAs not previously confirmed as functional. On the other hand, protoplasts transfection is faster method, but protoplasts isolation and regeneration of transgenics remain bottlenecks. Therefore, we established a protocol, which consists of the design of CRISPR/Cas9 constructs, transient transfection of protoplast to select functional sgRNAs, followed by stable transformation of potato explants. This was achieved through the optimisation of protoplasts isolation from potato, protoplasts transfection and high-resolution melting analysis (HRM) to confirm functionality of tested constructs. The highest yield and viability of protoplast was obtained using young developed leaves of three to four weeks old potato plants. We obtained the highest transfection efficiency (up to 40 %) using high concentration of protoplasts (~10^6 protoplasts/ml) and 8 minutes-incubation of protoplasts with 30% PEG4000 following gradual dilution of protoplasts-PEG mixture. Protoplasts transfection with designed constructs resulted in generation of mutants. Already one week after transfection, the mutations were confirmed by HRM. In the last step, functional constructs were used for stable transformation. Thus, by targeting miRNAs in potato, we confirmed our hypothesis that protoplasts transfection followed by HRM is an optimal strategy to test functionality of designed knock-out constructs prior to stable transformation.

The unique binding properties of aegerolysins and their application as biopesticides

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Aegerolysins ostreolysin A (OlyA), pleurotolysin A2 (PlyA2) and erylysin A (EryA), proteins from the mushroom genus *Pleurotus*, preferentially bind to ceramide phosphoethanolamine (CPE), the main sphingolipid in invertebrate cell membranes. Upon binding to CPE-containing membranes, these aegerolysins can recruit proteins pleurotolysin B (PlyB) or erylysin (EryB) with the membrane-attack-complex/perforin domain to form multimeric bi-component transmembrane pores.

We provide new insights into the unique interaction of *Pleurotus* aegerolysins (OlyA, PlyA2 and EryA) with membranes containing CPE. Spectral FRET analysis showed that monomers of fluorescently labeled OlyA pack closely together only on CPE-containing membranes. These aegerolysins bind to insect cells and artificial lipid membranes at as low CPE concentrations as found in insect cell membranes. Moreover, aegerolysins form pores in CPE-containing artificial and biological membranes when combined with PlyB or EryB. OlyA/PlyB, PlyA2/PlyB and EryA/PlyB complexes have shown a selective toxic effect on Colorado potato beetle (CPB) larvae and Western corn rootworm (WCR), and not to other tested insect pests. Exposure of CPB larvae to leaf disks treated with protein mixtures caused significant larval mortalities on day 5 after initiation of the experiment, and exposure of WCR larvae to artificial food mixed with OlyA6/PlyB and PlyA2/PlyB resulted in significant mortalities on day 5 after the initiation of the experiment. The current study highlights the unique binding of aegerolysins on CPE-membranes and their possible use as biopesticides.

PI 41

Deciphering the binding and disaggregating potential of New Methylene Blue towards fibrillation of lysozyme

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Binding interaction of phenothiazinium derivative new methylene blue (NMB) with lysozyme (LYZ) was investigated by multifaceted biophysical approaches. Also, the effect of NMB in rupturing the fibrillar assemblies of LYZ was examined. NMB exhibited hypochromic changes in presence of LYZ. The fluorescence intensity of NMB regularly decreased and the maximum emission wavelength did not apparently shift with the increase of LYZ concentration. Synchronous fluorescence spectra revealed that binding with NMB caused polarity changes around the tryptophan (Trp) moiety of LYZ. The interaction caused significant loss in the helicity of LYZ. Isothermal calorimetric titration revealed the interaction between LYZ and NMB to be exothermic and entropy-driven. Differential scanning calorimetric studies showed no appreciable change in the melting temperature of LYZ-NMB complex compared to LYZ.

Blocking amyloid-like LYZ aggregates and disturbing the fibrillar self-assemblies stand among top therapeutic strategies in tackling amyloidosis. The morphology of the formed fibril of LYZ, as scanned by atomic force microscopy and confocal microscopy revealed that the compact and more matured fibril gets converted to shorter and more matured ones in presence of NMB. Based on far-UV CD spectral data, the conformational changes associated with fibril formation conclusively proved that NMB can delay the α to β transition of LYZ very potently. Fibrillogenesis kinetics was studied by staining agents like thioflavin T and congo red. There was a noticeable time delay in ThT fluorescence enhancement and hyperchromicity of CR absorbance in presence of NMB. A decline in fluorescence intensity was observed when NMB compete with hydrophobic probe ANS for the hydrophobic regions of LYZ, again indicating towards a delay in amyloid fibrillogenesis. Detailed information gathered from here may furnish an enhanced vision to the discovery of potential small molecules for curbing amyloid diseases.

Evaluation of qPCR and dPCR Instruments Performance using Single Control DNA

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Calibrated and properly maintained equipment is the prerequisite for reliable measurements. Maintenance and operational qualification procedures and their time intervals for instruments have to be defined by each laboratory according to its needs, manufacturer recommendations, frequency of use and knowledge about the instruments and can of can become very costly, particularly when (1) there are several instruments in a laboratory, (2) when checking needs to be done more than once per year or (3) when instruments are rarely used. However, complete operational qualification is not always necessary.

Here we present an approach that enables regular in-house checks of instruments for real-time quantitative PCR (qPCR) and digital PCR (dPCR).

We have prepared a control DNA that enables us to check the performance of different qPCR (LightCycler® 480 System, QuantStudio™ 7 Flex, ViiA 7™ and 7900HT Fast Real-Time PCR Systems) and dPCR platforms (Bio-Rad QX100™ and QX200™ Droplet Digital™ PCR Systems and Fluidigm Biomark™ HD), after minor repairs, as an intermediate check up between operational quantifications or as a yearly check. Following DNA extraction from the reference material (MON87769, AOCS 0809-B), using CTAB method, two validated assays targeting (1) soybean reference gene - lectin, and (2) genetically modified soybean line - MON87769, were used for evaluation of control DNA. Copy number per 1 µL of DNA solution for both amplicons has been assessed with QX100™ Droplet Digital™ PCR System and criteria for acceptance were determined.

To check the performance of dPCR platforms, control DNA is analysed with both singleplex and duplex assays. To check the performance of qPCR platforms, standard curve is prepared from control DNA and analysed using singleplex assays only.

The presented approach serves as a complement to the external service, enabling us to maintain instruments performance between external qualifications.

PI 43

Targeting tumor cell lines with lactic acid bacterium *Lactococcus lactis*

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Food-grade lactic acid bacterium (LAB) *Lactococcus lactis* represents an attractive host for recombinant protein expression and a promising vector for *in vivo* delivery of bioactive proteins by secretion or surface display. Display of recombinant proteins on the surface of LAB has already been exploited in therapy to prepare mucosal vaccines, to display binding molecules directed against pro-inflammatory cytokines in inflammatory bowel disease and to deliver antioxidant molecules in prevention of colorectal cancer. By displaying proteins targeting tumor antigens on the bacterial surface, directed binding of *L. lactis* to cancer cells could be achieved, essentially enabling targeted treatment with fewer side effects.

In the present study, we focused on the development of a system for targeted binding of *L. lactis* to colorectal tumor cell lines. We applied proteins with affinity for three tumor antigens, which are typically overexpressed in tumor cells of colorectal cancer, EpCAM, Her2 and Gb3. Genetic constructs for surface display included genes for affitin with affinity towards EpCAM, for affibody with affinity towards Her2 and for B subunit of Shiga toxin with affinity towards Gb3. Besides specific binding to colorectal cancer cell lines by using targeting proteins, we aimed at concomitant imaging of bound bacteria. We therefore simultaneously expressed infrared fluorescent protein (IRFP) in bacterial cytoplasm, by using plasmid for double protein expression. Surface display of FLAG-labelled targeting proteins was confirmed by flow cytometry, while expression of IRFP fluorescent protein was determined by measuring fluorescence. Furthermore, we confirmed, with flow cytometry, binding of soluble tumor antigens, EpCAM and Her2, to bacteria displaying their respective targeting proteins affitin and affibody. *L. lactis* displaying targeting proteins and IRFP were able to selectively recognize selected human tumor cell lines, indicating their promising targeting ability.

Antibiotic resistant bacteria in waste waters from Slovenian general hospital

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Hospital wastewaters are highly complex effluents acting as a hotspot for antibiotic resistant bacteria and generating environment for the exchange of antibiotic resistance genes. Especially Gram-negative bacteria bearing multiple antibiotic resistant genes are increasingly found in hospital wastewaters. The aim of study was to evaluate the presence of ESBL and Carbapenemase producing Enterobacteriaceae in hospital wastewaters from Slovenian general hospital as well as the occurrence of antibiotic resistant genes encoding for VIM, KPC, NDM, CTXM and OXA beta-lactamases in isolates from hospital wastewater. Samples were taken at main outflow form Slovenian general hospital in two consecutive years, July 2017 and July 2018. Results indicated high levels of ESBL and carbapenemase producing Enterobacteriaceae in hospital wastewater in ranges up to 10^7 cfu/mL. Carbapenemase producing Enterobacteriaceae and OXA 48-type producing CPE were present in ranges up to 10^5 cfu/mL. Although all colonies for further resistant patter identification were selected from selective chromogenic media (ChomID ESBL agar, chromID CARBA SMART), only 17 amplicons of different β-lactamase gene families out of 41 samples testedwere obtained with PCR. Among those only 3 isolates were identified as multiresistant bearing the VIM/CTXM and CTXM/OXA resistant genes. The CTXM ESBL family were represented by genotypes CTXM-1 in 6 different isolates and CTXM-9 in 4 different isolates. This was followed by 10 VIM positive isolates and 1 OXA-48 positive isolate. KPC or NDM carbapenemases were not identified with PCR screening of the isolates. Hospital wastewaters serves as a reservoir for nearly all clinically important antibiotic resistances. The importance of evaluating such potential environmental reservoirs is especially evident when outbreak cases could not be linked to an epidemiological source.

PI 45

Intracellular expression of cytotoxic fluorescent protein KillerRed in *Lactococcus lactis* for imaging and treating colorectal cancer

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Photodynamic therapy is a new method for cancer treatment in which a photosensitive compound – the photosensitizer is administered and induced by light to kill cancerous cells through generation of reactive oxygen species. Currently available chemical photosensitizers, such as different porphyrines and their mixtures, are administered exogenously and preferentially accumulate in tumors compared with normal tissue. However, they are not selective enough; thus, treated patients frequently suffer from long-term skin sensitivities caused by the retention of photosensitizers in their skin and subsequent exposure to light. KillerRed, a red fluorescent protein, is the first engineered genetically-encoded biological photosensitizer. It has 1000-fold more intense photo-induced cytotoxicity than other fluorescent proteins and can therefore be utilized to destroy cancer cell (photodynamic therapy) as well as to localize the tumor position using fluorescence imaging. The selectivity towards cancer cells is achieved by expression of this protein in bacteria, which are targeted against cancer tissue.

In this study we genetically engineered probiotic lactic acid bacterium, *Lactococcus lactis,* to express KillerRed protein and explore their potential use for detecting and destroying colorectal cancer cells. KillerRed-encoding gene was successfully expressed in *L. lactis* using nisin-controlled expression system. The level of expression was tested by measuring fluorescence intensity. Phototoxicity of KillerRed on bacteria-producer cells was determined by counting number of viable bacteria using DropPlate method. The amount of generated reactive oxygen species was quantified using nitroblue tetrazolium assay. KillerRed-expressing *L. lactis* will be tested on cancer cell lines to assess whether a reactive oxygen species produced by the bacteria can kill cancer cells.

Poster Session II

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Development of an Open Information System for Sample Management in Biomedical Research Studies and in a Biobank Repository

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Research and clinical laboratories need to implement harmonized standards in data and sample management and comply with the specific requirements for biobanking. This will facilitate collaboration and enhance the credibility and safety of processed data. Since developing a biobank is expensive our goal is to offer an alternative solution by implementing an open information system for management of biological samples in a biobank repository. The system specifications were based on MIABIS 2.0 and in accordance with ISO 20387:2018 standard. An emphasis was made on compliance of our system with the General Data Protection Regulation (GDPR), as well as with other safety, ethical, legal and social requirements for biobanking. We upgraded and adapted the InfoGEN information system to comply with the newest standards and requirements. The system was developed to fit especially the needs in research of the ongoing studies, however it can be customized to fit specific requirements of future research projects.

Our system will enable sample exchange among research groups and will therefore provide new opportunities for collaborations in biomedical research. This information system could present a solution for small laboratories, further improving interdisciplinary data exchange, importantly helping with the diagnosis of rare and poorly understood disease, discovery of new diagnostic tools and new treatment possibilities.

Acknowledgement: This work was partially supported by the projects “Establishment of open information system for the management of biological samples in the biobank repositories” and “Standardization of procedures for obtaining biological samples and information system for biobanks” co-financed by the Republic of Slovenia and the European Union under the European Social Fund.

Pharmacogenetics of postoperative pain management with tramadol

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Background: Tramadol is a centrally acting opioid analgesic, commonly used in the treatment of postoperative, dental, cancer, neuropathic and acute musculoskeletal pain. It is predominately metabolized in liver cells by CYP2D6, forming two active metabolites M1 and M5. M1 metabolite can be further inactivated by UGT2B7 glucuronidation, while ABCC2 and ABCB1 transporters play and important role in drug efflux. Our aim was to investigate if common functional polymorphisms in these genes predispose patients to adverse effects of tramadol.

Methods: The study included 112 female patients treated with tramadol after breast cancer surgery. *CYP2D6* (*3, *4, *5, *6, *10, *41, *2xN), *ABCB1* (rs1128503, rs2032582, rs1045642), *ABCC2* (rs2804402, rs717620, rs2273697) and *UGT2B7* (rs7668258, rs7668258) polymorphisms were genotyped using polymerase chain reaction methods including KASP and TaqMan assay. Appropriate statistic tests were used to analyse the influence of individual polymorphisms on treatment withdrawal and adverse effects such as nausea, vomiting, constipation and dizziness within the first month of treatment.

Results: Polymorphic homozygous *UGT2B7*rs7668258 TT genotype was significantly associated with treatment withdrawal (P =0.015). Other investigated polymorphisms were not significantly associated with the risk for tramadol adverse effects within one month of postoperative treatment. Similarly, *UGT2B7*, *ABCB1*and *ABCC2*haplotypes were not associated with tramadol adverse effects.

Conclusion: Patients carrying polymorphisms in *CYP2D6*, *ABCB1*, *ABCC2*and *UGT2B7* genes do not have a higher risk of adverse effects compared to patients with wildtype alleles within the first month of postoperative treatment after breast cancer surgery.

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Gene ontology of anti-TNF therapy response biomarkers in rheumatoid arthritis

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Objectives: Disease control in rheumatoid arthritis has been significantly improved by the development of biological drugs, such as anti-TNF agents. However, a significant fraction of rheumatoid arthritis patients do not have a satisfactory response to anti-TNF biological drugs or lose response over time. Such non-response usually represents loss of disease control in patients with extensive and severe inflammation, moreover it is an inefficient use of biological therapeutics and needlessly exposes the patient to potentially severe side effects of anti-TNF therapy. Currently, the mechanisms underlying non-response to anti-TNF therapy remains largely unknown.

Aim: The aim of our paper is employ gene ontology to elucidate the biological processes underlying non-response to anti-TNF therapy in rheumatoid arthritis using previously published biomarkers predictive of anti-TNF therapy response and/or non-response.

Methods: A literature search was performed to gather biomarkers predictive for non-response and/or response to anti-TNF therapy in rheumatoid arthritis and we elucidated enriched gene ontology terms in biomarker subgroups using gene ontology software tools.

Results: Several gene ontology terms are significantly enriched. The most statistically significant term is *proteasome* (p = 4,89 × 10^{-60}). Other enriched terms include *blood microparticle*, *Epstein-Barr virus infection* and *negative regulation of leukocyte chemotaxis*.

Conclusions: Our results help elucidate biological processes underlying response and non-response to anti-TNF therapy in rheumatoid arthritis. Furthermore, our results outline new therapeutic targets and encourage additional investigation of highlighted biological processes in rheumatoid arthritis.

Development and improvement of insect sex pheromone bioproduction in plants using systems biology approaches

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Use of insect sex pheromones has become an important part of integrated pest management as they provide a sustainable, species-specific and non-toxic control of insect pests in agriculture. Despite of the clear advantages, their widespread use is still limited due to unsustainable and not cost-effective manufacturing by chemical synthesis.

To answer the demand for alternative production of insect sex pheromones, SUPSHIRE project has set a goal to develop a plant and fungi-based bioproduction system with a commercial value. The project is focusing on upgrading an already developed system, The Sexy Plant v1.0 - transgenic *Nicotiana benthamiana* plants that produce moth pheromones. We have set to improve the metabolic flow in the exogenous metabolic pathway in the SxP v1.0 as well as to find and try to eliminate metabolic bottlenecks in the host organism metabolism. At the National institute of Biology, we are working on transcriptomic data-based network analyses of SxP v1.0 lines with different production and growth phenotypes, enabling us to determine possible metabolic perturbations in lines with high production yields and find the corresponding genes that would serve as targets for genetic improvement of the *N. benthamiana* as a chassis for insect sex pheromone production.

Due to lower commercial value of moth pheromone biomanufacturing, we also wish to develop biosynthetic pathways for production of sex pheromones whose organic synthesis is currently not viable. One such example are monoterpenoid pheromones of mealybugs (Coccoidea), synthesized by rare irregular coupling mechanisms. Since no biosynthetic pathways for their production have been identified in mealybugs, we are comparing transcriptome data from pheromone producing and not producing females, in order to identify genes involved in sex pheromone synthesis, which could be used in synthetic genetic circuits implemented in the new generation of the SxP v1.0.

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Microfluidic capillary electrophoresis for next-generation sequencing (NGS) library quantification and quality control

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NGS has revolutionized molecular biology, enabling studies that were previously impossible or unfeasible. With improvements in NGS chemistry and sequencing capacity, per base sequencing cost has fallen dramatically. Nevertheless, total cost of a single sequencing run remains relatively high. Therefore, it is worthwhile to implement careful quality control of NGS library preparation workflow, as it greatly reduces chances of sequencing failure or sub-optimal yields.

We present several cases of issues with NGS library quality, which were identified using a microfluidic system (LabChip GX, PerkinElmer). Libraries can be classified into three categories according to their quality.

The largest category includes libraries of good quality or with the following minor issues: nonideal size distribution, incomplete adapter monomer removal and, in multiplexed amplicon sequencing, nonspecific higher molecular weight products. In these cases, we can proceed to sequencing, but care should be taken to quantify only the part of the library expected to amplify in the templating step.

Second category includes libraries that can be salvaged using additional purification – most commonly libraries with adapter dimers or inadequate size distribution. We also present a viral sequencing project, where unwanted parts of library stemming from the host organism were reduced.

Third category is the smallest and encompasses libraries which cannot be salvaged and their construction must be repeated – typically libraries where DNA concentration is too low to allow additional purification, or libraries that are too short.

Fluorometry and qPCR are also widely used in NGS workflows to assess final library concentration. However, no sizing information is obtained using these techniques and consequently they offer virtually no possibility to detect problems before sequencing. Therefore, in our opinion and experience, microfluidic capillary electrophoresis is the method of choice for library evaluation.

The role of small RNA regulatory networks in tolerance and resistance responses of potato to PVY infection

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In the past decades, plant sRNAs have been shown to be essential for regulation of numerous biological processes. Recent studies showed that certain plant sRNAs play a central role in gene expression reprogramming and fine-tuning of defence responses against various pathogens, including viruses. The aim of our study was to identify key players in sRNA-regulatory networks controlling the establishment of tolerant and resistance responses of potato to PVY, one of the most important viruses affecting potato, responsible for huge economic losses in worldwide potato production. Employing high-throughput sequencing technology, we have identified and quantified sRNAs in the two tolerant (cv. Désirée, Pentland-Squire), in a resistant (cv. Rywal), as well as in two sensitive potato genotypes, impaired in accumulation of salicylic acid (NahG-Désirée, NahG-Rywal). This information was linked to expression profiles of their target transcripts to link sRNA level responses to physiological processes. Besides the already described regulation of immune receptors, we discovered an interesting novel sRNAs gibberellin (GA) regulatory circuit being activated only in tolerant plants of Désirée. By comparing responses between two tolerant genotypes we identified six commonly responding sRNAs. One of these sRNAs has been shown to be represent an additional regulatory path directed towards the suppression GA signalling to promote tolerance. Additionally, several sRNAs have been found to be regulated in resistant plants but not in tolerant ones, showing that different sRNA-regulatory networks are activated for establishment of tolerance and resistance. Taken together, we identified several sRNAs associated with either tolerance and/or resistance response of potato to PVY, which represent good candidates that can be further utilized as a valuable tool to manipulate target gene expression to improve crop immunity, impair pathogen virulence, and increase disease resistance of potato to PVY.

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Robust *de novo* assembly of tetraploid potato transcriptomes

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Although the genome sequence of *Solanum tuberosum* ssp. *phureja* double monoploid clone is available since 2011, its sequence and gene content are considerably different from cultivated tetraploid potato varieties (*S. tuberosum* ssp. *tuberosum*). To advance potato research and genomics-assisted breeding, it is necessary to determine the gene pool and genetic variation of potato varieties and breeding clones. *De novo* transcriptome assemblies of short RNA-seq reads are a cost-efficient way to achieve this, particularly if complemented with long reads. However, due to different *de novo* assemblers' properties, using a single assembler will result in an incomplete reference transcriptome, with many partially assembled transcripts. This, in turn, affects downstream analyses e.g. differential gene expression.

Using in-house and publicly available Illumina, SOLiD and PacBio reads, we have assembled three reference potato transcriptomes, for cv. Désireé, cv. Rywal and breeding clone PW363. For each genotype, over-assemblies, covering large parameter space, were produced using multiple *de novo* assembly algorithms optimized for short RNA-seq reads (Trinity, Velvet/Oases, CLC, and rnaSPAdes). In addition, long PacBio RNA reads of cv. Rywal were processed by Iso-Seq pipeline. *De novo* assembled contigs (i.e. transcripts) of each genotype were integrated into a non-redundant transcriptome using tr2aa workflow from EvidentialGene. We have additionally identified and removed contaminant sequences, performed homology-based annotation and assessed the completeness of transcriptomes with BUSCO.

Lastly, we combined the three *de novo* transcriptomes and the DM genome-derived transcriptome into a pan-transcriptome, to determine core and accessory genes. These transcriptomes are a valuable asset for *in silico* analyses, differential expression analysis and identification of gene-based genetic markers.

Landscape exploration of motifs in Arabidopsis thaliana and Solanum tuberosum networks

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The need to better understand stress-mitigating mechanisms in crop plants is increasing rapidly. Most often discussed biological systems are networks of genes or proteins. In our previous work, we have demonstrated the power of network generation and analysis approaches[1] for generation of novel hypotheses. Large-scale analyses of these networks can be complemented with network decomposition approaches, that divide the network into smaller units, that can be analysed independently. Motifs are structurally conserved simple building blocks found in analyses of real-world networks, implicating their importance in gene functional regulation. Pathogen infection of a plant triggers a complex interaction between both players involved, resulting in changes of the complex signalling network, such as changes in gene activity or reprogramming of the cell metabolism. In order to understand the mechanisms and dynamics, we generated environment specific networks of *Arabidopsis thaliana* under biotic stress by *Botrytis cinerea* and *Pieris rapae*[2] and *Solanum tuberosum* under biotic stress by *Potato virus Y*[3]. For each system, two separate networks were generated (with and without the stressor(s)), and analysed for the presence of network motifs unique or used by both. This in combination with experimental transcript expression data enabled us to form novel hypotheses on the condition specific responses of a model and crop plant to environmental cues, appropriate for testing in the laboratory.

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Identification of Novel Splicing Factors influencing Circular RNA Expression in Hepatocellular Carcinoma

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Circular RNA (circRNA) represent a new class of RNA which are gaining an increased appreciation in the field of RNA biology. They have already been implicated in pathological and physiological processes, especially in cancer, as oncogenes or tumour suppressors and are viewed as a new class of biomarkers. However little is known about the splicing regulation of this class of RNA molecules. Most circRNA are exonic and produced from protein coding genes. Different systemic analyses have so far shown that circRNA/mRNA pairs from the same host gene locus can have different patterns of change in expression. The identified differences are indicative of an active regulation in the splicing of circRNA/mRNA pairs. The aim was to identify the regulators of circRNA splicing and hence expression, in hepatocellular carcinoma, by identifying enriched motifs around differentially expressed circRNA splice sites. First, we identified statistically significant differentially expressed circRNA (GSE94508, GSE97332) and mRNA (TCGA database) in hepatocellular carcinoma and compared the pattern of expression across pairs. By using MEME Suite software, we successfully identified enriched motifs of RNA splicing regulators around circRNA splice sites. Different RNA splicing regulators were identified between circRNA/mRNA pairs depending on the concordance of their expression. Some of the identified splicing factors have already been shown to be upregulated in hepatocellular carcinoma. By analysing publicly available CLIP-seq and ChIP-seq data we confirmed binding for some of the splicing regulators to the sequences surrounding circRNA splice sites in HepG2 hepatocellular model cell line. Furthermore, we tested this hypothesis experimentally by overexpression and silencing of identified splicing regulators in a model cell line. In conclusion, we propose that differential expression of circRNA/mRNA pairs from the same locus can be actively regulated by splicing factors.

Association between response to anti-TNF treatment with adalimumab and genetic variants within the TLR signalling pathway in Crohn's disease

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Introduction: In several autoimmune complex diseases, an increased secretion of proinflammatory cytokine tumour necrosis factor (TNF) plays an essential role in the initiation and propagation of the disease. Therefore, anti-TNF monoclonal antibodies have shown an increased efficacy over conventional therapies. However, therapy with TNF inhibitors is ineffective in up to 30% of patients and the variety of therapeutic effects may reflect individual genetic background. It has been shown that variants within the TLR and NFκB signalling pathways could influence response to anti-TNF therapy. The aim of our study was to analyse association between *TLR* genes and response to anti-TNF inhibitor adalimumab (ADA) in well-defined cohort of Slovenian Crohn's disease (CD) patients.

Materials and Methods: We enrolled 102 CD patients on ADA for which biological and clinical response has been defined after 4, 12, 20 and 30 weeks of treatment. SNPs in three *TLR* genes (*TLR2*, *TLR4* and *TLR9*) were genotyped and statistical analysis has been performed.

Results: We found statistically significant association between response to ADA and all three *TLR* genes. The strongest statistically significant association has been found between SNP rs4696480 in *TLR2* gene and biological response measured with CRP after 20 weeks of treatment (p=1.48E-03). Patients with genotype AT or TT had significantly lower CRP after 20 weeks of treatment compared to patients with AA genotype. For *TLR4* and *TLR9* association was significant also only after 20 or 30 weeks of treatment, indicating that *TLR* genes are associated with long-term response/non-response to anti-TNF therapy.

Conclusion: Our results confirm association between *TLR* polymorphisms and particularly long-term response/non-response to therapy with ADA in Crohn's disease patients.

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Modelling of Cell Growth

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In order to maintain growth and maximize the utility of available nutrition, bacterial cell must precisely regulate the allocation of available energy into different metabolic compartments. The exact target of evolutionary pressure in a development of a bacterial cell remains unknown. We examined a simple coarse-grained model of bacterial cell (*E. coli*) undergoing moderate growth, which was developed by Maitra and Dill. The central energy value in the model is represented by ATP, which is produced by the catabolic machinery of the cell and is afterwards distributed to synthesis of ribosomes and proteins. The model successfully recreates some of the basic growth laws and predicts that the evolution targets the maximization of energy efficiency of fast-growing cells. By using more experimental data and fitting it to the model we exposed some inconsistencies and proposed a modified model.

Proteomic identification of legumain physiological substrates provides a novel link with innate immune response in mice

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Legumain or asparagine endopeptidase is an evolutionary conserved lysosomal protease with a distinct specificity for cleavage after asparagine and aspartate (1). Studies on legumain knock out mice demonstrated that its absence leads to splenomegaly, progressive kidney pathologies and disorders resembling hemophagocytic syndrome. Broad evolutionary conservation and several past studies on legumain knock out mice suggest important roles of this unique protease in lysosomal function and organ homeostasis but lack the molecular explanation for the resulting phenotype. To address this question we aimed our research to discover legumain proteolytic substrates and subsequently to identify signaling pathways involving legumain.

In our work we investigated legumain knock out mice by performing a global proteomic analysis of several mouse tissues/cell types. Identification and relative protein quantification revealed a group of lysosomal proteins significantly upregulated in knock out mice. We additionally implemented a two-dimensional PROTOMAP approach that provides semi quantitative topographical maps of proteins in the examined samples. With this approach we were able to screen for potential legumain substrates on a set of several thousand identified proteins. We determined a handful of physiological substrates, partially confirming known targets (cathepsin L, H) and importantly, identified a set of previously unknown substrates. Novel targets show a link between legumain, specific substrate cleavage events and native immune response providing further evidence for legumain as an important player in pathophysiological processes.

1. R. Vidmar, M. Vizovisek, D. Turk, B.Turk, M. Fonović. 2017. EMBO J. 36, 2455-2465.

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Preemptive pharmacogenomic testing for preventing adverse drug reactions – PREPARE study in Slovenia

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Background: Pharmacogenomics explores associations between patient's genetic profile and response to drug treatment. The aim of PREPARE study is to introduce preemptive pharmacogenomic testing for safer personalized treatment.

Methods: PREPARE is prospective cross-over clinical study ongoing in seven European countries, Slovenia included. In Slovenia, enrollment of patients in PGx Guided Prescribing group lasted from May 2017 to September 2018. Since October 1st 2018 patients are enrolled in Standard of Care group.

The main inclusion criteria is the first prescription of one of the 33 index drugs in the last 12 months. All patients are genotyped for 46 clinically relevant PGx markers in 12 pharmacogenes using allele specific real time PCR (KASP assays) on SNPline platform (LGC, UK). Treatment recommendations are sent to MD within 3-5 days from inclusion. Patients get a QR code card with a summary of results.

Results: In Slovenia a total of 317 patients were enrolled in the PGx Guided Prescribing group. The major drugs of incluson were: hypolipemics (29%), antidepressants (24%), immunosuppressants (17,7 %) and oncological drugs (13,3%). In total 44% of patients had polymorphisms in *CYP2D6*, 43% in *CYP2B6*, 36% in *CYP2C19* and *SLCO1B1*, 31% in *CYP2C9*, 14% in *CYP3A5*, 13% in *VKORC1*, 9% in *UGT1A1*, 5% in *F5* and *TPMT*, 4% in *HLA-B5701* and *DPYD*. A total of 78 (24,6 %) patients had treatment recommendations for inclusion drug.

Conclusions: Within the PGx Guided Prescribing arm of PREPARE study showed that 95,6 % of included Slovenian patients had at least one polymorphic pharmacogene with treatment recommendations. This indicates the importance of preemptive pharmacogenomic testing for personalized medicine.

A transcriptome-wide approach to identifying RNA targets of the Prader-Willi locus snoRNAs

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Prader-Willi syndrome (PWS) is the most common known genetic cause of life-threatening obesity in children. It is a complex genetic disorder with implications for the neurologic, metabolic, endocrine, and behavioral impairments. The PWS results from lack of expression of paternally expressed genes from 15q11.2-q13 genomic region. In a close up, a deletion of paternally expressed SNORD116 gene cluster (a group of C/D-box snoRNAs located in PWS region) was shown to be the primary genetic determinant of the PWS phenotype. Since molecular mechanism instigating PWS still remain unknown, there is an urgent need to study their identification.

SNORD116 (HBII-85) family consists of 29 homologues snoRNAs (small nucleolar RNAs), which display no apparent sequence complementary to canonical RNA targets and are considered as orphan snoRNAs with yet unknown function. Still, ectopic overexpression of SNORD116 in HEK293T cell line, endogenously not expressing this gene, resulted in the changed expression of more than 200 protein coding genes. This implies on SNORD116 cluster to play a regulatory role in mRNA stability. Whether the SNORD116 RNAs play a direct role in that or indirect by regulating trans-acting factors remains to be resolved. Thus, we study the function of SNORD116 snoRNAs by primarily identifying its interacting RNA targets.

In our SNORD116 cell models, the combination of transient interaction captured via psoralen cross-linking and subsequent enrichment of cross-linked RNAs, followed by high-throughput sequencing are allowing us to efficiently detect interacting events. We are using the PARIS protocol with our own modifications that support targeted approach. NTERA-2 cell line, with high endogenous expression of SNORD116, was used for hybrid library construction and for identification of SNORD116-RNA hybrids. We detected 147 508 hybrids among which there are 1694 unique interacting partners.

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Cholecalciferol ameliorates insulin signalling in the male rat heart

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The present study is an attempt to shed new light on molecular mechanisms of vitamin D effects on cardiac insulin signalling in the heart. Physiological conditions, rather than complex diabetic milieu, are convenient to clarify it. Considering vitamin D role in the regulation of insulin signalling in extracardiac tissues, as well as general importance of this vitamin for the heart, cholecalciferol (1000IU/kg) was administered to three months old male Wistar rats for 6 weeks, to study its effects on insulin signalling in the healthy heart. Effects of cholecalciferol on cardiac insulin signalling were estimated at the level of protein expression and/or phosphorylation of proteins of insulin receptor (IR), insulin receptor substrate-1 (IRS-1), protein phosphatase 1B (PTP1B), Akt/protein kinase B, p70 S6 kinase and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2). Although there were no changes in expression and/or phosphorylation of IR, PTP1B, p70 S6 kinase and ERK1/2, cholecalciferol administration decreased inhibitory phosphorylation of IRS-1 at serine 307 in the heart, indicating a better transmission of insulin signal. Insulin-stimulated phosphorylation of Akt/protein kinase B at threonine 308, which is necessary for Akt/protein kinase B activation, was increased following cholecalciferol treatment. Obtained results suggest that 6-weeks long cholecalciferol administration is sufficient for improvement of cardiac insulin signalling in the male rat heart.

Aberrant miR137 Host Gene methylation at the CpG island that encompasses miR137 in head and neck cancer patients

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Background: Head and neck cancer, including oral squamous cell carcinoma (OSCC), is one of the most common malignancies worldwide. Despite numerous advances in diagnosis and treatment, OSCC prognosis is still poor due to its aggressive nature, which includes strong local invasiveness and lymph node metastasis. The 5-year survival rate is among the lowest in cancer patients at <50%. Elucidation of molecular mechanisms underlying this type of cancer is crucial for early detection and treatment and for increasing patient survival. MicroRNAs are endogenous small RNAs that regulate gene expression in diverse cellular and metabolic pathways. Their dysregulation can lead to tumor development and cancer. MicroRNA miR137 has been shown to have a role in OSCC. We have set out to elucidate whether epigenetic regulation of the CpG island that encompasses miR137 and downstream sequence plays a role in head and neck cancer.

Patients and methods: Paired tumor and normal adjacent tissue samples were collected from 118 Head and neck cancer patients that were treated at the University Medical Centre in Maribor from 2016 to 2019. Specimens included oropharynx (36%), hypopharynx (21%), cavitas oris (19 %) and larynx (11%) primary tumors. Medical history and clinical data was collected for each patient, including tumor TNM classification, perivascular/perineural invasion, cancer recurrence and patient survival. The methylation levels of segments of the miR137 Host Gene were determined by combined bisulfite restriction analysis (COBRA) of bisulfite-modified tissue DNA. Precursor microRNA levels were determined by qRT-PCR.

Results: MiR137 Host Gene methylation was elevated in OSCC specimens compared to healthy adjacent tissues at the CpG island that contains miR137 and downstream sequence. The aberrant methylation levels in OSCC were examined in correlation with miR137 expression levels and patient survival and cancer recurrence.

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Expression of CB1, CB2 and GPR55 Receptors in Selected Breast Cancer Cell Lines

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Background: Approximately 70-80 % of all known breast cancers are estrogen receptor alpha (ERα) positive (1). Common endocrine therapy includes selective estrogen receptor modulator (SERM) tamoxifen, which binds to ERα and inhibits estrogen dependent signaling. Cannabinoids have recently been demonstrated to induce anti-tumor responses in certain cancer types, including breast carcinoma. Cannabinoid receptors (CBRs) are G-protein coupled receptors found in the central nervous system (CB1) and in the periphery (CB2). Cannabinoids can also activate other receptors, like GPR55. Recent studies have shown that tamoxifen acts as inverse agonist on CBRs (2) and may interfere with potential application of cannabinoids in breast cancer treatment.

Objectives: The aim of the study is to evaluate the expression of CB1, CB2 and GPR55 receptors in selected breast cancer cell lines of different subtypes and with different hormone status (ER+/PR+/HER2-, ER-/PR-/HER2+ and triple-negative).

Methods: The expression levels of CBRs in breast cancer cell lines were analyzed using immunocytochemistry, comparing two different primary antibodies (Abcam, Santa Cruz Biotechnology). Optimal concentration range and specificity of selected antibodies against CBRs was determined by Dot blot and Western blot, respectively.

Results: We tested one of the most used polyclonal antibodies against CBRs (Abcam), and were among the first to test the new monoclonal antibodies (Santa Cruz Biotechnology). Cell lines T-47D and MDA-MB-231 are positive for CB1 and CB2, while SK-BR-3 is positive for CB1. CBRs expression results differ among the cell lines. Only T-47D is positive for GPR55.

Conclusion: Hormone positive T-47D, triple-negative MDA-MB-231 and HER2 positive SK-BR-3 express one or more CBRs and are thus susceptible to manipulation of the endocannabinoid system.

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The influence of tramadol pharmacogenetics on treatment outcome in patients after breast cancer surgery

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Background: Breast cancer patients usually receive tramadol for pain management after surgery, however, some patients can experience insufficient pain relief or adverse events. Genetic variability of CYP2D6 and UGT2B7 metabolizing enzymes or ABCB1, ABCC2 and SLC22A1 drug transporters may affect efficacy and toxicity of tramadol. Our aim was to assess the association of genetic variability in tramadol pharmacokinetics pathway on long-term outcome of tramadol treatment after breast cancer surgery.

Methods: Within a double blind randomized clinical trial at the Institute of Oncology Ljubljana (KCT 04/2015-DORETAonko/si), breast cancer patients were randomized and treated with either 75 or 37.5 mg of tramadol for pain relief after breast cancer surgery. Data on long-term treatment outcome was available for 102 patients that were genotyped for 14 polymorphisms in *ABCB1*, *ABCC2*, *CYP2D6*, *SLC22A1* and *UGT2B7* genes and for *CYP2D6* duplication and deletion. The association of genetic factors with treatment outcome was evaluated with logistic regression and Mann-Whitney test.

Results: One year after treatment, CYP2D6 poor metabolizers were significantly more likely to experience chronic and neuropathic pain (OR=5.96, 95% CI=1.22-29.13, P=0.027 and OR=9.31, 95% CI=1.65-50.59, P=0.011, respectively), even after adjustment for tramadol dose (P=0.032 and P=0.016, respectively). CYP2D6 poor metabolizers also had higher average pain intensity compared to others regardless of tramadol dose (P=0.042). *ABCB1* rs1128503, rs2032582 and rs1045642 were also associated with more chronic pain in patients receiving lower tramadol dose (P=0.004, P=0.004 and P=0.047, respectively).

Conclusion: Genetic variability in tramadol pharmacokinetics pathway may influence treatment outcome in breast cancer patients. Pharmacogenetic testing could facilitate a more personalized treatment with better long term outcomes.

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Molecular mechanism of NLRP3 inflammasome assembly and self-regulation

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NLRP3 inflammasome is a multiprotein complex mediating inflammatory response in a variety of metabolic and degenerative diseases, yet the molecular mechanism of NLRP3 inflammasome initiation remains elusive. In this study, systematic truncation of NLRP3 and reconstitution of NLRP3 variants in NLRP3-deficient macrophages revealed that LRR domain is dispensable for activation and self-regulation. A minimal NLRP3 truncation variant was found fully responsive to various canonical NLRP3 activators. Using bioluminescence resonance energy transfer we showed that further truncation led to the variant that still changed conformation in response to canonical NLRP3 inflammasome trigger yet failed to form a functional inflammasome, even when a disease-causing point mutation was introduced. Substitution of the pyrin domain of NLRP3 with the CARD domain of NLRC4 or ASC led to a constitutive activation, demonstrating that the pyrin domain restricts NLRP3 in an inactive conformation. Further we show that pathological mutations of NLRP3 failed to engage wild-type NLRP3 in a self-catalytic oligomerization, demonstrating that the activating signal is not enhanced at the level of NLRP3 oligomerization, representing an additional level of NLRP3 regulation. These results contribute to the understanding of the molecular basis of NLRP3 inflammasome activation and demonstrate the versatility of recognition and regulation mechanisms of the innate immune receptors.

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PII 64

Obstacles in studying human myeloid-derived suppressor cells

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Myeloid-derived suppressor cells (MDSC) are main drivers of immunosuppression in cancer. During last decade numerous studies provided important insights into the key features of MDSC such as their expansion, immunosuppressive mechanisms and phenotype. It is becoming clear that mouse *in vivo* models and therefrom isolated MDSC are not adequate counterparts of human MDSC. A lot of effort was put in harmonization of MDSC nomenclature, which is indispensable for comparing studies that describe correlation between MDSC, cancer progression and response to therapy. However, as much as this contributed to the formation of the ground rules for studying MDSC in preclinical studies, recommendations for studying human MDSC are only beginning to emerge. There are not many publications that cover the isolation of human MDSC. In addition, methodological sections often lack key information, thus hindering reproducibility of the results. Due to ethical and technical issues it is difficult to obtain sufficient number of MDSC from cancer patients; therefore *in vitro* generation of human MDSC from peripheral blood mononuclear cells of healthy donors represents an important tool for studying their properties. Our results show great interindividual variation as myeloid cells from different donors do not respond uniformly to GM-CSF or GM-CSF/IL-6 stimulation. In fact, in some cases GM-CSF/IL-6 stimulation failed to induce immunosuppressive behavior in myeloid cells in contrast to stimulation with GM-CSF alone (negative control). Next, we tested four tumor cell lines (MDA-MB-231, U87, PC-3 and HOS) and found that MDA-MB-231 and U87 cell lines were capable of generating immunosuppressive myeloid cells, although the later to a lesser extent. In conclusion, tumor cell lines enhance the preparation of MDSC *in vitro*; however, further studies are needed to uniformly identify human MDSC and to fill the gap in knowledge on their role in cancer progression.

Diagnostic and/or prognostic value of plasma concentration of angiogenic factors in endometrioid endometrial cancer

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Endometrial cancer (EC) is the most frequent gynecological malignancy in more developed countries. Although the majority of patients are diagnosed at an early stage and cured by hysterectomy, the cancer recurs in about 20% patients with no sign of advanced/metastatic disease with limited response to systemic therapy. Novel biomarkers for EC would be invaluable for preoperative stratification of patients as low/high-risk, detection of primary and recurrent disease and thus enabling personalized therapeutic approaches. Angiogenesis plays an important role in the development and progression of several types of cancer, including EC. This process is controlled by angiogenic factors (AF). Currently, AF have been studied in some malignancies but their role in EC has not been determined yet. We analyzed plasma concentration of 20 different AF in patients with the endometrioid type of EC in prospective case – control study of women who underwent surgical treatment at the University Medical Centre Ljubljana (38 patients with endometrioid EC, 38 control patients with prolapsed uterus or myoma). Plasma samples were measured using commercially available assay that enables simultaneous determination of 20 different AF with the Luminex xMAP multiplexing technology. Plasma levels of soluble angiopoietin-2 showed significantly decreased levels in patients with EC compared to the control group ($p<0.05$). Tenascin C and angiostatin showed decreased levels in patients with EC and present lymphvascular invasion or deep myometrial invasion (DMI), respectively, while osteopontin levels were increased in patients with EC with DMI, but these differences did not reach statistical significance. Based on different grade of endometriod EC neuropilin showed significant difference. Results of our pilot study indicate that plasma levels of different AF might have a diagnostic and/or prognostic value in endometrioid EC. Further statistical evaluation is currently ongoing.

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Sepsis, non-canonocal inflammasome activation and cystatins

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Sepsis is a life-threatening organ dysfunction caused by a dysregulated response to infection. Its severity depends on pathogens as well as on host factors. Lipopolysaccharide (LPS), an endotoxin present in the cell wall of Gram-negative bacteria. I We reported that mice deficient in stefin B (cystatin B) are significantly more sensitive to LPS induced sepsis due to increased caspase 11 expression. Upon sensing LPS, caspase-11 forms a higher order structure - the non-canonical inflammasome that enables the activation of caspase-11 protease function, leading to gasdermin D cleavage and cell death.

In our precent study we used stefin B-deficient mice, as well as mice with an additional copy of stefin B gene, stefin B- trisomic mice and compared the signaling pathways upon LPS challenge. Treatment with LPS induced autophagy in wild-type but less in stefin B deficient macrophages. In stefin B-trisomic macrophages, we determined diminished caspase-11 expression upon LPS stimulation.

Circulating plasma placenta specific miR-518b could serve as a potential biomarker for discriminating pre-eclampsia and healthy pregnancies

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Introduction: Pre-eclampsia (PE) is defined as hypertension developing after 20 weeks' gestation associated with proteinuria, maternal organ dysfunction or fetal growth restriction. It is one of the leading causes of maternal and perinatal mortality and morbidity worldwide. Biomarkers for more sensitive detction of PE are needed. Evidence show abnormal circulating microRNAs in pregnancies affected by pre-eclampsia, both placenta specific and non-specific microRNAs.

Aim: The aim of this study was to evaluate circulating hypoxia-related miR-210-3p and placenta specific miR-518b in preeclamspisa and helathy pregnancy by digital droplet PCR (ddPCR).

Material and methods: This study included 36 pregnant women, from 20-39 gestational weeks, form Clinic for Gynaecology and Obstetrics "Narodni front", Belgrade, Serbia. There were 17 healthy pregnancies (control group, (CG)) and 19 PE patients. Circulating plasma miR210-3p and miR-518b, along with cel-miR-39 as a spike-in control were measured by ddPCR.

Results: Plasma miR-518b was significantly elevated in PE patients compared to CG (P=0.034; 0.302(0.217-0.421) vs. 0.171(0.110-0.266)). Plasma miR-210-3p showed no significant difference between the two groups (P=0.805). The adjustment of plasma miR-518b was made for gestational age and smoking status and the difference between the PE group and CG was unaffected (P=0.036; 0.293(0.208-0.412) vs. 0.173(0.121-0.245)). The potential of plasma miR-518b for discriminating PE patients from CG was investigated by ROC curve analysis. Plasma miR-518b was able to significantly discriminate between PE and CG, yielding AUC of 0.779 (95% CI:0.612-0.946), P=0.006.

Conclusion: In this study circulating plasma microRNA were measured for the first time in PE and healthy pregnancies with ddPCR. Circulating plasma placenta specific miR-518b could serve as a potential biomarker for discriminating PE and healthy pregnancies, while this study has failed to confirm the same potential for miR-210-3p.

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Insight into genome methylation patterns of suicide victims: potential association with brain plasticity

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With nearly a million lives lost each year, suicidal behaviour is a multifactorial, polygenic phenomenon affecting people worldwide. Slovenian population has one of the highest suicide rates in the world. Despite extensive knowledge of suicidal behaviour, the molecular mechanism leading to suicide is not known. However, there is an increasing amount of evidence associating changes of the DNA methylation pattern and suicidal behaviour. DNA methylation can have an effect on gene expression, with high percentage of methylated cytosines normally resulting in lower amount of expressed gene and vice versa.

In order to identify differentially methylated cytosines of two brain regions, Brodmann area 9 and hippocampus, we studied a small yet homogeneous group of Slovenian suicide victims who died by hanging (n = 9) and control group (n = 9). Using next generation sequencing we discovered numerous differentially methylated cytosines in suicide victims compared to controls for both brain regions (> 25% methylation difference and q-value <0.01). Gene ontology analysis revealed terms enriched in cell structural integrity and nervous system regulation, which could further support the hypothesis of altered brain plasticity in suicidal behaviour. Further validation of the methylation results with gene expression analysis confirmed the association between DNA methylation patterns and gene expression for two potentially regulatory genes, zinc finger protein 714 (*ZNF714,* p-value = 0.002) and nuclear receptor interacting protein 3 (*NRIP3,* p-value = 0.046).

The obtained results provide a new insight into altered epigenetic states in suicide victims and are serving as a starting point for further studies of new potential candidate genes, which we will investigate on a larger sample set.

Understanding metabolic changes in schizophrenics through AKT/FOXO signaling pathway

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Schizophrenia is mental illness that requires lifelong treatment with antipsychotic drugs, resulting in many adverse drug reactions such as metabolic syndrome and type II diabetes. At the same time, some antipsychotics proved to be toxic to the liver, which is crucial in regulating glucose and drug metabolism, including the metabolism of antipsychotics, and detoxification processes. Aripiprazole (Ari) and Olanzapine (Ola) are among the second-generation antipsychotics that have greater efficacy in the treatment of schizophrenia than the first-generation medicines, but the effectiveness of long-term treatment, especially with Ola, is hampered by different metabolic adverse reactions.

Protein kinase B (AKT/PKB) and *Forkhead box O protein* (FOXO) could play a role in adverse metabolic reactions since they are involved in apoptosis, cell proliferation, glucose metabolism and transcription and could represent a way to understand the link of drug catabolism with hepatic alterations. AKT protein phosphorylates FOXO transcription factors which are released from the nucleus and, consequently, increase the expression of oxidative phosphorylation proteins, which improve the efficiency of energy production. Phosphorylation of FOXO also increases levels of protein folding chaperones, heat shock proteins and the production of antioxidants that lower the level of ROS. Thus, removal of FOXO from the nucleus reduces signalization for apoptosis and drives cells into the cell cycle.

In concert with these processes, our observations upon addition of antipsychotics and hydrogen peroxide as an inducer of oxidative stress imply, that the effects of Ari and Ola increase the signaling of the AKT/FOXO signalling pathway. These findings are important to differentiate drug effects in acute stress conditions and might be helpful to design targeted strategies to either promote or decrease protein activation.

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Diagnosis of familial erythrocytosis in clinical practice

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Introduction: Rare blood disorder familial erythrocytosis (FE) is defined by increased haematocrit, haemoglobin and red blood cell number. Variants in genes involved in oxygen sensing pathway (including *EGLN1*) or genes affecting haemoglobin oxygen affinity results in eight different types of FE. Current practice revealed that etiology of FE is problematic, therefore our aim was to develop an effective diagnostic strategy for identification of FE in Slovenian patients.

Materials and Methods: National diagnostic algorithm for the evaluation of FE in Slovenia was developed in collaboration with Clinical Department of Haematology, University Medical Centre Ljubljana. Using next generation sequencing (NGS), patients were screened for variants in 39 genes involved in erythrocytosis. Libraries were prepared using Illumina Nextera DNA Exome, enriched with pre-designed probes and sequenced on MiniSeq sequencer. Identified variants were validated by Sanger sequencing.

Results: Retrospective analysis using national diagnostic algorithm revealed about 40 patients indicative for FE, including five families. Targeted exome-sequencing identified heterozygous missense substitution c.471G>C in *EGLN1* gene in two affected but not in unaffected members of one family. The described diagnostic algorithm was also established within the in-house developed ViDis platform, enabling visualisation and sharing of medical algorithms (http://vidis.fri.uni-lj.si). An open information system is developed for better management of samples from erythrocytosis patients.

Conclusions: We successfully established diagnostic algorithm for FE in Slovenia and facilitated its broad accessibility within medical society via ViDis platform. Implementation into clinical practice identified an *EGLN1* variant in one studied family, indicative for FE type 3 (ECYT3).

Supported by Slovenian Research Agency grant no. L3-9279, Young Researcher founding and University Medical Centre grant no. 20170073.

Extracellular vesicle cargo and morphology as potential diagnostic tools for neurodegenerative diseases and cancer

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Introduction: Extracellular vesicles (EVs) are a heterogeneous population of membrane vesicles, which are shed from cells. EVs characteristics reflect the composition and the (patho)physiological state of the parental cell. EVs can be isolated from easily accessible body fluids and thus have great potential for human diagnostic applications. In our study, we evaluated the biomarker potential of EVs' cargo and morphology for diagnosis of neurotropic fungal infections and pancreatic ductal adenocarcinoma (PDAC).

Methods: To study neurocytotoxicity, EVs were isolated from the culture of neurotropic fungus *Exophiala dermatitidis* and tested *in vitro* on neuroblastoma cells SH-SY5Y. For the PDAC clinical study, EVs were isolated from the blood plasma of 50 subjects, monitored longitudinally at four time-points. All subjects provided informed consent and the study was approved by the NMEC. EVs were characterized for the presence of marker proteins and melanin (WB, A_{400}) and for their morphology, size and concentration (TEM, NTA, AF4-MALS).

Results: Fungal EVs had typical characteristics: the average size of 90 nm (*R*g), the concentration of 2.8×10^{10} EVs/ml and marker proteins HSP70 and GAPDH, however, EVs showed higher buoyant density, probably due to melanin. EVs containing melanin were neurocytotoxic for SH-SY5Y cells (20.9% viability compared to control), with reduced effect in the presence of EVs without melanin (viability of 78.6% at twice the EVs concentration). EVs isolated from the plasma of PDAC patients showed the mode size range from 78.8–170.4 nm and the concentrations from $4.3–16.2\times10^{10}$/ml with some variability between the PDAC patients at zero time-point, however, changes were also observed longitudinally over time. Currently, we are evaluating the association of EV morphology to the clinical data of the PDAC cohort.

Conclusions: We showed that EVs cargo and morphology can relate to certain pathologies and could therefore be used as biomarkers of disease.

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Cannabidiol and Δ9-tetrahydrocannabinol affect human glioblastoma cell proliferation and survival

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Background: Glioblastoma (GB) is the most aggressive and therapeutically non-responsive primary brain tumour. Survival of patients ranges from 12 to 15 months after the diagnosis despite of improved treatments using irradiation and chemotherapy. This is mainly due to inefficient targeting of therapeutically resistant glioblastoma stem cells (GSCs), therefore new adjuvant treatment options against GSCs are urgently needed. Increasing number of pre-clinical studies have shown that cannabinoids induce the processes leading to anti-tumour responses in some types of cancer, including GB**.**

Objectives: The first objective of this in *vitro* preclinical study is to evaluate the expression of cannabinoid receptors CB1 and CB2 in a small cohort of glioblastoma tumour tissues, patients-derived GB cells and GSCs. Second objective is to identify most effective ratios of Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) to alter GB and GSC cell viability, apoptosis and proliferation.

Methods: Primary GB and GSC cell lines were established from a set of patient-derived GB tumours, as described [1]. We analysed protein expression profile of CB1 and CB2 receptor on GB tissue sections, patient derived glioblastoma cells and GSCs by immunolabelling. The effect of cannabinoids on GB cell viability, apoptosis and proliferation was studied using MTT assay, AnnexinV/PI flow cytometry assay and Ki67 immunostaining.

Results: CB2 receptor was highly expressed in all tumour tissue samples while differentiated GB cells express moderate level of both receptors, in contrast to high levels of CB1 and CB2 in GSCs. Both CBD and THC alone and in combination affect cell viability, proliferation and apoptosis of differentiated GB cells and GSCs.

Conclusion: Targeting GSCs with cannabinoids at optimal concentration for each patient is promising for personalized adjuvant GB therapy.

Antisense RNA transcript from C9orf72 gene mutation binds RNA binding proteins

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Mutation in *C9orf72* gene is the main genetic cause of two incurable neurodegenerative diseases - amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). ALS and FTD share clinical, neuropathological and genetic characteristics. Causes of development and progression of these diseases are still not entirely known. It has been shown that the mutation in *C9orf72* gene causes up to 40% of hereditary ALS cases and 25% of hereditary FTD cases. The mutation occurs in non-coding region of the gene in the form of increased number of polymorphic hexanucleotide repeats of GGGGCC sequence. Up to 23 repeats occur in healthy individuals, while in disease there are several hundred or several thousand repeats present. There are three potential mechanisms of action proposed for the mutation. First, repeats could cause reduction in expression of the *C9orf72* gene and, consequently, to haploinsufficiency of C9orf72 protein. Second, hexanucleotide repeats can be transcribed to RNA in sense and antisense direction, both transcripts form different secondary structures and are proposed to sequester different RNA binding proteins, which are important for normal cell functioning. Third, RNA transcripts of hexanucleotide repeats can be non-canonically transcribed to proteins with dipeptide repeats (DPRs), for which toxic effects on cells have been suggested.

The aim of our research is determination of proteins binding to antisense RNA transcripts from the *C9orf72* gene mutation and defining the role of this interaction in the development and progression of ALS and FTD. So far, we have identified several proteins binding to the antisense (C_4G_2) transcripts, which are part of cell protein synthesis pathway and cytoskeleton stability. Both mechanisms are important for cell survival and normal function of neurons. We will present the latest findings in regard to their importance in disease development and progression.

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Synergistic activity of 15-lipoxygenase and sPLA2 promotes inflammation by formation of TLR4 agonists from extracellular vesicles

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Damage-associated endogenous molecules (DAMPs) induce innate immune response, thus making sterile inflammation medically important. They can signal through innate immune receptors such as Toll-like receptors (TLRs) thus promoting inflammation.

During oxidative stress conditions, stress-derived EVs (stressEVs) were found to activate (TLR4) with a gene profile different from bacterial lipopolysaccharide (LPS), resulting in expression of different immune response proteins/cytokines underlying the differences between pathogen-induced and sterile inflammation. Additionally, synergistic activity of 15-lipoxygenase (15-LO) and secreted phospholipase A_2 ($sPLA_2$) was needed for the formation of TLR4 agonists, which were identified as lysophospholipids (lysoPLs) having oxidized unsaturated acyl chain. Hydroxy, hydroperoxy and keto products of 20:4 lysophosphatidylinositol (lysoPI) oxidation were determined by mass spectrometry and they activated the same gene pattern as stressEVs, including the Il-23, an inducer of the Il-23/Th17 signaling pathway. Furthermore, $sPLA_2$ activity, which was also detected in the synovial fluid from the rheumatoid arthritis and gout patients, promoted formation of the TLR4 agonists.

Both 15-LO and $sPLA_2$ are induced during inflammation, therefore these results confirm the role of oxidized lysoPLs which constitute stressEVs in the sterile inflammation that promotes chronic diseases. The formation of TLR4 agonists is enzyme driven so it provides an opportunity for the therapy without compromising innate immunity against pathogens.

The effects of *Cimicifuga racemosa* extract on hormone-dependent malignant gynaecological diseases

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In postmenopausal women estrogen levels depend exclusively upon the local formation from steroid precursors, dehydroepiandrosterone-sulfate and estrone-sulfate (E1-S). The reduced estrogen levels are associated with menopausal symptoms, which often occur in peri- and postmenopausal women. Usually, hormone replacement therapy (HRT) has been prescribed to improve life quality of these women. Due to the reported correlation with increased risk of stroke and breast cancer, the use of HRT has declined in the last decade. Women are therefore choosing natural "HRT", as extracts from *Cimicifuga racemosa* (CE). While CE treatment is viewed as safe, little is known about its effects on hormone-dependent malignancies like endometrial or ovarian cancers that arise in this population of women.

The purpose of this study was to evaluate the effects of CE (BNO 1055, a gift from Bionorica SE) on local formation of estradiol from the steroid precursor E1-S in the ovarian and endometrial cancer cell lines (CLs). We exposed high-serous ovarian carcinoma (COV362 and Kuramochi), and moderately (RL95-2) and poorly differentiated (KLE) endometrial carcinoma CLs to CE and examined the effects on the expression of 6 genes encoding estradiol biosynthetic and metabolic enzymes and 13 E1-S transporter genes.

After CE treatment we observed decreased and increases expression of genes involved in estradiol biosynthesis and metabolism in individual CLs. The most significant upregulation was observed for *HSD17B2* in KLE and downregulation for *SULT1E1* in COV362. *STS* was significantly downregulated in all CLs. Genes encoding *ESR1* and *ESR2* were significantly upregulated only in KLE. The E1-S transporter genes were upregulated, with the highest differences seen for *SLCO4A1* in KLE and COV362 CL.

Our research presents an insight on CE effects on ovarian and endometrial cancer CLs at the mRNA level. The effects at the protein level and at the level of E1-S metabolism still needs to be evaluated

PII 76

Kinase mediated Y526 phosphorylation of FUS afflicts its inclusions formation

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Abnormal cytoplasmic accumulation of Fused in Sarcoma (FUS) in neurons defines subtypes of amyotrophic lateral sclerosis (ALS) and fronto-temporal lobar degeneration (FTLD), both accompanied by progressive neuron loss. But the underlying pathological mechanisms leading to FUS mislocalization and aggregation in both diseases appear different. FUS is predominantly a nuclear protein that possesses non-classical PY-type nuclear localization signal (NLS) at its extreme C-terminus. In ALS but not in FTLD, the mutations in the NLS of FUS are responsible for its impaired nuclear transport mediated by nuclear import receptor transportin 1 (TNPO1). We reported on posttranslational modification - the phosphorylation of C-terminal tyrosine at position 526 in NLS of FUS, which abolished FUS interaction with TNPO1 and impaired its transport into the nucleus. As various kinases were shown to phosphorylate Y526, our aim was to elaborate on cell compartment specific phosphorylation of Y526 in mutated, truncated and full length FUS, by kinases of the Src-family. Custom antibodies against phosphorylated FUS Y526 were developed and their specificity confirmed by siRNA silencing, immunoprecipitation, phosphatase assays and in knock-out mice. We showed that mutated FUS, C-terminal fragments of FUS and full length FUS, when phosphorylated at Y526 form morphologically distinct aggregates with kinase-dependent localization within the cells. Moreover, in mice brain slices the signal for phosphorylated FUS showed to be cell type specific. Together our data suggest on kinase-type mediated FUS inclusion formation in the cells, possibly associated with distinct progressive modes of neurodegenerative diseases. The low level of cytoplasmic phosphorylation of Y526 may nonetheless serve to fine tune the nucleocytoplasmic shuttling of FUS to ensure that a small amount of FUS always remains present in the cytoplasm possibly for dendritic mRNA transport.

Serum Adiponectin, Resistin and Leptin Levels in Peripheral Arterial Disease (PAD) and Buerger's Disease

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Peripheral Arterial Disease (PAD) is a most common atherosclerotic and occlusive disease in associated with chronic limb ischemia. It occurs as a result of secretion of pro- and anti-atherogenic cytokines and adipokines such as adiponectin, resistin and leptin. There are studies about adipokines investigated their relations to obesity, metabolic syndrome, hypertension, type 2 diabetes and vascular diseases. In our study, we investigated serum adiponectin, resistin and leptin levels in patients with PAD and Buerger's. Buerger's disease is a non-atherosclerotic and occlusive inflammatory disease. The using tobacco is an important factor of Buerger's disease.

The study group were consisted of 127 PAD and 24 Buerger's disease. In our patients, we measured ancle-brachial index (ABI) values. The serum adipokines levels were measured by ELISA method. Statistical analyses were performed by using SPSS version 21. Serum adipokines were evaluated by Student-t test. Clinical features of groups were compared with chi-Square test. P values less than 0.05 were considered as significant.

ABI values were lower in patients with Buerger's disease in compared with PAD. Both adiponectin and leptin levels were lower in Buerger's disease than those of the PAD. There is no significantly difference in resistin levels between groups. When we evaluated the patients according to the presence of hypertension and diabetes, we found that adiponectin and leptin levels were significantly lower in Buerger's disease than those of the PAD group.

As a result of this study, we think that the presence of diabetes and hypertension in patients with PAD and Buerger's disease could be important factors on adiponectin and leptin levels.

Our study was supported by Istanbul University Research Projects (Project No: 24592).

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PII 78

PPAR-γ serum levels and PPAR-γ Pro12Ala polymorphism in Peripheral Arterial Disease

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Peripheral arterial disease (PAD) caused by stenosis or occlusion of lower extremity arteries is a progressive atherosclerotic disease. PAD affects over 200 million adults in world. Intermittent claudication is the most common symptom of PAD. The traditional risk factors of PAD are diabetes, smoking, age, hyperlipidemia and hypertension. Patients with PAD have a higher rate of myocardial infarction (MI), stroke and cardiovascular death. Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a ligand-activated transcription factor belonging to the nuclear hormone receptor superfamily. PPAR-γ plays a role in various cellular functions such as glucose, lipid metabolism and oxidative stress.

PPAR-γ is involved in the regulation of all steps of the onset of atherosclerosis. Several studies have shown an association between PPAR-γ polymorphism and microvascular and macrovascular complications of coronary and carotid arteries in patients with Type 2 diabetes. In a study, the prevalence rate of the Ala12 allele was significantly higher in PAD patients than those of the controls.

The aim of our study is to investigate serum PPAR-γ levels and its relationship with PPAR-γ gene Pro12Ala polymorphism in patients with PAD.

Our study group were consisted of 47 patients with PAD and 46 control subjects. Serum PPAR-γ levels were determined by ELISA method. PPAR-γ gene Pro12Ala polymorphism was determined by Real time-PCR method. Statistical analyses were performed by using SPSS version 21. Genotype and allele distributions were compared with chi-square test. PPAR-γ genotypes and serum levels were compared by using Student-t test. P values less than 0.05 were considered as significant.

Serum PPAR-γ levels were significantly lower in patients with PAD than those of the controls ($p<0.05$). There were no differences in genotype distributions of PPAR-γ Pro12Ala between PAD and control group.

In conclusion, we suggest that the further studies are needed to investigate serum PPAR-γ levels and PPAR-Y genotyping in larger populations.

Our study was supported by Istanbul University Research Projects (Projects No: 25856, 27020).

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Important roles of OATP, OAT and ABC transporters in endometrial cancer

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Endometrial cancer (EC) is associated with increased estrogen actions. Estrogens can be formed locally via the sulfatase pathway from estrone-sulfate (E1-S) after its transport into cells by organic anion-transporting polypeptides (OATP), sodium-dependent organic anion transporters (SOAT) or organic anion transporters (OAT). The efflux of E1-S from cells is enabled by ATP-binding cassette transporters (ABC) and organic solute transporter (OST) αβ. Currently, 19 transporters are known to transport E1-S but their roles in EC have not yet been determined. Here, we analyzed the expression of these transporters in model cell lines of EC, Ishikawa and HEC-1-A, and in paired samples of EC and adjacent control tissue using qPCR, imunocytochemistry or imunohistochemistry.

Results showed that 15 genes were significantly differentially expressed where *SLC51B, SLC51A, SLC10A6* and *ABCC4* were up-regulated and *SLCO3A1* was down-regulated in both EC cell lines compared to control endometrial cell line HIEEC. The highest difference in expression was seen for *SLCO1B3* (28930.7-fold up-regulation) and *ABCG2* (30.2-fold down-regulation) in HEC-1-A compared to HIEEC. Imunocytochemistry revealed significantly higher levels of OATP1B3 (*SLCO1B3*) (6.3-fold) in HEC-1-A compared to Ishikawa and low concentrations of ABCG2 in both EC cell lines.

In EC tissue significant up-regulation of *ABCC1* (1.6-fold) and down-regulation of *ABCG2* (3.2-fold) and *SLC51B* (2.1-fold) was observed compared to adjacent control tissue. In patients without lymphovascular invasion gene *SLCO1B3* was 15.6-fold up-regulated. Tumor grade had significant effects on expression of *SLC51B*, with lower levels seen in high grade cancers. Immunohistochemistry revealed significantly lower levels of ABCG2, OSTβ (*SLC51B*) and OATP1B3 (*SLCO1B3*) in EC compared to control tissue.

Our results suggest that OATP, OAT and ABC transporters have important roles in EC. Detailed investigation of E1-S uptake and efflux is thus in progress.

PII 80

Is the action of amyloid beta peptide in SH-SY5Y cells associated with profilin 1?

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Profilin 1, a 15 kDa promoter of actin polymerisation, is involved in a large number of physiological and pathological cell processes. As the central protein involved in cell cytoskeleton organisation and consecutive cell adhesion and migration processes it is of vital importance in brain development. Its role is important also for proper myelination in peripheral nervous system. On the other hand, irregular expression and action of profilin 1 can lead to various neuronal pathologies.

Amyloid β peptides, soluble and insoluble, are important players in the pathology of Alzheimer's and Parkinson's diseases. They may have important physiological functions and become toxic when there is an imbalance between their production and degradation. Our experiments on SH-SY5Y cell line demonstrated that the presence of the amyloid β peptide (25-35) decreases the protein level of profilin 1 in the cells. A consequence is lower actin polymerisation which affects cell adhesion properties. Regulation of profilin 1 function by amyloid β peptide provides new view on neurodegenerative processes that deserves to be further investigated.

Resolving the puzzle: How NLPs disrupt plant plasma membranes?

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Major diseases of crop plants that are caused by microbial plant pathogens are a considerable threat to global food security and lead to enormous economic loss. Nep1-like proteins (NLPs), secreted by taxonomically unrelated microorganisms – bacteria, fungi and oomyces, cause necrotic lesions of plant tissue and facilitate eudicot plant infection, but are not active against monocots. Recently, we identified glycosylinositol phosphorylceramides (GIPCs), a major class of plant sphingolipids, as target molecules for NLP's association with plant plasma membrane[1]. NLP from oomycete *Pythium aphanidermatum* (NLP_{Pya}) undergoes structural changes after binding to the terminal hexose moiety of GIPC. We proposed a model of early steps of NLP_{Pya} - membrane interaction and provided explanation for toxin's clade-specific selectivity[1]. However, the overall molecular mechanism by which NLP induce plant plasma membrane damage remains unknown. Identification of NLP's membrane receptor enabled exploitation of various GIPCs-containing lipid model systems. The data obtained by liposome sedimentation assay, dynamic light scattering, native triptophane fluorescence and giant unilamellar vesicles (GUVs) imaging contribute to our understanding of the nature and mechanism of NLP - membrane association, by either via pore-forming mechanism or other type of membrane integrity disruption.

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PII 82

Effects of acute versus long-term treatment with antipsychotics in liver cell model

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Patients often require medication over long time or for life, however, mechanisms of drug effects are often studied as the acute effects of high drug doses in cellular models. An example are metabolic effects associated with antipsychotic drugs that are mainly linked with long-term antipsychotic treatment. Therefore, long-term cell treatment seems more suitable choice for studying cellular antipsychotic effects.

To study metabolic dysfunctions associated with antipsychotic drugs, we use rat hepatoma cells (Fao) to compare differences between acute and long-term treatment by using antipsychotics with different metabolic effects in patients. The most prominent changes are observed in proliferative ability and in survival after acute exposure to an additional stressor. Therefore, long-term treatment is more appropriate method for studying antipsychotic effects on the hepatocyte cell line compared to the acute treatment.

Cytotoxicity of cytotoxic T cells is affected by endogenous as well as extracellular cystatin F

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Cytotoxic T cells (CTLs) can kill cancer and virus infected cells by releasing cytotoxic molecules, such as granzymes and perforin, from cytotoxic granules. Both, perforin and granzymes, are synthesised as an inactive pro-form and need to be proteolytically activated, a process were cysteine cathepsins are involved. Cathepsins C and H are responsible for activation of granzymes, while cathepsin L is implicated in perforin activation. Activity of cysteine cathepsins is regulated by their endogenous inhibitors, the cystatins, in cytotoxic cells most notably by cystatin F. Indeed, we demonstrated that levels of endogenous cystatin F are increased in hyporesponsive CTLs, characterised by decreased cytotoxicity. In addition, cystatin F interacted with cathepsins C and H and increased cystatin F levels correlated with decreased activities of cathepsins C and H and importantly, with decreased activity of the final cytotoxic effector molecule, granzyme B. Furthermore, we demonstrate that extracellular recombinant cystatin F can attenuate activities of cathepsins C, H and L in CTLs, leading to decreased activities of granzymes A and B. In addition, we tested processing of cathepsin C and perforin, neither of which was affected by extracellular cystatin F. Nevertheless, the effect on granzyme activation led to reduced ability of CTLs to kill target cells. Interestingly, when target cells, treated with cystatin F, were used in cytotoxicity assay, they were more resistant to killing by CTLs, confirming that in addition to endogenous and extracellular cystatin F even cystatin F present in the target cell can affect the ability of CTLs to kill target cells. To conclude, our results show that cystatin F is an important regulator of cytotoxicity of CTLs, regulating their ability to eliminate transformed or virally infected cells and thus affecting the immune response in cancer and infectious diseases.

PII 84

Identification of dipeptide repeat proteins interacting protein targets by proximity-dependent labeling

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Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) are debilitating neurodegenerative diseases that represent two ends of a complex disease spectrum. Aggregation of RNA binding proteins is one of the hallmark pathological features of ALS/FTLD, defining them as proteinopathies. Mutations of more than 30 different genes were linked to familial ALS/FTLD, including a hexanucleotide repeat expansion in the *C9orf72* gene. One of the major current hypotheses proposed for the pathogenicity of the expanded *C9orf72* repeat mutation is the accumulation of aggregates of dipeptide repeat (DPR) proteins: poly(GA), poly(GR), poly(PR), poly(PA), poly(GP), which are neurotoxic *in vitro*. Poly(GA) is the most hydrophobic and forms distinct perinuclear aggregates, whereas the highly charged poly(PR) and poly(GR) tend to accumulate in nucleoli and cytoplasm respectively. They all have been previously shown to interact with ribosomes, stress granules and low-complexity proteins. In the present study we aimed to identify the interactome of all five DPR proteins overexpressed in HEK293T cells using BioID2 proximity labeling. This technique harnesses the ability of the enzyme biotin ligase (birA) to biotinylate proximal endogenous proteins. We prepared constructs of the five DPR proteins consisting of 125 repeats conjugated to BioID2 enzyme by a flexible linker and transiently expressed them in cells. Bioinformatic analyses of proteomic data identified interaction candidates involved in protein translation, signal transduction pathways, protein catabolic processes, amide metabolic processes and RNA-binding. Selected protein candidates, including valosin-containing protein (VCP), which has been previously linked to ALS, were validated by immunoblotting. Altogether our results imply novel molecular mechanisms underlying the neurotoxicity of DPR proteins.

Genetic variation in cell cycle related genes and gastric cancer risk

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Introduction: Gastric cancer is the third most common cause of cancer-related deaths in the world. Non-specific symptoms lead to late diagnosis and poor outcome. A combination of genetic and environmental factors contributes to the disease development and progress. Cell cycle kinases are crucial for cell cycle progression and accurate cell division. Mutations in these genes lead to genetic instability and aneuploidy, which in turn contribute to carcinogenesis. In addition to driver mutations, which are highly penetrating changes, other genomic features, such as single nucleotide polymorphisms may contribute to gastric cancer susceptibility. These low penetrating changes may accumulate over time and in combination with other factors play a role in disease development and progress. The aim of this study was to determine whether selected polymorphisms in candidate cell cycle genes *AURKA*, *AURKC*, *BUB1B*, *CDC20*, *PLK2*, *PLK3* and *TTK* contribute to gastric cancer risk in Slovenian population.

Methods: We included a group of 620 patients diagnosed with gastric cancer and control subjects in the study. We used TaqMan allelic discrimination assays for SNP genotyping and analysed the results using generalized linear regression analysis to determine whether genotypes of selected variants increase the risk of gastric cancer in Slovenian patients in comparison to the control group.

Results: *PLK2* rs15009 (c.*58454523C>G) heterozygous genotype was significantly more represented in the control group than expected. Under the dominant model, carriers of at least one polymorphic allele had significantly lower odds for developing gastric cancer compared to carriers of two wild-type alleles (OR = 0.67, 95% CI = 0.48–0.92, P = 0.014).

Conclusion: In conclusion, the study revealed significant association between *PLK2* rs15009 genotypes and gastric cancer risk.

PII 86

Identification of biomarkers for early detection of kidney allograft dysfunction

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Background: Kidney transplantation is the optimal treatment for patients with chronic kidney disease. Compared to dialysis, successful transplantation prolongs and significantly improves the quality of patients lives. However, despite the considerable progress made, long-term survival of allografts has not been significantly improved. Renal biopsy is used as a "golden standard" to assess the function of allograft but it is an invasive method which cannot be used in a serial manner. Numerous studies focus on the detection and validation of various non-invasive biomarkers using an alternative method, i.e. liquid biopsy, where by identifying the presence or by measuring specific markers e.g. nucleic acids (DNA, RNAs) and extracellular vesicles (EVs) in body fluids, they can predict the course or outcome of the disease and eventually replace the classic invasive renal biopsy.

Methods: We are planning a prospective clinical study in which we will collect samples of renal biopsy, peripheral blood and urine. In the prospective part, we will isolate miRNA and cell-free DNA (cfDNA). We will also isolate EVs, check and quantify their cfDNA and miRNA content using both digital and quantitative real-time PCR. Blood and urine sampling will be performed every month during first six months after kidney transplantation, then every other month until one year, and finally at two years post operation. Additional sampling will be performed in case of complications.

Implications and conclusions: Our aim is to identify biomarkers for early detection of allograft dysfunction. We particularly want to assess the association of cfDNA, RNAs as well as various types of EVs with an early impairment of the transplanted organ, and the clinical utility of these biomarkers in monitoring and early detection of worsening renal function. We also want to establish a biobank and a database of patients with transplanted kidney that will enable future studies.

Research grants: P3-0323 and P1-0170.

Risk factor assessment for cryptorchidism in Slovenian population using array comparative genomic hybridisation (aCGH)

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Cryptorchidism is a common urogenital abnormality that manifests in about 2-4% of full term male births. It is associated with increased risk of infertility development. The mechanism for development of cryptorchidism is still unknown. It is a multifactorial disease that is at least partially genetically predetermined. Most studies in this research field were performed using candidate gene approach. Genome-wide studies of copy number variations (CNVs) in particular have been limited to a handful of studies. The aim of the present study was to analyse CNVs in 14 patients with non-syndromic cryptorchidism in Slovenian population. The analysis was performed using array comparative genomic hybridisation (aCGH) Microarrrays SurePrint G3 Unrestricted CGH (4x180K) which includes 16,650 genes included in RefSeq database. Results were analysed using data from DECIPHER, OMIM, ISCA, USCS genome browser and PubMed. Results revealed 16 CNVs in 11 patients. CNVs were located on 13 chromosomes and included 22 copy number loses and 5 copy number gains. Regions within CNVs overlap with 12 genes, including PGEM1, OTX1, FOXD1, MGAT1, and NEIL2. Four CNVs were intergenic and overlapped with regulatory regions. Additional functional studies are needed to determine whether these variations represent de novo CNVs and to further asses their clinical significance.

PII 88

Characterization of cell lines with CRISPR-Cas9 mediated deletion of late genes from cholesterol synthesis

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Cholesterol is the major sterol in mammalian cells and is crucial for its viability. Its synthesis includes over 20 reactions, starting from Acyl-CoA. We focus on the post-squalene part, where cholesterol is synthesized from lanosterol, the first sterol intermediate. Recently a variety of physiological functions are connected to these non-polar sterols, like role in spermatogenesis, oligodendrocyte remyelination and activations of nuclear receptor RAR Related Orphan Receptor C (RORC). Specific non-polar cholesterol intermediates can activate RORC *in vitro*, while the *in vivo* evidence and mechanistic proofs are lacking. It is proposed that by sterols activated RORC can modulate the expression of genes under control of RORE response DNA elements, that include also the circadian clock genes. To investigate the sterol-RORC signaling, we used CRISPR-Cas9 system on human immortal hepatocyte HepG2 cells, to knock-out four genes from the late part of cholesterol synthesis (*CYP51, DHCR24, SC5DL* and *HSD17B7*). Cells with knock-out in one of these genes are cholesterol auxotrophs and accumulate upstream sterol intermediates. Our hypothesis is that due to deletion in the genes from cholesterol synthesis the RORC signaling is altered, which will result in changed expression of genes under control of this nuclear receptor. For knock-out generation early exons were targeted to cause early stop codons and to eliminate translation of the proteins. The knock-outs were confirmed on single cell colonies with sequencing, western blots and with sterol metabolomics analysis. Since sterol intermediates from lanosterol towards cholesterol have very similar molecular structures and some even share the same molecular mass, we developed a new LC-MS method with which we can separate and quantify all the major sterol intermediates (10) in one run. The experiments to determine the mechanisms of RORC-sterol signaling and which of sterol intermediates are bound to RORC *in vivo*, are in progress.

Expression of potassium ion channel and calcium handling proteins in aorta of fructose and walnuts fed rats

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Aim: Fructose rich diet related insulin resistance induces endothelial dysfunction and alters basal vascular tone in blood vessels. Walnuts, high in monounsaturated fatty acids, may help optimize fat ingestion, but regular consumption might increase total fat and energy intakes. The recent studies have reported that eating nuts frequently is associated with reducing CVD risk. This study was performed to investigate the potential beneficial effects of daily walnut consumption on aortic ATP-sensitive potassium (KATP) channel, calcium channel and sodium-calcium exchanger protein.

Methods: Male Wistar rats were divided into two groups, control and fructose (10% fructose for 9 weeks). After 9 weeks, both groups were divided into two subgroups, with or without walnuts (2.4 g/day/animal for 6 weeks). Food, liquid and energy intake were determined. The expression in aorta lysate of KATP subunits (Kir6.1 and SUR-2B), α1C subunit of L-type Ca^{2+} channel (LTCC) and Na^{+}/Ca^{2+} exchanger 1 (NCX1) proteins were detected by Western blot.

Results: Fructose rich diet decreased food intake but increased liquid intake. Fructose and walnuts increased total energy intake alone and had greater effect in combination. Walnuts diet increased level of SUR-2B subunit of potassium channel without effect on expression of Kir6.1 subunit. Fructose alone as well as walnuts with or without fructose, decreased expression of LTCC compared to control group and walnuts-fructose combination decreased expression of NCX1 content in comparison with fructose group.

Conclusions: Our data suggest that walnuts selectively alters aortic ion channels, increasing expression of KATP and decreasing expression of Ca^{2+} handling proteins.

PII 90

Association of polymorphisms in selected matrix metalloproteinase genes with histological subtype and TNM stage of malignant mesothelioma

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Background: Malignant mesothelioma (MM) is a rare disease with a relatively short overall survival. Metalloproteinases (MMPs) have a vast biological effect on tumor progression, invasion, metastasis formation, and apoptosis. MMP expression was previously associated with survival in MPM. Our aim was to evaluate if genetic variability of *MMP* genes could serve as a biomarker associated with the TNM stage and the histological subtype of MM.

Methods: Among 181 patients with MM, 128 patients had epithelioid histological subtype and 115 patients had a higher tumor stage (III, IV). Fluorescence-based competitive allele-specific assay (KASPar) was used to genotype ten single nucleotide (SNPs) polymorphisms of selected *MMPs*: *MMP2* rs243865, rs243849 and rs7201, *MMP9* rs17576, rs17577, rs2250889 and rs20544, and *MMP14* rs1042703, rs1042704 and rs743257. The additive and dominant genetic models were used in statistical analyses. The association of genetic polymorphism with TNM stage and histological subtype was examined by logistic regression to calculate odds ratios (ORs) and their 95% confidence intervals (CIs).

Results: Carriers of polymorphic *MMP9* rs2250889 allele were significantly more likely to have a biphasic or sarcomatoid histological subtype (OR=3.54, 95% CI=1.24 - 10.07, p=0.018). Among the other tested SNPs none was significantly associated with the histological subtype. The tested *MMP* polymorphisms were not significantly associated with the TNM tumor stage.

Conclusions: The *MMP9* rs2250889 may predict a predisposition for a more aggressive mesothelioma subtype and therefore guide the potential treatment of MM in the era of targeted therapies and immunotherapy.

Glucose transport and metabolism in the heart of vitamin D treated rats

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The role of vitamin D and vitamin D receptor in maintenance of cardiovascular homeostasis is well documented, but despite diverse contributing mechanisms suggested, beneficial effects of vitamin D on the heart are still debatable. Furthermore, except the traditional role in regulation of calcium homeostasis, vitamin D has been also suggested to affect insulin signaling and glucose metabolism in extracardiac tissues. Based on these facts, vitamin D (cholecalciferol) was administered to adult male Wistar rats during 6 weeks to study its effects on molecules involved in cardiac glucose transport and metabolism. Expression, phosphorylation and/or subcellular localization of glucose transporter 4 (GLUT4), 6-phosphofructo-2-kinase (PFK2) and glycogen synthase kinase 3 (GSK3) were studied. Although the increased level of vitamin D was not accompanied with alterations in biochemical parameters and physical characteristics of rats, expression, phosphorylation and/or subcellular localization of glucose metabolism proteins in the heart were changed. The total level of major cardiac glucose transporter GLUT4 showed trend to increase subsequent to vitamin D administration, while the level of GLUT4 in plasma membrane in insulin-stimulated conditions was not altered. Protein level of GSK3 enzyme, important negative regulator of glycogen synthesis, was at the control level in vitamin D treated rats, but its insulin-induced phosphorylation at serine 9 was increased indicating reduced activity of GSK3. In addition, protein level of cardiac PFK2 enzyme involved in glycolysis was elevated after vitamin D treatment. On the other hand, vitamin D did not influence its serine 466 phosphoform. These findings encourage vitamin D supplementation as a cardiobeneficial protocol in numerous people with vitamin D deficiency.

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Microscopic imaging of the activity of cathepsin B and K in human glioblastoma tumors with metabolic mapping (enzyme histochemistry)

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Glioblastoma is the most lethal brain tumor, at least partly due to therapy-resistant glioblastoma stem cells (GSCs) that are protected in hypoxic peri-arteriolar GSC niches. We have found that cathepsin K (catK) is one of the highest differentially-expressed proteases in glioblastoma and that its expression is preferentially associated with peri-arteriolar GSC niches as we detected with immunohistochemistry in glioblastoma tissue sections. CatK expression was also found in glioblastoma cells and endothelial cells. However, we hardly found any activity of catK in glioblastoma tissue sections using metabolic mapping (Fig. 1A). Besides catK, catB was immunohistochemically detected in glioblastoma tissue sections (Fig. 1B). Its expression was ubiquitous and found in glioblastoma cells, endothelial cells, macrophages and microglia cells. In contrast to the absence of cathepsin K activity, metabolic mapping showed high catB activity in cells within peri-arteriolar GSC niches (Fig. 1C). CatB activity was inhibited by the cathepsin inhibitor leupeptin. We conclude that catK activity is involved in the release of GSCs from their niches by cleavage of the chemokine stromal-derived factor-1α (SDF-1α) that keeps CXCR4-positive GSCs in their niches. We conclude that catK protein is present in GSC niches in its inactive pro-form because catK is an aggressive protease and, therefore, its activity is tightly regulated in GSC niches. On the other hand, catB protein and its activity is localized in peri-arteriolar GSC niches (Fig. 1B,C) indicating that catB also has a yet unknown functional role in GSC niches in glioblastoma.

Nanobodies Anti-TufM (Nb225) and anti-vimentin (Nb79) are cytotoxic to glioblastoma (stem) cells and reduce cell migration

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Glioblastoma is one of the most fatal cancers, as most of the patients die within two years after diagnosis despite receiving standard therapy. One of the main reasons why therapy fails is the presence of glioblastoma cancer stem cells, that share characteristics with normal stem cells such as self-renewal and expression of stem cell markers. In glioblastoma, they can form new tumour and are resistant to chemo- and radiotherapy. One of the possibilities to target glioblastoma stem cells are nanobodies, antigen-recognizing parts of heavy-chain only antibodies produced by very few animals, such as llamas and camels. In our study, we determined the effect of two nanobodies, anti-TufM (Nb225) and anti-vimentin (Nb79) on survival and migration of glioblastoma cells.

At first, we determined the effect of temozolomide and nanobodies on survival of glioblastoma stem cells (NCH), glioblastoma mature cells (U251MG and U87MG) and astrocytes as a control after 24, 48 and 72 hours of incubation. Temozolomide had minor effect on glioblastoma cells and none to the astrocytes. Both of nanobodies, Nb225 and Nb79 showed no cytotoxic effect to astrocytes but decreased survival of NCH by 15-30%. Next, we determined the effect of two doses of both, temozolomide and nanobodies. Cells were treated with temozolomide or nanobodies for 72 hours when another dose was applied, and incubation was prolonged for another 72 hours. Two doses of temozolomide showed no effect on NCH and astrocytes. Two doses of Nb225, on the other hand, decreased survival of NCH644 by around 80%. Lastly, we determined effect of nanobodies on cell migration. Nb79 completely inhibited migration of U87MG. To conclude, we propose Nb225 as an agent to target glioblastoma stem cells and Nb79 as an agent to reduce glioblastoma cell migration.

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The characterization of DNA sequence containing G4C2 repeats

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The most common mutation in neurodegenerative disorders, amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), is an increased number of G_4C_2 repeats in the *C9orf72* gene. Healthy individuals have 2 – 23 G_4C_2 repeats, whereas more hundred to more thousand repeats are typically found in patients with ALS and FTD. The neurodegeneration mechanism mediated by mutation in *C9orf72* gene is not fully known. It has been shown that G_4C_2 DNA sequence forms G-quadruplexes. This is a secondary DNA structure made of planar G-quartet units held together and stabilized with a monovalent cation, usually potassium. A complementary DNA chain (C_4G_2) can also form secondary structures, so-called i-motives. So far, formation of G-quadruplexes has been shown for sequences of up to 8 G_4C_2 repeats. However, it remains a challenge to confirm G-quadruplex formation within DNA molecules containing longer sequences of G_4C_2 repeats.

The main goal of our work is to determine presence of G-quadruplex in DNA construct containing from 8 up to 48 G_4C_2 repeats and their biophysical and biochemical properties. Sequences containing 48 G_4C_2 repeats are of greater biological relevance, as ALS and FTD symptoms may be caused by 23 or more repeats. First, we studied the effects of temperature, pH and different ions on before mentioned constructs. We observed noticeable differences between constructs containing G_4C_2 repeats and control constructs, which are random DNA sequences of similar length to studied constructs. Despite the sequence being 100% G_4C_2, we successfully PCR amplified shorter regions of repeats with optimized polymerase chain reaction. This is crucial for obtaining modifications of the sequence for further research. One of such goals is to isolate single strands containing either G_4C_2 or C_4G_2 repeats and identification of proteins that bind to individual strands and secondary structures.

Autophagy is a Prosurvival Pathway in Chronic Lymphocytic Leukemia

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Chronic lymphocytic leukemia (CLL) is the most prevalent hematologic malignancy in the Western world. It is characterized by progressive accumulation of mature-appearing monoclonal CD5+, CD19+, CD20+ and CD23+ B-lymphocytes.[1] Since autophagy and ubiquitin-proteasome system (UPS) are two major systems involved in protein degradation and cellular homeostasis, we aimed to elucidate the anticancer potential of UPS inhibition and autophagy modulation in CLL.[2]

First, we determined the potency of FDA-approved proteasome inhibitors carfilzomib (CFZ), bortezomib, and ixazomib on CLL cell line MEC-1 with MTS assay. EC_{50} values for CFZ, bortezomib, and ixazomib were 7 nM, 11 nM and 46 nM, respectively. In addition, CFZ elicited synergistic antileukemic activity in combination with targeted therapy, demonstrating potential for its implementation in the treatment of CLL. On the other hand, autophagy activation with rapamycin resulted in increased survival of CFZ-exposed cells. Contrary to rapamycin, autophagy inhibitor bafilomycin A1 synergized with CFZ to enhance killing of malignant B lymphocytes. This synergistic effects can in fact be explained by CFZ's ability to modulate autophagy in MEC-1 cells, evidenced by changes in BECN1 and p62 protein levels.

To conclude, since autophagy elicits prosurvival activity during UPS inhibition, we suggest an inhibitor of autophagy pathway should be used in combination with proteasome inhibitors to prevent autophagy fostered resistance in CLL.

1. Eichhorst B, et al. *Ann Oncol*. 2015.
2. Kocaturk NM, et al. *Front Cell Dev Biol*. 2018.

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Effect of Ouabain on IL-6 Signaling in Human Skeletal Muscle Cells

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Cardiotonic steroids (CTS), which were used for centuries to treat heart failure, are endogenous hormones that play a role in regulation of cardiovascular system, salt and water homeostasis, metabolism, and immune system. Exercise is a major physiological stimulus that increases their secretion, indicating a role for CTS in adaptation to exercise. During exercise contracting skeletal muscles secrete interleukin-6 (IL-6). Once secreted, IL-6 exerts autocrine, paracrine, and endocrine effects by activating the JAK/STAT3 pathway via the IL-6Rα/gp130 receptor. We determined whether ouabain, which is thought to be a major endogenous CTS, modulates IL-6 action in human skeletal muscle cells.

Ouabain triggers biological effects by binding to Na^+, K^+-ATPase, an ion pump and a signal transducer that can act independently of ion transport. In pharmacological concentrations, ouabain triggers biological effects mainly by inhibiting Na^+,K^+-ATPase, thereby altering intracellular ion concentrations. In physiological concentrations, ouabain can activate intracellular signalling pathways without perturbing ion homeostasis. Here we examined whether ouabain modulates the IL-6/JAK/STAT3 pathway in human skeletal muscle cells.

We estimated expression of phosphorylated (Tyr705) and total STAT3 in the presence or absence of IL-6. Ouabain markedly suppressed basal and IL-6-stimulated STAT3 phosphorylation. Conversely, tocilizumab, a monoclonal antibody, which binds to IL-6Rα and blocks IL-6 action, reduced only stimulated STAT3 phosphorylation. While 10 nM ouabain did not alter total STAT3 expression, 50 nM ouabain markedly suppressed it. These results suggest that ouabain modulates the IL-6/JAK/STAT3 signalling pathway in human skeletal muscle cells via at least two mechanisms, which involve regulation of phosphorylation and expression of STAT3.

Collectively, our findings provide new insights into molecular mechanisms underlying ouabain action in skeletal muscle.

5-hydroxymethylcytosine alterations in peritumoral melanocytes and in normal melanocytes after skin exposure to UV irradiation

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Melanoma is the main cause of death from skin cancer. Excessive sun exposure is established as its major environmental risk factor. In addition to primary genetic changes, epigenetic alterations are now increasingly recognized as potentially reversible regulators of genomic anomalies. Loss of DNA hydroxymethylation mark 5-hydroxymethylcytosine (5-hmC) is significant in melanomagensis, but whether decreased 5-hmC is driven by UV irradiation remains unknown.

70 human excisions of chronically UV exposed skin and matched controls were evaluated. In addition, human foreskins were exposed to biologically relevant doses of UVB (302 nm) or UVA (365 nm) respectively and a dual anti-5-hmC and anti-MART-1 immunohistochemical staining was performed. Semiquantitative immunoreactivity scores were analyzed statistically and significant p-values ($p<0.05$) determined.

When comparing sun-protected versus chronically sun-exposed sites, lower levels of 5-hmC immunoreactivities were determined in melanocytes of sun-protected specimens ($p = 0.017$). Moreover, a more significant difference ($p = 0.0053$) was found between 5-hmC staining in normal skin melanocytes and atypical melanocytic hyperplasia of sun-damaged skin that surrounds melanomas. Finally, we sought to determine whether we could induce *in vitro* a UV effect on 5-hmC level similar to that seen in chronic sun-damage. Significantly decreased immunoreactivity for 5-hmC was observed in foreskin melanocytes after UV irradiation - a finding that replicated that observed in patient samples with chronically UV-altered melanocytes.

Our results suggest that UV irradiation effects on the epigenome may play a crucial role in the development of melanoma. Implementation of the dual staining for anti-5-hmC and anti-MART-1 may be helpful in defining melanocytes altered in the direction of melanomagenesis, and thus may serve as a practical assay in determining potential melanoma precursor fields amenable to surveillance and prophylactic therapies.

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Efficacy of C87, a small molecular inhibitor of Tumour necrosis factor, in human cells

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Tumour necrosis factor (TNF) represents an attractive therapeutic target in many inflammatory diseases including severe cases of Inflammatory Bowel Disease. Currently, anti-TNF therapeutic approaches are based on biologicals, which are complicated to produce and thus expensive to use. Therefore, these treatments are a considerable burden for the healthcare systems. To bypass these problems, small molecular inhibitors of TNF are also being developed. One such promising candidate is C87, which was successfully tested *in vitro* and *in vivo* on mice. So far, however, we found no reports of using C87 on human cells.

Here, we test the efficacy of C87 on several human cell lines of haematopoietic and epithelial origin. We first show that the C87 concentrations reported to efficiently block TNF signalling in murine cells are toxic for human cells. We next determine the non-toxic concentrations of C87 for human cells. Further, we test the non-toxic C87 concentrations for their efficiency to block direct (via TNF treatment) and LPS-induced TNF signalling in human cells.

Effects of alanosine and mercaptopurine on AMPK activity in skeletal muscle cells

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Type 2 diabetes has become a global epidemic. Insulin resistance in skeletal muscles, the main site of postprandial glucose uptake, is a major pathophysiological mechanism underlying type 2 diabetes. Activation of AMP-activated protein kinase (AMPK) reduces insulin resistance in skeletal muscles. Pharmacological compounds that activate AMPK may therefore bridge the gap in current therapeutic approaches, which are mostly ineffective in tackling insulin resistance, and lead to novel treatment options that oppose pathophysiological underpinnings of type 2 diabetes.

We previously showed that inhibition of *de novo* purine synthesis pathway with methotrexate promotes AMPK activation in skeletal muscle by reducing synthesis of inosine monophosphate (IMP) from ZMP, a direct AMPK activator and active form of the most widely used experimental AMPK activator AICAR. In our current study, we tested whether alanosine, inhibitor of adenylosuccinate synthetase, and mercaptopurine, inhibitor of IMP dehydrogenase, adenylosuccinate synthetase and adenylosuccinate lyase, which suppress synthesis of AMP and/or GMP, can activate AMPK and/or enhance AICAR-induced AMPK activation in skeletal muscle.

We tested effects of alanosine and mercaptopurine on primary human and rat L6 skeletal muscle cells. AMPK activation was analysed by measuring phosphorylation of AMPK (Thr172) and phosphorylation of a direct AMPK substrate acetyl-CoA carboxylase (Ser79) with western blotting. Alanosine and mercaptopurine both markedly suppressed proliferation of myoblasts, indicating effective inhibition of purine synthesis. Alanosine did not activate AMPK or enhance AICAR-induced AMPK activation. Conversely, mercaptopurine stimulated AMPK activation with or without AICAR.

Collectively, our results indicate that mercaptopurine promotes AMPK activation in skeletal muscle cells, suggesting inhibition of enzymes in the final steps of synthesis of AMP and GMP may represent a new strategy to activate AMPK.

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Effect of fructose enriched diet and walnut consumption on iNOS and phospho-ERK1/2 protein expression in rat aorta

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Aim: Excessive consumption of fructose is associated with development of obesity and CVD. On the other hand, studies have shown that walnut consumption reduces the risk of developing CVD. In inflammation, upregulation of inducible nitric oxide synthase (iNOS) produces large amounts of NO leading to endothelial dysfunction. Upregulation of iNOS by IL-1β in VSMC is mediated through extracellular signal–regulated kinase (Erk1/2) activation. We analyzed effects of fructose-rich diet and walnuts, alone and in combination on obesity development and Erk1/2 activation and iNOS protein expression in rat aorta.

Methods: Three weeks old male Wistar rats (n=) were divided into control group (C) fed by standard chow and fructose group (F) fed by the same food with addition of 10% fructose solution instead of tap water. After 9 weeks half of the animals from both groups consumed walnuts (2.4 g/day/animal) for additional 6 weeks (C+W group and F+W group). Protein content of iNOS and Erk1/2 phosphorylated on Thr^{202} /Tyr^{204} in aorta lysate were analyzed by Western blot.

Results: Body mass gain was higher in C+W compared to C group, and in F+W compared to C and F group. Animals in F+W had higher visceral fat mass compared to C and F group. Phosphorylation of Erk1/2 was increased in F+W compared to C group. Similary, iNOS protein expression was increased in F+W compared to C and F group.

Conclusions: Combination of fructose-rich and walnut diet results in upregulation of iNOS which coincide with Erk1/2 activation in rat aorta and development of obesity.

Targeting lipid droplets and autophagy to reduce cancer cell resistance to stress

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Lipid droplets (LDs) are fat storage organelles present in most eukaryotic cells. They are composed of a core of neutral lipids surrounded by a phospholipid monolayer and proteins. LD biogenesis is induced in cells exposed to excess nutrients and lipids and is characteristic of many diseases, such as obesity, diabetes and cancer. Paradoxically, their formation occurs also in cells fully deprived of nutrients and oxygen, suggesting that LDs are an integral part of the cellular stress response. LDs engage in a complex and as yet poorly defined relationship with autophagy, the major cellular recycling machinery and stress response pathway. First, autophagy may drive LD biogenesis by providing lipids recycled from other membranous organelles. Second, autophagy may participate in LD breakdown through a selective form of autophagy named lipophagy. Third, LDs may promote autophagy by providing lipids or signals that support the formation of autophagosomal membranes. We aim to discover the principal ways in which LDs and autophagy cooperate to promote the resistance of cancer cells to stress. We have found that lipid droplets are dynamically synthesized and broken down in cancer cells depending on the length and severity of nutrient deprivation. Autophagy is required for their biogenesis under acute starvation conditions, whereas lipolysis seems to be involved in their breakdown under milder conditions of starvation. By manipulating the activities of the major enzymes involved in LD metabolism in the context of activated or inhibited autophagy, we are currently examining the links between these two processes and their roles in cancer stress resistance. Using a xenograft mouse model, we are evaluating for the first time the feasibility of targeting the LD/autophagy axis to reduce tumour growth *in vivo*. Our work may open new perspectives in cancer research by providing important clues on the function of the recently recognized stress-associated organelle – the lipid droplet.

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Increased intracellular production and cell-to-cell transfer of serum amyloid A1 in IL-1β stimulated coronary endothelial cells

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Background: Serum amyloid A (SAA) is an acute-phase protein with important, pathogenic roles in the development of atherosclerosis. We aimed to determine whether induced human coronary artery endothelial cells (HCAEC) modulate SAA1/2/4 at the mRNA and protein levels, and influence intracellular location of SAA isoforms and intercellular transfer of SAA1.

Methods: HCAEC were stimulated with 1 ng/ml IL-1β, 10 ng/ml IL-6, and/or 1 μM dexamethasone for 24h. QPCR, Western blots, ELISA and fluorescence immunolabeling were performed for detection of SAA1/2/4 mRNA and protein levels, respectively. In SAA1 transfer experiments, a mixture of labeled (FITC or Cy3) and non-labeled SAA1 was added to HCAEC for 24h, followed by a combined incubation of SAA1-FITC and SAA1-Cy3 positive cells (10^5/well), with IL-1β for 4, 24 or 48h and analyzed by flow cytometry.

Results: IL-1β upregulated SAA1/2 mRNA expression levels, while expression of SAA4 was not affected. No difference was observed in intracellular SAA1/2 protein levels between stimulated and non-stimulated HCAEC. Intracellular SAA1 was found mainly as a monomer, while SAA2 and SAA4 formed octamers. In cells, SAA1/2/4 located mostly to the perinuclear area and tunneling membrane nanotubes. Co-culturing of SAA1-FITC and SAA1-Cy3 positive cells for 48h showed a significantly higher percentage of cells becoming double positive in IL-1β-stimulated (mean±SD; 60±4%) vs. non-stimulated cells (48±2%; $p<0.05$).

Conclusion: IL-1β induces SAA1 expression in HCAEC and promotes its intercellular exchange, suggesting an interesting impact on SAA synthesis, expression and transfer between cells that could contribute to atherosclerosis development.

Oestrane Derivatives as New Inhibitors of Steroidogenic Enzymes and as Anti-Proliferative Agents

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There is an ongoing demand for substantial research aimed at the development of new compounds with selective antitumorigenic activity. In the present study, 43 compounds structurally based on the 13ß-oestrone, 13α-oestrone or 17-deoxy-13α-oestrone skeleton were characterised in terms of their inhibitory properties against aldo-keto reductases AKR1C1, AKR1C2 and AKR1C3 and hydroxysteroid dehydrogenases HSD17B1 and HSD17B2, affinity for oestrogen receptor (ER) α and ß and anti-proliferative effects against human endometrial cancer (EC; HEC-1-A, KLE and Ishikawa) and ovarian cancer (OC; COV362 and Kuramochi) cell lines. The most potent inhibitors of AKR1C enzymes were C2, C4 di-halogenated oestrone-derived compounds (AKR1C1, IC50=0.73 μM; AKR1C3, IC50=6.31 μM) and an oestrone derivative containing an O-allyl group at the C15 (AKR1C2, IC50=0.485 μM). At 100 μM concentration, oestrone derivatives containing an azide (-N3) or O-allyl group at the C15 completely inhibited HSD17B1, and 2 compounds belonging to the series of C2/C4 mono-halogenated 17-deoxy-13α□oestrone derivatives showed >90% inhibition of HSD17B2. The most potent inhibitor of AKR1C2 and HSD17B1 showed binding affinity for ERß only (relative binding affinity (RBA)=20.53%) and low affinity for oestrogen receptors (RBA(ERα)=4.42%, RBA(ERß)=5.01%), respectively.

Cell viability was evaluated using alamarBlue metabolic assay. At 100 μM concentration, 9 compounds showed an anti-proliferative effect on EC cell lines, 4 on OC cell lines and 13 on both EC and OC cell lines. Determinations of EC50 values are currently in progress.

Our results suggest that chemical modifications of the A- and D-ring of the oestrane core may represent a promising strategy for design and development of more potent and specific inhibitors acting on steroidogenic enzymes and as anti-proliferative agents for hormone-dependent endometrial and ovarian cancers.

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Functional characterization of Tyr10 phosphorylation site of the Na^+, K^+-ATPase α1-subunit in skeletal muscle cells

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Na^+, K^+-ATPase (NKA) is an ATPase and a transmembrane pump that transports Na^+ and K^+ ions across the plasmalemma against their concentration gradients and plays a central role in preservation of ion homeostasis. In skeletal muscle transitions between resting and actively contracting state are coupled with rapid increases in energy consumption and transmembrane ion transport. Thus, tight regulation of NKA is mandatory for skeletal muscle function and whole body homeostasis.

Phosphorylation of its catalytic α-subunit, which has four isoforms (α1-4), plays an important role in regulation of NKA. In the α1-subunit, the isoform with the broadest tissue distribution, several Ser/Thr phosphorylation sites were functionally characterized. On the other hand, little is known about the Tyr10 phosphorylation site on the cytoplasmic N terminus of the α1-subunit. It was previously shown that acute insulin stimulation increases Tyr10 phosphorylation and activates NKA in kidney cells. However, the physiological role of Tyr10 (de)phosphorylation and signalling pathways that regulate it are largely unknown. Further, regulation of Tyr10 phosphorylation in skeletal muscle cells has not been established.

We determined whether AMP-activated protein kinase (AMPK), a cellular energy sensor, modulates Tyr10 in cultured human skeletal muscle cells. AMPK activator AICAR induced a time-dependent decrease in Tyr10 phosphorylation. Other pharmacological AMPK activators, such as A769662, which activates AMPK directly, FCCP (mitochondrial uncoupler), ionomycin (Ca^{2+}-ionophore), and 2-deoxyglucose (inhibitor of glycolysis), also decreased phosphorylation of Tyr10. In addition, we found that Try10 and the surrounding amino acid sequence are well-conserved in jawed vertebrates (*Gnathostomata*), suggesting functional importance of Tyr10 in the vertebrate lineage.

Taken together, our results highlight a link between AMPK activation and regulation of NKA in skeletal muscle cells.

Hormonal and pharmacological regulation of AMP-activated protein kinase in cultured skeletal muscle cells

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Skeletal muscles, one of the most metabolically active tissues and a major site of insulin-dependent glucose uptake, are important for maintaining normal blood glucose levels. Insulin resistance in skeletal muscle can lead to increased blood glucose levels and type 2 diabetes (T2D). AMP-activated protein kinase (AMPK), an intracellular energy sensor and regulator of energy metabolism, is a promising target for the treatment of insulin resistance and T2D. AMPK activity is modulated by various physiological stimuli, such as energy stress and hormones, and various pharmacological agents.

AMPK is activated allosterically and by phosphorylation of Thr172. AMPK has other phosphorylation sites, which have received much less attention than Thr172, including Ser485, which inhibits AMPK activation after phosphorylation. In our study, we investigated the effect of metabolic hormones (insulin and adrenaline) and various pharmacological AMPK activators on phosphorylation of Thr172 and Ser485 in cultured rat L6 skeletal muscle cells, a standard and widely used *in vitro* model for investigation of skeletal muscle. Activation of AMPK was estimated by measuring phosphorylation of acetyl-CoA carboxylase (ACC, Ser79), a direct downstream target of AMPK.

We found that adrenaline and insulin stimulated Ser485 phosphorylation and decreased Thr172 and ACC phosphorylation, indicating that both hormones inhibit AMPK activity. Pharmacological AMPK activators AICAR and A-769662 stimulated phosphorylation of Thr172, but had no effect on phosphorylation of Ser485, while 2-deoxy-glucose and H_2O_2 stimulated phosphorylation of both sites.

Our results highlight that metabolic hormones and pharmacological AMPK activators differentially modulate phosphorylation of Thr172 and Ser485, indicating existence of multiple regulatory signalling pathways. Dissection of these pathways may open new opportunities for identification of pharmacological targets and/or strategies for treatment of T2D.

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Quorum sensing and quorum quenching in *Campylobacter jejuni* – selection of the reporter strains of *Vibrio harveyi* for optimized studying

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In the last decade, bacterial communication and their coordinative behavior gained considerable attention. Sending, receiving and processing the information, in the form of signal molecules enables one cell organisms to have a multicellular entity, as well as better survival in a complex environment. Different studies have shown that *Campylobacter jejuni* contains quorum sensing (QS) mechanism, which are regulated via AI-2 signal molecules. Measuring the bioluminiscence of the reporter strain *Vibrio harveyi* BB170 (mutation in *luxN*, AHL receptor) is the most commonly used model for studying QS in *C. jejuni*. Two strains of *C. jejuni* (81-176 and cj11) have been shown to stimulate the bioluminiscence of the reporter strain *V. harveyi* BB886 (mutation in *luxP*, AI-receptor). We decided to study the QS of *C. jejuni* NCTC 11168 with different reporter strains of *V. harveyi*. We wanted to optimize the method and find the most appropriate strain for further study of QS and quorum quenching (QQ), which is triggered by plant inhibitors in *C. jejuni*. We used the wild type of *V. harveyi* (BB120), as well as three different mutants of *V. harveyi*: BB152 (mutation in *luxM*, AHL synthase), MM30 (mutation in *luxS*, AI-2 synthase) and BB170. Although *V. harveyi* BB170 is the most commonly used strain for studying QS, our results showed that it has significantly lower magnitude of response to spent medium of *C. jejuni* than *V. harveyi* MM30. We showed that *V. harveyi* MM30 has the best response to spent medium of *C. jejuni*. In conclusion, *V. harveyi* MM30 could potentially be used to study QS and QQ in *C. jejuni,* but appropriate controls have to be included.

Distribution of purinergic receptors and transient receptor potential channels in urinary bladder wall – the role in urothelial signalling

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The urinary bladder addresses fundamental physiological challenges of micturition cycle: storage and voiding. Either can go wrong, leading to one of the numerous lower urinary tract symptoms. To sustain these challenges, the urinary bladder wall is organized into mucosal, muscular and serosal/adventitial layers. The mucosal layer consists of urothelium, which forms a tight blood-urine barrier, and a lamina propria that contains interstitial cells of Cajal, vasculature and nerve terminals. For normal function of micturition cycle intercellular signalling must be tightly coordinated. This is accomplished by a complex bladder sensory web, which includes plasma membrane channels and receptors as well as released mediators. Currently the role of purinergic P2X receptors and transient receptor potential (TRP) channels in the urinary bladder wall is under special attention since they provide promising therapeutic targets. However, there is no consensus on their expression and localization in the urothelium. We have tested an array of antibodies on normal human samples by western blotting. Selected antibodies against P2X1, P2X3, P2X6, P2X7, TRPV1, TRPV4, TRPV6 and TRPM7 showed positive reaction and appropriate molecular weights. Only these antibodies were used for immunohistochemistry. P2X1 and P2X3 was detected on afferent neurons and interstitial cells of Cajal. P2X6 was not detected in the normal human bladder. P2X7 was detected in urothelium, mainly in intermediate and basal cell layers. TRPV1 was seen only in detrusor. TRPV4 was positive in lamina propria, but surprisingly most clearly on leucocytes. Antibodies against TRPV6 and TRPM7 stained urothelium, with increasing intensity toward the apical surface. Our results show that some purinergic receptors and transient receptor potential channels are present also in urothelium, confirming that this tissue has not only the role in permeability barrier but is also a part of bladder sensory web.

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The variation of intracellular calcium concentration in different endothelial cells in response to several vasoactive substances

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Calcium (Ca^{2+}) is an important second messenger involved in signal transduction and has a wide-ranging physiological role, also in endothelial cells. This study was aimed to characterize putative *in vitro* models for tumor and normal vasculature i.e. human microvascular endothelial cell line HMEC-1 and HUVEC-derived endothelial cell line EA.hy926, respectively in regards to variation in intracelluar Ca^{2+} activity $[Ca^{2+}]_i$ induced by different vasoactive agonists. Confluent cells were trypsinized, resuspended in Ca^{2+}-supplemented or Ca^{2+}-free PBS and loaded with 2.5 µM Fura2-AM. The dye was removed and cells were seeded in a black 96-well plate. The F_{340}/F_{380} ratio was used as an index of the variation of $[Ca^{2+}]_i$. For an initial 40 s period, the baseline fluorescence was determined. Then, different compounds i.e. bradykinin (BK; 10 µM), angiotensin II (ATII; 10 µM), ATP (100 µM), acetylcholine (300 µM) ionomycin (10 µM) and thapsigargin (TG; 1 µM), were injected automatically and their effect on the fluorescence intensity was recorded for an additional 300 s period. In some experiments, the cells were pre-treated either with selective Ca^{2+} channels inhibitors, i.e. 0.5 mM $LaCl_3$ or 0.5 µM $GdCl_3$ or 1 µM TG. Obtained results indicate that both cell lines have comparable intracellular Ca^{2+} stores; however HMEC-1 cells empty their stores faster in Ca^{2+}-free conditions. There were differences in endothelial Ca^{2+} response evoked by ATII and BK, to which HMEC-1 cells did not respond. When intracellular Ca^{2+} stores were depleted by TG, there was no response to ATP, indicating purinergic signaling through metabotropic P2Y receptors. In conclusion, differences in the vasoactive agonists-induced $[Ca^{2+}]_i$ suggests difference in the expression level of membrane receptors and the rates of Ca^{2+} stores unloading in tested endothelial cells, which is in compliance with differences between tumor and normal vasculature.

NLRP3 inflammasome is triggered by trimerisation of pyrin domains and can be specifically inhibited by designed peptides

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NLRP3 inflammasome is a multiprotein complex which forms within cells in response to various stress-associated triggers in order to activate the production of proinflammatory cytokines. The mechanism of NLRP3 inflammasome activation including the stoichiometry of NLRP3 oligomerisation is not fully understood, which hinders the design of effective and specific NLRP3 inflammasome inhibition. We aimed to determine the minimal NLRP3 oligomerisation state necessary to initiate inflammasome complex formation and to design specific peptide based inhibitors of NLRP3 inflammasome. In order to define the minimal activating NLRP3 oligomerisation state, we prepared retrovirus-transduced stable macrophage cell lines which express NLRP3PYD bound to various oligomerisation domains (dimerisation and trimerisation) under the doxycycline control and observed that trimerisation but not dimerisation of NLRP3 promoted inflammasome activation. We also designed putative inhibitory peptides which could potentially interfere with the protein interactions. The design was based on the crystal structures of the PYD and CARD interaction domains and on the pathological mutation hotspots in the NLRP3 NACHT domain. We identified peptides that were inhibiting the activation of caspase-1 and the release of IL-1β. The inhibition occured with various types of NLRP3 inflammasome triggers. We found that some of the peptides specifically inhibited the NLRP3 inflammasome. Peptides also effectively inhibited IL-1β in cell lines with NLRP3 mutations linked to cryopyrinopathies. Most promising peptide inhibitor was equipped with peptide sequence which allows transfer through the blood-brain barrier. It localised inside the cells as well as within the brain of mice after intravenous injection Designed peptides with the identified minimal oligomerisation state provide an insight into the mechanism of NLRP3 inflammasome assembly and provide the basis for the development of novel antiinflammatory strategies.

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The effect of sulfasalazine on the regulation of glucose metabolism in skeletal muscle

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Type 2 diabetes is the most common form of diabetes and major public health challenge in modern society. Skeletal muscles, a major site of insulin-stimulated postprandial glucose uptake, are important target tissue for treatment of insulin resistance and hyperglycemia in type 2 diabetes. Sulfasalazine (SSZ), an aminosalicylate and sulfapyridine, is anti-inflammatory and immunosuppressive drug used for treatment of chronic inflammatory bowel disease and inflammatory rheumatic diseases.

Salicylic acid and some of its derivatives ameliorate glucose homeostasis in type 2 diabetes, suggesting SSZ may exert similar metabolic effects. Consistent with this idea, clinical observations suggest that SSZ may increase the risk of hypoglycaemia in patients with rheumatic diseases and type 2 diabetes. Furthermore, animal studies showed that SSZ may block development of diabetic neuropathy and retinopathy. Here we examined whether SSZ modulates insulin signalling in cultured skeletal muscle cells.

To assess the effects of insulin, we used cultured rat L6 skeletal muscle cells, a standard *in vitro* model in skeletal muscle research. Activation of insulin signaling pathway was assessed by measuring phosphorylation of serine/threonine kinase Akt (aka protein kinase B, PKB) at Ser473 and its 160 kDa substrate AS160 (aka TBC1D4) at Ser588, a Rab GTPase activating protein. Phosphorylation of AS160 relieves suppression of GLUT4 translocation to the plasma membrane, thus leading to an increase in glucose uptake. Effect of SSZ on the Akt-AS160 pathway was assessed in the presence of insulin or platelet-derived growth factor (PDGF). We found that SSZ tends to suppress insulin-stimulated and PDGF-stimulated Akt phosphorylation in cultured skeletal muscle cells. SSZ markedly suppressed phosphorylation of AS160 in the presence or absence of insulin or PDGF.

Collectively, our results do not support the notion that SSZ improves insulin action and stimulates glucose uptake in skeletal muscle.

Regulation of LPS/TLR4/IL-6 signalling pathway in human skeletal muscle cells

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Interleukin 6 (IL-6) is a pleiotropic cytokine that is secreted from a wide spectrum of cells and tissues, including immune cells, adipocytes and contracting skeletal muscle. IL-6 that is chronically secreted from immune cells and adipocytes is mainly associated with proinflammatory effects and insulin resistance. Conversely, IL-6 that is acutely secreted from contracting skeletal muscles suppresses inflammation and exerts beneficial metabolic effects. Differences in biological action of IL-6 secreted from different tissues in different contexts, suggests existence of distinct signalling pathways linking physiological and pathophysiological stimuli to regulation of IL-6 expression.

Expression of IL-6 under inflammatory conditions is driven by NF-κB, a transcription factor that plays a central role in stress-induced immune and inflammatory responses. Classical NF-κB signalling is induced by LPS-dependent activation of TLR4 receptor. The expression of TLR4 and activity of the LPS/TLR4/IL-6 pathway is also modulated by contractile and metabolic activity of skeletal muscle. Here we determined whether TLR4 might be involved in regulation of spontaneous secretion of IL-6 from skeletal muscle cells in the absence of LPS.

We estimated phosphorylation of NF-κB, IκB and ERK1/2 and expression of *IL-6* mRNA under basal and LPS-stimulated conditions with or without inhibitors that suppress TLR4 (lipid IVa and CLI-095), NF- κB (parthenolide) or ERK1/2 (PD184352) signalling. Lipid IVa, CLI-095 and parthenolide suppressed LPS-induced NF-κB activation and *IL-6* mRNA expression in skeletal muscle cells, while PD184352 had no effect. Lipid IVa and CLI-095 also tended to reduce, but did not abolish, basal IL-6 mRNA expression, indicating TLR4 may promote IL-6 secretion even in the absence of LPS treatment. Collectively, our results suggest that TLR4 may play a role in regulating constitutive IL-6 secretion from cultured skeletal muscle cells.

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Mycophenolate mofetil promotes activation of AMP-activated protein kinase in cultured skeletal muscle cells

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Mycophenolate mofetil (MMF), an inhibitor of inosine monophosphate (IMP) dehydrogenase and an immunosuppressive drug, is used for prevention of organ rejection after transplantation and treatment of autoimmune diseases. Transplant recipients have increased risk for development of post-transplant diabetes mellitus (PTDM). While several mechanisms contribute to its development, immunosuppressive therapy is a major contributing factor. Unlike many immunosuppressive drugs, MMF has not been linked to PTDM, indicating it exerts protective metabolic effects.

Activation of AMP-activated protein kinase (AMPK), a cellular energy sensor, in skeletal muscle improves dysregulation of glucose homeostasis in type 2 diabetes. ZMP (aka AICAR-monophosphate) is an endogenous purine precursor and a direct AMPK activator. ZMP, synthesized in the de novo pathway of purine synthesis, is converted to IMP, which is further metabolized by IMP dehydrogenase to xanthosine monophosphate or by adenylosuccinate synthetase to adenylosuccinate. Here we determined whether inhibition of IMP dehydrogenase by MMF promotes ZMP accumulation and AMPK activation in cultured skeletal muscle cells.

We treated rat L6 skeletal muscle cells with MMF and/or AICAR, a precursor of ZMP and the most widely used exogenous AMPK activator. As estimated by phosphorylation of AMPK and its downstream target acetyl-CoA carboxylase, AICAR-stimulated AMPK activation was markedly enhanced by MMF, indicating MMF effectively suppressed IMP dehydrogenase. Conversely, MMF alone did not activate AMPK, which suggested endogenous ZMP does not sufficiently accumulate due to conversion of IMP to adenylosuccinate. In line with this notion, combined treatment of skeletal muscle cells with MMF and alanosine, an inhibitor of adenylosuccinate synthetase, promoted AMPK activation.

In summary, we showed that MMF promotes AMPK activation in cultured skeletal muscle cells, suggesting a role for AMPK in protection against PTDM.

Engineering atypical tetracycline formation in *Amycolatopsis sulphurea* for the production of modified chelocardin antibiotic

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To combat the increasing spread of antimicrobial resistance and the shortage of novel anti-infectives, one strategy for the development of new antibiotics is to optimize known chemical scaffolds. Our strategy focusses on the biosynthetic engineering of *Amycolatopsis sulphurea* for derivatization of the atypical tetracycline chelocardin and its potent broad spectrum derivative 2-carboxamido-2-deacetyl-chelocardin. Heterologous biosynthetic genes were introduced into chelocardin producer to modify functional groups and generate new derivatives. A number of modified chelocardin analogues were generated and we thus demonstrate cooperation of chelocardin polyketide synthase with tailoring enzymes involved in biosynthesis of oxytetracycline from *Streptomyces rimosus*. An interesting feature of chelocardin, compared with oxytetracycline, is the opposite stereochemistry of the C4 amino group. By incorporating genes involved in *N,N*-dimethylation from oxytetracycline gene cluster we also generated chelocardin derivatives with opposite stereochemistry at C4. Analysis of the antimicrobial activities of the modified compounds demonstrated that the primary amine in the *R* configuration is a crucial structural feature for activity of chelocardin. Unexpectedly, C10 glycosylated chelocardin analogues were identified, thus revealing the glycosylation potential of *A. sulphurea*.

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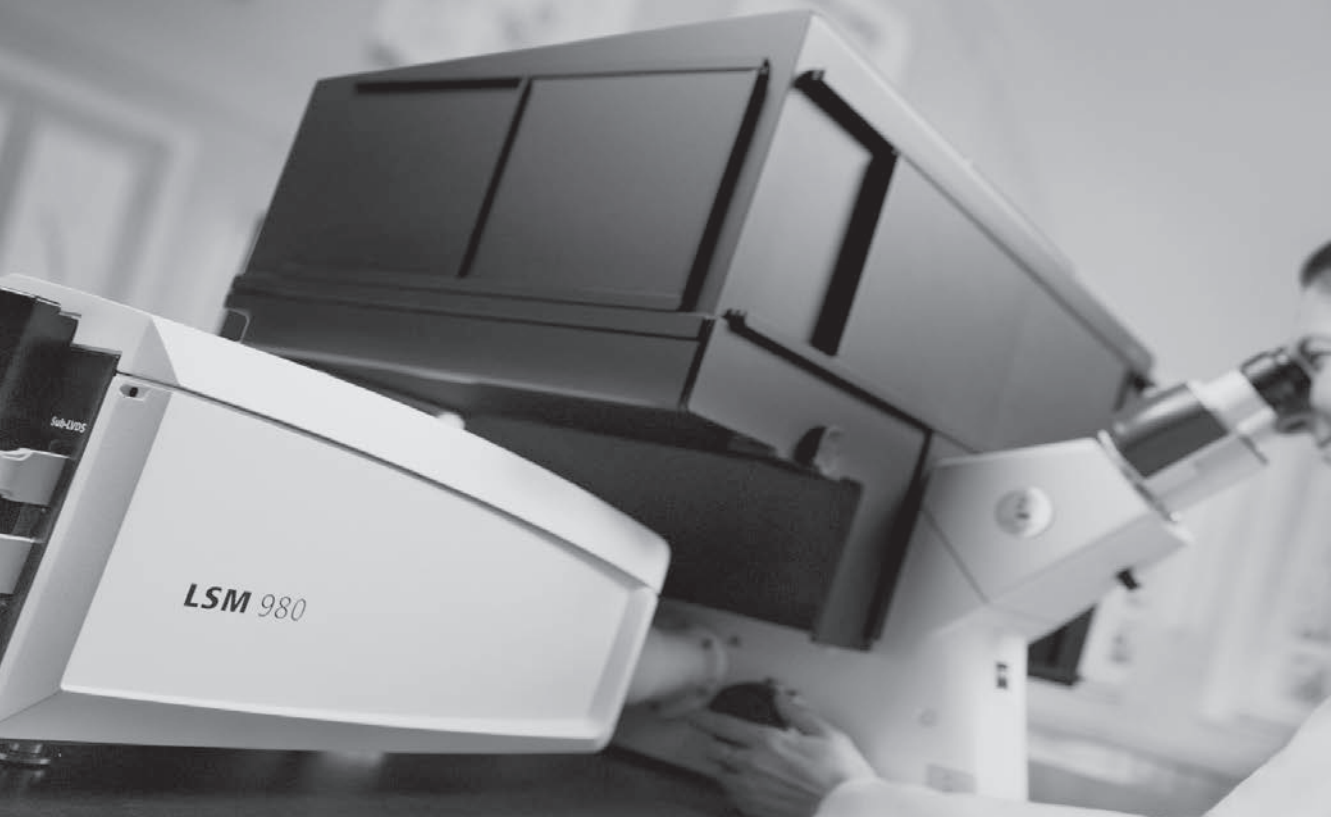

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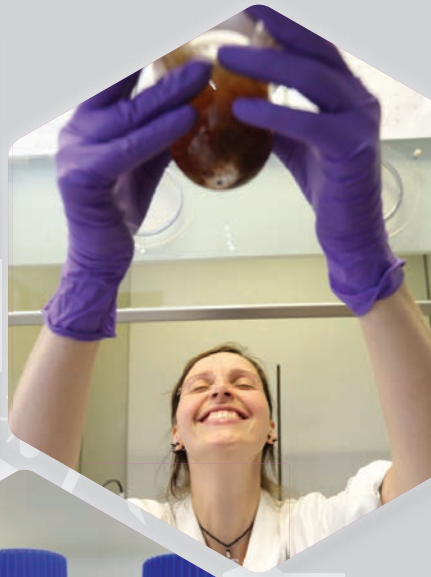

Basic and applied research on biotechnology, environmental protection, structural and theoretical chemistry, analytical chemistry, materials research, and chemical engineering.

In line with the priority areas of the EU Research and Innovation: genomics and biotechnology for health, nanotechnology, quality and safety of food, as well as nutrition, sustainable development, and global change.

We expand knowledge and transfer it to the domestic chemical, pharmaceutical, tire, and food industries.

We are aware of the power of youth so we transfer our knowledge on younger generations with providing many means of collaborations. mladi@ki.si

Cutting-edge science ...

Everything we do, we do for the good of people.

Quality is the basis of our dedication to the patients and drives our commitment to health. Our work is based on extensive knowledge and experience, mutual trust, inclusion and valuing diversity, as well as the highest ethical values.

Constant investments into research, innovation and the development of our production enable us to offer high-quality, safe and accessible medication. With our long-term plan of development, we are creating the conditions for new job openings, as well as providing education and enabling experts to develop into top scientists.

As a responsible employer, we care for the development of our employees, act responsibly toward local communities and contribute to the sustainable development of the environment.

Lek is a valued member of Novartis, the leading global company in the pharmaceutical industry.

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