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Medicinska fakulteta

Molekularna medicina in biotehnologija
Molecular Medicine and Biotechnology

Simpozij z mednarodno udeležbo ob 40. obletnici Inštituta za biokemijo in 20. obletnici Medicinskega centra za molekularno biologijo

International symposium at 40th anniversary of Institute of Biochemistry and 20th anniversary of Medical Centre for Molecular Biology

Book of Abstracts

Ljubljana, Faculty of Medicine

June 27-29, 2012

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Molecular Medicine and Biotechnology
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Foreword

Dear, guests, speakers and participants!

This year, we are proud to hold the Symposium at 40th anniversary of the Institute of Biochemistry and 20th anniversary of the Medical Centre for Molecular Biology. Institute of Biochemistry is a research institute which has in its 40-year history contributed to the development and transfer of new methods and skills in clinical practice and established successful cooperation with top research institutes from around the world, thereby contributing to the wealth of knowledge in medical and life sciences. The mission of the Institute is to promote excellence in research and graduate / undergraduate education in molecular genetics and biochemistry, to foster collaborations between researchers from different institutions, and to provide an interdisciplinary forum for experimentalists and theoreticians combining their expertise and reaching new frontiers in the molecular sciences. The infrastructure at the Institute has in the past supported the research of over 50 graduate students and numerous undergraduate students in different fields of biochemistry and molecular genetics.

Medical Centre for Molecular Biology, dwelling at the Institute of Biochemistry, has introduced research on human genome into Slovenian medical research and practice. It was established in 1992 by a consortium of institutes of the Faculty of Medicine at University of Ljubljana and some clinical institutes in Ljubljana, in order to coordinate research and teaching activities in the field of medical molecular biology / genetics among institutes of the Medical Faculty, departments of the University Medical Centre Ljubljana, and the Institute of Oncology in Ljubljana. Many experts trained here over the past years are now leaders of research and diagnostic laboratories in Slovenia.

Both institutions wish to mark these anniversaries with a Scientific Symposium, which will reflect research advances and present new directions of research through an attractive program presented by professors from both institutions, alumni fellows, leading researchers from collaborating research centers, and medical doctors, who have been trained in our facilities over the years and are today running prominent medical diagnostic laboratories. Furthermore, the program will be enriched with world-famous guest-speakers who will share their scientific proficiency in key speeches with the audience.

We are privileged to thank the President of The Republic of Slovenia, dr. Danilo Türk, for his support by granting his honorary Patronage.

Finally, we wish to thank the organizing team and the sponsoring institutions, agencies and companies for their invaluable contributions to Molecular Medicine and Biotechnology 2012.

Prof. dr. Ana Plemenitaš
Head of Institute of Biochemistry

Prof. dr. Radovan Komel
Head of Medical Centre for Molecular Biology

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Matija Peterlin (US)
Damjana Rozman (SI)
Jure Stojan (SI)
Mike Taussig (GB)
Jernej Ule (GB)
Matjaž Zorko (SI)

General Information on Symposium

Conference Venue

Faculty of Medicine, University of Ljubljana, Korytkova 2, Ljubljana, Slovenia.

Registration and Information Desk

All days of the symposium the registration desk will be located in the lobby of the Faculty of Medicine. The certificate of attendance will be issued at the registration desk.

REGISTRATION AND INFORMATION DESK OPENING HOURS:

Wednesday, 27 June 2012, 8.30 – 18.00
Thursday, 28 June 2012, 8.30 – 12.00
Friday, 29 June 2012, 8.30 – 17.00

Name Badges

All participants will receive a name badge upon registration and are kindly requested to wear badges at all times during the symposium.

Presentation Preview and Deposition

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

Poster Display

There will be only one poster session held on Wednesday, June 27, 2012 from 18.30 to 20.00 in the lobby and seminar of the Faculty of Medicine.

Presenters are kindly asked to mount their posters by 9.00 on Wednesday, 27 June 2012, and remove them on Friday, 29 June 2012 by 12.00.

Presenters should look-up the numbers assigned to their posters in the program book 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 the posters.

Authors are kindly asked to stand with their poster for the duration of the session.

Commercial Exhibitions

All sponsors exhibiting at the symposium will have their desks ready in the lobby of the Faculty of Medicine by Wednesday, 27 June 2012 at 8.00. All tables will be assigned in advance.

Social Program

Social program for all participants includes Welcome Reception on the first day of the Symposium.

Walk to the river port through the Old Town of Ljubljana and boat tour on Ljubljanica & Picnic are limited to invited speakers and members of IBK only.

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15.00 – 16.35	**Genetic Factors and Genetic Analysis**
16.35 – 17.00	Coffee Break
17.00 – 18.30	**Pharmacogenomics, Pharmacogenetics & Therapeutic Issues and Drug Design**
18.30 – 20.00	**Poster Session & Welcome Party with Snacks**

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Friday, 29 June 2012

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09.00 – 09.10	Short Break
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18.00 – 21.00	**Dinner for Foreign Invited Speakers and Program Committee at Ljubljana Castle**

Detailed Program

Wednesday, 27 June 2012

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10.00 – 11.00 **Opening Ceremony**

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Sir Richard J. Roberts, EMBO Member, *New England BioLabs, US*

Genomes and bioinformatics as key discovery tools in the 21st century

11.45 – 12.00 **Short Break**

12.00 – 13.30 **Magic Challenges**

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Translational control mechanisms in metabolic regulation: critical role of RNA binding proteins, microRNAs, and cytoplasmic RNA granules

12.30 – 13.00 Mauro Giacca, *ICGEB, IT*

"How can you mend a broken heart (Bee Gees, 1971)" – Searching for novel genes and microRNAs inducing myocardial protection and regeneration

13.00 – 13.30 Francisco E. Baralle, *ICGEB, IT*

hnRNPs dysfunction and neurodegenerative diseases

13.30 – 15.00 **Lunch**

15.00 – 16.35 **Genetic Factors and Genetic Analysis**

Chair: Vita Dolžan

15.00 – 15.30 Pier Franco Pignatti, *University of Verona, IT*

Coronary artery disease: the search for genetic factors

15.30 – 15.50 Tadej Battelino, *University of Ljubljana, SI*

Molecular biology and biochemistry in clinical routine

16.50 – 16.05 Janja Marc, *University of Ljubljana, SI*

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17.00 – 18.30 Pharmacogenomics, Pharmacogenetics & Therapeutic Issues and Drug Design

Chair: Jurij Stojan

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Pharmacogenomics and pharmacoepigenomics of drug treatment

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18.30 – 20.00 Poster Session & Welcome Party with Snacks

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12.30 – 14.00 Walk to the Port through the Old Town

14.00 – 17.00 Embarkment at the River Port Mesarski most for Ljubljanica Trip & Picnic

17.00 – 18.00 Retour to Ljubljana

Friday, 29 June 2012

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13.45 – 14.50 Molecular Mechanisms & Biomarkers of Disease

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14.15 – 14.35 Tea Lanišnik Rižner, *University of Ljubljana, SI*

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15.20 – 16.45 Biochemistry, Microbial World & Biotechnology

Chair: Matjaž Zorko

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18.00 – 21.00 **Dinner for Foreign Invited Speakers and Program Committee at Ljubljana Castle**

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EMBO Opening Lecture

Wednesday, 27 June 2012

11.00 – 11.45

Sir Richard J. Roberts, EMBO Member, *New England BioLabs, US*

Genomes and bioinformatics as key discovery tools in the 21st century

GENOMES AND BIOINFORMATICS AS KEY DISCOVERY TOOLS IN THE 21ST CENTURY

Richard J. Roberts[1]

[1]NEW ENGLAND BIOLABS, IPSWICH, MA, USA

In the last 30 years our ever-increasing ability to sequence DNA has taken us from knowing almost nothing about the genetic make-up of the organisms living on this planet to being able to sequence almost any organism's genome at will. The complete DNA sequence of a bacterium can now be sequenced over lunch while even a human's genome requires less than a day of data acquisition. However, progress in understanding the meaning of that DNA has been much slower. The bioinformatic tools for this purpose are still being developed and offer major opportunities for discovery. In this talk I will provide a brief history of the field and show some specific examples of both the problems and the opportunities that await well-prepared minds. In particular, I will show that the interplay of experimental science and computational ingenuity are likely to be the key to many major discoveries in biology in the years ahead. The impact of these discoveries on the practice of medicine will be profound.

Magic Challenges

Wednesday, 27 June 2012

12.00 – 13.30

Khosrow Adeli, *University of Toronto, CA*

Translational control mechanisms in metabolic regulation: critical role of RNA binding proteins, microRNAs, and cytoplasmic RNA granules

Mauro Giacca, *ICGEB, IT*

"How can you mend a broken heart (Bee Gees, 1971)" – Searching for novel genes and microRNAs inducing myocardial protection and regeneration

Francisco E. Baralle, *ICGEB, IT*

hnRNPs dysfunction and neurodegenerative diseases

TRANSLATIONAL CONTROL MECHANISMS IN METABOLIC REGULATION: CRITICAL ROLE OF RNA BINDING PROTEINS, MICRORNAS, AND CYTOPLASMIC RNA GRANULES

Khosrow Adeli[1]

[1]RESEARCH INSTITUTE, THE HOSPITAL FOR SICK CHILDREN, UNIVERSITY OF TORONTO, TORONTO, CANADA

Regulated cell metabolism involves acute and chronic regulation of gene expression by various nutritional and endocrine stimuli. To respond effectively to endogenous and exogenous signals, cells require rapid response mechanisms to modulate transcript expression and protein synthesis and cannot, in most cases, rely on control of transcriptional initiation that requires hours to take effect. Thus, co- and posttranslational mechanisms have been increasingly recognized as key modulators of metabolic function. In this talk, I will highlight the critical role of mRNA translational control in modulation of global protein synthesis as well as specific protein factors that regulate metabolic function. First, the complex lifecycle of eukaryotic mRNAs will be reviewed, including our current understanding of translational control mechanisms, regulation by RNA binding proteins and microRNAs, and the role of RNA granules, including processing bodies and stress granules. Second, the current evidence linking regulation of mRNA translation with normal physiological and metabolic pathways and the associated disease states are reviewed. A growing body of evidence supports a key role of translational control in metabolic regulation and implicates translational mechanisms in the pathogenesis of metabolic disorders such as type 2 diabetes. The lecture will also highlight translational control of apolipoprotein B (apoB) mRNA by insulin as a clear example of endocrine modulation of mRNA translation to bring about changes in specific metabolic pathways. Recent findings made on the role of 5'-untranslated regions (5'-UTR), 3'-UTR, RNA binding proteins, and RNA granules in mediating insulin regulation of apoB mRNA translation, apoB protein synthesis, and hepatic lipoprotein production are discussed.

"How can you mend a broken heart (Bee Gees, 1971)" - Searching for novel genes and microRNAs inducing myocardial protection and regeneration

Mauro Giacca[1]

[1]International Centre for Genetic Engineering and Biotechnology ICGEB, Trieste, Italy

The identification of novel genes and pathways controlling prenatal cardiomyocyte proliferation or regulating cardiomyocyte survival during the adult life holds paramount interest in view of developing new therapeutic approaches for patients with ischemic cardiomyopathy and heart failure. We are undertaking two complementary approaches in this respect. The first one exploits the capacity of viral vectors based on the Adeno-Associated Virus (AAV) to transduce myocardial cells in vivo with high efficiency and to promote the expression of their transgenes for prolonged periods of time. Using these vectors, we have undertaken an exhaustive approach to select factors exerting beneficial effect upon myocardial damage by the construction of library of AAV vectors delivering the whole mouse secretome (1700+ mouse secreted proteins). Preliminary screening of a subset of this library has led to the identification of peptide hormone ghrelin as a powerful cardioprotective agent in vitro and in vivo.

A second approach entails high throughput screening of microRNAs promoting primary cardiomyocytes proliferation in vitro. Out of a library consisting of 1000+ human microRNAs, we identified a few microRNAs with outstanding activity in promoting expansion of cardiomyocytes in cell culture, massive cardiac hyperplasty in the neonate and remarkable improvement of cardiac function after infarction in the adult.

HNRNPS DYSFUNCTION AND NEURODEGENERATIVE DISEASES

Francisco E. Baralle[1]

[1]INTERNATIONAL CENTRE FOR GENETIC ENGINEERING AND BIOTECHNOLOGY, TRIESTE, ITALY

The pathways to maintain correct protein homeostasis (proteostasis) act at different steps of the gene expression process. The control of mRNA levels is often seen for RNA binding proteins (RBDs) because of their potential ability to bind their own pre-mRNA and/or mRNA molecules. The binding of the protein to its own pre-mRNA may trigger specific alternative splicing that introduces a stop codon in the mRNA. This in turn activates Non Sense Mediated Decay (NMD), a process that contributes to maintain constant expression levels within the cellular environment. In addition to NMD, other mechanisms have also been described to play a role in auto-regulation of mRNA levels in other RBD proteins such as SRFS1 and TDP-4.

The currently available evidence shows that the level of the major TDP-43 mRNA isoforms is regulated through a pathway that does not significantly involve alternative splicing and NMD, significantly contribute to the regulation. The binding of TDP-43 to the 3'UTR of its pre-mRNA triggers a rapid lowering of mRNA levels. There are several possible pathways that may be involved in this process. The contribution of exosome activation, polyA site variation, RNApolII staling, etc. will be discussed in view of recent evidence.

The self-regulation loop of TDP-43 is likely to have a role in the growth of the TDP-43 brain aggregates identified as the end point of many neurodegenerative diseases. These aggregates may act as a TDP-43 "sink" that captures the protein in the cytoplasm, lowering TDP-43 concentrations in the nucleus and signaling to the cell to increase TDP-43 production. Models to study the effect of aggregate formation in the disruption of TDP-43 self-regulation both in cell lines and reat cortical neurons will be presented.

Genetic Factors and Genetic Analysis

Wednesday, 27 June 2012

15.00 – 16.35

Pier Franco Pignatti, *University of Verona, IT*
Coronary artery disease: the search for genetic factors

Tadej Battelino, *University of Ljubljana, SI*
Molecular biology and biochemistry in clinical routine

Janja Marc, *University of Ljubljana, SI*
Genetics of osteoporosis

Katja Drobnič, *National Forensic Laboratory, SI*
Perspective of alternative markers in forensic genetics

Irena Zupanič Pajnič, *University of Ljubljana, SI*
DNA identification of ancient skeletal remains in Slovenia

CORONARY ARTERY DISEASE: THE SEARCH FOR GENETIC FACTORS

Pier Franco Pignatti

[1]DEPARTMENT OF LIFE AND REPRODUCTION SCIENCES, UNIVERSITY OF VERONA, VERONA, ITALY

Introduction: Coronary Artery Disease (CAD) is a progressive inflammatory-metabolic disease in which atherosclerotic plaques cause stenosis on the coronary arteries, leading to acute clinical complications such as MI. Family and twin studies indicate that there is a strong genetic component: genetic risk is variously estimated to account for about 40-60% of susceptibility to CAD. This talk will indicate the role of genetic studies, including examples from our own work, in unraveling the etiopathogenesis of this common and complex disease.

Results: First, candidate gene studies will be mentioned, indicating how they have been useful in hypothesis-driven detection of genetic variants in CAD, and of their relationship with environmental factors.

A few specific examples will be cited. Apolipoprotein CIII has an important role in hypertriglyceridemia. An APOC3 gene variant correlates with triglyceride concentration and CAD risk as defined by coronary angiography [1], or CAD risk in Metabolic Syndrome patients [2]. Moreover, the CAD patients with the at-risk genotype show no response to unsaturated fatty acids in the diet, while the others respond as expected and lower their ApoCIII concentration in red blood cell membranes [3]. This is an indication of a genotype/phenotype correlation which may be taken into consideration in the management of the disease in this group of individuals.

Another example involves paraoxonase 2, an enzyme that prevents cell mediated lipid peroxidation. A PON2 gene variant correlates with CAD risk in smokers, but no so in non-smokers [4]. Therefore, it appears that PON2 may be one of the most important genes involved in predisposing subjects to smoking-induced oxidative damage.

A last example indicates the combined effect on MI risk of different genes involved in blood coagulation in patients with advanced coronary atherosclerosis [5]

Then, Genome Wide Association Studies (GWAS) will be mentioned, a hypothesis-free method of genetic analysis, indicating their role in the discovery of new genetic markers related to CAD. As an example, the 9p21 locus identifies a DNA region associated with CAD and MI that regulates transcription rather than being in a gene coding region [6].

Conclusions: The common variants so far discovered explain only a small percentage of the total heritability of CAD: larger GWAS collaborations will map further variants, and resequencing experiments might identify low-frequency variants in the near future [7]. Functional analysis of the variants may be a prerequisite for their clinical applications [8].

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MOLECULAR BIOLOGY AND BIOCHEMISTRY IN CLINICAL ROUTINE

Tadej Battelino[1,2]

[1]UMC-UNIVERSITY CHILDREN'S HOSPITAL, LJUBLJANA, SLOVENIA, [2]FACULTY OF MEDICINE, UNIVERSITY OF LJUBLJANA, LJUBLJANA, SLOVENIA

State-of-the-art clinical care has become increasingly dependent on newest techniques developed in research laboratories by molecular biologist and biochemists. Precise diagnosis of monogenic diseases, syndromes caused by genomic rearrangements, imprinting or posttranslational defects is based on meticulous molecular and biochemical tests. Moreover, selection of appropriate treatment and long-term prognosis are often influenced by the knowledge of the molecular basis of a particular disease and by other genetic characteristics of a particular individual.

Congenital adrenal hyperplasia is an example of a well-researched monogenic disorder where routine clinical work-up depend on precise molecular diagnosis, including prenatal diagnosis and in selected cases also prenatal treatment. Adrenoleukodystrophy is another example where molecular diagnosis represents a cornerstone in clinical decision-making despite the fact that we still lack the understanding of its etiopathogenesis. Finally, polygenic approaches may be useful in determining patient-specific profiles related to selected therapies or to potential risk for long-term complications. Cancer and several chronic diseases like diabetes are currently among the most intensively researched in this group of disorders.

In inborn errors of metabolism, the fastest way to establish a diagnosis runs through identification of specific metabolites. The use of tandem-mass spectrometry in biological samples helps us screen for rare disorders and establish biochemical diagnosis in time for immediate life-saving therapy.

Close collaboration between scientist and clinicians in the field of molecular biology and biochemistry is therefore essential for the excellence in clinical care as well as for the continuous progress in researching molecular bases of human diseases.

GENETICS OF OSTEOPOROSIS

Janja Marc[1]

[1]FACULTY OF PHARMACY, UNIVERSITY OF LJUBLJANA, LJUBLJANA, SLOVENIA

The increased life expectancy is leading also to higher incidence of degenerative disorders, including osteoporosis. Osteoporosis is a metabolic bone disease, characterized by low bone mineral density (BMD) and microarchitectural changes, both leading to an increased incidence of fractures. Enormous effort is put into research to discover the molecular mechanisms of pathogenesis of osteoporosis in order to obtain novel targets for the osteoporosis treatment or prevention, early diagnostic markers for identification of individuals at higher risk for the development of osteoporosis and prognostic markers for an effective individualized therapy. Current research results (studies) cannot explain the influence of genetic factors, demonstrated already 2 decades ago in twin studies [1]. The genes influencing the osteoporosis-related phenotypes, like bone mineral density, fracture risk, onset of the menopause and bone geometry, remain still largely unknown [2], mainly due to the polygenic and multifactorial nature of osteoporosis. Several approaches have been applied: linkage analysis in humans and in animals, as well as association studies. Taking into account a substantial genetic influence, numerous new genes are still expected to be identified. In an international GEFOS consortium, the meta-analysis of genome-wide analysis data was used as a tool for the identification of new possible osteoporosis-related candidate genes (3). Our research group participated in GENOMOS consortium for replication of GEFOS identified SNPs on 50.993 participants. In this study 56 loci (32 new) were found to be associated with BMD (4). In our study, a different approach using gene expression microarrays was applied for a stepwise identification of candidate genes. One thousand six hundred six genes were found to be differentially expressed, indicating increased demand for protein synthesis and decreased cell proliferation rate in osteoblasts from osteoporotic tissue as compared to osteoblasts from non-osteoporotic tissue. Further biostatistical analysis of the microarray data by gene set enrichment analysis suggested oxidative stress may have an important part in the pathogenesis of osteoporosis. Thus, secondly, we tested it by an in vitro assay on human osteosarcoma cell line cells treated with hydrogen peroxide (4). Our results presented a novel list of genes and metabolic pathways that may be associated with the pathogenesis of osteoporosis. PTN, CXCL2, COL15A1, IBSP, AOX1, MT1G, GSR and TXNRD1 could be candidate genes for further studies in the assessment of the genetic susceptibility to osteoporosis.

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PERSPECTIVE OF ALTERNATIVE MARKERS IN FORENSIC GENETICS

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Introduction: In forensic genetics the most common approach for determining the identity of a person who left traces is non-informative autosomal STR typing [1]. The Slovenian National Forensic Laboratory has been using this strategy since 1996. We successfully typed DNA in thousands of cases, sometimes from very difficult samples such as bones or penile swabs, by using different genetic markers [2-5]. However, the donor of biological traces will be impossible to find although some markers have a very high discrimination capacity when there is no DNA match between traces and persons. Moreover, none of these markers provided any information about the body fluid of origin. To overcome these problems, the alternative markers have been developed, which allow prediction of externally visible traits of an individual such as eye and hair color [6] or the identification of body fluids solely from traces [7]. The aim of this presentation is to provide information about the advantages and limitations of these markers for the purpose of forensic investigation.

Results: We managed to develop a sensitive and reliable single multiplex genotyping assay for predicting eye and hair color [8]. We demonstrated that the identification of vaginal secretion and menstrual blood by using mRNA markers can be a reliable method [9].

Conclusions: Soon DNA markers for predicting externally visible traits will be used as a “genetic eyewitness”, while mRNA profiling will be the method of choice for identifying the body fluid of origin.

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DNA IDENTIFICATION OF ANCIENT SKELETAL REMAINS IN SLOVENIA

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Introduction: The most common type of samples that are preserved in archaeological records and old mass grave sites are bones and teeth. Many ancient samples contain no endogenous DNA, where DNA is still preserved it is degraded to small average size [1]. The skeletal remains are usually genetically analysed for ancient mitochondrial and Y-chromosome sequences and autosomal microsatellites [2-4]. We will present the application of molecular genetic methods for identifying ancient skeletal remains in Slovenia. The genetic typing of five skeletons from 18th century found in the Auersperg chapel archaeological site will be presented and DNA identification of skeletal remains of 88 victims of World War II killings found in the Konfin I mass grave will be shown.

Results: We cleaned the bones and teeth, removed surface contamination, and ground them into a powder using liquid nitrogen [5,6]. Prior to DNA isolation bone or tooth powder was decalcified. We extracted up to 100 ng DNA/g of bone powder from Konfin I mass grave bones [7] and up to 10,7 ng DNA/g of teeth powder from Auersperg chapel archaeological site skeletal remains. Autosomal genetic profiles, mtDNA and Y-chromosome haplotypes were obtained for bones and teeth and for reference persons. For traceability in the event of contamination, we created an elimination database including genetic profiles of the nuclear and mtDNA of all persons that had been in contact with the skeletal remains.

Conclusions: We managed to obtain nuclear DNA for successful microsatellite typing from skeletal remains that were over 300 years old. We obtained complete genetic profiles of autosomal DNA, Y-STR haplotypes, and mtDNA haplotypes from almost all bone and teeth samples. When comparing genetic profiles, we matched 32 bones with living relatives (brothers, sisters, sons, daughters, nephews, or cousins). The statistical analyses showed a high confidence of correct identification for all 32 victims in the Konfin I mass grave [7].

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Pharmacogenomics, Pharmacogenetics & Therapeutic Issues and Drug Design

Wednesday, 27 June 2012

17.00 – 18.30

Magnus Ingelman-Sundberg, *Karolinska Institutet, SE*
Pharmacogenomics and pharmacoepigenomics of drug treatment

Vita Dolžan, *University of Ljubljana, SI*
Pharmacogenetics of rheumatoid arthritis treatment

Matjaž Zorko, *University of Ljubljana, SI*
Side effects of the cell – penetrating peptides

Stanislav Gobec, *University of Ljubljana, SI*
Discovery of enzyme inhibitors by virtual screening

PHARMACOGENOMICS AND PHARMACOEPIGENOMICS OF DRUG TREATMENT

Magnus Ingelman-Sundberg[1]

[1]KAROLINSKA INSTITUTET, STOCKHOLM, SWEDEN

Pharmacogenomic research has focused on understanding the molecular mechanisms behind Adverse Drug Reactions (ADRs) and finding biomarkers that identify people at risk as well as finding genetic causes to altered efficacy of drugs. Serious ADRs have been shown to cause/contribute to 6-7% of all primary hospitalizations, 20 % of readmissions and 30 % of hospitalizations among elderly. During recent years the number of reported ADRs and ADR-related fatalities have actually increased, both by about 2.6-fold. At least 34 drugs were withdrawn from the market during 1995-2006, mainly due to hepatotoxic or cardiotoxic effects.

A review of pharmacogenomic biomarkers reveals that markers with significant specificity and sensitivity encompass variable genes encoding drug metabolizing enzymes and transporters in relation to treatment with some anticancer drugs (irinotecan, 6-mercaptopurines, iressa), codeine, antidepressants, clopidogrel, tamoxifen, simvastatin and warfarin. Highest specificity is seen amongst the HLA allelic variants where more specific interactions occur and where the ADRs caused by e.g. carbamazepine, flucloxacillin, ximelagatran and abacavir can be predicted at a relatively high specificity and sensitivity.

Continuous studies based on GWAS analyses of subjects susceptible for specific ADRs reveal many associations with genetic alteration in genes encoding Class I-II antigens. An additional risk assessment can be done using risk combinations of variant genes, e.g. those encoding HLA antigens, with the incidence of ADRs.

At present we do understand a major part of the true genetic reasons to variability in expression of ADME genes or drug targets such as copy number variations, in/dels and SNPs. However, in addition, epigenetic and ncRNA dependent regulation of these genes are important and future direction in this novel research field is outlined with respect to our understanding of interindividual differences in drug action.

The lecture will give an update in the field of current and future pharmacogenomic biomarkers of importance for prediction of drug metabolism, drug action and ADRs focusing on the most clinically relevant examples as well as provide with an update regarding the epigenetic regulation of drug metabolism.

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PHARMACOGENETICS OF RHEUMATOID ARTHRITIS TREATMENT

Vita Dolžan[1]

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Rheumatoid arthritis (RA), a chronic autoimmune disease, leads to progressive joint deformation, dysfunction and disability. The treat-to-target approach aims to achieve full remission of the disease and thus prevent structural damage, and a rational selection of the DMARD and early and aggressive treatment play an important role.

Although the evidence supports the efficacy of DMARDs such as low dose methotrexate (MTX) and leflunomide in RA, there are significant differences between patients regarding the probability of failure of a particular drug. These differences may in part be due to individual variability in the metabolic pathways associated with specific drugs. The aim of our study is to identify genetic polymorphisms within these pathways that may influence drug metabolism, drug efficacy and toxicity and that may be used in directing treatment choices in RA.

MTX is usually the first chosen DMARD as the median time to discontinuation is 4.2 years compared to 2 years for other DMARDs. Still, 20 – 30% of patients discontinue MTX treatment due to inefficacy or adverse events. As the anti-inflammatory action of MTX is due to its antagonistic effect on folate pathway as well as its effect on intracellular adenosine metabolism, we investigated functional SNPs in MTX transport and folate and adenosine pathway in a clinically well characterized group of 213 RA patients treated with MTX. We observed several associations between polymorphisms in genes for MTX transporters *RFC1*, *SLCO1B1*, *ABCB1* and *ABCC2* and MTX toxicity. Within folate pathway, significant associations were observed between *MTHFD1* and treatment response. Similar to previous reports on adenosine pathway *AMPD1* and *ITPA* conferred to treatment response and *ATIC* was associated with increased risk for MTX toxicity. Our most recent studies showed a role of adenosine receptor *ADORA2A* polymorphisms in MTX toxicity, while genetic variability of *ADORA3* influenced treatment response. A clinical-pharmacogenetic model that will account for genetic variability in15 genes involved in MTX action is under construction.

Leflunomide is a relatively new DMARD. Although it is comparable to MTX in terms of clinical response rate, in routine clinical use the rate of withdrawal was higher than in clinical trials and ranged between 40 – 70 % within the first year. Leflunomide is a prodrug, activated by liver cytochromes P450 (CYP). At therapeutic plasma levels the active metabolite inhibits dihydroorotate dehydrogenase (DHODH) and inhibits cell proliferation by blocking de novo pyrimidine synthesis. In a study including 105 RA patients treated with leflunomide we found that *CYP1A2* and *DHODH* polymorphism increased the risk for leflunomide toxicity. Although independent confirmation is needed, our study identified the first pharmacogenetic predictors of leflunomide toxicity.

In conclusion, our results suggest that pharmacogenetic markers could be used in directing treatment choices in RA in clinical practice. Rational selection of DMARDs could improve the efficacy and safety of treatment of RA in clinical practice.

Acknowledgements: The contribution of clinical collaborators Blaž Rozman, Dušan Logar and Matija Tomšič from Department of Rheumatology, University Medical Centre Ljubljana and my former PhD student dr. Petra Bohanec Grabar as well as undergraduate students Sabina Rojko, Nadja Kobold and Barbara Jenko is greatly acknowledged.

SIDE EFFECTS OF THE CELL-PENETRATING PEPTIDES

Matjaž Zorko[1]

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For the last 20 years research work in our group was aimed mainly to clarify mechanisms of enzymes (cholinesterases and 17b-hydroxysteroid dehydrogenases), mechanisms of G-protein interaction with receptors (galanin, glucagon-like peptide, membrane steroid, and histamine receptors), mechanisms of direct interaction of G-proteins with some naturally occurring substances, and most intensively to studies of the side effects of the cell-penetrating peptides (CPPs), which are the topic of this presentation.

CPPs are peptides composed of less than 30 amino acids and can enter cells and organelles by penetrating through the lipid bilayer. In doing this, CPPs can carry into the cell different cargoes that can be many hundred times larger than themselves. They can be used as vehicles for the transport of different molecules including proteins and oligonucleotides in the cells for the research purposes and also as the drug delivery vectors. Our work was concentrated mainly on the action of the transportan (TP) family of CPPs that was invented by Ülo Langel from Stockholm. A problem in practical use of CPPs is their potential side effects. We have shown that TP inhibits G-proteins of Gs type by direct binding to their alpha subunit. More detailed studies revealed the importance of the first six amino acids of TP for this binding. By their deletion a new CPP transportan-10 (TP10) was generated which does not interact with G-proteins but retains the ability to transport cargo in the cell. Another side effect of virtually all CPPs from TP family is the formation of pores in the lipid bilayer. This is not surprising since a part of TP is mastoparan, a lytic peptide from the wasp toxin. We have shown that TP induces pores that are of toroid type with the diameter of the pores being TP concentration dependent. An important finding was also that the introduction of cholesterol into the lipid bilayer protects the membrane and prevents formation of the pores. Recently we were working with TP10 analogues PepFect 3 and 6 (PF3 and PF6) specifically designed for the non-covalent translocation of small RNAs into the cell in order to modify protein expression processes. In delivering oligonucleotides and small RNAs into the cell, PF3 and PF6 are more efficient than a well-known commercially available transfection tool lipofectamine. We have shown that PF3 and PF6 are not interfering with G-proteins but they still induce pores into the lipid bilayer albeit less effectively than TP10. In binding to the lipids, they show some specificity for the membrane composition in terms of better binding to the zwitterionic then to the negatively charged lipid monolayer. The presented studies are helping us to design more efficient and less harmful CPPs with the potential to be used as drug vehicles *in vivo*.

DISCOVERY OF ENZYME INHIBITORS BY VIRTUAL SCREENING

Stanislav Gobec[1]

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The discovery of new lead molecules with potential to interact with specific target receptors or enzymes is of central importance to early-stage drug discovery. The high cost and low hit rate of high-throughput screening have stimulated the development of computational (or *in silico*) screening methods [1]. Virtual screening (VS) is an automated technique that is used for computational screening of large databases of compounds. Its aim is to reduce large numbers of compounds to smaller subsets that are more likely to contain biologically active compounds. Ligand-based VS uses 2D or 3D structure of active ligand and compares it with the structures of compounds present in a database. Structure-based VS applied to the discovery of new enzyme inhibitors involves docking, the computational fitting of structures of compounds to the active site of an enzyme, and scoring and ranking each compound. The highest ranked compounds are then tested for their activities in a biological or biochemical assay.

At the Faculty of Pharmacy, University of Ljubljana, we have used these *in silico* tools to search for small-molecule hit inhibitors of different pharmacologically important enzymes. Enzyme inhibitory activities were then evaluated in collaboration with the Institute of Biochemistry, Medical Faculty, where enzymes were also overexpressed, purified, and the enzyme inhibition assays were set up. Promising inhibitors of fungal trihydroxynaphthalene reductase [2], 17β hydroxysteroid dehydrogenase [3], aldo-keto reductases 1C1 and 1C3 [4], and butyrylcholinesterase were discovered. These initial hit inhibitors represent an important starting point for development of new drug candidates for different diseases, including fungal diseases, endometriosis, hormone dependent and independent forms of cancer, and Alzheimer's disease.

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Plenary Lecture

Thursday, 28 June 2012

08.30 – 09.00

Francis Lévi, *INSERM, Hopital Paul Brousse, FR*

Systems chronopharmacology approaches for the personalization of cancer chronotherapeutics

SYSTEMS CHRONOPHARMACOLOGY APPROACHES FOR THE PERSONALIZATION OF CANCER CHRONOTHERAPEUTICS

Francis Lévi[1]

[1]UMRS776 INSERM AND PARIS SUD 11 UNIVERSITY, PAUL BROUSSE HOSPITAL, VILLEJUIF, FRANCE

Chronotherapeutics aim at improving treatment outcomes through the delivery of medicines according to the Circadian Timing System (CTS), a complex hierarchical and dynamic network system involving all cells in the body. As a result, circadian timing modifies up to 10-fold the tolerability of anticancer medications in experimental models and in cancer patients (*Lévi et al. Annu Rev Pharm Toxicol 2010*). However, sex, circadian disruption and tumor protein expressions are independent determinants of the optimal chronotherapeutic schedule, in international studies involving large number of patients with metastatic colorectal cancer. Such clinical data have driven experimental confirmation studies in mice. Moreover, human cancer chronotherapeutics constitute a unique paradigm for cancer therapy, where “the lesser the toxicity, the better the efficacy”, based on several landmark analyses of a randomized clinical trial involving 564 patients. Stochastic and deterministic mathematical models help analyze the dynamic interactions between circadian clocks, cell cycle and drug pharmacodynamics from single cell to whole organism. Biosimulation leads to the design of model-based optimal chronotherapeutic schedules, through the exploration of a wide range of parameter values, as shown for irinotecan. Systems chronopharmacology further reveals that optimal chronotherapeutics require circadian entrainment to be robust in healthy cells and disrupted in cancer cells. In practice, non-invasive reliable circadian biomarkers are critical for modeling CTS dynamics, for increasing CTS robustness through intervention measures, and for effectively personalizing circadian drug delivery schedules.

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CFGBC: Functional Genomics

Thursday, 28 June 2012

09.10 – 10.35

Michael Taussig, *Babraham Bioscience Technologies, GB*
Affinity proteomics: analyzing the human proteome with protein–binding reagents

Damjana Rozman, *University of Ljubljana, SI*
From mouse models to human diseases: the example of CYP51 from cholesterol synthesis

Simon Horvat, *University of Ljubljana, SI*
Obesity: tackling a disease by focusing on obesity resistance rather than susceptibility genes

Damjan Glavač, *University of Ljubljana, SI*
Genomic "dark matter": implications for understanding human diseases

AFFINITY PROTEOMICS: ANALYZING THE HUMAN PROTEOME WITH PROTEIN-BINDING REAGENTS

Michael Taussig[1]

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In affinity proteomics, specific protein-binding molecules (aka binders), principally antibodies, are applied as reagents in proteome analysis. In recent years, advances in binder technologies have created the potential for an unprecedented view on protein expression and distribution patterns in plasma, cells and tissues, and increasingly on protein function (1). Particular strengths of affinity proteomics methods include detecting proteins in their natural environments of cell or tissue, high sensitivity and selectivity for detection of low abundance proteins, and exploiting binding actions such as functional interference in living cells. To maximize the use and impact of affinity reagents, it will be essential to create a comprehensive, standardized binder collection. The EU FP7 Affinomics program (2) aims to extend affinity proteomics research by generating a large-scale resource of validated protein-binding molecules for characterization of the human proteome. Current activity is aimed at producing binders to about 1000 protein targets, primarily in signal transduction and cancer, by establishing a high throughput, coordinated production pipeline. An important aspect of the program is the development of highly efficient recombinant selection methods, based on phage, cell and ribosome display, capable of producing high quality binders at greater throughput and lower cost than hitherto. The program also involves development of innovative and sensitive technologies for specific detection of target proteins and interactions. In my own group, high density protein arrays are generated using cell free transcription and translation, through which it is possible to copy DNA into protein quickly in an array format. An aim is to display sequence-specified proteomes by linking genomic DNA sequences and their expressed transcriptomes with the DAPA protein array system (3,4) and to use them to analyze antibody responses, protein interactions and alterations due to mutation. Such a system could have wide applicability, regardless of species or cell type.

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FROM MOUSE MODELS TO HUMAN DISEASES: THE EXAMPLE OF CYP51 FROM CHOLESTEROL SYNTHESIS

Gregor Lorbek[1], Monika Lewinska[1], Matina Perše[2], Jera Jeruc[2], Peter Juvan[1], Jeffery C. Murray[3], Rok Keber[4], Simon Horvat[4], Damjana Rozman[1]

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Introduction: Cholesterol synthesis is an essential metabolic pathway of most mammalian cells. It is essential in embryonic development as evidenced from the mouse knockout models and from human malformations linked to defects in cholesterol synthesis genes. Lanosterol 14alpha-demethylase (CYP51) demethylates lanosterol in the late portion of the pathway. The full *Cyp51* knockout is embryonically lethal in mice at day 15.0, with a phenotype similar to other defects in the sonic-hedgehog signaling pathway. Due to essentiality of the pathway, the homozygous malformations linked to cholesterol synthesis are rare while the role of hypomorphic alleles that influence the involved enzyme activities are poorly investigated. The goal of this work is to get a deeper insight into potential allele effects of *Cyp51* and to reveal the role of CYP51 and cholesterol synthesis in the liver as the major organ of body cholesterol homeostasis.

Results: Heterozygous *Cyp51*+/- mice are viable with normal outer appearance. The *Cyp51* gene dosage effect is observed, with gender-specific differences in organ weight and blood parameters in response to the diet. The *Cyp51*+/- males seem to be more affected, with hepatomegaly and higher blood cholesterol on the western diet compared to wild types. Since deficiency of CYP51 activity associates with Antley–Bixler syndrome we investigated *CYP51A1* polymorphisms in a cohort of preterm babies and in mothers that gave premature birth. So far we identified only rare, heterozygous *CYP51* variants. To assess the liver effect of cholesterol synthesis we produced two lines of liver *Cyp51* conditional knockout mice (*Cyp51* Cre+ lox/lox or lox/-; LKO). Novel gender related differences that connect the liver *Cyp51* genotype and response to the diet have been identified. More affected are males, especially on diets with low cholesterol content (altromin and high fat diet with no cholesterol). The most striking is severe hepatomegaly with lower blood cholesterol in LKO males on low cholesterol diet compared to the wt. Interestingly, the western diet shows no obvious differences between the genotypes (wt *versus* LKO) within each gender. 4% of the progeny, almost exclusively males, show cholangiopathy with jaundice and eventually die before week 10. The sex-dependent changes aggravate by aging.

Conclusions: The mouse *Cyp51* transgenic models proved to be essential tools in revealing the tissue-specific and gender-specific roles of the cholesterol synthesis pathway *in vivo.* Due to observed differences between *Cyp51*+/- and wt mice we believe that two functional alleles of C*yp51* are required in the entire body for normal function. Defect of *Cyp51* alleles in the liver has severe consequences with potentially fatal outcomes especially for males. We currently investigate whether this results from the disrupted cholesterol synthesis and/or the disrupted gender-divergent bile acid homeostasis.

OBESITY: TACKLING A DISEASE BY FOCUSING ON OBESITY RESISTANCE RATHER THAN SUSCEPTIBILITY GENES

Simon Horvat[1,2], Zala Prevoršek[1], Gregor Gorjanc[1], Jasmina Beltram[1], Mathieu Laplante[3], Nicholas M. Morton[4]

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Introduction: Obesity results from interactions between numerous genes and modern »obesogenic« environment and presents a severe risk factor for a number of chronic diseases including diabetes, cardiovascular diseases and cancer. Forms of obesity with simple Mendelian inheritance are rare. Therefore, attention has turned to searching for genes of the more common polygenic form of obesity by genomics approaches such as quantitative trait locus (QTL) mapping and bioinformatics analyses. Additional genes need to be identified to elucidate the mechanisms of susceptibility or resistance to obesity development. We previously identified a large segment on mouse Chromosome 15 (*Fob3*) affecting obesity-related traits in lines of mice selected on high (Fat line) and low (Lean line) body fat content that represent a unique model of polygenic obesity [1]. Follow up studies revealed that *Fob3* consists of at least three separate linked QTLs *Fob3a*, *Fob3b1* and *Fob3b2* [2] and the that some obesity resistant mechanisms exist in the Lean mouse model as indicated by the liver microarrays and muscle physiological and expression studies [3].

Results: The first objective was to select candidate genes and fine map the genetic intervals of *Fob3a* and *Fob3b2* QTL using methods such as F_2 crosses of congenic lines, analyses of interval-specific haplotypes and between species comparative mapping. The second objective was to identify candidates for *Fob3a* and *Fob3b2* that were differentially expressed by employing microarray genome wide expression study and a follow up Real time-PCR validation. Using these approaches, we found only a few positional candidates with demonstrated differential expression and with additional hits from genomic and bioinformatics analyses. Our results provide evidence for existence of a handful of candidate genes likely to be causal for the phenotypic effects of *Fob3a* and *Fob3b2* QTLs on mouse chromosome 15. Physiological characterization of these top candidate genes is under way – we will present some of the current genetic, functional and physiological experiments that point to functional relevance of studied candidates for the observed phenotypic effects.

Conclusions: We identified strong positional candidates inherited from the Lean line that exhibit expression and physiological pattern as “leanness” genes by decreasing fatness, by acting as gain-of-function genes and by showing adipose tissue specificity. Such “lean” genes may represent novel therapeutic targets in white fat tissue for the treatment of obesity and related metabolic disorders.

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GENOMIC "DARK MATTER": IMPLICATIONS FOR UNDERSTANDING HUMAN DISEASES

Damjan Glavač[1]

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The predominant focus of disease research has traditionally been placed on the protein-coding regions of the human genome, which account for only a few % of its total sequence complexity. Recent studies indicate that the cell actually uses the remaining 97-98% of the genome to produce stable RNAs – the so-called "dark matter" RNA. There are at least 16,000 ncRNAs among which over 3,000 are lncRNAs. Three types of naturally occurring small RNAs have also been described, based on their origin or function: short interfering RNAs (siRNAs), repeat-associated short interfering RNAs (rasiRNAs) and microRNAs (miRNAs).

It is estimated that there could be as many as a thousand miRNAs, thought to regulate 30-90 % of genes within the human genome. Dysregulation of lncRNAs and miRNAs is associated with many types of human diseases, highlighting their potential as biomarkers and therapeutic targets. Our aim was to identify some lncRNAs and miRNAs that might play an important role in contributing to pathogenesis of cardiovascular diseases and brain tumors. Using a high performance microParaflo biochip platform and real-time polymerase chain reaction qPCR miRNA and lncRNA profiling was performed on tissue samples of myocardial infarction patients and patients with glioblastoma tumors. We used a qPCR based disease-related human lncRNA profiler which allows us quantification of differential expression of 83 preselected disease related lncRNAs. We identified that a substantial number of lncRNAs and miRNAs were dysregulated, suggesting that they might play an important role in pathogenesis of myocardial infarction and glioblastoma tumors. Moreover, some of miRNAs and lncRNAs might be involved in the recurrence and malignant progression of gliomas. Bioinformatics analysis of the predicted target genes (gene ontology, pathway and network analysis) was performed to elucidate the role of some lncRNA and miRNA in disease processes. Due to high tissue specificity of some lncRNAs and microRNAs and their involvement in different biological processes some of those molecules can lead to the development of diagnostic tools and perhaps to a novel class of therapeutic targets.

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CFGBC: Molecular Basis of Disease – New Views

Thursday, 28 June 2012

11.00 – 12.15

Roman Jerala, *National Institute of Chemistry, SI*
Molecular mechanism TLR4 and MyD88 – mediated signaling and inhibition

Jernej Ule, *MRC Laboratory of Molecular Biology, GB*
How does defective regulation of RNA processing lead to neurologic diseases?

Metka Ravnik-Glavač, *University of Ljubljana, SI*
States of mind in connection with gene expression

Gregor Majdič, *University of Ljubljana, SI*
Hormone independent sex differences in brain and behavior

MOLECULAR MECHANISM TLR4 AND MYD88-MEDIATED SIGNALING AND INHIBITION

Ota Fekonja[1], Monika Avbelj[1,2], Mateja Manček Keber[1], Simon Horvat[1,3], Roman Jerala[1,2,4]

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Introduction: Innate immune response plays an essential role for the defense of all multicellular organisms. TLR4 is in many ways an exception among the Toll-like receptors as it is the only one that signals through the two signaling pathways. TLR4 has a role in many chronic and infectious diseases. Cellular signaling of most TLRs is mediated by an adapter protein MyD88 through TIR domain interactions. Monomeric TIR domains inhibit activation of TLR signaling, which is exploited by pathogens to suppress the innate immune response. Whereas the structure of the Death-domain inflammasome comprising death domains of MyD88-IRAK4 and IRAK-2 has been determined, the mechanism of TIR domain mediated activation is not understood.

Results: We discovered the important role of TLR4 in sensing the oxidative stress, which is particularly relevant in chronic inflammation. Partially oxidized phospholipids in microvesicles from patients with rheumatoid arthritis mediate activation of TLR4 signaling pathway. Activation of TLR4 by MVs mimics the molecular mechanism of activation by LPS, demonstrated by the effects of MD-2, mutations, inhibitors and receptor complex dimerization. We reconstituted the biologically active MVs from synthetic phospholipids by partial oxidation. Signal from pathogens (LPS) and endogenous danger signal (MVs) induced significantly different expression profile response in mouse BMDMs with strong inflammation resolving component induced by the endogenous signal.

In order to investigate the molecular mechanism of TLR activation mediated by TIR-domain interactions we prepared a tethered TIR dimer. Dimeric TIR domain platform has a unifying role both for the immunosuppression by bacterial virulence factors TCPs (TIR domain-containing proteins) and for the proinflammatory signaling in cancer. Coiled-coil dimerization segment present in many bacterial TCPs such as the TcpB from *Brucella* is required for the potent suppression of TLR/IL1R innate immunity signaling. The addition of an artificial coiled-coil dimerization segment conferred superior inhibition of broad spectrum of TLRs and prevents the constitutive activation by a dimeric TIR platform. On the other hand oncogenic MyD88 mutants within its TIR domain, observed in B-cell lymphoma, displayed formation of similar cytosolic aggregates as tethered TIR domains and the same pattern of MyD88-dependent NF-kB activation, underlying generation of the cancer proinflammatory survival signal.

Conclusions: Microvesicles containing partially oxidized phospholipids thus represent a ubiquitous endogenous danger signal released under the oxidative stress, which underlies the pervasive role of TLR4 signaling in inflammation. Further we propose a molecular model of activation of TLR signaling based on the dimeric TIR-domain platform as the rate-limiting step of activation.

HOW DOES DEFECTIVE REGULATION OF RNA PROCESSING LEAD TO NEUROLOGIC DISEASES?

Julian König[1], Kathi Zarnack[2], Nicholas Luscombe[2], James R. Tollervey[1], Laura Easton[1], Mojca Tajnik[1,3], Gregor Rot[4], Črtomir Gorup[4], Tomaž Curk[4], Blaž Zupan[4], Boris Rogelj[5,6], Christopher E. Shaw[6], Jernej Ule[1]

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Defective regulation of RNA processing is implicated in several neurologic diseases. Recent renetic and neuropathological studies identified defects in two RNA-binding proteins (RBPs), TDP-43 and FUS, in frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS). We wish to understand how defective RBPs, or mutations in their RNA binding sites, can lead to neurologic diseases. We took three approaches to achieve this goal. First, we characterized the physiological RNA targets of TDP-43 and FUS. We found that the two proteins use distinct mechanisms to regulate pre-mRNA processing. However, both proteins predominantly regulate expression of genes with functions in neuronal development. Second, we analysed RNA isolated from the temporal cortex of individuals affected by FTLD or Alzheimer's disease (AD). Our results indicated that multiple RBPs contribute to the disease-related splicing changes, and revealed a relationship between the changes seen in disease and aging. Finally, we showed that specific mutations leading to neurologic disorders act by disrupting the RNA-binding sites of RBPs. I will present an overview of these studies, and give an outline of our current work.

STATES OF MIND IN CONNECTION WITH GENE EXPRESSION

Metka Ravnik-Glavač[1], Sonja Hrašovec[1], Jure Bon[2], Jurij Dreu[2], Damjan Glavač[1]

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Introduction: Meditation can be considered to be a universal human capacity, which, it is proposed, fosters clear thinking and open-heartedness, thereby developing a greater sense of emotional balance and well-being (1). However, there are reports of further states of the human mind, known to oriental thinkers for many centuries, in which the brain can transcend the boundaries of logic and reason, and experience states of extended awareness, commonly unrecognized (2). These higher states of consciousness are envisioned as natural to the experience and potential growth of every human being. A few individuals in any generation may spontaneously experience one or more of the higher states, but during the present age, for most people these states are accessible only as a result of regular practice of meditation technique. Long-term meditators of transcendental meditation techniques are described who have experienced periods of pure consciousness characterized by breath suspension episodes without compensatory hyperventilation, accompanied by high intra- and interhemispheric EEG coherence in alpha and theta frequencies (3).

Results: We designed this preliminary study to show whether differences in the subjective perception of a precisely experienced and defined higher states of consciousness are connected with significant and specific molecular genetic changes. In addition to phenomenological reports of the long-term meditation practitioners participated in this study the generated higher states of consciousness were also EEG recorded. Whole genome microarray expression analysis revealed significant differential expression of up to 1600 genes in higher state of consciousness compared to ordinary state of consciousness. Gene ontology enrichment analysis found significant down regulation in regulation of biological and cellular metabolic processes, signaling, protein transport, ubiquitin cycle, regulation of gene expression. Up-regulated GO enriched terms included immune system process, oxygen transport and homeostasis, intracellular signaling pathway, regulation of cell adhesion. Cell death and apoptosis, and response to stress were both up and down regulated in association with higher states of consciousness. More specifically, observed increase in mitochondrial genes expression together with neurotransmitter uptake might be connected with potential initiation of neuroplasticity process during higher state of consciousness.

Conclusions: The impact on health and well-being of the detected complex gene expression changes associated with higher state of consciousness is important to discover. It would also be interesting to discover whether individual genetic background could influence the ability to achieve a higher state of consciousness among long-term meditation practitioners.

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HORMONE INDEPENDENT SEX DIFFERENCES IN BRAIN AND BEHAVIOR

Tomaz Budefeld[1], Neza Grgurevič[1], Tanja Španič[1], Gregor Majdič[1,2]

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Mammalian sexual differentiation starts from the perspective of two sexes that correspond to sex chromosomes (X and Y) leading to individuals with sex typical characteristics [1]. Beside obvious external differences, sex differences exist in many other organs such as liver, immune system and brain. While sex chromosomes are usually credited as the trigger for generating sex differences, most sex differences in mammals are thought to arise due to differential exposure to sex steroid hormones secreted by gonads during development. More than 50 years ago a study of guinea pigs exposed to testosterone during pregnancy [2] led to a large number of studies showing that sex steroids play important roles for the sexual differentiation of brain and behavior [3]. Sex steroids, in particular testosterone and its metabolite estradiol, influence brain development and plasticity throughout the lifespan. Although sex steroid hormones account for most aspects of brain sexual differentiation, a growing literature has raised important questions about the direct role of genes on sex chromosomes [1, 4]. Sex chromosomes obviously differ by sex, but it has been controversial as to what extent the genes on these chromosomes might affect brain development directly and cause differences in the brain between males and females. In our studies, separate roles of sex chromosomes and sex steroid hormones in the brain development, utilizing SF-1 knockout mouse model, are studied. These mice are born without gonads and are never exposed to endogenous sex steroids [5], representing an excellent model to study sex differences in the absence of sex steroid hormones. Several sex differences in brain morphology and behavior have been established during our studies, confirming a direct role of genes on sex chromosomes in development of sex differences in the brain [6]. Results of these studies are important to better understand how sex differences in the brain develop, and how this leads to sex differences in many psychiatric disorders.

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Plenary Lecture

Friday, 29 June 2012

08.30 – 09.00

Ananda Chakrabarty, *University of Illinois, US*

Eradicating cancer in our lifetime: taking the first steps

ERADICATING CANCER IN OUR LIFETIME: TAKING THE FIRST STEPS

Ananda Chakrabarty[1]

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Cancer is a complex disease with a network of multiple metabolic pathways that are interlinked to promote growth and resist immune surveillance. Such a network is efficiently maintained through acquisition of multiple mutations in the human genome that result in the escape from normal cellular growth regulation and formation of lumps of fast growing cells known as tumors. The varied pathways through which cancer cells grow and inhibit their own cell death have made it difficult to develop effective drugs either to prevent the emergence of tumors or to check their rapid growth. Current anticancer drugs are either small molecules or monoclonal antibodies that target and inhibit a key important step in cancer progression pathway, thereby significantly inhibiting their proliferation. No effective drug or vaccine exists to prevent cancer initiation and drug resistance and toxicity are major problems in cancer chemotherapy. I will talk about our recent attempts to develop bacterial proteins that are used as weapons by certain pathogenic bacteria with long term residence in human bodies to prevent invasion of their habitat by invaders such as cancers, viruses or parasites. In one instance, such a protein, termed azurin, has been shown not only to have entry specificity in cancer cells and prevent cancer cell growth by interfering in multiple pathways by which cancer cells grow, but also to prevent induction of pre-cancerous lesion formation triggered by a potent carcinogen. A 28 amino acid peptide derived from azurin, p28, also shows similar anticancer and cancer preventive activity. In phase I human clinical trials, chemically-synthesized p28 has shown very little toxicity but significant beneficial effects, including partial and sometimes complete regression of metastatic refractory solid tumors in 15 advanced stage (stage IV) cancer patients where no conventional drugs were working. A second such protein, termed ATP-01, very different from azurin and obtained from a different bacterium, has shown similar anticancer and anti-HIV/AIDS activity and a 30 amino acid peptide derived from it has anticancer activity similar to p28. It would be of great interest to test these two proteins, should they prove to be non-toxic and non-immunogenic in humans, and the peptides derived from them, for their efficacy in cancer therapy and prevention. Such efficacies can be tested, singly or in combination, in vulnerable people such as people with predisposition to cancer (women with BRCA1/2 mutations, for example) or in people exposed to accidental leakage of radiation from nuclear reactors.

Complex Disease and Therapeutic Approaches

Friday, 29 June 2012

09.10 – 10.30

Borut Peterlin, *University Medical Centre Ljubljana, SI*
Integrative 'omic' approach towards understanding nature of human diseases

Janko Kos, *University of Ljubljana & Jožef Stefan Institute, SI*
Cysteine carboxypeptidase cathepsin X regulates neurotrophic activity of gamma enolase

Gregor Serša, *Institute of Oncology Ljubljana, SI*
Translational research on electrochemotherapy and gene electrotransfer in treatment of cancer

Ksenija Geršak, *University of Ljubljana, SI*
Hormone replacement therapy and risk of postmenopausal breast cancer

INTEGRATIVE 'OMIC' APPROACH TOWARDS UNDERSTANDING NATURE OF HUMAN DISEASES

Borut Peterlin[1], Ales Maver[1]

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Introduction: The combination of improving technologies for interrogation of global molecular alterations in human diseases along with increase in computational capacity, has enabled unprecedented insight into understanding of human disease. Although a large body of data from global high-throughput profiling studies has accumulated recently, complexity of data complicates reliable interpretation and translation to clinical use. To demonstrate additional value of integrative approach to interpretation of such data, we performed comprehensive integration of data from heterogeneous 'omic' studies in multiple sclerosis (MS).

Results: Data for inclusion was collected from 39 studies or bioinformatic sources. Altogether, 158.520 distinct significant signals discovered on 16 different biological levels were included in integration. Custom rank product prioritization approach based on genomic position of included results was utilized for data synthesis (Figure 1). In total, 381 genomic regions were characterized with significant accumulation of results, reaching local permutation p-value minima below 0.001.

Conclusions: Although integrative analysis of MS datasets identified number of genes already associated with MS, many novel genes were identified, whose involvement in MS is suggested by evidence in a complex body of data from omic studies, but their direct role in MS is not yet characterized and thus present plausible targets in further validation studies.

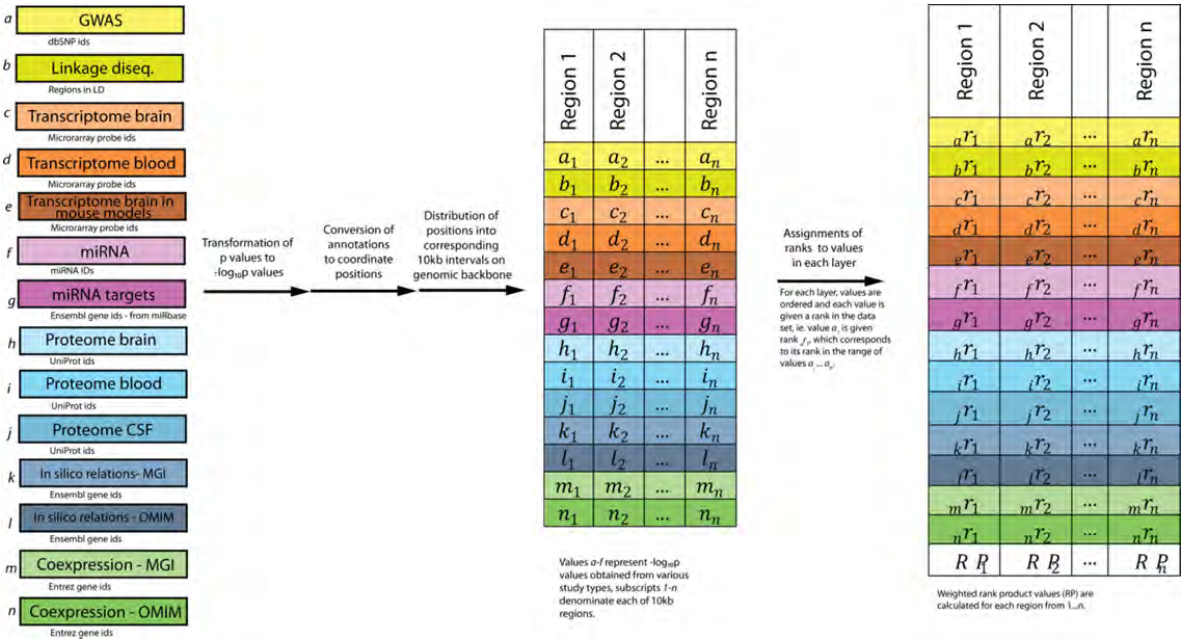

FIGURE 1: The integration process utilized in the synthesis of data from omic studies performed in MS.

CYSTEINE CARBOXYPEPTIDASE CATHEPSIN X REGULATES NEUROTROPHIC ACTIVITY OF GAMMA ENOLASE

Anja Hafner[1], Nataša Obermajer[2], Gordana Glavan[3], Marko Živin[3], Janko Kos[1,2]

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Cathepsin X is a cysteine carboxypeptidase expressed predominantly in immune cells. It is involved in immune response regulation, such as signal transduction, growth, maturation, adhesion, cell-cell communication, proliferation and migration of immune cells and in phagocytosis. By proteolytic cleavage of C-terminal amino acids, cathepsin X regulates β2 integrin functions and the role of CXCL-12 in adhesion of hematopoietic stem and progenitor cells to osteoblasts. Up-regulation of cathepsin X was associated also with the inflammatory processes in patients infected with *Helicobacter pylori*, those with multiple trauma, tuberculosis and Huntington disease.

Cathepsin X was also suggested to play a role in inflammation induced neurodegeneration. In mouse brain high levels of cathepsin X were detected in glial cells and aged neurons, its overexpression was determined also around β-amyloid plaques of transgenic Tg2576 mice model for Alzheimer's disease by using *in situ* hybridization and immunofluorescence. Gamma enolase was identified as a possible link between cathepsin X carboxypeptidase activity and neurodegeneration. As a multifunctional glycolytic enzyme, localized predominantly in neurons and neuroendocrine cells gamma enolase exhibits neurotrophic activity enhancing neuronal cell survival and neuritogenesis. Its neurotrophic function is associated with the intact C-terminal end and plasma membrane localization. We proved that cathepsin X subsequently cleaves two a.a. residues at C-terminal end of gamma enolase and that truncated gamma-enolase does not exhibit neurotrophic activity. Additionally, we demonstrated that gamma enolase acts as a neurotrophic factor if it binds via PDZ motif to gamma1-syntrophin, a scaffold protein, which translocates gamma enolase towards plasma membrane. The cleavage of C-terminal end of gamma enolase disrupts the PDZ binding motif, preventing the binding to gamma1-syntrophin and abolishing neurotrophic function. Our results further show that gamma enolase enhances cell survival and neuritogenesis through activation of PI 3-K/Akt and MEK/MAPK signaling pathways. PI 3-K/Akt pathway is involved in gamma enolase dependent pro-survival effect, whereas for neurite outgrowth the involvement of both, ERK1/2 and PI 3-K is necessary. The recognized mechanism of regulation of neurotrophic action of gamma enolase by cathepsin X indicates its importance in neurogenesis and implies a potential of cathepsin X inhibitors in treatment of neurodegenerative disorders.

TRANSLATIONAL RESEARCH ON ELECTROCHEMOTHERAPY AND GENE ELECTROTRANSFER IN TREATMENT OF CANCER

Gregor Serša[1]

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Introduction: Electroporation is a physical method for delivery of various molecules into cells by transiently increasing permeability of the cell membrane, using application of a controlled external electric field to the cells or tissues. Electroporation, *i.e.* application of electric pulses to the tumors, can be used for increased uptake of the drugs (electrochemotherapy), or as an alternative to viral gene delivery for transfection of therapeutic genes into different tissues (gene electrotransfer). Both applications have reached translation into the clinics.

Results: Electrochemotherapy is used predominantly in palliative intent for melanoma skin metastases and metastases of other histologies. The response rate is high; approximately 80% of objective responses and 70% long lasting complete responses [1]. Due to its simplicity the method is already used in European countries in routine setting [2]. However the development of the technology for deep seated tumors is still on-going; for liver and bone metastases, as well as for colorectal tumors and brain tumors. In Ljubljana a clinical trial developing technology for the treatment of liver metastases is on-going. High response rate and safety of the procedure have been recorded [3]. Gene electrotransfer of interleukin-12 coding plasmid into the tumors has proven its effectiveness on melanoma metastases, also in clinical trial [4]. It is our intention to collaborate on newly launched trial. Besides, this treatment approach has already been used in treatment of primary tumors in horses and dogs. In collaboration with Veterinary Faculty in Ljubljana several dogs have been successfully treated [5]. Furthermore, the first gene therapy trial using AMEP, an anti-angiogenic plasmid introduced intratumorally by electrotransfer into melanoma skin metastases was completed, with obvious antitumor effect. This was the first patient treated by gene therapy in Slovenia.

Conclusions: Electroporation based treatment approaches have reached their clinical applications. Electrochemotherapy has already reached prominent stage in Europe, while gene electrotransfer still needs further testing and development.

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HORMONE REPLACEMENT THERAPY AND RISK OF POSTMENOPAUSAL BREAST CANCER

Ksenija Gersak[1], Jasmina Ziva Cerne[1], Srdjan Novakovic[2]

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The major known risk factors for breast cancer are associated with prolonged exposure to increased levels of estrogens and their reactive metabolites. Several genetic polymorphisms within estrogen metabolic genes have been shown to have functional effects on the catalytic properties of their corresponding enzymes. These person-to-person differences define subpopulations of women with higher lifetime exposure to estrogens and/or estrogen metabolites. The aim of the association study was to examine the influence of estrogen metabolism genotypes (CYP1B1 c. 1294C>G, COMT c. 472G>A, GSTP1 c. 313A>G, MnSOD c. 47T>C) and of their interactions on postmenopausal breast cancer risk.

We studied 530 breast cancer cases and 270 controls of the same age and ethnicity. Genotyping was conducted by PCR-based RFLP analysis and TaqMan®allelic discrimination method. Adjusted odds ratios (OR) and 95% confidence intervals (CI) were calculated using logistic regression analysis.

None of the 4 genetic variants examined contributed to breast cancer risk individually. Significant associations with breast cancer risk were observed among women with two high-risk genotypes in CYP1B1 and COMT (OR 2.0, 95% CI 1.1-3.5) and in COMT and MnSOD (OR 2.0, 95% CI 1.0-3.8) as well as three high-risk genotypes in CYP1B1, COMT and GSTP1 (OR 2.7, 95% CI 1.1-6.8) and in CYP1B1, COMT and MnSOD (OR 12.2, 95% CI 1.4-102.3), compared to those with low-risk genotypes.

Our results show that not only the commonly accepted breast cancer risk factors and BRCA genes but even more, the individual susceptibility to the local toxic metabolites is of significant importance. In 4 selected genes within the estrogen metabolic pathways, we have confirmed the increased combined effects on breast cancer risk.

Cancer Research and Clinical Practice

Friday, 29 June 2012

11.00 – 12.15

Tamara Lah-Turnšek, *National Institute of Biology, SI*
Cancer associated stem cells in glioblastoma and their clinical relevance

Radovan Komel, *University of Ljubljana & National Institute of Chemistry, SI*
Roots of cancer: possible role of genetic variants in chromosome segregation

Srdjan Novaković, *Institute of Oncology Ljubljana, SI*
BRCA1 and BRCA2 sequence variations in Slovene population

Tadej Pajič, *University Medical Centre Ljubljana, SI*
Molecular – genetic analysis in leukaemias

CANCER ASSOCIATED STEM CELLS IN GLIOBLASTOMA AND THEIR CLINICAL RELEVANCE

Tamara Lah Turnšek[1], Helena Motaln[1], Kristina Gruden[2], Matjaž Hren[2,3], Ana Rotter[2], Christian Schichor[4]

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Introduction: Glioblastoma is one of the most fatal tumors and most abundant type of the CNS malignancies. The major reason for the treatment failure is high infiltrative invasion of the tumor cells, and its high resistance of the tumor initiating and propagating cells with stem cell characteristics, termed GBM stem cells (GSC). Further dimension to the complexity of tumor progression is due to the role of tumor micro-environment, recognized today as playing an equally important, if not even a decisive role in glioma progression. Among various types of stromal cells, ere is strong the evidence has recently accumulated that bone marrow derived mesenchymal (BM-MSC) stem cells infiltrate the glioma, presumably to repair the tumor perturbed brain tissue, but may finally result even in an increase of GBM malignancy. The complexity of all above mentioned interactions can only be revealed by the so called "omics" approaches and using systems biology tools.

Results: Here, we investigated the indirect interactions of MSC obtained from bone marrow and cultivated GBM cells under *in vitro* conditions, which would mimic the introduction of MSC to the tumors. Four different MSC clones and three different GBM cell lines (U87, U373 and U251 lines) were used to study their mutual paracrine interactions in co-cultures compared to their monocultures. The effects on cell growth, proliferation and invasion were quantified. The cellular cross- talk was monitored by the cytokines arrays and the gene expression in each of the interacting cell type was determined by cDNA microarrays (Illumina). Further, bioinformatics tools were used to relate these results to identify the key proteins and genes significantly involved in the cellular crosstalk.

We demonstrated that MSC are responsible for the impairment of GBM cell invasion and proliferation, possibly *via* induction of their senescence. On the other hand, U87-MG cells even more strongly inversely affected growth and invasion of MSCs. The CCL2/MCP-1 was collectively identified as the most significantly up regulated chemokine in MSC cell line and its role in U87-MG cell invasion was functionally confirmed. This and some other chemokines convey the intercellular signaling in U87 cells, affecting the genes associated with proliferation (Pmepa-1, NFkB, IL-6, IL-1b), invasion (EphB2, Sod2, Pcdh18, Col7A1, Gja1, Mmp1/2) and senescence (Kiaa1199, SerpinB2).

Conclusion: Here we propose a novel mechanism of CCL2/MCP-1 anti-migratory effects on GBM cells, distinct from its immunomodulatory role. Other key molecules relevant to understand the GBM microenvironment, may also be used in clinical settings as biomarkers. Significant alterations of GBM phenotype in the presence of MSC should encourage further studies on the use of naive or modified MSC for GBM treatment.

Acknowledgements: Supported by the INREMOS -SYSTHER (#3211-06-000539) and by ARRS Program P1-0245.

ROOTS OF CANCER: POSSIBLE ROLE OF GENETIC VARIANTS IN CHROMOSOME SEGREGATION

Radovan Komel[1], Petra Hudler[1], Marija Rogar[1], Carlo V. Bruschi[2], Lawrence Banks[3]

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Introduction: Malignant transformation of normal body cells is a complex and multistep process, involving different genomic, genetic and epigenetic changes. The most widely accepted view of how cancer begins has been that mutations of special genes eliminate tumor suppressor proteins and activate oncoproteins. However, the early instability theory tells us that cells, to accumulate a sufficient number of mutations to form a tumor, should suffer a malfunction in DNA duplication and repair machinery and thus dramatically accelerate the mutation rate. Opposite to this view, all-aneuploidy hypothesis predicts that the emergence and progress of a tumor are more closely connected to the appearance of abnormal number of chromosomes and/or aberrant chromosomes with truncations, extensions or swapped segments. Aneuploidy could lead to early genomic instability and consequently to a later serial mutation of known cancer genes leading to inactivation of tumor suppressors, activation of oncoproteins, and inducing changes in genes, implicated in normal homeostasis of cells. Recent studies revealed that subtle changes in mitotic segregation genes, controlling the chromatid separation, are the most probable candidates for inducing the slow process of accumulation of genetic changes, leading to chromosomal instability. This paradigm is further supported by the fact that this process is, as mentioned, slow, and explains the late onset of sporadic epithelial cancers.

Methods: Our approach is based on the determination of polymorphic genes which could be responsible for genome instability, using case-control and genome wide association studies. Gastric cancer, the model disease in this study, is a major health problem worldwide; it is often diagnosed at an advanced stage when the prognosis is poor, and molecular mechanisms of its development are complex and still largely unclear.

Results: We analysed distribution of functionally important polymorphisms (SNPs) - minor sequence variations that only subtly change protein expression, activity or function, and rarely cause disease but can predispose individuals to develop disease - in TTK and BUB1B genes, which are implicated in chromosome segregation. Genotyping was carried out using real-time PCR, DNA sequencing and high-throughput high resolution DNA melting (Roche). We evaluated the association between the coding SNPs in gastric cancer patients by comparing them with healthy controls. We determined the association between polymorphisms and clinicopathological features of the patients.

Conclusion: The selected polymorphisms showing close association with the disease are in the process to be evaluated in a larger cohort of patients, and the effect of polymorphisms and their combinations functionally assessed in controlled environment in cell lines and yeast. The analyses of candidate genes and determination of their possible effect on gastric cancer development could contribute to better clinical diagnostics and better prediction of the disease progression.

BRCA1 AND *BRCA2* SEQUENCE VARIATIONS IN SLOVENE POPULATION

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Introduction: Breast cancer is the most frequent type of cancer in Slovene female population. Between 2004 and 2008, there were per average 1127 new breast cancer patients registered yearly. The average incidence rate during this period was therefore 110.2/100000. Together, breast and ovarian cancers represent as much as 23.9% of all cancers in Slovene female population. The majority of these two types of cancer are sporadic and only 5%-10% are known to be caused by dominantly inherited susceptibility genes. Around 50% of the hereditary breast and ovarian cancer cases have their underlying cause in germline mutations of *BRCA1* and *BRCA2* susceptibility genes. Mutations of *BRCA1* and *BRCA2* are highly penetrant and confer an increased risk of breast and ovarian cancer to carriers. Since 1999, genetic counseling and testing for hereditary breast and ovarian cancer have been offered at the Institute of Oncology Ljubljana. The aim of this article is to present *BRCA1* and *BRCA2* mutation spectrum and mutation detection rates according to different family histories in Slovenian population for the 10 years' period.

Results: Until January 2009, approximately 1100 individuals have attended genetic counseling and 521 individuals from 322 families opted for genetic testing. *BRCA1* and *BRCA2* genes were screened using DGGE, PTT, HRM, MLPA and direct sequencing. Eighteen different mutations of *BRCA1* and 13 of *BRCA2* gene were found. Among those, 5 mutations of *BRCA1* and 4 mutations of *BRCA2* have not been described in other populations until now. Mutations of one or the other gene were found in 96 unrelated families. The most common mutations of *BRCA1* gene in families with an increased risk of breast and/or ovarian cancer in Slovenia are c.181T>G, c.1687C>T, c.5266dupC, c.844_850dupTCATTAC and c.181T>A. The most frequent mutations of *BRCA2* are c.7806-2A>G, c.5291C>G and c.3975_3978dupTGCT. The cumulative share of these frequent mutations of *BRCA1* and *BRCA2* genes in Slovenian families affected by the mutations is 68.7%.

Conclusion: In Slovene population, the mutation detection rate seems to be influenced by the presence of ovarian cancer history, the age of the individual at the diagnosis of breast cancers in the family and the number of breast cancer cases in the family. Moreover, the mutation detection rate might further be influenced in the future – namely, it might increase when the character of some of the unclassified variants will be clarified as pathogenic or decrease with the increasing number of tested individuals.

MOLECULAR-GENETIC ANALYSIS IN LEUKAEMIAS

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The discovery of the Ph chromosome in the 1960s, the hallmark of chronic myeloid leukaemia (CML), was the first consistent chromosomal abnormality associated with any malignancy. Since then, cytogenetics and molecular genetics techniques have played a critical role in the diagnosis, prognosis and management of many hematological neoplasms. Leukaemia represents a clonal disease, which is acquired due to genetic alterations such as chromosomal rearrangements that include fusion genes as a consequence of translocations, rarely deletions, over expression of oncogenes and sequence variations. These can be detected by polymerase chain reaction-based (PCR) techniques that complement and add values to the results obtain by standard karyotyping.

The novel quantitative assay, real-time quantitative PCR (qPCR), permits sensitive detection of low levels of minimal residual disease (MRD) below the level detectable by morphological examination and cytogenetic analysis. The rise of MRD level indicate leukaemia burden and could offer to treat the patient prior to clinical relapse.

As an example, recognition of the tyrosine kinase (TK) activity of the BCR-ABL1 proteins led to the discovery of a series of compounds with inhibitory activity against *BCR-ABL1*-encoded proteins. One of them, imatinib mesylat, was rapidly introduced into clinical practice and has revolutionized the treatment of CML, *BCR-ABL1* positive patients. Today, there are two other approved BCR-ABL1 TKIs for newly diagnosed chronic phase-CML patients, nilotinib and dasatinib. Many CML patients in chronic phase treated with IM or other drugs, nilotinib or dasatinib, achieve a complete cytogenetic response. To evaluate the response of such treatment in more detail, a more sensitive test, such as qPCR, is required. The measurement of BCR-ABL1 transcript level by qPCR has become important for evaluating treatment success in CML patients.

Molecular Mechanisms & Biomarkers of Disease

Friday, 29 June 2012

13.45 – 14.50

Nick A. Bersinger, *University of Bern, CH*
Importance and usefulness of biomarker determination in endometriosis

Tea Lanišnik Rižner, *University of Ljubljana, SI*
Roles of steroid hormones in hormone dependent diseases

Katarina Trebušak Podkrajšek, *University Medical Centre Ljubljana, SI*
Genetic and immunological markers in patients with autoimmune polyglandular syndrome type 1

IMPORTANCE AND USEFULNESS OF BIOMARKER DETERMINATION IN ENDOMETRIOSIS

Nick Bersinger[1,2], Brett McKinnon[2], Michael Mueller[1]

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Endometriosis is an extremely prevalent condition that is accompanied by chronic pain, reduced fecundity and an increased risk of ovarian cancer later in life – all of which are related to inflammation. The most widely accepted theory of endometriotic lesion development is retrograde menstruation, a process by which viable endometrial cells are refluxed backwards to the peritoneal cavity. These cells attach to the underlying tissue and via hormonal and inflammatory stimulation continue to grow. As retrograde menstruation has been shown to occur in >85% of women additional factors supporting the attachment of endometrial tissue in endometriosis patients must be involved. We are studying the presence of inflammatory cytokines, angiogenic, neurogenic, and growth factors in the peritoneum at both the gene expression and functional protein level on eutopic (intra-uterine) and ectopic (extra-uterine endometriotic lesion) tissue.

This presentation focuses on the determination of cytokine and growth factor protein concentrations in peritoneal fluid (PF) of women undergoing laparoscopic investigation for reasons of pain or unexplained infertility in the aim of identifying the best marker (or marker panels) for the biochemical diagnosis of endometriosis. An ideal molecule should present significant concentration differences between cases and controls, but at the same time vary as little as possible with patient age and menstrual cycle phase. A correlation with the extent of pain experienced by the patient should identify the markers with a potential neurostimulatory action and which may later become treatment targets.

Microplate immunometric assays, either as single ELISA or based on multiplexed double fluorescence (x-map) technology, are performed since 2005 in our laboratory on PF of women with and without endometriosis, and reporting different levels of experienced menstrual or other types of pain. Analyses on matched PF and serum samples have been initiated recently in a collaborative project with the University of Ljubljana.

Preliminary results show that:

1. Concentrations of IL-6, IL-8, IL-18, RANTES, PAPP-A, eotaxin and MCP-1 were increased in patients with endometriosis, for most of these markers as a function of the severity of the disease (rAFS stage) and without menstrual cycle variations;
2. Levels of IL-12(p70), ICAM-1, and GRO-α were higher in the secretory phase, while eotaxin concentrations were lower;
3. The presence of dysmenorrhoea was positively correlated with the PF levels of TNF-α, glycodelin A and OPG;
4. Hormonal treatment of endometriosis with steroids or GnRH analogues affected the PF levels of many cytokines and growth factors.

First comparisons indicate that serum and peritoneal fluid levels of most markers show only a poor correlation; the panels of diagnostically useful molecules are therefore likely to differ between the two compartments.

We conclude that some PF and hopefully serum markers have the potential to be used in the detection of endometriosis and the identification of patients who would benefit from hormonal or surgical treatment. The “miracle” molecule, however, has not been identified to date.

ROLES OF STEROID HORMONES IN HORMONE DEPENDENT DISEASES

Tea Lanišnik Rižner[1], Neli Hevir[1], Nataša Beranič[1], Maša Sinreih[1]

[1]FACULTY OF MEDICINE, UNIVERSITY OF LJUBLJANA, LJUBLJANA, SLOVENIA

Steroid hormones have important roles in human physiology, and their disturbed actions can lead to the development of hormone-dependent cancers and several benign diseases. Hormone-dependent cancers comprise around 30% of all cancers, and include prostate, breast and endometrial cancers. Worldwide, more than 2 million people are affected and more than 700,000 die annually by different types of hormone dependent cancers. Benign hormone-dependent diseases are also very common. Endometriosis, for instance, affects, and significantly impairs, an estimated 176 million women worldwide, and is diagnosed in up to 15% of pre-menopausal women and in 35% to 50% of women with infertility or pelvic pain. These enormous numbers of patients and deaths that are attributed to these hormone-dependent diseases demonstrate the uttermost importance for a detailed understanding of their pathophysiology, including the roles of the steroid hormones.

Estrogens can stimulate gene expression and cell proliferation, and are thus implicated in endometriosis. With the enhanced proliferation estrogens further increase the number of DNA replication errors, and recent data show that estrogens do not act solely as mitogens but can also cause DNA and protein damage via their oxidation products, the catechol estrogens and quinones. In this manner, the estrogens are implicated in the development of breast cancer and endometrial cancer. In addition to estrogens, progesterone also has an important role in breast cancer, endometrial cancer and endometriosis. Studies have shown that progesterone can either prevent cell proliferation and have differentiating effects, or can stimulate cell proliferation. These opposing effects can be explained by differences in progesterone metabolism. The disturbed ratio between progesterone metabolites can result in increased levels of 5a-pregnanes, which activate membrane-bound receptors and stimulate cell proliferation and migration, and decrease apoptosis. In this lecture the novel mechanisms of estrogen and progesterone actions in breast cancer, endometrial cancer, and endometriosis will be presented and the application of steroid hormone metabolites as potential biomarkers will be discussed.

GENETIC AND IMMUNOLOGICAL MARKERS IN PATIENTS WITH AUTOIMMUNE POLYGLANDULAR SYNDROME TYPE 1

Katarina Trebušak Podkrajšek[1], Kai Kisand[2], Anthony Meager[3], Nina Bratanič[1], Pärt Peterson[2], Tadej Battelino[1]

[1]UNIVERSITY CHILDREN'S HOSPITAL, LJUBLJANA, SLOVENIA, [2]INSTITUTE OF GENERAL AND MOLECULAR PATHOLOGY, UNIVERSITY OF TARTU, TARTU, ESTONIA, [3]BIOTHERAPEUTICS, THE NATIONAL INSTITUTE FOR BIOLOGICAL STANDARDS AND CONTROL, SOUTH MIMMS, HERTS, UK

Autoimmune polyglandular syndrome type 1 (APS-1) is a rare autosomal recessive disorder associated with mutations in *AIRE* gene. It presents as s combination of various autoimmune disorders. The current clinical diagnosis is based on the presence of at least two commonest clinical manifestations (chronic mucocutaneous candidiasis, hypoparathyroidism, Addison's disease). The clinical presentation along with the associated organ-specific tissue specific autoantibodies is extremely variable. In addition, APS-1 patients develop high-titer neutralizing autoantibodies against type I interferones and Th17-related cytokines, with a prevalence of 100% for IFN-ω and more than 90% for IL-22. With the exception of thymomas, IFN-ω autoantibodies are not found in numerous other infectious, autoimmune or neoplastic disorders. IFN-ω autoantibodies measurement is recommended as first diagnostics test when APS-1 is suspected and are followed by *AIRE* gene mutational analysis. More than 90 different mutations in APS-1 are described so far, but the mutations itself are usually not predicting the clinical presentation in the patient. Exception is the common p.Arg257X associated with higher prevalence of chronic mucocutaneous candidiasis. This mutation was detected in more than 70% of mutated Slovenian APS-1 patients alleles.[1] Additionally, 3 novel mutations restricted to Slovenian populations were identified.

APS-1 various endocrine features are resulting from defects in thymic self-tolerance induction due to *AIRE* gene defect. But the immunodeficiency underlying chronic mucocutaneous *Candida* infection is a long-standing puzzle. Multicenter survey including Slovenian APS-1 patients revealed neutralizing autoantibodies against IL-17A in 41%, IL-17F in 75% and/or IL-22 in 91% of over 150 included APS-1 patients, especially those with chronic mucocutaneous candidiasis. The autoantibodies preceded chronic mucocutaneous candidiasis.[2] Furthermore, production of IL-22 and IL-17F in patients peripheral blood mononuclear cells restimulated with *Candida* preparations were significantly reduced. Surprisingly, these reductions strongly associated with neutralizing autoantibodies to IL-17F and IL-22, whereas responses were normal and autoantibodies infrequent in APS-1 patients without candidiasis. Therefore, IL-22 and IL-17F are strongly suspected to be key natural defenders against chronic mucocutaneous *Candida* infection in APS-1 and the immunodeficiency underlying to have an autoimmune basis.

References:

1. Trebusak Podkrajsek K., et al. (2005) J Clin Endocrinol Metab 90(8):4930-5.
2. Kisand K., et al. (2010) J Exp Med.207:299-308

Biochemistry, Microbial World & Biotechnology

Friday, 29 June 2012

15.20 – 16.45

Jurij Stojan, *University of Ljubljana, SI*
The significance of low substrate concentration measurements for mechanistic interpretation in cholinesterases

Ana Plemenitaš, *University of Ljubljana, SI*
Molecular mechanisms of adaptation to extreme environments

Uroš Petrovič, *Jožef Stefan Institute, SI*
***Saccharomyces cerevisiae* cell – a molecular biologist's test tube**

Andreja Plaper, *KRKA d.d., SI*
Managing of genotoxic impurities in pharmaceutical products

Barbara Kunič Tešović, *Lek d.d., SI*
Intellectual Property: recent case law in the field of biomedicine

THE SIGNIFICANCE OF LOW SUBSTRATE CONCENTRATION MEASUREMENTS FOR MECHANISTIC INTERPRETATION IN CHOLINESTERASES

Jure Stojan[1]

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Cholinesterases do not follow the Michaelis Menten kinetics. In the past many reaction schemes were suggested to explain their complex interactions during the substrate turnover. Covalent catalysis was recognized very early and therefore, double intermediate traditional reaction scheme for the hydrolysis of good substrates at low concentrations was postulated. However, at intermediate and high substrate concentrations homotropic pseudocooperative effects take place in all cholinesterases, due to the nature of their buried active center. In an attempt to comprise all these events in a comprehensive catalytic cycle, we suggested that the catalysis starts with the binding of first substrate molecule at the entrance to the active site followed by it sliding to the bottom, where actual hydrolysis occurs. While covalent transformation is still in progress, another substrate molecule can influence it in two ways: depending on the size and plasticity of particular active site the product exit can be hindered or the accommodation of the substrate can be enhanced. At the extremely high substrate concentrations all cholinesterases get blocked when the enzyme's active site becomes fully occupied with several substrate molecules. We have constructed an appropriate reaction scheme and quantitatively evaluated each particular step for several types of acetylcholinesterases. Currently, efforts are made to develop a simple approach for fast characterization of all steps applicable to any cholinesterase. It is based on the evaluation of initial rate data measured in a very wide concentration range, combined with a subtle progress curve analysis at low substrate concentrations. A special emphasis is put on butyrylcholinesterase, the enzyme with the largest active site among cholinesterases, where the pseudocooperative effects appear at much higher concentrations then in acetylcholinesterases.

MOLECULAR MECHANISMS OF ADAPTATION TO EXTREME ENVIRONMENTS

Ana Plemenitaš[1], Metka Lenassi[1], Tilen Konte[1], Nina Gunde-Cimerman[2]

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Solar salterns and other similar hypersaline environments, are extreme habitats that prevent growth of most organisms except those, well adapted to extremely high salt concentration. In eukaryotic microorganisms, molecular studies of salt tolerance have been limited to the baker's yeast *Saccharomyces cerevisiae* and few other moderately halotolerant yeasts. Isolation of the extremely salt tolerant black yeast *Hortaea werneckii* and halophilic fungus *Wallemia ichthyophaga* in hypersaline waters of solar salterns enabled the introduction of new model organisms to study the mechanisms of salt tolerance in eukaryotes and also a promising source of transgenes for improvement of salt tolerance of industrially important yeasts and crops. While *H. werneckii* is unique in its adaptability to fluctuations in salt concentration and grows without NaCl as well as in the presence of up to 5M NaCl, *W. ichthyophaga* on the other hand requires the presence of at least 1,7M of NaCl and grows up to 5M NaCl is a true halophile. Strategies of adaptations on the physiological and molecular level in extremely salt-tolerant *H. werneckii* and halophilic *W. ichthyophaga* revealed novel, intricate mechanisms to combat fluctuating salinity. Observed physiological adaptations like increased membrane fluidity and maintenance of low intracellular sodium concentration over a wide range of salinity, accumulation of glycerol and other small molecules as compatible solutes correlate well with the changes at the molecular level in both model organisms. Sensing changes in sodium concentrations in the environment and responding to them is vital for cell survival. We have identified in *H. werneckii* and in *W. ichthyophaga* the components of pathway homologous to the HOG signal transduction pathway that senses and responds to changes in osmolarity in *S. cerevisiae.* Analysis of the proteins demonstrated important structural differences between the components of HOG pathway in halophilic fungi, isolated from solar saltern and salt-sensitive *S. cerevisiae,* as well as between extremely salt tolerant black yeast *H. werneckii* and halophilic *W. ichthyophaga.* Identification of HAL2 as promising transgene to improve halotolerance will be also discussed.

SACCHAROMYCES CEREVISIAE CELL – A MOLECULAR BIOLOGIST'S TEST TUBE

Uroš Petrovič[1], Mojca Mattiazzi[1,4], Petra Kaferle[1], Janez Kokošar[1], Igor Križaj[1], Toni Petan[1], Tina Kocjan[1], Andrej Bavdek[2], Tomaž Curk[2], Gregor Anderluh[2], Blaž Zupan[2], Klaus Natter[3], Heimo Wolinski[3], Sepp D. Kohlwein[3], Christoph F. Kurat[4], Igor Stagljar[4], Corey Nislow[4], Charlie Boone[4], Yidi Sun[5], David Drubin[5], Joe DeRisi[6]

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The genome of budding yeast *Saccharomyces cerevisiae* is the most thoroughly sequenced and the best annotated eukaryotic genome. Although a unicellular fungus, *S. cerevisiae* shares several key characteristics with animal cells and many of the biological processes are evolutionarily conserved from yeast to humans. Its unicellularity, alongside with the amenability for genetic and functional genomic experimental approaches, actually presents an advantage in using yeast as a model organism for studying basic biological processes in eukaryotes on the molecular level. I will present both our published and ongoing research on using *S. cerevisiae* to address three different biological questions. To understand the mechanism of action of a snake venom neurotoxin we used genome-wide synthetic dosage lethality method and identified, and later confirmed, that inhibition of the activity of amphiphysin is the major step causing reduction of endocytosis. Chemical genomic and transcriptomic studies helped us find the targets of an orphan drug which could be used for treatment of type 2 diabetes, neurodegenerative diseases and cystic fibrosis, and its major target was found to be AMP-dependent kinase. A high-throughput genetics method called X-QTL was improved and used to identify in a single experiment several genes which confer high lipid content phenotype in an industrial yeast strain. A new layer of regulation of energy metabolism was found in this latest study. In our hands, as well as in numerous laboratories world-wide, yeast proves to be an excellent model organism for biomedical and biotechnologically oriented studies.

MANAGING OF GENOTOXIC IMPURITIES IN PHARMACEUTICAL PRODUCTS

Andreja Plaper[1]

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Since 2007, when the finalized guideline on genotoxic impurities was formally published, the issue of genotoxic impurities (GTIs) has become one of the very important issues facing industry. To address the challenges posed in the guideline it was necessary to adopt a multidisciplinary strategy that draws in experts from many functions. Primary within these are the safety and chemistry functions. The fundamental issue of the successful strategy is a team which consists of chemist, toxicologist and analyst. Chemist produces a list of potential impurities (known and theoretical) for each active pharmaceutical ingredient (API) and the product. Toxicologist estimates the toxic potential of listed impurities with literature search for published data or *in silico* evaluation, and establishes the safety limit for each impurity. Analyst defines the levels of GTI with quantitative analysis. The action taken for each impurity depends upon its toxicity and the level present in API or product. The meaning of the toxicologist's work in the described strategy will be discussed in more details.

INTELLECTUAL PROPERTY: RECENT CASE LAW IN THE FIELD OF BIOMEDICINE

Barbara Kunič Tešovič[1]

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In European patent law, patents should not be granted for inventions which are contrary to the 'ordre public' or morality and in the field of biomedicine 'ethics and patenting' is one of the most discussed topics. Furthermore, with the development of modern biotechnology the question of industrial applicability became of most relevance which was not the case in other fields of technologies. In relation to these topics two interesting decisions were reached in 2011 at the European courts.

In October 2011 an important patent case relating to human embryonic stem cells (hES cells) was decided [1]. The case can have influence only on patenting and not on research relating to hES cells or any other even commercial exploitation. The background of the case lies in the fact that the research of the stem cells requires the use of embryos. The European Biotech Directive [2], however, excludes the patentability of inventions which claim the use of human embryos for commercial or industrial purposes. Mr. Brüstle held a patent for isolated and purified neural precursor cells derived from human blastocysts. Greenpeace filed a nullity action against this patent in Germany and the German Court referenced to the CJEU three questions, the first relating to the definition of 'human embryo', the second to the use of human embryo for commercial purposes, and the third question to the patentability of use of human embryos. With the answers of CJEU European Union gave an EU-wide definition of an embryo. The case has now returned to the German Court for the final decision to determine the patentability in light of CJEU decision. In further development it will be interesting to see the position of national courts especially on the patentability of pluripotent and multipotent hESCs.

The second interesting recent decision relates to the UK Supreme Court decision which overturned the key human genome patent ruling [3]. The provision of the European Patent Convention (EPC) in which Art 57 clarifies that an invention is susceptible of industrial application 'if it can be made or used in any kind of industry...' [4]. From the judgment several general principles of determining the conditions to be met that invention is susceptible to industrial application. This decision brings in line with the EPO Boards of Appeal decisions the UK case law and is clear indication that the UK courts are now prepared at least to some extent to follow the EPO case law.

References

1. Judgment of the Court (Grand Chamber); 18/10/2011. O. Brüstle v Greenpeace. Case C-34/10.
2. Directive 98/44/EC of the European Parliament and of the Council; 6/7/1998; O. J. L 213 , 30/07/1998 P. 0013 - 0021
3. Human Genome Sciences Inc v Eli Lilly & Co [2011] UKSC 51
4. European Patent Convention

Closing Lecture

Friday, 29 June 2012

16.50 – 17.35

Matija Peterlin, *University of California, US*

HIV and eukaryotic biology

HIV AND EUKARYOTIC BIOLOGY

Matija Peterlin[1,2]

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Since the discovery of the AIDS virus, HIV, it has contributed greatly to our understanding of eukaryotic biology. Following its replicative cycle from entry, it has given us TRIM28 and APOBEC3 family of cytidine deaminases, which restrict its passage through the cytoplasm in the absence of viral capsid (CA) and viral infectivity factor (Vif), respectively. It requires a cellular protein, LEGF, to integrate into actively transcribed genes and open chromatin. There, the virus can remain latent because of transcriptional interference and lack of host cell transcription factors. Chief among these is P-TEFb, which consists of cyclin T1 and CDK9 that phosphorylates negative elongation factors and the CTD of RNA polymerase II. As a result, transcription elongates and nascent transcripts are processed efficiently. The viral transactivator Tat binds to P-TEFb directly as well as to the transactivation response (TAR) RNA stem loop, thus increasing greatly viral transcription and HIV replication. Indeed, investigations into Tat revealed P-TEFb, which has since garnered great interest as the co-factor of cMyc and NF-kB. After 46 different HIV transcripts are made, those that are not spliced or are singly spliced are exported from the nucleus to the cytoplasm with the help of Rev, another viral regulatory protein. It binds CRM1 (aka exportin1), RanGTP and components of the nuclear pore. Tat and Rev revealed not only these proteins but began these fields of investigation. Although the assembly process of HIV remains poorly understood, it has highlighted the roles played by RNA helicases, miRNAs and translational silencing, as well as of the ESCRT complex, which is required for the release of new viral particles into the supernatant. Several viral accessory proteins are also important in this release, which include Vpu that counteracts tetherin. Nef also increases the formation of viral particles and Nef exosomes, which have deleterious effects on bystander cells. Finally, the accessory Vpx protein from HIV-2 and SIV revealed the role/s of innate nucleic acid sensors on the replicative cycle of these retroviruses. From these careful analyses have emerged drugs that not only inhibit the replication of HIV but have become useful in the treatment of HBV, HCV, other viral infections in humans and of autoimmune diseases in mouse models. I will highlight studies of the HIV replicative cycle where we contributed during the past 25 years.

Poster Abstracts

Poster Session

Wednesday, 27 June 2012

18.30 – 20.00

P1

INFLUENCE OF STEARYL AND TRIFLUOROMETHYLQUINOLINE MODIFICATIONS OF THE CELL PENETRATING PEPTIDE TP10 ON ITS INTERACTION WITH A LIPID MEMBRANE

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Cell-penetrating peptides (CPPs) are defined as amphipathic peptides composed of less than 30 amino acids that are able to enter the cell. In doing this, they can carry into the cell a large number of different cargoes, from small molecules to large proteins and plasmids, that themselves cannot access the cell interior. Mechanisms of cell uptake are divers, mainly endocytotic but also direct passage of the membrane has been observed. For all types of internalization the interaction with the membrane is essential. We have investigated the interaction of transportan-10 (TP10) and its modified analogs PepFect 3 and 6 (PF3 and PF6) with different model lipid monolayers and bilayers using phase contrast and fluorescence microscopy, differential light scattering, plasmon surface resonance, and others, in order to gather new data on the behavior of membranes in contact with the selected CPPs. Both PF3 and PF6 were designed by addition of a stearyl moiety to TP10 to improve the delivery of nucleic acids across plasma membranes. PF6 was further modified by addition of the complex organic structure composed mainly from trifluoromethylquinoline moieties in order to gain the ability of endosomal escape. We show that the addition of a stearyl moiety to TP10 increases the amphipathicity of these molecules and their ability to insert into a lipid monolayer composed of zwitterionic phospholipids. The addition of negatively charged phospholipids into the monolayer results in decreased binding and insertion of the stearylated peptides, indicating modification in the balance of hydrophobic versus electrostatic interactions of peptides with lipid bilayer, thus revealing some clues for the selective interaction of these CPPs with different lipids. Surprisingly, the large trifluoromethylquinoline moieties in PF6 make no significant contribution to membrane binding and insertion. TP10 actively introduces pores into the bilayers of large and giant unilamellar vesicles, while PF3 and PF6 do so only at higher concentrations. This is consistent with the lower toxicity of PF3 and PF6 observed also in previous studies.

P2

MEASUREMENTS OF MICROVESICLES ISOLATED FROM BLOOD BY FLOW CYTOMETRY

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Objective: Microvesicles (MVs) are 0.1 to 1 µm-sized membrane–enclosed particles which are pinched off from cells of all types and are detected in isolates from body fluids. Flow cytometry is the most widely used method for measurement of MVs in blood isolates; however, the accuracy of the method is limited.

Results: Blood was taken from human donors into vacutubes. MVs were isolated by repeated centrifugation and washing of the samples and counted by flow cytometry. Forward scattered light (FS) and side scattered light (SS) was detected and analyzed by flow cytometry on a Beckman Altra flow cytometer and on Miltenyi MACSQuant Analyzer. While forward scatter resolution and low background noise allow us to discriminate between 0.5 µm and 0.9 µm beads (Figure 1C), the shape of the region corresponding to MVs (Figure 1) indicates that particles smaller than 0.4 µm cannot be detected.

Conclusion: Flow cytometry does not detect the smallest MVs which are also expected to be abundant in patients with various diseases.

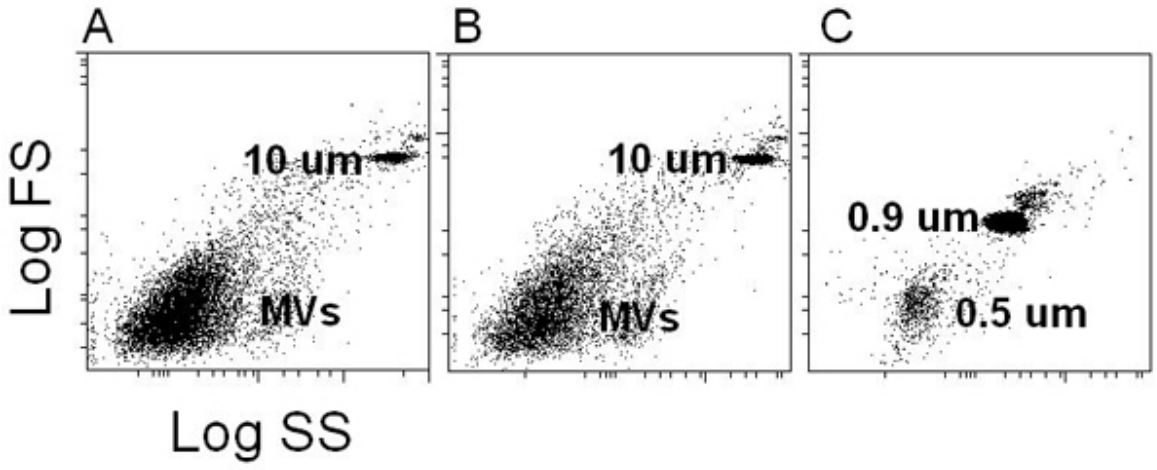

FIGURE 1: Flow cytometric diagrams of FS/SS corresponding to the samples from a patient with gastrointestinal cancer (A; calibrating beads are added), healthy donor (B; calibrating beads are added), and Megamix beads (C).

P3

PROGESTINS CONTRIBUTE TO INCREASED PROGESTERONE AND DECREASED ESTROGEN ACTION IN PERITONEAL ENDOMETRIOSIS

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Introduction: Endometriosis is a very common gynecological disease with disturbed hormone action. The diminished protective effects of progesterone (P4) are often explained with lack of progesterone receptors (PGR) while our unpublished data suggest that they are also related to P4 metabolism at the pre-receptor level. In peritoneal endometriosis estradiol (E2) can be synthesized locally in the endometriotic lesions predominantly via the aromatase pathway. These increased E2 levels stimulate proliferation of endometriotic tissue while disturbed P4 action prevents differentiation and thus common medical therapies are focused either on lowering E2 levels or stimulating P4 action. Among these safer but still effective option is application of PGR agonists, progestins, agents that have been used in endometriosis treatment for more than 40 years, but their pharmacological action is still not understood in detail. In the present study, we therefore aimed to determine the effects of progestins on the local P4 and E2 actions in a model epithelial cell line of peritoneal endometriosis, Z-12.

Results: We examined the effects of three progestins most commonly used in the therapy of endometriosis medroxyprogesterone acetate (MPA), dydrogesterone (D) and dienogest (DN) on expression of all genes encoding enzymes of P4 metabolism, E2 biosynthesis and corresponding steroid receptors, by qPCR and Western blot analysis.

Among investigated progestins D had the greatest effects on the pre-receptor P4 metabolism. Although all progestins down-regulated *SRD5A1*, which catalyzes the conversion of P4 into its inactive metabolite 5α-dihydroprogesterone, DHP, only D resulted in significant reduction. Additionally, D and DN significantly decreased expression of *AKR1C1* and *AKR1C2*, which both catalyze the reduction of DHP into their corresponding 3α/β- and 20α-hydroxy- 5α-pregnane metabolites. Lower effects on *AKR1C1* and *AKR1C2* were observed for MPA.

Our results also indicate that MPA, D and DN exert similar effects on genes of E2 biosynthesis. The most important effect of progestins was observed for *HSD17B1* that catalyzes the reduction of estrone to the potent E2. In contrast, all compounds significantly up-regulated expression of *HSD17B2* which catalyzes oxidation of E2 to estrone. D and DN also significantly decreased nuclear receptors ERα and ERβ with greater effect seen at the protein than mRNA levels, while MPA and D significantly reduced the mRNA levels of the membrane bound *GPER*.

Conclusions: Our results suggest that the beneficial effects of progestin treatment observed in peritoneal endometriosis patients may be explained by decreased pre-receptor metabolism of protective P4 and decreased local E2 biosynthesis, which may lead to enhanced P4 and decreased E2 mediated signaling.

P4

EXPLOITING BENZOATE 4-MONOOXYGENASE INHIBITION IN ANTIFUNGAL THERAPY

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Introduction: Adverse effects, toxicity, and resistance to currently available antifungal drugs are the limitations that advance antifungal drug research and development. New methods like comparative genomics in combination with high-throughput technologies and large compound libraries, will reveal a variety of new lead structures not only for the already well-accepted antifungal drug targets such as CYP51, but also for the CYPs, which have not yet been addressed as targets. Highly conserved enzymes of fungal CYP53 family are involved in detoxification of benzoate, a key intermediate in metabolism of aromatic compounds in fungi. Their high specificity for the narrow array of closely related phenolic substrates and the absence of homologue(s) in higher eukaryotes offer advantage in designing successful antifungal agents for effective treatment of fungal infections.

Results: In the work presented here, we explored chemical properties of isoeugenol for ligand-based similarity searching and CYP53A15 homology model for structure-based virtual screening of composite chemical library. Highest scoring compounds were analyzed in the spectral binding titration with CYP53A15, a benzoate 4-monooxygenase from *Cochliobolus lunatus* [1]. Furthermore, they were assayed for antifungal activity against *C. lunatus*, *Aspergillus niger*, and *Pleurotus ostreatus*. Finally, eight compounds with antifungal potential were evaluated as inhibitors of CYP53A15 activity.

Conclusions: Based on potent antifungal activity and good enzyme inhibition compounds I26 and I30 were selected for optimization of new lead structures with respect to specificity, selectivity, efficacy, and absence of toxicity in mammals, possibly resulting in compounds, suitable to enter the pre-clinical and clinical antifungal drug development programs.

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P5

UNUSUAL NEUTRAL LIPID COMPOSITION OF THE PLASMA MEMBRANE AND MITOCHONDRIA OF THE RECYCLED BOTTOM-FERMENTING BREWER'S YEAST AS ADAPTATION TO GROWTH CONDITIONS

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Introduction: Strain of *Saccharomyces cerevisiae* yeast used for the production of lager beer is known as the bottom-fermenting brewer's yeast. In the industrial process of beer production it undergoes propagation and fermentation during which it is exposed to various types of stresses, among which anaerobic conditions and ethanol toxicity have the strongest effect on the cells. Plasma membrane (PM) largely determines cell growth and survival ability, and it is known that membrane lipids make a part of an effective adaptation mechanism. The mitochondria (MI) are essential for brewing yeast physiology and fermentation performance, and ethanol tolerance is closely related to their lipid composition.

The aim of this study was to investigate how brewer's yeast copes with the shift from aerobic to anaerobic conditions when ergosterol and unsaturated fatty acids cannot be synthesized. We have determined the composition of neutral lipids and fatty acids in PM and MI of the pure culture (zero generation) grown aerobically and 1st recycled generation, grown anaerobically.

Results: The highly purified plasma membranes and crude mitochondria were isolated after enzymatic disruption of the cell wall. Lipids were extracted by Folch method. Neutral lipids were separated by two-step TLC of total lipid extract and quantified by densitometry, while fatty acids were determined by GC of the corresponding methyl esters.

In PM unsaturated acids accounted for 39% of total identified fatty acids in the zero and 54% in the 1st generation, respectively, while in MI they prevailed in both generations (51% and 56%, respectively). C16-acids prevailed in both generations of PM and MI, as well, accounting between 64 and 70%. Palmitic acid was the main one in all preparations except in the MI of the 1st generation.

Among neutral lipids of PM and MI only ergosterol and squalene were present in significant amounts. Ergosterol accounted for 18.4 µg/mg proteins in the zero and 11.0 in the 1st generation of the PM, respectively. The concentration of ergosterol was higher in the zero than in the 1st generation of MI, as well (9.7 and 7.0 µg/mg proteins, respectively). Interesting finding was that the PM and MI of both generations, the one grown anaerobically, but also the one grown aerobically, contained substantial amounts of squalene, PM 10.4 and 19.4 µg/mg proteins and MI 4.0 and 7.1 µg/mg proteins, respectively.

Conclusions: Fatty acid and neutral lipid composition of PM and MI of the zero and 1st recycled generation of the bottom-fermenting brewer's yeast differ significantly. We assume that the accumulation of squalene in these membranous systems is not just a way of storing it, but that it has an assigned function in the regulation of their fluidity and permeability as a part of adaptive mechanism.

P6

AMINO ACID RESUIDES INVOLVED IN CHOLINESTERASE INHIBITION WITH METAPROTERENOL AND ITS BISDIMETHYLCARBAMATE

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Introduction: Selectivity of cholinesterases and their interaction with various compounds, especially drugs, is a subject of many studies in the field of biochemistry and pharmacology, due to important roles of cholinesterases in organism and their involvement in metabolism of many drugs. Metaproterenol is a bronchodilator used in the treatment of asthma. Metacarb (bisdimethylcarbamate of metaproterenol) is structurally related to bambuterol. Bambuterol, a prodrug of bronchodilator terbutaline and one of the most selective butyrylcholinesterase (BChE) inhibitor. Metacarb, like bambuterol, carbamylates the active site serine and progressively inhibits cholinesterases. Metaproterenol, a final product of decarbamylation, is reversible cholinesterase inhibitor. The aim of the work was to identify amino acids that govern the inhibition with metaproterenol and metacarb by determining dissociation (K_I) and inhibition rate (k_i) constants, respectively.

Results and discussion: Metaproterenol and metacarb were studied as inhibitors of mouse recombinant acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and six AChE mutants. Mutations in the choline-binding site (Y337A) combined with those in the acyl pocket (F295L/Y337A, F297I/Y337A, and F295L/F297I/Y337A) or those in the peripheral site (Y124Q and Y72N/Y124Q/W286R) were employed to mimic BChE active site residues. Competitive inhibition was observed for AChE w.t., BChE w.t., choline binding site and acyl pocket mutants, while non-competitive inhibition was noticed in case of peripheral site mutants. All studied cholinesterases displayed poor affinity ($1/K_I$) for metaproterenol binding (K_I=0.32 – 13 mM). However, even than 3 times higher affinity of BChE than AChE was observed. Although mutations in choline binding site and acyl pocket mimics BChE, affinities of these mutants were up to 4 times higher than that of BChE. Peripheral site mutants had the same or a 3 times lower affinity than AChE w.t. The inhibition rate constant of BChE inhibition by metacarb was 280 times higher than that of AChE. All mutations employed, except those including F297I, increased inhibition rates comparing to AChE w.t. (up to 30-times for F295L/Y337A and Y124Q) and therefore decreased selectivity seen for the wild type enzymes.

Conclusions: Cholinesterase selectivity for metaproterenol reversible binding and carbamylation selectivity of metacarb is dictated with amino residues in choline-binding site and peripheral site residues.

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P7

MEDICAL DIAGNOSTICS REAL-TIME PCR WORKFLOW AUTOMATION SYSTEM

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The field of medical diagnostics utilizes the field of nucleic acid based molecular diagnostic. qPCR is an upgrade of the conventional PCR method which was developed approximately 15 years ago, and it allows not only the detection of specific sequences of DNA / RNA, but also their precise quantification (Bustin, 2005; Ginzinger, 2002; Kubista et al., 2006). The core advantages of qPCR are its dynamic detection range and its high sensitivity and specificity (Ginzinger, 2002). qPCR is therefore useful for diagnosing diseases and determining predispositions to these diseases based on changes in the hereditary record, for determining the efficacy of medications, pathogen detection and gene expression analysis (Klein, 2002). These benefits ensure qPCR's broad applicability in laboratory medicine.

Sample preparation and data analysis are complex qPCR steps that are performed by high-level experts in different programming environments for the purposes of designing analyses, making templates and laboratory worksheets, interpreting results and preparing reports. There is a clear demand for the automation, unification and simplification of the qPCR procedure. Our solution is the qPCR workflow management and quality assurance system. With integrated expert knowledge, the system unifies the entire qPCR workflow inside the organizational unit (laboratory, department, organization etc.). It is an easy to use, wizard-like software system that will lead lab experts through every step of the qPCR workflow. It will calculate reagent concentrations for the chosen analyses, prepare wet lab outputs and schemes for lab work and qPCR machines, analyze final raw data and interpret the results while taking into account the hierarchical positions between lab employees whilst confirming the final reports.

Adjustments are possible according to customer requirements; it also covers different qPCR platforms and is compatible with local systems for data organizing (LIMS). The core benefit of our solution is simplicity. It has been designed to turn complex and unconsolidated approaches into one simple, controlled environment with a basic purpose of easing pressure on high-level scientists. At the moment, the system has been developed for qPCR diagnostics.

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P8

FORMATION OF BLOOD CELL NANOVESICLES BY SHEAR-INDUCED RUPTURE

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Introduction: Isolation of cell fragments from peripheral blood by centrifugation yields sub-micron-sized particles which are created mostly after blood sampling by fragmentation of blood cells during the isolation process. The population of fragments in the isolate reflects the fragility of blood cells. These fragments are clinically relevant and are considered potential biomarkers of homeostasis. Concentration of nanoparticles in blood isolates was found increased in many diseases (e.g. cancer, autoimmune disorders). We enhanced the effect of shear-destruction by shaking blood cells in a bead-beater.

Results: Blood was centrifuged to separate erythrocytes from platelet-rich plasma. Samples (erythrocytes and platelet-rich plasma) were shook at 15Hz or 30Hz with the amplitude 5 cm in a bead beater MillMix20 (Domel d.o.o., Železniki, Slovenia) for 5 minutes at room temperature in the presence of 106 nm-sized glass beads. Then, nanoparticles were isolated from supernatants of centrifuged samples. Sediments and isolates were imaged by a scanning electron microscope. Regularly-shaped (globular and spherical) fragments were found in isolates (Figure 1).

Conclusions: Destruction of cells by shear forces yields nanovesicles. Shape and size of these nanovesicles is influenced by the properties of the membrane and also by the isolation process.

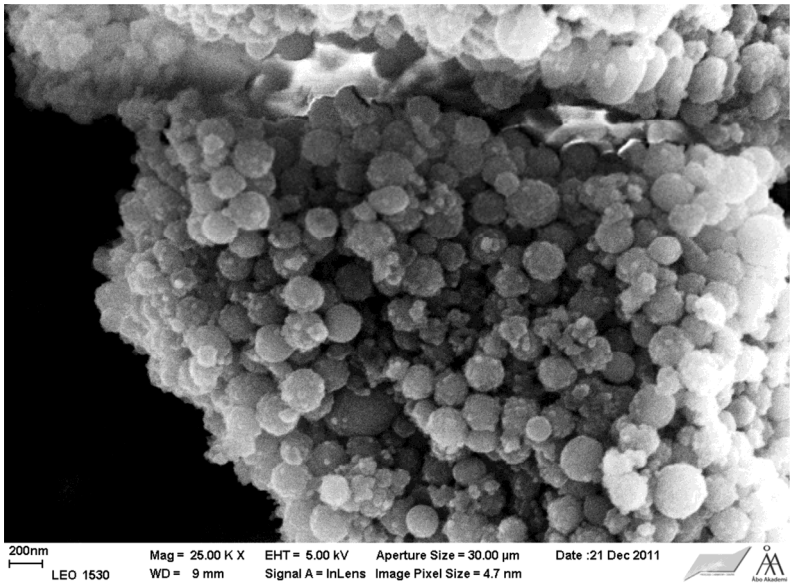

FIGURE 1: Isolated nanovesicles created by shaking the sample of platelet-rich plasma.

P9

CYSTEINE 341 OF THE THIRD INTRACELLULAR LOOP OF GLUCAGON LIKE-PEPTIDE-1 RECEPTOR AS AN ALTERNATIVE MONO-ADP-RIBOSYLATED AMINO ACID RESIDUE

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The glucagon-like peptide-1 receptor (GLP-1R) is a G-protein-coupled receptor and it is a member of secretin/vasointestinal peptide receptor family B [1]. Our previous studies confirmed that mono-ADP-ribosylation of the third loop of GLP-1R is possible *in vitro* and, very likely represents a novel type of receptor activity regulation *in vivo* [2]. The most credible candidate for modified amino acid residue, Arg348, was verified; mutant peptide was tested and, in comparison with the wild type peptide, its mono-ADP-ribosylation was reduced significantly but not eliminated completely [2]. Therefore, an alternative amino acid residue modification was suggested, in particular Cys341.

To further study the signaling mechanisms of GLP-1R, we investigated the effect of the third intracellular loop-derived peptides (IC_3, IC_3(C341A), IC_3(R348A,C341A)) on endogenous mono-ADP-ribosyltransferase mediated mono-ADP-ribosylation of G-proteins β subunit in CHO cells. Results showed an inhibitory effect of IC_3(C341A) peptide on mono-ADP-ribosylation of β subunit, similar to the effects of IC_3 peptide [3]. IC_3(C341A) peptide exerts its competitive inhibition on mono-ADP-ribosylation of β subunit in micro molar range around 50 μM [3], which is also the active amount of IC_3 peptide in other biological processes *in vitro* [2]. It is also evident that IC_3(C341) peptide is indeed mono-ADP-ribosylated, although to a greater extend as IC_3(R341A) [2,3], which means that Arg348 is modified predominantly. The sum of mono-ADP-ribosylation of IC_3(R348A) and IC_3(C341A) peptides correlates with IC_3 peptide modification which suggests no additional modified amino acid residues [3]. The latter suggestion is further supported with no significant effect of double mutant IC_3(R348A,C341A) peptide on mono-ADP-ribosylation of β subunit and that mono-ADP-ribosylation of double mutant IC_3(R348A,C341A) peptide is nearly completely lost [3].

We showed that Cys341 is, besides Arg348, an alternative amino acid residue of mono-ADP-ribosylation of GLP-1R and that they are most likely the only one. Modification of Cys341 possibly evolved recently as an alternative mechanism of receptor desensitization in the case of Arg348 mutation. Our findings can be supportive in development of new treatment procedures for diabetes mellitus type 2 and obesity. In future, different biochemical approaches *in vivo* must be used to elucidate the role of GLP-1R regulation by ART in normal and pathological physiology in mammals.

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P10

CASK IS A NEW INTERACTOR OF THE P2X3 RECEPTOR TO MODULATE ITS FUNCTION IN TRIGEMINAL SENSORY NEURONS.

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Introduction: P2X3 receptors are ligand-gated ion channels that transduce nociceptive stimuli mediated by extracellular ATP. Research into the molecular mechanisms underlying modulation of P2X3 receptors activity is an important step to develop new analgesic drugs. In this framework, the essential role for the Cdk5 kinase in modulating P2X3 receptor function has recently been demonstrated.

The calcium/calmodulin-dependent serine protein kinase (CASK) is a member of the membrane-associated guanylate kinase protein family (MAGUK) implicated in controlling vesicular release and synaptic maintenance via a Cdk5-dependent process. Since CASK supports trafficking of NMDA receptors to the neuronal membrane, we explored the possibility that CASK could also modulate P2X3 receptors in sensory neurons. To test this hypothesis, we performed immunoprecipitation, immunofluorescence and functional experiments with trigeminal neurons from C57 black mice.

Results: Our data demonstrated that CASK co-precipitated with P2X3 receptors and was expressed by P2X3-immunopositive trigeminal neurons. The co-precipitation of CASK with P2X3 receptors was enhanced by a short (5 min) application of NGF (50 ng/ml), whereas a brief (30 s) application of a large concentration (100 mM) of the P2X3 receptor agonist a,b-methyleneATP to produce receptor desensitization, significantly decreased the CASK/P2X3 receptor association. Silencing of CASK in trigeminal ganglion cultures reduced P2X3 receptor expression and, on patch-clamped sensory neurons, it depressed current responses evoked by a,b-methyleneATP, particularly those of small amplitude. Recent evidences demonstrated a peculiar expression distribution of CASK, that is maintained in trigeminal organotypic cultures in vitro.

Conclusions: Our data suggest that, in sensory neurons, CASK was preferentially associated with P2X3 receptors especially under resting or moderate activation conditions, implying that this kinase was perhaps modulating receptor activity in a state-dependent fashion. In view of the multifarious CASK roles, its function points to a broad range of P2X3 receptor modulation processes to tune-up pain transduction efficiency.

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P11

IMIDACLOPRID EFFECTS IN PERIPHERAL SENSORY NEURONS

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Introduction: Long-term exposure to environmental chemicals is a predisposing factor for the development of chronic diseases, neurodegeneration, hypersensitisation or ageing. Among pesticides, imidacloprid is a new generation insecticide, belonging to neonicotinoids family, with higher affinity for insects over mammalian nicotinic acetylcholine receptors (AChR). Nicotinic AChR have important role in control tissue physiology of the cardio-vascular system, muscle and sensory neurons. Little is known about potential hazard effects of imidacloprid on human health, especially in terms of potential occupational toxicity. To this aim, discovery of new molecular events evoked by new generation chemicals in mammalian models is interesting to generate more risk assessment data.

Results: Sensory neurons have the unique characteristic to sense environmental chemical-physical stimuli, and are highly prone to develop chronic sensorial hypersensitisation. In this work we studied the effects of imidacloprid (0.1 - 4 mM) on sensory neuron-derived cells F11 in vitro. Significant cytotoxicity was observed only at concentrations above 1 mM after 48h exposure, thus confirming overall low toxicity of imidacloprid. Nevertheless, early molecular events such as activation of p38 intracellular pathway, was partly necessary to generate reactive oxygen species and long-lasting lipid peroxidation. While p38 inhibitors were only partially sufficient to block imidacloprid cytotoxicity, anti-oxidants confer significant protection. Differential toxicity of imidacloprid, commercial formulation and its metabolites are also considered.

Conclusions: These results contribute to new risk assessment of imidacloprid effects on mammalian tissue and suggest caution for chronic sub-threshold exposure doses of imidacloprid in terms of potential long-term sensitization of peripheral sensory neurons.

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P12

RETINITIS PIGMENTOSA AND DEAFNESS AS A RESULT OF NOVEL LEU130PRO MUTATION IN *PRPH2* GENE AND MOST COMMON HOMOZYGOUS DELETION 35DELG IN *GJB2* GENE; DIFFERENTIAL DIAGNOSIS OF USHER SYNDROME

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Purpose: In a larger study of Usher syndrome in Slovenia, attempt was made to characterize atypical cases that presented with combination of recessive hearing impairment and autosomal dominant retinitis pigmentosa (RP).

Methods: Affected patients underwent ophthalmologic exam, including assessment of visual acuity with Snellen charts and color vision with Ishihara plates, Goldmann visual fields, optical coherent tomography (OCT), fundus autofluorescence imaging (FAF; Spectralis, Heidelberg, Germany) and Microperimetry (MP1). *GJB2* and *PRPH2* genes were sequenced in index patient (P3). Affected family members were confirmed with sequencing (*GJB2*), high resolution melt analysis (*GJB2*) or restriction enzyme (*PRPH2*).

Results: Father, two sons and a daughter (ages 71, 46, 36 and 41, respectively) presented with RP. Visual acuity was 1,0 in all, father had reduced color vision. FAF revealed parafoveal hyperautofluorescent rings and OCT showed loss of photoreceptors outside the rings. Goldmann showed constricted visual fields with II/1 radii of 4°, 8°, 6° and 4°, respectively. MP1 showed loss of retinal sensitivity outside the rings (Figure 1). Both sons also presented with profound hearing loss, mimicking Usher syndrome. *GJB2* gene sequencing revealed homozygous c.35delG mutation in both, the most common cause of hereditary hearing loss. *PRPH2* sequencing revealed a heterozygous mutation c.389T>C (p.Leu130Pro) in all patients with RP. This novel mutation occurs at highly conserved site and it is predicted by Polyphen as probably damaging.

Conclusion: Combination of RP and hearing loss in this family was found to be a result of mutations in two different genes, separately affecting hearing and vision. Such combination can be considered as important for differential diagnosis of Usher syndrome.

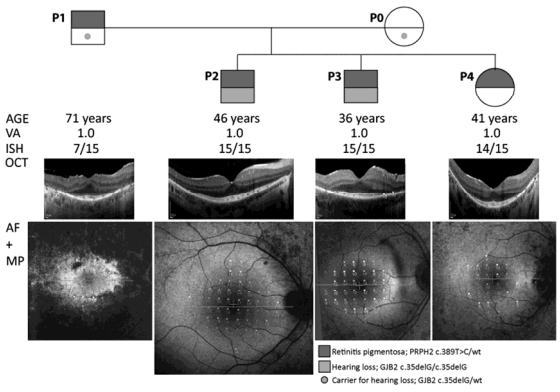

FIGURE 1: Family tree and phenotype data of family members presenting with retinitis pigmentosa and hearing loss. VA = visual acuity, ISH = Ishihara color vision test, OCT = optical coherence tomography, AF = fundus autofluorescence, MP= microperimetry.

P13

FLOW CYTOMETRIC / IMAGE ANALYSIS REVEALS DISTINCT DIFFERENCES IN STAT SIGNALING BETWEEN CONVENTIONAL AND REGULATORY CELLS

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Introduction: Regulatory T (Treg) cells, defined by the expression of forkhead family transcription factor (Foxp3), are essential for the maintenance of peripheral tolerance. Many human autoimmune diseases, including systemic lupus erythematosus (SLE), show altered numbers or dysfunction of Treg cells. Due to potential clinical significance of Treg cells, there is an intense interest in understanding how they differ from conventional T cells also in intracellular signaling pathways that lead from surface receptors to gene regulation. Cytokines and interferones, acting through JAK-STAT pathways, are regarded as essential factors in the development of autoimmunity.

Molecular translocation of STAT transcription factors from the cytoplasm to the nucleus is a pivotal event in cytokine signaling processes. Existing methods, used to measure STAT phosphorylation and nuclear translocation, require cell isolation and have significant limitations.

Results: To study multiple activated signaling pathways in complex populations of cells flow cytometry based biochemical analysis systems for kinase and phosphoprotein profiling have been developed. We used a novel multiparametric flow cytometry assay to measure basal and cytokine (IFN and IL-2)-induced phosphorylation of various STAT proteins in Tregs and conventional CD4+ T cells.

For the first time, by using multispectral imaging flow cytometry (Amnis), we could study translocation of STAT transcription factors immediately ex vivo in Tregs.

IFN-alpha stimulation resulted in increased levels of phospho STAT1 (tyr701) in all T cells, whereas IL-2 stimulation induced pSTAT5 (tyr694).

Analysis of the intensity of phospho-STAT5 staining revealed that ex vivo Treg cells have increased capacity to phosphorylate this protein relative to other-conventional CD4 T cells.

Our first results show higher STAT1 responses to IFN-alpha in terms of nuclear translocation in SLE Treg cells as compared to other lymphocytes.

Conclusions: Phosphoprofiling in whole blood can identify signaling aberrations in a given autoimmune disease such as SLE. Multispectral imaging flow cytometry reveals specific perturbations of STAT signaling cascades in Treg cells.

Type I INF's may represent the most important molecules in pathology of SLE and for the first time we describe higher STAT1 signaling responses to IFN-alpha in SLE T reg cells.

Monitoring signaling pathways on the single cell level can lead to developments in new diagnostic tools, especially in monitoring of disease activity. Results can also identify new targets of more specific and less toxic therapy with kinase inhibitors.

P14

THE ASSOCIATION OF DNA REPAIR POLYMORPHISMS WITH SUSCEPTIBILITY TO CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction: Genetic factors that influence DNA synthesis or repair capacity may play an important role in susceptibility to childhood acute lymphoblastic leukaemias (ALL). The aim of our study was to evaluate the associations of genetic polymorphisms, haplotypes and gene-gene interactions in DNA repair genes with susceptibility to ALL.

Results and discussion: In total, 121 children with ALL and 184 unrelated healthy controls of Slovenian origin were genotyped for eleven polymorphisms in five genes of base excision repair and homologous recombination repair (*OGG1*, *XRCC1, NBN*, *RAD51*, and *XRCC3*). Nine polymorphisms were in Hardy-Weinberg equilibrium in controls and their genotype frequencies were in agreement with those reported in other Caucasian populations. We did not observe significant differences in genotype distributions between ALL cases and controls for any of the investigated polymorphisms. However, after stratification by immunophenotype, a significantly decreased susceptibility to B-lineage ALL was observed in carriers of *NBN* 185Gln variant allele (p=0.037). Moreover, significantly decreased susceptibility to ALL was observed for *RAD51* GTT haplotype (p=0.016). We also observed significant influence of *OGG1* Ser326Cys – *RAD51* -61G>T (p=0.042) and *NBN* Glu185Gln – *RAD51* -98G>C (p=0.021) interactions on susceptibility to ALL. As the change conferred by a single common variant is probably small, it is not surprising that combinations of several polymorphisms, investigated in haplotype and gene-gene interaction analysis, have a greater influence on susceptibility to ALL. In addition, our previous study has shown that *NBN* Glu185Gln and *RAD51* -61G>T significantly affected the level of DNA damage, detected with comet assay [1], suggesting their influence on susceptibility to ALL is also biologically plausible.

Conclusions: Our results suggest that combination of polymorphisms influencing DNA repair may have greater effect on susceptibility to childhood ALL than individual polymorphisms.

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P15

THE HUMAN PRIMARY HEPATOCYTE TRANSCRIPTOME REVEALS NOVEL INSIGHTS INTO ATORVASTATIN AND ROSUVASTATIN ACTION

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Objectives: With particular emphasis on interactions between cholesterol homeostasis and drug metabolism we investigate the transcriptome of human primary hepatocytes treated by two commonly prescribed cholesterol lowering drugs atorvastatin and rosuvastatin and by rifampicin that serves as an outgroup as well as a model substance for induction of nuclear pregnane X receptor.

Methods: Hepatocytes from human donors have been treated with rosuvastatin, atorvastatin, and rifampicin for 12, 24, and 48 h. Expression profiling with cholesterol and drug metabolism enriched low density Steroltalk cDNA and whole genome Affymetrix HG-U133 Plus 2.0 arrays has been applied. Differential expression (DE) of genes and gene set enrichment analysis of KEGG pathways were performed. Lists of differentially expressed genes and gene sets were cross-compared. Selected genes were confirmed by quantitative real-time PCR.

Results: Statins lead to: (a) upregulation of cholesterol related genes indicating an increased LDL uptake and storage of esterified cholesterol, elevated bile acid/drug export and lower capacity to form HDL; (b) perturbation of genes in glucose and fatty acid homeostasis, influencing acetyl-CoA pools, promoting gluconeogenesis and glucose export; (c) elevated expression of ADIPOR2 suggesting increased sensitivity to adiponectin; (d) perturbations in genes of lipoprotein particle formation, differently for each statin; (e) perturbed expression of many metabolic genes that are directly controlled by nuclear receptors constitutive androstan and/or pregnane X.

Conclusion: These data provide a novel global insight into hepatic effects of statins, offering biochemical explanations for higher blood glucose in statin-treated patients, and for drug-induced secondary fatty liver disease.

P16

THE CO-EXPRESSION OF THE CLASSICAL AND G-PROTEIN-COUPLED ESTROGEN RECEPTORS IN ENDOMETRIAL CANCER

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Introduction: Endometrial cancer (EC) is the most frequent malignancy of the female genital tract in developed world. Many of the established risk factors for developing EC, especially that of the endometrioid type, are associated with excess exposure to estrogens unopposed by progesterone. Estrogens exert their biological effects through the estrogen receptors (ER). To date, there are three recognized ERs: the two classical ERs, ERα and ERβ, that function traditionally as ligand-activated nuclear transcription factors, and the G-protein-coupled receptor, GPR30 (also known as GPER) that is involved in rapid signaling events. The cellular effects of estrogens depend on the specific receptors expressed in the tissues. It is thought that the ER status constitutes independent prognostic factor but the precise role of each ER type is not yet clear. Since many cells and tissues co-express classical estrogen receptors and GPR30, we aimed to examine their status in cancer and control endometrium.

Results: In the present study, immunohistochemistry analysis was carried out to evaluate the ERα and ERβ cellular status. We investigated 21 specimens containing both neoplastic and morphologically normal or simple hyperplastic endometrial glands and endometrial cancer/ adjacent paired tissue microarrays (TMA; EMC241, Pantomics Inc., USA). Both ERα and ERβ were detected in in the nuclei and cytoplasm of the glandular cells of the tissue specimens, but for ERα the staining was stronger in nuclei. The scores for nuclear staining for ERα were significantly lower in cancerous glands compared to control tissues ($p = 0.0009$), while there were no difference in cytoplasmic staining. Comparable average scores for ERβ staining in nuclei and cytoplasm were seen in cancer and control specimens. TMA analysis showed significantly lower scores for ERα nuclear and cytoplasmic staining ($p = 0.0070$ and 0.0192, respectively) in the cancer sections than control and the average score for the cytoplasm was higher than for the nuclear staining. No differences were seen for the ERβ nuclear and cytoplasmic scores in TMA.

The decreased levels of the ERα protein in EC suggested that E2 might act via GPR30. We thus examined the *GPR30* mRNA levels in 30 samples of cancer and control endometrium using qPCR. We separately amplified *GPR30* gene variant 2 and variants 3 and 4. The expression of *GPR30* variant 2 was unchanged in the cancer endometrium *versus* the control tissue, while the expression levels of variants 3 and 4 were 2.4-fold decreased ($p = 0.0352$).

Conclusions: Our data show that estrogens may exert their effects through unchanged levels of GPR30 variant 2, but also through lower levels of ERα and unchanged levels of ERβ. Nonetheless, the roles of different ERs in EC, are still not clearly understood, and should, therefore, be further studied.

P17

GENETIC POLYMORPHISMS ASSOCIATED WITH DIABETIC RETINOPATHY IN TYPE 1 DIABETES

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Introduction: Several studies have suggested that reactive oxygen species (ROS) are implicated in the aetiology of type 1 diabetes (T1D) as well as in development of severe microangiopathic complications like diabetic retinopathy (DR) and diabetic nephropathy (DN) (2). The aim of our study was to evaluate the association of polymorphic markers in genes encoding antioxidant enzymes which share a common detoxification pathway (MnSOD, CAT, GSTM1 and GSTT1) with DR or DN in a cohort of patients with T1D.

Methods: One hundred and twenty four patients with T1D were investigated in this case-control study. All subjects were matched for sex, age and duration of diabetes. Genotyping was conducted using real-time PCR for p.Val16Ala polymorphism in MnSOD gene and c.C(-262)T in the promoter region of CAT gene. Multiplex PCR method was used for determination of GSTM1 and GSTT1 polymorphic deletions.

Results: A positive association of MnSOD genotype Val/Val (OR = 2.49, 95% CI = 1.00-6.16, P = 0.045) and GSTM1-1 genotype (OR = 2.63, 95% CI 1.07-6.47, P = 0.031) with diabetic retinopathy but not with diabetic nephropathy was demonstrated. Additionally, the combination of the two genotypes conveyed an even higher risk (OR=4.24, 95% CI 1.37-13.40, P=0.009). No other investigated genetic polymorphisms were associated with either DR or DN.

Conclusions: Selected polymorphisms in genes encoding MnSOD and GSTM1 could be useful genetic markers for identification of individuals with T1D at an increased risk for developing DR.

P18

POLYMORPHISMS OF THE SEGREGATION GENES ARE ASSOCIATED WITH RISK OF GASTRIC CANCER IN A SLOVENIAN POPULATION

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Introduction: Gastric cancer remains one of the major health problems in Slovenia. Although its incidence is in decline, the relative 5-year survival is only 21 % in the male population and 27 % in female population. The pathogenesis of gastric cancer is a poorly understood and complex process. Several environmental and genetic factors have been associated with gastric cancer, however, it became evident that only a minority of exposed individuals ultimately develop gastric cancer, which implies that host genetic susceptibility plays an important role gastric carcinogenesis. Such various susceptibilities could be explained, in part, by single nucleotide polymorphisms (SNPs) in mitotic segregation genes. Segregation is one of the fundamental processes in cells, which are rapidly dividing, such as gastric epithelial cells. Therefore, if regulation mechanisms governing this process are damaged, the cells might proceed through cytokinesis with DNA or spindle errors and could inherit unrepaired mutations or gain an abnormal number of chromosomes (aneuploidy). The objective of this study was to screen SNPs in segregation genes in gastric tumor tissues obtained from Slovenian patients.

Methods and results: Genotyping was carried out using real-time PCR (Applied Biosystems), RFLP, sequencing and high resolution DNA melting (Roche). We evaluated the association between coding and non-coding SNPs in gastric cancer patients by comparing them with healthy controls. We also determined the association between polymorphisms and clinicopathological features of the patients. We identified different distributions of genotypes for two polymorphisms in TTK and AURKA genes, respectively. Haplotype analysis revealed a weak contribution of these genes to gastric cancer susceptibility.

Conclusions: Our results indicate the importance of AURKA and TTK genotypes in the gastric carcinogenesis. Linking genotyping data with clinicopathological features of patients could also offer a broader insight into gastric carcinogenesis and identify potential biomarker genes, which could be useful for the development of new approaches for non-invasive screening methods.

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P19

EFFECT OF IMATINIB ON CONCENTRATION OF MICROVESICLES IN BLOOD ISOLATES. A 32 MONTH FOLLOW-UP STUDY.

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Introduction: Clinical studies have shown that the concentration of microvesicles (MVs) in blood isolates is a potential indicator of clinical status and can be used to monitor the development of the disease [1]. Gastrointestinal stromal tumors (GIST) express c-*kit* (CD117), a tyrosine kinase receptor specific to the interstitial Cajal cells. Imatinib (STI 571, Glivec, Gleevec; Novartis Pharmaceuticals) competitively binds to the binding site for ATP of some tyrosine kinases and thereby inhibits signaling [2] to suppress proliferation and survival of cancer cells. Imatinib improved the prognosis of locally advanced inoperable or metastatic GISTs as well as of stable disease and is currently considered standard therapy for GIST. We have monitored the effect of the treatment with imatinib on the MVs concentration in 12 patients after removal of GIST, 2 patients with metastatic GIST and in a healthy control, for 32 months.

Results: Patients treated with imatinib in the Department of Gastroenterology, University Medical Centre Ljubljana from 2008 and a healthy volunteer were assessed for the concentration of microvesicles in peripheral blood during 32 months. Blood samples were repetitively centrifuged and washed. Isolated MVs were counted by flow cytometry. In patients in whom gastrointestinal stromal tumor was previously removed, the concentration of MVs before treatment was increased with respect to the healthy subject (on the average for a factor 3,5 (2.6 to 9.2)). The first week after the initiation of the treatment, the concentration of MVs considerably increased with respect to the healthy subject in all patients (for a factor 13 on the average (5.9 to 21.2)) while it on the average decreased to the level of the healthy subject after the 4^{th} week and remained at that level during the following 128 weeks. In those GIST patients who had been receiving imatinib already before entering the study, the concentration of MVs remained at the level of the healthy donor 128 weeks of monitoring. We observed no remission of the disease in patients in whom the tumor was removed while the disease was suppressed in both patients with metastatic GIST. Tumor marker S-CA 19-9 increased with increasing concentration of MVs in blood isolates in patients treated with imatinib. This indicates that the marker is most probably located in microvesicles. Concentration of MVs in isolates increases with increasing concentration of platelets in blood in patients treated with imatinib which explains that majority of MVs in blood isolates derives from platelets.

Conclusions: Treatment with imatinib caused transient but not long-term increase of concentration of MVs in blood isolates. Low values of concentration of MVs in blood isolates reflect favorable effect of treatment with imatinib. MVs in isolates are a clinically relevant artifact.

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P20

FUNCTIONAL NETWORK LINKED TO CATHEPSIN F IN CERVICAL CANCER

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Lysosomal cysteine cathepsins have been known to play an important role at various stages of cancer progression and metastasis in selective types of cancer [1,2]. More recently it was found that cathepsin F is significantly upregulated in cervical carcinoma, one of the most common cancers among women worldwide [3].

Therefore, in order to get an insight into the molecular network associated to cervical cancer, Hela cells were transfected with human cathepsin F and further evaluated by real time quantitative PCR with a Human Cancer PathwayFinder™ RT² Profiler™ PCR Array (SABiosciences). The later contains 84 genes representative of six biological pathways involved in transformation and tumorigenesis, namely, a) cell cycle control and DNA damage repair, b) apoptosis and cell senescence, c) signal transduction molecules and transcription factors, d) adhesion, e) angiogenesis, and f) invasion and metastasis. Interestingly, two genes were significantly upregulated after cathepsin F overexpression. Namely, granzyme A, an enzyme involved in target cell lysis in cell-mediated immune responses was found upregulated 2.69-fold whereas integrin beta-3, also known to participate in cell adhesion and cell-surface-mediated signaling, was found upregulated 2.58-fold. On the other side, the metastasis suppressor protein 1 which may be involved in cancer progression or tumor metastasis was found downregulated 1.57-fold. Therefore, in order to get a more comprehensive view of the involvement of cathepsin F in cervical cancer, a functional network was built-up using several 'omics' tools. A minimal network, thus linking cathepsin F, granzyme A, integrin beta-3, and the metastasis suppressor protein 1 involves 44 nodes and 281 edges. The network topology reveals an average-degree of 12.77, average clustering coefficient of 0.57, an average shortest path length of 1.92, average eccentricity of 3.61 and average betweenness of 19.8.

In conclusion, we firstly present a biological network, thus highlighting the participation of cathepsin F in cervical cancer. This approach provides a new framework to analyze complex biological systems.

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P21

LLAMA HEAVY-CHAIN ANTIBODIES AS MEANS TO FIGHT BRAIN CANCER

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Introduction: A glioma, arising from glial cells, is a malignant tumor that starts in the brain or the spine. Glioblastoma multiforme (GBM) is the most common and most aggressive human brain tumor. It can be resistant to treatment (radiation, chemotherapy), drugs cannot always enter the brain because of the blood-brain barrier and surgery is not always an option. It can appear in both adults and children, and the survival period is around a year, after the tumor is diagnosed. Recent studies have reported the existence of normal brain stem cells and brain tumor stem cells (BTSCs) which have self-renewal properties, unlimited growth, high migration rate, resistance to chemotherapy and are able to renew the tumor cell population [1].

The camelids, besides the classical heteromeric antibodies composed of two heavy (H) and two light (L) chains, have the so called “heavy-chain” antibodies (Abs) which consist only of two heavy chains (HCAbs) [2]. HCAbs are easily purified from serum. Single-domain antibodies (VH and VHH) can be used in the treatment of acute coronary syndrome, Alzheimer’s disease, treatment of diseases of the gastrointestinal tract, prevention of thrombosis and brain cancer, as they might cross the blood-brain barrier.

Workflow: A pool of human brain tumors cultivated as spheroids will be prepared as a single cell suspension and used to immunize a llama to obtain antibodies directed against membrane proteins. RNA will be isolated from blood leukocytes and a single step PCR method will be used to construct a llama VH–VHH mixed library [3]. Next, immunoaffinity chromatography will be used to select specific phage display binders: first with proteins from tumor and non-tumor tissue sequentially bonded to the stationary phases and the displayed antibodies used in the mobile phase and then vice versa, with selected phages attached to the stationary phase. In the last step, two-dimensional gel electrophoresis and mass spectrometry analysis will be performed on tumor and non-tumor proteins from the last round of immunoaffinity chromatography.

Conclusions: With this work, we expect to identify protein markers of brain tumor (stem) cells that could serve as biomarkers and suggest new targets for therapeutic drugs.

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P22

ERYTHROPOIETIN RECEPTOR EXPRESSION REGULATION IN A CELL MODEL OF NON-HEMATOPOIETIC ORIGIN

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Introduction: Erythropoietin (EPO) is a glycoprotein, whose effects on proliferation, differentiation and survival of erythroid precursor cells are well known. Large amount of data also show additional, tissue protective effects on different tissues - neural, myocardial, skin, endothelial etc. Expression of the erythropoietin receptor gene (*EPOR*) in hematopoietic tissues is tightly regulated throughout erythropoiesis, while detailed molecular mechanisms involved in other tissues are not yet entirely known and even the identity of the cytoprotective EPOR in neuronal cells is controversial. Current hypothesis in the field is that metabolic stress and tissue damage induce TNFα which causes several effects, some of them being inhibition of EPO synthesis and activation of expression of tissue-protective receptors. Aims of our study were to determine if metabolic stress, TNFα and EPO itself regulate the expression of *EPOR* and to study the tissue protective effects of EPO in cell lines.

Results: We tested the effect of EPO, TNFα and serum deprivation on gene expression in two cell lines of neural origin: PC12 and SH-SY5Y. We treated PC12 cells for 24 h with 5 and 100 U/mL of EPO or 0% serum (FBS and HS) before and after 7-days differentiation with NGF (nerve growth factor). SH-SY5Y cell line was grown in two different media (DMEM/F12 or DMEM) and cells were treated for 24 h with 10 and 100 ng/ml of TNFα, 100 U/mL of EPO or 0% serum (FBS). qRT-PCR analyses showed that metabolic stress, *i. e.* serum deprivation, leads to twofold increase in *EPOR* expression. However, treatment with TNFα had no effect. We also tested whether EPO itself was expressed in the two cell lines - and it was not detected in any of the conditions. We did not observe EPO regulation of its own receptor gene expression in either cell line. In both cell lines serum deprivation has also been described as a trigger of apoptosis. We measured the expression of pro-apoptotic genes *BAD* and *BAX*. No change was detected in any of the tested conditions in SH-SY5Y cell line. However, in 7-days differentiated PC12 cells with NGF we observed almost two fold increase in gene expression of *Bax* and *Bad* after 24 h withdrawal of NGF.

Conclusions: Serum deprivation affects *EPOR* gene expression and is also known to induce apoptosis in the studied cell lines. This condition will be used for future studies of neuroprotection of EPO and also to determine which signaling pathway regulates *EPOR* expression.

Acknowledgments: We thank Janko Kos for the kind gift of PC12 cell line.

P23

SPECIFICITY IMPROVEMENT OF RECOMBINANT ANTIBODY BINDERS AGAINST CANCER RELATED HUMAN PROTEINS

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Highly similar ERK2 and ERK1 MAP kinases play a critical role in the regulation of cell growth and differentiation. Their upstream activation pathways are highly similar, and also many of their known downstream targets are shared. However in diseases, ERK1 and ERK2 can display distinct cellular functions, as has been shown for the development of cancer (1). A general approach to improve cross-reactive antibody binders to abolish the recognition of a highly similar antigen is of high value for molecular identification in research and in the clinic. Here we describe a strategy for identification of improved cross-reactive binders by rational mutation library design and high density protein microarray screening.

A cross-reactive recombinant anti-ERK2 scFv fragment was chosen as the starting template on which diversity was introduced by computational modeling of the HCDR3 loop using the Rosetta Antibody server (http://antibody.graylab.jhu.edu). Top-scoring models were retrieved and superposed to gain information about conformationally stable and flexible parts of the loop. Finally, 6 residues were identified as being potentially highly contributing to HCDR3 flexibility. Residues were mutated with an overlapping “megaprimer” PCR method encoding the original residue or Ala/Thr/Gly in selected residues. Extracts of a designed mutant library was first screened for expression and differential specificity on single antigen protein arrays. Identified active clones were further tested for cross reactivity on spotted protein microarrays with a set of non-relevant proteins. Specificities of selected mutants for ERK2 compared to ERK1 were improved from 5 to almost 9 times when compared to wild-type. Also their overall specificity (against other-non related proteins) were significantly improved.

Identification of residues which are responsible for antibody cross-reactivity facilitates antibody design and engineering. In addition it enables the handling of smaller mutant libraries which can be directly screened on high throughput microarrays without the additional need for technically challenging and time consuming selection procedures.

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P24

GENETIC POLYMORPHISMS MODIFYING OXIDATIVE STRESS AND RESPONSE TO ACUTE ANTIPSYCHOTIC TREATMENT

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It has been suggested that genetic polymorphisms modifying oxidative stress may influence the response to antipsychotic treatment. In the present study we investigated the influence of polymorphisms in the *Catalase* (*CAT*), *Superoxide dismutase* (*MnSOD*) and *Cytochrome P450 17-hydroxylase* (*CYP17*) genes on the response to acute antipsychotic treatment of schizophrenia in 74 patients acutely treated with haloperidol or risperidone.

CAT C-262T (rs1001179) , *SOD2* T1183C (MnSOD Ala-9Val) (rs4880) and CYP17 T-34C (rs743572) were genotyped by real time PCR assay and sequence specific PCR. Psychopathological symptoms were assessed with BPRS and CGI scales twice: 8-12 and 36-40 days after the first dose of antipsychotic. Extrapyramidal side effects (EPS) as common adverse events were assessed with the SAS (parkinsonism), BARS (akathisia) and AIMS (acute dystonia) scales.

The respective genotypes frequencies were: *CAT* C-262T: CC 0.384; CT 0.507 and TT 0.110; *MnSOD* Ala-9Val: ValVal 0,338; ValAla 0,568; AlaAla 0,095 and *CYP17*: TT 0,243; TC 0,527 and CC 0,230. When controlled for age, gender, BMI, illness duration, number of previous hospitalizations, drug type and dosage patients with at least one *CAT* T allele (CT + TT genotype) had significantly higher total AIMS scores (P=0.022) and total BARS scores (P=0.010) than patients with two CC genotype. We did not observe any statistical significant association between *CAT* C-262T genotype and the efficacy of treatment although it did affect the baseline BPRS score (P=0.044). *MnSOD* Ala-9-Val and *CYP17* (A2 allele) gene polymorphisms did not significantly influence neither the EPS neither the efficacy of treatment.

Our results support the impact of *CAT* C-262T polymorphism on the occurrence of side effects in acute antipsychotic treatment in Slovenian schizophrenia patients.

P25

EFFECT OF THE PD447 INHIBITOR ON THE HWHOG1 KINASE IN FUNGUS *HORTAEA WERNECKII*

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The aim of my research was to analyze the Hog1 kinase inhibitor activity on the HwHog1 in fungus *Hortaea werneckii*. Black yeast *H. werneckii* is a model organism for studying molecular mechanisms of adaptation to environments with extremely high salt concentrations in eukaryotes. It was isolated from Slovenian Sečovlje salterns and it is an extremely halotolerant organism, which can grow in medium with up to almost saturated sodium chloride concentration. Despite several previous studies, the key role of the MAP kinase HwHog1 remains unsolved. HwHog1 activity is regulated by the HOG signaling pathway, through which the signal is transferred from the membrane osmosensors to the MAP kinase cascade inside the cell. This triggers HwHog1 phosphorylation, which leads to its activation and regulation of expression of target genes.

We constructed a yeast plasmid with inserted *HwHOG1* gene with no introns and transformed it into *Saccharomyces cerevisiae* strain with deleted *HOG1* gene. Western blotting of the whole cell lysates with anti-Hog1 antibodies confirmed cell expression of Hog1 but not HwHog1. The latter was successfully detected in phosphorylated form with antibodies against phosphorylated human homologue (p38), additionally showing the activation of the HwHog1 protein. Functional complementation tests showed that HwHog1 is able to complement the MAP kinase Hog1 function in *S. cerevisiae* mutants, because growth of strain expressing HwHog1 is similar to the growth of control strain expressing Hog1. Media used contained different molar concentrations of osmolytes NaCl and sorbitol.

The system described above enabled us to study the effect of PD447 inhibitor on HwHog1 in the same genetic background as was used for testing the effect on *S. cerevisiae* Hog1. Inhibitor plate tests showed that PD447 efficiently inhibits the HwHog1 kinase when expressed in *S. cerevisiae*. The effect of PD447 was also studied in *H. werneckii* background, by inhibitor plate tests and by measuring growth curves in 96-well microtiter plates. Although slight negative effect on the growth of *H. werneckii* in media with NaCl was observed, there was an absence of complete growth inhibition. Obtained results are in agreement with our findings from recently sequenced genome of *H. werneckii*. The functional annotation of the genome namely showed huge abundance in ABC multidrug transporter genes. This findings suggest that PD447 is being actively pumped out of the *H. werneckii* cells.

P26

GENETIC ANALYSIS OF THE *LDLR* GENE IN CHILDREN WITH HYPERCHOLESTEROLEMIA

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Introduction: Hypercholesterolemia (HCH) is considered to be one of the main risk factors for the development of the atherosclerosis (ATS). The development of the ATS begins in the early childhood, but manifests itself clinically only few decades later as a cardiovascular disease (CVD). The HCH in the childhood is associated with the ATS and CVD in the adulthood. The rapid progression of the ATS usually depends on the simultaneous presence of many risk factors (genetic predisposition, dyslipidemia, arterial hypertension, diabetes, smoking etc.), or alternatively on the presence of one major risk factor such as monogenic HCH. In the great majority of the cases, the etiology of the HCH is polygenic or multifactorial. Much rarer is the monogenic HCH, which mostly is heterozygous familial HCH (FH). FH is an autosomal co-dominant inherited disease which is characterized by the 2- to 3-fold increase in the LDL-C values, from the birth on. The incidence of the heterozygous FH is around 1 in 500. Three different genes are known to cause the FH. Mutations are most frequently found in the LDL-C receptor gene (*LDLR* gene), the frequency of the heterozygotes is around 1 in 500. Around 10% of all cases of the FH are caused by the mutations in the apolipoprotein B100 gene (APOB gene). Still much rarer are mutations in the proprotein convertase subtilisin/kexin 9 gene (PCSK9).

The aim of the study was to analyze the *LDLR* gene in the Slovenian population of the patients with the HCH.

Results: In this study, we have used various molecular genetic methods (PCR, Denaturing High Pressure Liquid Chromatography - dHPLC, High Resolution Melting – HRM and sequencing). We have compared the spectrum of the causative mutations for the FH in our population with those in the previously reported populations. On the basis of the mutational analysis we have prepared the protocol for the genetic diagnostics in patients which could have FH. 50 patients with HCH recruited form Department of Endocrinology, Diabetes and Metabolic Diseases at University Children's Hospital have been included into the study. According to our preliminary results, 36 out of 50 patients have 17 different mutations in LDLR gene, all known causative mutations for HCH. Among them, 2 related patients have the same novel mutation in acceptor splicing site of intron 10 c.1587-1G>C.

Conclusions: The results are in accordance with the mutational spectrum reported in neighboring populations. We will continue with the genetic characterization of patients with HCH for *PCSK9* and *APOB* gene.

P27

POTENTIAL BIOMARKERS OF OVARIAN ENDOMETRIOSIS

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Endometriosis is a common benign gynecological disorder associated with pelvic pain and infertility. It is inflammatory disease associated with an abnormal immune response stimulated by various major cytokines. The symptoms are often non-specific and a surgical procedure is required to make a definitive diagnosis. Facilitating the diagnosis in a patient by a less invasive method would be highly beneficial. In many studies elevated levels of cytokines in the peritoneal fluid of endometriosis patients were found. We have measured the levels of secreted cytokines using enzyme-linked immunosorbent assays in single manual mode or using a multiplexed system based on Luminex dual fluorescence technology (Bioplex, Bio-Rad Laboratories). We have determined interleukins (IL)-6, IL-8, IL-15, IL-18, interferon gamma-induced protein (IP-10), monocyte chemotactic protein 1 (MCP-1), vascular endothelial growth factor (VEGF) and Regulated upon activation, normal T-cell expressed and secreted (RANTES), growth regulated oncogene alpha (GRO-alpha), inter-cellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) using these methods. We have also measured other proteins which are involved in the immune and inflammatory response such as C-reactive protein (CRP), ficolin-2 (a human lectin which activates immune cells to secrete cytokines), endometrium-derived glycodelin (PP14, involved in early placental development, inhibiting activation and proliferation, and inducing apoptosis of T-cells) and leptin, which can promote the adhesion, implantation and growth of ectopic endometrial glands and stroma in the peritoneal cavity. Leptin also regulates the immune activity of peritoneal cells and promotes the secretion of inflammatory and angiogenic factors. We have measured these proteins in a total of 99 matched pairs of serum and peritoneal fluids, and the results were analyzed as a function of the presence (N=58) and absence (N=41) of endometriosis. We found PP14 to be significantly elevated and leptin significantly decreased in both serum and PF in the endometriosis group. Amongst the other analytes determined in the serum, only IL-8 showed a significantly lower concentration in the case group, others did not differ. In PF we found statistically significant differences detected for IL-6, IL-8, and MCP-1. IL-15 and IP-10 were decreased. PF seems to be the better compartment than serum for this type of analysis.

P28

TWO-DIMENSIONAL GEL ELECTROPHORESIS OF ALKALINE PROTEINS IN GASTRIC TISSUE

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Introduction: Two-dimensional gel electrophoresis (2-DE) in combination with mass spectrometry is a powerful tool for resolving mixtures of proteins. Alkaline pH ranges, however, require some more optimization then other pH gradients. In this study we aimed to optimize 2-DE in alkaline pH range for gastric tissue samples and apply the optimal conditions as found to analyze pooled non-tumor and pooled tumor samples for differentially expressed proteins in patients with gastric adenocarcinoma (GA).

Results: We optimized 2-DE in alkaline pH range for gastric tissue samples by trying out different sample loading procedures, reducing agents and/or additives to the rehydration solution. We then used the best conditions we determined to analyze pooled non-tumor and pooled tumor gastric samples for differentially expressed proteins. We successfully identified 38 spots as 24 different proteins. Four were chosen for further validation with immunoblotting on individual paired samples (n = 28) based on their putative relevance. Our results show that LGALS4 ($p = 0,028$) and HNRNPM ($p = 0,0299$) are statistically significantly over-expressed in GA. The results from immunoblotting correlated with those from 2-DE. On the other hand, as concerning mitochondrial trifunctional protein, we found both subunits of the protein under-expressed in our 2-DE analysis in tumor pooled samples. However, after validating these subunits on individual samples, HADHA was found statistically significantly under–expressed ($p = 0.0034$) and HADHB over–expressed ($p = 0.0007$) in GA. We observed correlation of HNRNPM with location of the tumor ($p = 0.0312$); differential expression was higher in samples from cardia/gastroesophageal border ($p < 0.01$).

Conclusions: Future work will be focused on translating the research to blood samples, so the proteins would ultimately be useful as biomarkers for early detection of the disease.

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OBLIGATE HALOPHILIC FUNGUS *WALLEMIA ICHTHYOPHAGA* AND ITS HOG SIGNALING PATHWAY

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All living cells must maintain optimal water potential and turgor to assure normal physical and chemical environment for cellular processes. Sensing and reacting to changes in osmolarity is of essential importance to organisms, especially if they inhabit environments with either fluctuating or high osmolyte concentrations. Signaling pathway sensing osmolarity in *Saccharomyces cerevisiae* is known as High-Osmolarity Glycerol (HOG) and has been studied extensively in the past decades. However, similar signaling pathways with homologous proteins are also present in other fungi, among them in extremely halotolerant fungus *Hortaea werneckii.* Our research is aimed at HOG components in extremely halophilic basidiomycetous fungus *Wallemia ichthyophaga*, which is the most halophilic eukaryote known to date. Compared to *H. werneckii,* which does not require salt to remain viable, *W. ichthyophaga* needs at least 10% NaCl (w/v) in the media and is metabolically active even at saturated salt concentration. In this extremophilic fungus we successfully identified three sequential MAP kinases and a putative homologue of Sho1 transmembrane protein, which is considered as part of sensory complex of HOG pathway. Comparison of *S. cerevisiae*, *H. werneckii* and *W. ichthyophaga* protein sequences revealed high conservation of key motifs and domains, which are responsible for their structure and function. However, in contrast with *H. werneckii*, where duplications of *HwSte11*, *HwPbs2* and *HwSho1* genes were observed, we noticed only duplication of the *WiHog1* kinase gene in *W. ichthyophaga*. Expression of *W. ichthyophaga* kinases in *S. cerevisiae* deletion mutants successfully rescued osmosensitivity of the mutant strains. On the other hand, when WiSho1 was expressed in functional complementation experiments, we did not get positive results. Localization pattern of proteins was monitored by microscopic observation of GFP fusion constructs. By identifying HOG pathway MAP kinase module (WiSte11 (MAPKKK), WiPbs2 (MAPKK) and two paralogous WiHog1 (MAPK)) and WiSho1 transmembrane protein we confirmed the existence of HOG signaling pathway as well as its putative role in osmosensing in *W. ichthyophaga*.

P30

COMPARISON OF DRIVING FORCES FOR ENTRAINMENT OF CIRCADIAN CLOCKS IN MOUSE LIVER AND ADRENAL GLAND

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Circadian clocks are endogenous oscillators that regulate the temporal organization of physiology, metabolism, and behavior. Cell-autonomous oscillations are established by transcriptional feedback loops that involve regulatory elements E-boxes, D-boxes, and RREs [1]. In peripheral organs, circadian rhythms are additionally affected and entrained by systemic cues, although their exact influence remains unknown [2]. We tackled the question of driving forces for entrainment using a combined experimental and theoretical approach.

We measured gene expression of clock genes in the liver and adrenal gland of two groups of mice; one was entrained to 12h:12h light-dark conditions and the other was kept in constant darkness. Based on carefully normalized gene expression data and confirmed transcription factor binding sites we developed a simplified gene regulatory network that served as a basis for our two models; the liver [3] and the adrenal mathematical model. The models are firmly rooted in experimentally known facts. By using delay-differential equations, we drastically reduced the number of unknown parameters without losing biological information.

Our models reproduce phases, amplitudes, and waveforms of circadian rhythms in mouse liver and adrenal gland. The quantitative agreement of measurements and simulations suggests that the proposed intrinsic gene regulatory network drives the circadian clock in the liver as well as in the adrenal gland and that E-box- and RRE-induced transcriptional regulation is the strongest driving force. Systemic cues such as light-dark cycles are merely fine-tuning the rhythms and are responsible for differences in gene expression between constant darkness and light-dark cycles. However, model analysis shows that there are fundamental differences in the underlying mechanism of entrainment in the two tissues.

Mathematical models help us to study general design principles of molecular clocks: we have shown that non-linear transcriptional inhibition and overcritical delays generate oscillations, and a synergy of inhibition and activation enhances the amplitudes. Our generic concept of combinatorial gene regulation allows quantitative predictions on how transcriptional regulation, post-transcriptional delays and half-lives control phase differences between regulators and target genes.

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ROBUSTNESS OF CIRCADIAN GENE EXPRESSION IN MOUSE LIVER AND ADRENAL GLANDS DEPENDS ON THE GENOTYPE AND LIGHT

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Circadian rhythms are important adaptation processes of organisms to periodic diurnal changes of day and night. Coordinated action is essential for homeostasis of the whole body, as evidenced by mutations in circadian clock components that lead to pathologies, such as metabolic syndrome. Molecular mechanisms of the core clock are well understood, although little is known regarding the influence of genetic background (genotype) on circadian rhythms and metabolism. To address this question, we investigated the circadian gene expression in liver and adrenal glands of two strains of mice; i.e. 129/SvPas plus C57BL/6J or pure C57BL/6J, in two light conditions: dark-dark (DD) or 12h light-12h dark (LD). Mice were sampled in 2–4h intervals over 24h, with at least 5 mice per time point. The 24 h gene expression profiles were fitted using various trigonometric functions (with 24 h and 12 h harmonics) or a conditional-mean-type smoother. These fits were evaluated to obtain the amplitudes and phases of the circadian rhythms. We show that the robustness of the circadian expression depends on the tissue and genotype. In adrenal glands under LD conditions many genes differ in circadian expression profiles between the mouse strains. Steroidogenic genes (*Cyp11a1, Cyp7b1, Cyp17a1, Cyp21a1, Cyp51* and *Cyp39a1)* are phase-shifted between the strains at least in one of the lightening conditions. Majority of steroidogenic genes and core clock genes is expressed at higher levels (mesinor) with higher amplitudes in 129/SvPas containing mixed strain, with the exception of *Arntl* and *Cry1.* Liver seems to maintain more robust circadian expression compared to adrenal glands since less differences are observed between the strains. Particularly interesting are the profiles of nuclear receptors which show higher amplitudes in 129/SvPas plus C57BL/6J compared to pure C57BL/6J. In general, we identified that light has greater impact on circadian expression of core clock and metabolic genes in 129/SvPas background. The light and genotype influence primarily the amplitudes of core clock genes while the amplitudes and also phases are affected for the metabolic genes. In conclusion, genotype has a role in maintaining the robustness of the circadian expression under different lighting conditions. This is important for further understanding of human diseases that are provoked by rhythm disturbances, such as shift work.

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OPTIMIZED HIGH RESOLUTION MELTING (HRM) ANALYSIS ALGORITHM IMPROVES DETECTION RATE OF GENETIC VARIANTS

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The detection of genetic variants with HRM analysis of double stranded DNA fragments became possible with the commercialization of improved saturating DNA dyes (EvaGreen, Syto 9, etc.). HRM analysis of DNA amplicons is cost-effective and fast method that enables detection of genetic variants through dissociation of DNA dyes from denaturing double stranded DNA and consequential loss of fluorescence intensity. The changes in the DNA sequence (SNPs and mutations) cause the change in the melting profile (shape of the melting curve) of the analyzed DNA fragment compared to the wild type DNA and the comparison algorithm of those melting profiles is crucial to distinguish between different genetic variants. After the PCR, the DNA product is gradually melted, and the emitted fluorescence is measured on a specialized qPCR instrument. Characteristics of the DNA amplicon such as sequence, GC content, length, and heterozygosity will influence the melt curve profiles for each amplicon. The resulting profiles can provide valuable information about present mutations, SNPs, methylation profiles, etc.

To improve the detection rate of hard-to-detect genomic variants (SNPs class IV for example), we introduced additional steps to the mathematical analysis and comparison of melting profiles of analyzed DNA. First of all the principal component analysis (PCA) of melting profiles is performed followed by execution of two different unsupervised clustering algorithms – density-based spatial clustering of application with noise (DBSCAN) and expectation maximization (EM) clustering algorithm. DBSCAN algorithm is used to detect complex cluster structures, while EM clustering algorithm is used to predict number of different genetic variations in the analyzed population and clustering of simply shaped clusters (spheres).

The quality and accuracy of genetic variant calling was tested by analysis of *SOD1*, *SOD2* and *SOD3* genes of 192 different samples. Whole blood samples were taken from each individual and DNA isolation was performed according to established laboratory protocols. We used Type-IT HRM mastermix (Quiagen) and 7500 Fast RT-PCR system (Life Tech.) for the HRM experiment. The melting curve alignment and normalization was performed with HRM 2.0 software (Life Tech.) while PCA and clustering was performed with RapidMiner statistical software (Rapid-I GmbH) the results were visualized with Tableau public visualization software (Tableau).

We successfully identified 6 different genetic variants which were confirmed with DNA sequencing on ABI 310 (Life Tech.).

The results confirm that optimized HRM analysis is fast and reliable method for detection of genetic variant, with clustering algorithms that can distinguish between complex clusters of genetic variants and give us the number of different genetic variants in the analyzed samples without prior knowledge of this number, thus improving detection rate of rare and new variants.

P33

STABLE REFERENCE GENES FOR REAL-TIME QPCR FOR NORMALIZATION OF TARGET GENES IN PHARMACOGENOMICS STUDIES IN ASTHMA PATIENTS.

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Objective: The aim of this study was to determine a set of reference genes whose gene expression is stable during therapy and could be used for normalization of target gene expression measured in asthma patients during anti-asthmatic therapy.

Methods: qPCR was used to determine the expression level of 7 candidate reference genes (*18S rRNA*, *ACTB*, *B2M*, *GAPDH*, *POL2AR*, *RPL13A* and *RPL32*) and 7 target genes in leukocytes from asthma patients before and after treatment with corticosteroids and anti-leukotriens as well as in control group of healthy individuals Variance of Cq values was analyzed and stability ranking was determined with geNorm. We further investigated how different panels of reference genes and normalization strategies affect the consistency of conclusions if the specific investigated target gene is downregulated or upregulated during anti asthmatic therapy.

Results: Cq values of *ACTB*, *B2M* and *GAPDH* were shown to be stable across samples from patients obtained before and after treatment. These were also the top-ranking reference genes determined by geNorm when all samples were included. Different normalization strategies on 7 target genes expression revealed inconsistency in conclusions which genes are down or up regulated during treatment or when compared to healthy controls. However when using the geometric mean of the three most stable and top-ranking reference genes for normalization the consistency in target gene expression was brought up to 90%.

Conclusions: We developed set of reference genes that could be used for normalization of target genes in pharmacogenomics studies in asthma patients during anti asthmatic therapy.

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EFFECT OF ENGINEERED NANOPARTICLES ON HUMAN AND DOG BLOOD CELL MEMBRANES

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Introduction: With increasing use of industrial nanoparticles, questions about their effect on health of human and animals have been raised. Here we studied the effect of three types of nanoparticles (carbon black (CB), nano-sized TiO_2 and nano-sized ZnO) on human and dog blood cells.

Materials and methods: Blood samples were incubated with nanoparticles for 1, 3 and 24 hours, respectively. Then, platelet-rich plasma was separated from erythrocytes by centrifugation of samples. Erythrocytes and platelets were observed by phase contrast microscope and fixed for scanning electron microscope imaging.

Results: Nanoparticles caused segregation of erythrocytes which was more pronounced in samples with longer exposition to nanoparticles. We observed invaginations in red blood cell membranes which could correspond to pores or encapsulated nanoparticles. ZnO but not TiO_2 or CB caused activation of platelets which was more pronounced for longer time of incubation.

Conclusions: Engineered nanoparticles interact with erythrocyte and platelet membrane, and affect cell segregation and platelet activation**.**

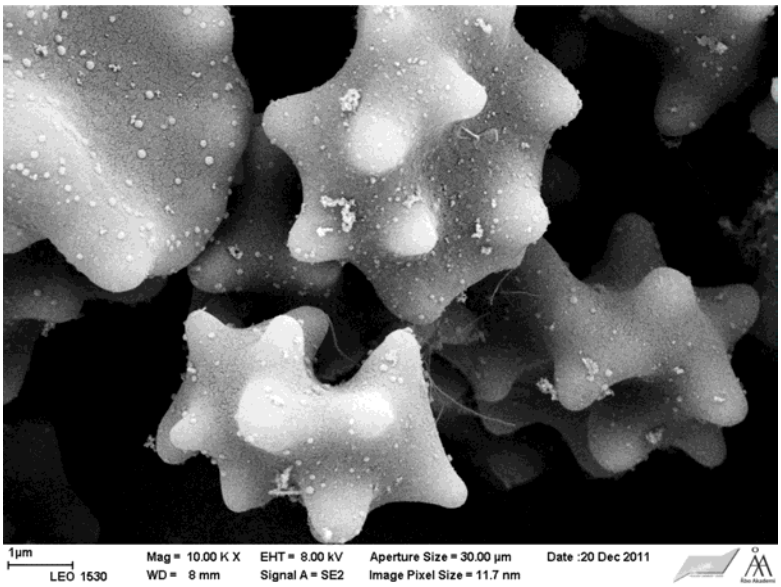

FIGURE 1: Echinocytes in the sediment of a sample of dog blood incubated with ZnO as observed by a scanning electron microscope. Aggregates of nanoparticles that adhered to the membrane are visible.

P35

HUMAN AMNIOTIC MEMBRANE SCAFFOLDS PROMOTE UROTHELIUM FORMATION: A POTENTIAL APPLICATION IN RECONSTRUCTIVE UROLOGY

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Introduction: Tissue engineering (TE) is an increasingly evolving field that enables tissue formation, its maintenance and restoration. Yet a challenge in TE still remains to establish a fully functional tissue that could be used to replace damaged or defective tissues or organs. An important component of TE is a scaffold, which supports cell growth and promotes cell differentiation and its formation into a new tissue [1]. The amniotic membrane (AM), due to its unique biological and mechanical properties, presents an excellent scaffold for TE [2]. The aim of our study was to determine whether AM enables normal urothelium formation and to further analyze UC growth and differentiation using differently prepared AM scaffolds.

Materials and methods: Cryopreserved human AM was used as a scaffold in three different ways. Normal porcine UCs were seeded on the amniotic epithelial monolayer (intact AM), the basal lamina (denuded AM) or the stromal matrix (AM stroma) and were cultivated for 3 weeks. UC growth on AM scaffolds was monitored daily. After 3 weeks, differentiation of UCs was determined by electron microscopy and immunofluorescence of urothelial differentiation-related markers (uroplakins, occludin).

Results: Using all three AM scaffolds the normal urothelium composed of basal, intermediate and superficial cells expressing uroplakins was established. The growth of UCs was the fastest on the AM stroma scaffold, where also the basal lamina between stromal matrix and urothelium was formed de novo. Accordingly, also the highest differentiation stage of UCs was demonstrated on the AM stroma scaffold. Superficial UCs cultured on AM stroma were large, with the apical plasma membrane shaped into microridges, with numerous discoidal vesicles in the apical cytoplasm, well developed tight junctions and the highest fluorescence intensity of uroplakins.

Discussion: The amniotic membrane offers a new approach for stimulation of UC growth and differentiation. In all three AM scaffolds a normal urothelium with superficial UCs expressing uroplakins was established. Most importantly our findings indicate that a human AM stroma scaffold enables the development of tissue-engineered porcine urothelium with molecular and ultrastructural properties comparable to native urothelium, making such an urothelium an excellent research tool for difficult-to-source human urothelium. Moreover, the highly differentiated urothelia on the AM stroma scaffold provide an important experimental model for developing tissue-engineering strategies considering that subtle differences are identified before translation to the clinical settings.

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P36

COMPARISON OF SIGNAL TRANSDUCTION PROPERTIES AND TUMOR FORMATION ABILITY OF HUMAN EPSTEIN-BARR VIRUS (EBV)-ENCODED BILF1 AND ITS PORCINE HOMOLOGS

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EBV is a virus infecting more than 90% of the human population worldwide. After primary infection it persists in B cells and is able to transform them, which can result in the development of malignant lymphomas and post-transplantation associated lymphoproliferative disorders (PLTD). PTLD also occurs in association with enhanced replication of porcine lymphotropic herpesvirus, that has homology with human EBV. EBV open reading frame BILF1 encodes a seven-transmembrane (7TM) G protein-coupled receptor. Recently receptors in connection to porcine virus were discovered: PLHV1, PLHV2 and PLHV3, which are also important for PTLD. The main aim of the presented research is to understand the action of EBV, connection to BILF1 and to determine connection between human and porcine homologs, through characterization of these receptors in the aspect of their cell surface expression, mechanism of constitutive activation of reporter genes, b-arrestin interaction, ERK1 signaling, their internalization pathways and receptors transforming potential in connection to cancer.

By using ELISA technique to study cell surface expression and assays to evaluate ability to stimulate transcription factors of BILF1 and PLHV1-3 receptors study showed that all receptors are expressed on the cell surface and are functional 7TM receptors, able to constitutively activate endogenous Ga_i, by the inhibition of forskolin-stimulated CREB activity, NFAT and NFκB, but not SRE. NFAT and NFκB activation is suggested to be mediated through Ga_i and Ga_q activation. By the means of confocal microscopy it was shown that BILF1 and PLHV1 have significantly different internalization pattern and b-arrestin interaction than PLHV2 and PLHV3, which corresponds to differences in ERK1 activation pattern, which was performed by immunoprecipitation to HA-tagged ERK1 and Western blots. In addition in BILF1 and PLHV1 transforming potential was investigated *in vitro* in foci formation assay using retrovirally transduced NIH3T3 cells, as well as *in vivo* by using nude mice. BILF1 revealed a transforming potential that was dependent on constitutive signaling, as a signaling-deficient mutant completely lost its ability to transform cells *in vitro*. In nude mice, BILF1 promoted tumor formation in 90% of cases.

Data suggest that BILF1, when expressed during EBV infection, could be involved in the pathogenesis of EBV associated malignancies, but not PLHV1-3 receptors. This is important especially in the relation to homeostasis in the organism and in relation to develop specific treatment for EBV cancers in the state of organism immunodeficiency. Similarity of human and porcine homologs is extremely important in the view of xenotransplantations.

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P37

IDENTIFICATION OF CONSERVED MOTIF IN THE THIRD INTRACELLULAR LOOP IN DOPAMINE RECEPTOR TYPE 2 (D_{2L}-R) RELATED TO INTRACELLULAR RETENTION OF D_{2L}-R

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Introduction: The effects of nervous system transmitter dopamine on its targets are mediated by D_1, D_2, D_3, D_4 and D_5 forms of dopamine receptors, members of seven transmembrane receptors (7TMR), also known as the G protein coupled receptors (GPCR). These receptors are important targets for therapy of Parkinson disease and some other motor and physiological diseases. The gene encoding the D_2 receptor gives rise to two alternatively spliced isoforms, termed the short (D_{2S}-R) and the long isoform (D_{2L}-R). The D_{2L}-R has an additional 29 amino acids insert within the third cytoplasmic loop and its sequence is well-conserved across the species. The presence of an additional insert in the putative third cytoplasmic loop of the D_{2L}-R might determine i) G-protein specificity ii) post-translational modification, iii) intracellular traffic and iv) receptor localization. The D_{2L}-R is mostly retained inside the cell, presumably in the endoplasmic reticulum (ER). Considering that the 29-amino acid addition contains potential arginine-based intracellular retention signal of the RXR type, we aimed to investigate whether the conserved motif RRR controls receptor surface expression and intracellular trafficking in heterologous HEK-293 cells. To address this question we used a combination of site-directed mutagenesis and immunocytochemical techniques to monitor cell surface expression, internalization and intracellular localization of the wild-type and mutants of D_{2L}-R.

Results: Seven mutants of the D_{2L}-R were generated by substituting arginine residues within the conserved RRR motif with glutamic acid and expressed in the HEK-293 cells. Confocal microscopy and colocalization studies with ER, enodosomes and lysosomes markers showed preferential retention of the wild-type D_{2L}-R in ER, while mutants displayed lower degree of colocalization with the ER marker. ELISA measurements of receptor surface expression confirmed that disruption of the RRR motif increased receptor plasma membrane expression and affected internalization pattern of individual mutants. However, the importance of individual restudies varied inside the motif.

Conclusions: The obtained results suggest that the evolutionarily well conserved intracellular RXR type ER retention signal present within the amino acid insert of the D_{2L}-R could serve as a regulator of receptor surface transport and trafficking.

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P38

THE KEY ADAPTATIONS TO EXTREMELY SALTY ENVIRONMENTS BASED ON THE NEW *HORTAEA WERNECKII* GENOME DATA

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The black yeast *Hortaea werneckii* has an outstanding ability to overcome the turgor loss and sodium toxicity that are typical for hypersaline environments. When analyzing the draft *H. werneckii* genome sequence, many of its 23 000 putative proteins have predicted transporter activity. When specifically looking at the cation transporters, which regulate the Na^+, K^+ and H^+ intracellular concentrations, many are found even in 8 to 10 copies. The tightly regulated expulsion of Na^+ by the transporter network could be responsible for the low intracellular Na^+ concentration observed in *H. werneckii*. Adaptation to Na^+ could also be observed on the protein catalytic activity level, as in the case of PAP phosphatase HwHal2, which tolerates higher Na^+ concentrations. When exposed to high Na^+ concentrations, *H. werneckii* also has to maintain the cell turgor by synthesizing glycerol in high concentrations, helped also by the activity of two *GPD1* homologues, which are highly expressed in response to high environmental osmolarity. The expression is dependent on HwHog1, the key kinase of the *H. werneckii* HOG pathway, activated by a range of histidine kinases forwarding the signal into the Sln1 branch. HwHog1 modulated **c**hanges of membrane lipid composition and cell-wall structure also help maintain the integrity and functioning of the stressed cells, also preventing the glycerol leakage. The negative impact of a hyperosmolar environment is counteracted by an increase in the energy supply that is needed to drive the energy demanding export of ions and synthesis of compatible solutes. Many genes associated with energy supply were highly upregulated in cells growing at high NaCl, including genes coding for components of the electron-transport chain, ATP production, for conveying excessive cytosolic NADH into the mitochondrial respiratory chain and others. A supplemental energy source might also be the function of the homologue of the *L. maculans* rhodopsin, the first proven case of a fungal light-driven transmembrane proton pump.

P39

NOVEL POLYMORPHISMS IN THE *CYP51* GENE FOUND IN CAUCASIAN MOTHERS AND NEONATES WITH POTENTIAL CONTRIBUTION TO SPONTANEOUS PRETERM BIRTH

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Introduction: Cholesterol is an essential component of cellular membranes, a precursor of steroid hormones, oxysterols, and bile acids. It is involved in many signaling pathways including the sonic hedgehog (Shh) pathway. Sterols play crucial role in maintaining pregnancy, and a large amount of cholesterol is required during ontogenesis and embryogenesis. Maternal cholesterol and triglycerides levels and their increase rates during the pregnancy contribute risk to preterm delivery (PTD) and later in life risk to cardiovascular diseases. Defects in cholesterol synthesis or intracellular transport result in serious fetal malformations and mutations in some cholesterol synthesis genes are associated with PTD.

Results: Here we investigated for the first time variants in fetal and maternal lanosterol 14α-demethylase (*CYP51A1)*, a key gene of post-squalene cholesterol synthesis, and we examined their contribution to PTD. Ten amplicons covering exons, untranslated regions (UTR) and intron-exon borders have been investigated in 188 Caucasian women who had a spontaneous preterm delivery and 188 unrelated preterm infants born at a gestational age <37 weeks. The study included neonates from singleton pregnancies, 94 of each gender.

Conclusions: We identified 22 polymorphisms, where 11 represent rare, novel variants. Three novel variants are heterozygous missense mutations in exons 1, 3 and 4. According to PolyPhen2 the mutation in exon 3 causes a probably damaging amino acid substitution in the substrate recognition site, resulting in a T/G change causing a Y145D transversion. This amplicon was sequenced further in 1000 premature infants. The low frequency of the novel *CYP51* polymorphism suggests that this polymorphism has little contribution to PTD. TaqMan genotyping of common variants in larger population is in progress, together with further sequencing of the 5' and 3'-UTRs. We are also investigating selected common variants in *CYP51A1* gene in over 700 pregnant women and their correlation with cholesterol and triglycerides levels and increase rates between first and second trimester of pregnancy and their association with PTD.

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P40

DEATH RECEPTOR SIGNALING AND DNA DAMAGE RESPONSE MECHANISMS ARE ALTERED IN EBS DOWLING-MEARA KERATINOCYTES

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It has been recently reported that epidermolysis bullosa simplex (EBS) patients with the severe Dowling-Meara phenotype have an increased cumulative risk for basal cell carcinoma development after the age of 40 (Fine et al., 2009). Using three previously described EBS-DM patient-derived and two control keratinocyte cell lines (Morley et al., 2003; Chamcheu et al., 2009; Chamcheu et al., 2011) we tested the effectiveness of their DNA damage response mechanisms after exposure to ionizing radiation (IR). This was measured as cell metabolic activity before and after exposure to IR, and followed up to 7 days after treatment. All cell lines showed a marked decrease in metabolic activity till 4 days after treatment. However, while control cells recovered between the 4th and 7th day, the EBS-DM mutants still displayed low cell count (only 10-50% of initially plated cells) and decreased metabolic activity (around 50% of the initial values). To evaluate the differences between these cell lines, we used the Sigma-Aldrich Panorama Antibody-XPRESS Profiler 725 microarray to compare protein extracts from one wild type and one mutant cell line. This panel contains antibodies directed against about 500 proteins with a key role in MAPK signaling, the cell cycle, apoptosis, DNA damage response and gene regulation. In the EBS-DM mutant, out of the resulting 46 proteins with significantly altered expression levels, one third involved components of the death receptor signaling pathway (TRAIL, DR3, DR4, DR5, DcR1, DcR2, TNFRII, etc.), a number of pro-survival and pro-apoptosis components of the intrinsic apoptotic signaling pathway (BID, Bcl-XL, etc) and a number of DNA damage response proteins such as P53 BP1 and PUMA. Because of the known interplay between these pathways it is difficult to predict their biological consequences to EBS-DM keratinocytes. Therefore, we tested EBS-DM (and control) keratinocytes' sensitivity to TRAIL and TNF treatment. In a "normal" situation a decrease in metabolic activity would be expected after treatment. However, all three severe EBS-DM cell lines displayed lower sensitivity to death receptor ligands, with up to 100% higher metabolic activity than the control cells. An in depth analysis of the components of these signaling pathways is underway.

Low sensitivity to apoptotic stimuli and increased sensitivity to ionizing radiation are distinct features of cancer cells. Our data suggests that in order for EBS-DM keratinocytes to overcome the structural and functional consequences of severe keratin (5 or 14) gene mutations, their metabolism needs to adjust/modify to the extent that they also acquire traits in common to cancer cells. Taken in this context, it is plausible that when DNA damage mechanisms fail, EBS-DM keratinocytes may become more prone to embark on the way to tumorigenesis than wild type cells.

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CONDITIONAL DELETION OF HEPATIC *CYP51* GENE REVEALS SEX-SPECIFIC IMPACT ON DEVELOPMENT AND PLASMA CHOLESTEROL HOMEOSTASIS

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Introduction: Cholesterol, being one of the centerpiece molecules in our bodies, is in certain aspects of synthesis and homeostasis still poorly understood. The majority of research today is focused mainly on the detrimental conditions of hypercholesterolemia and little attention is favored by situations of faulty cholesterol synthesis. We are investigating the *in vivo* role of lanosterol 14α-demethylase (CYP51), one of the key enzymes in the late stage of cholesterol biosynthesis. Complete disruption of *Cyp51* causes embryonic lethality in mice. To gain further knowledge about the importance of CYP51, we generated two conditional knockout (KO) mouse models: liver-specific *Cyp51* KO mice (*Cyp51*$^{lox/lox}$;Alb-Cre^{+}) and mice with one *Cyp51* allele absent in the entire organism and the other allele absent only in the liver (*Cyp51*$^{-/lox}$;Alb-Cre^{+}).

Results: Both transgenic strains are viable and normal in outer appearance. Approximately 10 % of male mice of both KO genotypes experience growth arrest with jaundice and hepatomegaly between 6 and 9 weeks of age. Initial histological studies of the liver revealed proliferation of small bile ducts accompanied by singular cases of mitosis and apoptosis of hepatocytes. Biochemical analysis of the plasma indicate disrupted cholesterol homeostasis with lower HDL- and total cholesterol compared to liver KO mice that develop normally as well as to wild types. Cholangiocyte proliferation and hepatomegaly is also observed in KO mice without jaundice. Immunohistochemical staining of the liver with pan-cytokeratin antibody confirmed liver stem (oval) cell response. Challenging *Cyp51*$^{lox/lox}$;Alb-Cre^{+} and *Cyp51*$^{-/lox}$;Alb-Cre^{+} mice with different diets showed lower body weight and increased liver-to-body weight ratio compared to the wild types on standard chow and on high-fat diet without cholesterol but not on high-fat diet with added cholesterol. Interestingly, both male KO mouse models on high-fat diet without cholesterol show significant decreases of plasma cholesterol, triglycerides and free fatty acids compared to wild types. These differences are more prominent in *Cyp51*$^{-/lox}$;Alb-Cre^{+} mice, whereas females are unaffected.

Conclusions: The initial data indicate that the hepatic disruption of *Cyp51* and consequently the disruption of cholesterol synthesis causes oval cell response with cholangiocyte proliferation and can in male mice lead to early growth arrest with severe jaundice. Male mice also seem to be incapable of sustaining normal cholesterol homeostasis when cholesterol is not present in the diet. Molecular mechanisms leading to these sex-specific differences are presently under investigation.

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MODELING THE VESICLE RESPONSE TO TRANSPORTAN-LIPID INTERACTION

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Lipid-peptide interactions play an important role in different cellular processes, among others also in enabling internalization of drugs into the cell interior [1]. Giant vesicles are simple system, where pure lipid-peptide interaction can be studied through observation of changing of the vesicle morphology or of detecting short-lasting phenomena as it is the occurrence of transient tension pores in the membrane. The dimensions of giant vesicles are very close to the dimension of cells, so are the characteristic times of the processes governed by diffusion. We have used this system for monitoring the pore formation in the lipid bilayer by transportan (TP), the cell penetrating peptide which is a promising drug delivery vector.

After a single vesicle containing sucrose solution was transferred into the isomolar glucose solution with added TP, four different vesicle responses were observed in dependence on increasing peptide concentration: a) the vesicle inside solution remains unchanged; b) the vesicle inside solution is exchanged through sequentially occurring short-lasting transient tension pores; c) disintegration and disappearance of the vesicle and d) vesicle survival with just one, long lasting tension pore. The model presented describes osmotic responses of the vesicle due to the fact that permeability of peptide pores for different solutes is different. All four observed responses can be explained if it is assumed, that the average size and the number of peptide pores are TP concentration dependent. The time dependence of the radius of the transient tension pore is governed by the flows of solutes into and out of the vesicle, including the diffusion flow through the open tension pore. Analyzing the images of the tension pore, the line tension was determined. In the regime of short-lasting transient tension pores, their number needed to completely exchange the vesicle content is determined by the effective radius of the peptide pores, sizes of solutes and vesicle critical volume. Using the model predictions, the average radius of peptide pore was determined, where the critical volume of the vesicle was obtained from the measured time dependence of the vesicle radius. Lower critical volume and line tension (1.8% and 3 pN) than for intact POPC membrane (6% and 13 pN) indicate that due to the TP vesicle membrane is destabilized. This and that the average radius of the peptide pores are TP concentration dependent (from smaller than 2 nm at 20 mM to larger than 5 nm at 50 mM) lead to the conclusion that TP pore is most likely toroidal one.

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INFLUENCE OF POLYMORPHISM -13910C>T, LCT GENE EXPRESSION AND DAILY LACTOSE INTAKE ON SYMPTOMS CHARACTERISTICS FOR LACTOSE INTOLERANCE AND RISK FOR INFLAMMATORY BOWEL DISEASE

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Introduction: Lactose intolerance is an inability to break down lactose into glucose and galactose and is associated with a substitution of C to T at position -13910 bp upstream the LCT gene (rs4988235).[1,2,3] The aim of our study was finding a connection between the medical, CC, genotype for lactose intolerance, the symptoms and lactose intake.

Results: In the survey we evaluated 607 randomly chosen individuals in which we examined the frequency of individual lactase genotypes. Through the questionnaire, which included 42 questions, we checked the nutritional habits of randomly selected healthy individuals. Genotyping was performed by PCR-RFLP technique and by TaqMan assay using real-time PCR. Gene expression was also measured by TaqMan assay using real-time PCR.

The prevalence of the CC genotype was 36,6%, CT genotype 46,6% and the TT genotype 16,8%. We also examined the frequency of individual lactase genotypes in patients with inflammatory bowel disease (IBD) and control groups.

The lactose intake was among subjects with symptoms after ingestion of milk and dairy products lower, than in subjects whose symptoms do not occur after ingestion of milk and milk products ($p = 0.035$). The lactose intake was also lower among subjects with symptoms of lactose intolerance, than in subjects who are of the opinion that the symptoms occur after ingestion of milk and milk products ($p = 0.031$). In this study we also measured the LCT gene expression. LCT gene expression was among the subject with symptoms significantly lower ($2^{-\Delta\Delta Ct}=27,87$) than among subjects without symptoms characteristic for lactose intolerance ($2^{-\Delta\Delta Ct}=37,25$). The comparison has proved to be statistically significant ($p=0,046$).

Conclusions: Our study is one of the few studies that don't examine only the genotyping, but also the dietary habits of the subjects.

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P44

THE EFFECT OF NATURAL SUBSTANCES FROM WINE AND SOME FRUITS ON VASCULAR TONUS VIA INTERACTION WITH G-PROTEINS

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Protected effect of substances from wine and from some fruit preparations on cardio-vascular system has been frequently attributed to their ability to relax vascular tonus. This process is controlled by a number of mediators and intracellular signaling pathways that frequently include G-proteins. The aim of our work was to correlate the effect of dealcoholized whole wine extract, extract of fractions of wine separated according to the polarity, and extract from blueberries on G-proteins from rat brain membranes and on Gs and Gi types of G-proteins overexpressed in sf9 cells with the effect of these substances on blood-vessel contraction/relaxation. We showed that whole wine extract and extract from blueberries activated G proteins from rat brain cortical membranes with the maximal effect being higher with wine extract than with blueberry extract. Use of different fractions of wine extract revealed that non-polar substances in wine activate while polar substances inhibit G-proteins. With further experiments we have shown that Gs proteins and Gi proteins show similar pattern of effects than rat brain G-proteins, however, non-polar fractions were activating Gi more efficiently than Gs and polar substances were better inhibitors of Gs than Gi. It is interesting that whole wine extract and all fractions were able to relax porcine coronary arteries that were previously contracted by KCl, but only whole wine extract and non-polar substances were doing this in the same concentration range in which they were effective toward G-proteins. This speaks for two distinct mechanisms of relaxation, probably mediated by different types of G-proteins. These results were obtained in scope of the diploma work (Nastja Miglar, Faculty of pharmacy, University of Ljubljana) and the rewarded student research project (Katja Štern and Lovro Vidmar, Medical Faculty, University of Ljubljana).

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EFFECT OF GLUTARALDEHYDE AND FORMALDEHYDE ON STABILITY OF CELL AND ARTIFICIAL MEMBRANES

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Introduction: Microvesicles (MVs) in blood isolates are mostly an artifact due to fragmentation of platelets[1]. We address the effect of fixatives on membranes as a possible method for suppression of this artifact.

Results: Human and horse blood was drawn into anticoagulated tubes and incubated in PBS citrated buffer (as a control) or in buffer with added 0,2% glutaraldehyde (GA), 4% formaldehyde (FD), or 0,2% GA + 4% FD at 4 or 37 °C. Samples were centrifuged and washed. Isolated MVs were counted by flow cytometry or observed by scanning electron microscopy (SEM). The effect of fixatives was studied on giant phospholipid vesicles (GPVs). Populations of GPVs were assessed by a computerized method from phase contrast micrographs[2]. MVs were more numerous after blood incubation in fixatives (Figure 1). We observed no effect of fixatives on GPVs.

Conclusion: Microvesiculation during isolation cannot be suppressed by fixatives glutaraldehyde and formaldehyde.

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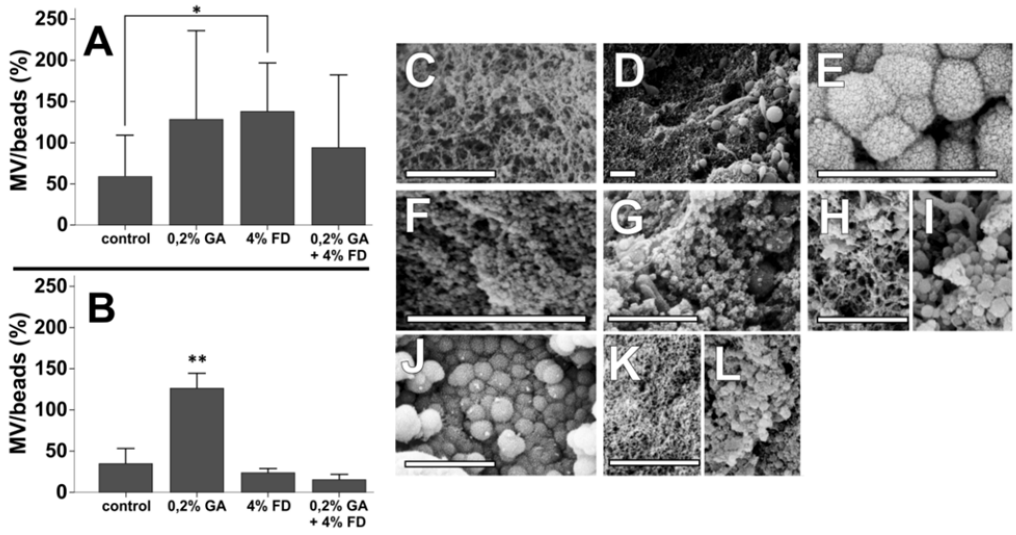

FIGURE 1: MV concentration in isolates of blood incubated at 37 °C (A) and at 4 °C (B). SEM of MVs from blood incubated in PBS citrate (human C, horse D) with added 0,2% GA (human E, horse F) 4% FD (human G, horse H,I) or 0,2% + 4% FD (human J, horse K,L). Bars represent 1 um.

P46

THE RS8111699 POLYMORPHISM IN THE *STK11* GENE AS A RISK FACTOR FOR POLYCYSTIC OVARY SYNDROME

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Introduction: Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age [1]. Clinically, PCOS manifests from mild to severe disturbances of reproductive, metabolic and hormonal functions [2]. Recent studies show evidence that gluconeogenesis could be upregulated in PCOS patients. One of the key down-regulators of hepatic gluconeogenesis is the AMP-activated protein kinase (AMPK), whose action is activated by serine/threonine kinase 11 (STK11) [3]. We hypothesized that genetic polymorphisms in the *STK11* gene could influence the PCOS biochemical and consequently its clinical picture.

The aim of the present study was to examine possible associations of the rs8111699 (c.290+2512C>G, intron) genetic variants in the *STK11* gene with the occurrence of PCOS.

Results: Preliminary, 97 Slovenian PCOS patients were included in the study. The DNA from peripheral blood leukocytes was extracted for all women and the rs8111699 was investigated by real-time PCR using TaqMan® Genotyping Assay C___2830633_20. The rs8111699 distribution of CC, GG and CG genotypes was 0.381, 0.134 and 0.485, respectively. The frequency of the allele C was 62 % and the frequency of the allele G was 38 %. Minor Allele Frequency (MAF) for rs8111699 published in the PubMed SNP database was C=0.359/785. So far, we can predict that the allele C is more frequent in Slovene PCOS patients compared to the previously estimated frequency in the general population.

Conclusion: To confirm the rs811699 as a risk factor for PCOS, it would be necessary to compare the frequency of rs811699 alleles between PCOS patients and healthy volunteers. Furthermore, correlations between the genotypes and clinical/biochemical characteristics of PCOS should be established.

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P47

EMERGING ROLE FOR NEURAL AGRIN IN HUMAN MYOBLASTS PROLIFERATION

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The principal role of the heparan sulphate proteoglycan agrin in the formation of synapses at the neuromuscular junction (NMJ) has been clearly documented. Till now neural agrin has mainly been studied to be a synaptic organizer which induces acetylcholine receptor clustering. However, agrin function is not limited only to the NMJ, but is involved in a broader spectrum of signaling pathways in various tissues. Indeed, the latest studies revealed that agrin is directly implicated in the organization of the cytoskeleton in skeletal muscle. Furthermore, it has also been identified that agrin functions as a co-stimulatory molecule in T-cell activation and formation of an immunological synapse. Only recently it has come to the fore its possible involvement in satellite cells regeneration. Interestingly, we have found out that agrin positively affects proliferation of human myoblasts. Growth curves showed that agrin enhanced satellite cell proliferation. These results were confirmed by bromodeoxyuridine assay. Desmin detection showed that agrin did not affect myogenicity and myoblasts fusion into myotubes. This is the first evidence for a function of agrin in myoblast proliferation. We will further attempt to identify the signaling pathway triggered by agrin and to investigate the possible involvement of muscle-specific kinase (MuSK) in controlling the proliferation process. Our preliminary observations indicate the important role of agrin in the regeneration of skeletal muscle and support the recently discovered therapeutic potential of agrin in muscle dystrophy. The characterization of the mechanisms underlying its proliferative activity could contribute to the identification of novel therapeutic protocols for muscle diseases.

P48

ASSOCIATION OF POLYMORPHISMS AND GENE EXPRESSION OF IL12B, IL13 AND IL23R WITH ASTHMA BEHAVIOR AND THERAPY RESPONSE

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Introduction: Asthma is the most common serious chronic disease in children [1,2]. Recently, single nucleotide polymorphisms (SNPs) in the cytokine gene cluster on 5q31-33 region, was associated with asthma development [3]. However, the role of those SNPs in asthma behavior and response to therapy is poorly characterized. The aim of our study is to analyze the role of IL13, IL12B and IL23R genes in asthma pathogenesis and determinate their role in response to different therapies.

Results: We have included 288 children with asthma and 186 healthy individuals. Genotyping was performed by PCR-RFLP technique. Gene expression was measured by TaqMan assay using real-time PCR. The data were statistically analyzed. We were the first to found the higher expression of IL12B in asthmatics compared to the control group ($p<0.001$). The expression of IL12B was reduced after treatment with anti-leukotriene drug montelukast ($p=0.015$). Montelukast affect the expression of IL23R gene, which significantly increased after the therapy. In eQTL analysis, we found that the expression of IL12B is significantly higher in carriers of at least one allele of SNP rs6887695 G, which was in preliminary studies identified as a risk factor for chronic immune-mediated diseases. In our study, we were the first to found an impact of allele G on airway obstruction in asthma ($p=0.006$).

Conclusions: The results of our study suggest that genes coding interleukins IL12B and IL13 have an important role in the pathogenesis of asthma. The finding of a higher IL12B expression in asthmatics may in future serve as diagnostic marker for asthma and the finding that montelukast decrease its expression may become an important fact in choice of therapy.

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P49

CTCAG – RECOGNITION

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CTCAG – recognition is a meticulously structured performance aiming to shed light on the many aspects of genetic testing. Its title comes from the restriction recognition sequence of the enzyme *Dde*I, which is used in the process of distinguishing between the wild type BRCA2 gene and the allele harboring a mutation, which ultimately exposes the carrier of such a mutation to a high risk of developing breast or ovarian cancer. This particular mutation is characteristic of the Slovene population.

An introduction to the discourse is made in the gallery by a representative of GenePlanet, a Slovene based commercial company that offers genetic testing. The introduction ends by offering to test a few members of the audience at the gallery's expense.

Two days later, the performance is held in a laboratory at the Institute of Biochemistry, MF, UL. A member of the audience is asked to perform the final step of the procedure which reveals if the author's DNA contains the IVS16-2A>G BRCA2 mutation in real time using agarose gel electrophoresis.

Simultaneously, a video lecture that focuses on conceptual issues of genetics in society is shown. Besides giving a short historical overview of the changing view on biotechnology, it explores the transformation of individual and social attitudes under the influence of genetic knowledge and technology. The lecture is juxtaposed by an intimate view of the author cleaning her apartment. She talks of the life circumstances, which lead to her decision to test her DNA for the BRCA2 mutation. She speaks of her mother's two-year battle with ovarian cancer and the implications a genetic defect in the BRCA2 would have on her future health. The performance ends with the technician's interpretation of the test results, which show the author's BRCA2 gene does not contain this particular mutation.

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FIGURE 1: CTCAG -recognition. Photo by Miha Fras (left two images) and Jurij Krpan (right image).

P50

GENOME WIDE ASSOCIATION STUDY IN SLOVENIAN INFLAMMATORY BOWEL DISEASE PATIENTS AND FUNCTIONAL CHARACTERIZATION OF ASSOCIATED SNPS WITH EQTL ANALYSIS

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Introduction: Analysis of published Inflammatory bowel disease (IBD) genome wide association (GWA) data suggested that only 9,2% of most disease significantly associated single nucleotide polymorphisms (SNPs) were located in coding regions of the genes and the rest 90,8% were non-coding SNPs, including 36,8% SNPs located in introns and 54,0% of SNPs in regions between genes or even in “gene deserts”.

Methods: We have genotyped 200,000 SNPs using Immunochip (iCHIP) in 256 Slovenian Inflammatory bowel disease patients (IBD) and 236 healthy individuals. For the selected disease associated loci with multiple candidate genes we attempted to link SNPs to the causal gene using expression quantitative trait locus (eQTL) analysis in which we correlated SNPs to gene expression of candidate genes in the region. We finally used a number of most significantly associated SNPs to build up and evaluate genetic profiles according to specificity and sensitivity to predict risk for IBD, clinical parameters and treatment response.

Results: Association analysis using genotypes from 169.774 SNPs that passed strict quality control procedure has identified 134 SNPs associated with Slovenian IBD patients with borderline significance after strict adjustment for multiple testing commonly used in GWA analysis. These SNPs were further evaluated in replication study where total of 632 Slovenian IBD patients and 312 controls were analyzed. The replication study confirmed association with the previous known major IBD genes, including NOD2/CARD15, IL23, ATG16L1, IRGM, PTGER4, IL10 and identified new candidate genes including TNFSF11 on chromosome 13q14. The eQTL analysis of chromosome 5 IBD locus where SLC22A4, SLC222A5 and IRF1 are located showed SLC22A5 as the best candidate IBD gene. The predictive models using combination of 10-12 associated SNPs with highest OR showed above 95% specificity in patients with 90% disease alleles however only a few patients would have 90% disease alleles resulting in low sensitivity usually below 20% of such genetic test. On the other hand, most of the patients would have between 50%-60% of disease alleles however the specificity of genetic test in this case would be less than 15%.

Conclusions: Our data suggested that up to 50% of candidate regions with unknown gene could be resolved by eQTL analysis. Our results suggest more complex predictive models need to be developed for better risk prediction in IBD.

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CIRCADIAN EXPRESSION OF *ICER* AND *PERIOD* ON THE PERIPHERY: THE ROLE OF ICER IN *PERIOD* REPRESSION.

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Introduction: cAMP responsive element modulator CREM encodes multiple activators and repressors. The best known are activator CREMt with essential role in spermatogenesis (*Crem-/-* males are infertile) and repressor ICER involved in circadian regulation of melatonin synthesis in the pineal gland. Expression of many genes in peripheral organs differ in phase of circadian oscillation. This suggests that apart from core circadian regulators, additional fine-tuners are needed. Role of CREM in regulation of circadian events in the adrenal gland and liver is poorly understood and our aim was to establish the role of ICER on the circadian expression of *Per1* and *Per2* (core circadian regulators) *in vitro* and *in vivo*.

Results: By applying *Crem* knockout (KO) mice we show that in complete darkness (DD) CREM isoforms contribute to circadian expression of only a few genes in liver and adrenals *in vivo*: slight phase-delay is indicated for *Per1, 2* in *Crem* KO adrenals. *Icer* mRNA is circadian in the wild type adrenal with maximum at CT 12. The amplitude of *Icerg* (lacking exon gamma) is higher compared to *Icer* but with identical phase. *In vivo,* ICER proteins are lower at dusk compared to the mid-day in both organs. To evaluate whether ICER is involved in cAMP-dependent regulation of *Period* genes the forskolin and cycloheximde experiments have been performed. *Icer* and *Icer*g are induced by forskolin in an immediate early fashion and *de novo* protein synthesis is important for repression of *Per1* (and less of *Per2*) after the forskolin induction. ICER binds CRE1 of the *Per1* promoter and *Icer* antisense RNA increases *Per1* reporter activity.

Conclusions: These data show for the first time the circadian nature of *Icer* on the periphery and direct involvement of ICER in repression of *Per1* which might be important for the *Period* related re-entrainment *in vivo*.

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P52

THE CONSTRUCTION OF *HAL2* DELETION MUTANTS OF EXTREMELY HALOPHILIC *WALLEMIA ICHTHYOPHAGA* AND HALOTOLERANT *AUREOBASIDIUM PULLULANS*

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Salt toxicity in *Saccharomyces cerevisiae* results from PAP phosphatase (Hal2) activity inhibition by sodium or lithium ions, causing accumulation of PAP produced during sulphur assimilation important for methionine synthesis. Broad sodium tolerance range is of special importance in fungi from extreme saline environments, where sodium concentrations reach up to saturated levels. Two of the fungi thriving in these conditions are the extremely halotolerant *Aureobasidium pullullans* and halophilic *Wallemia ichthyophaga*.

In the present study, we wanted to examine whether Hal2 is the key enzyme of halotolerance also in the above mentioned fungi, by constructing *HAL2* deletion mutants. To this purpose we designed a knock-out vector, which disrupts the fungal HAL2 gene by homologous recombination. The knock-out vector is composed of three fragments. The outermost fragments are specific to the HAL2 gene from each fungi (referred to as the 5' and 3' arms), while the middle fragment is the hygromycin resistance cassette. We then transformed *A. pullullans* and *W. ichthyophaga* protoplasts with the prepared constructs using electroporation. The transformant selection was done on agar plates containing hygromycin. To confirm the stable integration of the constructs into the fungal genomes, we performed PCR on transformants able to grow on hygromycin plates. Preliminary results show that Hal2 is indeed vital for halotolerance of *A. pullullans* and *W. ichthyophaga*, as *HAL2* deletion mutants demonstrated reduced tolerance for sodium and lithium ions.

P53

CHANGES IN miRNA EXPRESSION IN HUMAN MUSCLE TISSUE FOLLOWING SUSTAINED REST AND / OR HYPOXIA

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Introduction: The main aim of the project is to study the effects of hypoxia and sustained recumbency (bedrest) on human physiological systems. Changes in the transcriptome are part of overall physiological changes in organisms. In this study we aim to look for changes in alternative splicing and miRNA expression in human muscle tissue after bed rest and/or hypoxia. Studies in rodents already indicate involvement of microRNAs in development of muscle atrophy and studies in humans show global changes in mRNA expression in humans after bed rest.

Results: We have extracted total RNA, including RNA less than 18 nucleotides long, from skeletal muscle biopsies from human male subjects subjected to 10 day bed rest in normoxia or hypoxia. Samples for evaluation of basal expression before the start of the treatments were also extracted. Using Nanostring technology we have analysed microRNA expression in 6 subjects subjected to bed rest and normoxia. We have detected 154 miRNA in human muscle tissue and 16 miRNAs were differentially expressed using Wilcoxon signed rank test for paired samples with alpha<0.05. All of them with exception of two were down-regulated. Among them are: miR-206, a muscle-specific miR, involved in regulation of cell cycle, cell differentiation and neuromuscular junction; miR-23a involved in insulin-independent glucose transport activity and protein degradation; let-7 family involved in cell cycle, cell differentiation and glucose homeostasis. Some of them have been shown by other to change expression in denervation or wasting of skeletal muscle in mice. In order to analyze changes in transcription level and changes in alternative splicing we are also in the process of whole transcriptome analyses in all samples using next-generation sequencing (RNA-Seq).

Conclusions: Results from miRNA analyses indicate that changes associated with glucose homeostasis, cell cycle, protein degradation and neuromuscular junction are also observed at the miRNA level. With mRNA-Seq analyses, we expect to confirm our results from miRNA expression analyses as well as determine the splicing changes associated with sustained recumbency and hypoxia.

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P54

ASSOCIATION BETWEEN ANEUPLOIDY AND GASTRIC CANCER IN SLOVENIAN PATIENTS AND IDENTIFICATION OF FUNCTIONAL POLYMORPHISMS IN SEGREGATION GENES

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Introduction: Stomach cancer is the fourth most common cancer and the second leading cause of cancer-related death in the world, because in its early stages the symptoms are very nonspecific. The main focus of novel genetic studies of gastric cancer is genomic instability, because the underlying mechanisms of gastric cancer are quite unknown and the pathogenic processes leading to the development of malignant cells are complex.

Genomic instability is broadly classified into microsatellite instability (MIN) and chromosome instability (CIN) leading to aneuploidy. Chromosomal instability is a hallmark of most solid human neoplasms. It was reported that DNA aneuploidy has been observed in 85% of colorectal cancers. The CIN pathway was typically associated with the accumulation of mutations in tumor suppressor genes and oncogenes. Lately, it has been defined that DNA aneuploidy occurs more likely due to disorders in the spindle assembly checkpoint. Aneuploidy phenotype of cancer cells has a high proliferative activity, is highly invasive and also has a huge metastatic potential.

Methods: Our first aim is to find out the proportion of aneuploid cells in our samples of gastric tumors (Slovenian population), because we want to establish a link between the incidence of aneuploidy and cancer of the stomach. We will isolate nuclei from frozen tissue with enzymatic disaggregation and then we will use flow cytometry for cell count determination and analysis of the cell cycle. Our next goal is to identify relevant polymorphisms in the selected genes, such as TTK, BUB1B, ZW10, ZWINT and TPX2. All these genes are involved in mechanisms to ensure proper chromosome segregation during cell division.

Results and Conclusions: These polymorphisms may be useful as potential biomarkers which could be used for detection of gastric cancer in early stages and could thus improve the prognosis of patients suffering from this type of cancer.

P55

VESICLE SIZE DETERMINATION IN ASTROCYTES – USE OF DIFFERENT FLUORESCENCE MICROSCOPY TECHNIQUES

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Astrocytes are glial cells which provide important metabolic support to neurons, actively tune synaptic activity and influence brain microcirculation. One of the key processes which sustain astrocyte communication with neighboring cells is regulated exocytosis of membrane bound vesicles which may contain gliotransmitters (peptides, amino acids), membrane transporters, channels and other molecules. During exocytosis, a vesicle fuses with the plasma membrane and forms an intermediate state (fusion pore). The diameter of the fusion pore is related to the diameter of the vesicle [1]. To measure vesicle diameter in live cells, our aim is to compare different fluorescence microscopy techniques which allow observation of subcellular structures inside a living cell in real-time; confocal microscopy, structured illumination microscopy (SIM) and stimulated emission depletion (STED) microscopy.

Various vesicle diameters in different cell types were reported in the past by electron or fluorescence microscopies; from around 30-700 nm. The use of different techniques and different cell types aggravates the comparison. Therefore, we are measuring the size of several vesicle types in only one cell type, astrocytes. With immunocytochemistry we label various vesicles with primary antibodies: against atrial natriuretic peptide (ANP), brain derived neurotrophic factor (BDNF), vesicular glutamate transporter 1 (vGLUT1), D-serine and lysosomal-associated membrane protein 1 (LAMP1) and with secondary antibodies with conjugated dyes of high photostability and large stimulated emission cross sections in the visible to near infrared range. The average diameter (mean ± SE) of vesicles measured with confocal microscopy was 311 ± 8 nm (ANP), 319 ± 11 nm (BDNF), 281 ± 7 nm (VGLUT1), 292 ± 9 nm (D-serine), 357 ± 14 nm (LAMP1). However, confocal microscopy has the diffraction limit ~200 nm. With recent development in the field of microscopy the resolution can be improved [2]. Therefore, we are now measuring and comparing vesicle diameters of the same types of vesicles recorded by a super-resolution technique, structured illumination microscopy (SIM), which results in ~100 nm resolution. The preliminary data show that the average diameter of vesicles is smaller and is expected to be even smaller when the stimulated emission depletion (STED) microscopy with resolution < 50 nm [3] will be employed.

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EVALUATION OF SCORING FUNCTIONS USE IN CASE OF ACETYLCHOLINESTERASE INHIBITORS

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Introduction: Molecular modeling tools are recognized as helpful in a drug design and medicinal chemistry. Proper evaluation of receptor-ligand interaction is main goal in drug design and development. Next step will be a prediction of receptor-ligand interaction with respect to affinity, required for a ligand structure optimization. Recognition of key interactions is beneficial for developing selective and high affinity ligands. Scoring functions are introduced for this purpose. They are developed by correlating interactions from numerous crystal structures of various different receptor-ligand complexes with matching inhibition constant.

Results: Here we evaluated several high affinity acetylcholinesterase inhibitors using scoring functions to determine correlation between reported inhibition constant for following inhibitor complexes PDB ID: 1E66, 1EVE, 1H22, 1H23, 1U65, 1ZGB and 1ZGC, and score values derived from scoring functions. We used following scoring functions available in Accelrys DiscoveryStudio software: PLP1, PLP2 [1], PMF, PMF04 [2], Jain [3], LigScore1 and LigScore2 [4]. Deviation between inhibition constants was 10% while deviation between matching scores were from 12 to 40%.

Conclusions: Scoring function LigScore2 and PLP2 evaluated well acetylcholinesterase-inhibitor complexes with lowest score deviation. Although LigScore2 predicted logarithmic value of affinity in two cases (1H23 and 1ZGC), some disagreement was noticed due to 25% lower value of predicted affinities.

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P57

PROGESTERONE METABOLISM IN ENDOMETRIAL CANCER CELL LINE ISHIKAWA

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Endometrial cancer is the fifth most common malignant disease in women in Slovenia and the sixth most common cancer in the world. For year 2011, 303 new cases are predicted for Slovenia and 288 387 new cases worldwide. It is widely accepted that chronic exposure to estrogens and insufficient concentration of protective progesterone (P4) can lead to endometrial hyperplasia and endometrial cancer. In our previous work we have demonstrated that the genes involved in metabolism of P4 are expressed in endometrial cancer cell line Ishikawa and in endometrial cancer tissue samples [1, 2, 3]. Other authors described biosynthesis of P4 in peripheral tissues including endometrium [4]. Since P4 can be formed and metabolized in endometrium we hypothesized that its altered metabolism may be implicated in development of endometrial cancer. Our aim is thus to clarify the metabolism of P4 in endometrial cancer cell line Ishikawa (endometrial adenocarcinoma cell line) in comparison with control endometrial cell line HES (cell line of proliferative endometrium) [5].

Ishikawa cells and HES cells were treated with ^{3}H labeled P4 in a medium without phenol red and FBS. Samples were collected after 4, 8 and 24 h. After extraction of metabolites from the medium, samples were vacuum-dried and analyzed by HPLC and TLC. The conjugated metabolites were determined by treatment of the extracted metabolites with a mixture of enzymes β-glucuronidase/arylsulphatase for 24 h at 37°C. Metabolites were again extracted with organic solvent and analyzed by HPLC. These experiments have been performed also in the presence of inhibitors of P4 metabolizing enzymes.

Our data show that different metabolites are produced in Ishikawa cells when compared to HES cells and that the rate of conjugation is more than two fold faster in endometrial cancer cells. Although the final identification of P4 metabolites is still in progress our results suggest that in endometrial cancer cells P4 is first metabolized to 20α-hydroxy metabolite by AKR1C1. This metabolite is then reduced to 3α/β, 20α-dihydroxy metabolites by the actions of AKR1C3 and 3β-HSDs. Dihydroxy metabolites thus formed are then conjugated to glucuronidates or sulphates. This enhanced metabolism of P4 may lead to lower concentration of protective P4 in endometrial cancer. With the final identification of P4 metabolites and a thorough understanding of P4 metabolism we expect to shed light on the potential role of P4 in pathophysiology of endometrial cancer.

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P58

DIRECT INTERACTION OF GALNON WITH G-PROTEINS

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Galnon was first reported as a low molecular weight non-peptide agonist at galanin receptors [Saar et al. (2002) Proc. Natl. Acad. Sci. USA 99, 7136–7141]. Following its systemic administration, this synthetic ligand affected a range of important physiological processes including appetite, seizures and pain. Physiological activity of galnon could not be explained solely by the activation of the three known galanin receptors, GalR1, GalR2 and GalR3. Consequently, it was possible that galnon generates its manifold effects by interacting with other signaling pathway components, in addition to via GalR1-3, particularly because it has been shown that it can penetrate across the plasma membrane of cells. In this report, we present evidence that galnon can activate intracellular G-proteins directly and independently of receptor activation thereby triggering downstream signaling. In doing this, galnon demonstrates selectivity for different G-proteins. By using molecular modeling and docking approach, we have identified two potential binding sites for galnon on the surface of Gs and Gi type of G-protein alpha subunits. We have also demonstrated the physiological relevance of this binding by showing the relaxation effect of galnon on KCl contracted porcine coronary artery which could not be reversed by additional KCl treatment. We conclude that galnon has multiple sites of interaction within the GPCR signaling cascade which mediate its physiological effects. This work was started as a diploma work of Jure Škraban but was later expanded by other authors.

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INFLUENCE OF ARTHROSCOPY ON POPULATION OF MICROVESICLES IN ISOLATES FROM BLOOD

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Introduction: Although day care arthroscopy of knee is considered to be a relatively safe procedure, postoperative thrombotic complication have been reported [1]. Microvesicles (MVs) can be released by blood and vascular cells and are connected to a hypercoagulable state, leading to thrombotic complications. Our aim was to assess the effect of arthroscopy on population of MVs in isolates from blood and thereby assess their contribution to risk for thrombotic complications.

Results: 2.7 mL of blood was withdrawn from 20 consecutive outpatients before and after the surgery. Following isolation by centrifugation and rinsing, flow cytometry was used for counting MVs. 4 patients were excluded from analysis, because they ate lunch before postoperative blood sampling [2]. We found a decrease of the average MV concentration after the surgery, however this decrease was statistically insignificant.

Conclusion: No statistically significant changes in postoperative count of MVs were recorded. These preliminary results imply that a day care arthroscopic knee surgery is a safe procedure with respect to microvesiculation and that this process does not indicate a need for a routine antithrombotic prophylaxis.

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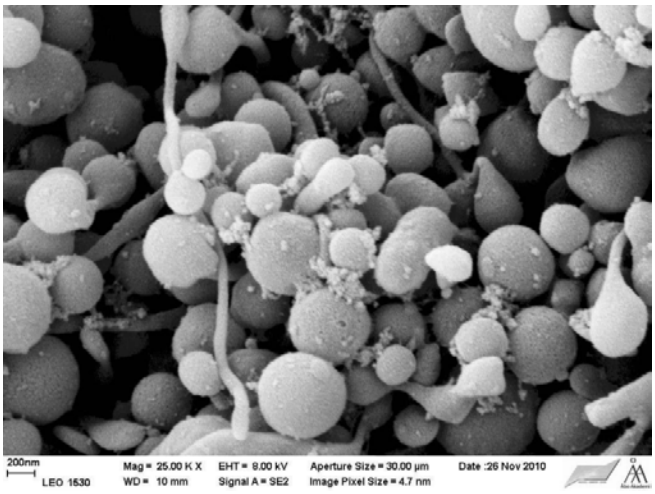

FIGURE 1: A scanning electron micrograph of a blood isolate showing mostly platelet-derived microvesicles.

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EFFECTS OF EXTERNAL PARAMETERS ON ISOLATION OF MICROVESICLES FROM BLOOD

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The most widely used method for assessment of MVs from blood consists of centrifugation and washing of samples, however, this method is highly sensitive to external parameters such as temperature during the process and the level of blood in vacutubes. The concentration of MVs in isolates from peripheral blood is an indicator of the platelet conditions which may be affected by the shear stress in the tube. Also, platelets may be activated in the shear flow through the needle, so they are more prone to fragment during the centrifugation. Here we investigate the effects of temperature, sampling needle and volume of samples on the concentration of MVs in isolates from blood.

Blood was collected from healthy human subjects with a 21-gauge needle (Microlance, Becton Dickinson, NJ) in 2.7 mL tubes containing 270 trisodium citrate at a concentration of 0.109 mol/L [1,2]. Evacuated tubes (BD Vacutainers, Becton Dickinson, CA) were used in all experiments, with the exception of the experiment where we investigate the effect of the sampling needle (Microlance™ and TIK d.o.o.). In that experiment the covers of the vacutube was removed prior to sampling and the blood allowed to drop freely into the tube. Different needles varying in thickness and length were used. The time needed to acquire the required volume of blood was measured. MVs were counted by flow cytometry[1].

In a study including 47 healthy human donors it was found that at 30 ^{o}C, the number of MVs in the isolate was twice the number at 37 ^{o}C, the difference being statistically significant ($p = 0,01$) while at 40 ^{o}C, the number was further decreased. In a study of volume effect three different volumes of blood (1.2 ml, 1.7 ml and 3 ml) were taken from 8 healthy donors. The isolation process was performed at 37 ^{o}C. We found that less blood in the vacutainer gives a lower concentration of MVs. A possible reason is that centrifugal force created in the centrifuge rotor exerted on the upper plasma is higher at lower volumes so platelets are more effectively cleared from the upper part of plasma which is then taken for isolation. In a study of the effect of blood sampling it was assumed that the concentration of MVs in isolates is proportional to energy dissipated during flow through the needle. The energy was calculated by using the Navier-Stokes equation. Concentration of MVs in two isolates from blood was found to positively correlate with the calculated dissipation energy. It is concluded that concentration of MVs is higher if the procedure is performed at lower temperatures, if larger volume of blood is taken for analysis, and if larger amount of energy is dissipated in the needle during sampling.

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MECHANISM OF ALU ELEMENTS EXONIZATION AND ITS EFFECT ON POST-TRANSCRIPTIONAL REGULATION

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Introduction: During co-transcriptional regulation cryptic exons have to be silenced to maintain correct mRNA processing. Majority of newly-emerged exons are represented by ALU elements. We found heterogeneous nuclear ribonucleoprotein C1/C2 (hnRNP C) functions as a silencer of ALU elements through its binding to long uridine tracts at the 3' splice sites of the pre-mRNA. Using genome-wide iCLIP and RNAseq methods, binding to polypyrimidine tracts was found to be mediated by direct competition with the core splicing factor U2AF65.

Results: Mechanism of competition was studied using reporter minigene assays. Long uridine tracts at 3'SS of the ALU elements were disrupted to avoid hnRNP C binding and on the same time maintain binding of the U2AF65. Introduced mutations lead to increased inclusion of cryptic ALU exons in hnRNP C control and knock-down conditions, what confirms its role in ALU exonization. By constructing minigene with disease sequence polymorphism, we identified hnRNP C to be important as well in cryptic ALU exonization that leads to progression of the disease. Further, regulation of cryptic ALU exonization by competition of hnRNPC with the core splicing machinery was found to be involved in maintaining correct mRNA processing, due to its effect on flanking alternative exons as well as on correct 3' end processing.

Conclusion: Genome-wide mechanism of ALU exonization was identified to be regulated by competition of hnRNP C with the U2AF65 for the polypyrimidine tract. The importance of this process was further shown to influence expression of flanking exons, as well as 3' end processing. Results show major progress in understanding exonization of transposable elements, important in disease and evolution.

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ESTIMATION OF SERUM CONCENTRATIONS OF CLOZAPINE BY CYP3A4 EXPRESSION (PRELIMINARY RESULTS)

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The incidence of schizophrenia in Hungary is approximately 1 % of the total population. According to the present directive, the therapy has to include antipsychotic drugs. First generation antipsychotics like chlorpromazine proved to be effective for treatment of schizophrenia, but can cause serious side effects, primarily extrapyramidal (EP) symptoms. The main advantages of the widely used second generation drugs are milder side effects or the lack of EP. One of these drugs, clozapine was proven to provide an effective treatment for nearly one third of the patients with schizophrenia and resistant to most antipsychotics. In rare cases, clozapine treatment can lead to serious side effects, thus monitoring of clozapine serum levels is strongly recommended by the present medical directive. The dosing of antipsychotics like clozapine is determined individually, where the dosage is slowly raised to therapeutic levels to avoid serious side effects. This practice unfortunately prolongs the symptomatic period in case of an ineffective drug, and the elevated serum concentrations increase the chance of unwanted symptoms. The majority of psychiatric drugs are metabolized by cytochrome P450 (CYP) enzymes, thus genetic polymorphisms may influence the blood levels of antipsychotics. However, the correlation between CYP genotypes and side effects or the lack of clinical response is only moderate. This may be due to the fact that CYP phenotype (gene expression) is one of the main factors affecting the current drug metabolizing capacity of patients. Determining CYP gene expression (CYP-phenotyping) and the presence of mutant CYP genes (CYP-genotyping) together may give a more detailed description of the patients' drug metabolizing capacity. A recent observation of the strong correlation between the CYP3A4 mRNA levels in leukocytes and hepatic CYP3A4 activity may lead to new opportunities in personalized medication. CYP3A4 is one of the two enzymes responsible for clozapine metabolism and the only enzyme in N-desmethylclozapine formation. In this clinical study, CYP3A4 mRNA levels in leukocytes were determined in patients treated with clozapine for at least two weeks. We found a strong relation between CYP3A4 expression and the serum concentration of clozapine normalized by body mass and dosage. These findings are obtained from a limited group of patients (n=21, avg. bw. 82,2kg, avg clozapine dose 263mg), and require further experiments with larger sample groups.

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TIME-DEPENDENT EFFECT OF ERYTHROPOIETIN ON BREAST CANCER CELL PROLIFERATION AND PROTECTION FROM CISPLATIN INDUCED CYTOTOXICITY

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Introduction: Severe anemia is a frequent side effect of cancer chemotherapy, resulting mainly from chemotherapy induced inhibition of erythroid cell maturation in the bone marrow and interference with the ability of kidney to produce Epo. In the early days, rHuEpo was shown to be a safe and effective treatment of choice, improving quality-of-life and reducing the need for blood transfusions. However, recent clinical trials gave conflicting results indicating that rHuEpo usage may exert unfavorable effects for the patient survival due to increased adverse events, potentially related to tHuEpo-induced cancer progression. In the present study, we investigated the effect of rHuEpo treatment on breast cancer cell proliferation and protection from cisplatin (cDDP) induced cytotoxicity.

Materials and Methods: MCF-7 and MDA-MB-231 breast cancer cell lines differing in estrogen (ER) and progesterone (PR) receptor and p53 status were used. Cells ware treated with rHuEpo for 24 h and 9 weeks, exposed to cDDP and analyzed for their proliferation and viability (colony formation, cell count). Expression of p53-dependent genes and bcl-2 gene family members (qPCR) was also addressed together with activation of MAPK and PI-3K signaling pathways (western blot). Differences in cell response to rHuEpo and cDDP were paralleled with expression of both steroid receptors and p53 status.

Results: MCF-7 cell proliferation is rHuEpo dependent and is reduced after short-term treatment but increased after long-term exposure. Cytotoxicity studies using cDDP showed protective effect of short-term rHuEpo for MCF-7 and MDA-MB-231 cells. In contrast, long-term rHuEpo exposure influenced only MCF-7 cell viability which was significantly lowered. MDA-MB-231 seems to be able to compensate for the long-term rHuEpo effect which was further confirmed in the lowered level of ERK phosphorylation when compared to short-term treated cells. Gene expression analysis of p53-dependent genes and bcl-2 gene family members confirmed differences between long and short-term rHuEpo effects, indicating the most prominent changes in expression of genes involved in apoptosis, like BCL2 and BAD.

Conclusions: MCF-7 cells proliferation and viability seem to be reversely influenced by the length of rHuEpo treatment, while MDA-MB-231 cells are almost irresponsive to the long-term rHuEpo. This could be explained in terms of ER/PR and p53 genetic signature. Speculatively, this signature may be used to predict the beneficial or maleficent effect of rHuEpo supportive therapy in the individual patient. In order to confirm this speculation, gene expression analysis of genes involved in p53 pathway will be employed.

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THE ROLE OF NOVEL KLOTHO PROTEIN LCTL IN ENDOCRINE FGF SIGNALING

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Introduction: The majority of fibroblast growth factors (FGFs) act in a paracrine or autocrine manner due to their dependence on glycosaminoglycans. FGF19 subfamily has reduced affinity for glycosaminoglycans that enables them to diffuse beyond their site of origin and act in an endocrine fashion. FGF15/19 is an essential component in a postprandial bile acid negative feedback loop. FGF21 is fasting hormone responsible for lipolysis in white adipose tissue and ketogenesis in liver. FGF23 regulates phosphate and calcium homeostasis and is implicated in the development of chronic kidney disease.

For efficient activation of FGF receptor (FGFR), endocrine FGFs require additional cofactor from the Klotho protein family. Klothos are type I transmembrane glycoproteins with extracellular regions that contain two β-glycosidase-like domains. Recently, a new member of Klotho family was identified and named lactase-like (Lctl) [1]. So far, the molecular function of Lctl is unknown. Klothos and Lctl exhibit overall sequence similarity to family 1 glycosidases, however two highly conserved glutamic acid residues, critical for the enzymatic activities of family 1 glycosidases, are not conserved. This suggests that these proteins might serve similar physiological functions. Unlike Klotho and beta-Klotho, Lctl has only a single beta-glycosidase-like domain, raising the question as to whether it functions as a coreceptor for the endocrine FGFs or rather inhibit their signaling.

Aim of the study: To determine the role of Lctl in signaling by endocrine FGFs.

Methods: Our preliminary data show that Lctl binds several FGFRs (2). In addition, molecular modeling based on published crystal structures predicted that Lctl can form a complex with FGFR4 and FGF19. In order to characterize the effect of Lctl on eFGF-FGFR binding and signaling, different soluble recombinant Lctl proteins will be prepared and protein-protein interactions analyzed by surface plasmon resonance, coimmunoprecipitation and pull down assay. Recombinant proteins will be also expressed in cell lines and responses to FGF19 treatment will be evaluated on the level of gene expression and ERK1/2 phosphorylation.

Results: We endeavor to characterize the role of Lctl, novel member of Klotho protein family in endocrine FGFs function. The results of our study have potential therapeutic implication in several metabolic diseases, such as diabetes, chronic diarrhea due to bile acid malabsorption, and chronic kidney disease.

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SUICIDE IN SLOVENIA: GENETICS

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Introduction: Suicide is complex, multifactorial phenomenon, and is an outcome of interplay of environmental, genetic, and epigenetic factors. Slovenia belongs to the group of countries with the highest suicide rate in the world; in the year 2011 ranked 7th with suicide rate of 30.9 suicide victims per 100.000 citizens.

Growing number of molecular genetic studies have primarily focused on serotonergic neurotransmitter system, and it has been suggested that it might be particularly involved in the pathogenesis of depression, suicidal behaviour, and also in aggression and impulsivity, which are important intermediate traits for suicide. Beside the well studied neurotransmitter systems new candidate genes have been determined through genome wide association and copy number variations studies. These genes are importnatly involved in regulation of neuronal growth, survival and repair, brain plasticity, neurodegeneration, mood, cognition, behaviour, and stress response.

It has also been estimated that more than 90% of suicide victims have a diagnosable psychiatric disorder at the time of death, and therefore many of the candidate genes of suicide overlap with psychiatric.

Methods and Results: Our study cohort included more than 600 subjects, both suicide victims and controls, that were genotyped for single nucleotide polymorphisms (SNPs) in the genes for serotonin receptor 1A (HTR1A), tryptophan hydroxylase 2 (TPH2), brain-derived neurotrophic factor (BDNF), reelin (RELN), and alpha-1 subunit of a voltage-dependent calcium channel (CACNA1C). Association of polymorphisms with suicide was determined. Different subgroups were formed to test impact of specific variables (gender, suicide method (violent and non-violent), alcoholism, psychiatric disorders) on suicide and genetic component.

Results showed association of polymorphism -1019 C>G in HTR1A with environmental stressors and impulsiveness. Association was determined also for some SNPs of TPH2 and alcohol-related suicide, with additon of impulsive and verbal aggressive behavior. For Val66Met of BDNF Met variant was proposed as risk factor in violent suicide female subjects and in suicide victims exposed to childhood trauma. RELN showed association with suicide in women. CACNA1C was not associated with suicide, however, it is an important candidate gene for suicide in depressed, bipolar, and schizophrenic patients.

Conclusions: Any genetic marker is likely to be influenced by epistatic, epigenetic and gene-environment interactions and it is thereore unlikely that any single common genetic polymorphism could be importantly associated with complex disorder like suicide. For determination of the influence of potentially aberrant genes more specific traits or enophenotypes should be formed and tested for association. Nevertheless our results are representative and could be important for future studies, because our research was conducted on a population with extremely high suicide rate.

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MOLECULAR AND ULTRASTRUCTURAL CHANGES REQUIRED FOR RAPID RESEALING OF UROTHELIAL INJURY

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Introduction: Epithelium of urinary bladder (i.e. urothelium) covers renal pelvis, ureters, bladder, upper urethra and glandular ducts of the prostate. There sits at the interface between the urine and underlying tissue and its primary function is to provide high resistance barrier to ion, solute as well as pathogens [1]. This exceptionally high resistance barrier results from a high transcellular resistance of a specialized apical plasma membrane (urothelial plaques) and surface glycans, from a paracellular resistance of tight junctions and greatly diminished apical endocytosis in superficial urothelial cells (UCs) [2]. When this barrier is injured (e.g. bladder stones), the rapid resealing of injury is crucial for the normal functioning of the organism. To investigate the mechanisms required for rapid resealing of the urothelial injury, the long-term study on hyperplastic and normoplastic urothelial models in vitro was performed.

Materials and methods: We established two hyperplastic in vitro models that resemble hyperplastic urothelium during wound healing in vivo and the normoplastic urothelial model by maintaining porcine UCs in different UroM media. The formation of the urothelial barrier was evaluated using molecular and ultrastructural analysis, flow cytometry and by measuring transepithelial resistance (TER) for two months.

Results: Flow cytometry analysis confirmed that up to nine-layered hyperplastic urothelia has a significantly higher percentage of cells in S and G2/M cell cycle phases (S=1.55 ± 0.08% and G2/M=6.5 ± 0.18%) than the up to five-layered normoplastic urothelia (S=0.91 ± 0.07% and G2/M=5.4 ± 0.17%). Hyperplastic urothelia achieve significantly higher TER (>10.000 Ωcm2) than normoplastic urothelia (>4000 Ωcm²). However, the expression of cell junctional (claudin-8, E-cadherin) and differentiation-related proteins (cytokeratin 20 and uroplakins) was weaker in hyperplastic urothelia. Ultrastructural analysis confirmed that superficial UCs in hyperplastic urothelial models were less differentiated compared to superficial UCs in normoplastic urothelial models.

Discussion: With the establishment of hyperplastic and normoplastic urothelial models, we here provide an unequivocal evidence that specific cell-cycle distribution and, consequently, the number of cell layers have a significant influence on the barrier function of urothelia. We demonstrate that the molecular and ultrastructural changes in UCs are indispensable for the rapid resealing of urothelial injury and maintenance of high TER. The established urothelial models provide an opportunity for further in vitro studies, including monitoring of the dynamic of wound healing process and testing potential new drugs in bladder-associated diseases.

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IL12RB1 POLYMORPHISM CONTRIBUTES TO THE RISK FOR THE DEVELOPMENT OF SOLITARY AND MULTIPLE UTERINE LEIOMYOMAS

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Introduction: Uterine leiomyoma (ULM) is a common female pelvic benign tumor. It occurs in ~40% of women and 25% of individuals develop clinical symptoms, e.g. pelvic pain, abnormal bleeding, infertility and pregnancy complications. The mechanism of ULM development is believed to be the result of complex interactions between genes and environment. The aim of this study was to investigate the role of single nucleotide polymorphisms (SNPs) in genes *IL12B* (rs6887695), *IL12RB1* (rs11575934) and *IL23R* (rs7517847) as the potential risk factors for ULM.

Methods: The association study was performed in 169 women with clinically and surgically diagnosed ULM and 92 women with verified absence of myomas used as the control group. The association study was performed in a whole ULM group and separately for women with solitary ULM and women with three or more (multiple) ULM. Polymorphisms were tested with PCR following RFLP genotype determination.

Results: Women with multiple ULM had higher prevalence of positive family history (40% vs. 19,5%; p=0,029), percentage of smoking (57,4% vs. 21,1%; p=0,001) and prevalence of GG genotype with G allele frequency in *IL12RB1* rs11575934 polymorphism (p=0,036; OR=0,13 and p=0,029; OR=0,46 respectively), lower age at menarche (0,8 year; p=0,015), number of miscarriages (60% less; p=0,005), age at first sexual intercourse (17,7 vs. 19 years; p=0,003), number of pregnancies (1,7 vs. 3,3; p=2x10-6) and parity (1,4 vs. 2,6; p=1x10-6) compared to healthy controls. Women with solitary ULM had lower parity (27% less; p=0,006) and higher prevalence of AG and GG genotypes in *IL12RB1* rs11575934 polymorphism (p=0,037; OR=2,54) compared to healthy control subjects.

Conclusions: In general women having ULM differed from healthy control subjects in IL12RB1 gene polymorphism, hormonal and external epidemiological factors. Our results clearly show an association of GG genotype and G allele frequency in rs11575934 with prevalence of multiple ULM and AG + GG genotypes with solitary ULM. The rs11575934 polymorphism, together with epidemiological factors can contribute to a higher risk for development of ULM.

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STEROID TOXICITY AND DETOXIFICATION IN FUNGI

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The research on exposure of fungi to steroid hormones is important for several reasons. The number of fungal infections is increasing due to larger numbers of immunocompromised patients because of AIDS, organ transplantation and other reasons. Steroid hormones have been found to play a role in host/fungus relationship. They affect growth, morphology, virulence and drug susceptibility of fungi [1]. In the present study we present our results of the effect of important human steroid hormones on growth of the halophilic yeast *Hortaea werneckii* in comparison to the model yeast *S. cerevisiae* and filamentous fungus *Aspergillus oryzae.* The contribution of the biotransformation, reduced uptake, increased efflux and vacuolar sequestration on steroid detoxification was examined. In addition the possible target of steroid hormone action in fungi has been under investigation.

Toxic effect of steroids was monitored through the impact on fungal growth, involvement of different proteins/enzymes in fungal response was studied with appropriate genetically modified *S. cerevisiae* strains. Possible inhibitory effect of steroid hormones on ergosterol biosynthesis was investigated by analyzing GC profiles of intermediate sterols from fungi grown in the presence of steroid hormones.

The same steroids, most efficiently dehydroepiandrosterone, were found to inhibit growth of the selected microorganisms. In the biotransformation with *S. cerevisiae* and *A. oryzae* mainly constitutive enzymes are involved (in *S. cerevisiae* Ayr1, Atf2 and Fox2) while in *H. werneckii* a large part of the enzymes is of an inducible nature. The biotransformation of steroid hormones is a relatively slow process which does not contribute very efficiently to steroid detoxification. Proteins Dan1 and Aus1 involved in sterol uptake were not found to be involved in steroid hormone uptake. On the other hand we confirmed that membrane ABC transporters Pdr5 and Snq2 and vacuolar transporters YBT1 and YCF1 are involved in lowering of the intracellular concentration of steroid hormones although they do not allow their complete detoxification. We were also interested in the cause of the toxicity of steroid hormones. We found out that steroids do not inhibit significantly the uptake of tryptophan by competing with its uptake but rather by inducing the degradation of the tryptophan permease Tat2. At the same time steroids were found to inhibit ergosterol biosynthesis in *H. werneckii* and *S. cerevisiae*, most probably at the level of Erg 6. Since the appropriate amount of ergosterol has to be present in yeast membrane for normal functioning of Tat2 [2] the reduced uptake of tryptophan could be the result of inhibition of ergosterol biosynthesis.

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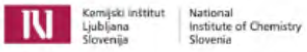

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