Charles University
Faculty of Medicine in Hradec Králové
13th INTERNATIONAL MEDICAL POSTGRADUATE CONFERENCE

New Frontiers in the Research of Ph.D. Students

Conference of Medical Schools

November 24 – 25, 2016

Organized by
Charles University, Faculty of Medicine in Hradec Králové

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Under the Auspices of His Magnificence
Rector of the Charles University
Tomáš Zima

Hradec Králové
Educational Center of the Faculty of Medicine
Location: University Hospital
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The publication has undergone neither linguistic editing nor proof reading.
It is printed from the author’s e-mail correspondence.
GENERAL INFORMATION

Venue:

Educational Center (Výukové centrum)
Charles University, Faculty of Medicine
University Hospital (Fakultní nemocnice) Hradec Králové

Conference Office:

The conference office is to be set up for information and registration at the Educational Center in the area of the University Hospital at the following opening hours:

Thursday, November 24, 9:00 – 17:40
Friday, November 25, 8:00 – 16:20

Official Language:

English

Presentation Time:

Lecture 15 min
Discussion 5 min

Accommodation of Participants:

Hotel Nové Adalbertinum
Velké náměstí 32
500 03 Hradec Králové 3
Dear friends and colleagues,

I would like to welcome you to the 13th International Medical Postgraduate Conference in Hradec Králové. The conference has been progressing well since its inception, and over the years it has turned into a real international meeting. We are proud to welcome participants not only from the Czech Republic and Slovakia, but also from Austria, Georgia, Hungary, Poland, Portugal, Croatia, the Netherlands and the United Kingdom. I personally believe that the position of this conference is well established, and it is a standard part of international activities of our faculty.

There are several reasons for organizing this conference. The first obvious reason is the opportunity to compare achieved results, to present one’s data and learn from others. Nevertheless, we consider this particular meeting of postgraduate students in biomedicine also very important as a tool for international harmonization of Ph.D. studies in the European area. We are very happy that ORPHEUS (Organisation of Ph.D. Education in Biomedicine and Health Sciences in the European System) is an active partner in the organization of this conference.

Another important reason for organizing this meeting is an opportunity for direct personal contacts. Dear participants, take advantage of this occasion not only to learn the news in other medical fields but also to think about the bits of knowledge in other medical areas which can be valuable for you and your postgraduate work. Though there is only “one medicine”, the mutual overlapping of its disciplines can result in great benefits for all.

Those of you evaluated as the best by an expert panel of judges will receive a financial award, yet this should be considered secondary. I am sure that the idea of our meeting is similar to the idea behind the Olympic Games – winning is not the most important thing. Taking part, learning scientific news, and above all meeting new colleagues and friends is of the utmost importance. If we succeed in this, the conference has fulfilled its purpose.

I wish you very successful scientific meeting and enjoyable time in our beautiful city!

Miroslav Červinka
Dean, Faculty of Medicine in Hradec Králové
Charles University
# PROGRAMME OVERVIEW

## Thursday – November 24, 2016

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SCIENTIFIC PROGRAMME

THURSDAY, NOVEMBER 24

Part I
Chairperson: Aleš Ryška

11:00 J. Brůha (Plzeň, Czech Republic): Effective of Selective Portal Vein Embolization and Intraportal Administration of Autologous Stem Cells on the Progresion of Colorectal Liver Metastases
11:20 I. Gullo (Porto, Portugal): Gastric Carcinoma With Lymphoid Stroma in the Era of the Immune Context and Immunotherapies
11:40 J. Joos (Dresden, Germany): Histone H3 Phosphorylation at Serine 28 Regulates Longevity, Heart Function and Heart Morphology in D. Melanogaster
12:00 N. Kobakhidze (Tbilisi, Georgia): Genetic Markers of Exfoliation Syndrome in Georgian Population
12:20 P. Kustán (Pécs, Hungary): Urinary Orosomucoid as a Potential Diagnostic Marker of Sepsis
12:40 M. Nováková (Prague, Czech Republic): Loss of B Cells and Their Precursors is the Most Constant Feature of GATA-2 Deficiency in Childhood Myelodysplastic Syndrome

Part II
Chairperson: Emil Rudolf

14:00 J. Biedermann (Dresden, Germany): STAT6 Regulates Ephrin-A1 in Human Macrophages, which Influences Cell Mechanics and Migration
14:20 R. Bower (Hull, United Kingdom): Head and Neck Cancer: ON-CHIP Culture Provides a Bespoke System for Monitoring the Response of Patient Samples
14:40 A. Krajčová (Prague, Czech Republic): Mitochondrial Pathogenesis of Propofol Infusion Syndrome in an In Vitro Model of Human Skeletal Muscle
15:00 J. Válka (Prague, Czech Republic): Differential Expression of the Homologous Recombination DNA Repair Genes in Early and Advanced Stages of Myelodysplastic Syndrome
15:20 K. Vašíčková (Brno, Czech Republic): Senescence is Modulated by the UPR Response in Ovarian Surface Epithelial Cells
Part III
Chairperson: Stanislav Mičuda

16:00  **T. Baka (Bratislava, Slovakia):** Cardiovascular Remodelling in L-NAME-Induced Hypertension: Effect of Ivabradine

16:20  **E. Fárková (Prague, Czech Republic):** The Relationship Between Chronotype and Psychosocial Phenomena

16:40  **Z. Hanusová (Prague, Czech Republic):** Establishment of Permanently Prion Infected Cell Lines for Studies of Prion Strains in Tissue Cultures

17:00  **T. Kazda (Brno, Czech Republic):** Post Whole-Brain-Radiotherapy Cognitive Impairment and Hippocampal Neuronal Depletion Measured by In Vivo Metabolic MR Spectroscopy

17:20  **M. Mešťaník (Martin, Slovakia):** Temperament, Character and Biomarkers of Cardiovascular Risk: Interaction Between Neurobiological Model of Personality and Cardiac Autonomic Control

FRIDAY, NOVEMBER 25

Part IV
Chairperson: Martina Řezáčová

9:00  **M. Arts (Maastricht, the Netherlands):** Matrix Assisted Laser Desorption Ionization Mass Spectrometry Imaging (MALDI-MSI) as a Tool to Map the In Situ Spatiotemporal Distribution of Amino Acids in Tumor Tissue: the Next Generation of Molecular Phenotyping

9:20  **M. Elmasry (Liverpool, United Kingdom):** Peri-Hepatectomy Pharmacological Induction of the Transcription Factor Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2): A Promising Novel Way of Enhancing Liver Regeneration

9:40  **A. Kvernadze (Tbilisi, Georgia):** Evolution of West Syndrome in Georgia, Predictors of Outcome

10:00 **O. Sobotka (Hradec Králové, Czech Republic):** Mitochondrial Functions in Steatotic RAT Liver

10:20 **L. Vokálová (Bratislava, Slovakia):** Acute Liver Injury: Role of Extracellular DNA

10:40 **H. Wilkinson (Hull, United Kingdom):** Exploring the Role of Metals in Wound Repair
**Part V**
Chairperson: **Jan Laco**

11:20 **F. Caisberger (Hradec Králové, Czech Republic):** Neuroprotective Effect of Oxime Therapy After Sarin Intoxication

11:40 **A. Jüngling (Pécs, Hungary):** The Effects of Early Environmental Enrichment and PACAP on Monoamine Levels in an Aging Rat Model of Parkinson's Disease

12:00 **J. Kosalka (Krakow, Poland):** Urinary Markers of Inflammation in Lupus Nephritis Patients

12:20 **M. Považan (Vienna, Austria):** Fast Ultra-High Resolution 3D Proton MR Spectroscopic Imaging of Human Brain at High Magnetic Fields

12:40 **I. Rausch (Vienna, Austria):** Lean Body Mass Estimation Form Standard DIXON Based Attenuation Correction in Integrated Positron Emission Tomography/Magnet Resonance Imaging

13:00 **P. Šteiner (Plzeň, Czech Republic):** Molecular Genetic Analysis of Adenoid Cystic Carcinoma of Salivary Glands

**Part VI**
Chairperson: **Jan Čáp**

14:20 **R. Adão (Porto, Portugal):** Urocortin-2 Attenuates Pulmonary Arterial Hypertension

14:40 **P. Krůpa (Hradec Králové, Czech Republic):** Experimental Treatment of the Spinal Cord Injury by Human Mesenchymal Stem Cells Derived From Wharton's Jelly (WJ-MSC)

15:00 **E. Neis (Maastricht, the Netherlands):** Effects of Gut Microbiota Manipulation by Antibiotics on Plasma Amino Acid Levels in Obese Humans

15:20 **K. Roženková (Prague, Czech Republic):** Genetics and Functional Analysis of Czech Patients With Congenital Hyperinsulinism

15:40 **T. Šimurda (Martin, Slovakia):** Congenital Afibrinogenemia: Novel Mutation Fibrinogen Martin Leading to Premature Termination Codon in Fibrinogen B Beta-Chain Gene

16:00 **M. Vajrychová (Hradec Králové, Czech Republic):** Label-Free Quantification of Endogenous Peptides Related to Infectious Inflammation in PPROM Pregnancies
EVALUATION COMMITTEE

Chairperson: Vladimír Palička
Vice-Dean for International Relations
Director of the University Hospital
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University of Pécs, Hungary

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Medical University of Vienna, Austria

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Chairman of the European Society of Parenteral and Enteral Nutrition (ESPEN)
Maastricht University Medical Centre, The Netherlands

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Charles University, Prague, Czech Republic
UROCORTIN-2 ATTENUATES PULMONARY ARTERIAL HYPERTENSION

Rui Adão
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Co-authors: P. Mendes-Ferreira, D. Santos-Ribeiro, C. Maia-Rocha, F. Potus, S. Breuils-Bonnet, M. Rademaker, S. Provencher, S. Bonnet, A. Leite-Moreira

Tutor: Carmen Brás-Silva

Introduction and Aims
Pulmonary arterial hypertension (PAH) is a rare and severe syndrome characterized by a progressive remodeling of small pulmonary arteries, leading to elevated pulmonary vascular resistance and right ventricular (RV) failure. Currently, drugs available to treat PAH are restricted to the application of endothelial vasomotor regulatory factors(1). However, the effectiveness of these drugs is limited, and despite significant advances in the treatment of PAH, the long-term prognosis is still poor. RV function is the main predictor of the outcome in PAH and new therapies should protect against RV failure(2).

Urocortin-2 (Ucn-2) is an endogenous vasoactive peptide of the corticotropin-releasing hormone (CRH) family that binds with high affinity to the type 2 CRH receptor (CRHR2), which is expressed abundantly in the cardiovascular system(3). A variety of studies in experimental heart failure (HF) have shown favorable effects of Ucn-2 treatment, which have demonstrated both acute and sustained beneficial actions on cardiac function and remodeling(4,5). Effects reported in healthy volunteers(6) and patients with acute and chronic HF(7,8) have further excited interest in the therapeutic potential of the Ucn-2 in human cardiovascular disease. However, the role of Ucn-2 in PAH and RV failure is still unknown.

Thus, this study aimed to analyze the expression of Ucn-2 in human and experimental PAH, and the effects of human Ucn-2 (hUcn-2) treatment in monocrotaline (MCT)-induced PAH animals. To distinguish cardiac-specific actions from effects on the pulmonary vasculature, hUcn-2 treatment was also studied in an experimental model of pressure overload by pulmonary artery banding (PAB), which results in RV loading without PAH.

Methods

Human Tissue Samples
Human RV samples were collected from patients with and without PAH at the time of cardiac surgery, heart transplantation or autopsy. Samples were categorized according to clinical history and the tricuspid annular plane systolic excursion, and designated as normal RV (NRV) or RV failure (RVF). Blood samples were also collected from PAH patients and age-matched controls.

Animal models and experimental design
Male wistar rats (7-8-weeks old) were injected with MCT (60mg/kg, s.c.) or saline. After two weeks, rats were treated with hUcn-2 (2.5ug/kg, bi-daily, i.p.) or vehicle for 10 days (n=15-22/group), resulting in four groups: Ctrl+vehicle (C); Ctrl+hUcn-2 (CU); MCT+vehicle (M) and MCT+hUcn-2 (MU). At the end of treatment, animals were submitted to exercise testing, echocardiographic and invasive hemodynamic, with subsequent sample collection. Another set of animals was submitted to PAB or sham surgery, and followed the same protocol (n=12-18/group), resulting in three groups: Sham+vehicle (S); PAB+vehicle (B) and PAB+hUcn-2 (BU).
**Statistical analysis**

Two-way analysis of variance (ANOVA) was used to analyze most parameters. Holm-Sidak’s method for post hoc comparisons between groups and Kaplan-Meier survival analysis (log-rank test) were performed. When appropriate, one-way ANOVA was used. Data are presented as means±SEM. Differences of p<0.05 were considered statistically significant.

**Results**

RV expression of Ucn-2 and its receptor was increased in MCT animals and patients with RVF. Plasmatic levels of Ucn-2 were increased in experimental and human PAH (Fig.1). hUcn-2 treatment of MCT-animals reduced PAH, resulting in decreased mortality, improved exercise capacity and attenuated pulmonary arterial and RV remodeling and dysfunction. We found attenuation in RV gene expression of hypertrophy and in failure signaling pathways. Moreover, hUcn-2 improved endothelial function (Fig.2). In the PAB model, hUcn-2 attenuated PAB-induced RV hypertrophy (Fig.3).

![Figure 1](image_url)

**Figure 1.** (A)mRNA expression of Ucn-2 in the RV of patients with NRV and RVF and MCT rats; (B)Representative blots of CRHR2 immunoreactivity in RV of human and rat samples; (C)Ucn-2 levels in the buffy coat of patients with PAH and in plasma of MCT-induced PAH rats. *p<0.05 versus CTRL/NRV.
Discussion
In this work, we evaluated the expression of the Ucn-2/CRHR2 system in experimental and human PAH, and the effects of hUcn-2 treatment in MCT-induced PAH rats. Our results show increased plasma and RV levels of Ucn-2 in both MCT animals and patients with PAH, possibly in response to myocardial stress. This is also the first study that shows an association between the expression of Ucn-2/CRHR2 system with RV dysfunction, suggesting a potential cardioprotective response in this condition.

Additionally, chronic hUcn-2 therapy in experimental MCT-induced PAH significantly attenuated the severity of this disease. Although not all characteristics of PAH are simulated by the MCT model, it shares many characteristics with pulmonary hypertension in humans, including pulmonary vascular remodeling, as well as RV and endothelial dysfunction(2). As Ucn-2 ameliorates most of these parameters, the peptide shows therapeutic potential in this context. Additionally, at 14 days after MCT administration animals exhibit RV hypertrophy and pulmonary flow compromise(9), suggesting that hUcn-2 reverses already established PAH. This is important as PAH in clinical practice is frequently diagnosed in a later stage(1). Furthermore, using a model of pressure loading of the RV without PAH, we also demonstrated that hUcn-2 treatment has direct beneficial effects on RV structure, which is the main predictor of the outcome in PAH.

Conclusions
In conclusion, this study shows for the first time that Ucn-2/CRHR2 signaling may have an important role in PAH and RV dysfunction, given that levels of Ucn-2 and its receptor are altered in human and experimental PAH, and hUcn-2 treatment in a rat model of PAH improves both cardiopulmonary structure and function. These data should encourage further studies to elucidate the underlying mechanisms through which Ucn-2 attenuates the pathophysiology of PAH.

Summary
Pulmonary arterial hypertension (PAH) is a devastating disease and current treatments are limited. Urocortin-2 (Ucn-2) is highly expressed in the cardiovascular system and has shown promising
therapeutic effects in experimental and clinical left ventricular heart failure (HF). Thus, our aim was to analyze the expression of Ucn-2 in human and experimental PAH, and investigate the effects of human Ucn-2 (hUcn-2) administration on right ventricular (RV) function and pulmonary vasculature in rats with monocrotaline (MCT)-induced PAH and RV hypertrophy. Blood and tissue samples were collected from patients with and without PAH and from rats with MCT-induced PAH; and hUcn-2 (5μg/Kg/day i.p. for 10 days) or vehicle was administered to rats subjected to MTC injection or pulmonary artery banding (PAB) (to induce RV overload without PAH). Ucn-2 levels were significantly elevated in the plasma of MCT-injected rats and in buffy coat from PAH patients, and expression of the peptide and its receptor (CRHR2) was increased in the RV of patients and rats with PAH. Ucn-2 treatment in MCT-rats reduced PAH, resulting in decreased mortality, improved exercise capacity and attenuated pulmonary arterial and RV remodeling and dysfunction. Underlying these changes were attenuation in RV gene expression of hypertrophy and failure signaling pathways. In addition, hUcn-2 significantly improved endothelial function. In the PAB model, hUcn-2 treatment attenuated PAB-induced RV hypertrophy. Ucn-2 levels are altered in human and experimental PAH. hUcn-2 treatment attenuates PAH in MCT animals, has direct anti-remodeling effects on the pressure-overloaded RV, and improves pulmonary vascular function.

References


Matrix Assisted Laser Desorption Ionization Mass Spectrometry Imaging (MALDI-MSI) as a tool to map the in situ spatiotemporal distribution of amino acids in tumor tissue: The next generation of molecular phenotyping

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Department of General Surgery, Maastricht University Medical Center+ in Maastricht, The Netherlands


Supervisor: Steven.W.M. Olde Damink
Co-supervisor: Zita Soons

Introduction
Mass Spectrometry Imaging (MSI) is a powerful high-performance analytical tool to simultaneously explore the spatially resolved molecular composition of hundreds of diverse biological molecules throughout tissues. MALDI-MSI relies on the desorption and laser-induced ionization of molecules present on the surface of tissue sections (1). Cancer among other diseases is characterized by the metabolic dysregulation of complex cellular processes. Metabolic reprogramming is common and very heterogeneous phenomenon in tumorigenesis and tumor progression (2). Amino acids are of particular interest since they represent key final effectors of the complex and dynamic interaction between the genomic and proteomic machinery tumor cells. Furthermore, amino acids represent the link between the proteome and metabolome due to their versatile nature as building blocks for proteins, active cellular signaling mediators and substrates for energy production. Stable isotopes of the indispensable amino acid L-phenylalanine (L-PHE) are commonly used in clinical studies to measure protein turnover. The liver also converts L-PHE into tyrosine (L-TYR), which is subsequently transported to the tumor where it may be further metabolized in the TCA cycle. Hence, L-PHE tracers can be used to visualize the metabolism and fluxes over time and are an ideal mechanism to study the active metabolic cellular processes (3, 4). However, spatial information about the dynamics of in vivo metabolism of L-PHE in relation to the tumor microenvironment is very limited. Additionally, certain molecular species like L-PHE are difficult to ionize and are therefore poorly detected in tissue by conventional MSI. On-tissue derivatization, based on the covalent binding of a positive charged group on amino acids, significantly enhances the detection of L-PHE and other amino metabolites in tissue sections (5). Hence, we aimed to develop a novel and robust method to visualize the spatiotemporal distribution and in-vivo metabolism of L-PHE and the isotopically labelled analogue by MALDI-MSI of on-tissue derivatized tumor sections.

Methods
A human NCI-H460 Non Small Cell Lung Carcinoma (NSCLC) xenograft model in adult female immune-compromised Nu-Fox1nu/nu mice (n=16) was used. When tumors reached a volume of 1000 mm³, the mice were injected with a bolus of ring 13C6-PHE (6). The mice were sacrificed at 4 time points after tracer injection (0 min, 10 min, 30 min and 60 min, respectively) followed by immediate dissection of the tumor and snap freezing in liquid nitrogen. Fresh-frozen tumor sections (10 μm) were covered with TAHS in acetonitrile (SunCollect, Sunchrome GmbH, Germany) for subsequent on-tissue derivatization. Next, DHB in 70% MeOH + 0.2% TFA matrix was applied (HTX-sprayer, HTX Technologies LCC, USA) for subsequent high resolution MALDI-FTICR-
MSI (9.4T Solarix XR, Bruker Daltonics, Germany) analysis in positive mode. MSI data were analyzed by implementing a newly developed MATLAB algorithm to process and visualize the spatial tracer kinetics. After MSI analysis, tumor sections were stained with hematoxylin and eosin (H&E), reviewed by a pathologist for tumor morphology, and co-registered with MSI images (Figure 1). Additionally, Principle Component Analysis (PCA) combined with Linear Discriminant Analysis (LDA) was used to identify spectral differential molecular regions within and between different tissues in relation to tissue morphology (7).

Results
We have optimized a method to visualize amino acids in tumor tissue enabling the visualization of spatial distribution of derivatized amino acids and other metabolites with an amine group in tumor tissue. Moreover, over 45 different amino compounds could be detected within one analysis. Furthermore, we were able to visualize the spatially resolved dynamics of the tracer in tumor metabolism. Hereeto, we developed a MATLAB algorithm to calculate and visualize the Tracer-to-Tracee ratio (TTR). The TTR after 10 min of tracer injection is reproducibly visualized for both 13C6-PHE and 13C6-TYR in consecutive tumor sections (Figure 2A, B). The TTR of L-PHE and L-TYR showed a heterogeneous distribution in the tumor tissue after 10 minutes of tracer injection (Figure 2C). Additionally, MALDI-MSI in conjunction with a principal component analysis facilitated the discrimination of differential molecular regions within the tumor, characterized by a unique spatial resolved tracer-related molecular fingerprint (Figure 2D).

Figure 1. MALDI-MSI workflow to simultaneously visualize the in-situ spatiotemporal distribution of L-PHE and other amino metabolites by on-tissue derivatization of fresh-frozen tumor tissue derived from a human NSCLC xenograft model.
Figure 2. Representative images showing the reproducibility of the visualization of the distribution of amino acids in tracer-infused consecutive tumor sections (t=10 min). 
(A) TTR of L-PHE calculated as the ratio of 13C6-PHE tracer divided by endogenous L-PHE. 
(B) TTR of L-TYR calculated as the ratio of 13C6-TYR tracer divided by endogenous L-TYR. 
(C) Co-localization of the 13C6-PHE and 13C6-TYR tracers in relation to their endogenous analogues, calculated as the TTR of L-TYR divided by the TTR of L-PHE. 
(D) Intra-tumor heterogeneity based on the first three principal components in Red (R), Green (G) and Blue (B).

Discussion
Tumors are heterogeneous tissues consisting of a mixture of cancer and stromal cells (e.g. immune and fibrotic cells). Additionally, tumors consist of a variety of clones, with clone expansion driven by different underlying mutations. Currently, cancer research towards personalized medicine mainly focusses on genomics, transcriptomics, and proteomics data, which are mostly static and does not address intra-tumor heterogeneity. Specifically targeting the different cancer cells is key to successful treatment, whereas current strategies to personalize treatment designed upon homogenate omics measurements may not, or only partly, target the actual cancer cells. MSI of isotopically labelled amino acids in tumor tissue is a potent tool to monitor tumor metabolism, thereby providing spatial and dynamic information about the active production and consumption of metabolites in the tumor tissue (8). Furthermore, those dynamic indicators may eventually lead to the discovery and identification of treatment targets, e.g. cellular transporters and metabolic enzymes (9). Hence, these preliminary data highlight the great potential MALDI-MSI for clinically relevant complementary applications with regard to cancer diagnostics, prognostics and better spatiotemporal knowledge of the metabolite dynamics in cancer and other diseases.

Conclusion
Here, we present a novel and reproducible method to explore the distribution and dynamics of amino acids in tumor metabolism. This is of great potential for clinically relevant breakthroughs in personalized medicine. Furthermore it provides a multimodal translational platform to combine traditional histology with morphology-related molecular information.

Summary
Currently, most cancer patients still receive a non-personalized generic treatment which might target the tumor cells only partially or not at all. This might be explained by the heterogeneous nature of tumors. However, research on personalized treatment mainly focusses on static homogenate based omics techniques (e.g. genomics, transcriptomics, proteomics), thereby lacking spatial and dynamic information about intra-tumor heterogeneity. Mass Spectrometry Imaging is
a potent tool to bridge the gap by visualization of the distribution of multiple molecules throughout the tumor tissue. Here, we show a novel and reproducible method to simultaneously visualize the spatiotemporal distribution of an isotopically labelled L-PHE tracer along with other amino acids in tumor tissue from human NSCLC lung cancer xenografts.

References


CARDIOVASCULAR REMODELLING IN L-NAME-INDUCED HYPERTENSION: EFFECT OF IVABRADINE

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Tutor: Fedor Šimko

Introduction and Aims
Hypertensive heart disease in terms of left ventricular hypertrophy and fibrosis is an independent risk factor of heart failure, myocardial infarction, sudden cardiac death and stroke (1). Thus, regression or prevention of left ventricular hypertrophy is the principle therapeutic aim (2). Elevated heart rate is a neglected risk factor in variable cardiovascular pathologies including hypertension. It is generally believed that heart rate reduction may bring reduction of cardiovascular morbidity and mortality (3). Ivabradine, the blocker of I_f-channel in sinoatrial node, exerts cardioprotection due to its heart rate reducing effect (4). However, there are some data suggesting that ivabradine-induced cardioprotection may involve also so called pleiotropic effects including antiremodelling action (5). The aim of our study was to show, whether ivabradine is or isn’t able to attenuate the remodelling of the heart, kidney and vessels in the L-NAME (N^ω-nitro-L-arginine-methyl ester)-induced hypertension.

Materials and Methods
Animals and drugs
12-week-old male Wistar rats were divided into 4 groups:
1st group: control (C; n=7)
2nd group: ivabradine (Procoralan®, Servier, France) [10 mg/kg/day] (Iv; n=7)
3rd group: L-NAME (Sigma-Aldrich, USA) [40 mg/kg/day] (L-NAME; n=8)
4th group: L-NAME+Iv [40 mg/kg/day and 10 mg/kg/day] (L-NAME+Iv; n=8)
Drugs were administered in drinking water for four weeks.

Systolic blood pressure (SBP) and heart rate (HR) measurement
During 4 weeks of experiment SBP and HR were measured by non-invasive tail-cuff plethysmography once a week.

Echocardiography
After 4 weeks of experiment, the heart function was assessed by echocardiography. Left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were quantified to determine systolic function of the left ventricle (LV). LV diastolic function was determined by the ratio of mitral peak velocity of early and late diastolic filling (E/A ratio) (6).

Tissue sampling
At the end of the experiment, rats were decapitated and heart, thoracic aorta and kidneys were removed. Relative LV weight (LV weight/body weight) was calculated. Samples of aorta and kidneys were fixed in 4% formaldehyde, embedded in paraffin and cut in 5 μm slices. Deparaffinised and dehydrated 5 μm slices were stained with hematoxylin and eosin (7).
Thoracic aorta morphometry
Stained samples of thoracic aorta were assessed by optical microscope and morphometrically processed by ImageJ software. Aorta wall thickness (WT) and cross-sectional area (CSA) were expressed in mm and mm², respectively (8).

Kidney morphometry
The same microscope and camera additionally equipped with 2-dimensional image analyser were used for kidney morphometry. Glomerular tuft area (AG) expressed in μm² and glomerular numerical density per area (N) expressed in count/mm² were determined (8).

Determination of hydroxyproline in LV
Hydroxyproline concentration in collagenous protein fractions was measured from samples of LV frozen at -80°C and expressed in μg hydroxyproline per 1 g of LV tissue (9).

Statistical analysis
Results are expressed as mean±S.E.M. Differences were considered as significant if the p-value was less than 0.05. For statistical analysis, one-way analysis of variance (ANOVA) and Bonferroni test were used.

Results
After 4 weeks of experiment, SBP was 122.2±0.9 mmHg in the control group. L-NAME increased SBP (41%, p<0.05). Ivabradine reduced (p<0.05) SBP in both groups, Iv (19%) and L-NAME+Iv (8%) (Fig. 1A).
In the control group, HR was 385±3 bpm after 4 weeks of experiment. L-NAME did not affect HR. Ivabradine reduced (p<0.05) HR in both groups, Iv (32%) and L-NAME+Iv (15%) (Fig. 1A).
Relative LV weight was 1.252±0.051 mg/g in controls. L-NAME increased relative LV weight (14%, p<0.05), while ivabradine reduced it (5%) (Fig. 1B).
In the control group, hydroxyproline concentration in the soluble collagen, in the insoluble collagen and the total hydroxyproline concentration was 0.13±0.01 μg/g, 0.42±0.04 μg/g and 0.57±0.04 μg/g, respectively. L-NAME increased (p<0.05) the hydroxyproline concentration in the soluble (38%) and insoluble (14%) collagen and also the total hydroxyproline concentration (18%). Ivabradine slightly reduced hydroxyproline concentration in the insoluble collagen (15%) and the total hydroxyproline concentration (9%) and slightly increased hydroxyproline concentration in the soluble collagen (11%) (Fig. 1B).
In the control group, LV ejection fraction and fractional shortening was 84±1.3% and 48±1.4%, respectively. L-NAME reduced (p<0.05) both, LVEF (14%) and LVFS (23%). Ivabradine slightly increased both, LVEF (10%) and LVFS (17%). E/A ratio was 1.24±0.02 in the control group. It was increased by L-NAME (7%) and decreased by ivabradine (15%) (Fig. 2).
In the aorta, L-NAME increased WT (30%, p<0.05) and CSA (32%). Ivabradine slightly reduced both, WT (3%) and CSA (4%).
In the kidney, L-NAME increased AG (24%, p<0.05) and decreased N (42%, p<0.05). Ivabradine reduced AG (13%, p<0.05) and increased N (46%, p<0.05) (Fig. 3).
Systolic Blood Pressure - SBP (mmHg)

Heart Rate - HR (bpm)
Fig. 1  
A Systolic blood pressure and heart rate during experiment.  
C – controls, Iv – ivabradine; * p<0.05 vs. C, # p<0.05 vs. L-NAME

B Hydroxyproline concentration in left ventricle and relative weight of left ventricle.  
BW – body weight, C – controls, Iv – ivabradine, LV – left ventricle, LVW – weight of left ventricle; * p<0.05 vs. C, # p<0.05 vs. L-NAME
Fig. 2 Echocardiographic assessment of left ventricular function. C – controls, Iv – ivabradine, LV – left ventricle, MV E – mitral peak velocity of early diastolic filling, MV A – mitral peak velocity of late diastolic filling; * p<0.05 vs. C, # p<0.05 vs. L-NAME.
Discussion
In our experiment, ivabradine significantly reduced HR in both, control and L-NAME group. Surprisingly, ivabradine reduced also SBP in both groups, analogically as described in rats previously by one research group only. It is suggested, that this unexpected hypotensive effect of ivabradine might have been related to the potential sympatholytic nature of ivabradine (10). Ivabradine improved systolic and diastolic function of the hypertensive heart. Antiremodelling effect of ivabradine in terms of LV hypertrophy and fibrosis reduction may underlie these functional benefits. Especially, the trend of ivabradine to reduce the insoluble (stiff) collagen might have participated on diastolic and systolic functional improvement. Moreover, the positive hemodynamic effects of ivabradine, such as HR and SBP reduction might be of additional benefit (1, 5).
Moreover, the slight antiremodelling action of ivabradine in the aorta and significant improvement of the structural parameters of kidney can be associated with the overall cardiovascular benefit of ivabradine (1, 5).

Conclusions
a) Ivabradine reduced not only heart rate but also systolic blood pressure in both, control and L-NAME group
b) Ivabradine reduced relative weight and fibrosis of the left ventricle, also reduced wall thickness of the aorta and protected glomerular loss in kidneys
c) Antiremodelling effect of ivabradine is suggested to underlie the improvement of systolic and diastolic function of the left ventricle

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Summary
Ivabradine, the blocker of If-channel in sinoatrial node, was shown to bring cardiovascular benefit. It is generally declared that cardioprotection by ivabradine is due to its heart rate-reducing effect. Pleiotropic effects of ivabradine, including antiremodelling action are suggested to be also involved in ivabradine-induced cardioprotection. The aim of our experiment was to show, whether ivabradine is able to attenuate the remodelling of the heart, kidney and aorta in the L-NAME (N\(^\circ\)-nitro-L-arginine-methyl ester)-induced hypertension. Four groups of adult male Wistar rats (n=30) were treated as follows for four weeks: untreated controls, ivabradine (10 mg/kg/day), L-NAME (40 mg/kg/day) and L-NAME (40 mg/kg/day) plus ivabradine (10 mg/kg/day). Systolic blood pressure and heart rate were measured by tail-cuff method. Relative weight of the left ventricle and hydroxyproline concentration in collagenous protein fractions were measured. Systolic and diastolic function of the left ventricle was assessed by echocardiography. Aorta wall thickness, cross-sectional area and glomerular tuft area and density were measured. L-NAME treatment led to hypertension, left ventricular hypertrophy and fibrosis with systolic and diastolic dysfunction. L-NAME induced aorta and kidney remodelling in terms of increased aorta wall thickness, cross-sectional area and glomerular tuft area and decreased glomerular density. Ivabradine reduced systolic blood pressure and heart rate in both, control and L-NAME group. Ivabradine prevented left ventricular hypertrophy and improved left ventricular systolic and diastolic dysfunction. Ivabradine reduced left ventricular hydroxyproline concentration in the insoluble (stiff) collagen and increased hydroxyproline concentration in the soluble collagen. Aorta and kidney remodelling was reduced by ivabradine. In conclusion, ivabradine reduced systolic blood pressure and heart rate and attenuated heart, aorta and kidney remodelling in L-NAME-induced hypertension.

References

STAT6 REGULATES EPHRIN-A1 IN HUMAN MACROPHAGES, WHICH INFLUENCES CELL MECHANICS AND MIGRATION

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Introduction

Atherosclerosis is an inflammatory disease and it is still the leading cause of death in developed countries.1 Currently, the role of macrophage polarization is highly discussed in context of atherosclerosis. Depending on microenvironment, macrophages can polarize towards two main types – classical activated M1-macrophages and alternative activated M2-macrophages. M1-macrophages have a more inflammatory, whereas M2-macrophages harbor a more anti-inflammatory phenotype.2 Both phenotypes are detected within atherosclerotic lesions.3 The members of the Eph/ephrin-system, the largest family of receptor tyrosine kinases, are membrane bound and typically interact via direct cell-cell-interaction. Depending on binding affinities and structure, the Eph-receptors as well as their ligands (ephrins) are divided into an A- and B-class.4 However, the role of the Eph/ephrin-system in atherosclerosis and especially in context of macrophage polarization is largely unknown.

The aim of the present study was to investigate the regulation and function of the Eph/ephrin-system, especially of ephrin-A1, during macrophage polarization and the function of ephrin-A1 in human macrophages in terms of atherosclerotic plaque development.

Methods

Human leucocyte concentrates from healthy donors were used for monocyte isolation by a two-step density gradient centrifugation. Culturing of monocytes for 7 days in M199 medium supplemented with glutamine, 10% FCS and 1% antibiotics/antimycotics leads to differentiation into M0 macrophages. The latter were subsequently polarized towards the M1 (20ng/ml INF-γ and 1µg/ml LPS) or M2a phenotype (20ng/ml IL-4).

Transfection of siRNA into human macrophages was achieved by using the Viromer® BLUE kit (Lipocalyx) according to manufacturer’s instructions.

Promoter sequences were obtained from a web resource (eukaryotic promoter database: http://epd.vital-it.ch/human/human_database.php) and screened for putative transcription factor binding sites using the Patch program provided by gene regulation (http://www.gene-regulation.com/pub/databases.html). Potential binding sites for STAT6 were identified and subsequently analyzed by ChIP. For chromatin isolation and immunoprecipitation, the ChIP-IT® Express Enzymatic Kit (Active Motif) was used according to manufacturer’s instructions.

Migration of macrophages was performed using transwell assay (8 µm pore inserts) in a 24-well plate format. Lower chamber contained M199 supplemented with 5% FCS. Macrophages resuspended in serum-free M199 and were transferred into the upper chamber (5*10⁴ cells per chamber). After 16h incubation at 37°C, cells were fixed using methanol and nuclei were
counterstained with DAPI. Counting of migrated cells, located on the lower site of the insert, was performed using fluorescence microscope images and ImageJ.

Cell mechanics of human macrophages were determined using real-time-deformability cytometry. This method is based on the deformation () of cells, which were flushed through a channel constriction in a PDMS based microfluidic chip. In real-time with a throughput of a flow cytometer, circularity () and cell size were determined by analyzing the cell contour.5

Results

In a first step, the expression of all known Eph-receptors and ephrin-ligands in human M0-, M1- and M2-macrophages was screened using real-time PCR. Whereas macrophages express almost all EphB-receptors (except EphB4) the expression of EphA-receptors was much more restricted (only EphA1, -A2, -A4, -A10 receptor). Macrophages express all ligands except ephrin-A2 and ephrin-B3. Polarization of macrophages also influences the expression patterns of the Eph/ephrin-system, e.g. ephrin-A1 was 15 fold upregulated by M2 polarization.

Further analysis revealed that induction of ephrinA1 is mediated by STAT6 as proven by ChiP and siRNA-mediated silencing of STAT6. Silencing of STAT6 abolished the IL-4 mediated upregulation of ephrin-A1 on mRNA and protein level. In silico analysis revealed several putative STAT6 binding sites within the human ephrin-A1 promoter. Using ChiP analysis a binding of STAT6 to a region between -666 and -304 upstream of the transcription start could be shown. Focusing the function siRNA-mediated silencing of ephrin-A1 in M2a-macrophages led to a decreased expression of typical M2 genes. The main transcription factor under M2a polarization, STAT6, was decreased on mRNA and protein level under ephrin-A1 silencing. Further, the downregulation of MRC1, a typical marker of M2a polarization, could be shown in ephrin-A1 silenced M2a-macrophages.

Using real-time-deformability cytometry the cell mechanics of macrophages was investigated. First, cell mechanics of the different macrophage polarization types were analyzed. Compared to M0 macrophages, M1 polarization led to an increase in stiffness, whereas M2a polarization led to significant softer cells.

Next cell mechanics were quantified under ephrin-A1-silencing conditions. Compared to the controls, ephrin-A1 silenced cells were significantly stiffer.

As cell mechanics can influence the migratory behavior of cells, macrophage migrations assays were performed. M2a-macrophages had a higher migration potential compared to M0-macrophages. Furthermore, silencing of ephrin-A1 significantly reduced migration in both, M0- and M2a-macrophages.

To investigate the expression of ephrin-A1 in atherosclerotic plaques, aortae of 1yr old ApoE−/− mice were explanted, macroscopically divided in areas with and without plaque and used for RNA isolation. The mRNA expression of ephrinA1 and macrophage-marker CD68 was measured using real-time PCR. We could show that ephrin-A1 expression is significantly higher in regions of macroscopically visible plaques and that this correlates with expression of CD68 (p<0.01, Spearman correlation r=0.819).

Discussion

The Eph/ephrin-system is influenced by macrophage polarization. The most impressive effect was seen in the upregulation of ephrin-A1 in M2a-macrophages. Further analysis identified ephrin-A1 as a new target of the transcription factor STAT6. In addition, ephrin-A1 influences the M2a polarization itself, suggesting a crucial role of this ligand and the Eph/ephrin-system in the process of macrophage polarization. Beside its role in regulation of the polarization, ephrin-A1 was shown to influence cell mechanics of macrophages. As it is known from other cell types that ephrin-A1 can influence the cytoskeleton, this effect might be based on this kind of modulation.6 Furthermore, analysis of migratory capacity of macrophages enlightened a crucial role of ephrin-A1 in this process. Especially the massive reduction of migration capacity upon ephrin-A1 silencing in M0-
and M2-macrophages might be a hint, that ephrin-A1 has a central role during migration and chemotaxis of macrophages. These processes are of fundamental importance for the process of atherosclerosis which is characterized by an accumulation of macrophages within the vessel wall.\(^7\) It could be possible that ephrin-A1 as a membrane bound ligand is a part of the leading edge of migration macrophages and direct these cells to sites of inflammation by interaction with other cell types.

**Summary and Conclusions**

The results of the present study showed, that ephrin-A1 is a STAT6 target in human M2a macrophages. Ephrin-A1 seems to be involved in the M2a-polarization itself and in the regulation of cell mechanics and migration of macrophages. Due to the fact, that ephrin-A1 is expressed in murine and human\(^6\) plaques macrophages and influences the phenotype of these cells, it might be promising to analyze the pro- or anti-atherogenic potential of ephrin-A1 *in vivo* and possibly in a cell-type specific manner.

**References**


HEAD AND NECK CANCER: ON-CHIP CULTURE PROVIDES A BESPOKE SYSTEM FOR MONITORING THE RESPONSE OF PATIENT SAMPLES

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Introduction
Head and neck cancers are a diverse group of malignancies arising from cells covering mucosal surfaces within the oral cavity, pharynx, larynx, salivary glands and nasal cavities. Head and neck cancer, is the sixth most common solid tumour worldwide with squamous cell carcinomas constituting 90% of all head and neck cancers (HNSCC). Chemo-radiation is the predominant primary treatment, but despite multiple advances over recent years, treatment resistance is a persistent problem contributing to the low overall 5 year survival figures of 40-60% for patients treated exclusively with radiotherapy or chemoradiotherapy. This highlights the need for screening technologies and personalised treatments.

On-chip culture systems involve the continuous perfusion and removal of waste from a biological sample ex vivo, in a fashion mimicking the circulatory system. The small geometries of these devices result in laminar flow within the channels, these unique characteristics in addition to low costs make on chip culture excellent candidates in biomedical applications. It is the capability of tissue response monitoring alongside the assertion of the clinical problem of treatment resistance that the concept of this technology seeks to address.

Aim
To develop tumour-on-chip culture by which personalised treatment could be achieved, by showing culture of HNSCC on-chip without loss of viability at the single cell level and by maintenance of tissue morphology.

Methods
Weight optimised (5-10mg) head and neck tumour samples (REC: 10/H1304/6) were analysed immediately or cultured in a microfluidic device (Figure 1; 37°C, 48h) with continuous media perfusion (2µL min⁻¹; volumetric flow rate). Haematoxylin and Eosin (H+E) staining was used to assess morphology. Mechanical and enzymatic dissociation (2h, 37°C incubation with 0.02% w/v Collagenase IV and 0.02% w/v DNase I) was used prior to cellular analysis. Cell suspensions were analysed using: trypan blue exclusion; Propidium Iodide (PI) incorporation by flow cytometry; and an MTS proliferation assay. Design of a new on-chip culture device was conducted on SolidWorks software. Statistical analyses by independent samples t-test (SPSS).

Results
Haematoxylin and eosin stained HNSCC tissue sections (n=15) were analysed before placement of the tumour in the microfluidic culture device (Figure 1, left) and following 48h of continuous perfusion within the device (Figure 1, right). Images are shown from two patients with laryngeal...
squamous cell carcinoma. The low magnification (x100) images show maintenance of the tissue structure. Infiltrating islands and interconnected cords of tumour cells are shown in the pre-microfluidic culture images and retained following 48h culture. Pale pink stromal areas of the tumour are also apparent before and after microfluidic culture. The higher magnification (x400) shows an intact basement membrane and the orientation of cell maturation seen in pre-microfluidic culture tissue, with more mature cells observed in the centre of tumour islands and nests present post 48h microfluidic culture. The chronic inflammatory mononuclear cell fraction (lymphocytes and macrophages) within the stroma are recognisable both before and after microfluidic culture.

**Figure 1.** Haematoxylin and Eosin stained 8 µM tissue sections, shown at x 100 and x 400 magnification. Two representative tumours are shown (n=15). Laryngeal squamous cell carcinoma, staging a) T4aN0M0 and b) T4N0M0. Each tumour is shown pre and post 48h microfluidic culture. White arrows show infiltrating islands and cords of tumour cells. Tumour area showing orientation of cell maturation is circled in white. Pale stromal areas with inflammatory cells are circled in black. Intact basement membrane is shown by black arrows.

Single cell suspensions generated from tumour tissue showed no significant difference in percentage viability, by trypan blue exclusion, between tumour tissue obtained following surgical resection (11-57.9%) and a sample from the same tumour cultured in the microfluidic device for 48 h. (Figure 2a; n=15). To consolidate these results, flow cytometry was utilised to quantify cell death by PI staining; no significant difference in percentage cell death was observed between the dissociated tumour cells before and after 48 h microfluidic culture (Figure 2b: n=8). No significant difference
was observed between the proliferative capacity of the dissociated tumour cells following 48h microfluidic culture (Figure 2c, n=7).

![Figure 2. Analysis of single cell suspensions from dissociated HNSCC samples. Mean (+SEM). A. Viability (%) of cell population by Trypan Blue Exclusion (n=15). B. Cells stained positive with PI flow cytometry, indicating cell death (n=8). C. Proliferation as shown by MTS assay and quantified as absorbance of formazan product normalised by tumour weight.](image)

In order to achieve improved perfusion of tumour tissue samples, a new tumour on chip culture device has been successfully designed and prototyped. A precision cut tissue slice is held between two sintered discs separating the fluid flow, conceptually mimicking *in vivo* vascular flow (Figure 3).

![Figure 3. Novel tumour-on-chip device for a precision cut tissue slice (350µM, 5mm spherical punch) A. Diagram detailing chip components and tissue slice placement. B. Photograph of total assembly with scale.](image)

**Discussion**

The biological data yielded promising results in terms of viability maintenance following microfluidic culture; the maintenance of morphology following microfluidic culture was demonstrated by H&E staining with the presence of distinct morphological characteristics of squamous cell carcinoma. This is in agreement with previous studies from the group, using both rat liver and HNSCC tissue and those of others who used H&E to demonstrate maintenance of tissue specific structures including single hair follicular units and human adipose tissue. The use of enzymatic and mechanical tissue dissociation to obtain a single cell suspension...
for subsequent analysis allows a more precise determination of cell viability and increases the scope of analytical methods that are achievable post treatment of the tissue.

**Conclusion**
The on-chip culture successfully maintains HNSCC tumour samples such that they are comparable to the pre culture sample in terms of morphology and viability; with the expected variation between patients. This maintenance is encouraging and invites subsequent studies applying treatment regimens to HNSCC tumour samples within optimised on-chip devices. The development of a novel device enabling improved perfusion of the sample will serve to further extend the applications of the technology.

**Summary**
Head and neck cancers have a poor prognosis which is linked in part to radioresistance. Culture of tumour-on-chip provides a novel platform for the study of tumour biology; maintenance of tumour opens the door for therapeutic assault to the tissue-on-chip and subsequent response monitoring. Ongoing work will deliver 5 irradiation fractions to tumour maintained on-chip and subsequent analysis will investigate radiation response markers and proliferation of the tumour.

**References**


EFFECT OF SELECTIVE PORTAL VEIN EMBOLIZATION AND INTRAPORTAL ADMINISTRATION OF AUTOLOGOUS STEM CELLS ON THE PROGRESSION OF COLORECTAL LIVER METASTASES

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Introduction and Aims

Extended liver resections for colorectal liver metastases (CLM) are limited by Future Liver Remnant Volume (FLRV). It is recommended to leave minimally 30% of Total Liver Volume (TLV). Prolonged chemotherapy needs increased FLRV (40%)1. Patients with small FLRV have higher risk of acute liver failure and death in postoperative period. Insufficient FLRV is a main cause of inoperability of patients with CLM2. Selective portal vein embolization (PVE) is well established to increase FLRV. The matter of recent discussion is the possible connection of this method with progression of CLM during the period of FLRV growth3,4. Stem cells (SC)- especially CD 133+ and CD 34+ cells- are able together with PVE to accelerate growth of FLRV5,6,7. There is a question if SC have any effect for the support of growth of malignancy after PVE. The aim of our study was to evaluate the progression of CLM after PVE and after PVE with administered SC. We also analysed growth of FLRV, DFI (Disease Free Interval), OS (Overall Survival) and number of radically performed procedures.

Methods

63 primary inoperable patients were included in the study. Cause of primary inoperability were inadequate FLRV (FLRV lower than 30%), other indication was prolonged chemotherapy (FLRV lower than 40%). PVE or PVE with administration of SC were used like a prevention of acute liver failure. All patients were without extrahepatic spreading of malignancy before PVE. The patients underwent PVE (PVE group) or PVE with contralateral intraportal administration of product with SC (group PVE SC) in the period 1/2004 - 7/2015. Group PVE had 43 patients, group PVE SC had 20 patients. All patients underwent PVE by percutaneous transhepatal approach. The stem cells were obtained by two approaches either from peripheral blood or from bone marrow. 10 patients from PVE SC group underwent stimulation by Granulocyte Colony Stimulating Factor (G-CSF). Stimulation was held for 4 days before PVE. SC were harvested from peripheral blood by leukapheresis after four days of stimulation. Product with stem cells was storage with anticoagulants ACD-A. Other 10 patients from PVE SC group received SC obtained from bone marrow. The procedure was performed in general anesthesia on the operating theatre 1st day after PVE. Stem cells were aspirated from bone marrow from the dorsal part of iliac crests. Isolation of SC from the aspirate was performed in dependence on density gradient by centrifugation on the SynGenX™ Stem Cell Processing System. Product with SC was administered into the portal vein immediately after centrifugation. Presence of CD 133+ and CD 34+ cells was verified by flow cytometry. Obtained products with SC (from peripheral blood and from bone marrow) were administered into the portal vein 1st day after PVE. We performed small laparotomy in right lower abdominal quadrant. Product was applicated via vena ileocolica under skiascopic control.
to the contralateral portal vein. All patients were controlled under the CT scans each week after procedure. TLV, FLRV and VLM (Volume of Liver Metastases) were measured and calculated by radiologists. All acquired data were statistically analyzed.

**Results**

Growth of FLRV was without statistically significant differences between group PVE a PVE SC. Trend of accelerated growth of FLRV (in two weeks after procedure) was observed in patients from PVE SC group with FLRV lower than 30%. Explorative laparotomy was performed at 3 patients in group PVE SC (15%) and at 17 patients in group PVE (39.5%). Progression of CLM was main cause of contraindication of radical liver surgery (p=0.044). But growth of VLM was without statistically significant differences between observed groups. In our study DFI and OS were non-statistically significant between observed group. DFI for PVE group was 25.6% (1st year), 2.0% (3rd year) and 0% (5th year). DFI for group PVE SC was 23.2%(1st year), 5.8-10.5% (estimated DFI for 3rd year) and 0.9-10.5% (estimated DFI for 5th year). Group PVE had OS 84.5% ((1st year), 59.4% (3rd year) and 6.7-14.7% (estimated OS for 5th year). Group PVE SC had OS for 1st year 81.3% (1st year), 29.9-49.9% (estimated OS for 3rd year) and 4.7-49.9% (estimated OS for 5th year). Patients without postoperative chemotherapy had significantly faster recurrence of malignant disease (groups PVE and PVE SC together, p=0.0086).

**Discussion**

FLRV is crucial factor that determined ability of liver surgery for CLM. PVE is method how to increased FLRV. This method could be also use for support of liver regeneration in patients with prolong chemotherapy. Risk of PVE is growth of CLM in period between PVE and liver surgery. Some studies demonstrated use of SC for increasing FLRV. But it is unclear if SC have effect on the growth of CLM. We didn’t prove acceleration of FLRV growth after PVE with administration of SC. We could observe trend of acceleration of FLRV growth especially in patients with FLRV lower than 30% in time before PVE. But this trend was without statistically significant differences, probably because of small number of patients in studied group. The growth of CLM after PVE was described in the literature. There is risk of acceleration of growth of CLM after SC administration. We didn’t prove this effect in our study. In combination with lower number of explorative laparotomies in group PVE SC it could be a good signal how to increased number of patients for radical liver surgery of primary inoperable CLM and significantly prolong the patients survival.

**Conclusion**

PVE with administration of SC is a new approach how to increased the FLRV before liver resection. We demonstrated that the use of PVE and SC could be advised for the patients with FLRV lower than 30%. Patients with PVE and intraportal administration of SC had decreased number of explorative laparotomies and progression of VLM wasn’t accelerated against PVE alone.

**References**


NEUROPROTECTIVE EFFECT OF OXIME THERAPY AFTER SARIN INTOXICATION

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Introduction
Nerve agents may induce progressive irreversible damage in the central nervous system associated with the dysfunction of irreversibly inhibited AChE and neuronal excitotoxicity and it seems to be largely responsible for persistent profound neuropsychiatric and neurological impairments in the victims of nerve agent exposure [1].

The standard treatment of nerve agent poisoning usually consists of three types of antidotes – anticholinergic agents, oximes and anticonvulsive drugs. Anticholinergic drugs such as atropine sulphate antagonize the muscarinic overstimulation, anticonvulsants such as diazepam are indicated to prevent centrally mediated seizures and secondary brain damage and the oximes disrupt the covalent bond between nerve agent and AChE and restore the physiological function of this enzyme [2]. Many studies have shown there is no potent broad-spectrum oxime able to sufficiently reactivate AChE inhibited by all nerve agents regardless of their chemical structure. HI-6, for instance, is considered to be a relatively strong reactivator of sarin, soman and cyclosarin inhibited AChE but its ability to reactivate AChE inhibited by tabun and less toxic organophosphorus pesticides is of low to moderate values [3]. Trimedoxime or newly synthetized oxime K203, on the other hand, seem to be effective against tabun and pesticides [3]. Since the effort to develop a potent broad-spectrum reactivator has failed so far, another way how to overcome this issue and possibly to increase the reactivating efficacy of antidotes is to combine the oximes in the antidotal treatment [4]. The main purpose of this study was to compare the neuroprotective efficacy of the oxime HI-6 with two oxime mixtures containing HI-6 and trimedoxime or the oxime K-203 in combination with atropine in sarin-poisoned rats. The neuroprotective potential was evaluated using histopathological examination with the help of Fluoro-Jade B fluorochrome used in neuroscience to label degenerating neurons and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay detecting DNA fragmentation during apoptosis.

Methods
Animals were divided into 5 groups (8 rats each): 1) a control group (administered with saline), 2) sarin poisoned group (108 µg/kg – 90% LD50), 3) sarin poisoned group treated with atropine sulphate (21 mg/kg – 5% LD50) and HI-6 (39 mg/kg – 5% LD50), 4) sarin poisoned group treated with atropine sulphate, HI-6 and trimedoxime (7.5 mg/kg – 5% LD50), and 5) sarin poisoned group treated with atropine sulphate, HI-6 and K203 (16.3 mg/kg – 5% LD50). The antidotes were administered 1 min after sarin challenge. Surviving rats were anesthetized by ether vapor 24 hours after intoxication. Samples were subsequently embedded into paraffin and 6µm thick coronary brain sections were cut at level between 3 mm and 4.2 mm from bregma. Selected brain sections were stained with hematoxylin & eosin, Fluoro-Jade B and TUNEL staining. The histological changes were scored using semi-quantitative criteria. Finally, individual animal brain damage scores were calculated representing a sum of histopathological, Fluoro-Jade B and TUNEL scores.
from all tested brain subregions in one animal. The Kruskal-Wallis test with subsequent multiple pairwise comparisons was used to evaluate differences between all groups within particular brain nucleus/subregion. The differences were considered significant when \( p \leq 0.05 \).

**Results**

The most extensive alterations were found in sarin-poisoned animals without antidotal treatment. Compared to the control group, significantly increased histopathological damage scores in cortex, hypothalamus and piriform cortex \((p = 0.023, 0.001 \text{ and } 0.020, \text{ respectively})\) was measured. In affected animals, cortex and piriform cortex displayed pseudolaminar necrosis in 1 and 3 cases, respectively. Hemorrhage was present in one case in hypothalamus. Single cell damage characterized by multifocal degenerative/necrotic changes accompanied with oedema was observed in the remaining positively scored rats. Especially, basolateral nucleus of amygdaloid body, the third and fifth layer of cortex (pyramidal cells), neurons in CA1 (> dentate gyrus > CA3 > CA2) zone of hippocampus, supramammillary hypothalamic nuclei, the third layer of piriform cortex, and medial and dorsolateral thalamic nuclei were markedly impaired.

Administration of antidotal treatment reduced the extent of damage. Any diffuse changes and/or hemorrhage in all groups treated with antidotes were not observed. However, significantly decreased histopathological scores were found in cortex \((p = 0.009)\) of animals administered with atropine and HI-6 and in cortex, hippocampus, hypothalamus, and piriform cortex \((p = 0.023, 0.010, 0.009, \text{ and } 0.020, \text{ respectively})\) in rats treated with atropine, HI-6 and K203. Significantly increased Fluoro-Jade B positivity in amygdaloid body, cortex, hypothalamus, piriform cortex, and thalamus \((p = 0.029, 0.032, < 0.001, 0.032, \text{ and } 0.029, \text{ respectively})\) was found in non-treated, sarin-poisoned rats. Diffuse fluorescent patterns were present in 3 of 5 surviving rats. In all six regions, maximal Fluoro-Jade B positivity correlated with histopathological findings. The antidotal treatment markedly reduced brain degeneration marked by Fluoro-Jade B. We found significantly decreased Fluoro-Jade B positivity in hippocampus \((p = 0.017)\) of atropine and HI-6 treated rats and in amygdaloid body and hippocampus \((p = 0.038 \text{ and } 0.026, \text{ respectively})\) of animals administered with atropine, HI-6 and trimedoxime. No hemorrhages were observed in both groups. In atropine, HI-6 and K203 treated group, Fluoro-Jade B fluorescence significantly decreased in cortex, hippocampus, piriform cortex, and thalamus \((p = 0.032, < 0.001, 0.032, \text{ and } 0.029, \text{ respectively})\). On the other hand, a small hemorrhage in the hypothalamic region was found. However, Fluoro-Jade B positivity in this area was scored as mild. TUNEL positivity was significantly increased in amygdaloid body, hippocampus and piriform cortex \((p = 0.012, 0.003 \text{ and } 0.041, \text{ respectively})\) of sarin-poisoned animals without antidotal treatment. The distribution of TUNEL staining pattern corresponds to the histopathological and Fluoro-Jade B findings except for hippocampal dentate gyrus. Atropine and HI-6 treatment did not significantly influence the outcome of sarin intoxication. In atropine, HI-6 and trimedoxime treated group, a decreased amount of TUNEL positive cells was only observed in amygdaloid body \((p = 0.016)\). The combination of atropine, HI-6 and K203 decreased positivity in amygdaloid body, hippocampus and piriform cortex \((p = 0.012, 0.003 \text{ and } 0.041, \text{ respectively})\).

**Discussion**

The alterations were particularly profound in cholinceptive subregions and/or subregions sensitive to hypoxic/ischemic stress. This observation is in accordance with generally accepted mechanism of nerve agent-induced acute brain injury and with previously published findings [5]. The distribution of histopathological changes correlated with Fluoro-Jade B and TUNEL staining pattern with one exception. In gyrus dentatus (hippocampus), histopathological findings and Fluoro-Jade B fluorescence showed the highest positivity in the polymorphic layer, whereas TUNEL positivity prevailed in the granular layer. Although both layers display different sensitivity to various stress stimuli [6], the reason of this discrepancy remains uncertain. An explanation may
lie in the limitations of TUNEL method. The method is used to detect DNA fragmentation in apoptotic and necrotic cells. On the other hand, DNA fragmentation occurs also in S phase of cell cycle and the method was reported to produce false positivity in proliferating tissues. Since the subgranular zone of dentate gyrus is considered to be one of the two main neurogenic zones in the adult brain, TUNEL positivity observed in this subregion could possibly represent the damage as well as its regeneration.

Antidotal treatment plays a pivotal role in acute phase of nerve agents’ toxicity [7]. In our model, all three therapeutic regimes mitigated the extent of sarin-induced brain injury. Although we did not find any statistically significant differences among groups treated with oximes, the neuroprotective efficacy of oxime mixtures was slightly higher compared to the group treated with a single oxime. Particularly, the combination of HI-6 and K203 appears to be the most effective to protect experimental animals from acute sarin-induced neuropathological changes in surviving animals. This conclusion corresponds to the previously published results demonstrating the benefit of combinations of oximes for the reactivating and therapeutic efficacy of antidotal treatment of sarin poisoning in rats and mice. Based on the described results, both trimedoxime and K203 do not interfere with HI-6 bioavailability but rather support its action. Therefore, combining the oximes in the antidotal treatment could be a promising step towards a broad-spectrum antidotal treatment of acute nerve agent-exposure regardless of the chemical structure of nerve agent. However, even in HI-6 and K203 group, animals displayed signs of neuronal damage, which tends to progress overtime. Limited therapeutic efficacy is probably related to low blood-brain barrier (BBB) penetration of bispyridinium oximes. Thus, the development and usage of oxime mixtures with better BBB penetration may further increase their ability to counteract the acute toxicity of nerve agents.

Summary
The aim of our study was to compare the ability of two combinations of oximes (HI-6 + trimedoxime and HI-6 + K203) to counteract acute sarin-induced brain damage with the efficacy of antidotal treatment involving single oxime (HI-6) using in vivo methods. Brain damage and neuroprotective effects of antidotal treatment were evaluated in rats poisoned with sarin at a sublethal dose (108 µg/kg i.m.; 90% LD₅₀) using histopathological, Fluoro-Jade B and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis 24 h after sarin administration. Both combinations of oximes reduce the number of rats that died before the end of experiment compared to non-treated sarin poisoning and sarin poisoning treated with HI-6 alone. In the case of treatment of sarin poisoning with HI-6 in combination with K203, all rats survived till the end of experiment. HI-6 alone was able to reduce sarin-induced brain damage; however, both combinations were slightly more effective. The oxime HI-6 in combination with K203 seems to be the most effective. Thus, both tested oxime combinations bring a small benefit in elimination of acute sarin-induced brain damage compared to single oxime antidotal therapy.

References


PERI-HEPATECTOMY PHARMACOLOGICAL INDUCTION OF THE TRANSCRIPTION FACTOR NUCLEAR FACTOR ERYTHROID 2-RELATED FACTOR 2 (NRF2): A PROMISING NOVEL WAY OF ENHANCING LIVER REGENERATION

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Introduction
The liver has a remarkable capacity for regeneration following hepatectomy; however, acute hepatic failure remains a significant and often fatal complication following major hepatectomy. The transcription factor Nrf2 plays a pivotal role as a master regulator of cyto-protection against oxidative stress1, nevertheless, its role in hepatic regeneration is still ill-defined2. We sought to investigate the prospect of Nrf2 as a potential enhancer of hepatic regeneration, which could pave the way for promising translational outcomes.

Methods
A murine model was used utilising C57BL/6J mice and two thirds partial hepatectomy was performed, followed by culling the mice at different time points. The liver tissue was collected at both the time of surgery and the time of cull. Pharmacological induction of Nrf2 was implemented by intra-peritoneal administration of CDDO-Me pre and post operatively. Nrf2 knockout mice were used as negative controls. Western blots for the proliferation marker PCNA were performed, and correlated to Nrf2 and Nrf2 downstream protein NQO1 as a surrogate marker for Nrf2 activity. Temporal changes in liver volume post-hepatectomy were measured using magnetic resonant imaging (MRI) hepatic volumetry. Liver function was monitored in-vivo by measuring the half-life of indocyanine green (ICG) clearance using Multispectral Optoacoustic Tomography (MSOT).

Results
A significant correlation between the increase of proliferation and Nrf2 activity was observed at 48 hours post-hepatectomy especially in the CDDO-Me treated mice as compared to the non-treated and knockout mice. MRI hepatic volumetry showed a significant increase in the liver volume at 48 and 72 hours post-operatively in the CDDO-Me treated mice as compared to the non-treated mice. Indocyanine green clearance half-life was reduced significantly in the treatment group denoting an improved liver function post-hepatectomy.

Discussion
Liver regeneration is a complex process that involves the activation of multiple cellular pathways ensuring the full recovery of this remarkable organ to retain its function. The advances in surgical techniques and patient care has allowed surgeons to remove as much as 70% of the liver e.g. in metastatic liver cancer, only for the liver to grow back almost to the same pre-operative size in less than one year in human. Post-hepatectomy liver failure (PHLF) is still major risk of morbidity and mortality increasing the post-operative mortality from less than 5% to almost 30% when it occurs3. The use
of techniques like segmental portal vein ligation or combining liver resection with radiofrequency ablation has helped to minimize the risk of PHLF with continuing research aiming to improve the capacity of the liver to regenerate.

Oxidative stress to the liver triggers the activation of the transcription factor Nrf2 which initiates the transcription of cyto-protective genes and their corresponding proteins. Nevertheless, the exact role of Nrf2 in hepatic regeneration is still poorly understood.

Our in-vivo animal studies using state of the art imaging modalities have allowed us to explore the effect of pharmacological induction of Nrf2 on hepatic regeneration following 2/3 partial hepatectomy in mice. Further work will be needed to expedite the Nrf2 activity in human following hepatectomy with the potential aim of applying this pre-clinical work into the surgical practice.

**Conclusions**
The transcription factor Nrf2 has a potential major role at the early stages of liver regeneration. Pharmacological or dietary induction of Nrf2 pre and post major hepatectomy could be a simple and effective way of decreasing the incidence of the devastating occurrence of post-hepatectomy liver failure.

**References**
THE RELATIONSHIP BETWEEN CHRONOTYPE AND PSYCHOSOCIAL PHENOMENA

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Introduction and Aims
Circadian rhythms are rhythmically repeating and genetically encoded changes in organisms working on many different levels. They are bound to changes in light/dark cycle and control individual sleep-wake timing, food intake and other processes (Berson et al., 2002). Their disruption may be cause by lifestyle habits such as exposition to artificial light after sunset or shift-work schedule (Hastings, 1997). Disruption of circadian rhythms is considered a risk factor for increased appetite, weight, blood pressure and decreased glucose tolerance or immune response and may result in development of cardiovascular, metabolic or immunological disorders (Karatsoreos, 2014; Walker et al., 2015). From this perspective, individual differences in circadian settings – called chronotypes represent a scientifically interesting area for further research.

There are three chronotypes: morning, evening and intermediate. Their distribution in general population is close to Gaussian and we can find differences between them on many levels (Roenneberg et al, 2007). For example: in reactivity to seasonal changes, tendency to store fat, in psychological characteristics, eating habits and health damaging behaviors or prevalence of lifestyle diseases. In many aspects, individuals with evening type (late chronotype or „owls“) are considered more risky (Hsu et al., 2012; Janečková, 2014; Walker et al., 2015).

In addition, human variability in photosensitivity resulting in a different response to changes associated with seasonal cycles seems to be related to chronotypes. People with late chronotype have three times higher probability to develop SAD (seasonal affective disorder) opposed to larks (morning chronotype). Even if they do not not suffer from SAD, they sleep longer and experience a worse mood during the winter months (Zhang et al., 2015).

The aim of the ongoing project is to describe in detail the human chronotype in the context of psychosocial determinants of health and its frequent complications (obesity, sleep disorders, etc.) in the adult Czech population. This field of chronobiology may greatly enrich clinical practice and disease prevention.

Methods
Data collection has been launched in September 2015 via an internet form. The questionnaire battery is anonymous and contains questions on demography, sleep hygiene and lifestyle as well as scaling questionnaires: MEQ (Morningness – Evenignness Questionnaire Self-Assessment Version), MCTQ (The Munich Chronotype Questionnaire) and SPAQ (Seasonal Pattern Assessment Questionnaire). In this communication we present the results of interim analyses. We used to Shapiro–Wilk test of normality and Pearson correlation for determining a relationship between age and the values in MEQ.
Results
Preliminarily, we can say that the distribution chronotype (MEQ score) in our sample (1071 respondents) corresponds with Gaussian curve (Graph 1). Using the Shapiro - Wilk test for normality data distribution further showed that both sexes divided into different age groups (18-25, 26-49, 50 and over) occupied a collected sample of individuals in terms of chronotype normal distribution (Graph 2).
It was found that there is a relationship between age and scores in MEQ (Pearson correlation), more precisely: the older the individual, the higher the MEQ score, i.e. the more morning chronotype (Tab. 1).

Graph 1: The number of respondents by MEQ score
1 and 5 stand extreme chronotype, 3 is intermediate type.

Graph 2: The distribution of respondents according to the MEQ score and their age.

Table 1: The resulting correlation between age and the MEQ score.

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<th>AGE</th>
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<td>MEQ score</td>
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Discussion
So far, no other similar work has been done in the Czech Republic. A previous Czech chronotype study focused on a narrowly defined group - university students. Similarly as in our sample, the distribution of chronotypes among university students according to MEQ data was very close to Gaussian distribution. International studies also report about Gaussian distribution of chronotypes. Moreover, our data revealed a positive association between age and morningness which is also in line with chronotype studies from other countries.

One of the objectives of the study was to determine whether people with an evening chronotype are more often obese and more likely to have diagnosed some of the metabolic and cardiovascular diseases. This will be subject of further data processing and assessing the relationship BMI score and the level of social jet lag, which is a strong indicator of the imbalance of biological and social rhythms. Therefore the further analyses will focus on the relationship between BMI score and the level of social jet-lag, which is a strong indicator of the imbalance of the biological clock and social rhythms. Furthermore, we intend to evaluate the association between MEQ score, social jet-lag and sensitivity to season. We hypothesize that MEQ and SPAQ scores will be related to the severity of social jet-lag and BMI.

Conclusions
The study builds on previous work that has been carried out in the Czech Republic and abroad. While also expanding the study chronotype by looking for connections between chronotype and health complications (primarily with obesity and metabolic disorders system). Understanding chronotypes, the associated individual habits and related health issues may help create targeted preventive and therapeutic strategies for diseases related to disorders of circadian rhythms.

Summary
Individual differences in circadian settings are called chronotypes (morning, evening, intermediate type). Different chronotypes are linked with differences at many levels and their distribution in general population approximates Gaussian curve. The aim of the ongoing project is to describe shuman chronotype in the context of psychosocial determinants of adult Czech population, which may have consequences for clinical practice and disease prevention. Data collection has been started in September 2015 via internet forms (a questionnaires battery: MEQ, MCTQ, SPAQ) and it is expected to be finished by the end of 2016. Preliminarily, we can say that the distribution chronotypes in our sample (1071 respondents) based on MEQ score is normal and corresponds to Gaussian curve. Moreover, our data showed that there is a correlation between age and scores in MEQ – higher age is associated with earlier chronotype (higher MEQ score).

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References


GASTRIC CARCINOMA WITH LYMPHOID STROMA IN THE ERA OF THE IMMUNE CONTEXT AND IMMUNOTHERAPIES

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Introduction and Aims
Gastric cancer (GC) represents a global health concern. Despite advances in prevention, diagnosis and treatment more than 900,000 GC cases are diagnosed per year, causing about 720,000. Molecular target agents have been recently approved, i.e. monoclonal antibodies against HER2 (Trastuzumab) and VEGFR2 (Ramucirumab). Still, these targeted therapies offer a survival advantage of only few months. Among biologic agents, novel cancer immunotherapies can selectively block the cancer evasion of immune surveillance. Phase II/III clinical trials with antibodies directed against CTLA-4 and PD-1/PD-L1 immune inhibitory checkpoints are currently ongoing in GC and are new attractive therapeutic strategies for GC patients [1,2,3,4]. Recently, The Cancer Genome Atlas (TCGA) network proposed a four-tiered molecular classification of GC [5], that could help to guide optimal selection of therapy. This classification defined Epstein-Barr virus (EBV)-positive, microsatellite instable (MSI-high), genomically stable (GS), and chromosomal instable (CIN) tumours. EBV+ and MSI-high GC are two molecular subtypes in which the PD-1/PD-L1 blockade may be particularly beneficial [5,6]. Moreover, a morphological subtype of GC has been associated to MSI-high status and EBV infection, that is Gastric Carcinoma with Lymphoid Stroma (GCLS) [7,8,9]. Hence, the abundant immune infiltrate and the molecular features of GCLS offer an attractive landscape to study tumour immune micro-environment, immune inhibitory checkpoints and their relationship with GC cells. The aim of this study was to analyse the clinico-pathological features, EBV infection, MSI, PD-L1 status and tumour immune microenvironment in GCLS.

Methods
Twenty-four GCLSs, selected from a series of 1088 surgically resected GC patients, were analysed by: RNA in situ hybridisation (EBER) for EBV, PCR/fragment analysis for MSI, and immunohistochemistry (IHC) for cytokeratin (CK) AE1/AE3, CD3, CD8 and PD-L1. PD-L1 immunoreactivity was evaluated separately for tumour immune and epithelial cells. The Immunoreactivity Scoring System (IRS) recently described by Boger C et al was applied [10]. Double immuno-fluorescence for CK/PD-L1 and CD68/PD-L1 was performed in selected cases. CD3+, CD8+ T cell densities and CD8/CD3 ratio (CD8/CD3R) were calculated both in the tumour centre (TC) and at the invasive front (IF) by digital analysis (Definines®). A tissue microarray (TMA) was constructed from a control group of 54 non-GCLSs and analysed by IHC for PD-L1 and by EBER.
Results

Figure 1 shows the morphologic characteristics (and diagnostic criteria) of GCLS. Regarding EBV and MSI status, 3 groups were identified: EBV+/microsatellite stable (MSS) (n=16), EBV-/MSI-high (n=4), and EBV-/MSS (n=4).

PD-L1 immunoreactivity was observed at the cell membrane of tumour epithelial cells (IRS>2) in 8/24 GCLSs (33.3%) and in immune stromal cells (≥1%) in 22/24 GCLSs (91.67%) (Fig. 2). IRS>2 was restricted to EBV+ (6/16; 37.5%) and MSI-high (2/4; 50%) GCLSs (Fig. 3).

Double immunofluorescence, performed in all cases with IRS≥7 (n=5), showed PD-L1 expression in both tumour epithelial (CK+) and stromal immune cells (CD68+). Membranous and strong PD-L1 expression was observed in tumour epithelial cells, whereas cytoplasmic, granular and dotted staining was observed in macrophages/monocytic cells.

Whole-Genome-Sequencing of one PD-L1+ case revealed PD-L1 amplification (the search for PD-L1 amplification in the remaining GCLSs is currently ongoing).

Overall, CD8/CD3R at the IF exceeded CD8/CD3R in the TC (p<0.001). CD8/CD3R was significantly higher (p=0.008) in EBV+ (n=16) than in EBV- (n=8) GCLSs. No significant association was found between CD8+ and CD3+ T cells counts/ratio and MSI status or PD-L1 expression.

By comparison with non-GCLSs, GCLSs were significantly associated with EBV infection (66.7% versus 5.56%, p<0.001) and PD-L1 protein expression (33.3% versus 13.0%, p=0.04). Moreover, GCLSs harbour distinctive clinico-pathological features: younger age (p=0.03), proximal location (p=0.04), indeterminate group of Lauren’s classification (p<0.001), lower lymphatic invasion (p=0.02), lower pTNM stage (p=0.002) and better overall survival (p=0.01).

Fig. 1 GCLS is a particular morphologic subtype of GC, characterised by an abundant lymphoid stroma that widely separates the tumour cells in the entire extent of tumour. Haematoxylin and eosin (H&E).

Fig. 2 PD-L1 expression in GCLS evaluated by IHC staining (clone E1L3N, CST®). Membranous, linear and strong PD-L1 expression was observed in tumour epithelial cells (arrows), whereas cytoplasmic dotted/granular pattern was observed in stromal immune cells (arrowheads). This GCLS case harboured PD-L1 amplification.
Discussion
This study focused on a particular morphological subset of GC, i.e. GCLS, and explored clinico-pathological, molecular characteristics and putative for immunotherapy. As already described in the literature, GCLS is more frequent in young patients, is localised in the proximal stomach, is frequently EBV infected and is associated, per se, to lower pTNM stages and better overall survival. Interestingly, as described in other studies \cite{7A,9}, none of the EBV+ GCLSs was MSI-H and vice-versa, suggesting that EBV positivity and MSI are distinct, and perhaps mutually exclusive pathogenic pathways of GCLS.
To the best of our knowledge, no further studies have been published, so far, addressing the tumour immune microenvironment and PD-L1 immune inhibitory checkpoint in GCLS. In this study, we described a higher CD8/CD3 ratio at IF than TC and in EBV+GCLS compared to EBV-GCLS. GCLSs harboured higher PD-L1 expression, compared to non-GCLS cases. Moreover, PD-L1 expression was restricted to EBV+ and MSI-high cases. All these findings suggest that GCLS is intimately associated to EBV+, MSI-high molecular subgroups and PD-L1 expression. Hence, this morphological subtype, easily recognised by pathologists on routine histological examination, deserves special attention as a predictive factor, to select patients for checkpoint blockade immunotherapies.

Conclusions
GCLSs are characterised by distinctive clinico-pathological features, EBV infection (66.7%), PD-L1 expression (33.3%) and high CD8/CD3R (at the IF and in EBV+ cases). In keeping with the recent molecular data, PD-L1 expression (IRS>2) was restricted to EBV+ and MSI-high cases, reinforcing the potential implications of immunotherapy in these molecular subtypes of GCLS.

Acknowledgements
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This study has been presented in abstract form at the 28th European Congress of Pathology and XXXI International Congress of the International Academy of Pathology, in Cologne, Germany, and was distinguished by the George Tiniakos Award for the best free-paper presentation in gastrointestinal, liver and pancreatobiliary pathology.
Summary

Introduction and aims: Novel cancer immunotherapies, such as PD-1/PD-L1 immune inhibitory checkpoint, can selectively block the cancer evasion of immune surveillance. EBV+ and MSI-high GC are two molecular subtypes in which the PD-1/PD-L1 blockade may be particularly beneficial. A morphological subtype of GC that has been associated to MSI-high status and EBV infection is Gastric Carcinoma with Lymphoid Stroma (GCLS). The aim of this study was to analyse the clinico-pathological features, EBV infection, MSI and PD-L1 status, and tumour immune microenvironment in GCLS.

Methods: Twenty-four GCLSs and a tissue microarray constructed from 54 non-GCLSs were analysed by RNA in situ hybridisation (EBER) for EBV and IHC for PD-L1. GCLSs were also analysed by PCR/fragment analysis for MSI, IHC for cytokeratin (CK) AE1/AE3, CD3 and CD8 and double immunofluorescence for CK/PD-L1 and CD68/PD-L1. CD3+, CD8+ T cell densities and CD8/CD3 ratio (CD8/CD3R) were calculated both in tumour centre (TC) and at invasive front (IF) by digital analysis.

Results: Regarding EBV and MSI status, 3 groups were identified: EBV+/MSS (n=16), EBV-/MSI-high (n=4), and EBV-/MSS (n=4). Overall, CD8/CD3R at the IF exceeded CD8/CD3R in the TC (p<0.001). CD8/CD3R was significantly higher in EBV+ than in EBV- GCLSs (p=0.008). PD-L1 expression in neoplastic cells was restricted to EBV+ and MSI-high GCLSs. By comparison with non-GCLSs, GCLSs were significantly associated with EBV infection (66.7% versus 5.56%, p<0.001) and PD-L1 protein expression (33.3% versus 13.0%, p=0.04). Moreover, GCLSs harbour distinctive clinico-pathological features.

Conclusions: GCLSs are characterized by EBV infection (66.7%), PD-L1 expression (33.3%) and high CD8/CD3R at the IF and in EBV+ cases. In keeping with the recent molecular data, PD-L1 expression (IRS>2) was restricted to EBV+ and MSI-high cases, reinforcing the potential implications of immunotherapy in these molecular subtypes of GCLS.

References


ESTABLISHMENT OF PERMANENTLY PRION INFECTED CELL LINES FOR STUDIES OF PRION STRAINS IN TISSUE CULTURES

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Introduction
Prion diseases, or transmissible spongiform encephalopathies (TSEs), represent a group of fatal neurodegenerative disorders that affect various mammals including humans. Pathology of prion diseases is closely associated with the conversion of normal cellular prion protein PrPC to its partially proteolytically resistant PrP \(^{\text{TSE}}\) isoform, and the prion infection is characterized by subsequent accumulation of PrP \(^{\text{TSE}}\) in the brain of affected individuals (1). At present, PrP \(^{\text{TSE}}\) is the only specific biochemical marker of human and animal TSEs. Diagnostic tests are mainly based on the detection of the resistant part of PrP (PrPres) after proteinase K (PK) digestion using various methods, such as Western blot or immunohistochemistry (2). Despite the fact that PrPC is encoded by the host genome, prions exist in many variants known as prion strains. Within the same species, phenotypically distinct diseases with biochemically distinguishable types of PrP \(^{\text{TSE}}\) can thus be observed (3). The pathogenesis of prion diseases is widely studied in animal models. However, the cost and complexity of \textit{in vivo} experiments led to the establishment of a few cell lines that are able to stably propagate infectious prion particles from diverse sources (4). Tissue cultures thus became useful experimental models for studying prion diseases, providing the opportunity to explore different biochemical aspects of these disorders in detail.

Aims
The aim of this study was to prepare new cell culture models for studies of prion strains originating from different hosts using available cell lines which are known to effectively propagate prions \textit{in vitro}.

Methods
Mouse neuronal cell lines CAD5 and PK1 susceptible for prion infection were incubated with brain homogenates (bh) of mice infected by different prion strains or with normal bh (CD1; negative control). Murine-adapted strains of scrapie (RML; positive control), variant Creutzfeldt-Jakob disease (mvCJD) and Gerstmann-Sträussler-Scheinker syndrome (Fu) were utilized for this study. Cells were maintained in culture for 15–20 passages after the infection and their ability to permanently propagate prions was continuously monitored by cell blot (5). Briefly, the cells were grown on plastic coverslips and after reaching the confluence, they were blotted directly onto the nitrocellulose membrane. PrPres-positive cells after PK treatment and denaturation with guanidine isothiocyanate were then identified by immunostaining using the anti-prion antibody. The presence of pathological PrP \(^{\text{TSE}}\) was evaluated by western blot after specific precipitation of PrP using sodium phosphothungstate (NaPTA) as described previously (6). The difference in PrP \(^{\text{TSE}}\) amount was assessed by conformation-dependent immunoassay (CDI) utilizing the dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) measurement and the anti-prion antibody recognizing the PrP \(^{\text{TSE}}\) epitope that is exposed only after its denaturation. The ratio between the signal of native (N) and denatured (D) samples giving the relative amount of PrP \(^{\text{TSE}}\) was determined for each infected cell line (7).
Results
As expected, the infection of cells with control RML prions led to the establishment of persistently infected cell lines in both CAD5 and PK1 cells, whereas cells incubated with CD1 remained uninfected. The detection of PrPres after PK treatment by both cell blot and Western blot confirmed that murine-adapted human prion strains mvCJD and Fu were both permanently propagated in CAD5 cells; the infection of CAD5 cells by murine-adapted cervid prions mCWD failed. Only Fu permanently infected PK-1 cells and PrPres after PK treatment was detected by cell blot and Western blot; mvCJD and mCWD prion strains were not propagated. In contrast to Western blot, conformation-dependent immunoassay could be used for PrP^TSE detection and for showing differences in the amount of PrP between various prion strains without the need of using PK treatment. The highest D/N ratio and thus relative amount of PrP^TSE was measured by CDI for RML-infected cells, whilst the D/N ratio was significantly lower for mvCJD- and Fu-infected cells and differed for CAD5 and PK1 cells.

Discussion
Novel CAD5-derived cell lines chronically infected by mvCJD and Fu became established and are suitable for further studies of these prion strains. Similarly, PK1-derived cell line propagating Fu prions was successfully developed. Further CDI measurements will be conducted to characterize these novel cell lines in more detailed manner. Our attempts to infect cells by murine-adapted cervid prions failed demonstrating peculiar propagation requirements of mCWD strain. Experiments heading towards the establishment of cell culture model to study mCWD are still in progress.

Conclusions
New CAD5/PK1-based model cell cultures for studies of mouse-adapted human prion strains mvCJD and Fu were established. mCWD prions were propagated neither in CAD5 nor in PK1 cells, further experiments are thus planned to investigate this strain and its requirements for efficient propagation in cell cultures.

Summary
Prion diseases (also called transmissible spongiform encephalopathies, TSEs) are neurodegenerative disorders characterized by accumulation of abnormally folded prion protein (PrP^TSE) in brain. These diseases can affect mammals including humans and are manifested phenotypically in many variants known as prion strains. The PrP^TSE molecule is partially proteolytically resistant, which could be used for its detection after proteinase K treatment by different methods. Traditionally, prions were studied in animals. However, a few cell lines able to effectively propagate prions in vitro recently became available and now represent a valuable tool to investigate prions. This study aimed to establish new cell lines permanently infected by prion strains originating from different hosts. We were able to derive CAD5 and PK1 cell lines stably infected by murine-adapted human prion strains. In contrast, murine-adapted cervid prions were not propagated in the selected cell lines.

Acknowledgements
CAD5 cells were donated by Charles Weissmann, PK1 cells by Peter-Christian Kloehn, RML by Adriano Aguzzi, Fu and mvCJD by Larisa Cervenakova. The study was supported by projects PRVOUK-P24/LF1/3 and SVV260260.
References


HISTONE H3 PHOSPHORYLATION AT SERINE 28 REGULATES LONGEVITY, HEART FUNCTION AND HEART MORPHOLOGY IN D. MELANOGASTER

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Introduction and aims
The structural units of chromatin, called nucleosomes, are composed of 146 bp of DNA wound around an octamer containing two copies of each of the highly conserved core histones (H2A, H2B, H3 and H4). Reversible modification of the N-terminal tails of specific core histones provides a major mechanism for regulating fundamental processes such as DNA replication and gene transcription. Of particular interest is the phosphorylation of histone H3 at serine 28 (H3S28) since it is considered a critical mediator of the transcriptional response to cellular stress1,2. Furthermore, it is strongly upregulated during mitosis3.

Our group recently observed hyper-phosphorylation of H3S28 both in cardiomyocytes of patients with end-stage heart failure and of animal models for cardiovascular disease (CVD; unpublished data). Given the central function of H3S28 phosphorylation and its upregulation in CVD, we intended to determine its impact on longevity, stress resistance, heart function and heart morphology. For this purpose, we used a D. melanogaster mutant ubiquitously expressing a histone H3 substituting serine 28 by alanine (H3S28A).

Material and Methods
D. melanogaster strains:
• H3wt: yw, hsp70-flp122; P{Ubi-GFP.D}33, P{Ubi-GFP.D}38, FRT40A; 6xHisGUH3wt
• H3S28A: yw, hsp70-flp122; P{Ubi-GFP.D}33, P{Ubi-GFP.D}38, FRT40A; 6xHisGUH3S28A

Since sole expression of H3S28A-mutated H3 is lethal, the endogenous H3 is preserved. All experiments were carried out with males.

Lifespan assays: Few hours after eclosion, 20-25 males were placed into vials containing standard medium. Flies were kept at room temperature (RT), deaths were scored daily.

Starvation assays: 7- to 10-day-old males were placed into vials (20-70 flies/vial) containing 1.3% low-melt agar with water. Flies were kept at RT, deaths were scored at least four times a day.

Oxidative stress assays: 7- to 10-day-old males were placed into vials (20-50 flies/vial) containing water with 1.3% low-melt agar, 5% sucrose and 15 mM paraquat. Flies were kept at RT, deaths were scored at least four times a day.

Optical coherence tomography (OCT): Following FlyNap® anesthesia, heart chambers of 2-day-old males were imaged for 5 sec at RT and, after a lead-time of 5 min, at 37°C. Heart rate (HR) and fractional shortening (FS, measured in the horizontal axis) were determined using custom made software.
Histological analysis: 7-day-old males were fixed in Telly’s fixation buffer (60% ethanol, 3.33% formalin, 4% glacial acetic acid) for at least 1 week at 4°C. Specimens were then dehydrated in sequential ethanol gradients and washed with Xylene before immersion in liquid paraffin. After solidification, blocks were sectioned in transverse orientation at 6 μm thickness. Sections were rehydrated and stained with hematoxylin and eosin. Heart dimensions were determined according to established criteria.

Statistical analysis:

- Survival and stress assays: Log-rank test
- HR and FS: Two-way ANOVA with Bonferroni post-test
- Heart muscle- and luminal area: Unpaired t test with Welch’s correction
- All data are presented as means±SEM; *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001

Results

Given the critical function of H3S28 phosphorylation in mediating the transcriptional response to cellular stress, we first determined whether the additional expression of H3S28A-mutated histones affects survival. Indeed, H3S28A mutants exhibited a substantially greater median lifespan than H3wt controls (~70%; Fig. 1A). In order to get an indication of potential physiological causes, we assessed resistances against starvation and paraquat-induced oxidative stress, two traits often correlated with increased longevity. Both were increased in H3S28A mutants (Fig. 1B,C), suggesting a critical role of H3S28 phosphorylation in regulating durability.

Fig. 1: H3S28A mutants exhibit a prolonged lifespan and an increased resistance to starvation and oxidative stress. (A) H3S28A mutants live 70% longer than H3wt controls (H3S28A n=96; H3wt n=56). (B) In H3S28A mutants, median survival during acute starvation is increased by ~17% (H3wt n=101; H3S28A n=152). (C) Exposed to 15 mM paraquat, H3S28A mutation enhances oxidative stress resistance by ~20% (H3wt n=70; H3S28A n=89). Each curve represents the average of at least 3 separate experiments.

In view of our preliminary data showing hyper-phosphorylation of H3S28 in CVD, we further assessed the impact of the H3S28A mutation on heart function (under basal and acute stress conditions) and morphology.

Heart function was determined using OCT, a laser based, non-invasive high-resolution imaging technique. Since HR is temperature-dependent in insects, increasing temperature to 37°C was chosen to induce acute cardiac stress. We found that H3S28A mutants exhibit significantly higher HR both at RT and at 37°C, while the stress response itself did not differ (Fig. 2A). Heartbeat regularity also increased with temperature, although, it behaved similar in both genotypes (data not shown). Another key parameter of cardiac function is the FS, which is defined as the percentage...
change in cardiac diameter during systole and therefore provides an indication of the contractility. At RT, the FS of both genotypes were equal, whereas at 37°C it increased more markedly in H3S28A flies (Fig. 2B). Together, these findings indicate a relationship between H3S28 phosphorylation and heart function.

**Fig. 2:** Heart rate (HR) and fractional shortening (FS) at room temperature (RT) and after thermal stimulation (37°C). (A) Both at RT and after five minutes at 37°C, H3S28A mutants have higher HR. (B) Thermal stimulation increases FS in H3S28A mutants more than in H3wt controls. (H3wt n=22, H3S28A n=24)

Heart function is directly related to its morphology. We therefore performed histological analyses to define luminal- and heart muscle area. The latter did not differ between genotypes (data not shown). Because H3S28A mutants showed significantly smaller luminal areas (Fig. 3A), the relative muscle area is substantially greater (Fig. 3B). Hence, mutation of H3S28 also affects heart morphology.

**Fig. 3:** H3S28A mutants exhibit (A) a smaller heart lumen and (B) a greater normalized heart muscle area compared to H3wt controls (H3wt n=11, H3S28A n=11). Heart dimensions were measured in two serial sections and the results were pooled.

**Discussion**

At least three pathways are known to mediate downstream effects of H3S28 phosphorylation: (1) binding of 14-3-3 proteins, a family of ubiquitously expressed adaptors mediating protein-protein interactions; (2) dissociation of histone deacetylases (HDACs); (3) prevention of the binding
of PRC2, a member of the transcriptional repressing Polycomb group proteins (PcG). As a common characteristic, these pathways largely lead to increased gene transcription. Since serine-to-alanine substitution prevents phosphorylation, it is commonly used to mimic the unphosphorylated state. Regarding the Polycomb system, however, the H3S28A mutation has been shown to also prevent PRC2 binding and is therefore considered phosphomimetic.

Given the fact that heterozygous mutation of several PRC2 subunits increases lifespan and resistance against starvation and oxidative stress in D. melanogaster, it is thus nearby that the extended durability observed in H3S28A mutants is due to impaired Polycomb function. Nonetheless, further research is needed to determine transcriptional consequences and the role of the other pathways.

H3S28A mutants exhibited an increased HR and a relative gain in heart muscle. These phenomena, at least in mammals, are both characteristic for heart failure. Therefore, the accelerated HR and the increased FS during cardiac stress could be deemed a compensatory reaction to the narrowed lumen. However, since mutants live significantly longer, it seems that these changes do not reach a level of pathophysiological relevance. Because chromatin remodeling regulates gene transcription, we are currently performing whole-genome expression studies of cardiac tissue to identify causative genes.

**Conclusion**

H3S28 phosphorylation regulates longevity and stress resistance in D. melanogaster. The importance of H3S28 phosphorylation to PcG regulation makes it highly likely that the increase in durability is due to an impairment of PcG-mediated gene silencing. Furthermore, we implicate H3S28 phosphorylation as a mechanism regulating heart function and morphology. Currently, we are defining causative mechanisms.

**Summary**

Phosphorylation of histone H3 at serine 28 is a highly dynamic chromatin modification involved in the regulation of fundamental cellular processes. Based on this fact and our preliminary data showing hyper-phosphorylation of H3S28 in cardiovascular disease, the present study aimed to determine the impact of H3S28 phosphorylation on longevity, stress resistance, heart function and morphology in vivo. Using a D. melanogaster mutant ubiquitously expressing a histone H3 substituting serine 28 by alanine (H3S28A), we showed that H3S28A mutation extends lifespan and increases resistance against starvation and oxidative stress, probably through inhibition of the Polycomb-mediated gene silencing. Concerning the heart, H3S28A mutants showed a generally accelerated beating frequency and a higher contractility after acute thermal stress. Future research will thus include the investigation of potential downstream signaling pathways.

**Acknowledgements**

This study was supported by the Else Kröner-Fresenius Foundation. D. melanogaster strains were a kind gift from Prof. Anne-Marie Martinez.

**References**


THE EFFECTS OF EARLY ENVIRONMENTAL ENRICHMENT AND PACAP ON MONOAMINE LEVELS IN AN AGING RAT MODEL OF PARKINSON’S DISEASE

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Introduction
The causative therapy of Parkinson’s disease (PD) is still under investigation. One of the well-studied effects of enriched environment and pituitary adenylate cyclase-activating polypeptide (PACAP) is the strong neuroprotective effect. Our research group has shown the protective effects of PACAP in animal models of Parkinson’s disease, Huntington chorea, retinal degeneration and traumatic brain injury. In vitro, we have proven that PACAP is protective against salsolinol-induced neuronal death, an in vitro model of Parkinson’s disease. Our earlier studies have proven that enriched environment is able to reverse some of the deleterious effects of glutamate toxicity on neurobehavioral development and protects the retina against excitotoxicity-induced degeneration. We have previously described the neuroprotective effects of PACAP and enriched environment in Parkinson’s disease in young animals. The aim of our present study is to investigate the protective effects of these factors in one-year-old rats after unilateral 6-OHDA-induced lesion of the substantia nigra, measuring the dopamine (DA) and serotonin (5-HT) levels in the brain.

Methods
Wistar rats were used in our experiment (n=15). Animals were divided into standard (n=7) and enriched groups (n=8) according to their environmental conditions. Animals of the standard group were placed under regular conditions. For environmental enrichment, during the first five postnatal weeks we placed pups in larger cages supplemented with toys, objects, running tunnels and rotating rods of different shape, size and material. Half of the toys were changed daily. In one-year-old animals PD was induced by unilateral injections of 6-OHDA (2 µl 6-OHDA (5 µg/µl)) into the left substantia nigra, control animals received 2 µl physiological saline to the same location. Following the 6-OHDA injections some of the standard-group-animals received 2 µl (1µg/µl) PACAP treatment. The right side of the animals always remained untreated, control side. On the 7th postoperative day the brain of the animals were collected and samples of the substantia nigra were taken with the help of brain matrix. Dopamine and serotonin levels were measured by HPLC-Q Exactive orbitrap MS system in control, 6-OHDA and 6-OHDA+PACAP injected rats of the standard and enriched environment.

Results
Physiological saline did not cause any significant decrease in DA levels in either of the animals. The substantia nigra of the 6-OHDA-treated standard and enriched animals showed significantly lower DA levels compared to the saline-treated animals of the same groups. Consistent with our previous studies in young animals the PACAP treatment could increase the DA levels with 15% after 6-OHDA induced lesion. The early environmental enrichment didn’t have any protective
effects in our present experiment. No significant differences could be observed regarding the serotonin levels of the substantia nigra.

Conclusions
Although the protective effect of early postnatal environmental enrichment is described in young animals, we could not prove it in our experiment on aging animals. However, similarly to younger animals PACAP could restore the decrease of DA levels, which could play a role in its neuroprotective effect in Parkinson’s disease.

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References


POST WHOLE-BRAIN-RADIOThERAPY COGNITIVE IMPAIRMENT AND HIPPOCAMPAL NEURONAL DEPLETION MEASURED BY IN-VIVO METABOLIC MR SPECTROSCOPY

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Co-authors: P. Pospisil, R. Janc alek, P. Slampa

Tutor: Radim Janc alek

Introduction
Despite the incredible advances in cancer treatment during last decades, patients with brain metastases (BM) have dismal prognosis and are treated with palliative intent. Radiotherapy has been the mainstay of their treatment for decades. Although the role of whole brain radiotherapy (WBRT) is in flux, WBRT continues to be an important tool in the treatment for patients with multiple BM, in prophylactic indications and in the salvage treatment after initial local therapy (Wen, 2015; Kazda, 2013). WBRT is, however, associated with adverse side effects leading to possible worsening of the quality of life, particularly in relation to the worsening of neurocognitive function, peaking in its acute phase 4 months after irradiation (Wen, 2015). Several strategies for preservation of memory function in patients with BM are studied, including investigation on sparing hippocampi during WBRT (Dye, 2015; Gondi, 2014; Kazda, 2014; Kazda, 2015). Radiation injury of the hippocampus is known to alter learning and memory formation, however, complete pathophysiologic explanation of all these processes is still lacking (Monje, 2002). Objective biomarkers of radiation injury can help elucidate this mechanism. Of the imaging biomarkers currently under investigation, magnetic resonance spectroscopy (MRS) is unique in that biologic correlation of sub-cellular molecules can be correlated with volumetric imaging. N-acetylaspartate (NAA) spectra are known as a marker of neuronal density and viability (Moffett 2007). We hypothesized that neuronal cell loss within the region of the hippocampus after WBRT would be measureable by changes in NAA concentration by MRS.

Aim
The aim of this prospective study is to evaluate post-WBRT changes in hippocampal concentration of NAA (h-NAA) as a marker of neuronal loss and to correlate those changes to neurocognitive function (NCF).

Methods
Patients referred to WBRT for newly diagnosed BM at our institution between May 2013 and February 2015 were screened for eligibility (Karnofsky performance status ≥ 70% and a favorable survival prognosis of more than 3.8 months as predicted by the graded prognostic assessment score (Kazda, 2015)). All enrolled patients underwent pre-WBRT hippocampal MRS and NCF testing. The examinations were repeated 4 months after completion of WBRT and changes of the h-NAA and memory were examined and correlated (Pospisil 2015).

External beam WBRT was delivered by standard two opposing, lateral fields defined employing an RTG 2D simulator (Varian Acuity iX). The prescribed dose 30 Gy in 10 fractions delivered in 2 weeks with 6 megavoltage photon beams of linear accelerator was uniform in all patients.
Single slice multi-voxel spectroscopic examination was performed using GE Medical Systems Discovery MR 750 3T (PRESS-CSI sequence with TE/TR = 135 ms/1690 ms, 12 averages, FOV 120 x 120 mm²) with region of interest placed through the whole bilateral temporal lobes with the voxel layer position adjusted based on the localization of hippocampi (voxel size set to 10 x 10 x 15 mm³). Raw spectroscopic data from both hippocampi were postprocessed for the calculation of the h-NAA absolute concentration [mM], see example at figure 1.

Brief quantitative measurement of cognitive status by Mini Mental State Examination (MMSE) as well as standardized tests focusing on memory were assessed by experienced psychologists: AVLT (Auditory Verbal Learning Test) and BVMT-R (Brief Visuospatial Memory Test - Revised). The AVLT includes memorizing 15 words for five consecutive attempts (Total Recall, TR), recalling them after 30 min (Delayed Recall, DR) and subsequently identifying these words from a list of related words (Recognition, R). The BVMT-R includes memorizing six geometric figures for three consecutive attempts (TR) and similarly as with the AVLT recalling them after 25 min (DR), and finally identifying them among those offered in the list of related figures (R). Quality of life was evaluated by official Czech translation of EORTC QLQ-C30 and EORTC QLQ-BN20 questionnaires.

Due to the exploratory nature of this study, no predetermined values for h-NAA at baseline or as a function of time were defined. Because normative standards were not known, each patient served as his or her own control for both analyses of MRS concentrations as well as cognitive testing.

Results
Eighty-four out of 185 screened patients met eligibility criteria; however, only 35 patients (42% of those eligible) agreed with participation, were enrolled and underwent baseline MRS and NCF testing. Eighteen patients (51% of those enrolled) underwent control examination after 4 months. Their basic characteristics are summarized in table 1.

On average, 9 voxels were analyzed per right and left hippocampus. Post-WBRT decreases in h-NAA are shown in table 2. The statistically significant decrease of h-NAA was observed in right (-12.9%) as well as in left (-12.0%) hippocampus with the median intrapatient standard deviation of ± 1.6 mM.

Statistically significant decline was observed in all AVLT and BVMT-R subtests with exception of AVLT_R. Mean absolute decrease in individual subtests are summarized also in table 2. Patients exhibited significant post-WBRT decline in AVLT_DR test with mean decrease from 7.4 to 5.6 points (p=˂0.01) while no significant change was observed in MMSE.

Positive moderate correlation was seen between left h-NAA decline and AVLT_TR (r=+0.32; p=0.24) as well as AVLT_DR (r=+0.33; p=0.22) declines but the results were not statistically significant with our sample size.

The overall subjective quality of life (questionnaire) declined after WBRT (mean Δ -14.1 ± 20.3 points in transformed 0 to 100 pointed scale; p=0.018). Cognitive function subscale declined from a mean 86.5 to 80.2 points (p=0.059) but the decline was not significant and no correlation to MRS changes was observed.

Discussion
In this study, we demonstrate a decline in left as well as right h-NAA concentration after WBRT. We also found declines in cognitive function as measured by AVLT_TR and AVLT_DR, which appear to correlate with declines in left hippocampal h-NAA changes. To the best of our
knowledge, this is the first work to describe correlation between NCF and in-vivo measured hippocampal metabolic changes after brain irradiation.

In contrast to Alzheimer disease, the experience with hippocampal MRS measurements after radiotherapy is very limited. Overall, the results of our study are consistent with previously published studies of hippocampal injury. Our results suggest hippocampal MRS is feasible and sensitive, non-invasive method for in-vivo description of post-WBRT metabolic changes. Despite the limitations, this study suggests MRS may have utility as an imaging biomarker of hippocampal neuronal loss after radiotherapy. Future directions and confirmation of these results will require studies evaluating the utility of MRS to detect differences in changes of h-NAA or other metabolites when hippocampal protection strategies are employed (Kazda, 2014; Pospisil, 2015).

Conclusion
Hippocampal N-acetylaspartate concentration decrease 4 months following whole brain radiotherapy. Correlations to neurocognitive function warrants further research. Hippocampal MR spectroscopy is feasible and sensitive method for non-invasive measurement of radiotherapy injury and hippocampal NAA concentrations decline after WBRT and MRS may be a useful imaging biomarker for monitoring neuronal loss after radiotherapy.

Summary
In this prospective study, we described post whole-brain-radiotherapy decrease of hippocampal concentration of N-acetyl aspartate, the neuronal marker, and observed some correlations to neurocognitive deficits after brain irradiation. These results add to the knowledge about treatment-related cognitive decline and enhance trends toward patient tailored personalized care in palliative radiotherapy of brain metastases.

Figure 1: example from java Spectroscopic Imaging PROcessing software (jSIPRO) for analysis of hippocampal MRS. It is possible to select each voxel within hippocampi and record pertinent values of metabolite (Cho – choline; Cr – creatine; NAA – N-acetyl aspartate) concentration.
<table>
<thead>
<tr>
<th></th>
<th>All enrolled patients n=35</th>
<th>Analyzable patients n=18</th>
</tr>
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<tbody>
<tr>
<td>Age mean ± SD</td>
<td>59.5±8.3</td>
<td>57.8±8.1</td>
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<tr>
<td>Sex Male (%)</td>
<td>68</td>
<td>63</td>
</tr>
<tr>
<td>KPS Median (range)</td>
<td>90 (70-100)</td>
<td>90 (80-90)</td>
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<tr>
<td>Location F/T/O/P/Crbl (%)</td>
<td>30/10/14/28/18</td>
<td>31/6/15/26/22</td>
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<tr>
<td>Primary diagnosis NSCLC (%)</td>
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<td>20</td>
</tr>
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<td>SCLC (%)</td>
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<td>6</td>
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<tr>
<td>Breast (%)</td>
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<td>RCC (%)</td>
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<tr>
<td>Melanoma (%)</td>
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</tr>
<tr>
<td>Other (%)</td>
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<td>6</td>
</tr>
<tr>
<td>Pre-WBRT surgery Yes (%)</td>
<td>32</td>
<td>44</td>
</tr>
<tr>
<td>Post-WBRT chemotherapy Yes (%)</td>
<td>41</td>
<td>75</td>
</tr>
<tr>
<td>Overall survival Median (95% CI)</td>
<td>7.48 (3.02-9.48)</td>
<td>9.80 (7.57 - NR)</td>
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**Table 1**: Patients basic clinical and survival characteristics
Table 2: Post-WBRT changes in the concentrations of h-NAA, in right hippocampus, left hippocampus and in bilateral hippocampi. P value is derived from Wilcoxon’s signed rank test. Abbreviations: WBRT, whole brain radiotherapy; CI, confidence interval; h-NAA, hippocampal N-acetylaspartate concentration; RH, right hippocampus; LH, left hippocampus; BH, bilateral hippocampi; AVLT, Auditory Verbal Learning Test; TR, total recall; DR, delayed recall; R, recognition; BVMT-R, Brief Visuospatial Memory Test – Revised; MMSE, Mini Mental State Examination.

<table>
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<tr>
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<th>Pre-WBRT N=18</th>
<th>Post-WBRT N=18</th>
<th>Absolute mean difference</th>
<th>p-value</th>
<th>Relative mean difference [%]</th>
<th>p-value</th>
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<tr>
<td>h-NAA</td>
<td>[mM]</td>
<td>[mM]</td>
<td>(95% CI)</td>
<td>p-value</td>
<td>(95% CI)</td>
<td>p-value</td>
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<td>RH</td>
<td>8.52</td>
<td>7.42</td>
<td>-1.1 (-0.66 to -1.54)</td>
<td>&lt;0.01*</td>
<td>-12.9 (-7.6 to -16.4)</td>
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<td>LH</td>
<td>8.64</td>
<td>7.60</td>
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<td>-12.0 (-7.9 to -16.2)</td>
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<td>7.46</td>
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<td>TR</td>
<td>42.8</td>
<td>34.1</td>
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<td>DR</td>
<td>7.4</td>
<td>5.6</td>
<td>-1.8 (-0.9 to -2.7)</td>
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<td>R</td>
<td>12.1</td>
<td>12.3</td>
<td>0.2 (1.1 to -0.86)</td>
<td>0.7</td>
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<tr>
<td>DR</td>
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<td>MMSE</td>
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<tr>
<td>score</td>
<td>28.9</td>
<td>28.7</td>
<td>-0.2 (0.26 to -0.5)</td>
<td>0.7</td>
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References


GENETIC MARKERS OF EXFOLIATION SYNDROME IN GEORGIAN POPULATION

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Purpose
The aim of this study was to identify susceptibility genes/loci for Exfoliation Syndrome and Exfoliation Glaucoma by a case control cohort study approach in Georgian population.

Materials and Methods
Eighty-three patients aged over 50 with XFS and 103 normal subjects over 60, having no clinical evidence of exfoliation material in anterior segment structures of the eye were included in this study. After signing an informed consent patients underwent venous blood sampling. Case control association study was performed by two-stage Genome-wide association study (GWAS). The genetic regions identified to harbor XFS susceptibility genes, were subsequently genotyped for the 750,000 Single Nucleotide Polymorphisms (SNPs) on Illumina chips in unrelated cases of exfoliation syndrome and controls. For replication, the Sequenom MassArray platform was used to test SNPs that were highly associated with the disease ($P < 1 \times 10^{-5}$).

Results
Principal component analysis of the Georgian XFS cases and controls showed the cases and controls to be well-matched genetically with minimal evidence of population stratification. Furthermore, the genome-wide data showed the Georgian XFS case-control collection to have an ancestry distinct from the other Northern, Western, Eastern, and Southern European collections. In Georgian, the frequency of LOXL1 rs3825942 was 95.8% in exfoliation cases and 84% in controls, $P = 0.0001$, OR=4.65. The frequency of CACNA1A rs4926244 was 20% in XFS patients and 17% in controls, OR =1.19 ($P = 0.4$ in Georgia and $P < 10^{-10}$ for global meta-analysis). The genetic effect sizes observed by us in Georgia were consistent with the data found in previously published studies for LOXL1 and CACNA1A.

Discussion
Exfoliation syndrome is an age-related disorder characterized by excessive production and progressive accumulation of extracellular fibrillar material in different structures of the eye. XFS is the most common identifiable cause of open-angle glaucoma and a very frequent cause of serious complications during cataract surgery (Ritch R, 2003). XFS patients are found to have three high-risk SNPs of LOXL1 gene on chromosome 15: rs2165341, rs10488661 and rs3825942 (Thorleifsson, G. 2007). Lysil oxidase is a member of copper-dependent enzymes, which plays role in genesis, stabilization and remodeling of elastic fibers. (Liu et al., 2004; Oleggini, 2007). It catalasates deamination of lysil remnants of tropoelastin, which is the most important reaction in elastogenesis (Ursula Schlötzer-Schrehardt et al., 2012). LOXL1 mutation causes overproduction of fibrillar material and its deposition in the anterior segment structures of the eye. SNP rs4926244 of the CACNA1A gene has also recently been linked to development of XFS. It encodes @ subunit of voltage dependent type P/Q calcium channels (Aung et al., 2015). These channels are involved in transmembrane transport of calcium ions.
and therefore in generation and transmission of electric signals. It was found that exfoliation material contains high concentrations of calcium (U Schlötzer-Schrehardt, 2001). It is also well known that fibrillin uses calcium for making stable aggregates. Interestingly, different SNPs have causative role in different populations. In Georgian population LOXL1 rs3825942 and CACNA1A rs4926244 are associated with high risk of disease development. The fact, that risk alleles are often “flipped”, implies that genetic architecture of XFS is very complex and needs further clarification.

Conclusion
SNPs of the LOXL1 and CACNA1A genes are associated with XFS in the Georgian population. Our data are consistent with previously reported findings in European and Asian populations. Although, further studies are needed to provide more insight into pathogenesis of the disease.

References


Introduction
Systemic lupus erythematosus (SLE) is a severe autoimmune disorder, characterized by unpredictable exacerbations and remissions. Clinical manifestations are variable from skin changes, arthralgia to internal organ involvement, most notably renal and central nervous system. Lupus nephritis (LN) develops in ~50% SLE patients, representing the main cause of morbidity and mortality (Kosalka et al, 2016). LN results from the activity of multiple biologic factors such as autoantibodies, inflammatory cells, growth factors, chemokines, cytokines, reactive oxygen and nitrogen species (Kaczmarczyk et al, 2014). Nevertheless the exact mechanism remains unclear. However, recent limited numbers of studies indicate a central role for the IL-23/IL-17 axis in the pathogenesis of LN (Zickert et al, 2015).

Aims
Renal involvement is the most severe manifestation of systemic lupus erythematosus. However assessing the inflammatory response in kidneys using non-invasive methods is still challenging. Thus the aim of this study was to define markers of active lupus nephritis (LN) using urine immune profiling.

Methods
Levels of cytokines (18-plex array), including the IL-23/IL-17 axis, and mRNA (40 immune and glomerular injury genes) were measured in urine samples of LN patients with active disease (n=17), during remission (n=16), and in healthy subjects (n=19). mRNA was quantified with a pre-designed Low-Density Array on 7900HT Real Time PCR platform. Relative quantities (RQ) of individual transcripts were calculated using 2-ΔΔCT method (with GAPDH-normalized median values). Urine levels of cytokines were assessed with pre-designed set of Procarta Luminex Immunoassays according to recommended protocol, using Magpix detection platform and xPonent software. Data were statistically analysed using GraphPad Prism 5.0.

Results
Urine levels of CCL2, CCL5, CXCL10 and IL-6 were elevated in active LN as compared to remission (best discrimination for CCL2), and correlated with LN activity [Fig. 1]. In the active disease, urinary cell transcriptome showed strong upregulation of proinflammatory cytokines (i.a. TNF, CCL2, CCL5, CXCL10), Th1 related genes (e.g. CD3G, CD4, TBX21, IFNG), and markers of glomerular damage (NPHS2 [podocin]) [Fig. 2]. Active pattern of gene expression was also observed in 5 patients in remission, who had moderately increased urinary leukocyte count, two patients from this group (40%) developed renal exacerbation during following 3 months. Markers of Th17 immune axis (e.g. IL-17A) were not significantly increased in active LN.
Figure 1. Urine levels of cytokines in LN patients. (A) Increased levels of CXCL10, CCL2, IL-6 and CCL5 in active-LN as compared to inactive (*P<0.05, **<0.01). (B) Urine levels of CCL2 and CXCL10 in LN and controls (*P<0.05, **<0.01).

Figure 2. Urine sediment cell transcriptome. Venn diagram representing the genes upregulated (red) or down regulated (black) in urine sediment cells from LN patients as compared to healthy controls. Only transcripts with >2-fold change in expression (and \(P<0.05\)) are shown. Normal letters - >2 fold increase in comparison to controls (\(P<0.05\)), bold letters - >2-fold decrease in comparison to controls (\(P<0.05\)).

Discussion
Most of the previously published analyses were performed in SLE patients with broad variety manifestations of the disease. It is known, that lupus nephritis is highly associated with a presence of anti-dsDNA antibodies produced in the course of autoimmune process. It seems to be necessary to check whether dysregulation of T regulatory and T effector cells is specific only in the pathogenesis...
of kidney involvement in the course of SLE. It is suggested that some cytokines play a crucial role in the pathogenesis of lupus nephritis, however those data are ambiguous. Recently published data (Zickert et al, 2015; Li et al, 2010) highlight main role of cytokines associated with the IL-23/IL-17 axis. Others (Esposito et al, 2009) observed increased level IL-6, what was also observed in our project. Surprisingly, our results showed that markers of Th17 immune axis (e.g. IL-17A) were not significantly increased in active LN. Some cytokines (e.g. CCL2) and chemokines (e.g. CXCL10) were elevated in the kidney exacerbation of SLE and correlated with an activity of the disease (Zhang X et al, 2012; Marie et al, 2014). Also in our results CCL2 and CXCL10 were best parameters correlating with SLEDAI (SLE disease activity index). Two from five (40%) patients with inactive LN, who displayed gene expression pattern similar to that of active disease, developed renal exacerbation during following 3 months; however these observed findings require further analysis among larger group of patients.

Conclusions
Active LN patients (also patients at risk of exacerbation) were characterized by marked increase of proinflammatory mediators and active profile of gene expression in the urine. We identified CCL2 and CXCL10 as most promising markers for monitoring of renal flare.

Summary
Systemic lupus erythematosus (SLE) is an autoimmune disorder, which etiology is still poorly understood. One of the most severe manifestations is kidney involvement representing the main cause of morbidity and mortality. It was proved that some cytokines and chemokines can play a crucial role in pathogenesis of the disease. We identified CCL2 and CXCL10 as most promising markers for monitoring of renal flare. Moreover 40% of inactive LN patients displayed gene expression pattern similar to that of active disease and developed renal exacerbation during following 3 months.

References
MITOCHONDRIAL PATHOGENESIS OF PROPOFOL INFUSION SYNDROME IN AN IN VITRO MODEL OF HUMAN SKELETAL MUSCLE

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Introduction
Propofol infusion syndrome is a rare, but serious adverse effect of a commonly used drug with a very high mortality rate (>50%) [1]. Symptoms can occur in various combinations and include: unexplained metabolic acidosis, arrhythmia, Brugada like pattern on electrocardiograph (elevated ST-segment and coved-T wave), cardiac and/or renal failure, rhabdomyolysis, hyperkalaemia, hepatomegaly and hyperlipidaemia. The mechanism of the syndrome is still unknown: experimental studies performed on animal models and clinical features of the syndrome are suggestive of its mitochondrial origin.

Aims
We hypothesize that propofol could decrease respiratory chain capacity, inhibit fatty acid oxidation and induce inner mitochondrial membrane uncoupling in a dose-dependent manner. Our study aims to test this hypothesis in vitro by exposing human skeletal muscle-derived cells to a range of propofol concentrations for 4 days.

Methods
Skeletal muscle cells were isolated from biopsies obtained from patients (n=16) undergoing hip replacement surgery and subsequently exposed to a range of propofol resembling clinical concentrations in human plasma during propofol infusion (0, 1, 2.5, 5 a 10 µg/ml) and to lipid vehicle (Intralipid® - IL). After 96 hours of exposure, mitochondrial metabolism was assessed by extracellular flux analysis (Seahorse Biosciences). Oxygen consumption rate (OCR) was measured at baseline and after addition of ATPase inhibitor, mitochondrial uncoupler and complex III inhibitor. Injection of these agents enables to calculate baseline OCR, ATP turnover rate, proton leak through inner mitochondrial membrane and respiratory chain capacity (uncoupled respiration). The capacity of fatty acid oxidation was measured as etomoxir-inhibitable OCR after adding of uncoupler and palmitate. Values presented in Table are expressed as % of baseline OCR.

Results
In human skeletal muscle cells exposed to propofol, respiratory chain capacity was decreased and uncoupling of inner mitochondrial membrane was increased. The most significant result was propofol-induced inhibition of fatty acid oxidation to 15%, respectively 11% of baseline values.
The influence of propofol on mitochondrial metabolism. Data are presented as median (interquartile range). Statistically significant results are signed as * if p-value < 0.05, ** p-value < 0.001.

<table>
<thead>
<tr>
<th>Propofol concentration [μg/ml]</th>
<th>0</th>
<th>1.0</th>
<th>2.5</th>
<th>5.0</th>
<th>10.0</th>
<th>Intralipid control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal OCR [pmol/min]</td>
<td>114 (91.5-181)</td>
<td>89 (52-159)</td>
<td>112 (87-155)</td>
<td>104 (58-179)</td>
<td>123 (63-207)</td>
<td>100 (73-124)</td>
</tr>
<tr>
<td>ATP turnover (% OCR)</td>
<td>77 (64-84)</td>
<td>74* (56-83)</td>
<td>76* (47.1-84)</td>
<td>65* (56-84)</td>
<td>80 (72-88)</td>
<td>74 (60-87)</td>
</tr>
<tr>
<td>Maximal respiratory capacity (% OCR)</td>
<td>311 (251-383)</td>
<td>190* (44.3-375)</td>
<td>175*** (78.8-290)</td>
<td>260** (150-305)</td>
<td>244 (196-358)</td>
<td>370 (293-433)</td>
</tr>
<tr>
<td>Fatty acid oxidation [pmol/min]</td>
<td>75 (40-134)</td>
<td>-</td>
<td>11** (2-34)</td>
<td>-</td>
<td>8** (1-27)</td>
<td>-</td>
</tr>
</tbody>
</table>

Table: The influence of propofol on mitochondrial metabolism. Data are presented as median (interquartile range). Statistically significant results are signed as * if p-value < 0.05, ** p-value < 0.001.

Discussion
The relation of these in vitro changes and clinical signs are yet to be proven. Propofol-induced changes in mitochondrial function can be caused by various mechanisms - inhibition linked with any of the complexes of respiratory chain or defect in electron transport between complexes. Mechanism of its molecular basis needs further investigation.

Conclusions
In clinically relevant concentrations, propofol is a potent inhibitor of fatty acid oxidation and induces changes in function of respiratory chain in an in vitro model of human skeletal muscle.

Grant acknowledgement: The work was supported by grants GAUK 270915 and PRVOUK P31.

Reference
EXPERIMENTAL TREATMENT OF THE SPINAL CORD INJURY 
BY HUMAN MESENCHYMAL STEM CELLS DERIVED 
FROM WHARTON´S JELLY (hWJ-MSC)

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Introduction

Spinal cord injury (SCI) remains one of the most physically, psychologically and socially 
debilitating medicine problems in the world. So far there is no specific or effective primary 
treatment yet available. Injury to the spinal cord results in motor and sensitive deficit, which 
severity depends on mechanism of the injury (primary damage) and on endogenous processes 
following the direct trauma in hours and days (secondary damage). While direct trauma can be 
hardly affected, modulation of the local inflammatory response, scavenging reaction 
and production of growth factors can result in better regeneration of the tissue.

In recent decade a variety of stem cells were used to affect the process of cell apoptosis, 
demyelination and axonal degeneration. Mesenchymal stem cells (MSCs) are a type of multipotent 
progenitor cells that can be differentiated into several cell types of mesodermal origin including 
osteocytes or cardiomyocytes. Major sources of MSCs are bone marrow and umbilical blood, but 
they are also found in adipose tissue or Wharton’s Jelly surrounding the umbilical vessels [1]. 
Their beneficial effect in spinal cord injury treatment is due to their ability to secrete trophic factors, 
which can affect secondary processes occurring after SCI, promoting axon regeneration, 
angiogenesis and reduce inflammatory cell activation and glial scar formation.[2]

Aims

The aim of the study was to determine, whether the human mesenchymal stem cells derived from 
Wharton’s Jelly could improve the functional outcome of the rat with ischemic-compression spinal cord 
injury. Secondary purpose was to evaluate, if the treatment is dose – responsive – e.g. if the repetition or 
higher doses of transplanted stem cells would result in better recovery.

Methods

Experiment was performed on 10-weeks-old male Wistar rats with the body weight 300g +/−15g. 
Ballon-induced spinal cord injury model was utilized as a ischemic-compression model of SCI. 
Rats were divided into five groups. Group A (single treatment of 0.5mil hWJ-MSC), Group B 
(single treatment of 1.5mil hWJ-MSC), Group C (repeated treatment of 0.5mil hWJ-MSC), Group 
D (repeated treatment of 1.5mil hWJ-MSC) and Group E (control group receiving saline). Seven 
days after SCI, hWJ-MSC (5 x10^5 or 1.5 x10^6/ 50 ml saline) were injected by the lumbar puncture 
into the subdural space through the L5-L6 intervertebral space. Groups with repeated treatment 
received another injection 14 and 21 days after SCI. Control rats were injected with 50ml of saline 
into the L5-L6 interspinous space.
Through the experiment the behavioural assessment was regularly performed. Locomotor function was tested by Basso, Beattie, and Bresnahan (BBB) open field test, flat-beam test and rotarod test every week after lesion.

Eight weeks after spinal cord injury all rats were perfused with 4% paraformaldehyde in PBS and their spinal cords were removed, embedded in paraffin and cut into cross sections. Samples were stained with Cresyl violet Luxol to visualise white and grey matter, with GAP43 to analyse axonal sprouting and with GFAP to recognize the glial scar.

Results

1. **Behavioural testing:**
   Group with single treatment of 1.5 mil hWJ-MSC and groups with repeated treatment of 3x0.5mil or 3x1.5mil hWJ-MSC recovered significantly better than control (two-way RM ANOVA, Treatment p<0.05). Treatment with single dose of 0.5 mil hWJ-MSC didn’t show any significant difference compared to control. Testing of advance motor function and limb coordination by flat beam test and rotarod test showed no or minimal differences between the treated groups and control.

2. **Histological assessment:**
   Grey and white matter sparing was measured as a remained tissue on cross section areas of the treated animals compared to the control group. Significant difference in preserved grey matter achieved only group with 3x1.5 mil hWJ-MSC (two-way RM ANOVA, Treatment p<0.05). No significant difference in preserving white matter was observed. Glial scar around the main cavity was measured and counted as a percentage of whole cross section tissue. Astrogliosis was significantly lower in higher single dose – 1x1.5mil hWJ-MSC and in both repeated doses – 3x0.5mil and 3x1.5mil hWJ-MSC. Axonal sprouting was counted as a number of fibers in GAP43 staining. There was significant higher number of GAP43+ fibers in all treated groups with exception of 1x0.5mil hWJ-MSC (one-way ANOVA, Treatment p<0.05)

Conclusions/Summary

In the presented experimental study we concluded, that treatment by single dose of 0.5 mil hWJ-MSC (group A) had no or minimal benefit on recovery from the spinal cord injury in rat model. We also proved that higher doses and repeated treatment (group B,C,D) of hWJ-MSC significantly improved overall functional outcome. Though there was no difference between the groups B,C and D in behavioural testing, group D had significantly higher number of GAP43+ fibers and had significantly larger area of spared grey matter in the centre of the lesion. These findings can lead to hypothesis that in long term recovery (> 9 weeks) rats in group D would have higher potential to improve than rats in group B and C.

References


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Introduction and Aims

Although the sepsis syndrome has been known since the ancient times it still remains a challenging healthcare problem nowadays. To improve disease outcome, early goal-directed therapy is necessary therefore prompt diagnosis is of utmost importance [1]. Laboratory parameters may be extremely useful in rapid clinical decision making and also in monitoring of sepsis. As an acute phase protein the serum levels of orosomucoid (ORM) can increase up to two-threefold during systemic inflammation [2]. Many studies investigated serum ORM but only scarce data are available on urinary ORM (u-ORM), however, ORM can also be found in the urine, as well. A few former studies reported slight elevation of u-ORM levels in diseases associated with chronic inflammatory activation. In spite of promising data, automated u-ORM assay is unavailable yet. For daily clinical utilization we developed and validated an immune turbidimetric assay for u-ORM measurements on a fully automated clinical chemistry analyzer. Our further aim was to determine reference range for u-ORM in healthy individuals. Furthermore our goal was to monitor u-ORM levels in critically ill patients hypothesizing that u-ORM is a useful marker of sepsis. We investigated the diagnostic and prognostic ability of u-ORM, too.

Methods

A particle-enhanced turbidimetric assay was validated for Cobas 8000/c502 analyzer to measure u-ORM levels by using orosomucoid immunoparticles (Dako A/S, Glostrup, Denmark). In order to determine a reference range for u-ORM, healthy volunteers (n=72) were recruited between the age of 10 and 60 years. We monitored u-ORM levels in patients with systemic inflammatory response syndrome (SIRS, n=13) and in septic patients (n=39) from our intensive care unit (ICU). To exclude the influence of comorbidities on u-ORM concentrations, a group of volunteers (n=30) served as matched-control group to ICU patients (similar in age, sex and medical history). Our study was approved by the Regional Ethics Committee of the University of Pécs, (no. 4327.316-2900/KK15/2011). Our patients were fully informed and written consent was obtained from all. Spontaneous random urine samples and venous blood were simultaneously obtained from the participants. To avoid the influence of urine volume, u-ORM concentrations were referred to urinary creatinine (u-CREAT) and expressed as u-ORM/u-CREAT (mg/mmol). Statistical analyses were performed by IBM SPSS Statistics for Windows, Version 22. For comparison of our groups Kruskal-Wallis or Mann-Whitney U tests were carried out. Receiver operating characteristic (ROC) curves were used for analyzing the diagnostic and predictive values. Our data are presented as median and interquartiles.

Results

Our new turbidimetric approach was set up to be fast (10 minutes) and sensitive with a detection limit of 0.02 mg/L. The working range of our assay was 0.16-5.25 mg/L. The intra- and inter-assay
imprecision as CV%, and also the inaccuracy were <5%. Within 10 to 60 years, a reference range for u-ORM/u-CREAT was found to be 0.08 (0.05-0.15) mg/mmol. Compared to these data a slightly (p<0.01) increased u-ORM/u-CREAT was found in the volunteers with comorbidities 0.2 (0.1-0.3) mg/mmol. On ICU admission, both SIRS and septic patient groups showed strongly elevated u-ORM/u-CREAT values compared to matched-controls (p<0.001). Furthermore, significantly higher (p<0.001) u-ORM/u-CREAT levels were found in sepsis than in SIRS (Table 1). The area under the ROC for distinguishing SIRS from sepsis was found to be 0.954 for u-ORM/u-CREAT, superior to se-ORM and hs-CRP (Figure 1). A cut off value for u-ORM/u-CREAT with 94.7% sensitivity and 90% specificity could be set at 6.75 mg/mmol to discriminate SIRS from sepsis. U-ORM/u-CREAT levels did not change during the 5-day follow-up period. We found no differences between the survivor and non-survivor septic patients (Figure 2). Significant (p<0.001) positive correlation between u-ORM/u-CREAT and se-ORM (ρ=0.693) and hs-CRP (ρ=0.600) levels were described.

Table 1: Patients’ characteristics

<table>
<thead>
<tr>
<th></th>
<th>Reference group n=72</th>
<th>Matched-controls n=30</th>
<th>SIRS n=13</th>
<th>Sepsis n=39</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females, n (%)</td>
<td>37 (51.4)</td>
<td>14 (46.7)</td>
<td>4 (30.8)</td>
<td>15 (38.5)</td>
<td>N/A</td>
</tr>
<tr>
<td>Age, (years)</td>
<td>23 (14-45)</td>
<td>58 (49-69)</td>
<td>62 (54-70)</td>
<td>67 (57-76)</td>
<td>N/A</td>
</tr>
<tr>
<td>Cardiovascular disease, n (%)</td>
<td>0 (0)</td>
<td>19 (63.3)</td>
<td>10 (76.9)</td>
<td>26 (66.7)</td>
<td>N/A</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>0 (0)</td>
<td>6 (20.0)</td>
<td>3 (23.1)</td>
<td>10 (25.6)</td>
<td>N/A</td>
</tr>
<tr>
<td>Pulmonary disease, n (%)</td>
<td>0 (0)</td>
<td>2 (6.7)</td>
<td>6 (46.2)</td>
<td>14 (35.9)</td>
<td>N/A</td>
</tr>
<tr>
<td>hs-CRP, (mg/L)</td>
<td>0.6 (0.3-1.5)</td>
<td>1.0 (0.5-2.2)</td>
<td>92.4 (56.4-227.9)</td>
<td>228.5 (138.6-336.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>u-ORM/u-CREAT, (mg/mmol)</td>
<td>0.08 (0.05-0.15)</td>
<td>0.20 (0.10-0.30)</td>
<td>2.10 (0.70-6.40)</td>
<td>19.20 (11.40-32.80)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Figure 1: ROC analysis for distinguishing SIRS from sepsis

Figure 2: Monitoring of u-ORM/u-CREAT levels in sepsis
Discussion
Our fully automated turbidimetric approach was described to be fast, sensitive and precise therefore it is ideal for routine u-ORM measurements. We determined a reference range for u-ORM/u-CREAT to be similar to others [3]. We found slightly elevated u-ORM levels in volunteers with comorbidities, which is in agreement with previous findings on diabetic patients [4]. This preclinical elevation is considered to be the result of chronic ongoing low-grade inflammation. Furthermore, we observed extremely increased u-ORM excretion in severe systemic inflammation compared to controls. We described an early and relevant u-ORM/u-CREAT elevation in sepsis with promising diagnostic performance to distinguish SIRS from sepsis. Although ORM/u-CREAT seems to be sensitive and early marker of sepsis with good diagnostic ability, it is unable to predict disease severity and mortality. The pathomechanism of u-ORM excretion is not well understood and the increased se-ORM level itself may not explain that alone. Alteration in kidney function, probably both glomerular and tubular dysfunctions may be involved. Furthermore, a possible local renal production due to systemic manifestation of inflammation is also suspected. The good correlations between inflammatory markers and u-ORM/u-CREAT support the hypothesis that elevated u-ORM is mainly due to systemic inflammation.

Conclusions
Our highly sensitive u-ORM assay is ideal for routine clinical utilization to measure u-ORM as a possible novel marker of inflammatory activity. U-ORM showed a considerably early elevation in sepsis with promising diagnostic performance in discriminating SIRS cases from true sepsis. Consequently, non-invasively obtained urine could be a possible alternative to blood sampling in diagnosis of sepsis, and u-ORM/u-CREAT might be suitable as a complementary marker of sepsis and could help clinicians in proper decision making.

Summary
We developed a fully automated u-ORM assay, which can be a novel diagnostic tool in management of severe systemic inflammation.

References


EVOLUTION OF WEST SYNDROME IN GEORGIA, PREDICTORS OF OUTCOME

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Tutor: Nana Tatishvili

Introduction. Aims

Infantile spasms is an age-specific devastating epileptic encephalopathy of early infancy, which is characterized by the combination of distinctive unique seizure type, which is accompanied by specific electroencephalographic pattern known as hypsarrhythmia and delay or arrest of psychomotor development. This is a prospective observational study about infantile spasms and predictors of outcome. This syndrome hasn’t been thoroughly investigated in Georgia and our purpose is to assess the evolution of West syndrome and its relation to patient characteristics, to study etiological peculiarities and its relation to the long-term outcome, to find out if time between initiation of spasms and treatment make the influence on the long-term outcome (lead time to treatment).

Methods

We evaluated 28 patients (16 male, 12 female) with infantile spasms at M. Iashvili Children Central Hospital, Institute of Neurology and Neuropsychology and I. Tsitsishvili Children New Hospital, Tbilisi, Georgia. Mean age - 6-7 months. Inclusion criteria were newly diagnosed patients, who met definition of West syndrome (IS, abnormal EEG), written informed consent of parents/ caregivers. We collected birth, family and seizure detailed history. All patients were examined neurologically, investigated with prolonged sleep and awake video-EEG, brain MRI, developmental testing (Bayley scales of infant and toddler development, III edition, ASQ - age and stage questionnaire) was done at admission. Spasm diary was given and filled by every parent/caregiver. Seizures were recorded on video and phenomenology of infantile spasms was studied. All patients were treated with adrenocorticotropic hormone (ACTH), hydrocortisone or antiepileptic drugs. Second and third years we are planning to do follow-up and assess: evolution of infantile spasms into other epilepsy (e.g. Lennox- Gastaut syndrome, symptomatic focal epileptic syndromes, etc.), treatment options with ACTH/antiepileptic drugs, electroencephalographic evolution after treatment and neurodevelopmental testing.

Results

Patients’ age below than 6 months, at the onset of spasms, was in 12 (42%) and above than 6 months in 16 children (58%). Etiologically symptomatic IS were present in 22 (79%) of cases. Among them - corpus callosum hypogenensis 6, hydrocephaly 6, lissencephaly 1 and various 9 cases. Cryptogenic in 6 (21%) cases. Symmetrical spasms were most common (82%). Most of the patients had spasms in cluster (85%). Hypsarrhythmia on EEG was seen in 20 (72%) patients, modified (atypical) hypsarrhythmia in 4 (14%) and other changes also in 4 (14%) patients. All patients were treated. After 5-8th injection with ACTH therapeutic response was satisfactory in 93%. Developmental delay was seen in 25 patients (89%), normal development -3 patients (11%). One patient died.
Discussion
Neurodevelopmental outcome, due to underlying etiology, is unfavorable. Among prognostic factors, the treatment lag and duration of spasms have been recognized as being significant. In our study number of patients isn’t much, though preliminary findings are consent to the other author’s studies. In symptomatic cases prognosis is unfavorable. Adrenocorticotropic hormone remains as a first choice treatment option.

Conclusion
Prognosis remains unfavorable and poor. Developmental assessment was done at the time of spasms initiation, most of the patients have rehabilitation course. Cessation of spasms was more likely in infants given hormonal treatments. This study is still in the process. We are planning to do clinical, EEG and neuropsychological follow-up one and two years after first assessment.

Summary
West syndrome is the object of a number of studies aimed at understanding the complex relationships between an epileptic disorder and neurodevelopment. Despite of such a huge interest and plenty of researches around West syndrome there still are too many questions that need to be addressed: unique seizure type, the phenomenon of spontaneous remission, pathophysiology, to date model of infantile spasms isn’t known yet, evolution of Infantile spasms, what factors determine the outcome, treatment remains problematic (3).
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TEMPEMANENT, CHARACTER AND BIOMARKERS OF CARDIOVASCULAR RISK: INTERACTION BETWEEN NEUROBIOLOGICAL MODEL OF PERSONALITY AND CARDIAC AUTONOMIC CONTROL

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Introduction
It has been known for several decades that people with certain personality traits and behavioural characteristics are more prone to cardiovascular diseases (CVD); in particular, type A personality characterized by competitiveness, time urgency and hostility was the most widely used construct related to cardiovascular morbidity (Booth-Kewley and Friedman, 1987). Recently, type D (distressed) personality associated with negative affectivity, social inhibition, anxiety and depression has been extensively studied as a risk factor for adverse cardiovascular outcomes (Cao et al., 2016). From this context, the role of personality and behaviour in the etiopathogenesis of CVD is universally recognized. However, the mechanisms underlying this relationship are still not fully understood. Therefore, we aimed to study the interaction between personality traits and physiological cardiac autonomic regulation at rest and in response to mental stress using a modern concept of personality - Cloninger’s neurobiological model and complex heart rate variability (HRV) analysis by conventional linear methods and novel nonlinear parameters in young healthy adults.

Methods
The studied group comprised 40 university students (20 women, age: 22.9±0.1 yr., BMI: 22.0±0.4 kg/m²) with excluded effect of underweight, overweight, recent acute illness, history of chronic cardiovascular, respiratory, endocrine, neurological, infectious diseases, mental disorders, smoking and use of medicaments and substances which could affect autonomic nervous system (e.g. alcohol, caffeine at least 12 h before the examination).

The examinations were performed under standard conditions in a sound-attenuated room with minimization of stimuli, between 9.00 a.m. – 12.30 p.m. After 15 min required to avoid a potential stress effect of laboratory environment, the continuous recording of ECG signal (DiANS PF8, Dimea, Czech Republic) was performed during stress assessment protocol: baseline (P1), Stroop test (P2), rest (P3), mental arithmetic test (P4), rest (P5), negative emotional stimulus (P6), and rest (P7). The duration of each period was 6 min. The RR-intervals were derived from ECG-recordings and following parameters of HRV were evaluated: RR-interval, spectral power in high frequency band of HRV (HF-HRV; reflecting cardiac vagal control), nonlinear HRV analysis - symbolic dynamics indices 0V% (cardiac sympathetic regulation) and 2LV% (cardiac vagal control), parameters indicating HRV complexity – normalized index of complexity (NCI) and normalized index of unpredictability (NUPI).

Personality traits were assessed according to Neurobiological model of personality, which integrates the neurobiological and psychological aspects of personality (Cloninger, 1999). Slovak version of the Temperament and Character Inventory – Revised was used. This inventory (240 items) allows to quantitatively assess the temperament and character dimensions.
of personality. Four temperament dimensions are considered to reflect functions of central neurotransmitters: 

- **novelty seeking** (associated with behavioural activation and function of dopamine),
- **harm avoidance** (associated with behavioural inhibition and function of GABA and serotonin),
- **reward dependence** (noradrenaline and serotonin), and
- **persistence** (glutamate and serotonin).

The temperament traits are supposed to be largely stable and inherited. Three character dimensions associated with social-learning and education (acquired dimensions) are **self-directedness**, **cooperativeness**, and **self-transcendence**.

**Results**

RR-interval was significantly shorter and lnHF-HRV and 2LV% were significantly lower in response to both cognitive (T2, T4) and emotional (T6) tasks (RR-interval: \( p<0.001 \) for T2 and T4, \( p<0.01 \) for T6; lnHF-HRV and 2LV%: \( p<0.01 \) for T2 and T6, \( p<0.001 \) for T4). Index OV% significantly increased in response to cognitive mental tasks (T2, T4; both \( p<0.001 \)). Parameters NCI and NUPI were significantly lower during cognitive mental tasks (T2: both \( p<0.001 \), T4: NCI \( p<0.05 \), NUPI \( p<0.01 \)). During recovery periods (T3, T5, T7), all the parameters returned to baseline values.

Correlation analysis revealed significant negative correlation between **harm avoidance** and OV% during mental arithmetic task (\( r=-0.356, p=0.024 \)) and significant positive correlation between **cooperativeness** and resting NCI and NUPI (\( r=0.391, p=0.013; r=0.383, p=0.015 \), respectively).

**Discussion**

We found that applied mental stressors were suitable to evoke a shift in dynamic autonomic sympathovagal balance assessed by linear and nonlinear HRV analysis. The nonlinear methods of HRV analysis revealed associations between personality traits and cardiac autonomic regulation in young healthy subjects, while conventional linear analysis was not sensitive to this relationship. The complex cardiac autonomic regulation is maintained by an integrated system of numerous mutually interconnected cortical and subcortical nervous centres which is known as **central autonomic network** (Benarroch, 2007). Existence of functional neural connections between cortical areas, limbic system and brainstem nuclei can result in fact, that parameters of HRV may reflect regulation of various psychophysiological processes, including personality and behaviour. In our study, subjects with more expressed behavioural inhibition marked by **harm avoidance** showed lower cardiac sympathetic reactivity to mental stressor. This interaction could be related to inhibitory GABA-ergic neurotransmission affecting the central processing of stress response (Nuss, 2015). Importantly, low stress reactivity has been recently considered to be a risk factor for CVD similarly as exaggerated physiological reactions (Lovallo, 2011). Interestingly, our study also revealed the association between acquired character trait and autonomic cardiac regulation. Participants with greater **cooperativeness** showed higher complexity and unpredictability of HRV, which are considered as novel biomarkers of effective physiological neuro-cardiac control and were found to be decreased under pathological conditions (e.g. in attention deficit/hyperactivity disorder, Tonhajzerova et al., 2016). Thus, the interaction between personality and autonomic regulation seems to be bidirectional with temperament personality traits being based on neurobiological properties of nervous system and vice versa autonomic nervous functions being affected by character dimensions of personality.

**Conclusions**

Application of novel nonlinear analysis of heart rate variability and Neurobiological model of personality seems to be a promising method to study the effect of personality on autonomic cardiac regulation. This study could help to understand the interaction between personality characteristics, autonomic nervous system, and risk of cardiovascular diseases. After more detailed study of the underlying mechanisms and long-term effects of personality traits, clinical application could include an improved assessment of the cardiovascular risk, individual adjustment
of prevention, diagnostics and treatment of CVD, and potential effect of intervention in character personality dimensions on future health outcomes.

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Summary
It has been known for several decades that people with certain personality traits and behavioural characteristics are more prone to cardiovascular diseases (CVD). However, the mechanisms underlying this relationship are still not fully understood. Therefore, we aimed to study the interaction between personality traits and physiological cardiac autonomic regulation at rest and in response to mental stress using a modern concept of personality - Cloninger’s neurobiological model and complex heart rate variability (HRV) analysis by conventional linear methods and novel nonlinear parameters in young healthy adults. The ECG recordings in studied group of 40 healthy young adults were performed during stress assessment protocol: baseline, Stroop test, rest, mental arithmetic test, rest, negative emotional stimulus, and rest. The dimensions of personality – temperament - neurobiological inherited traits and character - social acquired traits were quantitatively assessed using the Slovak version of the Temperament and Character Inventory – Revised. We found that applied mental stressors were suitable to evoke a shift in dynamic autonomic sympathovagal balance assessed by linear and nonlinear HRV analysis. The nonlinear methods of HRV analysis revealed associations between personality traits and cardiac autonomic regulation in young healthy subjects: significant negative correlation between harm avoidance and parameter 0V% (index of beta-adrenergic cardiac regulation) during mental arithmetic task and significant positive correlation between cooperativeness and resting measures of HRV complexity - normalized index of complexity (NCI) and normalized index of unpredictability (NUPI). This study could help to understand the interaction between personality characteristics, autonomic nervous system, and risk of cardiovascular diseases.

References


EFFECTS OF GUT MICROBIOTA MANIPULATION BY ANTIBIOTICS ON PLASMA AMINO ACID LEVELS IN OBESE HUMANS

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Introduction. Disruptions in gut microbiota composition and function are increasingly implicated in the pathogenesis of obesity, insulin resistance, and type 2 diabetes mellitus (T2DM). The functional output of the gut microbiota, including short-chain fatty acids (SCFAs) and amino acids, are thought to be important modulators underlying the development of these disorders. Gut bacteria can alter the bioavailability of amino acids by utilization of several amino acids originating from both alimentary and endogenous proteins. In turn, gut bacteria also provide amino acids to the host. This could have significant implications in the context of insulin resistance and type 2 diabetes mellitus, conditions associated with elevated systemic concentrations of certain amino acids, in particular the aromatic and branched-chain amino acids (BCAA). Moreover, several amino acids released by gut bacteria can serve as precursors for the synthesis of short-chain fatty acids, which also play a role in the development of obesity.

Aim. We investigated the effects of gut microbiota knock-down on arterial amino acid levels in humans.

Methods. 38 obese male subjects (BMI 31.2±2.6kg/m², age 59±7y, HOMA-IR 4.5±0.2) with impaired fasting glucose and/or impaired glucose tolerance participated in a randomized double-blind placebo-controlled trial.

Subjects were orally treated with 1500mg/day amoxicillin (AMOX; broad-spectrum antibiotic), vancomycin (VANCO; aimed at Gram-positive bacteria), or placebo (PLA; microcrystalline cellulose) for 7 days. Before and after treatment, arterial concentrations of 21 amino acids were measured using liquid chromatography.
**Results.** Baseline BCAA concentrations were high but did not differ between groups: 445.0±35.9µmol/L (VANCO), 423.3±42.7µmol/L (AMOX), and 440.6±44.7µmol/L (PLA), *P*=0.406. AMOX treatment specifically increased BCAA levels in comparison to PLA (464.3±60.7µmol/L vs. 434.6±66.5µmol/L; *P*=0.042), whilst VANCO treatment did not (441.9±30.3µmol/L; *P*=0.867). Within treatment groups, isoleucine (one of the BCAA) concentrations increased significantly upon both AMOX (from 68.4±8.1µmol/L to 79.3±10.9µmol/L; *P*=0.003) and VANCO treatment (from 70.3±7.7µmol/L to 78.3±9.6µmol/L; *P*=0.001), but not in the PLA group (from 75.6±10.5µmol/L to 78.1±12.3µmol/L; *P*=0.305). Besides, arginine concentrations increased significantly only upon AMOX treatment (from 89.9±20.1µmol/L to 101.5±21.2µmol/L; *P*=0.025). Other amino acids were not affected by any treatment.

**Conclusion.** The broad-spectrum antibiotic AMOX increases plasma BCAA concentrations.

**Summary.** The intestinal microbiota is involved in the utilization and catabolism of several amino acids originating from both alimentary and endogenous proteins. These amino acids can serve as precursors for the synthesis of metabolic end products produced by the microbiota including SCFAs. T2DM has been characterized by systemic elevations in some of these (precursor) amino acids. The altered bacterial composition in the gut as observed in obese subjects with T2DM may therefore play a major role in their metabolic derangements by influencing amino acid and SCFA bioavailability to the host. Similar, gut microbiota manipulation by antibiotics may affect parameters of insulin sensitivity and substrate metabolism by influencing amino acid levels in human. As shown by our dataset, the broad-spectrum antibiotic AMOX increases plasma BCAA concentrations. Current ongoing analyses will shed light on the nature of the gut microbiota alterations provoked by AMOX in relation to specific amino acid aberrations, parameters of insulin sensitivity, and substrate metabolism in obese subjects with impaired glucose tolerance.

**References**


LOSS OF B CELLS AND THEIR PRECURSORS IS THE MOST CONSTANT FEATURE OF GATA-2 DEFICIENCY IN CHILDHOOD MYELODYSPLASTIC SYNDROME

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Introduction
Myelodysplastic syndrome (MDS) is a rare disease in childhood. We distinguish several subtypes: the most common refractory cytopenia of childhood (RCC), followed by advanced forms with increased immature blasts in bone marrow or peripheral blood: refractory cytopenia with excess blasts (RAEB), RAEB in transformation (RAEB-t) and myelodysplasia related acute myeloid leukemia (MDR-AML). RCC has overlapping clinical and laboratory features with another acquired bone marrow failure syndrome, aplastic anemia (AA). The diagnosis is mainly based on histopathological examination of bone marrow biopsy. The utility of flow cytometry in diagnostic algorithm is limited, although it has been implemented in diagnosing MDS in adults by several groups.

Pathogenesis of both diseases is still not fully understood in all of the patients. Autoimmune destruction of bone marrow progenitors is presumed especially in patients with AA, but also in some patients with RCC. Recently a mutation in GATA2 gene was found in patients with several syndromes: MonoMAC (monocytopenia, mycobacterial infections), DCML deficiency (deficiency of dendritic cells, monocytes, B and NK lymphocytes), familiar MDS/AML and Emberger syndrome (lymphedema, multiple warts). Published data showed several abnormalities detectable by flow cytometry: e.g. decreased number of B cells, NK cells, monocytes, dendritic cells. However, these data were mainly gained from analyses of adult GATA2-mutated patients and patients predominantly suffering from immunodeficiency.

Aims
Our aim was to analyze nation-wide pediatric cohort of patients with MDS and AA to define prevalence of GATA2 mutation and to identify immunophenotypic profile characterizing this subgroup of patients with bone marrow failure.

Methods
We analyzed GATA2 mutation in a cohort of pediatric RCC and AA patients diagnosed in Czech Republic between 2005-2014 and in patients with advanced forms of MDS (assigned as “non RCC”) who were diagnosed between 1998-2014 to define prevalence of GATA2 mutation. In all of these patients we retrospectively or prospectively analyzed samples of bone marrow and peripheral blood by flow cytometry for composition of cell populations. Additionally, we included data from other patients with GATA2 mutation sent to our laboratory, either within collaboration with laboratories in Slovakia or Germany or from patients identified within family search or examined for hematological problems not fulfilling MDS/AA diagnosis. Overview
of patients may be found in Figure 1. We measured 4 to 8-color antibody panels identifying distinct myeloid and lymphoid populations including progenitors. As a control group we analyzed samples from patients with solid tumors without infiltration of bone marrow or healthy donors. Next, we analyzed excision circles of B and T cell receptors (KREC and TREC, respectively) using polymerase chain reaction (PCR), as surrogate markers of B and T cell production.7

Figure 1:

Results
In a consecutive cohort of 90 Czech pediatric patients with MDS/AA we found GATA2 mutation in 8 patients: 5 of them were diagnosed with RCC, 3 of them with advance form of MDS and none had AA. The prevalence of GATA2 mutation was thus 17% within RCC group, 14% within non RCC group and 0% within AA.

The most characteristic feature we found was deficiency of B cells including progenitors in GATA2-mutated patients: B cells in peripheral blood were decreased in 10 out of 12 analyzed patients. This correlated with decreased KREC levels in both bone marrow and peripheral blood, which were significantly decreased compared to all other patient groups and controls (Figure 2). Interestingly, we could analyze level of KRECs in newborn blood spots (Guthrie cards) in 4 of GATA2-mutated patients and in 3 of them the value was normal, in one patient KRECs were decreased already at birth. This patient developed MDS at age 4y and is the youngest patient in our cohort. On the contrary, we observed stable absolute and relative monocytopenia only in 2 patients, both of them suffered from interstitial lung disease. In some patients monocytosis was present. NK cells were decreased in only half of analyzed GATA2-mutated patients. Most of the patients also exhibited neutropenia. We performed receiver operating characteristic (ROC) curves to determine which parameter best identify GATA2-mutated patients within MDS/AA group, which confirmed our observation B cell lymphopenia as the most characteristic feature (Figure 3).
Discussion

Our finding of B cell deficiency as the most sensitive marker for identifying GATA2-mutated patients is in contrast to so far published studies. Commonly described monocytopenia was present steadily only in two patients, who both had interstitial lung disease and immunodeficiency. The difference in our study and others could be explained by cohort of pediatric MDS patient with more frequent advanced MDS diagnosis. The other studies contained mainly adult patients with immunodeficiency or patients with less frequent advanced MDS. Monocytosis as a feature accompanying progression of MDS was recently described by Wlodarski et al.\textsuperscript{8} Neutropenia reported in other studies\textsuperscript{9} was also observed in our patients, normal neutrophil count in two patients with lung disease could be explained partially by administration of corticosteroids. Differences between RCC and AA which we observed previously\textsuperscript{10} are diminished after exclusion of patients with GATA2 mutation, who are present exclusively in MDS group, but not in AA. Interestingly, we observed 3 patients with RCC with low B cells and KRECs with excluded mutation in GATA2, who are highly suspected of having so far unknown underlying genetic aberration.
Conclusions
In conclusion, we found that B cell production disorder is the strongest distinguishing biological feature in GATA2-mutated children with MDS. Evaluation of B cell count in peripheral blood and bone marrow with analysis of KREC levels can help to identify appropriate candidates for GATA2 mutation screening. Information on GATA2 mutational status in the family is of importance for several reasons: hematopoietic stem cell transplantation is considered as one of the main treatment options with preference of matched family donor, and for a need of careful immunological monitoring with early treatment of infections as GATA-2 deficiency is also an immunodeficient disorder which might lead to organ damage.

Summary
GATA2 mutation is a newly identified pathogenetic mechanism leading to pediatric MDS in so far largest subgroup of patients. The prevalence is 17% within RCC and 14% within advanced forms of MDS in a cohort of Czech pediatric patients. No patient with GATA2 mutation was identified within AA group. Although the clinical features in GATA-2 deficiency are broad and affecting many systems, pediatric patients with MDS share B cell production disorder that can be tracked by analysis of B cell counts in bone marrow and peripheral blood and by analysis of KREC excision circles.

References


Introduction

Magnetic resonance imaging (MRI) is one of the most common imaging modalities nowadays. Another application is a so-called magnetic resonance spectroscopy (MRS), where the MR spectrum is acquired from a localized volume of the body to provide additional biochemical information. The application of MRS and spectroscopic imaging (MRSI) as an adjunct to conventional MRI in clinical practice has been hindered by many factors. One of these factors is a low sensitivity, which may be improved by utilizing higher magnetic fields (>3T). However the transition to higher fields introduces many challenges such as B₀/B₁ inhomogeneities, chemical shift displacement errors (CSDEs), and shortened T₂ relaxation times. In addition, an increase in RF power deposition leads to increased demands on hardware as well as software of the MR scanner. Therefore, novel MRSI techniques based on free induction decay (FID) acquisition were introduced for ultra-high field (UHF, ≥7T)\(^1\,2\). Without any pre-localization and without the necessary power that is usually needed to suppress the unwanted signals of lipids, the lipid contamination posed a severe problem. To overcome this, we used high resolutions up to 23 ml and 128×128 matrix sizes in order to achieve a good point spread function and thus prevent the lipid signal from contaminating the brain region. Yet, for such resolutions, typical measurement times range from 30 min up to 2 hours - an unbearable time for any patient measurements. We therefore decreased the acquisition time significantly by acceleration using parallel imaging (PI). Reaching resolutions similar to conventional MRI at a clinically feasible time (~6-17 min) enables the analysis of smaller structures and lesions, which offers a high potential for research as well as for diagnostic applications.

Methods

Volunteers, tumor patients and MS patients underwent a measurement on a Siemens 7 T Magnetom scanner with a 32-channel head coil with different protocols. Written informed consent and IRB approval were obtained. All sequences were measured with an acquisition delay (TE*) of 1.3 ms, an FOV of 220×220 mm² and a slice thickness ranging from 6-12 mm. The signals from individual coil channels were combined using an in-house developed method (MUSICAL\(^3\)). To achieve a clinically feasible protocol, acceleration methods based on various undersampling patterns (GRAPPA\(^4\), 2D- and (2+1)D-CAIPIRINHA\(^5\)) were implemented and compared. The data from multiple voxels (often >10 000) per one volunteer were acquired, hence a fully automated processing pipeline with minimal user interaction was implemented. To reduce contaminant lipid signal from skull we utilized a regularized reconstruction according to Bilgic et al.\(^6\) The LCModel quantification software was used for data processing.

We adapted the sequence to different applications: 1. A double-inversion recovery sequence to obtain the macromolecular background\(^7\) and to suppress skull lipids\(^8\) for cases where the regularized reconstruction might not be enough. 2. For cases where bigger 3D-coverage was necessary, such
as tumor patients, pulse-cascaded Hadamard encoding together with (2+1)D-CAIPIRINHA were implemented. In this case, each additional Hadamard encoded slice had 1 ms increased TE*. 3. If very small lesions had to be measured, e.g. in MS patients, resolutions up to 128×128 were used (ultra-high resolution, UHR). In this case, acceleration was achieved by reducing the TR from 600 ms to 200 ms by optimizing the water suppression and the read-out module, in addition to 2D-CAIPIRINHA9.

Results
(2+1)D-CAIPIRINHA had smaller root mean square errors in comparison to 2D-GRAPPA (12.1 %) and 2D-CAIPIRINHA (6.9 %) with respect to the gold standard without any acceleration. Figure 1 shows metabolic maps obtained from a healthy volunteer. The higher resolution (128×128 matrix) allow to resolve a gray matter and white matter separation and even finer structures such as gyri (Figure 2). An average SNR for UHR was 24±8 over 3 volunteers compared to 24±8 (TR=200ms, 64x64, R=4) and 40±12 (TR=600ms, 64x64, R=4). Reducing the spectral sampling points affected CRLBs and FWHMs more than the smaller voxel volume, with average CRLB[%]/FWHMs[Hz] of 16±7/10±5 for UHR-MRSI. The qualitative assessment of fourteen MS patient data showed a decrease of (25.8±13.4)% of total signal of N-acetyl aspartate, and (2.3±15.7)% of total signal of creatine, while inositol increased by (41.8±34.2)% and total signal of choline by (2.3±16.5)%. In three patients, metabolic changes were visually not or only hardly seen on the 64x64 resolution maps, but were clearly visible on the UHR-MRSI (Figure 3).

Discussion and conclusion
We have successfully shown that fast MRSI with higher resolution in the brain at 7 T is feasible within a reasonable time for healthy subjects as well as patients. MRSI acquisition is always a trade-off between sufficient SNR and measurement time. By careful optimization of MRSI parameters, we are able to acquire high-resolution data in less than 20 minutes covering a large area of brain.

Summary
Since the existence of magnetic resonance imaging as a clinical imaging modality, the MR spectroscopy protocols were present on the MR scanners offering additional biochemical information from the tissue of interest. However, due to many limitations, MR spectroscopy is considered rather a powerful scientific tool than a robust method used in an everyday clinical practice. Therefore one of the aims of MR spectroscopists is to develop a protocol applicable in the day-to-day patient measurements in any hospital possessing an MR scanner. We tried to optimize the MR spectroscopic imaging sequence for various applications by utilization of available advanced methods of acceleration and data reconstruction. Afterwards, this sequence was tested on healthy volunteers, MS patients and tumor patients to assess the feasibility. We hope that we did another tiny step towards the utilization of MR spectroscopy in clinical routine.

References


Figures:

![Figure 1. T1-weighted image and three metabolic maps acquired from a healthy volunteer](image-url)

Figure 1. T₁-weighted image and three metabolic maps acquired from a healthy volunteer.
Figure 2. UHR-maps of one volunteer compared to maps with 64×64 matrix size and the same TR of 200 ms and TR of 600 ms. Finer anatomical details, especially the gyri structure can be seen on the UHR-maps.

Figure 3. Comparison of a 64x64 and a 100x100 total N-acetyl aspartate (tNAA) map with a FLAIR image. Two lesions are visible on the 100x100 tNAA map, which are hardly visible on the 64x64 image. This demonstrates that a resolution of 64x64 is sometimes not sufficient to resolve smaller MS lesions.
LEAN BODY MASS ESTIMATION FORM STANDARD DIXON BASED ATTENUATION CORRECTION IN INTEGRATED POSITRON EMISSION TOMOGRAPHY/MAGNET RESONANCE IMAGING

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Introduction
Positron emission tomography (PET) is a well-established method for in vivo measurement of metabolic processes. PET imaging relies on radioactive tagging of a metabolic compound, most commonly using fluorodeoxyglucose (¹⁸F-FDG), which substitutes the radioactive isotope fluorine-18 for the normal hydroxyl group in glucose. Once introduced into the patient’s bloodstream, the activity of the labelled compound can be measured to assess its distribution within the subject.

In practice a PET study is performed as a static measurement providing a snapshot of the activity distribution at a certain time point. This enables relative comparisons of the metabolic activity in selected regions of the body. Unfortunately, an absolute quantification of the metabolic activity is not a priori possible. The uptake of labelled tracer is dependent on the amount of available tracer and its ratio to the unlabelled analogue.

Kinetic modelling, which requires dynamic acquisitions, continuous measurement of the blood activity and extensive post-processing, could be used to overcome this limitation, but it is not clinically viable. Instead, the concept of the standardized uptake value (SUV) is used. SUV relies on the normalization of the PET signal with an estimate of tracer availability based on the injected radiation dose and a patient-specific statistic such as body weight (SUVbw), body surface area (SUVbsa) or lean body mass (SUVlbm) which is assumed to correlate with metabolic activity[2]. SUVlbm has been shown to give better estimates for true metabolic rate than SUVbw [3]. A number of LBM formulas involving body weight, patient height, body surface area and sex-specific correction factors have been developed, but none of them has found widespread clinical use [1,4].

Dual-modality systems combining computed tomography (CT) with PET (PET/CT) have become the clinical standard for acquisitions requiring co-localized metabolic and anatomic information. The effectiveness of PET/CT is due in part to the complementary role of CT in providing reliable, quantitative tissue attenuation values for PET reconstruction.

With the recent introduction dual-modality magnetic resonance (MR) and PET (PET/MR), novel clinical applications are being actively developed. PET/MR has the advantage of sparing patients the radiation dose from CT, while offering superior soft tissue sensitivity and a wide array of functional, morphological and even metabolic clinical imaging protocols. However, MR-based attenuation correction (MR-AC) is still an open area of research. The Siemens mMR system uses the Dixon water-fat MRI sequence to segment the patient into four discrete tissue types: soft tissue, fat, lung and air, each with a unique attenuation value. Despite the drawbacks of this attenuation correction method, it provides a standardized way of estimating the quantity of adipose tissue present for each PET scan performed on the mMR.
Therefore, the aim of this study was to evaluate if the Dixon-based fat quantification can be used to more accurately estimate patient-specific lean body mass than current estimations, which in turn will lead to more accurate SUV quantification.

Methods
This study included ten volunteers who underwent a body composition measurement using air displacement plethysmography (ADP) in a BOD POD system (BodPod; COSMED USA, Inc.). Volunteers were positioned in a fully-integrated PET/MR system (Siemens Healthcare AG, Germany) and three consecutive multi-bed acquisitions of the standard Dixon-based MR-AC image data were acquired. For each subject and MR-AC map, the following compartmental volumes were calculated: total-body (TB), soft tissue (ST), fat (F) and intermediate tissue (IM). To assess the reproducibility of intra-subject differences in TB and sub-compartmental volumes (ST, F, and IM) coefficients of variation (CV) were calculated for all MR-AC maps and excluding those with major artefacts.

ADP measurements were used to calculate a reference LBM (LBM_{ADP}). A second LBM estimate was derived from available MR-AC data using the formula:

\[ \text{LBM}_{\text{MR-AC}} = \frac{(V_{\text{ST}} \times \rho_{\text{ST}} + V_{\text{IM}} \times \rho_{\text{IM}} \times 0.5)}{(V_{\text{ST}} \times \rho_{\text{ST}} + V_{\text{F}} \times \rho_{\text{F}} + V_{\text{IM}} \times \rho_{\text{IM}} \times 0.5)} \times \text{BW}, \]

where \( V \) is the respective tissue volume, \( \rho \) is the tissue-dependent density and BW is the body weight. A third LBM estimate was obtained from a gender-specific formula (LBM\_formula). Pearson’s correlation was calculated for LBM_{ADP}, LBM_{MR-AC} and LBM\_formula. Further, linear regression analysis was performed on LBM_{MR-AC} and LBM_{ADP}.

Results
Mean CV of the TB volume for all 30 scans was (2.1±1.9)%. When excluding missing tissue artefacts, the CV was reduced to (0.3±0.2)%. Mean CV for the sub-compartments before and after excluding artefacts was ST: (0.9±1.1)% and (0.7±0.7)%, F: (2.9±4.1)% and (1.3±1.0)%, and IM: (3.6±3.7)% and (1.3±0.7)%, respectively.

Correlation was highest for LBM_{MR-AC} and LBM_{ADP} (r=0.99). Linear regression of data excluding artefacts resulted in a scaling factor of 1.06 for LBM_{MR-AC}.

Discussion
In general, the stability of the tissue volumes extracted from DIXON based MR-AC maps is comparable with advanced body composition measures. ADP is a well-established method for the assessment of body composition. Published CVs for the assessment of adipose tissue using ADP are between 1.7% and 3.7% [5], therefore showing a similar reproducibility as for the fat fraction extracted from the MR-AC maps. Moreover, excluding the most crucial artefacts (tissue swap and missing tissue) CVs were found to be in accordance with published CVs for advanced body composition measurements using MR [6]. The correlation between LBM_{ADP} and LBM_{MR-AC} was excellent (r=0.99) and better than the correlation between LBM_{ADP} and LBM\_formula. The lower correlation of LBM\_formula can be explained by the limited usability of predictive equations in individuals, especially for very lean or obese patients [7]. LBM_{MR-AC} underestimated LBM_{ADP}. Similar deviations were already reported by Ludwig et al. [6] for automatic tissue segmentations in DIXON based images and hence the deviations found in this study are likewise attributed to the properties of the segmentation algorithm. Nevertheless, due to the good correlation of LBM_{MR-AC} and LBM_{ADP} a simple scaling factor could be calculated to overcome this issue.

Conclusion
LBM estimation from DIXON MR-AC maps correlates well with standard LBM and thus offers routine SUV\_LBM-based quantification in PET/MR. However, MR-AC images must be controlled...
for systematic artefacts, including missing tissue and tissue swaps, which limit MR-AC reproducibility.

**Summary**

In positron emission tomography (PET) simplified measures like the standardized uptake value (SUV) can be used to estimate metabolic activity. Published data suggest that SUV normalized to lean body mass (LBM) is superior to SUV normalized to body weight. Nevertheless, due to difficulties in predicting LBM, SUV normalized to LBM is rarely used. In recently introduced positron emission tomography / magnetic resonance imaging (PET/MRI) systems attenuation correction is based on a segmentation of the body in different tissue classes, hence providing a measure of body composition. This study evaluates the usability of these attenuation correction maps for LBM prediction in 10 volunteers. Results show an excellent correlation of the extracted LBM with a standard LBM assessment and, therefore, reveal the possibility of a simple measure of LBM for SUV normalization in PET/MRI.

**References**


GENETICS AND FUNCTIONAL ANALYSIS OF CZECH PATIENTS WITH CONGENITAL HYPERINSULINISM

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Tutor: Štěpánka Průhová

Introduction
Congenital Hyperinsulinism of infancy (CHI) represents a group of heterogeneous disorders characterized by over-secretion of insulin from pancreatic β-cells causing severe hypoglycaemia [Mohamed et al., 2012]. Untreated hypoglycaemia in neonates and infants can lead to seizures, developmental delay, and subsequently to irreversible brain damage or death [Rozenkova et al., 2015].

The diagnosis is made on the basis of high insulin and C-peptide levels obtained at the time of hypoglycemia simultaneously with exclusion of other causes of hypoglycemia [Arnoux et al., 2011].

Genetically, recent advances in genetics have linked CHI to mutations in 9 genes that play a key role in regulating insulin secretion (ABCC8, KCNJ11, GLUD1, GCK, HADH, SLC16A1, UCP2, HNF4A, and HNF1A [Snider et al., 2013]. Mutations in all these genes account for about 50% of known causes of CHI.

Aim
The aim of our study was to collect clinical data and DNA samples from Czech patients with the diagnosis of CHI, to perform molecular genetics analysis and in case of novel mutations in the genes ABCC8, KCNJ11 and HNF1A to perform in vitro functional study to better understand the pathophysiological mechanism underlying the disease. In case of novel mutations to test if the novel mutations are pathogenic. The functional studies also have direct therapeutic consequences usable in the management of these patients as they determine the response to specific pharmacological agents.

Materials and Methods
We have systematically collected clinical information and DNA samples from 42 patients (14 females, 28 males) with the clinical diagnosis of CHI from all Czech centres caring for CHI patients from January 1997 till December 2014.

Individual genes were investigated by direct sequencing. Genomic DNAs were extracted from peripheral blood with a routine salting-out method or using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). We initially tested the complete coding region of the ABCC8 and KCNJ11 genes, including the intron-exon boundaries by conventional direct Sanger sequencing. Further, we analysed the ABCC8 gene using multiplex ligation-dependent probe amplification (MLPA, MRC Holland, Amsterdam, Netherlands) for possible deletions or duplications. In all subjects negative for ABCC8 and KCNJ11 mutations we performed molecular genetic analysis of the HNF4A, HNF1A and GCK gene. In case of no mutation detected, we further analysed
the coding regions and the intron-exon boundaries of the GLUD1, HADH, UCP2 and SLC16A1 genes. In addition, sequencing of relevant exons was also carried out in DNA samples from the patient’s parents in order to determine the source of the mutation.

The novel mutations found were created in vitro using site-directed mutagenesis. The mutations were confirmed by direct DNA sequencing.

In vitro functional study of novel mutations in the genes ABCC8, KCNJ11 was performed on HEK293 cells that were transfected with plasmids containing the wild type ABCC8 (WT) or KCNJ11 (WT) gene and the respective mutant gene. To compare the function of WT and mutant cells we used radioactive Rubidium (86Rb) efflux assay. Rubidium acts as a tracer for potassium movement across the cell membrane. HEK293 cells expressing wild type and mutant K-ATP channels were loaded with 86Rb, washed, and then incubated under experimental conditions: control (DMSO), 100µM diazoxide, 100µM diazoxide and 10µM glibenclamide, 2.5mM NaCN and 20mM 2-deoxy-D-glucose and 2.5mM NaCN, 20mM 2-deoxy-D-glucose and 10µM glibenclamide. The cell supernatant was then removed, and the remaining cell monolayer lysed. These two samples contain 86Rb that has moved out of the cell and 86Rb that remains in the cell, respectively. Measurement of the 86Rb content of these samples by liquid scintillation counter using Cherenkov radiation allows calculation of the 86Rb efflux percentage which informs us about the K-ATP channel function and activation by diazoxide.

In vitro functional study of novel mutations in the gene HNF1A was performed on HeLa cells that were transfected with plasmids containing the wild type HNF1A (WT) and mutant HNF1A. The Dual-Luciferase Reporter System (Promega AG, Wallisellen, Switzerland) was used for measurements of the HNF1A transactivation activity. To assess the HNF1A DNA-binding, we used electrophoretic mobility shift analysis (EMSA). Gels were analyzed by autoradiography.

The experiments were performed in triplicate in three independent experiments.

Results

The genetic cause was identified in 22 of 42 patients (52.4%). Mutations in the gene ABCC8 were identified in 12/42 patients (6 novel - F48delT, G389R, A478del, R657Q, Y1293D, K1373delAAG), in gene HNF1A in 5/42 patients (2 novel - N62fs, L254Q), in gene HNF4A in 2/42 patients (2 novel - S73del, R57P*13), in gene KCNJ11 in 2/42 patients (1 novel - R50W) and in gene GCK in 1 patient (M197I).

We performed in vitro functional study for 6 novel mutations in ABCC8 (F48del, G389R, A478del, R657Q, Y1293D, K1373del), 1 novel mutation in KCNJ11 (R50W) and 2 novel mutations in HNF1A (L254Q and N62fs). The activity measured using 86Rb efflux assay for mutant ABCC8 was reduced by 41-91.4% (median 83.6%) when compared to WT-ABCC8. The activity for the unique combination of heterozygous mutations in KCNJ11 and ABCC8 was reduced by 60.1% when compared to WT. The ability of mutant L254Q-HNF1A and N62fs-HNF1A to bind to a reporter gene was reduced by 78% and 97% when compared to WT-HNF1A. Moreover, the mutant L254Q-HNF1A and N62fs-HNF1A lose the ability to bind to DNA as proved by used electrophoretic mobility shift analysis (EMSA) [Rozenkova et al., 2015].

Conclusion

We report the biggest cohort of Czech patients with CHI published so far. The proportion of heterozygous mutations and mutations in HNF1A and HNF4A is much higher when compared to other published cohorts, most probably due to lack of consanguinity in the Czech population. Moreover, using in-vitro functional study, we have proved the pathogenic effect on the pancreatic
K-ATP channel function of 6 novel \textit{ABCC8} mutations, 1 novel \textit{KCNJ11} mutation and 2 novel \textit{HNF1A} mutations.

The results of our study help to evaluate the pathogenicity of these novel mutations and have a direct clinical application in the treatment regime of these patients and genetic counselling in the families.

**Summary**

**Introduction:** Congenital Hyperinsulinism (CHI) is a heterogeneous genetic disorder characterized by unregulated insulin secretion from pancreatic β-cells which leads to persistent hypoglycaemia in neonates and infants. To this day 9 genes have been identified whose mutations lead to the development of CHI. Genetic diagnosis affects the treatment strategy of these patients and helps to improve their prognosis.

**Aim:** The aim of our study was to collect clinical data and DNA samples from Czech patients with the diagnosis of CHI, to perform molecular genetics analysis and in case of novel mutations in the genes \textit{ABCC8}, \textit{KCNJ11} and \textit{HNF1A} to perform in vitro functional study.

**Materials and Methods:** The genetic material and clinical data were obtained from 42 patients with CHI. Individual genes were investigated by direct sequencing. The novel mutations found were created in vitro using site-directed mutagenesis. In vitro functional study for \textit{ABCC8}, \textit{KCNJ11} was performed on HEK293 cells that were transfected with plasmids containing the wild type \textit{ABCC8} (WT) or \textit{KCNJ11} (WT) gene and the respective mutant gene. To compare the function of WT and mutant cells we used radioactive Rubidium ($^{86}$Rb) efflux assay. $^{86}$Rb efflux was measured in a liquid scintillation counter using Cherenkov radiation. In vitro functional study for \textit{HNF1A} was performed on HeLa cells that were transfected with plasmids containing the wild type \textit{HNF1A} (WT) and mutant \textit{HNF1A}. HNF1A activity we measured using luciferase system. The experiments were performed in triplicate in three independent experiments.

**Results:** The genetic cause was identified in 22 of 42 patients (52.4%). Mutations in the gene \textit{ABCC8} were identified in 12/42 patients (6 novel), for \textit{HNF1A} in 5/42 (2 novel), for \textit{HNF4A} in 2/42 (2 novel), for \textit{KCNJ11} in 2/42 (1 novel) and for \textit{GCK} in 1 patient. We performed in vitro functional study for 6 novel mutations in \textit{ABCC8} (F48del, G389R, A478del, R657Q, Y1293D, K1373del), 1 novel mutation in \textit{KCNJ11} (R50W) and 2 novel mutations in \textit{HNF1A} (L254Q and N62fs). The activity measured using $^{86}$Rb efflux assay of mutant \textit{ABCC8} was reduced by 41-91.4% (median 83.6%) when compared to WT-\textit{ABCC8}. The activity for the unique combination of heterozygous mutations in \textit{KCNJ11} and \textit{ABCC8} was reduced by 60.1%. The ability of mutant L254Q-HNF1A and N62fs-HNF1A to bind to a reporter gene was reduced by 78% and 97%.

**Conclusion:** We report the biggest cohort of Czech patients with CHI published so far. The proportion of heterozygous mutations and mutations in \textit{HNF1A} and \textit{HNF4A} is much higher when compared to other published cohorts, most probably due to lack of consanguinity in the Czech population. Moreover, using in vitro functional study, we have proved the pathogenic effect on the pancreatic K\textit{ATP} channel function of 6 novel \textit{ABCC8} mutations, 1 novel \textit{KCNJ11} mutation and 2 novel \textit{HNF1A} mutations.

**References**


Introduction
Non-alcoholic fatty liver disease (NAFLD) is a frequent cause of chronic liver disease in western population. This hepatic disorder is characterized by increased accumulation of lipids in hepatocytes and by potential progression to inflammation called steatohepatitis (Loomba and Sanyal, 2013). This process can advance to the liver cirrhosis an eventual end-stage liver failure with a need of liver transplantation. Cirrhosis can be also complicated by the development of hepatocellular carcinoma. The prevalence of NAFLD is more than one-third of all people in western countries with more than 80% prevalence in obese population. NAFLD is mostly linked to lack of physical activity and unhealthy western type of diet (high cholesterol, high saturated fatty acids content). Liver steatosis is often accompanied by other pathological conditions and disorders such as atherosclerosis, metabolic syndrome and type II diabetes. The pathophysiology of NAFLD is associated with insulin resistance, impaired lipid metabolism and increase in reactive oxygen species (ROS) production (Satapati et al., 2015). However, the inflammation is also associated with lipid accumulation and the question of cause and consequence of NAFLD has not been sufficiently answered. The role of the liver mitochondria in NAFLD development and progression has not been fully elucidated yet and studies which would satisfactorily describe the changes in mitochondrial functions over the NAFLD progression are lacking. The vast majority of studies describe some kind of mitochondrial alterations, but facing the critical differences in study designs (type of animal, type and duration of high fat diet, assessment mitochondrial functions) we have to be careful with mitochondrial data interpretation (Kakimoto and Kowaltowski, 2016).

Aim
The aim of this study was to investigate the liver mitochondrial respiration and oxidative stress during high-fat and high-cholesterol diet for 1 to 6 weeks, which mimics NAFLD in humans.

Methods
Experiments were performed on male Wistar rats fed with a commercially prepared diet (Altromin) with high cholesterol and high fat content (HFD, 70 % of energy from lard enriched by 1.25 % cholesterol) for 1 and 3 weeks. Histological changes of liver tissue were evaluated by Hematoxylin eosin, Masson’s trichrome and Oil red O staining. Liver functions were estimated by measurement of biochemical markers (ALT, AST, ALP, bilirubin, urea) in serum. Triglycerides (TG) and cholesterol were assessed both in serum and in liver homogenates using commercial kits. Mitochondria were isolated from liver by differential centrifugation. Mitochondrial respiration was assessed using OROBOROS Oxygraph 2k with using comparative reference protocols. Tricarboxylic acid cycle, fatty acid oxidation (FAO) and glycero phosphatde dehydrogenase were evaluated by titrations of respective substrates and inhibitors.
Results
High fat diet did not influence the body weight or body weight gain even after 3 weeks in comparison to low fat diet control. Serum TG, HDL and LDL cholesterol as well as glycaemia were not different between both groups, but we detected progression of liver steatosis already after one week of HFD. Relative liver weight was significantly higher after 3 weeks of HFD feeding. Increased accumulation of lipids and micro vesicular steatosis were present in HFD group after staining by Oil red O and Hematoxylin eosin respectively. We also detected higher content of TG and cholesterol in liver homogenates. Mitochondrial respiration demonstrated significant increase in FAO capacity and relative inhibition of succinate stimulated respiration in HFD group. Maximal mitochondrial respiratory capacity was significantly increased after 3 weeks and mitochondria from HFD group exhibited more efficient oxidative phosphorylation.

Discussion
We described the development of early phase of NAFLD in rats fed by HFD. After the first three weeks of experiment we observed no significant biometrical changes even though the energy intake of HFD group was higher by more than 30% of controls. Already after one week of HFD there was a mild grade of fat accumulation in hepatocytes, but we did not observe any signs of impaired lipid metabolism or liver functional damage in serum biochemical markers. Liver mitochondria revealed higher capacity for FAO from the beginning of our study with more pronounced effect after three weeks of HFD. We may thus conclude that mitochondria started an adaptation process to reflect the diet with high fat and high cholesterol content by enhancing their capacity for FAO. To this day we were able to gain the results only from first and third week of the study, but the planned duration is 12 weeks.

Summary
We succeeded to induce liver steatosis by HFD in rats already after one week of feeding. We did not detect any changes in body constitutions but we observed higher TG accumulation in hepatocytes and mitochondrial modifications demonstrating increased FAO capacity.

Acknowledgements
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References
CONGENITAL AFIBRINOGENEMIA: NOVEL MUTATION
FIBRINOGEN MARTIN LEADING TO PREMATURE TERMINATION
CODON IN FIBRINOGEN B BETA-CHAIN GENE

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Introduction
Fibrinogen is a 340 kDa glycoprotein comprising pairs of three polypeptide chains termed Aα, Bβ and γ that are joined by disulfide bridging within the N-terminal E domain [1]. The three genes encoding fibrinogen Bβ (FGB), Aα (FGA), and γ (FGG), ordered from centromere to telomere, are clustered in a region of ≈ 50 kb on human chromosome 4q28 [2]. Diseases affecting fibrinogen may be acquired or inherited. Inherited disorders of fibrinogen are rare and can be subdivided into type I and type II disorders. Type I disorders (afibrinogenemia and hypofibrinogenemia) affect the quantity of fibrinogen in circulation (fibrinogen levels lower than 2.0 g L⁻¹). Type II disorders (dysfibrinogenemia and hypodysfibrinogenemia) affect the quality of circulating fibrinogen [3]. Congenital afibrinogenemia is an autosomal recessive bleeding disorder referring to the total absence of fibrinogen measured by an antigenic assay [4]. Afibrinogenemia is caused by mutations in the homozygous or compound heterozygous state in one of the three fibrinogen genes that affect the synthesis, assembly, intracellular processing, stability or secretion of fibrinogen [5,6].

Aim
In this study, we performed genetic analysis of FGA, FGB and FGG genes and coagulation tests in the patient with congenital afibrinogenemia and his immediate family, i.e. his parents and two older sisters.

Materials and Methods
The only patient with congenital afibrinogenemia reported in Slovakia is 26-year-old man. Umbilical cord bleeding and development of epidural hematoma and hygroma in the occipital region were the first signs of bleeding. In the patient's history there were many other bleeding episodes including repeated hemorrhage into muscles, joints, soft tissues (hematomas of the arms, forearms, right hypogastrium, left musculus gluteus medius, right thigh, right calf) and mucocutaneous bleeding. From the age of 15 years the secondary prophylactic dose of fibrinogen concentrate contributed to the substantial reduction of the frequency and intensity of spontaneous bleeding [7]. One of the most severe complications of the patients' disease was the coxitis developed probably due to the microbleeds in the joint capsule. The only possible treatment option was the implantation of a total hip endoprosthesis (performed repeatedly in the age of 15 and 26 years) [8]. To identify the genotypes in the Slovak kindreds of 26-year-old patient with congenital afibrinogenemia the samples of peripheral blood were collected from 5 subjects. The activated partial thromboplastin time, prothrombin time, thrombin time, reptilase time and fibrinogen (Clauss and Laurell methods) were tested. DNAs were isolated by using a DNA Blood Midi Kit. All the exons of three fibrinogen genes were amplified by using PCR and analysed by direct sequencing.
Results
All routine coagulation tests, i.e. activated partial thromboplastin time (aPTT), prothrombin time (PT) and thrombin time (TT) of the patient were infinitely prolonged (> 300s). Fibrinogen plasma level measured according to the Laurell and Clauss method was undetectable. Family members had reduced levels of fibrinogen below the normal range. Plasma fibrinogen according to the Clauss was 1.3 g.L⁻¹ in father and 1.5 g.L⁻¹ in mother (Table 1). We sequenced all exons of FGA, FGB and FGG genes including exon-intron boundaries to identify possible mutation leading to afibrinogenemia in the patient. Previously unknown homozygous C>T transversion in exon 4 of FGB gene at position 9661 (c.538C>T) resulting in the premature stop codon at position 180 (Q180X) was identified. Novel mutation in the heterozygous state was also detected in patient’s parents and his two older sisters (Figure 1).

Discussion
In our study we identified a novel mutation in the patient with afibrinogenemia. The mutation was localized in exon 4, nucleotide position 9661 of FGB and caused by the transversion C>T, leading to the switch of amino acid glutamine to stop codon [39]. The novel FGB mutation was subsequently also confirmed in the hypofibrinogenemic kindreds of the afibrinogenemic patient by direct sequence analysis of the three fibrinogen genes FGA, FGB and FGG. The kindreds, i.e. the patient’s parents and two sisters, were detected as heterozygous for the novel mutation which was later named “Fibrinogen Martin” after the town of its discovery. However, hypofibrinogenemia secondary to the novel mutation was present with only mild decrease of the fibrinogen level (activity and antigen between 1.0 g.L⁻¹ and 2.0 g.L⁻¹) in kindreds. Based on the results of the coagulation and functional tests of fibrinogen, as well as the results of genetic analysis of the patient kindreds, we have proposed the new pathological molecular mechanism underlying afibrinogenemia. The presence of the stop codon, caused by the substitution C9661T in exon 4 of FGB eliminates the aberrant mRNA encoding incomplete β polypeptide by the process of nonsense-mediated mRNA decay [9,10]. We presume that formation AαBβγ half-molecule is not present. Thus, the fibrinogen cannot be formed by the dimerization of two hexamers AαBβγ. Homozygous mutation in the patient with afibrinogenemia correlates well with the results of the coagulation assays whereas a total absence of fibrinogen was found in the patient.

Conclusion
The identification of the precise genetic defect of congenital afibrinogenemia is of value, to permit early testing of other at-risk individuals, to understand the correlation between genotype and clinical phenotype, to assist in therapeutic choices, and as an essential prerequisite for the development of new specific treatments, such as gene therapy.
Figure 1. Family pedigree of the patient with afibrinogenemia and electropherogram of the novel mutation fibrinogen martin. Functional and immunoreactive plasma fibrinogen levels, as well as the identified 538C > T mutations in FGB are depicted. The black filled-out symbol (black square) with arrow represents the homozygous patient. The half-filled-out, symbols with right half black represent the genotypically heterozygous kindreds.

Table 1. Phenotype of the patient and selected family members

<table>
<thead>
<tr>
<th>Member of family, age and control</th>
<th>PT (s)</th>
<th>APTT (s)</th>
<th>TT (s)</th>
<th>RT (s)</th>
<th>Fbg (Clauss method, g/L)</th>
<th>Fbg (Laurrell method, g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proband (26) II–3</td>
<td>&gt; 300</td>
<td>&gt; 300</td>
<td>&gt; 300</td>
<td>&gt; 150</td>
<td>Not detected</td>
<td>0.0</td>
</tr>
<tr>
<td>Older sister (32) II–2</td>
<td>12.4</td>
<td>31</td>
<td>15</td>
<td>20</td>
<td>1.6</td>
<td>1.4</td>
</tr>
<tr>
<td>The oldest sister (36) II–1</td>
<td>16.2</td>
<td>27</td>
<td>15</td>
<td>19</td>
<td>1.8</td>
<td>1.5</td>
</tr>
<tr>
<td>Father (62) I–1</td>
<td>12.3</td>
<td>28</td>
<td>16</td>
<td>19</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Mother (60) I–2</td>
<td>11.8</td>
<td>27</td>
<td>16</td>
<td>18</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Normal control</td>
<td>10.4–12.6</td>
<td>22–32</td>
<td>15–22</td>
<td>16–22</td>
<td>2.0–4.0</td>
<td>2.0–4.0</td>
</tr>
</tbody>
</table>

Legends: PT - prothrombin time, APTT - activated partial thromboplastin time, TT - thrombin time, RT - reptilase time – Fbg - fibrinogen
References


MOLECULAR GENETIC ANALYSIS OF ADENOID CYSTIC CARCINOMA OF SALIVARY GLANDS

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Co-author: L. Hauer
Tutor: Alena Skálová

Introduction
Adenoid cystic carcinoma of the salivary gland (AdCC) is slowly, but steadily growing and recurrent carcinoma which often results in fatal outcome. AdCC is well known for its absence of reliable prognostic markers and successful therapy. Translocation t(6;9)(q22-23;p23-24) resulting in the fusion of MYB and NFIB genes is typical for AdCC\(^1,2\). Also, deletion of 1p36 locus can serve as a prognostic marker in various malignancies\(^3,4\).

Aim
To identify the frequency of t(6;9)(q22-23;p23-24) and the frequency of 1p36 deletion in a cohort of 27 patients with AdCC and to compare these results with available clinical data.

Methods
Among 1696 salivary gland carcinomas in Registry of Šikl’s Pathology Department in Pilsen, 268 cases of AdCC have been identified. In this study, 27 patients with AdCC, who were treated in Faculty Hospital in Pilsen between years 1986 - 2016, were examined using FISH probes detecting breaks in MYB, NFIB genes, and MYB-NFIB fusion gene, respectively. The presence of fusion transcript MYB-NFIB was detected by RT-PCR. Deletion of locus 1p36 was also examined by FISH. These results were correlated with clinical data.

Results
Break of the MYB gene has been detected in 18/24 (75%) cases, break of the NFIB gene in 20/23 (87%) cases and fusion gene MYB-NFIB has been detected in 19/21 (90%) cases. Fusion transcript of the MYB-NFIB gene has been detected in 5/18 cases. 1p36 locus deletion has been detected in 3/23 (13%) cases. Results of this study are summarized in Table 1.

Discussion
Taken together, the MYB-NFIB fusion has been identified in 19/24 (79%) cases. In two cases, break of NFIB was present in absence of break of the MYB gene. This indicates the presence of other fusion partner than MYB. In one of these two cases, break of MYBL1 gene has been identified. Fusion partner in second case is currently unknown. The presence of MYB-NFIB fusion in concurrent absence of MYB and NFIB breaks indicates complex chromosomal rearrangement. All of three patients with deletion of 1p36 died of disease soon after diagnosis.

Conclusion
Detection of MYB-NFIB fusion can be used as a diagnostic marker of difficult cases of AdCC departing from conventional histomorphology. Considering the fact, that all three patients with 1p36 deletion has died of disease in a short time after diagnosis, the deletion of 1p36 locus
might be used as an adverse prognostic marker of AdCC. Further research should be accomplished to confirm our findings.

**Summary**

We have confirmed the role of t(6;9)(q22-23;p23-24) as differential diagnostic tool in the diagnosis of AdCC. Deletion of 1p36 was identified as a possible adverse prognostic marker of AdCC. This study was supported by grant SVV 2016 No.260 286.

**Table 1:** Molecular genetic analysis of salivary adenoid cystic carcinomas.

<table>
<thead>
<tr>
<th>No</th>
<th>1p36 deletion</th>
<th>MYB ba</th>
<th>NFIB ba</th>
<th>MYB/NFIB fusion</th>
<th>PCR MYBF</th>
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</thead>
<tbody>
<tr>
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<td>+</td>
<td>+</td>
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</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>Neg.</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Neg.</td>
</tr>
<tr>
<td>4</td>
<td>Neg.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>5</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>6</td>
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<td>+</td>
<td>+</td>
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</tr>
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<td>///</td>
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<tr>
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<td>Neg.</td>
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<td>+</td>
<td>///</td>
<td>///</td>
</tr>
<tr>
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</tr>
<tr>
<td>20</td>
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<td>Neg.</td>
<td>Neg.</td>
<td>///</td>
<td>///</td>
</tr>
<tr>
<td>21</td>
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<td>N/A</td>
<td>N/A</td>
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</tr>
<tr>
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<td>+</td>
<td>+</td>
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**Legend:**
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- **N/A** not-analyzable
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- **ba** break-apart
References


LABEL-FREE QUANTIFICATION OF ENDOGENOUS PEPTIDES RELATED TO INFECTIOUS INFLAMMATION IN PPROM PREGNANCIES

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Introduction
Infectious inflammation caused by a microbial invasion into the amniotic cavity followed by a histological chorioamnionitis (MIAC/HCA) complicates a significant portion of pregnancies with preterm prelabour rupture of the membranes (PPROM). Robust tools for prompt detection of MIAC/HCA are thus required to improve the management of PPROM pregnancies (particularly in low-gestational ages) and to prevent the fetal immune system from developing fetal inflammatory response syndrome (Gotsch et al., 2007). Amniotic fluid is a dynamic milieu closest to fetus, when protein and peptide presence makes it an attractive analytical material for a biomarker discovery using mass spectrometry-based proteomics. Our recent study revealed dysregulation in a large number of amniotic fluid proteins potentially associated with MIAC/HCA in PPROM, including several distinct proteases (Tambor et al., 2012). The up-regulation of proteases suggests that specific endogenous peptides might be generated due to the ongoing pathological process. Hence, the aim of our work was to develop a robust protocol for the enrichment of endogenous peptides from amniotic fluid and apply it for analysis of peptidome changes in PPROM women related to presence of MIAC/HCA.

Methods
The protocol for the endogenous peptides enrichment was based on denaturation of amniotic fluid samples and ultrafiltration. The procedure was exploited to characterize the amniotic fluid peptidome associated with MIAC/HCA in PPROM pregnancies in a cohort of 40 women. The endogenous peptides were analyzed by a LC-MS/MS using an UltiMate 3000 RSLCnano LC combined with a Q-Exactive Plus mass spectrometer (both from Thermo Scientific). Obtained data were processed using Proteome Discoverer (Thermo Scientific) with integrated algorithm Peakjuggler for label-free quantification. Statistical evaluation was accomplished using program R and Perseus.

Results and Discussion
More than 8000 distinct amniotic fluid endogenous peptides were identified during the LC-MS/MS analyses. The majority of the peptides were generated from collagen chains I and III, implying an ongoing reconstruction of the fetal membranes. A comparative analysis subsequently revealed significant changes in amniotic fluid peptidome due to MIAC/HCA. Endogenous peptides derived from histone H2B protein forms showed the most profound changes. Beyond its role in nucleosome forming, H2B was proved to act as an antimicrobial agent in human placenta (Kim et al., 2002). Similarly, dermcidin peptides dysregulated in the presence of MIAC/HCA were found (Burian and Schittek, 2015). Of note were further peptides resulted from insulin-like growth factor-binding protein that is directly associated with PPROM (Abdelazim, 2014).
Conclusions
More than 8000 endogenous peptides were identified across all amniotic fluid samples, making thus the enrichment protocol viable. Moreover, a portion of endogenous peptides was found to be significantly due to MIAC/HCA, deserving thus more targeted focus.

References
DIFFERENTIAL EXPRESSION OF THE HOMOLOGOUS RECOMBINATION DNA REPAIR GENES IN EARLY AND ADVANCED STAGES OF MYELODYSPLASTIC SYNDROME

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Background and Aims
The high incidence of mutations in patients with myelodysplastic syndrome (MDS) (1) strongly suggests an implication of defective DNA repair mechanisms (2) in the MDS pathogenesis. Based on the hypothesis that genetic changes are amplified during evolution of MDS-cell clone and disease progression (3); we investigated abnormalities in DNA repair gene expressions and monitored their possible development during MDS progression. Further, we focused on sequencing of selected DNA repair genes and searched for their mutations associated with the disease. (4)

Methods
First, gene expression of 84 DNA repair genes in bone marrow (BM) CD34+ cells of 18 MDS patients was measured by RT² Profiler PCR Arrays (Qiagen). Validation of expression data in selected genes was performed on a cohort of 100 MDS patients. Moreover, paired samples from 15 patients with disease progression were used for monitoring of \textit{RAD51} and \textit{XRCC2} gene expressions in the course of disease. Mutational analysis was performed on 84 DNA repair genes in 16 patients by targeting next generation sequencing (NGS) (SeqCap EZ System, NimbleGen). Detected mutation was confirmed by Sanger sequencing. Multivariate analysis using a Cox regression model to determine the independent impact of each variable (BM blasts, hemoglobin, neutrophils, platelet count, karyotype, and gene expressions of \textit{RAD51} and \textit{XRCC2}) examined for overall survival (OS) was done.

Results
\textit{RAD51} (p<0.0001) and \textit{XRCC2} (p<0.0001) genes showed differential expression between low-risk, high-risk patients and control samples (Figure 1A). Patients with high expression level of \textit{RAD51} gene had significantly longer OS to those with low level (median: 108.4 vs. 21.9 months; p<0.0001; HR=0.26) (Figure 1B). The expression of \textit{RP43} gene was decreased (FC=-2.65) in all MDS patients (p<0.0001). Down-regulated expression of \textit{XRCC2} gene, located on 7q36.1, correlated with monosomy 7 or partial deletion of the long arm of chromosome 7 (7q-) which are associated with a poor prognostic category. Thus, \textit{XRCC2} gene may be a candidate leukemogenic gene. Furthermore, expressions of \textit{RAD51} and \textit{XRCC2} genes were measured in paired samples of patients with disease progression; the expressions were gradually decreased along the stepwise progression (from initial to advanced stages of MDS) (Figure 2). Multivariate analysis identified high expression level of \textit{RAD51} gene (HR 0.49; p=0.01) and cytogenetic category (HR 1.68; p=0.002) as significant prognostic factors for OS. Interestingly, overexpression of \textit{RAD51} and \textit{XRCC2} was observed in patients with advanced disease progression to AML with myelodysplasia with 35-40% of BM blasts. This might indicate the evolution of highly resistant clone with an increased function of DNA repair.
A heterozygous frameshift mutation caused by a deletion of two base pair in codon 263 of XRCC2 gene (XRCC2: c.789_790delCA) was detected in one patient. CD3+ T-cells were used as control to distinguish germline mutation from somatic one. The result indicated the germline mutation. XRCC2 expression was significantly down-regulated in this case (FC= -5.04).

Conclusions and Summary
Our study demonstrates that an alteration of DNA repair factors, mainly RAD51 and XRCC2, key mediators in the homologous recombination repair of DNA double strand breaks, may contribute to the pathogenesis of MDS. The gene expression of RAD51 and cytogenetic category were shown to be the strongest independent prognostic factors for OS. The inappropriate function of DNA repair in CD34+ cells seems to be progressive in the course of disease, allowing formation and accumulation of new mutations during MDS clone evolution resulting in development of highly resistant leukemic cells. Notably, we described the new germinal mutation in XRCC2 repair gene associated with MDS.

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Figure 1: RAD51 gene mRNA expression
(A) Differential mRNA expression of RAD51 gene between MDS stages.
(B) Kaplan–Meier curves of overall survival (OS) according to mRNA expression of RAD51 gene.
Figure 2: *RAD51* mRNA expression in selected MDS patients with the progression.

References


SENEGENCE IS MODULATED BY THE UPR RESPONSE IN OVARIAN SURFACE EPITHELIAL CELLS

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Introduction and Aims

Endoplasmic reticulum (ER) is a multifunctional organelle responsible for transmembrane and secreted protein synthesis and folding. Disruption of ER function triggers a protective machinery called the unfolded protein response (UPR). UPR pathways then arrest the RNA translation and increase the production of ER chaperones to alleviate ER stress, or, if the stress cannot be resolved, UPR induces phenotypic change or apoptosis. We recently discovered a link between UPR activation and cancer progression in ovarian epithelial cancer1,2. This prompted us to investigate the role of UPR signaling in normal ovarian surface epithelium (OSE), a major source of ovarian malignancies.

Cellular senescence is a complex cell phenotype, whereby cells irreversibly cease to divide after a number of passages. It can be induced by telomere shortening, DNA damage, oxidative stress and activation of some oncogenes. Since senescence occurs as a stress response and was previously shown to be interconnected with ER stress3,4, in this work, we aimed to address the following questions:

• What are the expression patterns of key UPR mediators in mOSE cells upon senescence?
• Is ER stress the driving force of cellular aging and can it be used to modulate senescence?
• What are the molecular links between ER-stress, senescence and epithelial plasticity of mOSE cells?

Materials and Methods

Mouse ovarian surface epithelial (mOSE) cells were isolated from 8-10 weeks old female mice. Briefly, aseptically dissected ovaries were incubated in 0.2% trypsin at 37 °C for 30 minutes. Isolated mOSE cells were maintained in DMEM (high glucose, GlutaMAX) supplied with 10% FBS, 1% ITS, 500 ng/ml hydrocortisone and 10 ng/ml EGF. Identity of mOSE cells was confirmed by immunofluorescent staining with epithelial and mesenchymal markers (E-Cadherin, N-Cadherin, Vimentin and Cytokeratin 8).

Cells were passaged regularly each 3 or 4 days. Expression of UPR and senescence markers was assessed by immunoblotting (Fig. 1A) and qPCR (Fig. 1B) after each passage.

Next, ER stress was induced and/or modulated in isolated mOSE cells by treatment with tunicamycin (0.5 µM), salubrinal (20 µM) or TUDCA (500 µM) for indicated time periods. Expression of UPR, EMT and senescence markers was evaluated by immunobloting (Fig. 1C) and qPCR (Fig. 1D) after 24-hour treatment. To study the effect of ER stress on senescence modulation, mOSE cells of low (2) and high (4) passage were used and number of senescent cells was determined by senescence-associated β-galactosidase staining after 24 and 72 hours of treatment (Fig. 2). β-Galactosidase staining was performed using the Senescence Cells Histochemical Staining Kit (Sigma-Aldrich) according to the manufacturer’s instructions. Cell proliferation was evaluated by real-time impedance based xCELLigence RTCA assay2, cellular impedance was continuously measured for up to 119 hours.
Results
OSE forms a simple layer of squamous-to-cubic cells that express markers of both epithelial and mesenchymal phenotypes. We confirmed the identity of isolated mOSE by immunofluorescent staining. Next, we found, that aging mOSE tend to ectopically elevate the expression of UPR markers (BiP, CHOP, Ire1α, TUSC3) in correlation with increasing number of passages (Fig.1A, 1B). Induction of ER stress by tunicamycin also increased expression of these markers and neither TUDCA nor salubrinal had any significant impact (Fig. 2). Additionally, tunicamycin treatment increased markers of senescence (p21) and EMT (Slug, N-Cadherin) (Fig. 2C, 2D). Proliferation of mOSE cells as a function of cell index over time was determined by the xCELLigence RTCA system. Tunicamycin treatment significantly reduced the cell proliferation after 24-hour long incubation and both TUDCA and salubrinal also slightly lowered cellular proliferation. Surprisingly, β-galactosidase staining showed lower portion of senescent cells after ER stress induction by tunicamycin at both low and high passage, while TUDCA treatment resulted in decreased number of senescent cells only at higher passage and salubrinal caused increase percentage of senescent cells at low passage (Fig. 2).

Fig. 1: Representative images from immunoblotting (A, C) and qPCR results (B, D).
Discussion
OSE is a highly dynamic structure undergoing regular cycles of reparation during fertile period while it is also exposed to multiple intrinsic or extrinsic signals varying from oxidative stress and DNA damage to growth or stress factors. Endoplasmic reticulum integrates responses to diverse kinds of cellular stress and triggers UPR. Recent data reported correlation between UPR activation and replicative or stress-induced premature senescence in cultured human cells and cancer. Despite the fact that senescence has been considered a tumor suppressive mechanism, abnormal accumulation of senescent cells may strongly alter tissue microenvironment and even support cancer development.

Our results indicate that mOSE activates UPR signaling upon entering senescence while ER stress induction by tunicamycin halts cell proliferation and causes increased expression of senescent marker p21 responsible for inhibition of cyclin-dependent kinases and growth arrest. Surprisingly, β-galactosidase staining of senescent cells showed lower percentage of stained cells after both ER stress induction (tunicamycin) and inhibition (TUDCA). ER stress in mOSE also upregulated markers of epithelial-to-mesenchymal transition suggesting a link between ER stress, senescence and cellular plasticity.

Conclusion
In conclusion, our results indicate a biologically relevant link between UPR and senescence of OSE cells that may contribute to better understanding of ovarian and age-related pathologies extending the portfolio of druggable molecular targets.

Fig. 2: Percentage of β-galactosidase-stained cells in mOSE cells of low (A) and high (B) passage.

Fig. 3: Scheme of the overall hypothesis (A). Transmission electron micrograph of ER (indicated by white arrows) in mOSE cell (B).
Summary
Endoplasmic reticulum is a major cell organelle responsible for transmembrane and secretory protein synthesis. ER is also involved in maintaining cellular homeostasis in various physiological or pathological scenarios. The accumulation of misfolded or unfolded proteins in the ER triggers an evolutionary conserved cellular response, known as the unfolded protein response (UPR). The UPR then attenuates mRNA translation and increases the production of ER chaperones and mediators to abrogate ER stress or to evoke phenotypic change or apoptosis on cellular level. UPR may be evoked intrinsically by proteosynthesis overload or extrinsically by alterations of normal tissue microenvironment, by e.g. hypoxia, inflammation, or even cytotoxic therapy. We identified recently that genes coding for components of enzymatic machinery in ER are involved in tissue maintenance and epithelial cancer progression.

In our study, the ER-associated tumor suppressor candidate 3 (TUSC3) mediated ER stress and UPR in ovarian cancer (OC) cells and its loss correlated with shorter survival of OC patients and extensive tumor growth in mice model in vivo. Loss of TUSC3 in vitro was followed by epithelial-to-mesenchymal transition (EMT) and enhanced proliferation of OC cells. This prompted us to investigate the role of UPR signaling in normal ovarian surface epithelium, a major source of ovarian malignancies. Using mouse OSE we demonstrated that ER stress is linked to cellular senescence and phenotypic plasticity. Induction of senescence lead to upregulation of UPR hallmark proteins and interestingly, modulation of ER stress by various chemical compounds and chaperons, overridden the onset of senescence.

Acknowledgements
This work was supported by the project from Masaryk University no. MUNI/A/1352/2015, the project no. LQ1605 from the National Program of Sustainability II (MEYS CR) and by the project FNUSA-ICRC no. CZ.1.05/1.1.00/02.0123 (OP VaVpI).

References
ACUTE LIVER INJURY: ROLE OF EXTRACELLULAR DNA

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Introduction

DNA in cells is packed in the nuclei and mitochondria. When cells die, DNA can get outside of the cells and is called cell-free or extracellular DNA (ecDNA). Elevated concentrations of ecDNA in plasma are associated with tissue damage. It has been shown that trauma causes an increase in ecDNA and this can lead to sepsis-like syndrome.1 Besides nuclear genomic DNA ecDNA contains also mitochondrial DNA (mtDNA). Mitochondria are evolutionary prokaryotes and their DNA shares several characteristics with bacterial DNA including unmethylated CpG islands.2 Extracellular mtDNA as a damage-associated molecular pattern is recognized by pattern recognition receptors such as the toll-like receptor 9 and induces an inflammatory response.2 Deoxyribonuclease (DNase) is an enzyme that cleaves ecDNA and can prevent the inflammation induced by ecDNA.3 DNase can degrade neutrophil extracellular traps, but also mtDNA that is not protected by histones.4 Drug-induced liver injury causes hepatocyte necrosis and DNA release into the extracellular space. This DNA has been shown to induce sterile liver inflammation.5 However, it is not clear whether the released DNA increases plasma ecDNA that might potentially induce systemic effects and distant organ failure. The aim of this study was to quantify plasma ecDNA in an animal model of acute liver injury. An additional goal was to analyse renal consequences of TAA administration and to prove the effects of systemic DNase treatment in TAA-induced acute liver injury.

Methods

Animals. Adult male Wistar rats were used and kept under controlled laboratory conditions with ad libitum access to standard diet and tap water. The animal study was approved by the Ethics Committee of Institute of Molecular Biomedicine, Comenius University.

Model of acute liver injury. Animals were randomized into three groups, TAA (n=10), TAA+DNASE (n=10) and control group (n=10). TAA is hepatotoxic agent causing centrilobular necrosis widely used to model drug-induced liver injury, as described.5 TAA dissolved in warm saline was injected intraperitoneally twice 24 hours apart. DNase I (10 mg/kg) was administrated intravenously two hours after each TAA injection. Control animals were injected with saline intraperitoneally and intravenously. Rats were sacrificed six hours after the second TAA injection. Blood was collected from aorta into EDTA and heparin tubes. Samples of urine were taken from the urinary bladder by puncture.

Biochemical analysis. Plasma alanine aminotransferase (ALT) and creatinine were measured using COBAS C6000 analyzer at the Department of Clinical Biochemistry and Hematology, St. Michal Hospital, Bratislava. Urine creatinine was measured according to Jaffé.6 Urinary proteins were measured with the pyrogallol red-molybdate complex according to the method modified by Watanabe et al.7 Blood urine nitrogen (BUN) was measured using Urea nitrogen colorimetric detection kit and creatinine using Creatinine serum detection kit according to the protocol of the manufacturer.
Quantification of ecDNA. Cell-free plasma was obtained from whole-blood collected into EDTA tubes after centrifugation for 10 minutes at 10000 g. Total extracellular DNA was isolated from 200 µl plasma. Total ecDNA was quantified using the Qubit 2.0 Fluorometer. For SYBR green quantitative real-time polymerase chain reaction targeting circulating nuclear ecDNA were used primers for glyceraldehyde 3-phosphate dehydrogenase (forward – GAAATCCCCTGGAGCTCTGT and reverse – CTGGCACCAGATGAAATGTG). MtDNA was quantified using primers targeting the cytochrome B forward – CCTCCCATTCATTACGGCCCGCTTGC and reverse – ATTTTGCTCTGCGTCGGAGTT. Real-time PCR standard curves were created using mtDNA extracted from the isolated mitochondria using of liver tissues.

Statistical analysis. Comparisons between groups were analysed with one-way ANOVA and Bonferroni-modified post-hoc t-test using software Prism 6.0.

Results
TAA hepatotoxicity was proved using significantly elevated liver enzyme and bilirubin in TAA-injected rats in comparison with the controls (Fig. 1, a) ALT F=7.71, p=0.002 b) bilirubin F=15.1, p=0.001).

The TAA hepatotoxic effect was partially ameliorated by intravenous application of DNase. DNase treatment of TAA-injected rats resulted in lower average values of measured ALT when compared to the TAA group (ALT 46%). The results in the TAA+DNASE group were not statistically different from the control group (ALT t=2.27). DNase significantly reduced plasma bilirubin levels when compared with the TAA group (t=4.13, p=0.001).

Figure 1: Biochemical parameters of liver damage and inflammation. Alanine transaminase (ALT) (A), Bilirubin (B). Control (CTRL), thioacetamide (TAA), Deoxyribonuclease (DNase). *p<0.05; **p<0.01; ***p<0.001

Thioacetamide-induced kidney injury manifested as higher plasma creatinine in TAA-treated rats (Fig. 2, a) F=10.17, p<0.001) and increased BUN (Fig. 2, b) F=10.4, p<0.001). DNase treatment of TAA-injected rats caused 30% reduction of plasma creatinine (t=3.22, p=0.01) in comparison to TAA-group. Mean BUN was slightly lower in the DNase-treated group when compared to TAA-injected rats (t=1.01, p=0.95).
Figure 2: Biochemical markers of renal function. Creatinine (A). Blood urea nitrogen (BUN) (B). Control (CTRL), thioacetamide (TAA), deoxyribonuclease (DNase). **P<0.01; ***P<0.001.

Total ecDNA in plasma were higher after TAA-induced liver damage (Fig. 3, a) total ecDNA $F=7.06, p=0.003$, b) nuclear $F=5.02, p=0.01$, c) mitochondrial ecDNA $F=3.76, p=0.03$). DNase treatment of TAA-injected rats reduced the concentrations of total ($t=1.58, p=0.37$) and mitochondrial ($t=1.64, p=0.33$) ecDNA without statistical significance. Concentrations of total and mitochondrial ecDNA were not significantly different from control group (total ecDNA $t=2.11, p=0.13$, mitochondrial ecDNA $t=0.84, p>0.99$). DNase treatment did not reduced the ecDNA concentration of nuclear origin ($t=0.21, p>0.99$) in comparison to TAA group.

Figure 3: Concentrations of extracellular DNA (ecDNA). Total DNA(A). Nuclear DNA(B). Mitochondrial DNA (C). Control (CTRL), thioacetamide (TAA), deoxyribonuclease (DNase). *p<0.05; **P<0.01.

Discussion

We found that in thioacetamide model of acute liver injury the ecDNA in plasma increases in TAA group in comparison to the controls. The lower total ecDNA in rats treated with DNase must be caused by the marked decrease of mtDNA in plasma so that the treated group TAA+DNase did not significantly differ from the control group in plasma mtDNA concentrations. Our results do not reveal the cause for this differential effect of DNase treatment on the ecDNA fractions. It can only be hypothesized that the cause is that mtDNA is not protected by histones and can, thus, be more efficiently cleaved by the administered DNase.

The treatment with DNase had a hepatoprotective effect as shown by improved liver enzymes and lower bilirubin concentrations. This is in line with the published findings showing a DNase hepatoprotective effect in models of liver injury. Since the increased ecDNA in the liver does not help to explain systemic effects of the liver injury we have focused on circulating DNA rather than
on ecDNA in the liver. Our results suggest that the increased ecDNA in plasma could affect other organs and might, thus, be important for the development of the hepatorenal syndrome. Thioacetamide is known to cause renal toxicity. Rats in the TAA group showed marked renal impairment as seen on higher creatinine and BUN. The renal damage seemed to be ameliorated by DNase treatment. This confirms previous findings on the role of ecDNA especially of mitochondrial origin in a model of sepsis-induced acute kidney injury.

The increase of ecDNA might be due to the release from hepatocytes, but also due to lower clearance by the liver or the kidney.

**Conclusions**

Our results suggest that ecDNA, especially mtDNA plays a role in the pathogenesis of hepatorenal toxicity of TAA. We show for the first time that DNase ameliorates both, liver and renal injury induced by TAA administration, likely by reducing the circulating concentrations of mitochondrial rather than the nuclear ecDNA.

**Summary**

Several recent studies have shown that liver injury is associated with the release of DNA from hepatocytes. This DNA stimulates innate immunity and induces sterile inflammation exacerbating the liver damage. Similar mechanisms have been described in acute renal injury. Deoxyribonuclease degrades cell-free DNA and can potentially prevent some of the induced tissue damage. In this study the effects of thioacetamide-induced hepatorenal injury on plasma DNA in rats were analysed. Plasma DNA of both, nuclear and mitochondrial origin was higher in thioacetamide-treated animals. Administration of deoxyribonuclease resulted in a decrease of plasma DNA of mitochondrial and not of nuclear origin. This was accompanied by a marked decrease of liver enzymes as well as creatinine and urea as markers of renal function. In conclusion, our experiment confirmed the hepatotoxic and nephrotoxic effect of thioacetamide. The associated increase in cell-free DNA seems to be involved in the pathogenesis as treatment with deoxyribonuclease resulted in a partial prevention of the hepatorenal injury.

**References**


EXPLORING THE ROLE OF METALS IN WOUND REPAIR

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Introduction
In response to damage our skin invokes a highly dynamic repair programme. Essential for effective repair is activation of the innate immune response, where a diverse range of immune cells orchestrate healing and remove unwanted pathogens. Co-ordinated temporo-spatial regulation of keratinocyte, fibroblast and endothelial cell function leads to wound closure, restoration of dermal tissue and generation of new blood vessels (Broughton et al., 2006). While a plethora of studies have identified genomic, transcriptional and even epigenetic regulators of healing, few studies to date have explored the role of global metal profiles in the events leading to skin repair (Lansdown et al., 2007).

Metal ions and metal complexes are ubiquitous throughout the body, where they control diverse cellular signalling pathways. Indeed, alterations in the global tissue distribution of metals, known as the metallome, results in diabetes, cardiovascular, neurodegenerative and a host of other disorders (Carboni et al., 2015). These chronic degenerative conditions share defective cellular processes also linked to wound pathology, e.g. immune cell dysfunction (Elsholz et al., 2014). Pathological wound healing remains a major area of clinical unmet need. Chronic, non-healing wounds, prevalent in the elderly and diabetic, cause increased patient morbidity and significant financial costs to healthcare providers. Current treatments for chronic wounds are inadequate, often addressing secondary symptoms rather than the primary cause (Percival et al., 2015). At the tissue level chronic wounds are thought to be the result of defects to one or more cellular aspects of wound repair, however, much remains to be elucidated (Walsh et al., 2016). Here, we hypothesised that changes in the metallome play an important role in pathological wound healing. Thus, the aims of this project are to profile metals in normal and pathological wound healing, and functionally test the effects of manipulating specific metals during wound repair.

Methods
In vivo wounding. Female pathological healing (20 month old “aged” C57/BL6 and diabetic LEPR−/−) and normal healing (2 month old “young” C57/BL6 and lean LEPR+/+) control mice were anaesthetised and two full thickness 6mm excisional wounds made on the dorsum using sterile trace metal free titanium instruments. Wounds (n=3-5) were collected at 1, 3 and 7 days post-wounding, bisected and processed for subsequent analysis. Inductively coupled plasma mass spectrometry (ICP-MS). Samples were freeze-dried, digested overnight in trace metal free nitric acid/hydrogen peroxide with a certified reference material (DOLT-5 dogfish liver), and analysed using an Agilent 7500cx. Histological analysis. Paraffin -embedded samples were sectioned at 5µm. Haematoxylin and eosin (H&E) stained slides were used for wound measurements and Alizarin Red was used to visualise Ca. qPCR. RNA was extracted (TRIzol® reagent), reverse transcribed and amplified using MESA GREEN. Keratinocyte culture and wounding. HaCaT human keratinocytes were cultured in DMEM with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). Scratch wounds were performed on confluent cells using sterile 1ml pipette tips, stained with crystal violet at 24 and 48 hours post-scratching, and imaged microscopically. Macrophage culture and activation. Mononuclear phagocyte progenitor cells
were isolated from mouse bone marrow and cultured in DMEM with 10% FBS and 1% P/S. Differentiation was induced using L929 cell conditioned media. Macrophages were M1-polarised by stimulation with LPS and IFN-γ or M2-polarised by stimulation with anti-IFN-γ and IL-4. In vitro metal treatments. Metals were added directly to cell culture media at final concentrations ranging between 0.01mM and 10mM. Statistical analysis. One or Two-Way ANOVA with post-hoc analysis was performed as appropriate.

Results
Metallome profiling of normal and pathological repair. Recent methodological advances now permit highly sensitive tissue-specific quantification of metal ions (Dlouhy and Outten, 2013). In this study, ICP-MS profiling revealed that a number of metal species were temporally regulated during normal healing. Next, we profiled the metallome of two different mouse pathological healing models: A) 20 month old “aged” mice with corresponding 2 month old “young” controls and; B) diabetic (LEPR−/−) mice with corresponding lean (LEPR +/+) controls. Crucially, profiling metals in skin and wound tissue from these pre-clinical pathological wound models revealed major changes in the local metallome versus controls (Figure 1). Calcium, the most abundant metal in skin, was strongly induced upon acute wounding (P<0.01) yet substantially reduced in both chronic wound models (P<0.05; Figure 1). Profiling expression of calcium-regulated genes confirmed these observations. For example, calmodulin (Calm1) was strongly down-regulated in the wounds of aged mice but not young controls. Functional modulation of metal ions. In vitro studies revealed that exogenous treatment with specific metals directly modulates intrinsic cellular functions required for efficient wound repair. Calcium administration dose-dependently promoted keratinocyte scratch-wound closure (Figure 2). Moreover, calcium treatment effectively dampened pro-inflammatory M1 macrophage polarisation, promoting a tissue resolutory phenotype (Figure 3). Of note, the beneficial effects of calcium on M2 macrophage polarisation were attenuated in cells derived from chronic wound models (Figure 3).

Discussion
The majority of research pertaining to metal abundance within the skin is restricted to assessments of bioaccumulation following treatment or occupational exposure (Bianco et al., 2015). Only one prior study has attempted to characterise endogenous metal concentrations in wound tissue. Using flame atomic spectrophotometry on rat wounds, Lansdown et al., (1999) reported changes in zinc, copper, magnesium and calcium throughout normal healing. Here, using the far more sensitive technique of ICP-MS we demonstrate changes in a number of additional metals, and crucially show that metals are altered in murine pathological healing models. In the case of calcium, we note with interest that calcium-regulated genes follow the ICP-MS profile for the metal ion. One such gene, Calm1, encodes the cytosolic Ca2+–binding protein calmodulin. Intriguingly, binding of calmodulin to plectin 1a in keratinocytes is important for integrin phosphorylation, thus enabling cell motility (Kostan et al, 2009).

Conclusions
These studies demonstrate clear and significant changes in the skin metallome across normal wound repair. Altered levels of specific metals in mouse chronic wounds suggest a direct association with wound pathology, while in vitro studies demonstrate direct effects on cellular functions important in wound repair.

Summary
Metal ions and metal-containing compounds are widespread in the environment and are essential for life on Earth. For the first time, our results illustrate fascinating changes in the metallome of normal and chronic wounds, and link these to functional changes in skin cells following exogenous metal treatments. These data now provide an unprecedented opportunity to elucidate the crucial roles metals play in the orchestrated wound repair response. Future studies will ascertain
the temporo-spatial distribution of key metal species during acute and pathological repair, and explore the mechanistic links to healing regulation. Ultimately, findings from my PhD should aid the development of novel therapeutics for chronic wounds.

Figure 1. ICP-MS (inductively coupled plasma mass spectrometry) revealing substantial changes in the metal profile of delayed healing wounds. Representative fold change in the global profile of metals between young and aged (Y-axis) and lean and diabetic (Db, X-axis) skin (A) and wounds (B). Illustrative macroscopic wound images are also given (C), where the black, dotted line illustrates overall wound size. Metal sphere size demonstrates the abundance of each metal in a normal-healing young murine mouse model.

Figure 2. Modulation of calcium on human keratinocyte (HaCaT) scratch migration in vitro. Calcium treatment of HaCaTs (A) with representative scratch migration images (4X magnification) at 24 hours (B) and 48 hours (C). Black arrows depict “wound” edges (B and C). Mean +/- SEM, n=4 per group, bar = 500µm (B, C), * = P<0.05, ** = P<0.01 and *** = P<0.001.
Figure 3. Calcium alters macrophage polarisation in normal and pathological healing. Relative expression of macrophage polarisation markers is shown for young (A and B) and aged (C and D) models. Classical activation (M1) is illustrated by pro-inflammatory Nos2 expression (A and C), while alternative activation (M2) is demonstrated by anti-inflammatory Ym1 expression (B and D). “M0”, “M1” and “M2” represent non-polarised, M1-polarised and M2-polarised macrophages, respectively. Macrophages were treated with no calcium (no metal), 0.1mM and 1mM calcium. Calcium treatment significantly dampened pro-inflammatory responses in young macrophages. ND = not done. Mean +/- SEM, n=3 per treatment group, * = P<0.05, ** = P<0.01 and *** = P<0.001.

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