New Frontiers in the Research of PhD Students

Conference of Medical Schools

Hradec Králové
November 26 – 27, 2015
Charles University in Prague,
Faculty of Medicine in Hradec Králové
12th INTERNATIONAL MEDICAL POSTGRADUATE CONFERENCE

New Frontiers in the Research of PhD Students
Conference of Medical Schools

November 26 – 27, 2015

Organized by
Charles University in Prague, Faculty of Medicine in Hradec Králové

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Under the auspices of his Magnificence,
Rector of the Charles University in Prague
Prof. MUDr. Tomáš Zima, DrSc.

Hradec Králové
Educational Centre of the Faculty of Medicine,
location: University Hospital
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Venue:

Educational Centre
Charles University, Faculty of Medicine
University Hospital

Conference office:

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

Thursday, November 26, 9:30 – 17:50
Friday, November 27, 8:30 – 16:10

Official language:

English

Presentation time:

Lecture 15 min
Discussion 5 min

Accommodation of participants:

Hotel Nové Adalbertinum
Velké náměstí 32
500 03 Hradec Králové 2
Dear friends and colleagues,

I would like to welcome you to the 12th 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 and Slovak Republics, 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 PhD 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!

Prof. MUDr. RNDr. Miroslav Červinka, CSc.
Dean, Faculty of Medicine in Hradec Králové
Charles University in Prague
### Thursday – November 26, 2015

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<td>Meeting of the Evaluation Committee</td>
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<td>10:40 – 11:00</td>
<td>Opening of the Conference</td>
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<td>11:00 – 13:00</td>
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<td>16:10 – 17:50</td>
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<td>20:00 – 22:00</td>
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<td>19:30 – 22:00</td>
<td>Social evening, Awards, Closing ceremony</td>
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THURSDAY, NOVEMBER 26

Part I
Chair: prof. RNDr. Jan Krejsek, CSc.

11:00  R. Atkinson (Hull): PROBING MITOCHONDRIAL FUNCTION IN VIVO
11:20  J. Brůha (Plzeň): COMPARISON OF PERCUTANEOUS AND OPEN APPROACH OF RADIOFREQUENCY ABLATION FOR COLORECTAL LIVER METASTASES IN TEACHING HOSPITAL PILSEN IN YEARS 2001-2015
11:40  S. Smolárová (Martin): SURFACTANT PROTEIN B HAPLOTYPES AND RESPIRATORY DISTRESS SYNDROME IN NEONATES
12:00  P. Sauerová (Plzeň): DEVELOPMENT AND IN VITRO TESTING OF NEW COMPOSITE MATERIALS FOR BONE SURGERY APPLICATIONS
12:20  M. Viktorinová (Praha): qEEG CORRELATES OF EMOTIONAL PROCESSING IN PATIENTS WITH BIPOLAR AFFECTIVE DISORDER AND HEALTHY CONTROLS
12:40  S. Dodd (Liverpool): BOBO – BIOMARKERS OF BARRETT’S OESOPHAGUS

Part II
Chair: prof. MUDr. Martina Řezáčová, Ph.D.

14:00  T. Dorňák (Olomouc): POSTERIOR VS ANTERIOR CIRCULATION INFARCTION; DEMOGRAPHY, OUTCOMES, AND FREQUENCY OF HEMORRHAGE AFTER THROMBOLYSIS
14:20  B. Englinger (Vienna): RESISTANCE MECHANISMS OF FGFR1-DRIVEN SMALL CELL LUNG CANCER AGAINST THE FGFR INHIBITOR BIBF-1120
14:40  I. Fabrik (Hradec Králové – University of Defense): MAPPING OF DENDRITIC CELL SIGNALING EVENTS DURING THE FIRST HOUR OF INFECTION BY FRANCISELLA TULARENSIS
15:00  D. G. Ferreira (Porto): ADENOSINE A2A RECEPTOR: A PROMISSING TARGET IN PARKINSON’S DISEASE-RELATED COGNITIVE DEFICITS
15:20  V. Fisi (Pécs): IMPACT OF PROTEIN O-GLCNAC MODIFICATION ON CELL VOLUME REGULATION
Part III
Chair: prof. MUDr. Jan Čáp, CSc.

16:10 F. Istel (Vienna): A GENOME-SCALE COLLECTION OF GENE DELETION MUTANTS IN THE HUMAN FUNGAL PATHOGEN C. GLABRATA
16:30 M. Kolářová (Praha): QUANTIFICATION OF ENDOGENOUS ANTIBODIES AGAINST NEURONAL PROTEIN ASSOCIATED WITH ALZHEIMER’S DISEASE IN HUMAN SERUM AND CSF
16:50 K. Kratochvílová (Brno): TUSC3 LOSS ENHANCES THE TUMOR GROWTH OF OVARIAN CANCER
17:10 B. Krausová (Praha): NMDA RECEPTOR CHANNEL VESTIBULE: SITE OF ACTION FOR INHIBITORY NEUROSTEROIDS
17:30 Ch. Kypridemos (Liverpool): AN OPPORTUNITY TO REDUCE THE BURDENS OF CARDIOVASCULAR DISEASE AND GASTRIC CANCER CAUSED BY DIETARY SALT: IMPACTNCD MICROSIMULATION STUDY

FRIDAY, NOVEMBER 27

Part IV
Chair: prof. MUDr. Milan Bayer, CSc.

9:00 Z. Nedelská (Praha): SPATIAL NAVIGATION IMPAIRMENT IS PROPORTIONAL TO HIPPOCAMPAL ATROPHY IN SUBJECTS WITH ALZHEIMER’S DISEASE
9:20 M. Urík (Brno): CHANGES OF STRUCTURE OF THE TYMPANIC MEMBRANE DURING ITS TRANSFORMATION TO RETRACTION POCKET IN CHILDREN
9:40 E. Liluashvili (Tbilisi): PRIMARY LACTOSE DEFICIENCY AMONG MALNOURISHED CHILDREN WITH PERSISTENT DIARRHEA ADMITTED TO GLOBALMED PEDIATRIC CLINIC; TBILISI; GEORGIA
10:00 J. Maciag (Krakow): INFLUENCE OF DENTURE-RELATED STOMATITIS ON CLINICAL MEASURES OF VASCULAR DYSFUNCTION AND SYSTEMIC CELLULAR IMMUNE RESPONSE
10:20 N. Masiukovich (Tbilisi): MATERNAL STRESS INFLUENCE ON CHILD’S GLOBAL DEVELOPMENT
10:40 H. L. Moody (Hull): MICRONORMA-31 REGULATES CHEMOSENSITIVITY IN MALIGNANT PLEURAL MESOTHELIOMA VIA ALTERATIONS IN INTRACELLULAR ACCUMULATION
SCIENTIFIC PROGRAMME

Part V
Chair: prof. MUDr. Stanislav Mičuda, Ph.D.

11:30  I. Padjen (Zagreb): RENAL DISORDER IS MORE FREQUENT AMONG DECEASED PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) – RETROSPECTIVE ANALYSIS FROM A CROATIAN TERTIARY CENTER
11:50  O. Sobotka (Hradec Králové): TOXIC EFFECT OF 3-BROMOPYRUVATE ON RAT HEPATOCYTES IN VITRO
12:10  S. Školeková (Bratislava): MESENCHYMAL STROMAL CELLS PLAY AN IMPORTANT ROLE IN THE TUMOR MICROENVIRONMENT
12:30  R. Štichhauer (Hradec Králové): POSSIBILITIES OF LESIONAL VAS DEFERENS REPAIR IN THE EXPERIMENT
12:50  A. Šucúr (Zagreb): CHEMOKINE RECEPTOR PROFILE OF OSTEOCLAST PROGENITOR CELLS IN PATIENTS WITH RHEUMATOID ARTHRITIS

Part VI
Chair: doc. MUDr. Michaela Adamcová, CSc., Ph.D.

14:10  M. Vaváková (Bratislava): OXIDATIVE AND NITRATIVE STRESS MARKERS IN CHILDREN AND ADOLESCENT WITH DEPRESSION DISORDER (PRELIMINARY RESULTS)
14:30  K. Vlčková (Praha): SURVIVIN, A NOVEL TARGET OF THE HEDGEHOG/GLI SIGNALING PATHWAY IN HUMAN TUMOR CELLS
14:50  M. Voška (Hradec Králové – University of Defense): THE EFFICIENCY OF COLONIC CAPSULE ENDOSCOPY IN DETECTION OF COLORECTAL POLYPS AND CANCERS COMPARED TO COLONOSCOPY – FINAL RESULTS OF MULTICENTER, PROSPECTIVE, CROSS-OVER STUDY
15:10  L. A. de Wert (Maastricht): REDUCING SHEAR FORCES ON SKIN WITH WOUND DRESSINGS: A NEW STEP IN PRESSURE ULCER PREVENTION?
15:30  J. Zhao (Maastricht): MIR-21 AND MIR-150 DOWNREGULATION CORRELATES WITH OXALIPLATIN-RELATED SINUSOIDAL OBSTRUCTION SYNDROME AND IMPAIRED SURVIVAL IN PATIENTS WITH COLORECTAL LIVER METASTASES
15:50  Z. Zrínyi (Pécs): DIRECTIONS OF EMBRYO QUALITY ASSESSMENT DURING IN VITRO FERTILIZATION: THE REAL VALUE OF MORPHOLOGY
EVALUATION COMMITTEE

Chairperson:  
Professor Vladimír Palička  
Vice-Dean for International Relations  
Charles University, Faculty of Medicine  
Hradec Králové, Czech Republic

Members:  
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President of Association of Medical Schools in Europe  
Association of Medical Schools in Europe (AMSE)  
Berlin, Germany

Professor Margarethe Geiger  
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Research Centre of Physiology and Pharmacology  
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Vienna, Austria

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Experimental Biology Department  
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Portugal

Professor John Greenman  
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Great Britain

Professor Zdravko Lacković  
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University of Zagreb Medical School,  
Zagreb, Croatia

Professor Peter Soeters  
Emeritus Professor of Surgery  
Maastricht University Medical Centre  
Maastricht, the Netherlands
PROBING MITOCHONDRIAL FUNCTION IN VIVO

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School of Biological, Biomedical and Environmental Sciences,
University of Hull, United Kingdom

Co-authors: Christopher Cawthorne, Sunil Bhandari,
Steve Archibald & Anne-Marie Seymour
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Summary
Uraemic cardiomyopathy is a leading course of death in patients with chronic kidney disease and is characterised by left ventricular hypertrophy and cellular remodelling including mitochondrial dysfunction. The present study aimed to investigate myocardial glucose metabolism in vivo using 18F-fluorodeoxyglucose positron emission tomography and assess changes in mitochondrial morphology. 13 weeks post-surgery animals exhibited significant cardiac hypertrophy, uraemia and anaemia. Myocardial glucose uptake was markedly enhanced in uraemic animals at 5, 9 and 13 weeks indicating metabolic remodelling occurs early in UCM. At the cellular level, UCM mitochondria exhibit ultrastructural remodelling and elevated state 4 (uncoupled) respiration suggesting decreased efficiency. Overall these results highlight cardiac mitochondria in UCM are structurally and energetically remodelled and such changes may contribute to an increased susceptibility to cell injury and death.

Background and aims
Uraemic cardiomyopathy (UCM) is one of the leading courses of death in patients with chronic kidney disease (CKD). It is characterised by left ventricular hypertrophy (LVH), cellular remodelling and anaemia, features common to conditions of heart failure. LVH and consequent cardiac dysfunction are associated with altered myocardial bioenergetics (1). Previous studies in our laboratory have shown a significant remodelling of metabolism in the uraemic heart with a greater reliance on glucose using 13C NMR (2) together with evidence of mitochondrial dysfunction and an increased susceptibility to cell injury and death (3). Thus, the aims of this project are; (1) to investigate potential alterations in cardiac glucose metabolism over time in vivo during the progression of UCM using 18F-fluorodeoxyglucose positron emission tomography (18F-FDG PET) and (2) to determine the extent of mitochondrial dysfunction in UCM including assessing changes in mitochondrial morphology.

Methods
Experimental uraemia was induced in male Sprague-Dawley rats via a subtotal nephrectomy (2). Dynamic PET/CT scans were acquired in list mode at 5, 9 and 13 weeks post-surgery using a bolus intravenous injection of 40MBq 18F-FDG (4). 300µl of blood was collected at the time of scanning for subsequent serum metabolite measurements. Data were reconstructed using the 3D ordered subset maximisation algorithm and SUVs generated by manually drawing a region of interest around the left ventricle (LV) and myocardial blood pool (MBP). The rate and distribution of cardiac 18F-FDG uptake was determined using Patlak multiple time graphical analysis (4) and polar maps (5).
Using isolated mitochondria, respiration was measured with a Clark oxygen electrode and respiratory complex activities assessed spectrophotometrically \(^{(3)}\). Expression of the key mitochondrial fusion and fission proteins mitofusin 1 (MFN1), mitofusin 2 (MFN2), optic atrophy 1 (OPA1) and dynamin related protein 1 (DRP1) was probed using Western blotting \(^{(6)}\). Mitochondrial structure was investigated in left ventricular tissue using transmission electron microscopy (TEM) and mitochondrial size evaluated using auto-fluorescence flow cytometry and dynamic light scattering (DLS).

LVH was quantified using the heart weight-to-tibia length ratio (HW:TL). The extent of uraemia and anaemia was assessed from serum levels of creatinine, urea and the packed cell volume (PCV).

**Results**

Animals exhibited significant cardiac hypertrophy (HW:TL = 0,38±0,01 uraemic (n=24) vs 0,34±0,01 control (n=22) (P < 0,01)); uraemia (serum creatinine = 86,3µM ±4,09 uraemic (n = 13) vs 38,4µM ± 1,73 control (n=14) (P < 0,01)) and anaemia (PCV = 0,50 ± 0,01 uraemic (n = 21) vs 0,58 ± 0,02 control (n = 23) (P < 0,01)) highlighting impaired kidney function.

Preliminary SUV data highlighted enhanced glucose uptake \textit{in vivo} in uraemic hearts at all time, points consistent with previous \textit{ex vivo} studies (Table 1). Patlak multiple time graphical analysis also revealed an increased rate of cardiac \(^{18}\)F-FDG uptake, however, no differences in the distribution of \(^{18}\)F-FDG uptake in the heart were observed (Figure 1).

<table>
<thead>
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<th>Table 1. Myocardial (^{18})F-FDG uptake</th>
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<td>Uptake rate (Ki, ml/min/g)</td>
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* P < 0,05

UCM mitochondria exhibited significantly increased state 4 (uncoupled) respiration suggesting decreased mitochondrial efficiency (oxygen consumption (nA O/min/mg) = 51,2 ± 2,8 uraemic (n=9) vs 39,1 ± 2,9 control (n=10) (P < 0,01)). These changes were mirrored by substantial remodelling of mitochondrial cristae as revealed by TEM (Figure 2). However, the expression of key fusion and fission proteins and gross mitochondrial size were unchanged (hydrodynamic diameter = 1,64nm ± 0,1 uraemic vs 1,6nm ± 0,2 control).
Discussion
13 weeks post-surgery animals exhibited significant cardiac hypertrophy, uraemia and anaemia. The PET study reported here highlights enhanced in vivo myocardial $^{18}$F-FDG uptake in uraemic animals compared to controls, suggesting metabolic remodelling occurs early in UCM and, consistent with ex vivo studies (2) yet preceding contractile dysfunction. At the cellular level, UCM mitochondria demonstrated sparsely packed cristae in comparison to control mitochondria whilst maintaining gross size. As the cristae are the site of the respiratory chain, such changes can be considered indicative of altered mitochondrial efficiency as reported previously (7). Indeed, the significantly increased state 4 respiration observed in UCM mitochondria in this study is consistent with this.

Conclusions
Using $^{18}$F-FDG PET, this study has indicated myocardial glucose uptake is enhanced in uraemic animals from an early stage and is sustained throughout disease progression. In parallel, UCM mitochondria exhibit an altered cristae structure and are uncoupled suggesting reduced efficiency. Taken together, these results highlight cardiac mitochondria in UCM are remodelled both structurally and energetically and such adaptions may contribute to the increased susceptibility to cell injury and death previously reported.

References
Introduction
Radiofrequency ablation (RFA) is a palliative approach used for treatment of unresectable liver tumors. Special single or cluster RFA probes are leaded into the tumor. There RFA develops heat that cause cell death and tissue necrosis. RFA is performed by open (surgeons) or percutaneous approach (radiologists).

Aim
Compare open and percutaneous RFA approach at group of patients with colorectal liver metastases in retrospective study.

Methods
147 patients underwent RFA for colorectal liver metastases (CLM) from 1/2001 to 1/2015. There were 103 male and 44 female, average age was 65 years (38-82 years). Total 168 RFA were performed. 111 RFA were performed by open and 57 by percutaneous approach. Total 305 metastases were treated. One metastasis was treated at 95 patients, more than 1 metastasis were treated at 73 patients. Average length of metastases was 28.7 cm (5-90 mm). Patients with RFA and liver resection, patients with two-stage surgery or patients with duplicity were excluded. We observed factors affecting overall survival (OS), no evidence of disease (NED) and tumor’s non-ablation in relation to tumor’s size, number of metastases and type of used probes (single/cluster). Patients were followed up by ultrasonography or computer tomography scans every 3 months after procedure. All data were compared between percutaneous and open approach RFA groups.

Results
OS was influenced by high number of censors. OS for first year was 93.6%, 3 years 61%. There weren’t statistical differences between percutaneous and open approach. OS wasn’t influenced by size of metastases. NED was significantly shorter at patients with percutaneous approach (5 months at percutaneous approach and 6.6 months at open approach). NED wasn’t influenced neither size nor numbers of tumors. Higher risk of non-ablation was significantly observed at patients with percutaneous RFA (26.8%) against open approach (13.6%). Higher risk of non-ablation was also observed at largest metastases, but this wasn’t statistically significant. Patients with percutaneous RFA had shorter duration of hospitalisation (5.4 days versus 8.8 days) and less number of complications (5.5% versus 17.3%)
Discussion
RFA is used for palliative treatment of unresectable CLM. OS is reported in literature between 88-92% for first year, 65-80% for second year and 51-57% for third year at patient with CLM.1,2,3 Median for NED is reported 6,2-26 months.3,4,5,6 Our results for OS and NED are comparable with these studies. Postoperative complications and postoperative hospitalization were decreased in patients with percutaneous approach and therefore percutaneous RFA is often used in patients with many comorbidities. On the other hand percutaneous approach had higher risk of non-ablation. We could also observe increasing risk of non-ablation at patient with increasing size of metastases. Although this trend wasn’t statistically significant in our group of patients, it’s in accordance with previously reported risk factor for early recurrence that is a size of tumor more than 3cm in diameter.4
Previously there was also reported the same survival of patients after liver resection or RFA,7 but replacement of liver resection by RFA is still controversial. Liver resection with R0 margin and following chemotherapy is still the best way for the treatment of colorectal liver metastases.8

Conclusion
RFA is a palliative procedure for alternative treatment of only unresectable liver tumors. Open approach had lower risk of non-ablation and longer NED. In stead of percutaneous RFA has lower risk of complication and shorter duration of convalescence. That’s why is suitable for patients that are poor candidates for surgery.

Summary
RFA is established palliative method to treat unresectable liver tumors. We compared percutaneous and open approach of RFA in patients with colorectal liver metastases. Patients with open approach had lower incidence of non-ablation and longer period of NED against percutaneous approach. In stead of percutaneous RFA has lower risk of complications and shorter duration of convalescence and this approach is suitable for patients that are poor candidates for surgery. It’s necessary to emphasize that RFA mustn’t replacement liver resection that is still only one method providing radical treatment.

References
Introduction
Pulmonary surfactant is a mixture of lipids and specific proteins SP-A, SP-B, SP-C and SP-D, that reduces surface tension at the alveolar air–liquid interface and prevents lung collapse at end-expiration. Deficiency of pulmonary surfactant due to immaturity is the principal cause of the respiratory distress syndrome (RDS) observed in the prematurely born infants (Robertson and Halliday, 1998). In recent years it has been recognized that surfactant deficiency develops in full-term infants due to genetic abnormalities in surfactant metabolism. Hereditary SP-B deficiency was the first-recognized genetic cause of surfactant deficiency (Nogee et al., 1993). Up to now, thirty-four mutations (Kurath-Koller et al., 2015) and many polymorphisms have been published in the literature. Combinations of single nucleotide polymorphisms (SNPs), that are transmitted together along one stretch of DNA, are haplotypes. They enable to identify all other polymorphic part in the investigated gene region without sequencing of the entire gene (Clark 2004). The aim of our study was to focus on the possible relationship between haplotypes of SP-B and the development of neonatal RDS.

Methods
Premature infants with and without RDS (n=89), term neonates with respiratory problems of unknown etiology (n=20) and infants with non-respiratory disease (controls, n=20) were enrolled in the prospective study. The blood sample was examined by methods of molecular diagnostic. Seven tag single nucleotide polymorphisms (SNPs) in or near of the SFTPB gene (chromosome 2, position 85886500 to 85897800) rs9752, rs762548, rs2304566, rs893159, rs110866, rs3024791 and rs4616480 were chosen. For the tag selection, Hap Map SNP database with MAF˃0,1 in Caucasian population and Haploview software (Broad Institute, USA) were used. The DNA was genotyped using TaqMan Genotyping assay (Life Technologies, USA) for each SNP and TaqMan Genotyping Master Mix (Life Technologies, USA). The association of single SNP and haplotypes of SP-B with clinical variables as surfactant administration within 2 h (early), after 2 h (“late”), its repeated administration, early infection and gestational age were investigated. The statistical evaluation was done by STATISTICA 10 Cz. For detecting genetic associations of alleles calculation odd ratio (OR) and Hardy-Weinberg Equilibrium (HWE) were used.

Results
There was a significant correlation between the surfactant administration and gestational age (g.a.) (P<0,001) and the late surfactant administration with g.a. (P<0,01). Gestational age was related to the infection (P = 0,037) and to repetitive surfactant administration (P<0,001). Early infection was related
to the early surfactant administration within 2 h (P = 0.015). The SP-B polymorphism rs762548 was associated with the repeated administration of surfactant (P<0.033). Two types of haplotypes were associated with the repeated surfactant administration (CGTGATG P<0.048; GATTACG P<0.031).

**Discussion**

Surfactant proteins (SP) are important for the innate host defence and essential for a physiological lung function. Several linkage and association studies have investigated the genes coding for different surfactant proteins in the context of pulmonary diseases. One of the main causes of premature birth is early infection (Galinsky et al., 2013), that is linked with gestational age. In our study, we confirmed the significant correlation between gestational age and early infection, and between early infection and early surfactant administration. Also significant correlation between gestational age and the need of surfactant administration was confirmed. It is now generally accepted that genetic variants of surfactant protein B are associated with RDS (Marttila et al., 2003). For our study we have chosen 7 tag SNPs. We performed the analysis of the association of single SNP with clinical variables and found out that the rs762548 was associated with the repeated surfactant administration. Currently there is not many information about linkage between RDS and haplotypes of the SP genes. Haplotypes are more powerful discriminators between cases and controls in disease association studies. In 2006 Floros with co-workers reported that certain alleles or haplotypes of SP-B are susceptible or protective factors for the development of RDS in premature babies. In the study of Puthothu et al. (2007) haplotypes analyses revealed association of SP-B with severe RSV infection. In our study, two types of haplotypes were associated with the repeated administration of surfactant. The haplotypes were assigned with patients with atypical course of RDS.

**Conclusions**

There is no link between common haplotypes of SP-B with respiratory distress in neonates. The results warrant further search for rare haplotypes by next generation sequencing.

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**References**

DEVELOPMENT AND IN VITRO TESTING
OF NEW COMPOSITE MATERIALS FOR BONE SURGERY
APPLICATIONS

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Z. Sucharda, M. Hubálek Kalbáčová
Tutor: Doc. RNDr. Marie Hubálek Kalbáčová, PhD.

Introduction
Interactions of human mesenchymal stem cells (hMSCs) with collagen-based scaffolds were studied
with the aim of bone surgery application. The scaffolds should imitate an extracellular bone matrix and
support hMSCs adhesion, proliferation and their osteogenic differentiation. The scaffolds should de-
grade after appropriate time. Collagen-based scaffolds are widely researched due to collagen positive
effect on many cell types, its lattice-like organization ability and biocompatibility. However, the fast
biodegradation rate and the low mechanical strength of the untreated collagen very often complicate in
vitro and in vivo applications. The stability of collagen scaffolds can be enhanced by cross-linking.
Thus, in this study, various cross-linking agents were used, e.g. genipin, N-(3-dimethylamino propyl)-
N’-ethylcarbodiimide hydrochloride and N-hydroxysuccinimide in an ethanol solution
(EDC/NHS/EtOH) or EDC/NHS in a phosphate buffer saline solution (EDC/NHS/PBS) and their
impact on scaffold properties studied in vitro.

Experimental methods
The metabolic activity of cells cultivated in scaffolds infusions and/or on the scaffolds (Cell Titer 96
AQeuous One Solution Cell Proliferation Assay, MTS, Promega, USA), number of cells adhered on
scaffolds and fluorescence visualization of cell/scaffold interactions (nuclei and actin staining) were
determined. All procedures were performed after short (48 h) and long (168 h) cell cultivation. Tissue
culture treated polystyrene (PS) (TPP, Switzerland) was used as a positive control. hMSCs were
isolated from a bone marrow blood of three healthy donors to simulate a stem cell variability naturally
occurred among individuals.

Results
Composite scaffolds (consisting of natural collagen as matrix, poly(DL-lactide) electrospun nanofibers
as a scaffold, calcium phosphate as dispersed filler supplemented by sodium hyaluronate) cross-linked
by EDC/NHS/EtOH, EDC/NHS/PBS or genipin, were tested in vitro. The scaffold infusions were tested
to check a release of cytotoxic agents from individual scaffolds into cultivation medium. After 48 h,
the metabolic activities of hMSCs cultivated in all three scaffolds infusions were comparable and
slightly higher than the PS control. After 168 h, the metabolic activities of the cells treated by genipin
and EDC/NHS/EtOH cross-linked scaffolds infusions remained similar as at 48 h. However, the meta-
bolic activity of hMSCs treated by EDC/NHS/PBS cross-linked scaffold infusion markedly decreased
to 83% of PS control. Metabolic activity of the cells seeded on scaffolds was almost comparable among all three samples at both incubation times. Fluorescence visualization determined the cell ability to adhere on the tested scaffolds. After 48 h, hMSCs on the genipin cross-linked scaffold revealed the best morphology and symmetric distribution. Interestingly, after 168 h, cells on all scaffolds were similarly organized with comparable appearance. Thus, the genipin cross-linked scaffold provided the best conditions for cells in general. In addition, it maintained constant mechanical properties in contrast to the rest of cross-linked scaffolds.

Discussion
All scaffold infusions seemed to be non-cytotoxic; cytotoxicity is often determined by a cell metabolic activity decrease under 75%. However, the metabolic activities and penetrations of cells seeded on scaffold were comparable for all three scaffolds. The quality of cell morphology, adhesion and distribution was different during the testing. The results revealed, cross-linking agents can influence cell viability, cell conditions and scaffold behaviour. In this study, the genipin assigned the best features to other tested scaffolds for hMSC application.

Conclusion
The genipin cross-linked scaffold is the best scaffold with ideal mechanical and biological properties for subsequent in vitro 3D analyses and in vivo experiments.

Summary
The designed collagen-based scaffolds have the potential in bone surgery application – they can provide a support for hMSCs adhesion and preconditions for consequent osteogenic differentiation. The stability and structure of scaffolds is crucial for hMSC behaviour and can be enhanced by cross-linking – thus, genipin, EDC/NHS/EtOH or EDC/NHS/PBS cross-linked scaffolds were tested at 2D cultivation level. Scaffold cytotoxicity, cell metabolic activity, adhesion and morphology of cells were determined. Genipin cross-linked scaffold revealed the best properties for cell application and for advanced and subsequent in vitro and in vivo experiments.

References
Introduction
Accurate emotional processing is essential in order to function in the social world and is often reported as disrupted in patients with bipolar affective disorder (BAP) (1). While previous research has targeted brain regions involved in disrupted functioning during the acute phase of the illness (2), the neurobiology underlying the remitted state of the disorder remains to be poorly understood. Yet the clinical experience shows that even in times of remission patients with BAP remain to be very sensitive to various both internal and external emotional stressors. In order to fill this knowledge gap, present study had the following objectives.

Objectives
1. Identify vulnerability markers related to emotional processing in remitted patients with BAP by means of quantitative electroencephalography (qEEG). 2. Evaluate the differences of connectivity among emotion-regulating cortical regions during induced sadness vs. relaxed state in healthy controls.

Methods
Subjects: 29 remitted patients (aged 18-60 years) with BAP were recruited from the Prague Psychiatric Center with a clinician-assigned diagnosis of bipolar affective disorder based on the DSM-IV criteria. At the time of the assessment, all of the patients were euthymic for at least one month (Montgomery Asberg Depression Rating score, MADRS < 10, Young Mania Rating Scale score, YMRS < 7) and on a long-term, stable medication. The patients were matched with 30 healthy controls on gender and age. Exclusion criteria included organic brain disease, head trauma resulting in a loss of consciousness and substance or alcohol dependence. Data acquisition and processing: EEG recording (10/20 system with 21 EEG channels) was obtained with eyes closed during transient sadness and neutral mood state induced by a written, autobiographical script (3). Data was filtered (0.5 – 70 Hz) and artifacts were removed by means of visual and semiautomatic inspection (SW Neuroguide). Discrete, ten-minute, artifact free epochs of EEG activity were selected and divided into the following frequency bands: delta (1.5-6 Hz), theta (6.5-8 Hz), alpha-1 (8.5-10 Hz), alpha-2 (10.5-12 Hz), beta-1 (12.5-18 Hz), beta-2 (18.5-21 Hz) and beta-3 (21.5-30 Hz), gamma (35-44 Hz). 3D intracerebral current density distribution and cortical connectivity were calculated by exact Low Resolution Electromagnetic Tomography (eLORETA) (4).

Results
eLORETA revealed significant differences in brain activity during processing of sad emotions in patients with BAP and healthy controls. Compared to healthy controls, the induction of emotionally negative
mood state led to significant increases of gamma current densities in anterior cingulate gyrus (BA 24, 25, 33) as well as in parietal cortices (BA 7, 40). In healthy participants, the induction of transient sadness led to an increased neuronal activity in anterior cingulate, subcallosal gyrus and medial frontal gyrus. Group comparison of the differences between emotionally negative and neutral state revealed an increased gamma activity in posterior cingulate and a decrease of alpha-2 current density in the inferior and middle frontal gyri in patients with BAP.

Discussion
To our knowledge, this is the first qEEG study investigating the underlying neurobiology of transient self-induced emotional changes in remitted patients with BAP and healthy controls. Reported findings in healthy controls are in line with previous research describing the activation of the subgenual anterior cingulate region during the processing of sad emotions (5). The observed more pronounced activation of various limbic and frontal areas seen in remitted BAP patients can be interpreted within the network of aberrant subgenual cingulate-superior frontal gyrus connectivity (6, 7) and shows that although these patients are clinically stable and euthymic, they process intense emotions differently. The reported hyperactivity of posterior cingulate and a reduced activation of the inferior frontal cortex may represent an EEG trait marker of BAP.

Conclusions
Our finding of abnormal neuronal activity in subgenual cingulate and superior frontal gyrus is in accordance with previous studies of bipolar depression and could be interpreted within the framework of aberrant aberrant subgenual cingulate-superior frontal gyrus connectivity revealed during induced sadness in euthymia patients.

Summary
This study used a mood challenge paradigm in order to unmask possible trait-markers in remitted patients with BAP by means of qEEG. The findings of altered neuronal activity during processing of intense negative emotions could be a candidate trait marker of BAP. Future studies may operate longitudinally and include patients with unipolar depression in order to better characterize vulnerability which could lead to another relapse of illness.

References

Acknowledgements: This work was supported by grants IGA MZCR NT 12024 and NTI13337 and by the project PRVOUK P34.
**Introduction**

Adenocarcinoma (ACO) of the oesophagus is one of the fastest growing cancers in the western world (Flejou 2005), and develops on a background of Barrett’s oesophagus. Barrett’s oesophagus is an acquired condition characterised by replacement of the squamous epithelium of the lower oesophagus with columnar epithelium featuring gastric or intestinal metaplasia and occurs as a consequence of reflux of gastric acid and bile salts. The first line therapy to treat Barrett’s oesophagus is proton pump inhibitors (PPIs) which inhibit gastric acid secretion. A side effect of PPI treatment is increased serum gastrin concentration since acid inhibits gastrin release. Gastrin is known to be growth factor for gastric epithelial cells and previous studies have also identified expression of the CCK-2 receptor, through which gastrin acts, in Barrett’s tissue (Haigh, Attwood et al. 2003). Moreover, there was a significant association between high serum gastrin and high grade dysplasia in Barrett’s epithelium (Wang, Varro et al. 2010). Our hypothesis is that elevated serum gastrin concentration is a risk factor for progression of Barrett’s to ACO.

**Aims**

The overall aim of this project is to identify biomarkers for gastrin-stimulation of Barrett’s oesophagus. The specific objectives of the present study were (a) to determine whether the gastrin profile in Barrett’s patients is similar to controls, and (b) as a proof-of-concept study, determine whether chromogranin A (CgA) is an indicator of increased serum gastrin in Barrett’s as well as control subjects.

**Methods**

**Patients.** Control (*H. pylori* negative, no gastric pathology) or Barrett’s patients (diagnosed on the basis of oesophageal histopathology) were recruited from a cohort attending for diagnostic upper gastrointestinal endoscopy at the Royal Liverpool University Hospital. Serum and gastric biopsies for research were collected. Patients gave written, informed consent and the study was approved by the local ethics committee.

**Determination of serum gastrin.** Serum gastrin was analysed by radioimmunoassay (RIA) using an antibody reacting at the C-terminus common to the two major forms, G17 and G34; a standard of human G17 was used and 125I-labelled G17 was obtained from Perkin Elmer (NEX176010UC).

**Determination of serum CgA.** Serum CgA was determined by RIA utilising a proprietary kit (Eurodiagnostica, Cat No. RB321) according to the manufacturer’s instructions.
qPCR of tissue CgA cDNA. Real time qPCRs using an Applied Biosystems AB7500 system was employed to determine CgA in gastric biopsies of control and Barrett’s individuals using Taqman technology. Primer and probe sequences used were as follows: GAPDH - 5'-GCT CCT CCT GTT CGA CAG TCA-3’ (forward), 5’-ACC TTC CCC ATG GTG TCT GA-3’ (reverse), 5’-CGT CGC CAG CCG AGC CAC A-3’ (probe); CGA - 5’-GAT ACC GAG GTG ATG AAA TGC A-3’ (forward), 5’-TCC TTC AGT AAA TTC TGA TGT CTC AGA-3’ (reverse), 5’-CCA GCC CCA TGC CTG TCA GCC-3’ (probe).

Results
Serum Gastrin. Serum gastrin was significantly increased in both control and Barrett’s subjects on PPI’s (control N=19, Barrett’s N=34) compared with those not on PPI’s (control N=20, Barrett’s N=19). Serum gastrin was higher in Barrett’s than control patients on PPI’s.

Figure 1. Total serum gastrin concentration of control and Barrett’s patients either on -PPIs or not. Mean +/- SEM, p<0.05, 2-way ANOVA.

Figure 2. Abundance of CgA mRNA relative to GAPDH in control and Barrett’s oesophageal biopsies. Mean +/- SEM, p<0.05, 2-way ANOVA.

Figure 3. A comparison of the serum CgA concentration in normal and Barrett’s patients determined by RIA. Mean +/- SEM, p<0.05, 2-way ANOVA.
**Tissue CgA mRNA.** The relative abundance of CgA mRNA in gastric biopsies was higher in those control and Barrett’s patients on PPI’s compared with those not on PPI’s; there was no difference between matched groups of control and Barrett’s patients. Spearman’s rank correlation showed a significant (p<0.05) correlation between total serum gastrin concentration and CgA mRNA expression.

**Serum CgA.** Serum CgA was significantly elevated in both control and Barrett’s subjects on PPI’s compared with those not on PPI’s. There was no difference between matched groups of control and Barrett’s patients. Spearman’s rank correlation also showed a significant (p<0.05) correlation between total serum gastrin concentration and serum CgA concentration.

**Discussion**
As expected, PPI treatment was associated with a significant increase in serum gastrin concentration in control subjects. Importantly, there was also increased serum gastrin in Barrett’s patients which was greater than control. It is possible that the Barrett’s patients had been on PPI’s longer than the controls and further work will be needed to clarify this.

Chromogranin A is a previously identified biomarker of elevated gastrin in patients with neuroendocrine tumours. Our data suggest that it is also a useful biomarker of elevated gastrin in control subjects. Moreover, we show that the association between gastrin and CgA in serum or mRNA in biopsies is preserved in Barrett’s.

**Conclusion**
For most Barrett’s patients PPI’s are protective by reducing acid secretion; however in the important minority of patients with dysplasia, increased gastrin secondary to PPI usage may accelerate progression. Biomarkers of sensitivity to gastrin may therefore be useful in identifying the at-risk sub-group. Our data on CgA establishes the principle that biomarkers of gastrin sensitivity may be identified in both control and Barrett’s patients.

**Summary**
Serum gastrin in patients with Barrett’s oesophagus on PPI’s is elevated, and a marker of gastrin responsiveness (CgA) is also increased. The work establishes the concept that biomarkers of gastrin responsiveness can be applied to Barrett’s patients, which may help identify those at risk of gastrin-driven progression to adenocarcinoma.

**References**
POSTERIOR VS ANTERIOR CIRCULATION INFARCTION; DEMOGRAPHY, OUTCOMES, AND FREQUENCY OF HEMORRHAGE AFTER THROMBOLYSIS

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Tutor: prof. MUDr. Roman Herzig, Ph.D., FESO, FEAN

Introduction
Intravenous thrombolysis (IVT) is considered to be a standard specific reperfusion therapy in acute ischemic stroke in both, anterior and posterior cerebral circulation. Randomized trials showed that patients might benefit from IVT up to 4,5 h after symptom onset. Our aim was to evaluate 90-day outcome and rate of ICH after recombinant tissue plasminogen activator (rt-PA) administration in posterior circulation stroke (PCS), and subsequently to compare the risk of intracranial bleeding between PCS and anterior circulation stroke (ACS).

Patients and Methods

Patients
A single-center retrospective study was performed. Eight hundred and eighty two consecutive acute ischemic stroke patients (AIS) who underwent IVT with a standard dose of 0,9 mg/kg of alteplase in the Comprehensive Stroke Center of Olomouc University Hospital from July 2005 to November 2014 were identified. PCS was defined as symptomatic ischemia in the vascular territory of the vertebral, basilar, or the posterior cerebral arteries. ACS was defined as symptomatic ischemia in the territory of the internal carotid, middle or anterior cerebral arteries. A vascular territory with no lesion detected on CT/MRI was marked as uncertain and classified according to the clinical presentation.

Observed parameters
Data was collected including information on: baseline characteristics, stroke risk factors (arterial hypertension, diabetes mellitus, hyperlipidemia, atrial fibrillation, coronary artery disease, current smoking, recent neck trauma and dissection of vertebral artery), pre-event antiplatelet medication, pre-event anticoagulation, neurological deficit at time of treatment in the National Institutes of Health Stroke Scale (NIHSS), type and estimated time to therapy procedure initiation, blood glucose level before IVT, value of arterial pressure before and after IVT, need for intravenous antihypertensive therapy before and during IVT, recanalization rate, and clinical outcome at Day 90 (including mortality).

Outcome parameters
Post-treatment imaging was performed using CT or MRI and was carried out within 36 h after IVT initiation. Intracerebral haemorrhage (ICH) was classified according to ECASS I(2) protocol: hemorrhagic infarction (HI) was defined as a petechial infarction without a space-occupying effect; HI 1 - small
petechiae along the margins of the infarct and HI 2 - more confluent petechiae within the infarcted area, but without a space-occupying effect. Parenchymal hematoma (PH) was defined as a hemorrhage with mass effect; PH 1 - blood clot not exceeding 30 % of the infarcted area with some mild space-occupying effect and PH2 - dense blood clot(s) exceeding 30 % of the infarct volume with a significant space-occupying effect.

The clinical outcome on Day 90 was evaluated using the modified Rankin scale (mRS), with a good clinical outcome defined as a score of 0 to 2.

Results
Out of the 877 included patients, 100 (12.8 %) had PCS and 777 (87.2 %) had ACS. Baseline characteristics, stroke severity, time to treatment and IVT administration circumstances are shown in Table 1.

Table 1. Baseline Characteristic of patients suffering from PCS and ACS

<table>
<thead>
<tr>
<th>Intracranial hemorrhage</th>
<th>Posterior circulation stroke (n=100)</th>
<th>Anterior Circulation Stroke (n=777)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), median</td>
<td>67,5</td>
<td>71</td>
<td>0,006</td>
</tr>
<tr>
<td>Male gender, n (%)</td>
<td>65</td>
<td>54,5</td>
<td>0,047</td>
</tr>
<tr>
<td>Arterial hypertension, %</td>
<td>82</td>
<td>77,1</td>
<td>0,267</td>
</tr>
<tr>
<td>Atrial fibrillation, %</td>
<td>24</td>
<td>33,8</td>
<td>0,048</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>25</td>
<td>21,6</td>
<td>0,443</td>
</tr>
<tr>
<td>Coronary artery disease, %</td>
<td>22</td>
<td>29,7</td>
<td>0,108</td>
</tr>
<tr>
<td>Hyperlipidemia, %</td>
<td>56</td>
<td>35,6</td>
<td>0,0001</td>
</tr>
<tr>
<td>Current smoking, %</td>
<td>20</td>
<td>16,2</td>
<td>0,339</td>
</tr>
<tr>
<td>Pre-event, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antiplatelet medication</td>
<td>27</td>
<td>31,3</td>
<td>0,383</td>
</tr>
<tr>
<td>Anticoagulation</td>
<td>0</td>
<td>2,2</td>
<td>0,243</td>
</tr>
<tr>
<td>History of, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transient ischemic attack</td>
<td>3</td>
<td>5,7</td>
<td>0,266</td>
</tr>
<tr>
<td>Stroke</td>
<td>10</td>
<td>6</td>
<td>0,131</td>
</tr>
<tr>
<td>Intracerebral hemorrhage</td>
<td>0</td>
<td>1,4</td>
<td>0,624</td>
</tr>
<tr>
<td>NIHSS prior to IVT</td>
<td>8</td>
<td>10</td>
<td>0,262</td>
</tr>
<tr>
<td>Time to treatment (min), median</td>
<td>175</td>
<td>160</td>
<td>0,001</td>
</tr>
<tr>
<td>Blood glucose level prior to IVT (mmol/l), median</td>
<td>7,25</td>
<td>6,7</td>
<td>0,005</td>
</tr>
<tr>
<td>Systolic blood pressure, median</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior to IVT</td>
<td>172</td>
<td>170</td>
<td>0,573</td>
</tr>
<tr>
<td>After IVT</td>
<td>150</td>
<td>150</td>
<td>0,54</td>
</tr>
<tr>
<td>Intravenous antihypertensive therapy before and during IVT administration, %</td>
<td>40,4</td>
<td>34,7</td>
<td>0,304</td>
</tr>
<tr>
<td>Additional endovascular treatment, %</td>
<td>15</td>
<td>17</td>
<td>0,616</td>
</tr>
<tr>
<td>Control imaging used – CT/MR/none</td>
<td>30/68/2</td>
<td>436/329/12</td>
<td></td>
</tr>
</tbody>
</table>
Intracerebral hemorrhage was significantly less frequent in PCS than in ACS patients as presented in Table 2. The risk for ICH was 3.4 times higher in ACS than in PCS. Moreover, the risk for large hemorrhage (PH1 + PH2) was 5.2 times greater in ACS. The incidence of hemorrhage in PCS was significantly dependent on the presence of atrial fibrillation and additional endovascular therapy.

**Table 2. Rate of intracranial hemorrhage**

<table>
<thead>
<tr>
<th></th>
<th>All, n (%)</th>
<th>HI1 + HI2, n (%)</th>
<th>PH1 + PH2, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 (5.1%)</td>
<td>3 (3.0%)</td>
<td>2 (2.0%)</td>
</tr>
<tr>
<td></td>
<td>133 (17.2%)</td>
<td>51 (6.6%)</td>
<td>81 (10.4%)</td>
</tr>
</tbody>
</table>

The 90-day clinical outcome is shown in Figure 1. Stepwise binary logistic regression analysis identified higher, presence of severe neurological deficit and progression of ischemic lesion after 24 h as independent significant negative predictors of a good clinical outcome in PCS.

**Figure 1. 90–day clinical outcome in posterior circulation stroke**

Anterior circulation stroke was identified as significant predictor for both ICH and large ICH when multiple logistic regression, adjusted for age, gender, presence of atrial fibrillation, hyperlipidemia, time to treatment and blood glucose level prior to IVT was used.

**Discussion**

This is the largest single-center series assessing the safety and clinical outcome of IVT for PCS. Most of the previously published studies evaluating the safety and efficacy of IVT for PCS had small sample sizes (range, 9 to 84 patients), they assessed ICH using a different definition of SICH, or they investigated acute basilar artery occlusion only (3-8).
Unlike other studies that assessed symptomatic intracerebral hemorrhage per NINDS, ECASS II or SITS-MOST, the present study noted any ICH. To compare ICH risks, it is more relevant to include all ICHs than to select a smaller group according to the treating physician’s opinion. In the present study, PCS patients experienced bleeding significantly less frequently than ACS patients. Thus, a more aggressive approach should be considered for IVT administration in PCS.

Conclusion
Our study suggests that a lower risk of ICH is more associated with PCS than with ACS, indicating that administration of IVT is safer in PCS. Since these groups do not differ in essential clinical or demographic characteristics, an extension of the time window should be considered for PCS.

Acknowledgements
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References:
RESISTANCE MECHANISMS OF FGFR1-DRIVEN SMALL CELL LUNG CANCER AGAINST THE FGFR INHIBITOR BIBF-1120

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Introduction
Lung cancer is the major cause of cancer-related death worldwide. In small cell lung cancer (SCLC), relapse after surgical resection and rapid resistance development result in a poor patient outcome and to date no targeted anticancer compounds are approved for this tumor type. In a defined subgroup of non-small cell lung cancer (NSCLC) but also of SCLC, Fibroblast Growth Factor Receptor 1 (FGFR1) gene amplification is a driving oncogenic event and inhibition of the FGFR1 signaling axis exerts strong antitumor effects. The FGFR/Platelet Derived Growth Factor Receptor (PDGFR)/Vascular Endothelial Growth Factor Receptor (VEGFR) tyrosine kinase inhibitor (TKI) BIBF-1120 is currently being evaluated in clinical trials also for the treatment of SCLC. Despite initial treatment success, we anticipate -as observed in other cancer types- relapse due to acquired resistance development.

Aims
This study aims to characterize the molecular mechanisms that underlie resistance development of FGFR1-driven SCLC against BIBF-1120.

Methods
DMS114 SCLC cells were selected for BIBF-1120 resistance by constant in vitro exposure. A high-throughput anticancer compound screen was performed on a Perkin Elmer screening platform in collaboration with S. Kubicek at CeMM. Cell viability in response to drug exposure was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) vitality assay. Array Comparative Genomic Hybridization (aCGH) was performed on 4x44K oligonucleotide microarrays (Agilent). Total cell protein lysates were analyzed by Western blot analysis by SDS-PAGE and blotting on polyvinylidene difluoride membranes. To determine the effect of BIBF-1120 on the efflux of ATP-binding cassette transporter A1 (ABCB1) substrates, the calcein AM assay was performed. Intracellular levels of BIBF-1120 were measured by HPLC-MS. The effect of BIBF-1120 on the ABCB1-ATPase activity was determined by ATPase assay in ABCB1-enriched Sf9 crude cell membranes.

Results
Constant exposure of DMS114 to BIBF-1120 led to upregulated expression of the multidrug efflux pump ABCB1. Insensitivity of DMS114 to FGFR1 inhibition was mediated by efflux of BIBF-1120 via ABCB1. Intracellular levels of BIBF-1120 were strongly reduced in the selected subline as demonstrated by mass spectrometric analysis. This effect could be reverted by ABCB1 inhibition using the
third generation ABCB1 inhibitor Elacridar. BIBF-1120 increased the ATPase activity of ABCB1. Accordingly, intracellular accumulation of the ABCB1 fluorescence probe and substrate calcein AM was increased in the BIBF-1120-resistant subline in the presence of BIBF-1120, again proving BIBF-1120 as being an ABCB1 substrate.

Discussion and Conclusion
ABC transporters are transmembrane proteins that play a key role in the translocation of broad range of chemicals across cellular membranes. One of the most important functions of ABC transporters represents the protection of the body against xenobiotics. This becomes evident by the expression of different ABC transporter subtypes in various tissue types that function as protection barriers in the body, including amongst others the epithelial lining of the intestinal tract and the endothelial cells in the cerebral microvasculature. Substrates for ABC transporter-mediated efflux include a range of biological compounds, but also a distinct set of chemotherapeutic agents and small, targeted anticancer drugs, such as epidermal growth factor receptor (EGFR) TKIs. In cancer cells, induction of ABC transporter expression in response to therapy (especially of ABCB1, ABCG2 and ABCC1) is a well-characterized mechanism by which tumors develop a multidrug resistance phenotype. In contrast, knowledge of the impact of ABC transporters on the activity of FGFR inhibitors is limited. So far, reversal of ABCB1-mediated multidrug resistance by the FGFR inhibitors PD173074 or BIBF-1120 has been suggested. In this study, we show that FGFR1-driven DMS114 SCLC cells induce ABCB1 expression in response to chronic BIBF-1120 exposure and that activation of this pump mediates acquired resistance by actively exporting BIBF-1120 across the cell membrane. On the contrary, BIBF-1120 is also able to restore sensitivity of other ABCB1-substrate compounds leading to synergism in combination experiments in MDR cell models.

In conclusion, we identified the FGFR/PDGFR/VEGFR inhibitor BIBF-1120 as a substrate for the multidrug efflux transporter ABCB1. This finding is important for the development of treatment strategies including BIBF-1120 not only for SCLC but also for other cancers driven by FGFR/PDGFR/VEGFR.
activation. Tumors with acquired multidrug resistance and cancers of the central nervous system, where the efficient drug efflux activity of the blood brain barrier depicts a limiting factor for therapy success, might harbor intrinsic BIBF-1120 resistance. Switching to small-molecule inhibitors that also target FGFR but are not substrates of ABCB1 might be a feasible option to circumvent multidrug resistance-mediated unresponsiveness to BIBF-1120.

**Summary**

This study proves that the clinically approved FGFR/PDGFR/VEGFR inhibitor BIBF-1120 is a substrate and inhibitor of the MDR drug efflux pump ABCB1. Hence, this ABC transporter has to be considered as a factor underlying intrinsic and acquired BIBF-1120 resistance. In addition, BIBF-1120 within combination treatment schemes might function as a resistance reverser besides its action as TKI.

**References**

Introduction
Tularemia is zoonotic disease caused by Gram-negative coccobacillus Francisella tularensis. While the transmission to humans occurs mostly accidentally, the high infectivity and the severity of the disease raise concerns about the intentional abuse of the bacterium as a biological weapon. The infection effectivity of Francisella is mirrored by its intracellular life-style and its ability to survive inside sentinels of innate immunity, professional phagocytes. Although the host first engulfs Francisella, the bacterium escapes within 1 hour to the host cytosol, where it replicates. Francisella subsequently spreads from the site of infection to local lymph nodes and other organs. As for other intracellular pathogens, Th1 adaptive immunity is indispensable for the control of the infection. But while the infection strategy of Francisella is aggressive, the adaptive immunity response is relatively weak and delayed. The most efficient adaptive immunity-priming cells are dendritic cells (DCs). Although macrophages are the main target of Francisella in the infection site, DCs are also present and, as professional phagocytes, they are susceptible to infection. Importantly, Francisella-infected DCs do not seem to be properly activated. Such DCs would perform poorly in proinflammatory priming of naïve T-cells, thus providing the potential explanation for inefficient adaptive immunity response toward Francisella.
Deciphering the interaction events of DC-Francisella crosstalk would help to understand the outcomes of the infection. In particular, the first hour post infection (p.i.) seems to be the crucial phase during which Francisella needs to hijack intracellular trafficking of the host to escape from the phagosome. In the same time, the bacterium needs to avoid bactericidal mechanisms and the activation of DC in general. The nature of these rapid steps should be reflected in changes in membrane proteome and phosphoproteome of Francisella-infected DCs. Therefore, our goal was to analyze and to describe these events by global LC-MS-based proteomic approach in hope we will be able to put the results into the perspective of Francisella infection.

Methods
Bacterial strains – Virulent strain Francisella tularensis subsp. holarctica FSC200 was used in infection experiments.
Bone-marrow derived DC (BMDC) – Primary BMDCs generated from murine bone marrow were used as a host infection model. Isolated bone marrow cells were kept for 9 days in RPMI-1640 with 10% FBS in the presence of recombinant GM-CSF. On 9th day, about 80-85% suspension cells were CD11c+. For SILAC labeling (Stable Isotope Labeling by Amino Acids in Cell Culture), we developed a modified cultivation protocol yielding cells with proteome labeling efficiency ≥90%.
Isolation of membrane rafts proteome – BMDCs were lysed in a lysis buffer containing 0.5% TX-100. Lysate was overlaid with 30% and 5% sucrose and centrifuged for 24 h at 150,000g at 4 °C. Membrane raft fraction, separated as a band between 30% and 5% sucrose, was collected and proteins were digested by trypsin.

Isolation of phosphopeptides – Whole-cell lysate digest was subjected to HPLC fractionation based on HILIC-retention mechanisms. Phosphopeptides from collected fractions were enriched by the incubation with TiO2 microbeads.

LC-MS – Peptides were separated on C18 column by nano-scale LC system connected through nanospray with high-resolution mass spectrometer. Data were generated by cycling of MS and MS/MS scans. Precursors were selected dynamically from previous MS scan.

Results
Global changes in membrane proteome (here typified by proteome of membrane rafts which serve as signal-transducing scaffolds) and phosphoproteome of Francisella-infected primary BMDCs during the first 60 minutes of invasion were analyzed by shotgun proteomics employing SILAC-based quantitation. Moreover, to obtain time-dependent image of the process, these events were mapped in three time points within this early phase of infection. For early interval of 10 min p.i., BMDC membrane raft proteome and phosphoproteome were studied to track changes associated with the bacterial entry. In addition, host phosphoproteome was analyzed in 30 and 60 min p.i. to uncover processes connected with bacterial escape into cytoplasm. Overall, we were able to identify more than 1,200 proteins of membrane rafts and more than 17,000 phosphosites in Francisella-infected BMDCs. While changes in membrane raft proteome were rather subtle, response to bacterial invasion was apparent on the global state of phosphoproteome which showed two signaling maxima (10 and 60 min p.i.). Protein-protein interaction network based on STRING database was constructed to merge the results from membrane raft proteome and phosphoproteome datasets (2,725 nodes connected by 22,777 edges). Phosphosites quantified in all three time points were clustered by fuzzy c-means algorithm according their time profile. Kinase motif analysis was applied to these clusters to obtain activity profiles of potentially involved kinases.

Discussion
First 10 minutes of Francisella invasion of BMDCs is characterized by the regulation of host pathways connected to remodeling of cytoskeleton and vesicular trafficking. For instance, activation sites of PAK kinases were found to be phosphorylated and also PAK-kinase motifs were enriched in phosphosite cluster with maximum in 10 min p.i. Changes in proteome of membrane rafts and phosphoproteome also suggest regulation of Ras signaling together with the activation of Erk1/2 and Akt pathways. In 30 min p.i., the regulation of phosphosites returns to the level of control cells. However, in 60 min p.i., the second signaling “peak” occurs. Based on the state of phosphosites connected with kinase regulation, p38, JNK and Erks seem to be activated. Phosphosites in the cluster with the maximum in this time point show enrichment for AMPK kinase motif. Interestingly, AMPK activity follows the inverse trend than that of Akt kinase. Phosphorylation states of corresponding substrates confirm these results and also point to the down-regulation of mTORC1 complex activity and to the induction of autophagy in 60 min p.i.

Conclusions
Two important early events of Francisella invasion are reflected in the cellular signaling of infected BMDCs – cellular entry and the escape of the bacterium from the phagosome. The latter ignites pathways leading to the induction of autophagy. While the impact of the process on the fitness of cytosolic Francisella is still debated, our results show that this occurs in the same time frame as the appearance of first bacteria in the cytosol.
Summary

*Francisella* tularensis is highly virulent intracellular bacterial pathogen which is able to evade the effective immune response of the host. As the priming of the adaptive immunity relies on the antigen presentation and the most effective antigen-presentation cells – dendritic cells (DCs) – are susceptible to *Francisella* infection, it was suspected that the regulated interplay with this host cell may be actually beneficial for *Francisella*. Nevertheless, the interaction with DC must be tightly controlled in order to avoid host anti-bacterial mechanisms while establishing cytosolic replicative niche in the same time. To explore these early and rapid events in detail, we decided to map DC response during the first hour of *Francisella* invasion by the means of shotgun proteomics. States of membrane raft proteome and phosphoproteome in 10 min p.i. and the state of phosphoproteome in 30 and 60 min p.i. were analyzed. Interestingly, results showed two peaks of DC signaling. The first was associated with the entrance of the bacterium; regulated proteins were involved mainly in cytoskeleton rearrangements and vesicle trafficking and also Ras, Erk1/2 and Akt pathways signaling. The second wave of DC response occurred in 60 min after infection, i.e. during phagosomal escape of the bacterium. In this point, all MAPKs seemed to be activated. Also in correlation with the dampening of Akt signaling, AMPK activity increased. This pattern would suggest the inactivation of mTORC1 complex and the induction of autophagy-related processes.

References

Introduction and Aims
The deposition of alpha-synuclein (aSyn) in Lewy bodies is the neuropathological hallmark of Parkinson’s disease (PD)\(^1\). In addition to the characteristic motor symptoms, cognitive disturbances are also common in PD. These cognitive deficits, which can predict the development of dementia in later stages, do not respond to dopamine therapies, and represent an unmet need in the treatment of PD\(^2\).

Recently, adenosine A\(_{2A}\) receptors (A\(_{2A}\)R) emerged as an attractive non-dopaminergic target for the treatment of both motor as well as non-motor symptoms of PD. In fact, A\(_{2A}\)R deregulation was suggested to play an important role in aSyn mediated neurotoxicity, since aSyn induced damage to striatal neurons was clearly reduced in A\(_{2A}\)R KO mice\(^3\).

Nevertheless, the precise molecular mechanisms underlying neuroprotection and the effects of blocking A\(_{2A}\)R on synaptic plasticity and cognition in a PD context remain unknown. Therefore, the purpose of this study was to gain insight into the novel concept of a crosstalk between aSyn and A\(_{2A}\)R and to explore the ability of A\(_{2A}\)R to modulate aSyn-mediated synaptic dysfunction.

Methods
Electrophysiological recordings
Field excitatory post synaptic potentials (fEPSPs) were recorded as previously\(^4\) in the stratum radiatum of the CA1 area from the hippocampus of male Wistar rats (8–12 weeks old; Harlan Interfauna Iberica, SL). Long term potentiation (LTP) was induced by a theta-burst protocol (10 trains with four pulses each at 100 Hz, separated by 200 ms).

Co-immunoprecipitation and Western blot analysis
WT rat hippocampal slices were homogenized and immunoprecipitated with anti-PSD-95 antibody (1:50; Cell Signaling). Immunoprecipitation and western blot analysis were performed as previously described\(^5\). Immunoprecipitates were probed with anti-NMDA receptor subunit 2B (NMDAR2B; 1:1000; Cell Signaling), anti-NMDA receptor subunit 1 (NMDAR1; 1:1000; Millipore) and anti-PSD-95 (1:1000) antibodies.
Statistics
The values presented are mean ± SEM of n independent experiments. A one-way ANOVA, followed by a Bonferroni’s multiple comparison post-hoc test was used to test the significance of the differences between conditions. Values of P < 0.05 were considered to be statistically significant.

Results
We have previously demonstrated that aSyn oligomers, but not monomers or fibers, impair synaptic plasticity and increase basal synaptic transmission through NMDA receptor (NMDAR) activation4. Thus, we set out to investigate the role of A2AR on this impairment of synaptic function. For this, we pre-incubated rat hippocampal slices with aSyn oligomers (500nM, 90min) together with the selective A2AR antagonist SCH58260 (50nM, 110min; Tocris; Fig. 1), and induced LTP in Schaffer-collaterals/CA1 pyramid glutamatergic synapses. The LTP amplitude was significantly reduced in slices pre-incubated with aSyn oligomers when compared to control slices (LTP_{CTR}=58,1±7.4%; LTP_{aSyn olig}=7.7±2.3%; n=9; *P<0,001; Fig. 1). When A2AR were blocked by SCH58261, the LTP magnitude was reestablished to control values (LTP_{SCH58261+aSyn olig}=50,6±13,0%; n=5; *P<0,01 vs. LTP_{aSyn olig}; Fig. 1). SCH58261 alone did not affect LTP magnitude (LTP_{SCH58261}=52,5±10,6%; n=5; *P<0,05 vs. LTP_{CTR}; Fig. 1).

Figure 1. A2AR blockade rescues LTP impairment induced by extracellular aSyn oligomers.
Top panels: Schematic representation of hippocampal slices incubation protocol and representative traces (1) prior and (2) after LTP induction, composed of the stimulus artifact followed by the presynaptic volley and the fEPSP. Left lower panel: Changes in fEPSP slope upon LTP induced by theta-burst stimulation from hippocampal rat slices (CTR: control; aSyn olig: after pre-incubation with aSyn oligomers, 500 nM, 90 min; aSyn olig + SCH58261: after incubation with α-synuclein oligomers in the presence of the A2AR antagonist, SCH58261, 50 nM, 110 min). SCH58261 rescued LTP impairment induced by aSyn oligomers. *P < 0.01. Right lower panel: LTP magnitude after theta-burst stimulation (change in fEPSP slope at 50–60 min).
To assess the role of A2A R on NMDAR mediated effects, we evaluated the effect of the NMDAR antagonist APV (50µM; Abcam) on basal synaptic transmission. As expected, APV did not modify the fEPSP slope in control slices (Fig. 2). In contrast, the acute application of APV induced a progressive reduction of the fEPSP in aSyn oligomer-treated slices (fEPSP<sub>CTR</sub>=100.0±1.2%; fEPSP<sub>aSyn olig</sub>=87.6±2.1%; n=6-7; *P<0.001; Fig. 2), in agreement with the previously reported impact of oligomeric aSyn on NMDAR4. Interestingly, when slices were pre-incubated with SCH58261 together with aSyn oligomers, the effect of the NMDAR antagonist was prevented (fEPSP<sub>SCH58261+ aSyn olig</sub>=94.8±1.0%; n=4; *P<0.05 vs. fEPSP<sub>aSyn olig</sub>; Fig. 2).

Figure 2. A2A R blockade rescues NMDAR overactivation in basal conditions induced by extracellular aSyn oligomers. Left panel: Effect of NMDAR antagonist APV (50 µM, 30 min) superfusion on basal fEPSP slope. SCH58261 prevented the effect of aSyn oligomers on NMDAR contribution for basal synaptic transmission. *P < 0.05. Right panel: quantification of the effects observed in left panel (change in slope between baseline and the last 10 min of APV application).
Accordingly, we observed an increase in NMDAR2B (137.9±9.4%; n=3; *P<0.05 vs CTR; Fig. 3) in slices exposed to aSyn. This increase was prevented by co-incubation with SCH58261 (95.1±11.9%; n=3; *P<0.05 vs CTR; Fig. 3). These modifications in the levels of NMDAR2B were specific to this NMDAR subunit since NMDAR1 levels were not modified by aSyn oligomers nor SCH58261 incubation. SCH58261 alone did not alter NMDAR2B levels.

**Figure 3.** A2A R blockade rescues NMDAR2B increased levels induced by extracellular aSyn oligomers. Co-immunoprecipitation of PSD-95 in hippocampal slices. NMDAR subunit 2B (NMDAR2B) are enriched in aSyn olig pre-incubated slices while co-incubation with SCH58261 reestablished NMDAR2B subunit levels. NMDAR subunit 1 (NMDAR1) levels were not changed in any condition. Values were normalized to PSD-95. IgG was used as a negative control (Neg CTR). *P < 0.05.

**Discussion**

Our data demonstrate that the selective blockade of A2A R prevents synaptic plasticity impairment induced by extracellular aSyn oligomers. The ability to respond to theta-burst stimulation, when exposed to aSyn mature oligomers, is restored in WT hippocampal slices in the presence of a specific A2A R antagonist. As we have previously reported, aSyn oligomers promote an increase in basal synaptic transmission both by the activation of NMDAR and by the insertion of Ca2+-permeable AMPA receptors (AMPAR) in the post-synaptic membrane4, which leads to synapse saturation and consequent LTP impairment. Since Ca2+- permeable AMPAR are crucial for LTP maintenance6, the complete rescue of LTP impairment by A2A R antagonist suggests a reestablishment of this AMPA impaired trafficking. In fact, it has been described that A2A R have the ability to modulate the membrane levels of Ca2+-permeable AMPAR7, which can explain the observed effects. Furthermore, we show that the basal overactivation of NMDAR caused by aSyn oligomers, is also prevented by A2A R blockade, since NMDAR basal contribution is no longer observed.

Together our results suggest that aSyn-induced toxicity is associated with an increase in A2A R activation that mediates NMDAR overactivation which is a prominent synaptic event leading to excitotoxicity8. In fact, A2A R are known to increase NMDAR function in the hippocampus9, namely promoting Ca2+ entry through NMDAR, by (PKA)-dependent regulation10. Interestingly, the same mechanisms as Ca2+ entry dysfunction and NMDAR activation4 are involved in aSyn-associated neurotoxicity. This suggests that A2A R blockade is probably counteracting these aSyn-associated effects, which then translates into the prevention of synaptic dysfunction.
Conclusions
We have gathered evidence indicating that A2A R play an important role in modulating the deleterious effects of aSyn. Here, we show, for the first time, that pharmacological inactivation of A2A R fully prevents the aSyn-mediated toxic effects on synaptic function. This neuroprotective effect afforded by A2A R inhibition is due to the reestablishment of glutamate NMDAR signaling.
Currently, there are multiple specific A2A R antagonists, including caffeine, progressing through phase II and III clinical trials for PD treatment. The present findings strengthen the rationale for disease modification trials of A2A R antagonism and substantially broaden the potential use of A2A R as therapeutic targets in synucleinopathies.

Summary
The deposition of alpha-synuclein (aSyn) in Lewy bodies is the neuropathological hallmark of Parkinson’s disease (PD). In addition to the characteristic motor symptoms, cognitive disturbances are also common in PD. These cognitive deficits, which can predict the development of dementia in later stages, do not respond to dopamine therapies, and represent an unmet need in the treatment of PD. Recently, adenosine A2A receptors (A2A R) emerged as an attractive non-dopaminergic target for the treatment of both motor as well as non-motor symptoms of PD. Nevertheless, the precise molecular mechanisms underlying neuroprotection and the effects of blocking A2A R on synaptic plasticity and cognition in a PD context remain unknown.
Based on our previous data showing that aSyn oligomers impair long-term potentiation (LTP), we set out to investigate whether A2A R blockade prevented synaptic dysfunction. We found that hippocampal slices preincubated with the selective A2A R antagonist SCH58260 (110 min, 50 nM) did not lose the ability to sustain LTP when preincubated with aSyn oligomers (90 min, 500 nM) (n=5-9, P<0.01). This neuroprotection was achieved through the prevention of NMDA receptor (NMDAR) basal overactivation and NMDAR subunit 2B increased levels caused by aSyn oligomers.
Overall, these results reveal the involvement of A2A R in the aSyn-associated synaptic impairments by showing that long-lasting synaptic effects of aSyn oligomers can be reverted by targeting adenosine A2A R. These findings provide a novel evidence for the use of adenosine A2A R antagonists as potential therapeutic targets in PD-related cognitive deficits.

References


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Introduction, aims
Volume regulatory mechanisms influence the outcome of several physiologic and pathophysiologic processes such as cell proliferation, migration, adaptation to osmotic stress, metabolic and electrolyte balance. Recently, it has been shown that protein O-glycosylation (O-GlcNAcylation) has an influence on cell volume regulation under hypotonic stress (1).

O-GlcNAcylation is a dynamic post-translational modification, targeting the Ser/Thr moieties of proteins by the addition of an N-acetyl-glucosamine molecule by the enzyme called O-GlcNAc Transferase (OGT). The cleavage of O-GlcNAc moieties happens by O-GlcNAcase (OGA). The modification can be responsible for the control of proteins’ activity, conformation, interaction with molecular partners, homo-oligomerization, compartmentalization and stability (2,3).

In the present work, we studied whether the so called ICln protein (chloride channel, nucleotide sensitive) is influenced by O-GlcNAc. ICln is a highly conserved, ubiquitously expressed multifunctional protein that plays a critical role in the mechanisms counteracting cell swelling in hypotonia, which process is called regulatory volume decrease (4,5). ICln is thought to be transposed to the plasma membrane after hypotonic challenge and it is responsible for the outwardly rectifying chloride ion current (6). Recently, ICln has been identified as a candidate for O-GlcNAc protein (7).

Methods
Cell line and culture conditions. HeLa cells (human cervix carcinoma cell line) were grown in a 1:1 mixture of EMEM and Ham’s F12 medium supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, penicillin (100 U/mL) and streptomycin (100 µg/mL). O-GlcNAc levels were influenced metabolically by adding 10 mM glucosamine or enzymatically by adding 100 µM PUGNAc (O-(2-Acetamido-2-deoxy-D-glucopyranosylidene)amino N-phenyl carbamate), which inhibits OGA. Cells treated with DMSO (solvent of PUGNAc) and 10 mM mannitol served as controls. Hypotonic (200 mOsmol) and normotonic (300 mOsmol) treatments were performed by incubation in a modified Hank’s balanced salt solution (80 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1mM MgCl2, 1,2 mM CaCl2, 5 mM glucose,15 mM HEPES, +/- 100 mM mannitol; pH=7,35) for 15 minutes, at 37 °C.

Immunoblotting. Cell lysates were separated by 7,5% SDS-PAGE. Gels were electroblotted onto a PVDF membrane. Blots were incubated with CTD110,6 a monoclonal mouse IgM antibody, which is highly specific for O-glycosylated proteins, and polyclonal rabbit anti-ICln antibody, followed by respective HRP-conjugated secondary antibodies. The bands were visualized using Femto chemiluminescent substrate (Pierce) and signal was detected by Kodak Image Station 2000R. To verify that equal amount of protein was loaded in each lane, total protein staining of the membranes was performed with SYPRO Ruby Protein Blot Stain (Bio-Rad).
**Immunofluorescent staining.** The expression of ICln and the amount of O-glycosylated proteins were also analyzed by indirect immunofluorescence using the same primary antibodies (CTD 110,6 anti-ICln) and Alexa Fluor 594-conjugated anti-mouse IgM and FITC-conjugated anti-rabbit IgG as secondary antibodies. Photos were taken with Zeiss LSM 710 confocal microscope.

**Statistical analysis.** All data are presented as mean±SEM. One-way ANOVA followed by Bonferroni multiple comparisons test was performed using GraphPad Prism version 6,00 GraphPad Software, La Jolla California USA.

**Results**

Immunofluorescent staining of HeLa cells showed that elevated O-glycosylation caused a significantly higher intensity of ICln staining (Fig.1.A). However, analysis of cells exposed to hypotonic shock showed decreased amount of O-GlcNAc labeling (Fig.1.B.). This decrease was more pronounced in the case of elevated O-GlcNAc levels caused by PUGNAc and glucosamine pretreatment.

Western blot analyses with the same conditions confirmed our results gained by immunofluorescence, namely, that increased level of O-GlcNAc resulted in higher amount of ICln protein expression (Fig.2.A.). The amount of ICln showed parallel changes with O-glycosylation (Fig.2.B.). After glucosamine treatment, a marked increase of ICln was detectable but a further increase in O-glycosylation by the addition of PUGNAc caused a more significant elevation in the amount of ICln.

The difference in O-glycosylation between normo- and hypotonic cells observed with immunofluorescence was also detectable with western blot but differences were statistically not significant with this method (Fig. 3.A.B.).

![Figure 1](image-url)  
*Figure 1. Results of immunofluorescent stainings in various conditions. Diagrams show the relative pixel intensity, expressed as a ratio to the average of control, normotonic cells. A. Relative amount of positive pixels labeled with anti-ICln antibody. B. Relative amount of positive pixels labeled with CTD110,6 (anti-O-GlcNAc) antibody. Data are the cumulative sum from three independent experiments, average pixel intensity was collected from at least 15 cells. Data are shown as means ±SEM, ** p<0.001, *** p<0.0001.*
Figure 2. Representative western blots of cell lysates form control, glucosamine treated and PUGNAc + glucosamine treated HeLa cells. A. Anti-ICln staining B. O-GlcNAcylated proteins C. Total protein staining.

Figure 3. A. Western blot analysis using CTD110,6 antibody to detect O-GlcNAc modified proteins. B. Densitometric analysis of the CTD110,6 staining. Levels are expressed as a percentage of the baseline ratio. Each data point represents the average of at least 3 separate experiments. Data are shown as means ±SEM.
Discussion
Our results show that O-glycosylation seems to influence ICln, a protein strongly involved in cell volume regulation. On the other hand, we have also found that hypotonic challenge decreases the overall level of protein O-glycosylation in HeLa cells. The present data, taken together with our previous data (1) and the fact that ICln was spotted in a proteome wide screening study as a candidate for O-glycosylation strongly suggest ICln and O-glycosylation interaction is a key element in cell volume regulation. Our collaborators’ preliminary findings further support the notion; the function (chloride conductance upon cell swelling, measured with patch clamp) of ICln was inhibited by increased O-GlcNAcylation, however it was enhanced by lowered O-GlcNAc levels (data not shown). Our current experiments focus on enriching ICln by immunoprecipitation to subsequently analyze it by mass spectrometry and to pinpoint the O-GlcNAc site(s) on ICln.

Conclusions
Our current working hypothesis is that ICln is an O-GlcNAcylated protein and that the function of O-glycosylation is to inhibit ICln’s transport to the cell membrane and consequently inhibiting regulatory volume decrease. During hypotonic stress, O-GlcNAc modification decreases and ICln is released from the inhibition. We think this process could have important clinical implications if it proves to be a valid hypothesis. E.g. in diabetes, the balance of O-GlcNAc modification is altered and therefore its impact on volumetric, cellular morphologic and intracellular water regulatory mechanism should be considered.

Summary
Volume regulatory mechanisms influence the outcome of several physiologic and patho-physiologic processes. It has previously been shown by us that protein O-glycosylation influences the regulation of cellular volume under hypotonic stress condition. O-GlcNAc is a dynamic post-translational modification, targeting the Ser/Thr moieties of proteins by the addition of a single N-acetyl-glucosamine molecule. In the present work, we studied whether the so called ICln protein (chloride channel, nucleotide sensitive) is influenced by O-GlcNAc. ICln is a highly conserved, ubiquitously expressed multifunctional protein that plays a critical role in regulatory volume decrease. ICln is thought to be transposed to the plasma membrane after hypotonic challenge and it is responsible for the outwardly rectifying chloride ion current.
In cell culture models (HeLa) we exposed cells to normo-, or hypotonic conditions. O-GlcNAc levels were influenced metabolically by glucosamine or PUGNaC (O-(2-Acetamido-2-deoxy-D-glucopyranosylidene)amino N-phenyl carbamate), which inhibits the enzymatic removal of N-acetyl-glucosamine from proteins. We measured the amount of expressed ICln protein by western -blot and immunofluorescence.
We have found that increasing the level of O-GlcNAc also caused increased levels of ICln protein expression. However, when HeLa cells were exposed to hypotonic shock, the amount of O-GlcNAc labeling decreased. The patch clamp results of our collaborative partners -showing the function (chloride conductance upon cell swelling) of ICln was inhibited by increased O-GlcNAc levels, however it was enhanced by lowered O-GlcNAcylation- also support our findings. Taken together, we think that ICln is O-GlcNAcylated and its IClnswell function is inhibited under normotonic conditions, but it is released from the inhibition during hypotonic stress. This work was funded by PTE ÁOK-KA-2013/19 and supported by János Szentágothai Research Centre.
References
A GENOME-SCALE COLLECTION OF GENE DELETION MUTANTS IN THE HUMAN FUNGAL PATHOGEN C. GLABRATA

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Summary
Within the last decade, there has been a steady increase of invasive fungal infections in immunocompromised patients. The most common of these infections are caused by Candida spp., which have a high mortality in their disseminated form. C. glabrata (C.g.) now is the second most frequent cause and accounts for 15 – 20 % of all cases of Candidiasis. Importantly, C.g. is inherently tolerant to azole antifungals when compared to most other Candida spp. This is of clinical importance because of the wide use of azole therapy in fungal infections. Moreover, C.g. is unable to form true hyphae or secrete proteases, which are considered important virulence factors of C.a. These differences, as well as the high tolerance to conventional fungal therapies, leave the nature of virulence factors largely unknown. To identify candidate virulence and drug resistance factors, we initiated the construction of a large-scale collection of C.g. deletion mutants. This collection enables studies on the molecular functions of genes implicated in virulence and drug resistance, as well as those modulating signaling pathways and stress response. Hence, we use a reverse genetic approach to generate a bar-coded C.g. gene deletion collection, which is subsequently analyzed in vitro for their sensitivity to different environmental stress conditions including heat, pH and osmotic stress, as well as a variety of other conditions including antifungal drug susceptibility. The collection now comprises more than 700 deletion strains. We shall present a comprehensive data set on the phenotypic profiling of the deletion collection in comparison to data from clinical patients isolates. Interestingly, a number of C.g. clinical isolates display high-level resistance to azoles (Fluconazole, Voriconazole and Posaonazole) as well as echinocandins (Caspofungin). Based on the phenotypic data from the deletion collection, we have also generated deletion mutants in clinical isolates to delineate the contributions of specific genes to clinical drug resistance phenotypes.

Introduction
Within the last decade, there has been a steady increase of invasive fungal infections in immunocompromised patients. The most common of these infections are caused by Candida spp., which have a high mortality in their disseminated form. C. glabrata (C.g.) now is the second most frequent cause and accounts for 15 – 20 % of all cases of Candidiasis [1]. Importantly, C.g. is inherently tolerant to azole antifungals when compared to most other Candida spp. This is of clinical importance because of the wide use of azole therapy in fungal infections [2,3]. Moreover, C.g. is unable to form true hyphae or secrete proteases, which are considered important virulence factors of C. albicans. In addition, more than 95 % of the genome of C.g. is uncharacterized [4]. These differences, as well as the high tolerance to conventional fungal therapies, leave the nature of virulence factors largely unknown.
The aim of this study was to construct a genome-scale gene deletion library of bar-coded C.g. mutants. Each C.g. mutant lacks a single gene, therefore, creating the opportunity to study the role of a specific gene. The created deletion mutants were then to be analysed using large-scale screening for antifungal and other stress stimuli.

**Methods**

To construct the gene deletion mutants the HTL background strain was used. This strain is based on ATCC2001 (CBS138) and lacks HIS3, LEU2 and TRP1. The single genes were then replaced by gene deletion cassettes. These cassettes were assembled by fusion PCR and consist of a NAT1 marker for Nourseothricin resistance, two molecular barcodes and two flanking regions of 500 bp each. After transformation by electroporation the transformants were grown on agar plates supplemented with Nourseothricin [5]. In addition, transformants were analysed by colony PCR, ensuring that the cassette replaced the targeted gene completely. Figure 1 shows the construction of gene deletion mutants.

*Figure 1. Generation of gene deletion mutants and analysis of transformants. (A) Gene deletion cassettes were assembled by fusion PCR using the dominant marker NAT1 with two barcoded-markers and two flanking regions. (B) Correct integration of the gene deletion cassettes was ensured by colony PCR. The absence of the targeted gene was controlled by PCR as well. (C) Overview of the steps involved in the construction of gene deletion mutants. Figure taken from Schwarzmüller et al., 2014 [6].*
For phenotypic profiling the deletion strains were arrayed in a 96-well format. The strains were then plated on square YPD agar plates by a Singer RoToR HDA replicating robot (Singer Instruments, Somerset, UK). After growing the strains for 24 to 48 hours at 30 °C, the growth of strains on YPD agar plates supplemented with antifungals was compared to the growth on regular YPD agar plates. Strains that showed a reduced growth on plates supplemented with antifungals were subjected to a refined screening. This refined screening consisted of a solid screening with a dilution series of cells and a liquid screening to determine the minimal inhibitory concentration.

Results
The phenotypic analysis resulted in the identification of genes involved in the tolerance to Caspofungin, azoles and AmphotericinB. Figure 2 shows the results for all three antifungal classes as well as the overlaps. A total of 48 genes were shown to be involved in Caspofungin tolerance, 14 in azole tolerance and 13 in AmphotericinB tolerance. Several genes were involved in the tolerance to two or even three antifungal classes [6].

Figure 2. Result of the phenotypic analysis. Genes that are involved in tolerance to the tested antifungals are connected by lines. For genes with known homologs in Saccharomyces cerevisiae (S.c.) the gene name in S.c. is shown. For those without a known homolog the ORF number starting with CAGL is shown [4,7]. Figure taken from Schwarzmüller et al. [6].
Discussion
Most of the genes of C.g. are uncharacterized, meaning that their function is entirely unknown. Therefore, it is of great importance to develop tools to identify and to study putative virulence and resistance genes in C.g. In this study it was possible to identify genes related to the tolerance of C.g. to the antifungals currently in use. Several of the genes are implied in the tolerance to two or even three of these classes. Importantly, 28 genes were even implicated for the first time in the tolerance to the echinocandin Caspofungin. Further studies of those genes will bring new insights into resistance mechanisms of C.g. However, this deletion mutant collection is also a valuable tool to study other aspects of C.g. infections such as virulence and host-pathogen interactions.

References
QUANTIFICATION OF ENDOGENOUS ANTIBODIES AGAINST NEURONAL PROTEIN ASSOCIATED WITH ALZHEIMER’S DISEASE IN HUMAN SERUM AND CSF

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Introduction
Alzheimer’s disease (AD) is characterized as a protein conformational disorder. Two key molecules – Abeta peptide and tau protein participate in formation of specific aggregates in AD brains. Recently, naturally occurring antibodies against both proteins were detected in serum of AD patients as well as of healthy subjects and in intravenous immunoglobulin products. In a present study, we have focused on quantification of endogenous serum and CSF antibodies reactive with various forms of tau protein in four groups of individuals with different stage of cognitive impairment. At first, we isolated tau-reactive antibodies from plasma pool of healthy donors and verified their reactivity against recombinant and native tau proteins. Subsequently, the isolated antibodies served as a standard to evaluate the specific antibody levels occurring in biofluids of studied individuals.

Methods
Immunoaffinity chromatography
The isolation of plasma antibodies from intravenous immunoglobulin product Flebogamma was carried out against recombinant full-length tau protein. Plasma pool diluted with PBS buffer was applied to a column containing agarose resin with covalently bound tau protein. Retained IgG were recovered by acidic elution and concentration of eluate was established spectrophotometrically at 280 nm as 1.6 mg/ml.

Western blotting
To characterize the reactivity of isolated plasma antibodies, sections of left hemisphere hippocampi from 2 AD patients and 2 controls were homogenized in PBS or PBS containing 2% SDS in 1:3 w/v dilution. 25 μg of total protein/lane was loaded onto the Mini-Protean TGX 10% precast gels and separated proteins were transferred onto the nitrocellulose membrane. Membranes were blocked with 10% non-fat dried milk in PBS with 0.1% Tween-20 (PBST) and incubated overnight at 4°C with primary antibodies diluted with 1% BSA in PBST; rabbit polyclonal anti-tau antibody (1:250), mouse monoclonal Tau 5 antibody (1:60,000), isolated plasma anti-tau antibodies (1:125), rabbit polyclonal anti-pSer396 tau antibody (1:1,000). Incubation for 1 hour at RT with secondary antibodies; anti-rabbit or anti-mouse antibody HRP conjugates (1:3,000), anti-human IgG HRP conjugate (1:10,000) and chemiluminescence detection by WesternBright ECL HRP substrate followed.
ELISA assay
Isolated plasma antibodies were used as a standard in an ELISA assay for quantification of antibodies reactive to tau protein in serum and CSF of individuals with different stage of cognitive impairment (Table 1). 0.125 μg/well of full-length tau protein or fragment of tau 155-421 aa was coated to microplates overnight at 4°C. After blocking with 1% BSA in PBST, diluted serum (1:200, 1:600, 1:1,800), undiluted CSF and standard (36-0,05 μg/ml) were applied to wells for 1 hour in RT. Washed plates were incubated with F(ab')2-goat anti-human IgG HRP conjugate diluted 1:20,000 for 30 minutes. After washing, the chromogen substrate TMB was added and the plates were shaken in the dark for 25 minutes. The reaction was stopped by 1M H₂SO₄ and signal read at wavelength 450 nm. The concentrations of tau-reactive antibodies were interpolated from standard curve using GraphPad Prism software and for serum samples adjusted to the dilution factor.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>N</th>
<th>Female sex (%)</th>
<th>Age at sampling (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls</td>
<td>44</td>
<td>19 (43%)</td>
<td>66 ± 8</td>
</tr>
<tr>
<td>Mild cognitive impairment</td>
<td>32</td>
<td>14 (44%)</td>
<td>69 ± 8</td>
</tr>
<tr>
<td>Alzheimer disease</td>
<td>28</td>
<td>23 (82%)</td>
<td>75 ± 7</td>
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<tr>
<td>Other dementias</td>
<td>26</td>
<td>11 (42%)</td>
<td>66 ± 10</td>
</tr>
</tbody>
</table>

*Table 1. Description of groups of individuals enrolled in the measurement of tau-reactive antibodies levels.*

All experiments are in accordance with the Declaration of Helsinki. The research was formally approved by the local ethics commission of the National Institute of Mental Health/Klecany, Czech Republic.

Results
The reactivity of isolated plasma antibodies was compared with other selected antibodies using human brain homogenates (Fig. 1). Monoclonal Tau 5 antibody and rabbit polyclonal anti-tau antibody showed typical distribution of tau isoforms in the brain as well as higher molecular weight (HMW) protein bands. On the contrary, isolated plasma antibodies showed reactivity preferentially to HMW proteins. All used antibodies showed differences between control brain and AD brains. There was difference in the reactivity even between AD brains, especially for pSer396 antibody. The staining with pSer396 antibody point out to different stages of disease, because this antibody is specific to late stage of AD⁸.
To estimate the levels of endogenous antibodies reactive with tau protein, we performed ELISA assay. We detected tau-reactive antibodies in serum and CSF of all four analyzed groups. We found differences in levels of serum antibodies between control group and AD patients for tau fragment 155-421 aa (Mann-Whitney test, p value 0.047; Fig. 2).

**Figure 1.** Western blot analysis of human brain homogenates (25 μg of total protein/lane) was carried out using polyclonal rabbit anti-tau antibody (A); monoclonal Tau 5 antibody (B); polyclonal pSer396 antibody (C) and isolated endogenous antibodies reactive with tau protein (D). Left hemisphere hippocampi of two control brains and 2 histopathologically proven AD patients were homogenized in PBS buffer (1,7 - control brains and 3,5 - AD brains) or PBS buffer containing 2% SDS (2,8-control brains) and (4,6 - AD brains), respectively. Recombinant fragment of tau 155-421 with the theoretical molecular weight of 30 kDa (2 μg/well) (9) was included as a positive control. An error occurred in the preparation of one control brain homogenate (7,8) as was proven by staining with anti-tubulin antibody (data not shown).

To estimate the levels of endogenous antibodies reactive with tau protein, we performed ELISA assay. We detected tau-reactive antibodies in serum and CSF of all four analyzed groups. We found differences in levels of serum antibodies between control group and AD patients for tau fragment 155-421 aa (Mann-Whitney test, p value 0.047; Fig. 2).
Discussion

Immunotherapeutic interventions are now in the spotlight of AD research. The occurrence of natural antibodies against Abeta peptide and tau protein in serum has been proven. Therefore, we were interested in characterization of these natural endogenous antibodies.

We confirmed that the plasma of healthy donors contains antibodies reactive to tau protein. We compared the reactivity of these antibodies and other selected polyclonal and monoclonal anti-tau antibodies against brain homogenates containing different forms of tau protein ranging from presumable monomers of different isoforms (characteristic triplet) and fragments (Fig. 1A,B) to HMW protein forms (Fig. 1A,C and D). The HMW proteins were found mostly in AD brains in contrast to control brain tissue. Interestingly, the isolated plasma antibodies stained the HMW protein bands mostly. The most studied pathological forms of tau protein are now toxic soluble oligomers, which are believed to propagate the pathology of AD. The results with endogenous antibodies can point out to immune system involvement in controlling of the toxic aggregates occurrence.

Figure 2. The serum and CSF levels of endogenous antibodies measured by ELISA assay against two forms of tau protein (0.125 μg/well); recombinant full-length form of tau protein (C and D) and fragment of tau protein (155-421 aa; A and B) in groups of healthy controls, patients with Mild Cognitive Impairment (MCI), patients with Alzheimer’s Disease (AD) and with Other Dementias (OD). The graphs are expressed as median with a box of 25th-75th percentiles and whisiers showing 10th-90th percentiles. Statistical analysis was performed by Mann-Whitney test at significant p level 0.05 (*). The standard curves for each protein form are included as inserts.
Moreover, we have measured detectable levels of antibodies against tau protein forms in serum and CSF samples. The control group had higher levels of specific antibodies against fragment of tau 155-421 aa which decreased with progression of disease. Presumably, the used fragment can represent pathological form of tau protein which eventual occurrence could be regulated by the immune system under physiological conditions. Whilst in AD, the amounts of pathological tau molecules can increase which leads to form antigen-antibody complexes and thus lowering levels of detectable antibodies.

**Conclusions**

To conclude our study, we have found that specific antibodies against tau protein occur in the plasma of healthy donors (intravenous immunoglobulins product Flebogamma). This result was supported by our measurements of serum antibodies levels from healthy controls which were higher in contrast to AD patients. We found that these antibodies stained mostly the HMW proteins, which can indicate the presence of potential regulatory mechanisms of the immune system against toxic aggregates.

**Summary**

Current studies of Alzheimer’s disease are focusing on aggregates of tau protein and the possible interventions to disrupt the aggregation process of this protein. Antibodies specific to pathological forms of tau protein, which could protect against aggregation, are most desired. The purpose of this study was to characterize reactivity of endogenous antibodies against tau protein occurring in different biofluids of cognitively healthy subjects and patients with Alzheimer’s disease. We have isolated plasma antibodies reactive to tau protein from intravenous immunoglobulin product. These antibodies turned out to be reactive mainly with higher molecular weight proteins or aggregates from AD brains and control brain. Moreover, we have detected antibodies against various forms of tau protein in serum and CSF of healthy subjects, patients with mild cognitive impairment and AD patients. We have found significantly higher levels of serum antibodies against fragment 155-421 of tau protein in the control group in comparison to AD patients group. This study provides a basic screening of endogenous antibodies occurring in human blood and CSF.

**Acknowledgments**

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**References**

TUSC3 LOSS ENHANCES THE TUMOR GROWTH OF OVARIAN CANCER

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Introduction and aims
Ovarian cancer (OC) is the most lethal gynecological malignancy and fifth most common cause of cancer-related death in women¹. The high mortality of OC mostly contributes to the fact, that the majority of cases are diagnosed in an advanced stage (FIGO III and IV) and the 5-year survival rate at this stage is only 29% of patients¹. Another complication is the lack of reliable prognostic and predictive markers of OC. Recently, a high correlation between OC patients’ prognosis and epigenetic silencing of TUSC3 by promoter hypermethylation was found³.

Tumor suppressor candidate 3 (TUSC3), originally named N33, is a putative tumor suppressor located on chromosomal region 8p22, which is often lost in common epithelial cancers, such as breast, prostate, oral squamous or ovarian cancer⁴. It is localized in the endoplasmic reticulum (ER) as a subunit of an oligosaccharyltransferase (OST) complex, which is responsible for protein N-glycosylation². Latest structural and kinetic data indicates that TUSC3 regulates substrate specificity of OST complex and enhances N-glycosylation effectivity⁵. Modified glycosylation patterns can be found in most cancers and impaired glycosylation can lead to ectopic accumulation of unfolded or misfolded proteins in ER and causing the ER stress and consequent unfolded protein response (UPR).

The aim of this work was to clarify the role of TUSC3 in ovarian cancer development and progression, to confirm the tumor suppressive character of TUSC3 in vivo and to reveal its potential role in regulation of ER homeostasis.

Materials and methods
In all experiments, we used three different cell lines derived from human ovarian serous adenocarcinoma – SKOV3, TR-170 and H134. Using lentiviral transfection, 2 variants of each cell line were prepared – one control variant with unchanged TUSC3 expression and the other with down-regulated (in the case of SKOV3 and TR-170) or upregulated (in H134 cells) expression of TUSC3. Expression of TUSC3 was checked by western blotting and qPCR. In xenograft experiments, 5x10⁶ of control or TUSC3-altered cells were subcutaneously injected in contralateral flanks of 30 mice (10 mice per cell line) and tumors were regularly measured by external caliper and by high-frequency ultrasound Vevo 2100 system. In in-vitro experiments, ER stress was specifically induced by various concentrations of tunicamycin (0,2 – 1 μg ml⁻¹). Then transmission electron microscopy (TEM), immunoblotting, MTT viability test, spheroid experiment and real-time impedance based cell analysis were performed. All methods were described in detail in⁶.
Results
Cell lines with downregulated TUSC3 induced earlier and more massive formation of tumors (Fig. 1), confirming tumor suppressive status of TUSC3. Ultrastructural TEM analysis revealed dilations of ER cisternae in TUSC3-downregulated or tunicamycin-treated cells (Fig. 2). Then, we analyzed the expression of ER stress markers (BiP, CHOP, PERK, Ire-1α and Ero-1α) by immunoblotting and revealed that cells with attenuated TUSC3 induce rather the adaptive than apoptotic pathways of UPR upon ER stress condition. MTT and spheroid assays also confirmed the higher tolerance or TUSC3-downregulated cells to ER stress induction either in 2D or 3D culture setups. Moreover, we also found that cells with attenuated TUSC3 downregulated expression of crucial epithelial markers E-cadherin and ZO-1 and in parallel, expression of mesenchymal transcription factors Slug and TCF8/ZEB was increased, indicating epithelial-to-mesenchymal transition. In concordance with these results, the real-time impedance based xCELLigence analysis also revealed alterations in adhesion and migration capability of TUSC3-lacking cells.

Figure 1. Loss of TUSC3 promotes tumor growth in immunocompromised mice. Plots represent mean tumor volume and SD.
Figure 2. (A) TUSC3 prevents alterations of rough ER. Representative TEM images of the endoplasmic reticulum in SKOV3, TR170 and H134 cells. Scale bars: 2 mm. (B) Loss of TUSC3 alters the ER stress response and induces hallmarks of the EMT in ovarian cancer cells. Cells were cultured for the indicated times in varying concentrations of tunicamycin, and expression of protein markers of the ER stress response and EMT were measured using SDS-PAGE and immunoblotting.
Discussion
TUSC3 was recently reported as a robust biomarker correlating independently with disease-free and overall-survival in ovarian cancer patients, however, its molecular role remained unclear so far. Homeostasis or ER is tightly regulated process, involving complex in- and out-signaling. UPR is activated upon deregulation of proteosynthesis or alterations in ER, Golgi or secretory pathways. The ER stress is also activated by exogenous agents, e.g. cisplatin or chemotherapy in general. In our work we reported for the first time alterations of ER homeostasis, leading to better adaptation of TUSC3-attenuated cells to stress environment by PERK-mediated UPR. Together with high proliferation rate and activation of EMT pathways, the loss of TUSC3 leads to increased invasivity in-vivo.

In light of these findings, we conclude that the cumulative effect of (i) TUSC3 silencing and (ii) extrinsic microenvironment cues that trigger the ER stress response significantly contributes to the phenotypic changes involved in the EMT and tumor dissemination observed in ovarian cancer cells.

Conclusion
We confirmed TUSC3 as a novel ovarian cancer tumor suppressor in vivo using xenograft mouse model and we identified a possible mechanism enhancing malignant potential of ovarian cancer cells with attenuated TUSC3 expression. On both the molecular and morphological levels, we have demonstrated that downregulation of TUSC3 leads to manifestation of ER stress adaptive response and subsequently the EMT and enhanced cell migration in vitro and cancer progression in vivo. These data has already been published in International Journal of Cancer.
Summary
Ovarian cancer is the most lethal of gynecologic malignancies and, in spite of recent progress in cancer therapeutics and increased knowledge about the cellular and molecular biology of cancer, still presents a clinical challenge. Recently, it was established, that epigenetic silencing of TUSC3 correlates with poor prognosis in ovarian cancer patients, and it also serves as an independent biomarker of their overall and disease-free survival. TUSC3 is localized on the membrane of endoplasmic reticulum (ER) as a part of an oligosaccharyltransferase complex, which finalizes protein N-glycosylation. Despite its potential to become therapeutic or diagnostic target, the precise molecular role of TUSC3 in neoplastic transformation or in the biology of ovarian cancer, remains unclear.

In this work, we reported that loss of TUSC3 in ovarian cancer cells resulted in structural destabilization of ER and modified the ER stress response. Attenuation of TUSC3 expression also corresponded with more efficient adhesion and migration of cancer cells, higher tolerance to induced ER stress as well as loss of epithelial phenotype and epithelial-to-mesenchymal transition. In accordance with our in vitro results, our in vivo mouse xenograft experiments confirmed the tumor-suppressive character of TUSC3.

Acknowledgements
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References
Introduction and Aims

N-methyl-D-aspartate receptors (NMDARs) are glutamate-gated ion channels permeable to calcium that mediate excitatory synaptic transmission and synaptic plasticity. However, their over-activation leads to excitotoxicity, a specific type of cell death, which contributes to the pathology of several neurodegenerative diseases. The activity of NMDARs can be influenced by a variety of allosteric modulators, including endogenous neurosteroid pregnanolone sulfate (PAS), which has an inhibitory effect on NMDARs. Similarly to open channel blockers, PAS inhibits the NMDAR activity in a use-dependent manner, but unlike them, its effect is voltage independent (1). It has been shown that analogs of endogenous neurosteroids have a neuroprotective effect and, moreover, do not induce psychotomimetic behavior, which is typical for NMDAR antagonists (2). Although the modulation of NMDARs by neurosteroids has been a subject of intensive research, localization of their binding site has so far failed. The aim of our study was therefore to identify amino acid residues at NMDARs that are important for the inhibitory effect of PAS.

Methods

To study the effect of PAS on NMDARs we used electrophysiological recordings from human embryonic kidney (HEK293T) cells expressing recombinant GluN1-1a/GluN2B receptors. Whole cell voltage clamp recordings from transfected HEK293T cells were performed at room temperature, 24-48 hrs after Matra-A transfection (IBA, Göttingen, Germany). To identify amino acid residues on NMDARs we used molecular biological techniques in combination with molecular modeling. We used Quick-Change site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) to generate point mutations in specific regions with manually designed primers purchased from Sigma. For designing the homology model of the NMDAR we used the crystal structures of AMPAR and NMDAR (3, 4).

Results and Discussion

Subunits of NMDARs share the same topology and are composed of extracellular N-terminal domain (NTD), agonist binding domain, four membrane domains (M1-M4) and intracellular C-terminal domain (CTD). In the first set of experiments we tested truncated recombinant receptors with deleted NTD (354 amino acids) of GluN1 and GluN2B subunit and constructs with deleted CTDs (GluN1838stop and GluN2B844stop). The results indicate that the deletion of NTD or CTD of either GluN1 and GluN2B subunit, or their combination, does not affect the inhibition by PAS. These results support the idea that the membrane domains of NMDARs are important for the effect of PAS. This hypothesis is in accordance with our previous study suggesting the interaction with the plasma membrane as a route for neurosteroids to access their binding site on the NMDAR (5). Since the effect of PAS is voltage independent,
the potential binding site is probably not located in the selective pore of the NMDAR’s ion channel formed by the M2 domains. Likewise, we tested the effect of PAS on GluR0 receptors, which are prokaryotic glutamate receptors selective for potassium that lack M4 domains. These receptors were still inhibited by PAS, so we excluded the M4 domains from our systematic analysis. Previous experiments show that neurosteroids act only when extracellularly applied, since due to the charged group on carbon C3 they cannot readily cross the cytoplasmic membrane (6). In subsequent experiments, we performed a series of double and single point mutations in the extracellular portions of M1 and M3 membrane domains of both GluN1 and GluN2B subunits. Results of these experiments show that the inhibitory effect of PAS on NMDARs was significantly reduced in the case of two mutations (T648A and A649T) in the highly conserved SYTAN motif in the M3 domain of the GluN1 subunit (Fig. 1). These amino acids are located in the narrowest portion of the NMDAR channel vestibule and form the extracellular gate of the ion permeation pathway.

Figure 1. Mutations in the M1 and M3 membrane domains of GluN1 and GluN2B subunits alter the inhibitory effect of PAS. Graphs of relative PAS affinity for NMDARs mutated in M1 (A) and M3 (B) domain of GluN1 subunit and M1 (C) and M3 (D) domain of GluN2B subunit. Assumed from Vyklicky, 2015 (7).
Conclusions and Summary
Results of our experiments indicate that the extracellular vestibule of NMDAR’s ion channel pore, which is accessible after receptor activation, is the site of action for neurosteroids with an inhibitory effect. We assume that neurosteroid binding into the channel vestibule prevents permeation of small ions. These results were further supported by molecular modeling. Specifically, we proposed a model of the open state of the NMDAR, which explains different contributions of GluN1 and GluN2B subunits to the inhibition by PAS. Detailed understanding of the mechanism of the inhibitory effect of neurosteroids on NMDARs has therapeutic importance for the development of drugs with neuroprotective action for the treatment of diseases involving dysfunction of the glutamate system.

Acknowledgements
Supported by GA CR P303/12/1464, P303/11/P391, P304/12/G069; TA CR TE01020028; UNCE 204013; GA UK 800313/2012/2.LF; RVO 67985823; BIOCEV CZ.1.05/1.1.00/02.0109 and CBR CZ.1.07/2.3.00/30.0025.

References
AN OPPORTUNITY TO REDUCE THE BURDENS OF CARDIOVASCULAR DISEASE AND GASTRIC CANCER CAUSED BY DIETARY SALT: IMPACTNCD MICROSIMULATION STUDY

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Tutors: Dr. Martin O’Flaherty, PhD. Prof Simon Capewell, DSc. Prof Iain Buchan, PhD.

Introduction and aims
Excess salt consumption is associated with higher risk of cardiovascular disease and gastric cancer.1,2 Globally, more than 1.5 million cardiovascular related deaths every year can be attributed to excess salt intake.3 Further life loss may occur due to salt attributable gastric cancer related deaths. Hence, World Health Organisation recommends salt reduction as one of the ‘best buy’ strategies to prevent non-communicable diseases, highlighting its cost-effectiveness and feasibility.4 Since 2003, United Kingdom (UK) has one of the most well-implemented salt reduction strategies worldwide.5 The programme comprises of a mixture of policies, namely public awareness campaigns, food labelling, and voluntary reformulation of processed food by food industry. Between 2001 and 2011 the mean salt consumption in the UK dropped from 9.5g/day to 8.1g/day.6 A great achievement, however still far from the national target of 6g/day.7 Evidence from modelling studies consistently suggest that more structural interventions, like mandatory reformulation of processed food, can be more effective than the current UK policy.8,9 Other countries, including Argentina and South Africa, have already implemented the mandatory reformulation, proving its feasibility.5 Aim of this study was to estimate the impact of current UK salt reduction policy on cardiovascular disease and gastric cancer primary prevention on England’s population. We additionally explored a counterfactual scenario, modelling the addition of mandatory reformulation of processed food to current policy.

Methods
We used the validated IMPACTNCD model (discrete time, dynamic, stochastic microsimulation) to estimate cardiovascular disease and gastric cancer burdens between 2008 and 2030. The inputs of the model were informed by published meta-analyses, population representative surveys, and their estimated uncertainty.

Three scenarios were considered. The first assumed that salt consumption remained steady since 2003 at 8.9 g/day (baseline). The second was present policy, with the assumption that the recent observed downward logarithmic trend in salt consumption continues. The third was adding mandatory reformulation in 2015, with the assumption that mandatory reformulation of processed foods achieves a steeper, linear salt decline, reaching the national target (6 g/day) by 2020 and levelling out.

All scenarios assumed a 5 year time lag for cardiovascular disease (10 year lag for gastric cancer) and no risk for salt consumption of less than 3.8 g/day. Salt consumption was modelled after measurements of 24 h urinary sodium excretion, between years 2001 and 2011. Our definition of cardiovascular disease included coronary heart disease (angina pectoris and acute myocardial infarction) and any type of stroke. Only the effect on primary prevention was considered.
Uncertainty intervals (UI) were estimated by second order Monte Carlo simulation and reflect the probabilistic sensitivity analysis. Bootstrapped confidence intervals (CI) are reported for in-between scenario comparisons.

**Results**

The trajectories of salt consumption assumed in the three scenarios can be seen in Figure 1. For the baseline scenario, IMPACT\textsubscript{NCD} estimated approximately 2.1 (95% UI: 1.8 to 2.4) millions new cardiovascular disease cases and 1.1 (95% UI: 1.0 to 1.2) million deaths, among those aged 30 to 84. Similarly, for gastric cancer 120,000 (95% UI: 100,000 to 140,000) new cases and 70,000 (95% UI: 60,000 to 80,000) deaths were estimated.

Table 1 summarises the estimated impact of present policy and the addition of mandatory reformulation compared to the baseline scenario, in terms of new cases (and deaths) prevented or postponed from cardiovascular disease and gastric cancer.

The predictive validation of IMPACT\textsubscript{NCD} against the observed mortality is presented in Figure 2. The discrepancy from year 2011 is partly because of an update in the coding system of the death registry. Predictive validation remained good even when stratified by age, sex, and disease.

<table>
<thead>
<tr>
<th>Scenarios</th>
<th>Cardiovascular disease</th>
<th>Gastric cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases prevented / postponed</td>
<td>Deaths prevented / postponed</td>
</tr>
<tr>
<td>Present policy</td>
<td>83,000 (95% CI: 80,000 to 86,000)</td>
<td>19,000 (95% CI: 17,000 to 21,000)</td>
</tr>
<tr>
<td>Addition of mandatory reformulation</td>
<td>115,000 (95% CI: 112,000 to 118,000)</td>
<td>26,000 (95% CI: 24,000 to 28,000)</td>
</tr>
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</table>

**Table 1.** Estimated cases and deaths prevented or postponed as a result of present salt reduction policy and with the addition of mandatory reformulation, between years 2008 and 2030.

**Discussion**

The present salt reduction policy in the UK can potentially prevent or postponed more than 20 000 deaths by 2030 and approximately 10 000 more deaths can be prevented by the addition of mandatory reformulation. Further health benefits may arise from the prevention of other diseases, for instance kidney disease, and from improved survival of cardiovascular disease patients.

The Czech Republic has the highest salt consumption in the European Union (EU). Data from 2003/4 suggest that mean salt consumption was 16.6 g/day among men and 10.5 g/day among women; the highest gender gap in the EU.10 Therefore, the opportunity to decrease disease burden and gender inequality is the greatest among all EU counties. A recent study has estimated almost 5,000 cardiovascular related deaths attributable to excess salt consumption, could have been prevented annually in the Czech Republic.\textsuperscript{3} In fact, the Czech Republic has adopted policies similar to those of the UK; however, the evaluation of these policies is lacking and no data exist for years later than 2003/4.5,10 Consequently, their effectiveness is unknown.
The main strength of this study is the advanced modelling methodology that synthesises the best available evidence to simulate the life-course of individuals. Disease incidence and mortality are reconstructed in the synthetic population from well accepted epidemiological principles. Reassuringly, the predictive validation of IMPACTNCD is reasonably good. On the other hand, while our estimations regarding the effectiveness of present policy is based on empirical evidence, such data are not available for mandatory reformulation. Therefore, we had to rely on other modelling studies.

**Conclusions**

Present salt reduction policy in the UK is effective and may prevent or postpone more than 20,000 deaths by 2030. The addition of mandatory reformulation could prevent further 10,000 deaths. The potential benefits of salt reduction policies for the Czech Republic could be even greater, since salt exposure is much higher.

**Summary**

Excess salt consumption is associated with higher risk of cardiovascular disease and gastric cancer and the World Health Organisation recommends salt reduction as one of the ‘best buy’ strategies to prevent non-communicable diseases. In this modelling study we quantify the potential impact of current UK salt reduction policy on the burden of cardiovascular disease and gastric cancer by 2030. Furthermore, we explore the additional population health benefits from the adoption and implementation of more structural policies, like the mandatory reformulation of processed foods. Finally, we contrast our findings with the current situation in the Czech Republic.

**References**

Figure 1. Modelled trajectories of mean salt consumption for the three scenarios. Years 2001 to 2025 and ages 20 to 74. Vertical lines depict the uncertainty considered in our calculations.

Figure 2. Validation of IMPACTNCD. Observed vs. modelled number of deaths from cardiovascular disease (ICD10: I20 – 25, I60 - 69) and gastric cancer (ICD10: C16) in England for years 2002 to 2013 and ages 30 to 84. Vertical lines depict the 95% uncertainty intervals.
SPATIAL NAVIGATION IMPAIRMENT IS PROPORTIONAL TO HIPPOCAMPAL ATROPHY IN SUBJECTS WITH ALZHEIMER’S DISEASE

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Tutor: Professor Jakub Hort, MD, PhD

Introduction
The incidence of Alzheimer's disease (AD) has increased in western societies and there is a continuous need for timely diagnosis of the disease. Patients with AD and amnestic mild cognitive impairment (aMCI), who are at higher risk for developing AD, experience difficulties with spatial navigation. Based on the animal research, two basic navigation strategies that have different neural representation have been distinguished. Egocentric (self-centered) navigation uses our own distance and direction from the goal and is dependent on parietal cortices and caudate nucleus (White, 2002; Packard, 2002). Allocentric (world-centered) navigation uses external orientation cues, and has been associated with the hippocampus (O'Keefe, 1971). Previously, our group introduced a human analogue of Morris Water Maze (hMWM) and reported a distinctive allocentric navigation impairment in patients with aMCI and AD compared to normal controls (Hort et al., 2007). However, the structural background of the allocentric navigation impairment in humans has not been entirely elucidated.

Aims
Our primary aim was to measure the relationship between allocentric spatial navigation accuracy and hippocampal volume using hMWM test in patients with cognitive impairment (AD and aMCI) and cognitively intact subjects. Since human brain functions are lateralized, we hypothesized that allocentric performance would be associated with predominantly right hippocampal volume, and this relationship would be more pronounced in cognitively impaired than in cognitively healthy subjects. Furthermore, we aimed to contrast the results from two versions of hMWM, real-space and 2D computer version.

Methods
We included 42 consecutively recruited cognitively impaired patients with either aMCI (n=23; Petersen 2004) or mild and moderate AD (n=19; McKhan et al., 1984), and 14 cognitively intact older controls from Motol University Hospital between 2009-2011. All subjects had 1.5 Tesla MRI brain scans using T1-weighted 3D sequence. We used automated algorithm FreeSurfer v4.4.0 to calculate right and left hippocampal and total brain volumes. Volumes were adjusted by total intracranial volume to control for the variations in the head size (Jack, et al, 1992). After subjects received instructions, spatial navigation performance was evaluated using hMWM developed in house. The hMWM is a circular arena with 2.8m diameter covered in dark velvet. It is constructed in the real-space and in its simplified 2D version as a computer test. hWMW is designed to separately examine the allocentric and egocentric navigations. In the allocentric task comprising of eight trials, subjects were required to locate an invisible
goal using two external navigation cues. The distance error between subjects' choice and the correct position of the invisible goal determined spatial navigation accuracy, and was averaged across eight trials. Pearson's correlations were used to explore the bivariate relationships between volumetric measures and spatial performance. Multivariate linear regression models, adjusted for age, sex and education, were used to assess the relative contribution of the right and left hippocampal and total brain volumes to the spatial navigation impairment within the whole cohort and separately for cognitively impaired and intact subjects.

**Results**

Qualitatively, cognitively impaired subjects performed worse in allocentric hMWM task than cognitively intact subjects (Fig. 1). Within the entire cohort, we observed strong negative correlations between smaller hippocampal volumes and distance error in both real-space and computer hMWM (Fig. 2). Using the multivariate modeling in the whole cohort, smaller right hippocampal volume was associated with poorer allocentric navigation performance in both, the real-space (β= - .62; p<.001) and virtual (β= -.43, p=.026) versions, controlling for demographic variables, total brain and left hippocampal volumes. In separate analyses for patients and controls, these associations were significant in cognitively impaired (p’s≤.05) but not in cognitively healthy (p’s>.59) subjects. The respective real-space and virtual test scores strongly correlated with each other (r=.83, p<.001).

![Figure 1](image_url)

*Figure 1. Inside the circular hMWM arena, a distribution of subjects' choices of the invisible goal position (black dots) and the correct goal's position (grey circle) is shown. Two orientation cues on the perimeter (grey bars; thick and thin) are used to locate the invisible goal (allocentric navigation). A single dot represents one single trial of an individual subject. Cognitively intact subjects (left) showed good accuracy when locating the goal, whereas cognitively impaired subjects performed considerably worse (right).*
Using hWMW we found that allocentric navigation impairment in a real-space setting and in its corresponding 2D computer versions was proportional to the right hippocampal volume in older adults. These results appeared to reflect a link between the extent of right hippocampal atrophy and spatial navigation performance particularly in those with cognitive impairment represented by aMCI and AD. We concluded that smaller right hippocampal volume, irrespective of a total brain atrophy, as well as age, sex, education, and left hippocampal atrophy, is responsible for decline in allocentric navigation performance. Our results are consistent with previous studies indicating that the hippocampus is a key structure for allocentric navigation in animals and humans.

Conclusion. The study findings indicate that the right hippocampus plays a critical role in the allocentric navigation in older adults both in the real space and virtual setting. Together, the results can serve as a basis for future research to ascertain the ability of spatial navigation testing in order to identify patients in the preclinical stage of Alzheimer’s disease, where hippocampal impairment is among the primary symptoms. Additionally, the findings indicate that our 2D computer version of the hMWM can reasonably imitate navigation in the real world and can serve as a useful and inexpensive screening tool for early detection of hippocampal dysfunction in older adults.

Figure 2. A negative correlation between smaller right hippocampal volume in mm3 and larger distance error from the invisible goal in the real-space [left; (r=-.71; p<.001)] and 2D computer version of hMWM [right; (r=-.64; p<.001)] within the entire cohort.

Figure 3. Correlation between findings from the real-space and 2D computerized versions of the hMW in the entire cohort (r=.83, p<.001).
Summary
Cognitive deficits in older adults attributable to AD-type pathology are featured early on by hippocampal impairment. Among these individuals, deterioration in spatial navigation, manifested by poor hippocampus-dependent allocentric navigation, may occur well before the clinical onset of dementia. Using hMWM to evaluate spatial navigation impairment and hippocampal volumetry we suggested that right hippocampal atrophy was associated with poorer navigation performance in the real-space and 2D computer versions of hMWM in cognitively impaired patients (aMCI and AD) but not in cognitively intact subjects. The findings from the real-space and virtual versions strongly correlated with each other. Our findings suggest that the right hippocampus plays a critical role in allocentric navigation, particularly when cognitive impairment is present.

References.

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CHANGES OF STRUCTURE OF THE TYMPANIC MEMBRANE DURING ITS TRANSFORMATION TO RETRACTION POCKET IN CHILDREN

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Tutor: Prof. MUDr. Ivo Šlapák, CSc.

Introduction
The retraction pocket (RP) is an invagination of the tympanic membrane (TM) into the tympanic cavity. It is caused by recurrent or chronic negative pressure in the middle ear, which is secondary to dysfunction of the Eustachian tube (1,2). An alternative hypothesis for the pathogenesis is related to the persistence of an inflammatory reaction, secondary to otitis media (3). Retraction of the TM is one of the theories of genesis of the cholesteatoma (4,5). The aim of our study was to identify the morphological abnormalities of pars tensa RP and signs leading to the formation of cholesteatoma and to confirm that the RP is not only static invagination of the TM but it is an active process where inflammation plays a key role.

Methods
We examined TM taken during standard operations at Pediatric ENT Department Brno with diagnosis of RP of pars tensa in stages II or III by Charachon classification. The patients with cleft palate were excluded. All samples were immediately fixated after their removal in special plastic cells with foam rubber to prevent the distortion of RP and then were standardly processed for light microscopy. We prepared paraffin sections stained with hematoxylin and eosin (HE), Van Gieson (collagen), Verhoeff (elastic fibres), Alcian (acidic polysaccharides) and PAS (polysaccharides). We conducted evaluations of the continuity of the basement membrane, cellular proliferation and collagen stroma of the middle layer of the tympanic membrane. For statistical analysis we used the generalized linearmodel. For the descriptive data we used frequency tables and graphs.

Results
Epidemiological data
We operated on 25 children from 6 to 18 years old, the average age was 10 years. As to gender, 48% of the sample was made up of females. The mean followup of the patients by ENT specialists was 5.16 years. Only 20% of the children had an SOM (secretory otitis media). We found all the basic types of the tympanometry curves (A,B,C1,C2), but there was no correlation between the type of curve and grading of severity of RP. Up to 76% of the patients had a recurrent infections of upper airways. Only 36% of patients had normacusis before the operation, 56% had a mild conductive hearing loss (26-40dBHL) and only 2 patients had a moderate conductive hearing loss (41-55dBHL). 36% of all patients never had a ventilation tube and 20% never had adenotomy and 76% patients had recurrent otitis media in anamnesis (3 or more OMA in six months). We operated on 16 children with RP of the stage III and 9 children with RP of the stage II by Charachone.
**Histology**

The mean length of RP was 5262 μm and thickness 373 μm. We found the rete pegs in 80% of all RPs, papillomatosis of epidermis in 64%, parakeratosis in 4%, hyperkeratosis in 100% (lamelar in 100% and basket in 80%), intraepithelial spongiosis in 12%, intraepithelial inflammation in 52%, subepithelial inflammation in 80%, myxoid changes in 84%, hypervascularisation in 100% (immature capillaries in 88%), subepithelial elastic fibers in 96% (fragmented in 92%). We demonstrated the dependence of the density of capillaries on hyperkeratosis (p=0.00837) and on the thickness (p=0.00802), than the interaction of hyperkeratosis and thickness (p=0.00726) and the dependence of the subepithelial inflammation on the thickness (p=0.0188) and papillomatosis (p=0.0463). We observed changes in the continuity of the basement membrane. A continuum of progressive histological features akin to cholesteatoma was noted with increasing grades of RP by Charachon classification (II-III).

**Discussion and Conclusion**

In all samples of RP of pars tensa, we observed the morphological changes compared to the histological structure of the normal healthy TM in children. We found certain histological abnormalities in the external layer of TM (epidermis) such as hyperkeratosis, papillomatosis, intra and subepithelial inflammation infiltrate, and in the middle layer of TM myxoid changes, hypervascularisation with immature capillaries and fragmented elastic fibers. All these abnormalities are typical for cholesteatoma. Hyperkeratosis and papillomatosis are typical for matrix of cholesteatoma (keratinized, stratified squamous epithelium). Inflammation infiltrate, hypervascularisation and myxoid changes of connective tissue are typical for the perimatrix of cholesteatoma. Fragmented elastic fibers are probably responsible for the reduction of compliance and weakening of the TM (related to dysfunction of the Eustachian tube).

A continuum of progressive histological features akin to cholesteatoma was noted with increasing grades of RP by Charachon classification (II-III). We assume that the RP is an active and progressive disease. The incidence of the recurrent OMA and upper airways infections is a high risk for the formation of RP in children. SOM is not a crucial factor in our group of patients. Based on our findings and clinical experience, we consider that the RP is a pre-cholesteatoma stage and we believe that inflammation plays a key role in the formation of RP and cholesteatoma in children Due to these facts we prefer a surgical treatment of RP of pars tensa stage II or III by Charachon.

**Summary**

Objective: To study the histological and proliferation characteristics of the pars tensa retraction pocket (RP) in children. To identify the morphological signs of its transformation into cholesteatoma in children. Methods: We examined tympanic membranes taken during operations at Pediatric ENT Department Brno with the diagnosis of RP of pars tensa in stage II-III by Charachon classification. We prepared paraffin sections stained with hematoxylin and eosin, Van Gieson (collagen), Verhoeff (elastic fibers), Alcian (acidic polysaccharides) and PAS (polysaccharides). We conducted evaluations of the continuity of the basement membrane, cellular proliferation and collagen stroma of the middle layer of the tympanic membrane. Results: The following are the findings noted as frequent in pars tensa retraction pockets: (1) hyperkeratosis, papillomatosis, intraepithelial infiltration, spongiosis and parakeratosis in the external layer (squamous epithelium), (2) no changes in the continuity of the basement membrane (3) regressive changes in collagen stroma, (4) atypical hypervascularisation, (5) presence of fragmentation of elastic fibers and (6) presence of inflammatory infiltrate with a high concentration of T-lymphocytes. A trend was noted along progressive grades of retraction (II-III) for an increasing incidence of these morphological changes and abnormalities. Conclusion: The morphological and functional changes of the RP show that there is an active process in the tympanic membrane, potentially leading to the development of cholesteatoma. A continuum of progressive histological features akin to cholesteatoma is noted with increasing grades of retraction by Charachon classification (II-III).
References
Introduction

Around the world, diarrhea is the second leading cause of death in children under age 5, taking the lives of 760,000 children each year, according to the World Health Organization (WHO, 2013). In resource-limited ("developing") countries, persistent diarrhea is most common in children younger than two years of age, and especially in children under one year [1], but can also occur in older children. These prolonged episodes are important not only because of the unpleasantness of having diarrhea but because of the association with malnutrition and increased risk of death, especially in resource-limited settings [2]. Lactose intolerance is a common complication of diarrhea in infants with malnutrition and a cause of treatment failure [3]. Malnourished children commonly have a reduced activity of intestinal lactase, the enzyme responsible for the digestion of lactose [4], and it has been suggested that feeding this disaccharide can retard nutritional recovery. A large proportion of children with PD are seen in the wake of lactation failure and following the introduction of animal milk feeds [5]. The most common clinical problem encountered in dietary selection is one of possible lactose intolerance. The consequence of lactose malabsorption and continued milk feeding include osmotic diarrhea and increased stool output. Reduced intake of nutrients, maldigestion and malabsorption increases nutrient losses, and the effects of the inflammatory response are some of the factors involved. Given the propensity of PD in the younger age group, it is natural that milk forms a major part of the dietary intake in these children. However, some of these children may be lactose intolerant, possibly contributing to the high rate of unfavorable treatment outcomes.

Aim

This study was therefore designed to establish the prevalence of lactose intolerance and associated factors among malnourished children with persistent diarrhea.

Methods

This was a prospective cohort study. The study population consisted of mild malnourished children with persistent diarrhea aged 3-24 months admitted to Globalmed Pediatric Clinic between October 2014 and September 2015. The Gomez classification of malnutrition was used. A mild malnourished child was whose weight-for-height was less than 75%-90% of the median National Centre for Health Statistics (NCHS)/WHO reference median. The incidence of lactose intolerance in the study population was explored on the basis of a pre-coded and pre-tested structured questionnaire and a genetic test of lactose intolerance. EDTA blood samples were obtained and posted in Stuttgart Laboratory for lactose intolerance test. The test was considered positive if (examination of polymorphism – 13910C>T (relative to LCT gene)) homozygous CC genotype was consistent with primary lactose deficiency. Data was
coded and entered into a computerized database. All data collected was checked for completeness and accuracy and cleaned before analysis. The analysis was done using the Statistical Package for Social Sciences (SPSS 22) software. Continuous variables are expressed as mean ± SD, and categorical variables are expressed as frequencies. The analysis of continuous variables was performed using Student's t test and that of categorical variables was performed using the Fisher's exact. Voluntary informed consent was obtained from the parents/caretakers before participating in the study. Confidentiality was observed throughout the study.

Results
The study included 78 malnourished children with persistent diarrhea 3-24 months of age. 43 children of the study group were females and 35 children were males. The prevalence of primary lactose deficiency among the study children was 41.0%. Diarrhea episodes in the previous 3 month, abdominal distention, vomiting, ever had problems with cow’s milk and average frequency of stool in 24 hrs were significantly associated with primary lactose deficiency. Correlation analysis was done by Spearman rank correlation. Results were summarized in texts and tables. (Table 1) Other characteristics which were explored during the study period were child’s age, sex, birth order, duration of exclusive breastfeeding, immunization status, watery stool, antibiotic use during diarrhea and dehydration. The correlation analysis demonstrated that, primary lactose deficiency reveals a significant positive correlation with number of diarrhea episodes in the previous 3 month (r=0.556*, p=0.0000), abdominal distention (r=0.490**, p=0.0000), ever had problems with cow's milk (r=0.331*, p=0.0076) and Vomiting (r=0.294**p=0.0091)

Table 1. Characteristics associated with primary lactose deficiency

<table>
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<tr>
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<th>Lactose tolerant</th>
<th>Primary lactose deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>abs</td>
<td>Mean</td>
</tr>
<tr>
<td>Diarrhea episodes in the previous 3 month</td>
<td>12</td>
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</tr>
<tr>
<td>Abdominal distention</td>
<td>22</td>
<td>0.58</td>
</tr>
<tr>
<td>Ever had problems with cow's milk</td>
<td>20</td>
<td>0.53</td>
</tr>
<tr>
<td>Vomiting</td>
<td>21</td>
<td>0.55</td>
</tr>
<tr>
<td>Frequency of Stool in 24 hrs</td>
<td>5.29</td>
<td>1.088</td>
</tr>
</tbody>
</table>
Discussion
The 41.0% prevalence of primary lactose deficiency in the 78 mild malnourished children with persistent diarrhea is higher and is not significantly different in children’s age groups (3-12 month; above 12 month) in contrast to other studies [6]. That might be explained by the difference in sample size and study population. Children with primary lactose deficiency have a higher mean of stool frequency (≥8 motions in 24 hour period) \( [p=0.000] \) a finding consistent with that by Ozmert and colleagues in Turkey [7]. High prevalence of primary lactose deficiency among children having had two or more diarrhea episodes in the previous 3 months \( (p=0.000) \) as found in this study has also been reported elsewhere [6]. Recurrent episodes of diarrhea result in repeated disruption of the intestinal villi with shortened regeneration and maturation time, predisposing to intestinal lactase deficiency. Conversely, lactose intolerance prolongs and increases the severity of diarrhea [8].

Conclusion
The prevalence of primary lactose deficiency in mild malnourished children with persistent diarrhea in the study setting of 41.0% is relatively high. Clinical predictors of primary lactose deficiency in mild malnourished children includes higher mean stool frequency, having ≥2 diarrhea episodes in the previous 3 months, abdominal distention, vomiting and ever had problems with cow’s milk.

Summary
Persistent diarrhea became the major cause for diarrheal mortality in children from developing countries. The relationship between diarrhea and malnutrition is bidirectional: diarrhea leads to malnutrition while malnutrition aggravates the course of diarrhea. Lactose intolerance is a relatively common cause of persistent diarrhea. The most common cause of primary lactose deficiency is lactase enzyme non-persistence. Genetic testing is a new tool for the diagnosis of lactase persistence. In our study prevalence of primary lactose deficiency among mild malnourished children with persistent diarrhea was high. Diarrhea episodes in the previous 3 month, abdominal distention, vomiting, ever had problems with cow’s milk and average frequency of stool in 24 hrs were significantly associated with primary lactose deficiency. Prevalence of primary lactose deficiency among malnourished children with persistent diarrhea in different regions of Georgia is the main topic of our investigation and further studies.

References
Introduction and Aims

Oral inflammation is an important element in the pathogenesis of vascular disease. Large body of evidence has accumulated that chronic periodontitis is a potential risk factor for atherosclerosis and endothelial dysfunction. While in periodontitis systemic immune activation is important in mediating increased cardiovascular risk, the extent of systemic response to oral fungal infection - denture-related stomatitis (DS) is poorly characterized. Systemic inflammation may affect vascular dysfunction in number of ways, which include activation of monocytes and T cells with overproduction of cytokines such as IFN-γ, TNF-α, IL-6 or IL-17, subsequently leading to atherosclerosis and hypertension and increased cardiovascular risk. Despite affecting up to 70% of patients in the course of life, relationship between DS and systemic inflammatory response in context of vascular risk has not yet been studied. Therefore, the aim of this study was to determine whether the presence and the treatment of local inflammation caused by DS influence the clinical measures of vascular dysfunction or systemic immune response measured as activation of peripheral blood immune cells.

Methods

Consecutive patients using dental prostheses for at least 6 months were recruited and assigned to two groups: DS (n = 20) and control (n = 24). Patients were recruited to DS group when clinical symptoms (erythema, swelling) were confirmed by positive microbiological culture of Candida species from palatal tissue. Exclusion criteria included acute inflammatory disorders other than DS, neoplastic disease relapses or chemotherapy courses less than 5 years before the enrolment, antibiotics in the previous 4 weeks or anti-inflammatory drugs (steroids and non-steroidal, excluding aspirin in doses below 80 mg) in the previous 2 months prior to enrolment. Patients with history of myocardial infarction, acute coronary incidents, vascular inflammation, chronic haematological disorders and immunodeficiency or major medication changes during 5 weeks prior to enrolment or during study were excluded. Out of DS group 3 patients did not agree to participate in subsequent visits. 17 patients were treated for oral fungal infection with nystatin (100 000 IU/ml) applied every 6 h for 3 weeks on the infected area of the oral mucousa. Blood samples and blood pressure (24 hours measurement), flow-mediated (FMD) and nitroglycerine-mediated (NMD) vascular dilatation data (ultrasonographic measurement after 1, 2 and 4-5 minutes after manometer cuff deflation or sublingual administration of nitroglycerine, respectively) were collected once from control group and thrice from DS group: before introducing treatment regimen, immediately after therapy, and 2 months after completion of treatment. Peripheral blood
mononuclear cells (PBMC) were isolated from blood samples (density gradient separation), incubated with fluorescently labelled monoclonal antibodies and studied by flow cytometry. Intracellular cytokine production was assessed by flow cytometry after stimulation of PBMC with Leukocyte Activation Cocktail with BD GolgiPlug utilizing cell permeabilisation protocol (BD Pharmingen) in subgroup of 10 patients.

Results
DS and control groups were comparable in relation to age, gender and major clinical characteristics (Table 1). Compared to the general population, the proportion of females was higher in all groups. FMD measurements showed significantly reduced percentage of endothelium-dependent arterial dilation in the DS group in comparison with control patients (Figure 1) and significant improvement between visit before and 2 months after treatment (Figure 2A). At the same time there was no difference between groups and during treatment in endothelium-independent NMD (Figure 1 and Figure 2B) measurements. Systolic and diastolic blood pressure values in DS and control group and throughout treatment did not differ.

Percentages of lymphocytes and T cells in PBMC, as well as CD4+ and CD8+ T cell subsets of DS and control patients showed no differences, also after treatment. There were no differences between groups in percentage of CD4+ and CD8+ cells expressing CD69, CCR5 and CD28null cells. During treatment, percentage of CD4+CD69+ and CD8+CD69+ cells increased immediately after therapy and percentage of CD4+CCR5+ cells increased after therapy and decreased at the end of study. Percentage of CD25+ cells was lower in DS patients than in control, while no change during treatment was observed, as well as for CD28null cells. There was no significant difference in IL-4, IL-17, IFN-γ and TNF-α production by T cell subsets between healthy and DS group and during subsequent visits.

Table 1. Patient clinical characteristics

<table>
<thead>
<tr>
<th></th>
<th>DS group n = 20</th>
<th>Control group n = 24</th>
<th>Treated group n = 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M:F)</td>
<td>2:18</td>
<td>p &gt; 0,05</td>
<td>6:18</td>
</tr>
<tr>
<td>Age [mean (SD)]</td>
<td>63,9 (6,6)</td>
<td>p &gt; 0,05</td>
<td>65,9 (10,3)</td>
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<td>BMI [median (Q1;Q2)]</td>
<td>28,5 (24,9;33,6)</td>
<td>p &gt; 0,05</td>
<td>27,8 (24,3;29,3)</td>
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<tr>
<td>Smoking (%)</td>
<td>6 (30%)</td>
<td>p &gt; 0,05</td>
<td>3 (12,5%)</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>6 (30%)</td>
<td>p &gt; 0,05</td>
<td>2 (8,3%)</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>17 (85%)</td>
<td>p &gt; 0,05</td>
<td>19 (79,2%)</td>
</tr>
<tr>
<td>Hyperlipidemia (%)</td>
<td>13 (65%)</td>
<td>p &gt; 0,05</td>
<td>12 (50%)</td>
</tr>
</tbody>
</table>

*BMI - Body Mass Index, F - females, M - males, SD - standard deviation.
**Figure 1.** Vascular dysfunction in control and DS patients. Vascular endothelium-dependent dilation (FMD) and endothelium-independent dilation (NMD) parameters were assessed by ultrasonography. Results presented as median (Q1; Q2); * - p < 0.005

**Figure 2.** Vascular dysfunction in DS patients treated with nystatin. Vascular endothelium-dependent dilation (A) and endothelium-independent dilation (B) parameters were assessed by ultrasonography before starting nystatin therapy, immediately after finishing it and two months after therapy completion. Results presented as median (box: Q1-Q3; whisker: minimal and maximal measurements). # - p < 0.05 for comparison of 2nd and 3rd visit; ¶ - p < 0.05 for comparison of 1st and 3rd visit.
Discussion
DS is one of the most common oral diseases among elderly denture wearers, especially women, smokers and diabetic patients, what is reflected by study population structure. While numerous studies have shown increased cardiovascular risk in subjects with oral inflammatory conditions such as periodontitis, this is the first study focusing on elderly population of patients wearing dentures in context of vascular dysfunction and systemic inflammation.
As it is known that the severity of endothelial dysfunction correlates with the development of coronary artery disease and predicts future cardiovascular events, our results implicate that the presence of DS may be associated with negative cardiovascular outcomes. Thus, such patients should be particularly carefully monitored in relation to their cardiovascular risk. Considering that DS is common oral disorder in the elderly, our finding may have important implications for clinical care of denture wearing patients. We did not observe clear systemic cellular response in DS patients, what possibly point to other mechanisms linking it with vascular dysfunction.
Since our experiments are performed in relatively small number of patients, our results should be considered preliminary. It should be also kept in mind, that presence of confounders, such as diabetes or smoking, although not statistically significant, can affect the results. Another potential weakness of our study is lack of a control group of the DS patients who did not undergo treatment, caused by the fact that the local ethics committee considered it inappropriate to leave patients untreated for the duration of the study.

Conclusions
To conclude, DS patients are characterized by more pronounced systemic endothelial dysfunction, which improve with DS therapy, however common risk factors for oral and vascular diseases might confound interpretation of these results. On the other hand, we did not observe clear evidence of a systemic cellular response to the presence and treatment of oral C. albicans infection. While our study identifies certain very interesting and potentially very important cardiovascular aspects of DS, a larger study is warranted to finally confirm these observations.

Summary
Chronic oral inflammation has been linked with systemic immune response activation, which contributes to the pathogenesis of cardiovascular diseases. Since fungal oral infections were not studied in this aspect yet, the aim of our study was to determine whether the local inflammation caused by denture-related stomatitis (DS) is associated with the systemic inflammatory response and measures of vascular dysfunction, and whether DS treatment can lead to the improvement of these parameters. Peripheral blood samples were collected and blood pressure, flow-mediated (FMD) and nitroglycerine-mediated (NMD) vascular dilation were measured in DS (n = 20) and control patients (n = 24). 17 of DS patients agreed for 3-week local antibiotic treatment and were studied before treatment, one day and two months after conclusion of antifungal therapy. Activation of T cells from patients blood was assessed by flow cytometry.
FMD was significantly lower in DS than in non-DS subjects and showed significant improvement of endothelial function 2 months after treatment, while there was no difference in NMD values. Systolic and diastolic blood pressure as well as percentages of major immune cell populations were similar between groups and during treatment visits.
Presence of DS is associated with worsened endothelial function and DS treatment with its improvement. DS also does not seem to affect the general state of the cellular components of the immune system. Since endothelial dysfunction is known to precede the development of severe cardiovascular disorders, DS patients require close cardiovascular monitoring.
References
MATERNAL STRESS INFLUENCE ON CHILD’S GLOBAL DEVELOPMENT

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David Tvildiani Medical University “Aiety”, Tbilisi, Georgia
Tutor: Ketevan Nemsadze MD, PhD

“The integrated framework of childhood health and child’s early development makes the aim achievable - that a child may not only be healthy and well-fed, but every child to be given an opportunity to reach their highest potential” [1].

There is evidence from several prospective studies that stress during pregnancy can affect the behavioural, emotional and cognitive development of the fetus and the child. Animal models suggest that this may be mediated via the HPA axis [2, 3]. It is suggested that the function of fetal programming is linked with a predictive adaptive response. Environmental influences on the mother are preparing the child in her womb for the external environment it is likely to inhabit. This is different from the genetic changes and mutations which will be effective for many generations [4, 5, 6].

The gestational environment can impact fetal brain structure and function and increase long-term susceptibility to neurodevelopmental and neuropsychiatric disorders [7, 8].

Vast brain growth occurs during the first two years of postnatal life.

Overall brain size doubles during the first year of postnatal life; the brain is about 70 percent of adult size at one year of age, and 85 percent at two years of age. Structural and functional connectivity emerge over the first two years of life. [9, 10].

“Fetal Programming” significantly changed general understanding about the chronic diseases. Fetal Programming and the idea of trans-generative, non-genetic diseases’ formation takes a very important part in modern Psychobiology.

The term “Predictive Adaptive Response” makes it plausible, that the developing human organism grows strong according the environment he/she grows; fetus uses mother’s relevant hormonal changes for that. [11].

Long before the birth fetal development influences on learning, thinking and stress-reaction forming. The children developmental problems are frequent all around the world (WHO, 2012; Gottlieb et al. 2009).
Every year, more than 200 million children under five years old fail to reach their full cognitive and social potential. Basically, it’s the fate of the low and middle income countries, which is largely due to simultaneous existence of multiple disturbing risk-factors, such as: harmful effects of the environment, adverse effects of social factors - poverty associated nutritional deficit, unstable relationships, stressful situations and violence among them; low birth weight, preterm labors, unfavorable environment.

Thus, supporting the child’s early global development is of essential importance for the stable relationships (e.g. mother-child attachment/bonding, parent-child relationship quality/stability/proximity), for proper, safe and reliable environment provision.

**Research aim**
Elaboration of the early childhood global development supporting strategies for diseases prevention in the future; for establishment of optimal adaptation ability having generation to physical and social environments; for formation of healthy generation - having optimal behavior and decision-making skills in difficult situations.

### Assessment of child’s communication, gross motor, fine motor, problem solving, personal-social and social-emotional skills in case of existing maternal emotional stress

<table>
<thead>
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<th>Below Cutoff</th>
<th>Beyond Cutoff</th>
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<th>P</th>
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<tr>
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<td>0.48</td>
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</table>
Methods
In the retrospective research 122 children of age 23-24 months were included. They had been assessed by the ASQ-3 questionnaire; Pregnancy ongoing and it’s features had been evaluated by use of adapted questionnaire. The analysis was done using Statistical Package for Social Sciences (SPSS 22) software. Continuous variables are expressed as mean ± SD, and categorical variables are expressed as frequencies. The analysis of continuous variables was performed using Student's t test and that of categorical variables was performed using the Fisher’s exact. Confidentiality was observed throughout the study.

Results
In case of maternal stress the Communication “below cutoff” data was reliably more, than “near cutoff” and “beyond cutoff”; Gross Motor developmental indicator “near cutoff” was reliably more, than that - “beyond cutoff”; Assessing Fine Motor data - “below cutoff” indicator was reliably more, than “near cutoff” and “beyond cutoff”; Problem-solving data - “below cutoff” indicator was reliably more, than „near cutoff” and “beyond cutoff” and in the same case - “near cutoff” was reliably more, than “beyond cutoff”. In case of maternal stress existance the difference for ASQ-3 Assessment/ Personal Social data hadn’t been observed. In case of maternal stress the difference for Social-Emotional Development data hadn’t been observed.

Discussion
Prenatal maternal depression symptoms were not only directly associated with lowered cognitive postnatal function of the child, but they also did so indirectly via the nutritional environment. For some time it has been recognised that children are affected not only by the symptoms of different maternal stress (anxious, worried, unhappy, crying), but it affect the immediate rearing environment of a child. Relatively fewer studies have focused on how maternal different maternal stress might lead to poor global development of a child. The researches around Fetal Programming in the largest biomedical research engine PubMed are not numerous (2349) and non of them discusses Fetal Programming in context of it’s effect on child’s global development.

Results
Maternal stress during pregnancy basically affected child’s Communication Skills, Fine Motor and Problem-Solving Skills as well.

Summary
Mother’s negative emotions, stress on early period of pregnancy leads to high rate of Stress-related Diseases. Prenatal programming in the context of it’s effect on child’s global development had not been yet highlighted. This is the main theme of our investigation and further studies.

References


11. Colin Blakemore / British Neuroscience Association/Fetal exposure to excessive stress hormones in the womb linked to adult mood disorders, April, 2013.
Introduction
Malignant pleural mesothelioma (MPM) is a cancer of the body cavities surrounding the lungs, with exposure to asbestos fibres being its primary aetiological factor. MPM is associated with an extremely poor prognosis and many patients are unresponsive to treatment, particularly platinum-based chemotherapy, which represents the current standard of care (Favoni and Florio, 2011). MicroRNAs (miRNA/MiR) are a family of small, non-coding RNAs that function to pleiotropically regulate gene expression at the posttranscriptional level, have tissue and cell type specific activities, and have been demonstrated to alter cellular sensitivity to cytotoxic agents in a variety of cancers (Maher et al., 2015). MiR-31 is encoded within a fragile site on chromosome 9p21.3 in the human genome and is deleted in ~54% of MPM tumours, representing the most frequent deletion in these patients (Ivanov et al., 2010). Of the limited number of studies examining miR-31 in MPM, there is an equally divided controversy as to whether miR-31 loss is prognostically advantageous or disadvantageous (Ivanov et al., 2010; Matsumoto et al., 2014). Here, we hypothesized that miR-31 loss promotes resistance to chemotherapy in MPM, and that synthetic miR-31 replacement would enhance cellular sensitivity to chemotherapy.

Aims
To determine whether the dysregulation of miR-31 contributes to MPM chemoresistance; to explore the effect of miR-31 modulation on biological endpoints relating to chemoresistance, and to identify functionally relevant targets that are modulated by miR-31.

Methods
The miR-31-negative NCI-H2452 and miR-31-positive P31 cell lines were used as in vitro models of MPM. Stable miR-31 re-expression in NCI-H2452 and silencing in P31 cells was achieved via liposomal-based transfection. Vector and vehicle controls were employed for all experimental comparisons. Clonogenic assay was employed as a gold-standard measure of cellular sensitivity to cisplatin and carboplatin. A basic cumulative proliferation assay was used to assess proliferative capacity. Inductively coupled plasma mass spectrometry (ICP-MS) was utilised to quantify cellular cisplatin flux. Subcellular fractionation via sucrose gradient centrifugation was employed to isolate intracellular organelles. Immunofluorescence staining and microscopy was used to visualise and quantify intracellular protein localisation and density/expression. Differential gene expression was assessed by qPCR and protein expression and phosphorylation status by Western blot. Gene promoter bioinformatics analysis was performed using the DECODE database. Data were statistically analysed using InStat3 software.
Results

Unexpectedly, re-introduction of miR-31 into NCI-H2452 cells significantly increased cellular resistance to the chemotherapeutic agents cisplatin and carboplatin; conversely, and supporting this finding, suppression of miR-31 in P31 cells significantly increased cellular sensitivity to chemotherapy [Fig. 1]. Furthermore, miR-31 re-introduction mediated a delay in the cytotoxic activity of cisplatin. To identify if the miR-31-mediated enhancement of chemoresistance was due to alterations in cellular uptake of chemotherapeutics, relative intracellular platinum levels were assessed. Paradoxically, a higher intracellular concentration of platinum was observed in NCI-H2452 cells upon miR-31 re-expression relative to vector controls. This can be explained, at least in part, by a miR-31-mediated increase in expression of the copper influx transporter CTR1, which is largely responsible for facilitating cellular influx of platinum agents. There were no significant alterations in the expression of ATP7A and ATP7B, which facilitate the efflux of platinum agents from cells. Subsequently, it was identified that miR-31 re-expression resulted in a significant decrease in relative intranuclear accumulation of platinum [Fig. 2]. Supporting this, chemotherapy-induced DNA damage, as measured by H2A.X phosphorylation, was demonstrated to be substantially reduced in miR-31 expressing NCI-H2452 cells [Fig. 2]. This was further supported by a substantial enhancement of chemotherapy-induced DNA damage in P31 cells upon miR-31 suppression. These data firmly indicate that miR-31 expression promotes resistance to chemotherapy via reduced trafficking of platinum agents to the nucleus due to extranuclear accumulation or sequestration.

In other cancer types, it has previously been demonstrated that sequestration of chemotherapeutic agents in lysosomes promotes a resistant phenotype (Gorden et al., 2015), which may be partially mediated by enhanced expression of the lysosomal associated drug transporter ABCB9 (Ween et al., 2015). In our model, we identified that miR-31 re-expression in NCI-H2452 cells significantly increased the expression of lysosomal ABCB9, which was independent of an increase in lysosome number, as determined by comparable expression of the lysosomal marker LAMP-1 [Fig. 3]. Consequently, analysis revealed that lysosomes isolated from NCI-H2452 cells with miR-31 re-expression had higher concentrations of platinum compared with controls.

It was considered that as miRNAs function to regulate gene expression via translational repression, the observation that miR-31 re-expression paradoxically increases CTR1 and ABCB9, indicated that miR-31 might be indirectly regulating CTR1 and ABCB9 through an unidentified intermediary. Bioinformatic analysis revealed the bipotential transcription factor OCT1 as a candidate factor that potentially facilitates the miR-31-mediated alterations in ABCB9 and CTR1. Supporting this, OCT1 expression was found to be downregulated with miR-31 re-expression in NCI-H2452 cells relative to vector controls [Fig. 3]. Taken together these data indicate that miR-31 expression in MPM cells promotes therapeutic resistance via reduced trafficking of DNA damaging platinum agents to the nucleus, which is likely a result of increased trafficking and sequestration of drug in lysosomes.
Figure 1. MiR-31 manipulation in MPM cells alters cellular sensitivity to platinum based treatment. Clonogenic analysis of miR-31 re-introduction in NCI-H2452 illustrated a significant increase in surviving fraction in cells treated with 1 µM cisplatin (IC$_{50}$ dose) (n=7) relative to control (miR-VC). Furthermore, there is a significant decrease in surviving fraction of P31 cells treated with 2 µM cisplatin (IC$_{50}$ dose) upon miR-31 suppression (Zip-miR-31) (n=3). Similar effects were observed with carboplatin treatment (n=3). Dashed line represents vehicle control (PBS) treated cells. Data are expressed as the mean ± SEM and were analysed by paired Student’s t-test. *p<0,05 **p<0,01.

Figure 2. MiR-31 modulation alters nuclear accumulation of cisplatin and DNA damage induction. (A) Quantification of intracellular cisplatin content using ICP-MS. There is a significant increase in whole cell levels of platinum in NCI-H2452 cells upon miR-31 re-expression compared to miR-VC control (n=3). Following subcellular fractionation, there is a significant decrease in platinum concentration within the nuclear fraction of miR-31 expressing cells following 50 µM cisplatin treatment for 24 hours. Data presented as the mean ± SEM and were analysed by paired Student’s t-test *p<0,05 **p<0,01 (B) The expression of miR-31 correlates with the extent of DNA damage induced upon cisplatin treatment. Representative Western blot for γH2A.X (n=3). The confirmation of a reduction in DNA damage with miR-31 can be viewed in the second band (left to right), with an increase in DNA damage evident in the P31 cell line upon miR-31 suppression.
Discussion

The role of miR-31 within differing tumour types remains unclear and there is evidence supportive of oncogenic and tumour suppressive functions (Hua et al., 2012). Within MPM, the fragile site encoding miR-31 has been correlated with poor prognosis (Ivanov et al., 2010), however, it has also been reported that miR-31 expression is allied with aggressive subtypes in patient cohorts (Matsumoto et al., 2014). Based on our previous findings that miR-31 regulates DNA repair gene expression and low miR-31 expression is associated with radioresistance in oesophageal adenocarcinoma (Lynam-Lennon et al., 2012), and considering there are supportive studies in MPM (Ivanov et al., 2010), we hypothesised that miR-31 loss may promote resistance to chemotherapy, potentially via alterations to the DNA repair mechanism. Surprisingly, we have determined miR-31 expression in MPM promotes resistance to platinum based therapy. Consequently, loss of miR-31 in MPM tumours might actually confer a chemosensitive phenotype. Contrasting with our hypothesis, the data support the alternative hypothesis that miR-31 loss in MPM confers a positive prognostic influence. The potential mechanism by which miR-31 mediates resistance appears to be reliant upon intracellular transport. Dependence upon nuclear transport has previously been noted in breast cancer (Kuusisto and Jans, 2015), with associations between altered transport of platinum containing agents within the cellular environment and resistance

Figure 3. Reintroduction of miR-31 affects lysosomal transport. (A) Representative Western blot illustrating an increase in ABCB9 expression level with miR-31 re-expression, with no apparent change in lysosomal marker LAMP-1. These data were also confirmed by fluorescent microscopy. (B) Densitometry analysis revealing significant a significant upregulation of ABCB9 (n=3). (C) Representative Western blot illustrating the downregulation of potential negative regulator of both CTR1 and ABCB9 expression, OCT1. Data are presented as the mean ± SEM and were analysed by paired Student’s t-test *p<0.05.
to therapy being comprehensively reviewed (Burger et al., 2011). Although unexpected, we have uncovered a potentially novel mechanism behind chemoresistance in MPM, which may precipitate a modified strategy of treatment in the future. Many MPM patients are inherently resistant to chemotherapy, and most have extremely poor prognosis; this has driven the field to find an alternative therapeutics, or indeed enhance the efficacy of the currently available therapeutics. Indeed, miRNA suppression and re-expression is already being clinically trialled, and miR-31 suppression may provide an interesting basis on which to develop future research to combat this aggressive disease.

**Conclusion**

MiR-31 expression in MPM facilitates resistance to platinum-based chemotherapy. Our data suggest that while deletions in chromosome 9p21.3 may be associated with an overall poor prognosis, the specific loss of miR-31 from this region may not contribute to the chemoresistance observed in MPM patients. Screening patients for miR-31 expression status and corresponding suppression of the miRNA may promote enhanced sensitivity to platinum based chemotherapeutics, improving patient outcomes.

**Summary**

MPM is an aggressive cancer with a poor prognosis and displays significant resistance to therapeutics. One of the most commonly deleted miRNA in MPM, miR-31, was found to negatively impact resistance to platinum based chemotherapy when manipulated in MPM cell line models *in vitro*. The mechanisms by which resistance is enhanced can be partially attributed to altered localisation of cisplatin within the intracellular environment. Future work will include further investigation into mechanisms underpinning miR-31 mediated regulation of chemosensitivity in MPM.

**References**

Introduction
Classification criteria for systemic lupus erythematosus (SLE) are primarily designed for the purpose of enrolling patients in studies. The American College of Rheumatology (ACR) criteria from 1982 (1) and their 1997 update (2) comprise eleven typical disease features. They serve not only for classification purposes but also as a helpful tool for disease phenotyping, although many of the disease features are not included in these criteria (3). According to the previous literature, renal and neuropsychiatric disorder are identified as features signaling worse prognosis (3, 4, 5).

AIM: Comparison of the frequency of ACR criteria between two groups of patients – deceased and not identified as deceased during a 10-year period in a Croatian tertiary center.

Methods
We identified patients with a clinical diagnosis of SLE with at least one visit to our institution during the 2002-2011 period. We also identified SLE patients under our follow-up that died during the same period. Deceased patients were identified by means of matching our institutional database of SLE patients (6) with the national death certificate database administered by the Croatian Institute of Public Health. Demographic data and data on the fulfillment of ACR criteria were extracted from patients’ medical charts.

We excluded non-deceased patients and deceased patients not residing in Croatia at the time of their last visit to our institution and at the time of death, respectively. We also excluded patients with an overlap syndrome of SLE and another systemic autoimmune disease. Since four ACR criteria are required to classify a patient as SLE (according to the criteria definition), patients not fulfilling four criteria were also excluded from the study. We compared the number of classification criteria and the frequency of each of them between deceased and non-deceased patients.

The chi-square test and Fisher’s exact test, as well as the Student t-test were used to evaluate differences between categorical and continuous variables, respectively. We also used descriptive statistics. Statistical analysis was performed in Statistica version 12.0 (Dell, Round Rock, TX, USA).

Results
We identified 967 patients (868 women and 99 men) with SLE: 818 were not identified and 149 were identified as deceased. 20 non-deceased patients were excluded due to their non-Croatian residence. We excluded 91 non-deceased patients with an overlap syndrome and 181 patients with less than four
ACR criteria. We also excluded 17 deceased patients with an overlap syndrome and 21 with less than four criteria. Finally, we identified 526 non-deceased (475 females and 51 males) and 111 deceased patients (88 females and 23 males) who were subjected to further analysis (Table 1).

Comparison between the two groups (Table 1) revealed a relatively higher proportion of males in the deceased group of patients ($p=0.001$, $\chi^2=10.85$), as well as a higher age at diagnosis ($p<0.001$, $t=-7.719$, $df=546$) in that patient group. We also observed a higher number of ACR criteria among the deceased group, but the difference is not clinically important.

The most frequently fulfilled criteria in both patient groups were antinuclear antibodies, immunological disorder, hematological disorder and serositis (Table 2). Comparison of both groups revealed a higher frequency of renal disorder and serositis in the deceased group of patients ($p<0.001$).

Discussion
The higher frequency of renal disorder among deceased patients is in concordance with previous studies that revealed worse prognosis of SLE patients with renal disorder (5). A higher proportion of serositis among deceased patients is in line with recent studies from Turkey and Iran, detecting serositis at diagnosis as a predictor of worse prognosis (7, 8). On the other hand, serositis is generally considered to be a mild feature not contributing to mortality (4). These contradictory findings require further elucidation.

A higher proportion of males among deceased patients may be associated with a worse disease course in men, but also with the observed age difference at diagnosis between the two groups. Since the deceased group has a significantly higher age at diagnosis and Croatian men are known to have a lower life expectancy than Croatian women, the higher proportion of men may actually be linked to the age difference rather than solely to the severity of disease.

The strength of this study is a rather large number of patients for a study conducted in a single center and a moderately long time period of observation. We enrolled only patients with at least four ACR criteria, trying to reduce the number of misdiagnosed or overdiagnosed cases. Furthermore, we were able to match our institutional data with data from the national death certificate database, which added to the accuracy of deceased patients’ identification.

However, our study suffers from several limitations. During the course of patients’ follow-up medical reports were not structured uniformly and different laboratory methods for measuring immunologic parameters were used. These weaknesses are associated with the retrospective design of the conducted study. We did not exclude patients that were not regularly followed-up and who were diagnosed in other institutions, so it is possible that the number of ACR criteria is underestimated due to lack of data recorded in medical charts archived in our institution. Furthermore, the study design does not include survival analysis and assessment of predictive variables. Finally, classification criteria are only a crude measure of disease phenotype. In our further studies we are also going to assess the role of damage and disease activity – their differences between the deceased and non-deceased group and their role as predictors of survival/mortality.

Conclusions
Renal disorder, serositis and male sex were more frequent in the group of deceased patients with SLE. These results are in line with previous findings except for serositis that is generally considered to be a mild disease feature.

Summary
We analyzed data of patients with SLE followed-up by our institution during the 2002-2011 period and compared the frequency of ACR criteria between patients identified as deceased during the observed period and those not identified as deceased. A higher frequency of male patients, renal disorder and serositis was observed among deceased patients.
References

Table 1. Basic characteristics of analyzed patients: non-deceased and deceased

<table>
<thead>
<tr>
<th>Features</th>
<th>Non-deceased</th>
<th>Deceased</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>526</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>Female to male ratio</td>
<td>475/51 (9,31)</td>
<td>88/23 (3,83)</td>
<td>0,0011</td>
</tr>
<tr>
<td>Age at diagnosis (mean, SD)</td>
<td>35,14; 13,77</td>
<td>46,94; 16,38</td>
<td>&lt;0,0012</td>
</tr>
<tr>
<td>Number of ACR criteria at diagnosis (mean, SD)</td>
<td>3,81; 1,35</td>
<td>4,01; 1,36</td>
<td>0,2123</td>
</tr>
<tr>
<td>Number of criteria during the disease course (mean, SD)</td>
<td>5,24; 1,22</td>
<td>5,56; 1,32</td>
<td>0,0144</td>
</tr>
</tbody>
</table>

SD – standard deviation; 1$\chi^2$=10,85; 2$t=-7,719$, df=546; 3$t=-1,251$, df=461; 4$t=-2,472$, df=635

Table 2. Frequency of ACR classification criteria in non-deceased and deceased patients

<table>
<thead>
<tr>
<th>ACR criteria</th>
<th>Non-deceased (N=526)</th>
<th>%</th>
<th>Deceased (N=111)</th>
<th>%</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malar rash</td>
<td>255</td>
<td>48</td>
<td>57</td>
<td>51</td>
<td>0,582</td>
</tr>
<tr>
<td>Discoid rash</td>
<td>108</td>
<td>21</td>
<td>30</td>
<td>27</td>
<td>0,131</td>
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<tr>
<td>Oral ulcers</td>
<td>70</td>
<td>13</td>
<td>13</td>
<td>12</td>
<td>0,65</td>
</tr>
<tr>
<td>Photosensitivity</td>
<td>243</td>
<td>46</td>
<td>42</td>
<td>38</td>
<td>0,108</td>
</tr>
<tr>
<td>Arthritis</td>
<td>351</td>
<td>67</td>
<td>78</td>
<td>70</td>
<td>0,47</td>
</tr>
<tr>
<td>Serositis</td>
<td>97</td>
<td>18</td>
<td>39</td>
<td>35</td>
<td>&lt;0,001</td>
</tr>
<tr>
<td>Renal disorder</td>
<td>150</td>
<td>29</td>
<td>54</td>
<td>49</td>
<td>&lt;0,001</td>
</tr>
<tr>
<td>Neuropsychiatric disorder</td>
<td>42</td>
<td>8</td>
<td>12</td>
<td>11</td>
<td>0,331</td>
</tr>
<tr>
<td>Hematological disorder</td>
<td>405</td>
<td>77</td>
<td>85</td>
<td>77</td>
<td>0,924</td>
</tr>
<tr>
<td>Immunological disorder</td>
<td>479</td>
<td>91</td>
<td>100</td>
<td>90</td>
<td>0,746</td>
</tr>
<tr>
<td>Antinuclear antibodies</td>
<td>512</td>
<td>97</td>
<td>107</td>
<td>96</td>
<td>0,586</td>
</tr>
</tbody>
</table>
Introduction

3-bromopyruvate (3-BrPyr) is a well-known alkylating agent with documented anticancer effects [1]. 3-BrPyr induced growth arrest of implanted tumors in vivo and caused necrosis and apoptosis of different transformed cell lines in vitro [2]. The inhibition of several intracellular enzymes influencing the homeostasis of the cell was described [3].

Aims

The main goal of this study was to assess the effect of 3-BrPyr on primary rat hepatocytes exposed for up to 20 hours. We focused on a viability, functional capacity, intracellular ROS production, type of cell death, morphological state of the culture and possible mitochondrial damage. The importance of this study is potentiated by the first use of 3-BrPyr in human cancer treatment [4].

Methods

WST-1 test was used to assess the activity of intracellular dehydrogenases. The damage of cell membrane was evaluated by the measurement of lactate dehydrogenase (LDH) activity in extracellular medium and by calculated LDH leakage. The functional state of cultured hepatocytes was estimated by measurement of total albumin production. The concentration of albumin was measured by ELISA method. ROS production was evaluated using DCFDA fluorescent probe. The presence of apoptotic cell death was assessed by the caspase 3 activity measurement. Phase contrast microscopy was used to evaluate cell morphology and fluorescent probes JC-1 and DAPI enabled to visualize the mitochondrial membrane potential (MMP) and to illustrate apoptotic changes respectively. After short term 3-BrPyr exposure, Oroboros O2k respirometry and Safranin O fluorescent probe were used to measure direct effect of 3-BrPyr on stimulated mitochondrial oxygen consumption and MMP respectively. The data were obtained from at least three independent hepatocyte isolations and statistical evaluation was performed using GraphPad Prism software.

Results and Discussion

We demonstrated toxic effect of 3-BrPyr on non-cancer rat hepatocytes in vitro. 3-BrPyr caused damage to the morphological structure and attenuated viability and functional capacity of cultured hepatocytes. Both the diminished activity of cellular dehydrogenases and greater plasmatic membrane damage exhibited dose- and time-dependent response relationship. Albumin production after 20 hours of exposure to 3-BrPyr was significantly decreased from concentration 150 µmol/l of 3-BrPyr in culture medium (p<0.001). The cellular damage was accompanied with increased ROS production. We found
significantly increased ROS production after one hour of incubation with 3-BrPyr at concentrations ≥100 µM (p<0.01). We proved higher activity of caspase 3 after 20 hours of incubation at concentrations 150 µM and 200 µM of 3-BrPyr, however at concentration 300 µM the activity of caspase 3 was under the detection limit. The finding was confirmed by the DAPI fluorescence staining. We observed chromatin fragmentations and condensations, which are changes typical for programmed type of cell death. Fluorescence staining JC-1 showed decrease in MMP.

Our results indicate possible connection with mitochondrial dysfunction. To differentiate if the mitochondrial dysfunction is the cause or consequence we further investigated the direct effect of 3-BrPyr on mitochondrial properties. 3-BrPyr induced decline in MMP for glutamate and malate stimulated respiration from concentrations ≥ 20 µM (incubation time 10 minutes; p<0.001). The respiratory rate for complex I initiated mitochondrial respiration was similarly reduced. However succinate stimulated respiration and MMP was even more sensitive starting at concentration 10 µM of 3-BrPyr (p<0.001) in suspensions of permeabilized hepatocytes. This effect of 3-BrPyr on electron transport chain was confirmed by the measurements on isolated rat liver mitochondria. The results on isolated mitochondria show stronger 3-BrPyr effect, confirming our assumption that mitochondria play significant role in 3-BrPyr effect on non-cancer rat hepatocytes.

We succeeded to provide the evidence of 3-BrPyr toxic action on primary hepatocytes in vitro. This toxic effect of 3-BrPyr on non-cancer tissue was pronounced even in concentrations comparable to doses used in experiments with HepG2 cell line [5]. We want to point out the fact that the toxic effect is connected with mitochondrial dysfunction and initiation of the apoptotic pathway. According to our results low doses of 3-BrPyr induce apoptosis and higher doses lead directly to necrotic damage. The results of this study need to be reconsidered in upcoming in vivo experiments or in future clinical trials.

Acknowledgements
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References
MESENCHYMAL STROMAL CELLS PLAY AN IMPORTANT ROLE IN THE TUMOR MICROENVIRONMENT

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Introduction
The discovery that mesenchymal stromal cells (MSC) are recruited to the tumor site has led to an increased interest in the function of MSC in tumors. Their preference for injured, inflammed and tumor tissue is exploited in the regenerative medicine (1,2). We have decided to analyze the effects of MSC-released factors on behaviour and morphology of tumor cells and also the changes in the gene expression and secretome of MSC after their exposure to treatment.

Material and methods
Adipose tissue-derived mesenchymal stromal cells (AT-MSC) were isolated from healthy individuals undergoing elective lipoaspiration and characterised based on the established minimum definition criteria. Each subject provided informed consent. Cells were maintained in high (tumor cells) or low (AT-MSC) glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with fetal bovine serum (Biochrom AG), 10 000 IU/ml penicillin (Biotica, Part. Lupca, Slovakia), 5 µg/ml streptomycin, 2 mM glutamine, and 2,5 µg/ml amphotericin (Sigma, St. Louis, MO). Tumor cells were cultured in conditioned medium of control or pre-treated AT-MSC. AT-MSC cells were cultured with or without 1 µg/ml cisplatin overnight. Total RNA was isolated from 4x10^6 cells. The expression of specific genes was analysed by qPCR. Proliferation, apoptosis and cytotoxicity were evaluated by established method with the IncuCyte Zoom™ Kinetic Imaging System (Essen BioScience, UK). Analysis of phosphorylation profiles of kinases and their protein substrates was done by the Human Phospho-Kinase Array (R&D Systems). We analyzed AT-MSC conditioned medium also for detection of secreted cytokines and chemokines after cisplatin exposure regarding Human Cytokine Array Panel A protocol.

Results
Adipose tissue-derived mesenchymal stromal cells were shown to induce specific morphological and phenotypical alterations in breast cancer cell line EGFP-Sk-Br-3 and EGFP-MCF7. We have shown that the cultivation of EGFP-MCF7 and EGFP-Sk-Br-3 cells in AT-MSC conditioned medium led to changes in morphology of tumor cells associated with epithelial-to-mesenchymal transition (EMT). We have decided to analyze what is the fate of MSC in the tumor microenvironment when they are exposed to chemotherapy. We have shown increased phosphorylation of ERK1/2, WNK1, RSK1/2/3, p53, c-Jun and STAT3 in AT-MSC overnight treated with cisplatin. The expression profile of genes which products were shown to be responsible for invasiveness, survival, pluripotency and mammosphere formation in breast cancer cells was also increased after AT-MSC pretreatment. Cisplatin pretreatment of AT-MSC partially elevated the expression of VEGF-B and the expression of CXCL12 (SDF-1α). The analysis of influence of the pretreatment on apoptotic related proteins in AT-MSCs revealed changes on both levels of pro- and anti-apoptotic proteins.
Discussion

AT-MSCs were described to integrate to the tumor microenvironment and exhibited multiple regulatory functions in the tumor-associated stroma (2-6). We have shown that AT-MSCs exposure to non-toxic drug concentrations activated several signalling cascades. AT-MSCs pre-treated with cisplatin showed increased phosphorylation of ERK1/2, CREB, STAT3, WNK1, RSK1/2/3, c-Jun and p53. These changes could also play a role in MSC-mediated changes of tumor cells as it was shown that e.g. Y705F-STAT3 drive the expression of many genes important in oncogenesis, cell cycle control and the immune response (7). The analysis of gene expression revealed increased expression of VEGF-B in pre-exposed AT-MSCs. Mylona et al. (8) showed the negative impact of VEGF-B expression on both disease-free and overall survival of the node-positive patients with breast cancer so we suggest that the level of expression of VEGF-B could play a role also in the resistance of breast cancer cells. MSCs were shown to be resistant to chemotherapy and also our experiments showed that commonly used concentration of cisplatin haven’t trigger apoptosis in AT-MSCs. Our experiments demonstrated that AT-MSCs exposure to cisplatin stimulated changes in gene expression and activation of different signaling cascades in AT-MSCs, which could result in increased resistance of tumor cells to chemotherapy. Our experiments present novel view on the role of MSCs in resistance of tumor cells to chemotherapy.

Conclusion

Understanding of the crosstalk of MSC with tumor cells and also the network of cytokines and secreted growth factors may lead to an important new therapeutic approaches in controlling the growth and metastasis of tumors through inhibition of tumor stroma. We also suggest that the intervention with the relevant signalling events could revert the chemoresistance and unravel a potential strategy to augment chemotherapeutic efficiency relevant for clinical application. The elucidation of the chemoresistance mechanisms and provision of essential context dependent information is critical for the development of more efficient combination of therapeutic strategies.

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References

POSSIBILITIES OF LESIONAL VAS DEFERENS REPAIR IN THE EXPERIMENT

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Tutor: Assoc. Prof. Dr. Milan Kaška, MD, PhD

Introduction
Reparation of inguinal hernia represents one of the most frequent general surgical operations both in adults and children. Some literature references regarding this theme speak about the fact that the tissues of resected hernia sacs can contain the tissue structures of the vas deferens in 0,1 – 1,7% in some analysed groups of the youngest children. This finding probably seems to be one of the most frequently occurring iatrogenic injuries which can happen in operations performed by paediatric surgeons. Surgical reparation of the lesional vas deferens in adults have been described in details, but recommendation for the appropriate therapeutic method for this injury in newborns and in a very young children group is missing. Microsurgery is not commonly performed at paediatric surgery departments for limitations in special apparatuses and special surgical devices equipment and in a surgeon’s experience. The main idea of the present experimental study is a creation of some useful algorithm for injured vas deferens reparation during inguinal hernia surgery under the conditions of our basic paediatric surgery departments with the use of magnifying glasses only.

Aims
The aim of this experimental study with a small laboratory animal is to perform and analyze the possibilities of new reparation methods for the lesional vas deferens in a standard paediatric surgery department without the use of a microscope.

Hypotheses
Injury of the small child vas deferens after its contusion by a surgical instrument can cause its substantial morphologic and functional changes. Methods of vas deferens dissection and its consequent reparation with the application of various techniques and materials can lead to the finding of their best combination recommendable for a daily clinical practice use.

Materials and methods
A pilot prospective experimental study was performed on the rat, Wistar strain, body weight 350-450 g, whose anatomical condition of the vas deferens is very similar to those in very small children. 36 animals were included into the study and they were divided in six subgroups according to the method of the vas deferens injury (contusion or transection) and its consequent reparation. The subgroups of animals according to the method of injury and sequent reparation method: 1. contusion of the vas deferens by pressing in a pean for 2 sec, 2. anastomosis of the vas deferens by single absorbable stitches (Vicryl R 8/0), 3. jointing of both ends each to other with the help of an intraluminally lead fibre of
absorbable sewing material (PDS 8/0) knotted externally, 4. = 3. jointing with a non-absorbable fibre of sewing material (Prolen 7/0) knotted externally, 5. anastomosis by absorbable sewing material (Vicryl R 8/0) with an intraluminally situated fibre of absorbable sewing material (PDS 7/0), 6. anastomosis by absorbable sewing material (Vicryl R. 8/0) with an intraluminally situated fibre of non-absorbable sewing material (Prolen 7/0). All above mentioned operations were performed in general anaesthesia (ketamin + xylazin solution intraperitoneally) using minor surgical instruments and with the help of magnifying glasses (enlargement 4x). The vas deferens was checked 3 months after the primary operation. An operated both other side were resected in a length of 20 mm with a line of artificial injury in the central part. These resected parts of the vas deferens were examined in function by the flow rate of methylene blue solution (mL/min). The pathologist performed morphologic evaluation of the resected vas deferens using histology with HE-staining. Findings on the injured vas deferens were compared with those on the second side without surgery.

**Results**

<table>
<thead>
<tr>
<th>Surgery/reparation</th>
<th>Mean stream rate in operated VD (mL / min.)</th>
<th>Mean stream rate in non-operated VD (mL / min.)</th>
<th>Histologic findings</th>
<th>Histologic findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contusion/non</td>
<td>17,885</td>
<td>17,95</td>
<td>Minimal changes</td>
<td>normal</td>
</tr>
<tr>
<td>cut off/anastomosis</td>
<td>9,032</td>
<td>17,875</td>
<td>Tight stenoses or dehiscence</td>
<td>normal</td>
</tr>
<tr>
<td>cut off/ jointing absorbable stitch</td>
<td>13,173</td>
<td>16,313</td>
<td>Small changes</td>
<td>normal</td>
</tr>
<tr>
<td>cut off/jointing non-absorb. stitch</td>
<td>4,004</td>
<td>13,328</td>
<td>Tight stenoses or dehiscence</td>
<td>normal</td>
</tr>
<tr>
<td>cut off/anastomosis + absorb. fibre</td>
<td>10,402</td>
<td>17,901</td>
<td>Stenoses or dehiscence</td>
<td>normal</td>
</tr>
<tr>
<td>cut off/anastomosis + non-absorb. fibre</td>
<td>6,879</td>
<td>17,827</td>
<td>Tight stenoses or dehiscence</td>
<td>normal</td>
</tr>
</tbody>
</table>

*VD - vas deferens, anastomosis-suture by Vicryl 8/0*

**Conclusions**

We found a normal liquid flow rate through the resected part of the vas deferens and morphological conditions in this subgroup with the contused vas deferens after 3 months. The best results in liquid flow rate through the transected and repaired vas deferens were evaluated in the subgroup with reparation performed by jointing the transected vas deferens with the help of absorbable sewing material (PDS 7/0) situated intraluminally and knotted out of the vas deferens. Similar results were found in the subgroup anastomosis performed with absorbable stitch (Vicryl - 8/0) and with intraluminally situated absorbable stitch (Vicryl 7/0) Examination of healed anastomoses carried out with absorbable sewing material 8/0 without an intraluminally situated stitch fibre as “a leader” and with “a leader” from non-absorbable sewing material showed the substantially worst results.
Acknowledgments
The authors thank for support of this study the Grant Agency UK, Prague, No. 160315.

References
Introduction
Rheumatoid arthritis (RA) is a chronic joint disease marked by persistent inflammation and osteo-
destruction. Mechanisms leading to joint destruction involve infiltration of osteoclasts (OCs), highly spe-
cialized multinucleated cells derived from monocyte/macrophage lineage which are capable of resorbing
mineralized matrix (1). Human osteoclast progenitors (OCPs), contained among myeloid hematopoietic
lineage, can be found among peripheral blood monocytes at low frequency even in healthy subjects
(2). OCPs are known to exhibit chemotaxis (3) and, furthermore, synovial compartment of RA patients
highly expresses different chemokines (4), indicating a possible mechanism for their accumulation in
affected joints where osteoclastogenesis is markedly enhanced by various proinflammatory factors (2)
released by other infiltrating cells (i.e. plasma cells, T and B lymphocytes), contributing to local bone
loss and typical symptoms of RA (joint pain, stiffness, swelling, structural changes).

Aim
The aim of our study was to define possible chemotactic signals for OCs by analyzing expression of
several chemokine receptors on OCPs in peripheral blood, levels of chemokines in serum and synovial
fluid of RA patients and to assess differentiation potential of isolated OCPs.

Materials and methods
Samples of 81 RA and 72 control patients admitted to Clinical Hospital Center “Sisters of Mercy” and
Clinical Hospital “Holy Spirit”, were collected in the study after obtaining approval from the Ethics
Committee and informed consent from patients. Control patients were admitted for non-inflammatory
conditions, with normal values for inflammatory indicators and without history of autoimmune or joint
diseases. RA was diagnosed according to the revised American College of Rheumatology (ACR). The
study was conducted in accordance with the Declaration of Helsinki. Peripheral blood samples were
taken from all patients, and, in case of a therapeutic knee puncture in RA, synovial fluid was also taken.
Age, sex were recorded, as well as clinical data for RA patients – duration of disease, levels of
C-reactive protein (measured by standard nephelometric assay) and erythrocyte sedimentation rate (de-
termined according to the Westergren method), disease activity score in 28 joints (DAS28). Mononu-
clear cells and serum were obtained by centrifugation in Histopaque (Sigma-Aldrich) density gradient.
Frequency and phenotype of OCPs, designated as CD3-CD19-CD56-CD11b+CD14+ from publications
(2), was determined using Attune flow cytometer (Thermo Fisher Scientific) for receptors CCR1, CCR2,
CCR4, CXCR4 and C5AR1. CCL2, CCL3, CCL4, CCL5, CXCL9, CXCL10 concentrations were meas-
ured in serum and synovial fluid using cytometric bead arrays (BD Biosciences). OCPs were sorted on
FACS Aria cell sorter (BD Biosciences), with sorting purity >98%, and cultured in 96-well plastic plates at density of 40000 cells/well, with 60 ng/mL M-CSF for the first 4 days to induce proliferation and subsequently for 8 days with 30 ng/mL M-CSF and 60ng/mL RANKL to induce OC differentiation. After two weeks, cells were stained for tartarate resistant acid phosphatase (TRAP) enzyme and positive cells with three or more nuclei were manually counted as mature OCs using light microscopy on an Axiovert 200 (Carl Zeiss). Total RNA was extracted from PBMC using TRIzol (Invitrogen), converted to complementary DNA and amplified in duplicates by qPCR in an ABI Prism 7500 Sequence Detection System (Applied Biosystems). Gene expression of CCL2, CCL3 and CCL4 was assessed using TaqMan Assays (Applied Biosystems) and presented as RNA relative quantity in comparison to the expression of housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Statistical analysis was performed using MedCalc software package. For all experiments, α-level was set at 0,05.

Results
OCPs accounted for 5% of peripheral blood mononuclear cells (PBMC) in both groups, with no significant difference (Fig.1A). Almost all OCPs expressed c-Fms, while a subset of OCPs expressed RANK (Fig.1B), with significantly higher frequency in RA. Cell culture revealed high differentiation potential, with no significant difference in osteoclastogenesis between groups, although RA OCs were larger with more nuclei per cell (Fig.1C).

Figure 1. Osteoclast progenitor frequency (A), crucial differentiation factor receptors’ expression (B) and osteoclastogenic in vitro potential (C).
OCPs similarly expressed chemokine receptors in both groups, with a high frequency of CCR2, CCR4, CXCR4, C5AR1 expression, and almost nonexistent expression of CCR1 (Fig. 2).

**Figure 2.** Osteoclast progenitor chemokine receptor profile.

Relative gene expression of CCL2, CCL3 and CCL4 in isolated PBMCs indicated very low expression of chemokines, with no difference between groups (data not shown). However, all chemokine ligands’ concentrations were higher in RA serum, and even higher in synovial fluid (with exception of CCL5), with significantly higher values of CCL4 and CXCL10 in synovial fluid compared to serum in RA, and for CCL2, CXCL9 and CXCL10 serum levels in RA patients (Fig. 3).

**Figure 3.** Chemokine concentrations in sera and synovial fluid.
Discussion
We assessed the subpopulation of monocytes expressing both CD11b and CD14 as presumably having highest osteoclastogenic potential (2,5). OCPs were of similar frequency in both groups, with almost all cells expressing receptor for M-CSF, the early crucial OC differentiation factor. RA OCPs had two times higher RANK expression, which is crucial for final differentiation into mature osteoclasts, possibly an osteoclastogenic effect of a systemically raised level of inflammatory cytokines (1). OCPs showed high osteoclastogenic potential, similar in both groups, with a morphology in RA indicative of higher resorption capability (6), possibly a result of higher RANK expression. We confirmed that OCPs highly expressed receptors normally present on bone osteoclasts (CCR2, CCR4, CXCR4, C5AR1) for which OCs exhibit chemotaxis (3). An almost non-existent expression of chemokine ligands in PBMC was indicative that the source is located outside circulation, presumably in joints. This was supported by the cytometric bead assay, in which chemokine ligands were higher in RA serum, and even more increased in synovial fluid (with exception of CCL5), indicating a rising blood-joint gradient which OCPs could follow and therefore home to the affected joints (2).

Conclusions
Although OCPs in RA and control have a similar phenotype and differentiation potential, levels of several chemokines are upregulated, indicating a possible chemotactic mechanism of OCP migration to affected joints. These results may help to reveal a migration mechanism of OCPs specifically associated with RA in order to develop more efficient therapeutic approaches.

Acknowledgement
This work has been fully supported by Croatian Science Foundation under the project 5699.

Summary
Rheumatoid arthritis (RA) is marked by persistent inflammation and joint destruction involving infiltration of osteoclasts. Human osteoclast progenitors (OCPs) are contained among peripheral blood monocytes at low frequency, exhibit chemotaxis and, furthermore, synovial compartment of RA patients highly expresses different chemokines. The aim was to define these chemotactic signals. Mononuclear cells were isolated from peripheral blood of control and RA patients. The phenotype of OCPs (CD3-CD19-CD56-CD11b CD14 ) was determined using flow cytometry for receptors CCR1, CCR2, CCR4, CXCR4, C5AR1. Chemokine ligand concentrations (CCL2, CCL3, CCL4, CCL5, CXCL9, CXCL10) were measured in serum and synovial fluid of RA patients using cytometric bead assay. OCPs were sorted and cultured with M-CSF and RANKL. After two weeks, the cells were stained for TRAP enzyme and mature osteoclasts were counted. Human peripheral blood OCPs similarly expressed chemokine receptors in RA and healthy subjects. However, CCL4 and CXCL10 concentrations were significantly higher in synovial fluid compared to serum levels in RA, while CCL2, CXCL9 and CXCL10 serum levels were higher in RA patients compared to the control group. Cell culture revealed no significant differences in mature osteoclast count between groups. Although OCPs in RA have a differentiation potential similar to controls, levels of several chemokines are upregulated, indicating a possible chemotactic mechanism of OCP migration to affected joints. These results may help to reveal a migration mechanism of OCPs specifically associated with RA in order to develop more efficient therapeutic approaches.
References
Introduction
Major depression is multifactorial disease affected by environmental, psychological and biological factors. Pathophysiology of major depression is associated with defects of neurotransmitters metabolism, (e.g. serotonin, dopamine), elevated inflammatory processes in organism, lowered level of antioxidants, elevated oxidative and nitrative stress level, neurodegeneration and mitochondrial dysfunction (Maes et al. 2011). Lowered levels of antioxidants are observed in depression disorder. Major depression is also associated with elevated lipid peroxidation (e.g. elevated level of malondialdehyde and 8-isoprostanates), elevated levels of pro-inflammatory cytokines (e.g. interferon α, interleukin 1) or elevated concentration of nitric oxide. Patients with major depression have also decreased length of telomeres and elevated oxidatively damaged DNA in comparison to healthy controls. On the other hand, there are inconsistent findings about activity of antioxidant enzymes in depression (reviewed in Vaváková et al. 2015). Aim of the study was to determine differences in levels of oxidatively damaged proteins, lipids and DNA, nitratively damaged proteins, pro-inflammatory markers and difference in activity of antioxidant enzymes in children and adolescents with major depression in comparison to healthy children and adolescents of similar age without history of mental disorders, including major depression.

Methods
A patient group consists of 28 children and adolescents diagnosed with major depression and/or anxiety disorder (F32.0, F32.1, F41.0, F41.2) at age 15.4±1.6 (M/F = 6/22) from Department of Pediatric Psychiatry, Medical Faculty of Comenius University and Child University Hospital, Bratislava. 20 healthy volunteers at age 14.3±2.6 (M/F = 8/12) from Paediatric Centrum Juvenalia, sro., Dunajská Streda was included in a control group. Venous blood samples were collected after 12-hours overnight fast. Within 1 h of collection, blood was centrifuged (1200 x g, 7 min), serum and plasma (EDTA as an anticoagulant) were obtained and frozen at -80°C until analysis. Urine was collected, alliquoted and storage at -20°C until analysis. Plasma samples were used for determination of levels of nitrotyrosine (Hycult biotech HK-501), thromboxane B2 (Cayman, no. 519031) and 8-isoprostane (8-iso-prostaglandine F2α) (Cayman, no. 516351) on the basis of commercially available ELISA/EIA kits. Advanced oxidation protein products (AOPP) (modified by Witko-Sarsat et al. 1996), lipoperoxides (El-Saadani et al. 1989), paraoxonase lactonase activity (PON-L) (Rainwater et al. 2009) and paraoxonase arylesterase activity (PON-A) (Gan et al. 1991) were detected in serum samples. Samples of erythrocytes lysates were used for detection of catalase activity (Bergmeyer, 1987). Level of 11-dehydro-thromboxanes B2 was determined in urine samples on the basis of spectrophotometry by EIA (Cayman, no. 519510). Fresh whole blood was used for determination of 8-oxoguanine (8-oxoG), marker of oxidatively damaged DNA, on
the basis of enzymatically modified comet assay (single cell gel electrophoresis) and incubation with enzyme formamidopyrimidine DNA glycosylase (Collins et al. 2008). Samples were evaluated by software LUCIA comet assay (version 7.0). Levels of oxidatively damaged DNA are expressed as 8-oxoG/106G (100 cells per sample) by visual evaluation according to ESCODD (Gedik et al. 2005). Results are expressed as mean ± SD in case that data are distributed normally. In case of non-normality, results are expressed as median (1. quartile; 3. quartile). Normally distributed data were computed by unpaired t-test, in case of non-normality we used Mann-Whitney test. Not complete set of control and patients samples were analyzed in every method on the ground of material availability and number of current samples in time of analysis. Samples collection and evaluation are not terminated and will continue in the future.

**Table 1. Parameters of oxidative and nitrative stress, pro-inflammatory markers and antioxidant enzymes**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SD or median (Q1; Q3)</td>
<td>n</td>
</tr>
<tr>
<td>AOPP (μmol/l)</td>
<td>42,08 (38,61; 44,54)</td>
<td>20</td>
</tr>
<tr>
<td>Nitrotyrosine (nmol/l)</td>
<td>55,63 (33,56; 104,37)*</td>
<td>9</td>
</tr>
<tr>
<td>Free 8-isoP (pg/ml)</td>
<td>34,09 (24,4; 61,53)</td>
<td>11</td>
</tr>
<tr>
<td>Total 8-isoP (pg/ml)</td>
<td>210,0 (176,5; 278,6) D</td>
<td>11</td>
</tr>
<tr>
<td>Lipoperoxides (mmol/ml)</td>
<td>52,32 (34,6; 76,8)</td>
<td>28</td>
</tr>
<tr>
<td>TXB2 (pg/ml)</td>
<td>320,3 (195,2; 502,2)*</td>
<td>12</td>
</tr>
<tr>
<td>11-dehydro TXB2 (ng/mmol creatinine)</td>
<td>162,1 ± 60,2</td>
<td>13</td>
</tr>
<tr>
<td>PON-A (U/ml)</td>
<td>129,7 (106,9; 141,9)</td>
<td>28</td>
</tr>
<tr>
<td>PON-L (U/ml)</td>
<td>9,85 (8,0; 11,2)</td>
<td>28</td>
</tr>
<tr>
<td>Catalase (μkat/g hemoglobin)</td>
<td>4,267 ± 0,96</td>
<td>20</td>
</tr>
<tr>
<td>8-oxoguanine (8-oxoG/106G)</td>
<td>0,04 (0,02; 0,17) *</td>
<td>16</td>
</tr>
</tbody>
</table>

Parameters are presented as mean ± SD in case of data with normal distribution, in case of non-normal distribution as median (1. quartile; 3. quartile). TXB2 – thromboxane B2, 11-dehydro TXB – 11-dehydro thromboxane B2, 8-isoP – 8-isoprostanes, AOPP - advanced oxidation protein products, PON-L - paraoxonase lactonase activity, PON-A - paraoxonase arylesterase activity, n represents total number of analyzed samples. * p < 0,05 and Δ p < 0,1 for significance between patients and control group.
Results
Markers of oxidative and nitrative stress, pro-inflammatory markers and activity of antioxidant enzymes are reported in Table 1. We detected elevated concentration of thromboxanes B2 and nitrotyrosines in plasma of patients with major depression in comparison to control group. We observed trend for elevation of total 8-isoprostanes (sum of free 8-isoprostanes and 8-isoprostanes esterified in phospholipids) in patients in comparison to control group. We didn’t detect significant differences in concentration of 11-dehydro thromboxanes in urine, free 8-isoprostanes in plasma, lipoperoxides, AOPP, PON-A, PON-L in serum and catalase activity in lysates of erythrocytes. We have also observed statistically significant decrease of oxidatively damaged DNA (8-oxoG) in patients determined by visual evaluation.

Discussion
Oxidatively damaged proteins were determinate on basis of AOPP in serum and nitrative protein damage on the basis of nitrotyrosine in plasma. We didn’t observe significant difference in patients and control group in AOPP levels, but we detected significant elevation of nitrotyrosine in patient group, similarly to Maes et al. (2011). As an example of marker of oxidative damage to lipids, we determined lipoperoxides and 8-isoprostanes in patients and controls. We detected trend to elevation of total 8-isoprostanes in patients group, but we didn’t observe statistical difference in free 8-isoprostanes in patients and controls. Lipoperoxides were not different in both groups. Observed elevation of thromboxanes B2 in plasma of patients in comparison to healthy controls is in agreement with Lieb et al. (1983). It indicates nonspecific initiation of inflammatory actions on the basis of elevated activation of thrombocytes in the blood. On the other hand, level of thromboxanes in patients’ urine was not statistically different from control group, similarly as was observed by Gehi et al. (2010). Activity of antioxidant enzymes (PON-A, PON-L, catalase) didn’t differ in patients and controls that are in agreement with mixed or unconvincing results from other studies (reviewed by Gałecki, 2014). Oxidative DNA damage measured by comet assay and expressed as 8-oxoG/10^6G was surprisingly significantly higher in control group than in patients that are not consistent with previous results (e.g. Czarny et al. 2015). We propose a possibility of influence of antidepressant treatment of patients or differences in DNA repair systems or enzymes in patients and healthy controls.

Conclusions
On the basis of evaluation of markers of oxidative and nitrative stress, activity of antioxidant enzymes and pro-inflammatory markers in depression disorder in children and adolescents, we detected elevation of thromboxanes and nitrotyrosine in plasma. On the other hand, we failed to find differences in activity of PON-A, PON-L and catalase. We have also paradoxically observed lower level of 8-oxoG mutations in DNA of patients in comparison to control group. Evaluation of markers of oxidative and nitrative stress together with antioxidant enzymes activity or pro-inflammatory markers in major depression can illustrate redox imbalance in patients and could be used as indicator of severity of disease.

Summary
Evaluation of oxidative and nitrative stress markers in patients with major depression indicate elevated nitrative damage to proteins and elevated concentration of pro-inflammatory eicosanoids in comparison to healthy controls. On the contrary, we failed to detect elevation of oxidatively damaged DNA in patients, oxidatively damaged lipids or changes in activity of antioxidant enzymes. Our preliminary findings confirmed and extended knowledge about oxidative and nitrative stress markers in major depression in children and adolescents. We will continue with further research.

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References
SURVIVIN, A NOVEL TARGET OF THE HEDGEHOG/GLI SIGNALING PATHWAY IN HUMAN TUMOR CELLS

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Introduction
Survivin is an important anti-apoptotic protein, which has no or very low expression in normal tissues but is expressed in the tumors. Its overexpression in the tumors has been associated with aggressive biological features of tumors such as resistance to chemotherapy and poor clinical outcome. The mechanism that activates survivin expression in tumors is unclear. It was suggested that basal transcription of the survivin gene is driven mainly by the transcription factors Sp. However, Sp transcription factors are ubiquitously expressed in mammalian cells and influence the expression of many genes. Since the survivin expression is increased almost exclusively in tumor cells, it is not possible to explain its aberrant expression only by the presence of Sp transcription factors. The aim of the work is to identify the new transcription factors that could activate the survivin expression in tumor cells. In the first place we focused on GLI transcription factors that are the effector proteins of Hedgehog signaling pathway.

Methods
Eleven non-consensus GLI binding sites were found in the survivin promoter. Reporter plasmids containing either different parts of survivin promoter or survivin promoter with mutated single or more GLI binding sites were prepared. Expression plasmids containing different GLI transcription factors were cloned as well. More than 40 cell lines of various tumor origin were used for the experiments. Promoter-reporter assays were performed with plasmids carrying different parts of survivin promoter. Promoter – reporter assay was also performed when cells transfected by reporter plasmid containing a part of survivin promoter were treated by inhibitor of GLI transcription factors GANT61 for 36 hour. Another promoter - reporter assays were carried out with cells cotransfected by plasmid containing a variant of survivin promoter and by GLI expression plasmid. In the other type of experiment various cell lines were treated by GANT61 for 24 hours. For measuring survivin mRNA level treated cells were harvested and real-time PCR was performed. To see whether the level of endogenous survivin protein were changed after GANT61 treatment, western blots were carried out. To be sure that GLI transcription factors activate survivin expression, non-tumor cells IMR 90, which normally do not express survivin and GLI2, were transfected by different GLI expression plasmids and endogenous level of survivin protein was subsequently detected by western blot.

Results
We have identified 11 potential binding sites for the GLI transcription factors (the effector proteins of Hedgehog/GLI signaling pathway) in the survivin promoter, albeit none of them have a fully consensus sequence. The proximal promoter (-990 to -10, numbered in relation to the start of translation) contains
5 GLI-binding sites. The mid-length promoter (-1814 to -10) containing 4 additional distal GLI-binding sites showed about 1.5-fold higher activity than the proximal promoter. Complete mutagenesis of each GLI-binding site in the promoter was carried out to recognize its relative importance in the promoter. The mutagenesis of 4 distal GLI-binding sites in mid-length promoter (-1814 to -10) decreases the promoter activity by half. Interestingly, the longest promoter (-1814 to +57) contains all 11 GLI-binding sites and have minimal activity. When GLI-binding site no. 10 was mutated in this promoter, the activity of the promoter strongly increased (activity was similar to non-mutated -990 to -10 promoter). (Fig.1) The survivin promoter-reporter has been found to be upregulated mainly by plasmids encoding GLI2 and ΔN-GLI2, a more active GLI2 truncated mutant. We also used GANT61 (inhibitor of GLI1 and GLI2) to see whether it is possible to inhibit the activity of survivin promoter by blocking GLI transcription factors. We tested eight cell lines. As suggested, GANT61 decreased the survivin promoter activity to 20 - 70% of the original activity. By analyzing 40 human tumor cell lines of various origin, we have found that the endogenous survivin protein level substantially decreases after the incubation with GANT61 in many cell lines (Fig.2). Real-time PCR determining the survivin mRNA level in GANT61-sensitive cell lines showed a decrease by up to about 60 per cent. To demonstrate that survivin expression can be activated by GLI transcription factors, human fibroblast cell line IMR 90 was transfected by GLI1, GLI2 and ΔN-GLI2. This cell line normally do not express neither survivin nor GLI2. Endogenous level of survivin protein was detected by western blot. It clearly shows that all GLI transcription factors activated survivin expression in IMR90 (Fig.3). ΔN-GLI2 was the most potent activator, suggesting that GLI2 is a direct survivin promoter activator in cells.

Discussion
It is known that aberrant signaling by the Hedgehog/GLI signaling pathway plays a critical role in many tumors. Hedgehog pathway regulates several genes important for cancer growth, e.g. Bcl2 is regulated via Hedgehog effector GLI2. We revealed that survivin, an important anti-apoptotic protein, is probably a target of Hedgehog/GLI signaling. We found GLI binding sites in survivin promoter. Mutagenesis of GLI binding sites revealed that one of these sites is important for repression of survivin activity. The high number of GLI binding sites made it difficult to determine the critical combination of sites important for survivin activity. This could be studied more in the future. Furthermore, we proved that GLI1/2 inhibitor GANT61 decreases endogenous mRNA and protein level in many cell lines. Some cell lines are resistant to GANT61, which can be caused by upstream mutations in Hedgehog pathway. Moreover, endogenous survivin expression can be evoked in normal human fibroblasts IMR90 (which do not express survivin and GLI2) by ectopic GLI2 and ΔN-GLI2 expression. These data suggest that survivin may be a novel important target regulated by the Hedgehog/GLI signaling pathway.

Conclusions
Our data suggest that survivin may be a novel part of Hedgehog/GLI signaling pathway. It seems that the main activator of survivin expression is GLI2 transcription factor. Survivin has been reported previously to be upregulated in virtually all tumor types. When taken together, both survivin and GLI factors may be targeted by specific drugs in the personalized therapy.

Summary
Survivin is an important anti-apoptotic protein overexpressed in many tumors. Currently, the mechanism of its activation is not sufficiently explained. Here we show that Hedgehog/GLI signaling pathway could be responsible for survivin aberrant activation in tumors. It is shown that GLI effectors activate survivin expression in non-tumor cell line and also that survivin expression can be decreased using GLI1/2 inhibitor GANT 61. GLI2 seems to be the main activator of survivin in tumor cells.
References
Background
The national colorectal cancer screening program in the Czech Republic (CR) is based on an examination by faecal occult blood test (FOBT) and colonoscopy. The total coverage of target population is still not satisfactory, in 2012 reached the level of 25.8%. For this reason, other screening modalities are examined.

Objective
To assess the accuracy of second-generation capsule colonoscopy (CCE2) in detection of colorectal neoplasia (polyps, adenomas, cancers) in comparison to optical colonoscopy (OC). The secondary aims were: comparison of colon cleansing, number of complications and target population acceptance of both methods (CCE2 and OC).

Methods
Consecutive individuals were examined prospectively in years 2011–2014 at four specialized endoscopy centers by capsule colonoscopy and optical colonoscopy. Only the screening population was included: asymptomatic persons aged over 50, with no personal or familial history of colorectal neoplasia. The primary outcomes were the accuracy of detection of all polyps (polyps ≥ 6 mm and ≥ 10 mm; adenomas ≥ 10 mm) and cancers. Colon cleansing was evaluated as adequate and inadequate. Complications were assessed as serious (bleeding, perforation) and mild.

Results
In total, 236 individuals (mean age 59 years) were enrolled. During optical colonoscopy polyps were diagnosed in 121 persons (51%), polyps ≥ 6 mm and ≥ 10 mm in 39 (17%) and 16 (7%) patients, respectively. In 63 (27%) patients the adenoma was diagnosed, in eleven (5%) its size was ≥ 10 mm. The sensitivity of CCE2 for polyps ≥ 6 mm and ≥ 10 mm and adenomas ≥ 10 mm reached 77%, 88% and 100%, respectively. The specificity for polyps ≥ 6 mm and ≥ 10 mm was 97% and 99%, respectively. Two cancers were diagnosed at both CCE2 and OC. There were no serious complications registered. Adequate colon cleansing was achieved in 87% (CCE2) and 91% (OC) individuals.
Conclusion
Colon capsule is a safe, non-invasive and sensitive method for diagnosis of colorectal neoplasia. It is acceptable as the primary test for colorectal cancer screening.

References

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REDUCING SHEAR FORCES ON SKIN WITH WOUND DRESSINGS: A NEW STEP IN PRESSURE ULCER PREVENTION?

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Tutor: Prof. Peter B. Soeters, MD, PhD

Background
The condition of pressure ulcers represents localized injury to skin and/or underlying tissue as a result of prolonged mechanical loading in the form of pressure, or pressure in combination with shear (EPUAP/NPUAP Guidelines 2014). Pressure ulcers are a major healthcare problem and associated with high mortality, morbidity, pain and a lower quality of life. Although shear force is understood to be a major contributing factor, pressure ulcer preventive strategies focus mainly on pressure relief. No preventive interventions are aimed at the reduction of shear forces. Therefore, the aim of this study was to investigate if shear force on skin in humans can be reduced with wound dressings and if so, what type of wound dressing performs best.

Methods
A physical model was developed to apply a combined loading of 2.4 kPa pressure and 14.5 Newton (N) shear force on skin in humans. Ten healthy volunteers could participate at this study. Loading was applied on the volar aspect of both forearms for 30 minutes. One arm, the dressing arm, received loading while a dressing was applied between the shear model and the skin (dressing arm). The other arm, which served as a control, received loading directly on the skin. The following parameters were determined before and after loading of the skin: IL-1α/Total Protein- ratio (ELISA, BCA), used as a measure of skin damage and collected non-invasively with Sebutape (Cuderm Corp, Dallas, TX); Cutaneous blood cell flux (Laser Doppler Flowmeter), used as a measure of reactive hyperaemia; Lactate (enzymatically analysed, Cobas Fara Centrifugal Spectrophotometer), used as a measure of tissue ischemia and collected non-invasively with Sebutape.

The research was carried out on three different test days, because three different dressings were tested (table 1). The order of dressing application, the dressing arm (left/ right) and start of the intervention (with or without dressing) were randomized.

Results
Ten healthy volunteers participated at this study. Mean (SEM) age was 22.5 (0.5) with a BMI of 22.3 (0.7). No drop outs were reported. The IL-1α/Total Protein- ratio of the skin was significantly lower after the application of pressure and shear when the Mepilex® (P<0.01), Allevyn (P<0.05) or AquacelTM dressing (P<0.01) was attached to the skin compared with their own control measurement. When the dressings were compared to each other, the Mepilex® dressing was more effective in reducing the post-load IL-1α/Total Protein- ratio compared to the Allevyn dressing (P<0.01). The cutaneous blood cell flux was significantly lower when the Mepilex® dressing or the AquacelTM dressing was attached to the
skin compared to their control measurement without a dressing (P<0.001) after loading of the skin. Although, the Allevyn dressing reduced the amount of post load reactive hyperaemia too, the mean (SEM) difference of 114.6 (54.7) arbitrary units (AU) was not significantly lower compared to its own control measurement (P>0.05). In addition, the Mepilex® and AquacelTM dressing induced significantly less post load cutaneous blood cell flux than the Allevyn dressing (P<0.01 and P<0.001, respectively). Lactate was not significantly increased after a combined loading of pressure and shear for 30 minutes (P=0.07). Therefore, lactate could not be used to evaluate the performance of the dressings with this model.

Conclusion
This is the first study to demonstrate that the effects of shear force on the skin in humans can be reduced with wound dressings. Therefore, they might be beneficial in pressure ulcer preventive strategies. Second, the multi-layered dressings performed better than the single-layered dressing.

Acknowledgement
This study was funded with a Kootstra Talent Fellowship (Maastricht University Medical Centre). The dressings were ordered online by the research team. The manufacturers of the dressings did not know that the study was carried out. Therefore, they had no role in the design of the study, acquisition of subjects, acquisition of data, analysis/interpretation of data and preparation of the manuscript.

Table 1. Dressings used in this study

<table>
<thead>
<tr>
<th>Brand name</th>
<th>Materials</th>
<th>Layers</th>
<th>Size</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mepilex® Border</td>
<td>Polyurethane foam, non-woven spreading layer,</td>
<td>3</td>
<td>10 cm x 10 cm</td>
<td>Mölnlycke, Healthcare AB, Göteborg, Sweden</td>
</tr>
<tr>
<td></td>
<td>polyacrylate fibres</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allevyn Adhesive</td>
<td>Hydrocellular foam</td>
<td>1</td>
<td>10 cm x 10 cm</td>
<td>Smith &amp; Nephew, London, England</td>
</tr>
<tr>
<td>Aquacel™ Foam</td>
<td>Polyurethane foam, hydrofiber</td>
<td>2</td>
<td>10 cm x 10 cm</td>
<td>ConvaTec inc, Skillman, USA</td>
</tr>
</tbody>
</table>
Background
Oxaliplatin-related sinusoidal obstruction syndrome (SOS) is associated with increased morbidity and impaired survival. Hence, identification of the molecular mechanisms underpinning the development of SOS is pivotal.

Methods
This case-matched cohort study studied patients who underwent partial hepatectomy for colorectal liver metastases between 2008 and 2009. Twenty-eight patients with preoperative oxaliplatin treatment and SOS grade 0, 2, or 3 were included; ten patients with severe SOS were matched with ten without SOS by number of oxaliplatin cycles, cumulative oxaliplatin dose, sex, age, body mass index, and liver transaminases. Non-tumor liver microRNA expression was analyzed using Agilent arrays in the 20 patients that could be matched and validated by quantitative PCR in the 28 patients included. MicroRNA expression was subsequently related to overall survival and recurrence free survival.

Results
SOS was not associated with upregulation of any microRNA. However, miR-21 and miR-150 were significantly downregulated in the severe SOS group (array: 1.34-fold, p=0.028; 1.46-fold, p=0.035, respectively; qPCR: 1.32-fold, p=0.001; 1.34-fold, p=0.014, respectively). Low hepatic expression of both miR-21 and miR-150 was associated with impaired overall survival (n=28, p=0.032 and p=0.010, respectively). Likewise, both overall recurrence free survival and liver recurrence free survival were reduced in subjects with low hepatic miR-21 (p=0.010 and p=0.046, respectively) and miR-150 expression (p=0.025 and p=0.031, respectively).

Conclusions
miR-21 and miR-150 may represent important regulatory molecules in the context of SOS, tumor recurrence, and survival in patients with colorectal liver metastases. Further studies are warranted to investigate molecular links between SOS and survival by targeting miR-21 and miR-150 regulated genes.
Introduction
Single embryo transfer becomes more popular during in vitro fertilization (IVF); therefore selecting the most viable embryo is required (1). Despite of evolving microsurgical technologies such as the intracytoplasmic sperm injection, the rate of live birth is surprisingly low, only 30-35% worldwide. The invasive preimplantation genetic diagnosis and screening is informative in assessment of embryo quality, but involves a higher risk to unsuccessful pregnancy. Several studies have focused on developing new non-invasive methods using spent embryo culture media for proteomics and metabolomics or gene expression profiling (2-6). Presently, the predominant and the oldest technique for selecting viable embryos is based on morphological approach. Numerous embryo morphology scoring systems have been developed throughout the years. While a few national consensus schemes exist in some countries, it also has been created an international consensus on embryo assessment (7). In Hungary, there is no national consensus for embryo grading and no adaptation exists of the Istanbul consensus principles, as yet. In this study, we aimed to improve the success rate of embryo transfer by adopting and further optimizing the Istanbul consensus.

Materials and methods
Samples
215 embryo photos were investigated derived from 116 IVF cycles between February 2011 and November 2014 in Assisted Reproduction Unit, Department of Obstetrics and Gynecology, University of Pécs, Hungary. Photos were recorded by Nikon Diaphot 300 inverted phase contrast microscope at 40-fold magnification and resolution of 1024x768. The pictures were always taken 68±1 hours post-insemination (Day-3) or 116±2 hours post-insemination (Day-5). Images were analyzed – in a blind setting - by two independent examiners. Out of 215 photos, 160 photos were selected for statistical analysis. In these 160 cases the clinical outcome of an implantation could be verified with a 100% certainty: single embryo transferred resulting in pregnancy or not, and two embryos transferred resulting twins or no pregnancy occurred. Freeze-thawed embryos were not included in the study. (Ethical approval No.: 5273-2/2012/HER and BAR/006/58-2/2014)

Morphological analysis
We used two versions of embryo quality assessment: the original Istanbul consensus workshop criteria (ICCS) on one hand, and our optimized criteria (OCS) on the other hand. The differences are summarized in Table 1. As regards the 3-day old embryos, the OCS highlights 3 modified or new parameters: fragmentation (with a more permissive criterion of <15% in the “good” category); symmetry (good -
full symmetry; fair - light asymmetry; poor - evident asymmetry) and the blastomere number (less than 7 and 7 or more). In addition, the blastomere size was evaluated according to the original Istanbul consensus. A scoring-map was created to facilitate the evaluation (Table 1.). As regards the 5-day old embryos (116±2 hpf), the ICCS does not express the viability of embryos with a single category (good, fair, poor). We tried to overcome this by using a scoring map (Table 1.). Embryos, which look like compact morula with 116±2 hpf were evaluated according to the ICCS described for a 4-day old embryo.

Results

3-day old embryo scoring

The scoring map for 3-day old embryos consists of four digits, expressing the quality of the embryo as measured by the fragmentation, blastomere size, symmetry and blastomere number, respectively. Theoretically the best quality is characterized by a score of 1111, while the worst quality by 3232. Fig. 1A. and B. depict the comparative analysis of the same group of 3-day old embryos evaluated both according to the ICCS (A.) and the OCS (B.). The difference was significant (Chi-square test: P<0.001). On Fig.1C. the left embryo got good grade in both criteria systems. The right embryo got a fair quality score in ICCS because of its high fragmentation rate. In the OCS scoring got a good grade (2121), because of the light asymmetric cleavage and the number of blastomeres. Both embryos resulted in pregnancies.

5-day old embryo scoring

The ICCS aspects (stage, ICM, TE) individually were not predictive to success transfer. Some 5-day old embryos are still in compact morula stage. These embryos were evaluated with the ICCS scoring described for the 4-day old embryos. 11/32 embryos received good score and 8 of them resulted in pregnancy. In the OCS scoring we included these 5-day old, but 4-day looking embryos. Fig. 2A. indicates the distribution of 5-day old embryos in the OCS scoring. Fig. 2B. demonstrates the microphotograph of four Day-5 old embryos. The first embryo is in a compact morula stage, it was evaluated as a good according to both criteria. According to the ICCS scoring, the second embryo would receive a difficult, non-uniform score. This expanded blastocyst has fair ICM (arrow) and good TE. Evaluated according to the OCS scoring the composite score of this embryo is good (321). Both embryos resulted in pregnancy. The third embryo has a poor quality according to both criteria. The fourth, an early blastocyst has poor ICM and poor TE. According to the composite score of the OCS scoring, the quality is poor (133). Embryos 3 and 4 did not result in pregnancy.

Discussion

Taken together, the OCS score as proposed in this study, is more sensitive for the evaluation of Day-3 embryos and gives a composite, more simple and uniform evaluation of both Day-3 and Day-5 embryos. Limitations of the study are related to the retrospective study composition, the relatively few number of cases, lack of Day-2 evaluation and lack of time-lapse observations.

Conclusions

The mentioned new non-invasive techniques may have several disadvantages yet, for example the sample heterogeneity and preparation, time-consuming measurement, or require special instruments and professionals. Although have not so far resulted in higher pregnancy or birth rates. However, during the use of fast and simple morphological assessment the parameters are evaluated mostly subjectively and hard to standardize, but it continues to be the best tool for assessment of embryo quality. In this study, our primary aim was the adoption and optimization of ICCS. The OCS score is more sensitive for the evaluation of the viability of Day-3 embryos and gives a composite, more simple and uniform evaluation for both Day-3 and Day-5 embryos.
Summary
An international consensus (the Istanbul Consensus) has been reached on embryo assessment. Our primary aim in this study was the adoption and optimization of ICCS. As a part of this process, the rate of fragmentation was evaluated differently. However, we observed that embryos with 10-15% fragmentation rate are still frequently resulting in pregnancy. Therefore, our OCS scoring is more permissive (good score if the proportion of fragmented cells is less than 15%). We also introduced two new parameters (symmetry and the number of blastomeres), which, however, has mentioned in the Istanbul workshop, but not included in ICCS. Finally, we constructed a composite score based on these parameters. As it is evident from the results, this composite score is more sensitive to evaluate viability than the original ICCS scoring. In addition to the above-described parameters, the original ICCS assessment is also based on the evaluation of multinucleation. This parameter has been taken out from our OCS scoring, primarily because multinucleation can be best evaluated on the second day of incubation. The Hungarian clinical protocols prescribe the first check and quality assessment on Day-3.
Quality assessment of Day-5 old embryos is not an easy task either. In the OCS, we do not propose to change the principles of the ICCS scoring for the Day-5 embryos. As a small step to optimize, we also introduced a composite score for the evaluation of Day-5 embryos (blastocyst stage, ICM, TE). This way the evaluation has become more simple and uniform. Some of the Day-5 embryos are still in a compact morula stage (look like Day-4 embryos) and cannot be evaluated according the ICCS score for the Day-5 embryos. Despite of it’s poor grading according to the strict timing ICCS, for these embryos we applied the ICCS score for Day-4 old embryos. They would have discarded if we had used a prospective study composition, now 22.1% of this resulted in pregnancy. This work was supported by OTKA 115394 and by the MTA-PTE Human Reproduction Scientific Research Group.

Key words: IVF, in vitro fertilization, embryo morphology, quality assessment, embryo scoring.

References
Table 1. The differences between the Istanbul consensus (ICCS) and the optimized criteria system (OCS)

**ICCS for cleavage stage embryos**

<table>
<thead>
<tr>
<th>GOOD</th>
<th>OCS for cleavage stage embryos</th>
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<tbody>
<tr>
<td>&lt;10% fragmentation</td>
<td>Fragmentation 1 Good (&lt;15%)</td>
</tr>
<tr>
<td>Stage-specific cell size</td>
<td>2 Fair (15-25%)</td>
</tr>
<tr>
<td>No multinucleation</td>
<td>3 Poor (&gt;25%)</td>
</tr>
<tr>
<td>FAIR</td>
<td>Blastomere size 1 Stage specific</td>
</tr>
<tr>
<td>10-25% fragmentation</td>
<td>2 No stage specific</td>
</tr>
<tr>
<td>Stage-specific cell size for majority of cells</td>
<td>3 Symmetric cleavage</td>
</tr>
<tr>
<td>No evidence of multinucleation</td>
<td>2 Light asymmetry</td>
</tr>
<tr>
<td>POOR</td>
<td>3 Evident asymmetry</td>
</tr>
<tr>
<td>Severe fragmentation (&gt;25%)</td>
<td>Number of blastomeres 1 ≥7</td>
</tr>
<tr>
<td>Cell size not stage specific</td>
<td>2 &lt;7</td>
</tr>
<tr>
<td>Evidence of multinucleation</td>
<td></td>
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</table>

**ICCS for blastocysts**

<table>
<thead>
<tr>
<th>Stage</th>
<th>OCS for blastocyst (Day-5) – Scoring map</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Prominent, easily discernible, with many cells that are compacted and tightly adhered together</td>
</tr>
<tr>
<td>2</td>
<td>Easily discernible, with many cells that are loosely grouped together</td>
</tr>
<tr>
<td>3</td>
<td>Difficult to discern, with few cells</td>
</tr>
<tr>
<td>4</td>
<td>Many cells forming a cohesive epithelium</td>
</tr>
<tr>
<td>TE</td>
<td>Few cells forming a loose epithelium</td>
</tr>
<tr>
<td>Good</td>
<td>Poor</td>
</tr>
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</table>

**POOR**

<table>
<thead>
<tr>
<th>OCS for Day-3 embryos - Scoring map</th>
</tr>
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<tbody>
<tr>
<td>GOOD</td>
</tr>
<tr>
<td>FAIR</td>
</tr>
<tr>
<td>POOR</td>
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</table>
(1) Good embryo quality according to both criteria system (1111 according to ICCS)

(2) Fair embryo quality according to ICCS, however good in OCS (2121 according to OCS).
Assessment of Day-5 old embryos according to OCS scoring
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