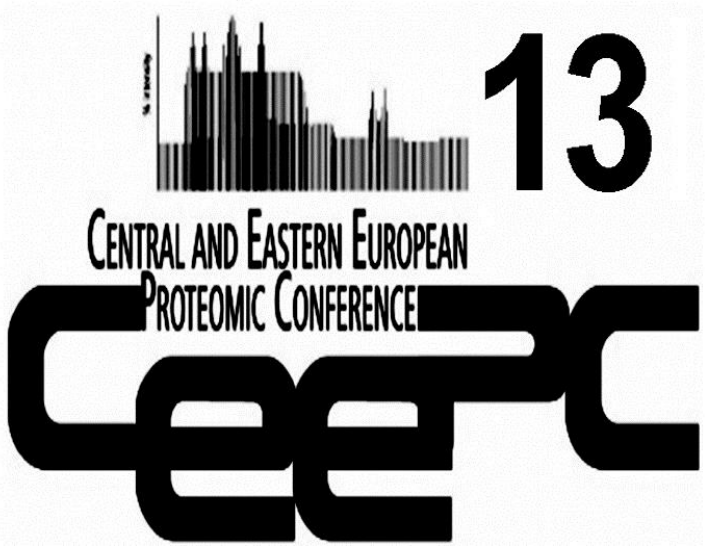

Central and Eastern European Proteomic Conference
13th CEEPC
23-25.09.2019, Ustron, Poland

Poland, Ustron-2019



13th Central and Eastern European Conference

23-25.09.2019, Ustroń, Poland

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Local Organizing Committee

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The Central and Eastern European Proteomic Conference has progressed to its present-day status and stature with an expansive network of proteomics in Central and Eastern Europe with links to international institutions worldwide to facilitate expert scientific interaction and collaborations. An informative website at http://ceepc.eu/ not only distinguishes CEEPC from other proteomic organizations but also underlines the uniqueness and individuality of its ethos and ideology. The birthplace of CEEPC together with advancing proteomics is captured by the CEEPC logo showing the ascending spires of the city of Prague outlined by the intensity of protein/peptide peaks of mass spectrometry depicting the pinnacles of excellence and cohesion of the CEEPC community.

The initial vision of a forum for enthusiastic scientists and researchers to meet and discuss their work in a relaxed manner in middle sized meetings remains unchanged to this day. Rotation of the meeting's venue each year to cultural cities of the world such as Prague, Vienna, Budapest, Jena, Poznan, Kosice, Bucharest and now Ustron, Poland, adds to the intertwining of 'cutting edge' research and the excitement that goes with it. CEEPC's success is not only due to different aspects of proteomics but also due to encompassed diverse proteomic topics as well as appreciation of the hot topics of the advancing science and medicine.

Demands to treat the individual rather than the average human and the advances in artificial intelligence (AI) is ushering in precision medicine or personalized medicine where tailoring predictive, preventive and treatment strategies to the individual is a priority. Since protein expression is dynamic and changes in relation to disease onset, severity or response to therapy are difficult to understand, proteomics stands to play a pivotal role in characterizing a disease at protein level. Proteomic technologies have progressed over the last decade allowing in principle the comprehensive analysis of expressed proteins in time and space. Until now, quantitative proteomics has been pin-pointing minor differences in the protein levels between normal and pathological samples. There is now an urgent need for sophisticated 'enabling technologies' to identify structural differences in proteins introduced by mutations or structural variations induced by post-translational modifications or protein truncation that are associated with a disease. Additionally, comprehensive characterization of the small molecule metabolites in biological systems and biological applications of the Metabolome together with the Proteome in precision medicine of the patient, stands to revolutionize global health. The complexity of the data generated has also been a stumbling block in understanding diseases because proteome analysis does not provide a simple 'yes/no' answer but rather requires deep interpretation. To this end, utilization of data and information from various 'multi-omics' studies including proteomics, metabolomics, together with AI in the hands of skilled researchers, can have significant societal impact.

CEEPC's careful balance between excellence and focus on societal needs holds the key to its success. It is evident from CEEPC's progress that it is ready for the next decade of excitement and expectations of multifaceted proteomics in Central and Eastern Europe. Additionally, in the era of emerging personalized medicine where treatment selection for each patient is becoming individualized, CEEPC and proteomics is expected to play a significant role moving forward for the benefit of mankind.

We acknowledge and are indebted to the CEEPC Community, the CEEPC Scientific Committee and the 13th CEEPC Organizing Committee – our sincere Thanks to all.

Suresh Jivan Gadher

Scientific Program

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September 23rd, 2019 (Monday)

16.00 – 18.00 **Registration**

18.00 – 19.00 **Opening of the Conference**

Opening welcome – Piotr Widłak (chairman of the local organizing committee)

Opening address – Suresh Gadher *(Carlsbad, USA)*: *The Central and Eastern European Proteomic Conference*: *Progress and Priorities* (OP-0; 15')

Opening lecture – Theresa L. Whiteside *(Pittsburgh, USA)*: *Tumor-derived exosomes as the emerging mechanism of cancer - induced immune suppression* (OP-1; 45')

19.00 – 19.30 Concert of the local folklore ensemble *Czantoria*

20.00 – Welcome Dinner

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September 24th, 2019 (Tuesday)

9.00 – 11.00 **Biotechnology and New Approaches**

Jacek R. Wisniewski *(Martinsried, Germany)*: Absolute quantitative proteomics of blood (BT-1; 35')

Joanna Bukowska *(Olsztyn, Poland)*: The effect of low oxygen on the proteomic signature and functional features of pig Adipose Derived Stromal/Stem Cells (pASCs): Implication in skin wound healing (BT-2; 20')

Aleksandra Bocian *(Rzeszów, Poland)*: Analysis of the antibacterial properties of Naja ashei venom components (BT-3; 20')

Mariola Słowińska *(Olsztyn, Poland)*: Isolation and immunohistochemical detection of cysteine-rich venom protein-like in turkey (Meleagris gallopavo) spermatozoa (BT-4; 20')

Robert Nawrot *(Poznań, Poland)*: Major latex protein (CmMLP) isolated from medicinal plant Chelidonium majus L. latex is accompanied by alkaloids – a proteomic study (BT-5; 20')

10.00 – 11.30 Coffee Break

11.30 – 13.30 **Clinical Proteomics**

Karoly Vekey *(Budapest, Hungary)*: Changes of IgG glycosylation in Rheumatoid Arthritis (CP-1; 20')

Aleksandra E. Lewandowska *(Gdańsk, Poland)*: Proteomic analysis of human follicular fluid from women undergoing in vitro fertilization procedure – combination of FASP and SWATH-MS methodology (CP-2; 20')

Piotr Widłak *(Gliwice, Poland)*: Molecular heterogeneity of papillary thyroid cancer: comparison of primary tumors and lymph nodes metastases by MALDI-MSI (CP-3; 20')

Magdalena Kowalewska *(Warszawa, Poland)*: Inflammation as a Driver of Vulvar Squamous Cell Carcinoma Progression (CP-4; 20')

Monika Pietrowska *(Gliwice, Poland)*: Proteomes of exosomes from HPV(+) or HPV(-) head and neck cancer cells: differential enrichment in immunoregulatory proteins (CP-5; 20')

Nick Morrice *(Redwood City, USA)*: Quantitative proteomics using fast LC gradients and its application to clinical proteomics (CP-6; 20')

13.30 – 14.30 Lunch

14.30 – 16.30 **Metabolomics**

Gwendolyn Barceló-Coblijn *(Palma, Spain)*: From MALDI-IMS lipidomic data to study cell signaling pathways: the importance of the lateral resolution (MT-1; 35')

Piotr Młynarz *(Wrocław, Poland)*: Metabolomics studies as a searching tool for therapeutic agents (MT-2; 20')

Anna Wojakowska *(Poznań, Poland)*: Metabolomic signatures discriminate normal human cornea from keratoconus and bullous keratopathy – a pilot GC/MS study (MT-3; 20')

Karol Jelonek *(Gliwice, Poland)*: Systemic effects of radiotherapy and concomitant chemo-radiotherapy in head and neck cancer patients – comparison of serum metabolome profiles (MT-4; 20')

Łukasz Marczak *(Poznań, Poland)*: Functional analysis in plants using metabolomic approach (MT-5; 20')

16.30 – 19.00 Coffee/Discussions/Outdoor Activities *(sunset 18.46)*

Meeting of the Polish Proteomics Society

19.00 – Dinner and Folklore at Skibowka on Mt. Rownica

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September 25th, 2019 (Wednesday)

9.00 – 11.00 **Young Guns** Session (part 1)

Magdalena Oroń *(Warszawa, Poland)*: Global proteomics and transcriptomics reveal proteasome machinery target networks and mechanisms of proteasome inhibitor resistance in human cancers (YG-1; 12')

Aurel Cerveanu-Hogaș *(Bucharest, Romania)*: Mass spectrometric analysis of thoracic aorta alarmin profile in experimental atherosclerosis (YG-2; 12')

Marta Woźniak *(Wrocław, Poland)*: Differences in metabolic profiles of primary and metastatic colon cancer cells upon hypoxia and glucose starvation conditions (YG-3; 12')

Anna Papież *(Gliwice, Poland)*: Proteomics validation analysis for ischemic heart disease in Mayak workers (YG-4; 12')

Joanna Suraj-Prażmowska *(Kraków, Poland)*: Comprehensive analysis of endothelial response to endotoxemia based on plasma profiling of protein biomarkers of endothelial dysfunction in mice; characteristics of endothelial effects of sulodexide (YG-5; 12')

Joanna Tracz *(Poznań, Poland)*: Proteomic analysis of monocytes in cardiovascular disease related to chronic kidney disease (YG-6; 12')

Konrad Hus *(Rzeszów, Poland)*: Fractionation and identification of Naja ashei venom proteins (YG-7; 12')

Mateusz Smolarz *(Gliwice, Poland)*: Comparison of protein profile of two fractions of serum nanovesicles (YG-8; 12')

Agata Abramowicz *(Gliwice, Poland)*: Characteristic of the proteome cargo of exosomes derived from radiochemo-resistant/sensitive HNSCC cells treated with genotoxic agents (YG-9; 12")

Aneta Żebrowska *(Gliwice, Poland)*: Is it possible to study the metabolome of exosomes? (YG-10; 12')

11.00 – 11.30 Coffee Break

11.30 – 13.30 **New Concepts and Methods**

Joanna Polańska *(Gliwice, Poland)*: Machine Learning in the processing of proteomics data (NC-1; 35')

Laszlo Drahos *(Budapest, Hungary)*: The effect of PTMs on proteomic searches (NC-2; 20')

Marta Gawin *(Gliwice, Poland)*: The pitfalls of sample preparation for MALDI mass spectrometry imaging of tryptic peptides in archival tissue specimens (NC-3; 15')

Martina Macht *(Bremen, Germany)*: Characteristics of MALDI-imaging on a new dual ion source QTOF with TIMS separation (NC-4; 20')

Peter Mowlds *(Edinburgh, UK)*: A modified Orbitrap™ Tribrid mass spectrometer with real-time search and advanced spectral processing enhances multiplexed proteome coverage and quantification accuracy (NC-5; 20')

13.30 – 14.30 Lunch

14.30 – 15.30 **Young Guns** Session (part 2)

Jakub M. Dąbrowski *(Warszawa, Poland)*: FANF-ic (Filter Assisted Nuclear Fractionation is cool) a novel approach for organelle fractionation for proteomics (YG-11; 12')

Katarzyna Frątczak *(Gliwice, Poland)*: Comparative classification study for pan-cancer mass spectrometry imaging data (YG-12; 12')

Anna Glodek *(Gliwice, Poland)*: Can the spatial distribution in MSI support the identification of the isotopic envelope? (YG-13; 12')

Agata Kurczyk *(Gliwice, Poland)*: Biostatistical approaches to assess molecular heterogeneity between and within histologically-defined regions of interest in tissue samples imaged by MALDI-MSI (YG-14; 12')

Maxim Domnich *(Essen, Germany)*: Tumor-derived exosomes influence the activity of neutrophils in head-and-neck cancer (YG-15; 12')

15.30 – 16.30 **Poster Session** and Coffee

Mariola A. Dietrich, A. Mostek, H. Karol, A. Ciereszko *(Olsztyn, Poland)*: Short-term storage of carp semen triggers oxidation of spermatozoa proteins (P-1)

A. Mostek, Anna Janta, A. Ciereszko *(Olsztyn, Poland)*: Differences in sperm protein abundance and carbonylation level caused by sex-sorting of bull spermatozoa (P-2)

Anna Kurpinska, J. Suraj, M. Stojak, Ł. Mateuszuk, E. Niedzielska-Andres, M. Smolik, D. Papiernik, J. Wietrzyk, I. Kalvins, M. Walczak, Chłopicki *(Kraków, Poland)*: Towards targeting protein disulfide isomerase (PDI) isoforms to treat cancer (P-3)

Joanna Ner-Kluza, A. Milewska, A. Dąbrowska, K. Pyrć, J. Silberring, P. Suder *(Kraków, Poland)*: TAILS based proteomic analysis of Zika virus infection (P-4)

Ciereszko, M. Dietrich, M. Słowińska, Joanna Nynca, M. Ciborowski, J. Kisluk, A. Michalska-Falkowska, J. Reszec, E. Sierko, J. Nikliński *(Olsztyn, Poland)*: Identification of protein changes in the blood plasma of lung cancer patients subjected to chemotherapy using a 2D-DIGE approach (P-5)

Joanna Nynca, M. Słowińska, A. Mostek, E. Liszewska, S. Judycka, H. Karol, S. Dobosz, A. Ciereszko *(Olsztyn, Poland)*: Acquiring potential for motility is accompanied by profound changes in sperm proteome (P-6)

Joanna Nynca, D. Żarski, A. Ciereszko *(Olsztyn, Poland)*: Comparative proteomic analysis of high quality eggs obtained from wild-caught and domesticated (Sander lucioperca) (P-7)

Kinga Piechura, P. Mielczarek, J. Silberring *(Kraków, Poland)*: Study of cysteine proteases using fluorescent probes (P-8)

Ľudmila Tkáčiková, E. Kaňová, A. Kulkarni, M. Bhide *(Košice, Slovak Republic)*: Comparative transcriptome analysis of the interaction between E.coli and IPEC-1 treated with exopolysaccharides isolated from Lactobacillus reuteri DSM17938 (P-9)

Gracjana Zając, K. Puszyński, P. Widłak *(Gliwice, Poland)*: Ionizing radiation activates the atypical NF-κB pathway in RKO cells derived from human colorectal cancer (P-10)

Anna Fel, A.E. Lewandowska, P.E. Petrides, J.R. Wiśniewski, S. Oldziej *(Gdańsk, Poland)*: Quantitative proteomic analysis of serum-derived exosomes from polycythemia vera patients (P-11)

I. Rubić, A. Horvatić, R Burchmore, C. Regnault, S. McGill, A. Monteiro, J. Gotić, R. Barić Rafaj, Vladimir Mrljak *(Zagreb, Croatia)*: Study of serum metabolom in canine babesiosis by mass spectrometry (P-12)

Viorel-Iulian Suica, L. Ivan, E. Uyy, R.M. Boteanu, A. Cerveanu-Hogas, F. Antohe, M. Simionescu *(Bucharest, Romania)*: Proteomic alterations revealed in the crosstalk of polymorphonuclear neutrophils and macrophages using dynamic SILAC methodology (P-13)

Wojciech Łabaj, J. Mika, M. Chekan, M. Pietrowska, P. Widłak, A. Polański *(Gliwice, Poland)*: Biological processes associated with mutations present in anaplastic and papillary thyroid cancers co-existing in the same thyroid gland (P-14)

Bartłomiej I. Łukasz, M. Karnasiewicz, R.T. Smoleński, I. Rybakowska *(Gdańsk, Poland)*: Determination of angiotensin levels in mouse and human serum samples with nanoLC/MS method (P-15)

16.30 – 18.10 **Proteo-genomics and Molecular Networks**

Maciej Lalowski *(Helsinki, Finland)*: Dissecting rare disorders: from compartmental proteomics towards affected pathways and identification of putative drugs (MO-1; 35')

Suresh Jivan Gadher (*Carlsbad, USA*): Simultaneously looking for molecular signature of human diseases at protein and gene level - power of 'proteo-genomics' (MO-2; 20')

Marek Rusin (*Gliwice, Poland*): Strong activation of p53 tumor suppressor protein is associated with coordinated upregulation of proteins protecting against infections (MO-3; 20')

Jadwiga Jablonska *(Essen, Germany)*: Identifying head-and-neck cancer biomarkers using proteomic analysis of neutrophils and neutrophil-derived exosomes (MO-4; 20')

18.10 – 18.20 **Closing of the Conference**

Presentation of plans for 14th CEEPC

19.00 – Farewell Dinner

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September 26th, 2019 (Thursday)

General departure after breakfast

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Folk Ensemble *Czantoria* –

conductor Danuta Zoń-Ciuk

The band was established in 1988. Marian Żyła was the first conductor, then it was Władysław Wilczak, and since 2011, Danuta Zoń-Ciuk has been in charge of the group. Apart from home and abroad concerts for example in the Czech Republic, Germany, Croatia, Italy, Slovakia, Lithuania, Latvia, Hungary and Bułgaria, Czantoria performs systematically in Ustroń, mainly at guest houses and sanatoriums. Since 1991, the ensemble has been presenting the harvest rite during the traditional *Święto Plonów* (Celebration of Harvest), and since 1994, it has performing during *Tydzień Kultury Beskidzkiej* (The Beskidy Week of Culture) in Wisła, Szczyrk, Oświęcim and Maków Podhalański. Czantoria is a laureate of numerous awards and distinctions, and the most valuable ones are: Silver Medal of the Polish Sejm (Parliament), distinction For the Merit of Bielsko Voivodeship and For the Merit of Ustroń. The band is now a representative ensemble of Ustroń town. Czantoria has given about 1000 concerts, performing mainly repertoire based on the folklore of Cieszyn Silesia and patriotic, religious and festive songs.

The recent successes of the group are the following: in 2014 the ensemble was awarded the 1st prize in its category at XXIII Regional Festival of Songs *Śląskie Śpiewanie* (Silesian Singing), the 1st prize in 2014 and 2016 at I and III edition of the European Competition *Rozśpiewany Śląsk* (Singing Silesia) in Koszęcin. In 2015 they won the 3rd prize at XXII International Festival of Regional Ensembles *Złoty Kłos* (Golden Crop) 2015 in Zebrzydowice. In 2017 they won the golden diploma at the international competition Slovakia Folk in Bratislava, Slovak Republic. Czantoria released a few CDs, and recently recorded carols and pastorals, which can be heard on *Beskidzkie kolędowanie* (Beskid Carolling) album. There is also a CD recorded live during a concert *Pieśni roztomiłych groni* (Songs of Beloved Mountains) in Czeski Cieszyn, in which choirs of the Institute of Art in Cieszyn University of Silesia in Katowice also participated. The album is entitled *Z Beskidów śpiewanie* (Singing from the Beskidy).

Program of the Concert:

1. *Polonez ustroński* (Ustroń Polonaise) - arranged by Władysław Wilczak
2. *Hej gróniczki* (Hey, Hills) – arranged by Karol Chmiel
3. *Beskidzie, Beskidzie* (Oh, Beskid) - arranged by Aleksander Niedoba
4. *Idóm gorole* (The Highlanders are Coming) – a medley of folk melodies arranged by Władysława Wilczaka
5. *Posłuchejcie siostrzyczki* (Sisters, listen) - arranged by Józef Firla
6. *Hej tam w dolinie* (Hey, There in the Valley) - arranged by Stanisław Hadyna
7. *Nie chodź kolo róży* (Don't Go Close to the Rose) - arranged by Wojciech Kilar
8. *Dysc* (Rain)-arranged by Tadeusz Mayzner
9. *Kaj żeś była Hanuliczko* (Where Have You Been, Hanuliczka) - a medley of folk melodies arranged by W. Wilczaka
10. *Sarna* (Roe-deer) - arranged by Władysław Wilczak
11. *Pije Kuba* (Jacob's Toast) - arranged by Władysław Wilczak
12. *Śpiewam ja śpiewam* (I Am Singing) - arranged by Marian Żyła

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Book of Abstracts

Tumor-derived exosomes as the emerging mechanism of cancer-induced immune suppression

T.L. Whiteside

University of Pittsburgh School of Medicine and UPMC Hillman Cancer Center, Pittsburgh, USA.

Introduction Tumor-derived exosomes (TEX) present in plasma of patients with cancer are emerging as an important inter-cellular communication system and as promising non-invasive correlates of cancer progression or response to therapy. Tumors produce excessive levels of exosomes which accumulate in patients' circulation. The molecular cargo of exosomes mimics that of parent tumor cells; hence, exosomes may serve as a tumor markers. Also, functions mediated by exosomes in vitro and in vivo parallel those mediated by tumor cells. Exosomes can be isolated from plasma, characterized and separated into subsets to study their molecular and genetic cargos and evaluate their contributions to reprogramming of the tumor microenvironment (TME), inhibition of host anti-tumor immunity and promotion of tumor progression.

Methods To study the impact of TEX on human immune cells and progression, we developed methodology for TEX isolation involving size exclusion chromatography, immune capture with mAbs specific for antigens carried by exosomes and on-bead flow cytometry for quantitative detection of proteins carried on the exosome surface. In addition, we used the shotgun LC-MC/MS proteomics approach to characterize proteomics profiles of these exosomes. Exosomes were isolated from plasma of patients with metastatic melanoma and plasma of 6 healthy donors. mAbs specific for the CSPG4 epitope overexpressed in most melanoma but not normal cells were used for immune capture from plasma of melanoma cell-derived exosomes (MTEX) and non-MTEX (exosomes derived from non-malignant cells). The molecular cargos and functions of MTEX and non-MTEX were compared.

Results MTEX were found to be highly enriched in immunosuppressive receptor/ligands. Non-MTEX were enriched in immunostimulatory proteins. The ratio of stimulatory/suppressive proteins in circulating exosomes in melanoma was the major determinant of their immunoregulatory functions, i.e., of the capability to modulate immune cell functions and to impact disease activity. The LC-MS/MS analysis identified 3,600 proteins in MTEX and 3,700 in non-MTEX; 300 proteins were present exclusively in MTEX. Most of these proteins were immunomodulatory. Quantitative proteomics analysis identified individual differences between melanoma patients, suggesting that MTEX have the potential to contribute to personalized analysis of proteins regulating immune competence of melanoma patients.

Conclusions MTEX isolated from plasma of melanoma emerge not only as surrogate markers of the tumor but also as biomarkers of the patients' immune competence. Preliminary data suggest that MTEX may also serve as predictive biomarkers of response to immunotherapy therapy in melanoma.

Absolute quantitative proteomics of blood

J.R. Wisniewski

Max Planck Institute of Biochemistry, Martinsried, Germany.

Introduction Determination of protein concentrations by mass spectrometry-based proteomic typically requires isotopically labeled standards, making any proteome wide quantification of proteins difficult and expensive. In contrast, the 'Total Protein Approach' (TPA) allows absolute protein quantitation without any biochemical input. In the TPA method, calculation of protein abundances is based on spectral intensities acquired in the large-scale proteomic analyses.

Results Blood tests are often used in health care to determine physiological and biochemical states, such as disease and organ function. Many tests include measuring of titers in plasma; however, the number of monitored proteins is limited. In contrast, proteomics has the capacity to provide quantitative information on hundreds of plasma proteins.

Conclusions Combination of the TPA method combined with MED-FASP-consecutive sample digestion strategy allows accurate quantification of plasma proteins. Analyses of unfractionated plasma cover protein concentrations spanning six orders of magnitude of protein abundance. In addition, this technology has been proven as a powerful tool for investigation of blood cells and vesicles.

The effect of low oxygen on the proteomic signature and functional features of pig Adipose Derived Stromal/Stem Cells (pASCs): Implication in skin wound healing

J. Bukowska, M. Słowińska, P. Cierniak, K. Walendzik, M. Kopcewicz, B. Gawrońska-Kozak

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Introduction Adipose Derived Stromal/Stem Cells (ASCs) are multipotent cells recognized for their potential to promote tissue restoration. Recent data show that human ASCs cultured in low O2 environment (hypoxia) improve wound healing. The use of ASCs in regenerative medicine routinely employs human donors, whereas their animal equivalents have received minimal attention. Since variety of pig organs and tissues reveal multiple similarities to its human counterpart, investigating pig ASCs (pASCs) is valuable for the evaluation of their efficacy in wound healing in human. The present study was to (1) characterize pig (Sus scrofa) ASCs; (2) evaluate the effect of hypoxia (1 % O2; 24 h) on pASCs proteome (a) and functional features (b) attributed to the wound healing in skin.

Methods Two-dimensional difference gel electrophoresis (2D-DIGE) and matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry, cell functional in vitro assays, flow cytometry, qRT-PCR, ELISA were employed.

Results Porcine ASCs showed expression of mesenchymal stem cell markers (MSC) including CD29, CD44, CD90, CD105. pASCs revealed rapid (2.25 ±0.24 days) population doubling time at seeding density 5.0x104 cells/well, whereas plated at low concentrations (10, 100, 200 cells/well) demonstrated potential to form colony-forming units (CFU). Furthermore, pASCs exhibited capacity to differentiate into adipocytes and osteoblasts. Proteomic analysis showed significant differences in protein profile between hypoxia- and normoxia-exposed pASCs. The 70 proteins with differences in abundance ($p < 0.05$) between pASCs cultured in low and ambient O2 environment were identified, including 41 up-regulated and 29 down-regulated upon hypoxia. Differentially abundant proteins were involved in cellular (38.60%), metabolic (34.11%) processes, and demonstrated catalytic function (45.01%). Proteins upregulated upon hypoxia were involved in glycolysis/gluconeogenesis, biosynthesis of amino acids and carbon metabolism. Majority of hypoxia up-regulated proteins (e.g. LDHA, ALDOA, ENO2, EROL1, PGK1) were linked with anaerobic metabolic pathways. With regard to proteins associated with wound healing, we found that prolyl 4-hydroxylase subunit alpha-1 precursor (P4HA1) was increased in response to low oxygen tension. Among down-regulated upon hypoxia proteins we found collagen I alpha 2 (COL1A2) the component of extracellular matrix (ECM) that is associated with scarring. Follow-up functional study revealed that hypoxia enhanced contractile ability of pASC ($p<0.01$).

Conclusions Differentiation potential, cell surface markers profile and ability to form CFU indicate that pASCs fulfill criteria for MSC. Furthermore, MS analysis revealed that pASCs are sensitive to oxygen level alterations. Most of differentially expressed proteins reflected cells adaptation to low oxygen tension that led to support pASCs survival and thus might enhance their regenerative potential in vivo. A minor proportion of proteins differentially abundant between hypoxia and normoxia- exposed pASCs were found to be associated exclusively with wound healing. Among them we identified ECM components P4HA1 and COL1A2. These suggest that ECM synthesis is oxygen-sensitive and oxygen level might act as a regulatory tool of wound healing via engagement in ECM deposition. Finally, analysis of functional features of cultured pASCs showed that hypoxia stimulates contractile abilities of pASC that are necessary for proper wound healing.

Acknowledgments This study was supported by the National Science Centre (NCN), Poland, Grant SONATA13 No. 2017/26/D/NZ5/00556.

Analysis of the antibacterial properties of *Naja ashei* venom components

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Introduction An increasing problem in the field of health protection is the emergence of drug-resistant and multi-drug-resistant bacterial strains. They cause a number of infections, including hospital infections, which currently available antibiotics are unable to fight. Therefore, many studies are devoted to the search for new therapeutic agents with bactericidal and bacteriostatic properties. One of the latest concepts is the search for this type of substances among toxins produced by venomous animals. Among them, special attention is paid to snake venom, because it has long been known that many of them have antibacterial properties. Thorough investigations have shown that the phospholipase A2 and L-amino acids oxidases, as well as fragments of these enzymes, are mainly responsible for the bactericidal properties of snake venoms. Less advanced research also suggests that fragments of 3FTx proteins are bactericidal. All three mentioned groups of proteins are present in the *Naja ashei* venom, which has antibacterial properties.

Methods African spitting cobra *Naja ashei* venom was fractionated using ion exchange chromatography (IEC) with the use of strong cation-exchange resin Resource S. Protein composition of the fractions was determined using LC/MS technique. Antibacterial activity of fractions was determined by appointing the minimum concentration inhibiting the growth of bacteria (MIC) and the minimum concentration of bactericidal (MBC) against certified bacterial strains (Staphylococcus aureus ATCC 6538 , Staphylococcus epidermidis ATCC 35984 and ATCC 12228) included in the collection of the Department of Biotechnology and Bioinformatics of the Faculty of Chemistry at the Rzeszów University of Technology.

Results The most interesting results were obtained for F2 fraction, which showed antibacterial properties against two S. epidermidis strains: ATCC 12228 (able to produce biofilm) and ATCC 35984 (not-able to produce biofilm). Moreover, this fraction exhibits additive (ATCC 12228) and synergistic (ATCC 35984) effects with ampicillin and tetracycline.

Conclusions The obtained results indicate that selected proteins from *Naja ashei* venom, present in fraction F2, are capable of both inhibiting bacterial growth and bactericidal activity. We strongly believe that these proteins could be used in bactericidal agents used externally, and in the future, they can also help in the design of new antibiotics effective against drug-resistant strains of bacteria.

Isolation and immunohistochemical detection of cysteine-rich venom protein-like in turkey (*Meleagris gallopavo*) spermatozoa

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Introduction The cysteine-rich protein identified as cysteine-rich venom protein-like isoform X2 (CRISP-V) is one of the most abundant turkey seminal plasma proteins. However this protein has not been isolated and identified in avian spermatozoa. In mammals, immunofluorescence experiments localized CRISP in the acrosome and tail of capacitated sperm cells and in the equatorial segment of calcium ionophore-induced acrosome reacted cells, suggesting the involvement of CRISP in gamete fusion. In contrast to mammals, there is no information regarding CRISP localization in the avian spermatozoa. In this study, we focused for the first time on the isolation, identification and detection of CRISP in avian spermatozoa.

Methods Semen were obtained from turkeys of the BUT Big-6 line. Polyclonal antibodies against CRISP-V were obtained by rabbit immunization with purified turkey seminal plasma CRISP-V preparation. The spermatozoa CRISP has been isolated by immunoaffinity with anti- CRISP IgG and detected using two-dimensional gel electrophoresis (2DE). The isolated proteins were identified using matrix assisted laser desorption/ionization time of flight/time of flight (MALDI TOF/TOF) mass spectrometer. For immunohistochemical study, sperm smears were fixed and incubated in the presence of anti- CRISP IgG, followed by species-compatibleCy3®-conjugated secondary antibodies. Coverslips were mounted with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) and examined with epifluorescence microscope Nikon Eclipse Ni (Nikon Instech Co.).

Results The sperm proteins bound to the anti-CRISP IgG were identified mainly (80%) as CRISP-V (PREDICTED: cysteine-rich venom protein-like isoform X2 and X1, accession numbers XP_010706464.1 and XP_003204693.1, respectively). Immunofluorescence staining revealed the presence of CRISP in turkey sperm. The signal of strong intensity was localized to the sperm head, that of moderate intensity was localized to the proximal part of the sperm tail, while a weak signal was observed along the rest of the sperm tail. No positive signal for CRISP was found in the sperm midpiece.

Conclusions For the first time, CRISP was identified and localized in the particular structures of avian spermatozoa. Localization of CRISP in the head and tail of spermatozoa suggests its role in sperm-egg interaction and regulation of sperm motility, respectively.

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Major latex protein (CmMLP) isolated from medicinal plant *Chelidonium majus L.* latex is accompanied by alkaloids – a proteomic study

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Introduction Greater Celandine (*Chelidonium majus L.*) is a perennial herbaceous plant from Papaveraceae family. Fresh milky sap (latex), which exudes from the plant, was externally applied to treat warts, papillae and condylomas, caused by human papilloma virus (HPV) infections, however the mechanism of this activity has not been fully elucidated. Our earlier studies showed that the biological activity of *C. majus* milky sap may result not only from the activity of alkaloids but also from proteins. Recent data suggest that defense-related activity of the *C. majus* milky sap against viruses is possibly connected with the presence of highly abundant protein of *C.majus* latex - major latex protein (MLP) (Nawrot R. Curr Protein Pept Sci. 2017;18:864-880). Its presence was reported using proteomic analysis against *C. majus* CDS sequence database after *C. majus* transcriptome sequencing and annotation (Nawrot et al. Plant Physiol Biochem. 2017;112:312-325). The goal of the present study was to isolate the MLP protein from *C. majus* milky sap, and to identify accompanying low-molecular compounds in the fraction samples with the use of bioinformatics approach of 3D modeling and molecular docking.

Methods To isolate MLP proteins from *C. majus* latex, affinity chromatography (HT Heparin, GE Healthcare) was employed. Stained protein bands were excised from the gel and analyzed by LC-ESI-MS/MS in the Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, PAS, Warsaw, Poland against the *C. majus* CDS database (http://webblast.ipk-gatersleben.de/chelidonium/), using MASCOT search engine (www.matrixscience.com). Nonprotein substances (small molecules) associated with proteins of nucleolytic activity were identified by LC-ESI-MS/MS in fractions separated by affinity chromatography on heparin column in Pharmaceutical Research Institute in Warsaw. Tertiary structure prediction and fold-recognition was carried out via the GeneSilico MetaServer gateway. For model evaluation two Model Quality Assessment Programs (MQAPs) were used: MetaMQAP and PROQ. For molecular docking alkaloid structures were retrieved from ZINC database (http://zinc.docking.org/).

Results Mass spectrometry analysis allowed identification of major latex protein (MLP) in protein bands with nucleolytic activity as their main constituents. The bioinformatic search of the database revealed the presence of 18 transcript sequences of different length coding for mlp-like proteins of different sizes with the common core of about 100 amino acids. For further analyses the shortest sequence was chosen coding for the CmMLP protein composed of 147 amino acids with calculated MW 16.77 kDa and theoretical pI 5.88. Bioinformatic analyses showed that the structure of CmMLP from C. majus contains conserved hydrophobic cavity for binding of small molecular compounds. Results showed that there is an association between the protein content in fractions and the presence of selected alkaloids. Moreover, results of molecular docking showed high affinity of several benzylisoquinoline alkaloids to C. majus MLP protein in its hydrophobic cavity with high docking affinity.

Conclusions We discovered a close relationship between CmMLP and low-molecular compounds from *C. majus* milky sap. Hence, low-molecular compounds from the latex might act synergistically with proteins, and proteins might facilitate their transport to cell or play the role of their transporters, what could be related to their biological and antiviral activity.

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Changes of IgG glycosylation in Rheumatoid Arthritis

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Introduction Immunoglobulins, and especially IgGs, are the prime components of the immune defense system. Immunoglobulins are glycosylated, and the glycan chains have a large influence on the mode of action. IgG glycosylation is known to change in disease, and we have explored this in Reumatoid Arthritis (RA). It is known that anti-citrullinated protein antibodies (ACPA) play a pathological role in RA. ACPA active IgGs were isolated from the sera of RA patients, and these were compared to IgGs isolated from healthy persons.

Methods Site-specific glycosylation profiles of IgGs were determined using LC-MS/MS, applying both low and high energy CID. Analysis was based on glycopeptide analysis, which is tolerant for impurities, allowed identification of IgG subclasses, and determination of glycosylation profiles for these subclasses.

Results Various IgG subclasses were separately studied, and their glycosylation patterns compared. Glycosylation profiles determined for IgG were found to be quite variable. Beside Rheumatoid arthritis, IgG glycosylation was found to depend on the age of the person, and varied among the subclasses as well. Surprisingly, glycosylation was more variable for RA patients, than for healthy persons. Fucosylation was found to be the most characteristic feature characterizing RA patients.

Conclusions We found that low energy CID studies were highly useful for glycopeptide analysis. Data evaluation based on a combination of MS/MS and MS1 studies was found to be efficient to deal with complex mixtures, and provided good quantitative markers. On the biological side, variability of glycosylation among RA patients was much larger, than among healthy individuals.

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Proteomic analysis of human follicular fluid from women undergoing in vitro fertilization procedure – combination of FASP and SWATH-MS methodology

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Introduction An oocyte in the ovarian follicle is encompassed by human follicular fluid (hFF) during all stages of development. hFF constitutes a selective filtrate of blood enriched by proteins secreted by the oocyte and accompanying follicle cells, i.e., cumulus and granulosa cells. Due to the intimate relation of the oocyte with the surrounding follicular fluid, analysis of hFF components, e.g., proteins, has been proposed as a non-invasive method of quality assessment of oocytes prior to the in vitro fertilization procedure (IVF).

Methods In this study we combined the FASP (Filter Aided Sample Preparation) technique and SWATH (Sequential Window Acquisition of All Theoretical Fragment Ion Spectra) mass spectrometry method utilizing variable windows to analyze hFF proteins in a quantitative manner. To extend the constructed spectral library, we employed ultrafiltration and affinity chromatography to separate high abundant proteins.

Results We analyzed 20 hFF clinical samples obtained from 4 IVF patients. We were able to identify 234 distinct proteins in spectral library database search. Constructed library allowed us to quantify 160 proteins. We were able to find proteins, which concentrations were at a comparable level in follicles of the same patient – patient-specific proteins; and proteins, which concentrations differed in single follicles of the same patient – follicle-specific proteins. Moreover, we compared obtained quantification results with the medical outcomes of the IVF procedure carried out on oocytes isolated from the tested follicles.

Conclusions Proteome of hFF is highly dependent on a patient, possibly because of the proteins migrated from blood, however it was possible to identify proteins unrelated to individual patients. This outcome allowed us to assume the possibility to establish a set of protein markers of oocyte quality located in the corresponding follicular fluid.

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Molecular heterogeneity of papillary thyroid cancer: comparison of primary tumors and lymph nodes metastases by MALDI-MSI

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Introduction Intratumor heterogeneity results from both genetic heterogeneity of cancer (sub)clones and phenotypic plasticity induced among others by interactions between cancer cells and different local microenvironments. The divergence between a primary tumor and a metastatic outgrowth is an important yet under-researched aspect of intratumor heterogeneity. Here we used a mass spectrometry imaging technique (MSI) to analyze phenotypic heterogeneity in papillary thyroid cancer (PTC) and compared molecular profiles of primary tumors located in the thyroid gland and synchronous metastases of cancer in regional lymph nodes.

Methods FFPE material (cancerous thyroid and at least 3 lymph nodes) from 11 patients with PTC was included and two types of cancer (primary tumor and metastasis) and two types of not cancer (thyroid gland and lymph node) regions of interest (ROIs) were defined by a pathologist. Distribution of tryptic peptides was analyzed by MALDI-TOF MSI. Molecular images were segmented using an unsupervised approach, similarities between spectra from different ROIs were estimated and discriminatory components were detected. In parallel, tryptic peptides were identified using an LC-MS/MS approach in lysates from corresponding tissue samples to enable hypothetical annotation of components detected by MALDI-MSI.

Results Separate clusters generated by unsupervised segmentation of cancer ROIs from all patients dominated in tumor ROIs and metastasis ROIs, indicating molecular differences between both types of cancer regions. Higher inter-patient similarity was observed for spectra from metastasis ROI than from tumor ROI. Intra-patient similarity between metastasis and lymph node ROIs was higher than intra-patient similarity between thyroid and tumor ROIs and between tumor and metastasis ROIs from the same patient (thyroid and lymph node ROIs were the most dissimilar). Most importantly, similarity between tumor and its metastasis from the same patients was lower than similarities among tumors and among metastases from different patients. The largest number of components with significantly different abundances was detected between normal thyroid and lymph node ROIs, yet discriminatory components were detected also for other pairwise compared ROIs. Matching molecular components detected by MSI with peptides identified by LC-MS/MS revealed thyroglobulin being specific for thyroid (and thyroid cancer) and proteins involved in blood and immune-related functions being characteristic for lymph nodes. Furthermore, proteins involved in gland development, chromosome organization and extracellular matrix differentiated between thyroid tumor and normal thyroid. Differences between thyroid tumor and its metastases included proteins involved in organization of the cytoskeleton and chromatin, as well as proteins involved in blood/immunity-related functions (which probably reflected the coexistence of metastatic cancer cells and immune cells in metastasis ROI).

Conclusions A marked molecular difference between primary thyroid cancer and its lymph node metastases was observed using mass spectrometry imaging. We concluded that phenotypical intra-tumor heterogeneity between primary tumor and lymph node metastases from the same patient was higher than inter-tumor heterogeneity between primary tumors from different patients.

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Inflammation as a Driver of Vulvar Squamous Cell Carcinoma Progression.

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Introduction The use of biomarkers to predict disease outcome for squamous cell vulvar carcinoma (VSCC) is obstructed by the limited knowledge on its biology. We aimed to identify protein markers of VSCC that would be indicative of a tumor that is more likely to progress.

Methods VSCC tumors and normal vulvar tissues were studied using iTRAQ analysis. The results obtained for tumor samples of VSCC patients that progressed (progVSCC) were compared to those obtained for samples of patients who were disease-free (d-fVSCC) during follow-up. Differentially expressed proteins (DEPs) were validated in solid tissues and blood samples of patients with VSCC tumors and vulvar premalignant lesions.

Results Immune response was the most over-represented Gene Ontology category for proteins differentially expressed in progVSCC and d-fVSCC tumors. Interestingly, pathway profiling suggested that bacterial infections may give rise to aggressive VSCC phenotypes, forming a novel hypothesis of vulvovaginal microflora disturbances as causes of inflammatory response that contributes to cancer progression. The top DEPs were validated using PRM and immunohistochemistry in VSCC and vulvar precancerous lesions. Correlation of the results with clinical parameters of the enrolled patients indicated that High Mobility Group AT-Hook 2 (HMGA2) and Proteinase 3 (PRTN3) should be considered as potential protein markers for the prediction of VC progression. Plasma and serum analysis with PRM assays and ELISA tests pointed towards PRTN3 and ANCA, antibody against PRTN3, as a potential blood markers of VSCC progression.

Conclusions Increased HMGA2 and PRTN3 tissue abundance and higher PRTN3 blood levels are associated with aggressive phenotype of VSCC. Their assessment holds promise as patient stratification tool.

Proteomes of exosomes from HPV(+) or HPV(-) head and neck cancer cells: differential enrichment in immunoregulatory proteins

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Introduction Human papillomavirus (HPV) is an etiologic factor in head and neck squamous cell carcinoma (HNSCC). HPV(+) cancers respond favorably to therapy potentially due to more robust anti-tumor immune responses. We hypothesized that tumor-derived exosomes (TEX) produced by HPV(+) or HPV(-) HNSCCs differentially modulate anti-tumor immune responses.

Methods Proteomes of exosomes from HPV(+) and HPV(-) HNSCC cell lines were compared in search for proteins putatively involved in the communication with immune system. TEX were isolated from supernatants of HPV(+) (SCC-2, SCC-47, and SCC-90) or HPV(-) (PCI-13 and PCI-30) cells by size exclusion chromatography. A comparison of proteome profiles was performed by high-resolution mass spectrometry. The presence and biological activity of selected immunoregulatory proteins were validated by flow cytometry and co-incubation assays.

Results Exosomes produced by SCC-90 and PCI-30 cells contained 711 proteins, including 80 proteins specific for HPV(+) exosomes and 77 specific for HPV(-) exosomes, associated with similar GO terms such as regulation of cell growth, metabolism, communication, and cellular signaling. Search for proteins localized in the membrane and involved in immune regulation identified a few proteins detected specifically in HPV(+) or HPV(-) exosomes. Only HPV(+) exosomes were enriched in immune effector cell-related CD47 and CD276 antigens; only HPV(-) exosomes contained tumor-protective/growth-promoting antigens, MUC-1 and HLA-DA. Flow cytometry and Western blots confirmed the reciprocal presence/paucity of these proteins in a whole panel of tumor cells and corresponding exosomes.

Conclusions The differential content of protein cargos in HPV(+) and HPV(-) exosomes might contribute to the disparity in immune responses that characterize HPV(+) and HPV(-) HNSCC.

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Quantitative proteomics using fast LC gradients and its application to clinical proteomics.

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Conclusion One of the challenges with clinical proteomics is the ability to analyze hundreds or thousands of patient samples in a meaningful timeframe. Traditional proteomics methods rely on long LC gradients which is not compatible with analyzing large sample cohorts. By combining fast microflow LC gradients (10min) and SWATH acquisition, around 100 clinical samples can be analyzed daily. Examples of SWATH analysis of plasma and FFPE biopsies will be given in this presentation.

From MALDI-IMS lipidomic data to study cell signaling pathways: the importance of the lateral resolution

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Introduction These are definitively exciting times for membrane lipid researchers. Considered for a long time as simple membrane building blocks, the important role these lipids play in cell physiology is steadily being acknowledged. Currently, it is possible to establish the precise lipid composition in a wide variety of biological contexts thanks to the advances in mass spectrometry techniques (MS). However, to fully understand the biological function of each lipid species, we need to know its spatial distribution within the tissue. In the last ten years, the MS field has experienced a profound revolution thanks to the development of imaging MS-based techniques (IMS). In its short lifetime, IMS has proven to be a powerful technique to unravel changes in cell and tissue homeostasis in the context of a disease, but also to understand the role that lipids and proteins play, even in the different cell types present in a given tissue. However, the information that can be withdrawn depends very much on the lateral resolution used during the analysis. Among the several desorption techniques used for IMS, MALDI (matrix-assisted laser desorption/ionization) offers a good compromise between sensibility and lateral resolution.

Methods Using MALDI-IMS to analyze human colon mucosa sections, we have demonstrated not only that the lipidome is cell-type specific but also that it is highly sensitive to any change in the pathophysiological state of the cell. Human colon mucosa consists of a single monolayer of colonocytes that invaginates into the stroma, generating the functional units called crypts. At the bottom of these structures reside the adult stem cells that divide and differentiate into fully mature colonocytes while ascending along the crypt. Thanks to the lateral resolution achieved during the IMS analyses (10 μm/pixel), it is possible to follow, pixel by pixel, the changes occurring in the lipidome along the colon crypt.

Results These analyses revealed how precisely a very specific set of lipids changes along the colon crypt. Impressively, this variation fits a mathematical equation: lipids containing monounsaturated fatty acids increase according to a first-degree equation ($y=ax+b$, $R2=0.95\text{-}0.98$) from the bottom to the top of the crypt, while those containing arachidonic acid decrease according to a logarithmic equation ($y=-\ln(x)+b$, $R2=0.95\text{-}0.98$). Furthermore, using this resolution and based merely on the lipidome, we were able to identify the colonocytes nuclei.

Conclusions While these techniques will help to place membrane lipids in the position they deserve, they also open the black box containing all the unknown regulatory mechanisms accounting for such tailored lipid composition. Currently, we are investigating the changes in gene expression occurring during colonocyte differentiation using a gene expression array (Human Clariom S Pico, Thermo Fisher Scientific). The preliminary analysis of the results reveals a gradual expression of enzymes involved in prostaglandin metabolism along the crypt. Altogether, these results indicate a complex interaction between membrane lipids and prostaglandin metabolism in colonocyte differentiation and tumorigenesis.

Metabolomics studies as a searching tool for therapeutic agents

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Introduction The investigation of cancer cells metabolism, by analytical chemistry methods (NMR and MS), allow monitoring metabolite levels and thus gives insight into cellular metabolism and its perturbations caused by external factors. However, cell growth and differentiation are dynamic processes, and thus may exhibit different metabolic profiles over time. Similarly, animal implanted xenografts may deliver many interesting information about the biochemical occurring reactions in the living organisms. This cancer model may provide the information about the tumour and its interaction with rest of the living system (organism).

Methods The most used way for tracking metabolic pathways, are based on metabolic flux analysis (MFA) performed by NMR and MS methods. These analytical methods use the 13C isotopically enriched substrates (metabolites), which are in medium for cancer cells culturing and introduced intravenously, or via oral route for animal xenograft models.

Results In this presentation, the capabilities of nuclear magnetic resonance NMR and mass spectrometry in MFA will be given basing on cell cultures and mouse cancer xenografts extracts.

Conclusions This method is very powerful in the cancer response analysis for designed therapeutic agents.

Metabolomic signatures discriminate normal human cornea from keratoconus and bullous keratopathy – a pilot GC/MS study

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Introduction Molecular etiology of two pathological states of the human cornea -keratoconus (KC) and bullous keratopathy (BK) remains unclear. The aim of this work was to perform profiling of metabolites and identification of features discriminating these two pathological conditions from the normal cornea.

Methods The combination of gas chromatography and mass spectrometry (GC/MS) techniques has been applied for profiling and identification of metabolites in corneal buttons from 6 healthy controls, 7 KC patients, and 6 BK patients.

Results An untargeted GC/MS-based approach allowed detection of 377 compounds, including 46 identified unique metabolites, whose levels enabled separation of compared groups of samples in unsupervised Hierarchical Cluster Analysis. There were 24 identified metabolites which level discriminated compared groups of samples. Downregulation of glucose, myo-inositol, and cholesterol was observed in both pathological states of the cornea when compared to normal control. Lactic acid was upregulated in KC and BK samples. Moreover, there were 19 identified metabolites whose abundances differentiated between KC and BK samples, which included sorbitol, myo-inositol, and gluconic acid upregulated in KC as well as oxalic acid, succinic acid, urea, cholesterol derivatives and several fatty acids upregulated in BK. Metabolic pathways associated with compounds that discriminated compared groups were involved in oxidative stress, sugar metabolism and energy production as well as lipid and inositol metabolism.

Conclusions Metabolic signatures discriminating keratoconus and bullous keratopathy from normal cornea were revealed. Observed signatures may reflect cellular processes involved in the development of pathological states of the cornea, including oxidative stress, apoptosis, and inflammation.

Systemic effects of radiotherapy and concomitant chemo-radiotherapy in head and neck cancer patients – comparison of serum metabolome profiles

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Introduction Radiotherapy and chemotherapy induce systemic molecular changes that could be detected at the level of bio-fluids. Understanding of how human metabolism is influenced by these treatments is crucial to predict the individual response and adjust personalized therapies. Here we aimed to compare profiles of metabolite in serum of head and neck cancer patients treated with concomitant chemo-radiotherapy (CCRT), radiotherapy alone (RT) or chemotherapy alone (ICT; induction treatment during sequential chemo-radiotherapy).

Methods Serum samples (10 μL) were analyzed by targeted quantitative approach using a combined direct flow injection and liquid chromatography (LC) tandem mass spectrometry (MS/MS) assay AbsoluteIDQ 180 kit (Biocrates, Innsbruck, Austria) according to the manufacturer's protocol. The method combines derivatization and extraction of analytes with the selective mass-spectrometric detection using multiple reaction monitoring and integrated isotope-labeled internal standards absolute quantification. This strategy allows simultaneous quantification of 185 metabolites: 40 amino acids and biogenic amines, 40 acylcarnitines, 90 glycerophospholipids and 15 sphingomyelins.

Results Obtained results showed that CCRT induced the most extensive changes in serum metabolome while chemotherapy alone did not change with statistical significance any measured analyte. Observed statistically significant changes were much common among phospholipids than among small metabolites. The prevailing number of statistically significant changes occurred faster in CCRT than in RT.

Conclusions Concomitant chemoradiotherapy induced the quickest and the most severe systematic changes observed in serum metabolome, which affected mostly its phospholipid component.

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Functional analysis in plants using metabolomic approach

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Introduction Metabolomics became an essential tool for functional analysis in living organisms. Plants produce a large number of small molecules of various structure and abundance. These metabolites play important roles in the growth, development and response of plants to the environment. Plant are also a valuable source of food and energy for human beings. Basically these metabolites are divided into primary and secondary metabolites. Primary metabolites are necessary for the growth and development of the plant, while the secondary metabolites are crucial for the plant to survive under stress conditions by preserving balance with the environment.

Methods The efficiency of plant metabolomics depends largely on used methodology and instrumentation for the comprehensive identification, quantification and localization of each metabolite. In fact, this task is difficult because of the complexity of the various metabolic characteristics and the abundance of molecules. Fortunately, although accurate and exhaustive analysis of the entire metabolome of the biological sample seems currently impossible, methodologies and instrumentation used in plant metabolomics are rapidly developing. Currently, large-scale analysis of highly complex mixtures is possible thanks to a number of integrated technologies and methodologies, such as non-destructive NMR spectroscopy, mass spectrometry (MS) methods, including GC-MS, LC-MS and metabolite fingerprinting using direct infusion high resolution MS.

Conclusions In this presentation we will show the review of plant metabolomics possibilities and future perspectives, as well as we will present examples of plant metabolites screening in use for functional analysis of plant metabolites performed in our laboratory.

Machine Learning in the processing of proteomics data

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Introduction Mass Spectrometry Imaging (MSI), as a powerful tool enabling untargeted investigations into the spatial distribution of molecular species in a variety of biological specimens, brings a tremendous amount of data that require the dedicated algorithms for signal analysis. The single results of low-resolution imaging weights as much as 8GB per sample and can be easily classified as the big data. Machine learning, a branch of artificial intelligence, support knowledge discovery at every stage of MSI signal processing. Mathematical models of the proteomic spectrum, finely tuned by the advanced algorithms, allow for feature extraction leading to the significant dimensionality reduction. Although the obtained number of mass-channel decreases outstandingly, the spatial spectrum redundancy remains untouched. The unsupervised machine learning techniques, mainly clustering algorithms, allow identifying the groups of spectra with a similar molecular profile. The tissue heterogeneity is then characterised by the set of the cluster centroids which can for further data dimensionality reduction. The information on the cluster spatial distribution and its molecular profile can be then utilised, for example, in the process of seeking for cancer tissue signature with the use of the supervised machine learning methods.

Methods We have developed the comprehensive MSI data processing pipeline, consisting of the Gaussian Mixture Model based feature extraction, intelligent stepwise divisive k-means clustering algorithm for tissue heterogeneity modelling and Monte Carlo family procedure for the identification of the most critical features and interactions between the features distinguishing healthy tissue from the tumour region.

Results The system performance will be demonstrated on the MSI proteomic data collected from the head and neck, thyroid and prostate cancer tissue samples.

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The effect of PTMs on proteomic searches

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Introduction Database search tools are commonly used to identify peptides and proteins in proteomics. Several input parameters are available to refine the identification, such as modifications. Theoretically, a longer modification list results in more sensitive peptide identification. The number of considered post-translational modifications (PTMs), however, also increases the search space, which may grow exponentially with the number of modifications. The bigger search space results in less sensitivity and gives worse results. Therefore when using classical search engines (like MASCOT) only very few (maximum 3-4) modifications are recommended. Newer search engines, like "lookup hybrid approach" used by Byonic, are better in this respect and modifications don't increase the search space so dramatically, but it is not straightforward how many modifications should be considered to maximize the number of reliable peptide hits.

Methods In the present talk we have systematically investigated the effect of PTMs on the number of identified proteins using Byonic and MASCOT search engines.

Results Several hundreds of searches were performed to discover general trends and determine when it is worth to consider a PTM in a protein search. In the presentation this topic and general conclusions will be discussed in detail.

Conclusions Our results show that Byonic search engine can handle a lot of modifications and one of its real advantage is to keep search space under control. It makes the usage of even tens of modifications possible without dramatically increasing the search space. In some cases 17 PTMs were needed to maximize the number of reliable peptide hits. Our results also show that as a rule of thumb it is worth to consider a PTM if its occurrence is more than 2.5% in case of Byonic searches.

The pitfalls of sample preparation for MALDI mass spectrometry imaging of tryptic peptides in archival tissue specimens

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Introduction Application of mass spectrometry imaging techniques to analysis of archival tissue specimens in the form of formalin-fixed paraffin-embedded (FFPE) material has opened broad possibilities in research on various diseases, including cancer research. However, the treatment of the resected material and the resulting chemical modification of the tissue imposes certain challenges which need to be faced at the stage of sample preparation.

Methods Typical MALDI mass spectrometry imaging workflow for tryptic peptides consists of tissue preparation, deposition of trypsin and matrix, and image registration. All steps of FFPE tissue sample preparation for MALDI mass spectrometry imaging analysis of proteins were discussed including examples of optimization procedures. Special attention was paid to breast cancer FFPE tissue being a particularly difficult material.

Results Proper adhesion of a tissue section to a glass slide must be maintained during consecutive steps of the whole analytical procedure, otherwise a section can be damaged or even lost. Application of coated glass slides and proper thermal treatment after tissue sectioning improved section adhesion, although heat-induced antigen retrieval (HIAR) process remained the most destructive step. Lowering the temperature of a retrieval solution along with extension of the incubation time helped prevent a section damage while maintaining sufficient yield of protein cross-linking reversal, i.e. resulting in S/N ratio of the most intense peak on a mass spectrum of at least 50.

Conclusions Analysis of FFPE tissue specimens with mass spectrometry imaging techniques enables transition of this methodology from basic science to clinical application. Nevertheless, it is of prime importance that preparation of tissue sections to MSI analysis is well-optimized on every step of the whole analytical procedure, with particular emphasis on heat-induced antigen retrieval process.

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Characteristics of MALDI-imaging on a new dual ion source QTOF with TIMS separation

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Introduction MALDI Imaging has a broad range of applications in Omics research. However, a gap exists between desired mass resolution capabilities and the acquisition speed of current instrumentation. We present initial results from the timsTOF flex system; a timsTOF Pro QTOF mounted with a high-throughput, high spatial resolution MALDI source and stage. The dual source design allows for SpatialOmX applications combining the spatial information from MALDI imaging and in-depth Omics examination of tissue extracts using LC-MS/MS in just one instrument.

Methods A timsTOF Pro was mounted with a MALDI source and 10 kHz smartBeam 3D laser featuring electronically controlled spot positioning and beam profile for imaging. Tissue samples for MALDI-MSI were mounted on conductive glass slides and coated with matrix using standard protocols on a TM Sprayer (HTX Technologies, Chapel Hill, NC, USA). Performance in ESI mode was evaluated by analyzing a commercially available HeLa digest (Pierce) using DDA PASEF approach. For protease activity experiments, stomach sections from a TCEA tumour bearing mouse and a wild type were treated with a protease inhibitor before spraying substance P as a substrate for endogenous proteases. Control sections were not treated with the inhibitor. After digestion, matrix was sprayed with a TM-sprayer and MALDI imaging data acquired in Q-TOF mode. For in-depth proteome analysis, tissue was excised, and extracts analysed by LC-MSMS. MALDI ion mobility imaging experiments were acquired on the system at a mobility resolution of 150 1/K0.

Results High spectral quality MALDI Imaging data could be acquired at a rate of up to 20 pixels/second in both positive and negative mode. A sagittal rat brain section consisting of approximately 370,000 pixels took ~5 hours to measure. Spatial resolution of 20 µm was confirmed by matching ion signals to specific cells and structures in rat brain. In experiments designed to stress the system, 20 hours of image acquisition or ~1.5 million pixels showed no decline in imaging dataset quality and a mass deviation of RMS 2.06 without lock mass. Trapped ion mobility imaging measurements removed isobaric interferences in lipid imaging. Proteomics analysis was used to assess if the dual source design and MALDI Imaging experiments affected LC-MS/MS performance. Injections of 200ng HeLa revealed over 5000 protein groups identified; this figure is maintained over the course of measuring 20 million MALDI pixels. MALDI imaging revealed differential distribution of protease activity depending on tissue background as demonstrated in experiments using a protease inhibitor cocktail to reduce endogenous protease activity. Overall, protease activity could be reduced when applying a protease inhibitor on tissue sections. Differences in protease activity was observed between tumour and non-tumour tissue from inhibited samples while in contrast, lipid signals stayed constant in protease inhibited and not inhibited samples. About 5000 proteins could be identified from tissue extracts of the same samples of which 7 peptidases could be detected as being differentially expressed in tumour samples as compared to wild type samples.

Conclusions The timsTOF fleX allows for fast, high-spatial resolution MALDI acquisition, and robust ESI performance.

A modified Orbitrap™ Tribrid mass spectrometer with real-time search and advanced spectral processing enhances multiplexed proteome coverage and quantification accuracy.

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Introduction Isobaric tagging strategies using Tandem Mass Tags™ (TMT™) are powerful tools for studying how proteins interact and function in biological systems. Up to 11 samples can be multiplexing in a single high-resolution LC/MS experiment to enable state-of-the-art quantitative analysis of peptide and protein abundance. However, co-isolated ion interference can suppress ratio quantification and thereby mask true differences in protein abundance. Multiple methods and hardware solutions help alleviate the negative influence of interfering ions (SPS, FAIMS Pro, statistical analysis). However, all of these solutions come with draw backs. Here we evaluate the benefits of a modified Orbitrap™ Tribrid™ mass spectrometer including real time search capabilities, advanced spectral processing algorithms, and modified hardware to enhance TMT quantification accuracy and proteome coverage.

Methods We present new hardware and software features that enhances proteome coverage and quantification accuracy for Tandem Mass Tags™ (TMT™). To assess the accuracy, precision, and sensitivity of the modified Orbitrap Tribrid mass spectrometer for TMT based quantitation, we utilized the TMT11plex yeast digest standard. Data was collected using Synchronous Precursor Selection (SPS) and with and without a FAIMS Pro™ interface. Raw data files were processed using Thermo Scientific™ Proteome Discoverer™ 2.3 software using the SEQUEST® HT search engine. To improve upon existing SPS methods, we implemented a Real Time Search (RTS) filter between the MS2 and MS3 scans. This feature benefits TMT SPS-MS3 methods in two distinct ways. First, MS3 scans are only triggered if a peptide-spectrum match (PSM) is identified from the preceding MS2. This increased the number of peptides identified with SPS-RTS-MS3 by 30%. Secondly, RTS identifies precursors for MS3 on-line that are generated from the identified peptide. Thus, TMT SPS-RTS-MS3 quantitation can be improved to be 95% isolation interference free. Next, we evaluated a new feature called TurboTMT, powered by the ΦSDM algorithm. ΦSDM is an advanced spectra processing algorithm that increases resolution within a range of the spectrum without requiring a longer transient. Applying ΦSDM specifically to the TMT reporter ions increased the resolution sufficient to baseline resolve TMT isotopologues even when using transients that produce a 30,000 or 15,000 resolving power MS2 scan. ΦSDM increased both the spectral acquisition rate for TMT11plex experiments and the number of identifications for SPS-MS3.

Results Overall, the modified Orbitrap Tribrid mass spectrometer includes unique features such as RTS for TMT SPS-MS3 based quantitation, theoretical precursor isotopic envelope fitting, and TurboTMT, which together allow for intelligent acquisition methods that improve quantitation accuracy, precision, and proteome coverage. Additionally, the modified Orbitrap Tribrid mass spectrometer has an optimized quadrupole that improves ion transmission. It is possible to use narrower isolation widths to improve TMT quantitation accuracy.

Conclusions We present a new hardware and software features that enhances proteome coverage and quantification accuracy for Tandem Mass Tags™ (TMT™).

Dissecting rare disorders: from compartmental proteomics towards affected pathways and identification of putative drugs

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Introduction Neuronal ceroid lipofuscinoses (NCL) are the most common inherited progressive encephalopathies of childhood, characterized by the association of epilepsy, blindness, dementia, motor impairment and premature death. Based on clinical, pathological and molecular criteria, fourteen different forms of NCL have been described, classified with nearly 400 mutations in thirteen genes (http://www.ucl.ac.uk/ncl). NCL5 is a rare late-infantile form of neuronal ceroid lipofuscinosis. The primary function of CLN5 protein and its physiological roles remain unresolved. Emerging evidence points to mitochondrial dysfunction in the onset and progression of several NCL forms, offering new insights into putative biomarkers and shared biological processes.

Methods To investigate the affected pathways, we implemented mitochondria-focused quantitative proteomics approach followed by set of functional validations in CLN5-knockout cell lines and Cln5 knockout mouse brain.

Results These analyses demonstrated an impairment of mitochondrial functions which correlates with disease progression, increased susceptibility to oxidative stress and damaged autophagy machinery consistent with activated mitophagy process as altered functional signatures of NCL5. Analysis of patients' primary cells allows translating organelle-specific proteomic studies into a disease scenario with potential targets for therapeutic intervention. Utilizing bioinformatic filtering, we specifically identified thioredoxin and its inhibitor PX-12 as being proficient in modulating protein overexpression, restoring protein levels and ameliorating oxidative stress, thus indicating putative molecular target for NCL5 therapies.

Conclusions These results demonstrate a functional link amid the impaired cellular respiration and the activation of autophagy or mitophagy pathways, and the ensuing induction of the processes of neuronal injury, which can be targeted pharmaceutically.

Simultaneously looking for molecular signature of human diseases at protein and gene level - power of 'proteo-genomics'.

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Introduction The ability to simultaneously measure multiple secreted proteins and the corresponding gene expression levels from a single sample is valuable for comprehensive analysis of any biological sample. Bottlenecks to traditional immunoassays and gene expression assays include large sample consumption, time consuming experimental procedures, and complex data analysis.

Methods Here, we demonstrate two high-throughput assays measuring both messenger RNA (mRNA) expression using QuantiGene assay and proteins using ProcartaPlex bead-based assay for a single biological sample on a multiplexing platform. Human peripheral blood mononuclear cells (hPBMCs) were treated with lipopolysaccharide (LPS) and harvested at 24 and 72 hours. Samples were assayed with the ProcartaPlex Human Immune Monitoring 65-plex Panel for protein and corresponding mRNA targets on a QuantiGene Human 80-plex Panel.

Results a) Cytokine and chemokine production from hPBMC The ProcartaPlex Human Immune Monitoring 65-plex Panel was chosen to include a number of known cytokines produced by hPBMCs when induced with LPS including interleukin-1β(IL-1β), interleukin 6 (IL6), tumor necrosis factor-α (TNF-α). As expected, at day 1 of LPS stimulation, there was an increase of IL-1β, IL-6, and TNF-α production in comparison to unstimulated day 1. The production of cytokine and chemokine molecules decreased by day 3 with LPS stimulation of hPBMCs. There was no significant change of cytokine and chemokine production from unstimulated day 1 to unstimulated day 3. Other proteins strongly detected included macrophage inflammatory protein-1b/CCL4 (MIP-1β) and neutrophil activating peptide/CXCL5 (ENA-78) in comparison to IL-1β, IL-6, and TNF-α protein levels. b) Comparison between ProcartaPlex assay and QuantiGene Plex assay results To test the fidelity between multiplexing two assays, we examined if proteins levels reflect their corresponding mRNA expression levels within the same sample. A custom QuantiGene Plex panel was developed to contain human genes that may translate into the 65 proteins found in the ProcartaPlex Human Immune Monitoring 65-plex Panel. The QuantiGene 80-Plex panel included sixty-eight encoding mRNA and 12 additional reference genes for data normalization. We found increased mRNA fold levels of markers IL-1β, IL-6, TNF-α, CCL4, and CXCL5 by day 1 and day 3 stimulation over unstimulated day 1. Other large changes included monocyte chemoattractant protein 1/CCL2 (MCP-1), chemokine (C-X-C motif) ligand 1 (CXCL1), Interleukin 8 (IL8), Interleukin 1A (IL1A), macrophage inflammatory protein 1alpha/CCL3 (MIP-1-alpha), and monocyte chemotactic protein 3/CCL7 (MCP3), all showed a greater than 2-fold expression level increase. We also compared protein and gene expression level trends. IL-1β, IL-6, TNF-α, MIP-1b, and ENA-78 protein and gene expression levels showed trend fidelity

Conclusions Interrogation of a single biological sample provided a broad survey of protein levels and gene expression networks. Complementing ProcartaPlex immunoassay with Quantigene mRNA assay provided a more holistic view of the investigation. Often measurement of mRNA expression or protein levels alone may not tell a complete story as mRNA expression levels may not translate to protein. Adding QuantiGene Plex assay to the ProcartaPlex immunoassay is an amenable solution for high level interrogation of both genomic and proteomic parameters for a single biological sample. Such 'proteo-genomic' approach may help screen large number of samples for molecular signature of human diseases at both protein and gene level. (https://doi.org/10.1016/j.ymeth.2019.01.018)

Strong activation of p53 tumor suppressor protein is associated with coordinated upregulation of proteins protecting against infections

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Introduction The p53 tumor suppressor protein is transcriptional regulator, which is best known as an inducer of cell cycle arrest and apoptosis. However, growing body of evidence indicates that it may also play major role in fighting against infections. This function of p53 is poorly studied. We decided to find out if an array of genes coding for proteins of the first line of defense against various viruses and bacteria is regulated in coordinated fashion by p53 protein.

Methods Various cancer cell lines were expose to camptothecin or actinomycin D with nutlin-3a. These anticancer drugs are strong activators of p53 tumor suppressor protein. Using the -omics methods, we identified the genes stimulated by these drugs. The genes regulated in p53-dependent fashion were identified using model cell lines with p53 expression knock-down by shRNAs or knocked-out using CRISPR/Cas9 technology. expression of selected genes at protein level was examined using immunodetection methods.

Results Treatment of cells with strong p53 activators caused at least 10-fold upregulation of approximately 500 genes coding for proteins belonging to several Gene Ontology functional terms. In addition to the expected proapoptotic proteins, the treatment induced expression of several dozens of genes coding for innate immunity proteins. We demonstrated for the first time that several of these genes are regulated in p53-dependent mode. Analyzing the known functions of upregulated proteins we generated several hypotheses concerning unknown functions of p53 tumor suppressor protein.

Conclusions (1) Strong activation of p53 primes the cells to undergo pyroptosis – programmed cell death caused by activation of caspase 1 and secretion of pro-inflammatory cytokines. (2) The p53 protein induces the expression of IFIT1, IFIT2 and IFIT3 proteins, which form multisubunit complex preventing the translation of mRNA molecules produced by some viruses. (3) Judging by the functions of other proteins induced by strong activation of p53, it can be concluded that p53 has the ability to fight infections caused by various pathogens including bacteria and viruses.

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Identifying head-and-neck cancer biomarkers using proteomic analysis of neutrophils and neutrophil-derived exosomes.

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Introduction Neutrophils play an essential role in the controlling of head-and-neck (HNC) tumor development. Depletion of neutrophils or inhibition of their migration during initial phases of tumor growth leads to impaired tumor development. Since neutrophils exhibit a high functional plasticity as they might maintain pro- or anti-tumor properties, regulation of neutrophil activation could lead to the changed predisposition of the host to develop tumors. As example, injection of tumor-promoting neutrophils into tumor-bearing animals leads to acceleration of tumor growth. In contrast, transfer of tumor-suppressive neutrophils efficiently represses tumor development in mice. This project aims to evaluate the exact mechanism that is involved in neutrophil-dependent regulation of tumor development and spread. We hypothesize that extracellular vesicles, mainly exosomes, released from neutrophils might be critical players in this process via mediating communication between tumor-associated neutrophils and target cells present in tumor- or metastatic niche. Therefore, evaluation of protein content of tumor-associated neutrophils and their exosomes could provide useful biomarkers to identify HNC and to predict patient outcome.

Methods Neutrophils from different sources were isolated. In addition, neutrophil-derived exosomes were isolated and purified. Global proteomics analysis of neutrophils and neutrophil-derived exosomes was done to reveal and compare their protein content. Moreover, flow cytometry of exosomes was performed. For functional studies, angiogenic assays and T-cell proliferation assays were performed with exosomes. The material was taken for qPCR and western-blot analysis.

Results We observed significant elevation of pro-angiogenic proteins in tumor-associated neutrophils. These proteins were especially up-regulated in tumor- and pre-metastatic lung neutrophils. Such isolated neutrophils are able to efficiently stimulate angiogenesis, what could be demonstrated in aortic ring assay. Moreover, neutrophils isolated from lymph nodes of tumor-bearing animals show elevated capacity to stimulate T cell proliferation. This capacity decreases along tumor progression. We could observe that neutrophil and their exosome content in tumor bearing hosts is increased, compared to healthy individuals. In parallel, the amount of exosome associated proteins is higher. Using flow cytometry, we confirmed the presence of surface markers involved in the regulation of immune responses, such as ICAM1 or PDL1, on exosomes. We expect that the interaction of such exosomes with T cells can regulate their apoptosis and activity. Analyses of neutrophil-derived exosome protein cargo revealed strongly reduced protein content, as compared to neutrophils. Moreover, neutrophil-derived exosomes seem to express different protein cargo as exosomes isolated from blood of tumor-bearing hosts. Further analyses will reveal proteins enriched in such EVs and processes modulated by them.

Conclusions Neutrophils regulate tumor development and spread mainly due to stimulation of angiogenic processes and the regulation of anti-tumor adaptive immunity. Exosomes are involved in both these processes. More studies are needed to understand mechanisms and pathways that are involved in this process.

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Global proteomics and transcriptomics reveal proteasome machinery target networks and mechanisms of proteasome inhibitor resistance in human cancers

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Introduction Proteasome machinery is the main factor responsible for controlled protein degradation in eukariotic cells. It is upregulated in the majority of human neoplasias of all tissues. Proteasome inhibitors - Bortezomib and Carfilzomib - are successfully used in multiple myeloma and mantle cell lymphoma therapies. In solid tumors these drugs are not efficient in clinics, even though they inhibit the proteasome activity in cancer cell lines in vitro and tumor tissues in vivo. We seek mechanisms which contribute to the resistance of solid tumors to the proteasome inhibition by differential identification of proteasome machinery targets in cancer cell lines vs. multiple myeloma cell lines.

Methods To find proteins and transcripts regulated in response to a proteasome inhibitor, we performed whole cell proteomics analysis and RNA sequencing in four pairs of cell lines of pancreatic, colon and lung cancers and multiple myeloma treated with Carfilzomib.

Results In the whole cell proteomics analyses we identified over 10000 proteins across all eight cell lines, filtered down to 7085 common proteins quantified in each cell line, and found on average 676 of these proteins significantly changing levels in the Carflizomib treated cells vs. the untreated controls. In the RNA sequencing among more than 34000 identified and quantified transcripts in each cell line, over 17000 were coding mRNAs, and on average 7300+ RNAs had significantly different levels in the Carflizomib treated cells vs. the untreated controls. In Carfilzomib-treated multiple myeloma cell lines a 50% decrease of the proteasome activity correlated with on average 50% of a decrease in cell line viability, while in solid tumor cell lines it decreased the viability on average by less than 10%. This suggested that solid tumor cells use mechanisms which compensate the proteasome inhibition to prevent cell death.

Conclusions Hierarchical clustering of large-scale analysis results reveled that either proteome or transcriptome changes upon proteasome inhibition cluster together in cancer cell lines, separately from multiple myeloma. We overlapped in silico analyses of molecular pathways associated with proteins differentially regulated in Carfilzomib-treated cell lines and identified pathways predicted to be affected specifically in cancer-derived or multiple myeloma cell lines as well as common to both neoplasia types. From 20 potentially most significantly regulated pathways we chose 44 genes as candidate key contributors to higher resistance of colon, pancreatic and lung cancer cell lines to Carfilzomib. Knockdown of these genes combined with the proteasome inhibition and its validation in patient-derived material allows us to understand the mechanisms enabling the tumor-derived cells to bypass the proteasome inhibition and to select the best targets for Carfilzomib therapy enhancement in solid tumors.

Mass spectrometric analysis of thoracic aorta alarmin profile in experimental atherosclerosis

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Introduction Atherosclerosis is a chronic multi-factorial inflammatory disease, whose manifestations lead to high levels of morbidity and mortality, especially in the well-developed countries. One of the main risk factors promoting the disease, the high fat diet, was successfully applied in our study to induce experimental hyperlipidemia in New Zeeland white rabbits (NZWR). Alarmins, also known as damage-associated molecular patterns, are molecules released by various cells as a result of a local or systemic insult, molecules that can initiate and perpetuate an inflammatory response. Various high fat diet intervals, corroborated with specific treatments allowed us to mimic human atherosclerotic plaque development and evaluate the associated marker potential of a selected alarmin protein panel in the thoracic aorta.

Methods The animal experiments comprised the following four groups: the control group (C) that received standard chow diet for 12 weeks; the early atherosclerotic group (Ae), which received a high fat diet with 0.5% cholesterol and 5% corn oil, for the first 4 weeks, after which they were switched to a standard diet for another 8 weeks; the stabilized atherosclerotic group (As) which received a high fat diet with 0.5% cholesterol and 5% corn oil for the first 4 weeks, after which they were switched to standard diet together with the simultaneous administration of atorvastatin and PCSK9 inhibitor for the following 8 weeks and the unstable atherosclerotic group (Au), which received the high fat diet for all 12 weeks . After euthanasia, thoracic aorta fragments were collected and suitably processed for mass spectrometric analysis using the Easy nLC II nano-chromatograph coupled to the LTQ Orbitrap Velos Pro ETD mass spectrometer. A shotgun approach was performed, where the top 15 most abundant precursor ions were fragmented through collision induced dissociation. The bioinformatic platform comprised Proteome Discoverer 1.4 for protein inference, Sieve 2.1 for precursor-based relative quantification and Protein Center 3.12 for file management, groups' comparison and statistical analysis.

Results High performance mass spectrometric analysis allowed us to accurately identify 5976 proteins in the C group, 4994 proteins in the Ae group, 4928 in the As group and 5124 in the Au atherosclerotic group. A total of 32 alarmin molecules were identified in all groups, out of which 24 were significantly differentially abundant versus control C in the Ae group, 15 in the As group and 11 in the Au group. 9 alarmin proteins were commonly significantly altered in all groups (vs. C), such as galectin 3, S100A8, calreticulin, Hsp70 or Hsp60.

Conclusions We have identified a panel of differentially abundant alarmin molecules that represents the basis for a selected reaction monitoring mass spectrometric absolute quantification strategy for developing rapid and multiplexed diagnosis and stratification of experimental atherosclerosis.

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Differences in metabolic profiles of primary and metastatic colon cancer cells upon hypoxia and glucose starvation conditions.

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Introduction Metabolomics is widely used to identify the changes in metabolite profile during cell development, growth and survival. Recent studies indicate that cancer cells adjust their metabolism to sustain proliferation in harsh conditions, such as hypoxia and nutrition deficiency, which are characteristic for a tumor microenvironment. Moreover, cancers are capable of aerobic glycolysis – a famous hallmark of cancer cells (the Warburg effect) which is the main source of their energy. Under hypoxia, most of glucose is converted to secretory lactate, which leads to the overuse of glutamine-carbon. The aim of this study was to compare the metabolic profiles from cell culture medium of two colon cancer cell lines derived from a primary tumor (SW480) and lymph node metastasis (SW620) from the same patient in response to hypoxia (1% oxygen) and glucose starvation conditions

Methods Viability of two colon cancer cell lines was measured by MTT assay after 24 and 48 hours in four cell culture conditions (21% oxygen, standard culture medium, 21% oxygen, glucose free medium, 1% oxygen, standard culture medium, 1% oxygen, glucose free medium). Subsequently, cell culture medium was investigated by proton nuclear magnetic resonance based on metabolomic methods

Results Here, we report that various cell culture conditions, especially hypoxia and glucose starvation, alter colon cancer cells metabolism in a different manner regarding to primary and metastatic site cells. 36 metabolites in cell culture medium were significantly changed, in particular arginine, glycine, phenylalanine, glutamine, glutamic acid, aspartic acid, proline, citric and lactic acid.

Conclusions Once the changed oxygen and glucose availability conditions perturb the cell homeostasis, re-building a new coordinative metabolism is required. Therefore, our results reveal how colon primary and metastatic site cells differ in their metabolomics profiles under hypoxia and glucose starvation. Furthermore, our studies show evidence that understanding of cancers metabolism will shed light onto metabolic pathways that are responsible for cancer cell survival in the tumor microenvironment and provide potential targets for colon cancer treatments.

Proteomics validation analysis for ischemic heart disease in Mayak workers

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Introduction Ionising radiation long term exposure produces many effects, which remain to be studied. It is of significant interest to unravel the mechanisms underlying disease caused by radiation exposure. In this study research is conducted on patients prone to exposure, monitored in specific conditions. Previous studies have pointed to pathways and processes linked to ischemic heart disease in Mayak nuclear facility workers. In this work new additional samples are examined for validation of the reported processes.

Methods In this study a group of workers at Mayak PA and unexposed controls who all died of ischemic heart disease has been assessed. Proteins have been quantified using LC-MS/MS spectrometry, which was performed on tissue samples gathered post-mortem from the cardiac left ventricles of the subjects. The data is studied against previously published samples in terms of radiation expose effects, as well as other factors such as age, smoking habits, alcohol consumption, and BMI. Moreover, a small group of additional female samples has been collected and analysed. The pipeline includes deregulated protein analysis, functional analysis and sample clustering.

Results Statistical data analysis presents the pathways and biological processes involved in response to ionising radiation occurring in the heart. A new factor of gender has been considered due to the presence of additional female samples. Previously reported pathways such as PPAR-alpha and oxidative phospholrylation have been reassessed. Moreover, newly found pathways have been examined.

Conclusions The clustering analysis identified key factors participating in chronic radiation exposure response. The use of a custom statistical analysis approach, adapted to the nature of the studied data served as an efficient validation procedure for the discovered proteomic processes.

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Comprehensive analysis of endothelial response to endotoxemia based on plasma profiling of protein biomarkers of endothelial dysfunction in mice; characteristics of endothelial effects of sulodexide

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Introduction Sepsis is a heterogenous disease related to disrupted inflammatory response caused by infection. Numerous studies indicate that endothelial dysfunction plays a key role in sepsis development and contributes to inflammation, loss of barrier function, dysregulated haemostasis and organ injury. Given the significant role of the endothelium in sepsis the aim of this study was 1) to define the changes occurring in vascular endothelium in mice in which endotoxemia was induced by intraperitoneal administration of lipopolysaccharide- LPS (3mg/kg, 5mg/kg, 10 mg/kg) and 2) to determine the endothelial effects of endotoxemia pretreatment with the single subcutaneous administration of sulodexide (40mg/kg).

Methods Endotoxemia-induced organ damage was evaluated on the basis of AST, ALT (liver injury), changes in the concentration of creatinine and urea (kidney injury), while the targeted proteomic analyses were performed using newly developed and validated microLC/MS-MRM method to assess endothelial phenotype. The panel of eighteen biomarkers measured in mouse plasma included: indicators of glycocalyx disruption (SDC-1, ESM-1), endothelial inflammation (sVCAM-1, sICAM-1, sE-sel, sP-sel), endothelial permeability (sFLT-1, Angpt-1, Angpt-2, Tie-2), markers of hemostasis (vWF, t-PA, PAI-1, THBS-1, TAFI) and other endothelial-related molecules (ADM, ADN, ANXA5).

Results In the murine model of endotoxemia the observed changes in the panel of protein biomarkers were dependent on the dose of administered LPS. Interestingly, in case of biomarkers specific for the development of endothelial inflammation (sE-sel, sP-sel, sVCAM-1), increased vascular permeability (sFLT-1, Angpt-2) and hemostasis processes (PAI-1, THBS-1, TAFI) the statistically significant changes have been reported after using lower doses of LPS (3 mg/kg and 5 mg/kg). Furthermore, changes in the response of protein biomarkers specific for glycocalyx disruption (SDC-1, ESM-1) were only visible after administration of LPS at a dose of 10 mg/kg. In addition, the used compound- sulodexide, in the studied murine model of endotoxemia slightly improved the condition of glycocalyx. Moreover, the application of sulodexide increased the number of blood platelets, decreased red blood cell count and hemoglobin.

Conclusions Comprehensive assessment of endothelium-related proteins using biomarker-oriented methodology seems to be the useful tool for better understanding the early and late endothelial response to endotoxemia.

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Proteomic analysis of monocytes in cardiovascular disease related to chronic kidney disease

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Introduction Chronic kidney disease (CKD) is defined as a progressive loss of renal function. The last stage of CKD is defined as the renal failure. Patients with CKD are exposed to strong risk of atherosclerosis and cardiovascular events. The formation of atherosclerosis plaques involves a chronic inflammatory response initiated by endothelial damage and inflammatory active cells. During the early stages of atherosclerosis, blood monocytes are recruited to the intima and subintima and differentiate into foam cells to form early plaques. We assume that molecular mechanism of cardiovascular disease related to CKD shows kind of differences in reference to classical atherosclerosis. To verify this hypothesis comparative proteomic analysis of monocytes of CKD and CVD was performed.

Methods We investigated the alterations in protein accumulation of CD14+ cells in patients with CKD in various stages compared to classical CVD and healthy control. Cells were isolated using immunomagnetic selection and obtained samples were analyzed by label-free proteomic approach using liquid chromatography and mass spectrometry techniques. All proteomic data were subjected to bioinformatic analysis to find physiological pathways disturbed in CKD and CVD during disease progression.

Results Mass spectrometric analysis of monocytes showed that the existence of some differentially expressed proteins between CKD and CVD patients are involved in distinct physiological pathways among other RNA metabolic process, RNA splicing and RNA/DNA binding. Also proteins involved in cell adhesion and leukocyte transendothelial migration were disturbed in different way. In particular, abundances of histone H1, H2A, H3 and H4 were significantly decreased only in CVD. On the other hand, level of vinculin, zyxin and talin-1, proteins related to cell adhesion, was evidently reduced in advanced stage of CKD compared to classical CVD.

Conclusions Obtained results strongly confirm the existence of some differences between molecular mechanisms of progression of atherosclerosis related and non-related to CKD. Further research should focus on detailed targeted analysis of proteins selected in this screening study and validation the selected proteins on the transcript level.

Fractionation and identification of Naja ashei venom proteins

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Introduction Snake venoms are very complex mixtures, which mainly comprise of enzymatic and non-enzymatic proteins and also a number of smaller organic compounds. In the last years, there has been a significant increase in understanding of function and composition of different snake species venoms. As it is known, venoms include various components which exhibit high specificity and low immunogenicity even at low doses. That is the reason why they possess great pharmacological potential. Despite the great variety of snake venom proteins, they are structurally divided only into several superfamilies. In general, proteins within one family show a high degree of similarity between the primary, secondary and tertiary structures, although they often differ in their pharmacological effect. Such high similarities between venom proteins cause that it is very difficult to separate them into single proteins or even individual groups. *Naja ashei* is an African spitting cobra which belongs to the *Elapidae* family of snakes. For a long time, there was no reliable information about the composition and function of this snake venom. Mainly because until 2007, this species was treated just as a different colour form of another spitting cobra – *Naja nigricollis*. Therefore, any information about this venom is valuable and brings hope to find new and interesting molecules with pharmacological potential.

Methods In this work, we conducted two-step chromatographic separation with the use of size exclusion chromatography (SEC) and ion exchange chromatography (IEX). After each separation step, the homogeneity of fraction was tested by gel electrophoresis and with LC-MS/MS.

Results After first chromatographic separation with SEC, we obtained 10 protein fractions with different level of sample complexity. LC-MS/MS analysis showed that at this step, none of the fraction was completely homogenous. These fractions were separated in the next stage on the cation exchanger, which allowed for further reduction of the complexity of the samples and, as a result, obtaining fractions ready for functional analysis.

Conclusions Two-stage separation of venom proteins using size exclusion chromatography in the first stage and ion exchange chromatography in the second stage allowed to separate proteins with different properties and to obtain protein fractions of different complexity. The obtained fractions will be tested for their catalytic and biological properties.

Comparison of protein profile of two fractions of serum nanovesicles

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Introduction Exosomes isolated from blood derivatives like serum and plasma are usually "contaminated" by other components such as lipoproteins and soluble proteins, which affects the quality of further results of proteomics analyses by mass spectrometry. One of the methods of purifying exosomes is size-exclusion chromatography (SEC). SEC is often combined with other preparative methods such as ultracentrifugation. However, such combined methods usually require a large amount of biological material, which reduces their applicability in clinics-oriented studies. Here we aimed to test a simple method of EV isolation from a small amount of human serum (<1 mL) using the micro-SEC standalone for the discovery of vesicle-specific proteins by the untargeted LC-MS/MS shotgun approach.

Methods Vesicles were purified from small volume (0.5 ml) of healthy donors' serum by centrifugation and ultrafiltration, then micro-SEC chromatography on Sepharose CL-2B. Size of the resulting vesicles was assessed by transmission electron microscopy (TEM) and dynamic light scattering (DLS). Protein markers were analyzed by Western blotting. The complete protein profile was analyzed by LC-MS/MS using an Orbitrap mass spectrometer.

Results Two SEC fractions containing nanovesicles were analyzed. The presence of markers typical for exosomes, i.e. CD63, CD81, CD9, and TSG101 was detected in both fractions. However, in both factions the relative proportion of these markers was different. In the fraction enriched in "large" vesicles (about 100 nm), CD63 and CD81 protein levels were proportionally higher. In the fraction enriched in "small" vesicles (about 30 nm) the level of CD9 and TSG101 proteins was proportionally higher. Immuno-gold TEM showed that only the "large" vesicles had on their surface the CD63 and lacked ApoB protein, which indicated that they represented actual exosomes. On the other hand, "small" vesicles were decorated by ApoB but lacked CD63, which indicated that they represented lipoproteins. LC-MS/MS analysis identified proteins encoded by 352 unique genes. Typical serum proteins identified in both fractions of vesicles constituted over 50% of common components. In the fraction enriched in exosomes, 92 proteins were absent in the lipoprotein-enriched fraction. GO analysis of proteins characteristic of the fraction enriched with "large" vesicles showed their relationship with the term "extracellular exosomes" and functions related to the immune system. This function is assigned to exosomes released by immune cells present in the blood.

Conclusions The SEC method used alone does not allow to obtain the fraction of exosomes fully purified from lipoproteins and other proteins contained in the serum. However, the SEC method could be recommended for pilot exploratory studies when a small amount of a serum/plasma specimen is available.

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Characteristic of the proteome cargo of exosomes derived from radiochemo-resistant/sensitive HNSCC cells treated with genotoxic agents

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Introduction Exosomes are the smallest members of extracellular vesicle family that are formed by budding into the endosomal lumen. Their specific cargo include proteins, lipids and nucleic acids. Currently it is very well known that exosomes are important players in intercellular communication. Although their release into extracellular space is an physiological process, they may have also some contributions towards pathological state development and drug resistance mediation. Here in our study we characterized two FaDu clonal cell lines different in sensitivity to cisplatin and irradiation and we examined the proteome content of exosomes released from these cell lines under stress conditions.

Methods Head and neck squamous cell carcinoma cell line FaDu and two clonal cell lines were characterized according to their radiochemo sensitivity using clonogenic and cell viability assays. Then cells were treated with 100 ng/mL of cisplatin or 2 Gy of ionizing radiation and after 24 h the cell culture supernatant was collected. Exosomes were isolated with size exclusion chromatography on Sepharose 2B columns. Exosomes from selected fraction were evaluated using Western blot and electron microscopy. After lysis of exosomes with SDS-based buffer proteins were digested with trypsin following standard FASP digestion protocol. After purification using a C18 StageTips tryptic peptides (1,7 µg) were separated on a reverse phase Acclaim PepMap RSLC nanoViper C18 column (75 µm x 25 cm, 2 µm granulation) using acetonitrile gradient (from 4 to 60%, in 0.1% formic acid) at 30°C and a flow rate of 300 nL/min (for 180 min) by Ultimate 3000 Nano system and analyzed on-line using a Q Exactive Plus mass spectrometer. Data analysis was performed in Protein Discoverer v.1.4. Protein identification was based on reviewed Swiss-Prot human database with a precision tolerance 10 ppm for peptide masses and 0.08 Da for fragment ion masses.

Results All samples were analyzed using untargeted shotgun LC–MS/MS approach. Analysis of 3 different cell lines treated with cisplatin or ionizing radiation allowed to identify over 1500 proteins in total. The mean number of identifications in each sample was around 375 proteins. About 20% of identified proteins were recognized as non-exosomal serum proteins and excluded from analysis. Among proteins upregulated in treated cells were proteins related mainly to extracellular matrix remodeling, histones and proteasome. In exosomes from all tested cell lines both stressors significantly increased level of agrin – protein correlated with aggressiveness and motility of cells in oral cancers. Exosomes derived from more resistant cell line had elevated level of potentially cell protective proteins like Ku70 (after irradiation) or HSP27 (after cisplatin treatment).

Conclusions The protein cargo of exosomes derived from clonal cell lines with elevated or decreased sensitivity to genotoxic agents is significantly different. Presence of proteins with potentially cytoprotective properties indicate the promising direction for the further study of exosomes in context of the development of drug resistance in some types of head and neck cancers.

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Challenges in analysis of metabolome of serum exosomes.

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Introduction Exosomes (EX) are considered as important source of cancer biomarkers in terms of diagnosis, prognosis and treatment response. It's also proved that EX participate in cancer cells - immunological system communication. Proteomics and transcriptomics of exosomes has already been widely investigated, while metabolomics of EX is at its early beginnings. Recent studies revealed disparities in metabolite profile of serum and exosomes between diseased and normal states in many types of cancer. Although area of metabolome studies is a growing field of research, metabolomic-based analysis of serum of head and neck cancer (HNC) patients are less represented in this group. One of the main issues in metabolome of EX studies is to find proper analytical pipeline with high level of detectability of metabolites abundant is small amount of sample. This study intended to gain the knowledge if GC-MS analytical platform will assert sufficient sensitivity to conduct comprehensive untargeted metabolomic profiling of exosomes from limited amount of material which is available after EX isolation.

Methods We collected serum of 10 healthy volunteers and 10 HNC patients (before and after RT). Exosomes were isolated with method combining differential centrifugation and size-exclusion chromatography (SEC). Exosomes were characterized by Western-blotting for EX markers and by transmission electron microscopy (TEM) and dynamic light scattering (DLS) measurement for their size and distribution. Low-molecular metabolites (LMWM) from serum (25 µL) and exosomes samples (500 µL) were extracted with 100% methanol (1:4 v/v). The metabolic profiles were further analyzed using GC-MS approach. Data analysis and statistics were performed on Perseus software platform.

Results By performing GC-MS analysis, we detected about 600 metabolites in serum. We identified 18 metabolites differentiating serum of healthy controls from HNC patients. Among them were: lactic acid, glucuronic acid, arachidonic acid, D-glucose, D-rybose. Analysis of metabolic profiles of exosomes revealed 31 metabolites, in between them was cholesterol trimethylsilyl ether, lactic acid, palmitic acid, stearic acid, arachidonic acid.

Conclusions Metabolites are directly connected with phenotype and, with proper analytical tools, may provide information on disease stage or treatment effect faster than it occurs at other 'omics' levels (proteome, transcriptome, genome). That's why determination of quantitative and qualitative changes in metabolic profiles of exosomes circulating in biofluids is of particular importance. But to be able to demonstrate significant results we need to further validate the instrumental analysis for a sensitive and specific detection and measurement of metabolites in small sample size which exosomes are. Another concern is to improve the techniques of exosomes isolation and sample concentration.

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FANF-ic (Filter Assisted Nuclear Fractionation is cool) a novel approach for organelle fractionation for proteomics

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Introduction Some proteins are hard to observe in crude biological samples (cell or tissue lysates). In such cases samples need to be simplified, to lower the background and spike up the desired signals. If the sought proteins tend to be located in a particular cellular compartment, cellular fractionation might be used. However currently used methods tend to demand specialist devices, to be time consuming (e.g. ultracentrifugation), or to have a high possibility of fractions carry over (detergent dependent fractionation). Here we propose a novel approach for nuclei fractionation. FANF-ic (Filter Assisted Nuclear Fractionation is cool) is a FASP-like method, that utilises centrifugal filtration units (concentrators) together with a detergent dependent membrane lysis.

Methods The MCF-7 cell line was used for all experiments. The sample (usually containing 2 million cells) was first incubated on ice with a buffer containing detergent (Triton X-100 or Igepal), then transferred on a filter membrane and centrifuged. A range of filtration units from different manufactures and with different characteristics were compared. Nuclei clamping (merging), their breakage and osmotic expansion are the main characteristics avoided while selecting the buffer for this step. The quality of the nuclei was controlled with the use of a microscope. The filtrate was assigned as the cytosol fraction. The material detained on the membrane was then incubated with Beznonsase in a buffer with high urea concentration (7 M) for the nuclei lysis and nucleic acids digestion. This step was followed with a second centrifugation concluding with a second filtrate – the nuclear fraction. Both fractions were digested using the FASP method and investigated by mass spectrometry (global analysis, PRM and MRM). Peptides from a set of 16 proteins (usually used in Western blot analysis, for a fraction quality assignment) were picked for the MRM/PRM analysis to indicate the fold enrichment.

Results A method for nuclei separation on a filter unit was devised. Filtration units from Merck Microfree 5.0 μm gave the best performance during fractionation. The initial cell lysis was most efficient in the buffer with a high level of salt (1 M) and 0.5 % of Triton. Such conditions allowed for nuclei fractionation as confirmed by MS.

Conclusions The obtained results show, that organelles might be fractionated with use of centrifugal concentrators. However, the thus proved only for cytosol/nuclear fractions method, might be enhanced to cover other organelles (e.g. mitochondria, ER).

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Comparative classification study for pan-cancer mass spectrometry imaging data

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Introduction The continually increasing amount of biological data causes the need for more advanced algorithms to perform the appropriate analyses. It applies in particular to MALDI-MSI imaging data where mass spectrum is measured for each raster point of the tissue preparation sample. The purpose of this work was to evaluate the performance of the standard classification algorithms in the problem of distinguishing normal tissue from tumour cores for six different types of cancer: head and neck, testicle, intestine, thyroid, prostate and stomach. The cross-cancer signature similarity and heterogeneity was also estimated.

Methods Biological samples for six different cancer types were collected from 10 patients in every group. The cores from tumour and healthy tissue were measured with the use of microarray tissue blocks. Molecular images were acquired by Brucker MALDI-ToF instrument. The preprocessing pipeline included: baseline removal, TIC normalization, spectra alignment and Gaussian mixture modelling (GMM) for peak extraction. The logistic regression with Bayesian information criterion (BIC) and forward feature selection algorithm and support vector machine (SVM) with the LASSO method for signature regularization were used for data classification. The classifiers were constructed for each patient separately and for all patients together within a given cancer type. Multiple random validation procedure with 70/30 split for training and testing set was applied. The classification quality indices as accuracy, sensitivity and specificity were calculated to compare all models.

Results The raw data included 60,617 spectra with 205,312 mass channels ranged from 700 to 3500 Da. After the GMM-based spectrum dimensionality reduction, 2,439 common peaks (features) for further analysis were achieved. The obtained classification accuracy for the LR technique was by average above 95% for all cancer type except for the prostate samples, where it was equal to 65% only. These results were much better than those obtained for SVM classifier. Additionally, the SVM algorithm gave significantly longer signatures compared to the LR method (from 10 to even 100 times longer). The highest accuracy was obtained for testicular cancer and the worst for prostate cancer for both LR and SVM classifiers. The patient molecular heterogeneity caused decreasing the classification accuracy (by 4% by average) when the classifier constructed for all patients was compared to the classifiers built for every patient individually. Although the patient-specific signatures seemed to included differet peaks, there were always among them a few features highly correlated ($r>0.8$).

Conclusions Studies have shown that regardless of the cancer types, it is possible to distinguish between healthy and cancerous tissue with high accuracy. The use of an appropriate classification algorithm in combination with a robust feature selection method plays a crucial role in obtaining reliable and accurate classification results.

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Can the spatial distribution in MSI support the identification of the isotopic envelope?

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Introduction One of the widely used technique in proteome studies is mass spectrometry for which the identification of isotopes plays a crucial role in accurate protein annotation. Nowadays exist a plethora of algorithms trying to cope with identification of the isotopic envelopes, but they are either highly-dependent of the experiment type (MALDI, ESI) or dedicated to the specific kind of molecules (lipids or peptides). Several existing algorithms for MALDI spectrum deisotoping return unsatisfactory results – a very few isotopic envelopes are found in total. According to that, there is a strong need to create a universal method for isotopic envelope identification, which can be used for all kind of molecules and many experimental platforms. We propose a unique approach combining the fuzzy inference and information on the molecule spatial distribution across the tissue sample.

Methods A novel algorithm consists of two steps. The first one is based on Mamdani-Assilan fuzzy-inference system, while the second one focuses on the spatial distribution of a molecule. The first step of the algorithm identifies the candidate peaks – peaks that are considered to be a part of an isotopic envelope. At the second step of data analysis, the heatmaps presenting intensities of peaks for particular m/z value on the whole tissue sample are taken into consideration. To evaluate the spatial distribution similarity between the candidate peaks, the chosen image texture metrics applied to the differential image are used. Only peaks with no significant differences in their spatial distribution are classified as belonging to the same isotopic envelope. The developed two-step algorithm was tested and validated on MALDI MSI data collected from the patients with head and neck cancer.

Results The results obtained demonstrate that both the non-overlapping and overlapping isotopic envelopes can be easily detected with high accuracy. The exemplary peptide MALDI spectrum reduced its dimensionality from the original more than 200,000 mass channels to 2,328 Gaussian peaks. After the first step of the algorithm, 492 isotopic envelopes were detected with 1,249 peaks included. The longest envelope consisted of 6 peaks. After performing the second step of the algorithm, 436 isotopic envelopes have been left (1005 peaks). The remaining peak sequences did not fulfil the requirement on similarity in spatial distribution. The identified envelopes were validated by the experienced spectrometrist.

Conclusions The proposed algorithm is a unique approach to the deisotoping problem which detection level outperforms the commonly used methods. As being independent of the reference databases, the system can be successfully used to process spectra from different species and experimental platforms.

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Biostatistical approaches to assess molecular heterogeneity between and within histologically-defined regions of interest in tissue samples imaged by MALDI-MSI

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Introduction Mass spectrometry imaging (MSI) detects, characterizes and visualizes molecular profile of tissue samples. A key advantage of MSI is its spatial registration, which allows to conduct comparative analyses between histologically-defined regions of tissue samples (region of interest, ROI) or analyze phenotypic heterogeneity within particular tissue region. Here we showed a few selected biostatistical approaches that can be applied for successful performance of both types of analyses.

Methods Normalized dot-product of two mass spectra (similarity index) was calculated to assess similarity of an examined pair of spectra. Spectra were labelled according to their location in tissue specimens and within one of previously defined, distinct histopathological tissue region. Pairwise similarity index was calculated between or within spectra from created spectra subsets in three different manners: (i) within the same type of ROI and within the same specimen (intra-patient intra-tissue similarity), (ii) between different types of ROI within the same specimen (intra-patient inter-tissue similarity), (iii) within the same type of ROI among different specimens (inter-patient intra-tissue similarity). Populations of computed similarity values were plotted as cumulative distribution functions to visualize similarities between spectra among and between analyzed ROIs. An effect size analysis was applied to indicate discriminatory molecular components and unsupervised spectra clustering was performed for exploratory data analysis to reveal possible molecular differences between ROIs based on number and size of clusters.

Results Imaging mass spectrometry dataset of 11 tissue samples derived from postoperative material of patients with papillary thyroid cancer was comprehensively analyzed to assess molecular differences between four types of histologically-defined ROIs (primary tumor, metastasis and two types of normal tissue: thyroid gland and lymph node). Pairwise similarity index was calculated to evaluate similarities of molecular profiles between different ROIs. The graphs of cumulative distribution functions evaluated at similarity values revealed the highest inter-patient heterogeneity for normal thyroid tissue and the lowest heterogeneity for normal lymph nodes. Furthermore, higher differences between primary tumor and its metastasis from the same patients in comparison to differences among tumors and among metastases from different patients were disclosed.

Conclusions A few biostatistical approaches and selected unsupervised learning techniques were successfully applied to assess molecular differences between histologically-defined regions of tissue samples imaged by MALDI-MSI. Particularly, the similarity approach allowed us to efficiently assess intra-patient tissue heterogeneity vs. inter-patient differences.

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Tumor-derived exosomes influence the activity of neutrophils in head-and-neck cancer.

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Introduction Tumor-derived exosomes (TEX) can be responsible for the reprogramming of immune cells. We previously showed that neutrophils can influence tumor angiogenesis and regulate anti-tumor immune responses. Accumulation of neutrophils in the tumors of patients diagnosed with head and neck cancer (HNC) was shown to correlate with a poor prognosis. However, it is not entirely clear how tumorigenic activity of neutrophils is regulated. We hypothesized that tumor-derived exosomes play a role in this process. We aimed to understand the role of TEX in the regulation of tumorigenic activity of neutrophils. Proteins associated with TEX should be identified to establish biomarkers that can facilitate patient diagnosis/prognosis, but also to identify molecules that could be targeted therapeutically.

Methods Exosomes were isolated and purified using size exclusion chromatography (SEC). Global proteomics analysis of exosomes was done to reveal their protein content using mass spectrometry approach. In addition, flow cytometry analysis of exosomes was performed. For functional studies, polymorphonuclear neutrophils (PMNs) were isolated from healthy donors and incubated with exosomes from HNC patients and healthy controls. The activity and survival of neutrophils was monitored. To confirm the presence of exosomes and validate the results, qPCR and western-blot analysis was performed.

Results We observed significant elevation of exosome content in HNC patient blood, compared to healthy individuals. Moreover, the amount of exosome-associated proteins was higher in patients. Using flow cytometry, we confirmed the presence of surface markers involved in the regulation of immune responses, such as ICAM1 or PDL1, on exosomes. We expect that the interaction of such exosomes with neutrophils supports their apoptosis and inhibits their cross-presentation activity. This is in agreement with the results showing elevated apoptosis of neutrophils after their incubation with patient exosomes, as compared to healthy exosomes. Of note, we observed that after incubation of TEX with neutrophils, the majority of exosomes was internalized.

Conclusions Our preliminary studies show that TEX can modulate the functionality of neutrophils in cancer. More studies are needed to understand mechanisms that are involved in this process. Targeted inhibition of TEX-mediated stimulation of pro-tumor neutrophils might be a novel therapeutic approach in HNC treatment.

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Short-term storage of carp semen triggers oxidation of spermatozoa proteins

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Introduction In aquaculture, refrigerated storage is a simple and inexpensive procedure often needed to deal with logistics of large-scale hatchery operations. Most fish spermatozoa have the advantage of being quiescent in the seminal plasma. Therefore, no energy is consumed for motility. This characteristic makes them most suitable for short-term storage. However, semen storage in chilling conditions (+4oC) causes the formation of free reactive oxygen species (ROS), resulting in decreasing of cell membrane stability, impaired functioning of the mitochondria and DNA fragmentation leading to decrease in sperm quality. The proteins that are oxidized during semen storage are unknown at present. The goal of this study was to investigate the oxidative modifications (measured as carbonylation level changes) of carp spermatozoa proteins triggered by the short term storage.

Methods The carp semen samples (n=6) of high quality (percentage of motile spermatozoa > 80% and a sperm concentration of at least 19 × 109sperm/ml) were diluted 10-fold in the immobilizing buffer (94 mM NaCl, 27 mM KCl, 50mM glycine, 15mM Tris–HCl, pH 7.5 supplemented with 200 U/ml penicillin) and stored for 120 h at 4^{O}C. All analyses were performed within 3 experimental groups of carp semen (n=6 in each group): fresh-diluted, stored for 48h and 120h. Flow cytometry (Muse® Cell Analyzer) was used to evaluate changes in ROS level of spermatozoa. The spermatozoa proteins that were specifically carbonylated were evaluated using DNPH labeling of carbonyl groups followed by two-dimensional electrophoresis, western blot (2D-oxyblot) and matrix-assisted laser desorption/ionization time-of-flight/time-of-flight spectrometry. Data were analyzed using one-way ANOVA and post-hoc Tukey's test at a significance level of 0.05 using GraphPad Prism software.

Results A steady increase in the sperm undergoing oxidative stress was observed during the storage; 2- and 5-fold increase of ROS content occurred after 48 and 120h, respectively. Short-term storage of semen resulted in increasing of carbonylation of 13 protein spots of spermatozoa, corresponding to 10 proteins, after 120h of storage. Three of the identified proteins occurred in two proteoforms (glutathione-S-transferase rho-GST, aldose reductase-AKR1B1 and radial spoke head protein 14-RSPH14). The majority of proteins were classified according to GO cellular function as proteins with oxidoreductase activity (GST, AKR1B10, NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial-NDUFS1 and glyceraldehyde 3-phosphate dehydrogenase-GAPDH), while GO biological process revealed their involvement in cellular detoxification (GST, AKR1B10), protein folding (transitional endoplasmic reticulum ATPase, heat shock protein A5, peptidyl-prolyl cis-trans isomerase), axoneme assembly (RSPH14, RSPH9, septin 5), energy production (NDUFS1, GAPDH) and transmembrane protein (sarcolemmal membrane associated protein 1).

Conclusions In conclusion, our results for the first time have shown that short-term storage induces the oxidation of carp sperm proteins involved mainly in cellular response to stress and sperm motility. The oxidative damage of these proteins may result in decrease of sperm quality after storage. Our results contribute to the knowledge concerning short-term storage injuries and to further development of a modified storage procedure aimed at minimizing oxidative damage of carp sperm proteins.

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Differences in sperm protein abundance and carbonylation level caused by sex-sorting of bull spermatozoa

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Introduction Sex-sorted semen is used especially in dairy cattle breeding, in order to increase the number of female calves. The technology of sperm sorting involves staining the male and female gamete DNA with fluorochrome and separating it by flow cytometry. Despite many benefits, the use of sexed semen is limited, mainly due to the high price and reduced fertilizing capacity caused by structural and functional sperm damage induced by the conditions of the sorting process (high pressure, unfavorable temperature, exposure to laser light, staining with fluorochrome). The aim of this study was to examine the effect of sperm sorting on the proteome profiles, protein carbonylation level, as well as on overall sperm quality parameters and acrosome integrity.

Methods The research material consisted of commercially available cryopreserved bull semen in the non-sexed and sexed variant (spermatozoa with X chromosome), (n = 8 in each group). Computer-Assisted Sperm analysis and flow cytometry were used to evaluate sperm quality parameters: motility, viability, ROS level and acrosome integrity. To assess differences in sperm proteomes, extracted protein samples were labelled with CyDye DIGE Fluor minimal dyes and separated by 2D SDS-PAGE. The level of protein oxidation was determined by labeling the protein carbonyl groups with dinitrophenylhydrazine (DNPH) and its detection with anti-DNPH antibodies using Western blotting, in conjunction with two-dimensional electrophoresis (2D-oxyblot). SameSpots software (Totallab, Newcastle, UK) was used to match and analyze the obtained protein patterns. Spots which showed changes ($P < 0.05$) in intensity on 2D-DIGE gels or carbonylation level on 2D-oxyblots were identified with MALDI TOF/TOF mass spectrometry (Bruker Daltonics) and classified into functional groups using STRING (https://string-db.org/).

Results Sex sorting of bull spermatozoa caused a 66.7% reduction of sperm viability, 67.7% reduction of sperm motility and 32.7% decrease in acrosome integrity. Our results showed that 68 protein spots corresponding to 52 sperm proteins underwent significant ($P < 0.05$) changes in abundance due to sex sorting. The identified proteins were localized mainly in mitochondria and flagella and were involved in energy metabolism, nucleotide metabolism and redox processes. Additionally, our results showed that sex-sorting caused increased carbonylation of 3 isoforms of outer dense fiber protein of a sperm tail, and decreased carbonylation of acrosin-binding proteins.

Conclusions The results suggest that mitochondria and flagella are the main sites of injury due to sex-sorting process, which may contribute to the reduced percentage of motile spermatozoa and disturbances in movement trajectory. Sex-sorting caused also disintegration of an acrosome. The obtained results may contribute to the optimization of the methods of cattle breeding in order to reduce damage within proteins and sperm cell membranes during sperm sorting.

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Towards targeting protein disulfide isomerase (PDI) isoforms to treat cancer

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Introduction Protein disulfide isomerases (PDI) are one of the newly proposed anti-cancer targets. Overexpression of the PDIs in cancer cells is correlated with invasiveness and metastasis but it is not clear which of the isoforms of PDIs are mainly responsible for invasiveness, as the PDI inhibitors reported to suppress tumor growth (PACMA31) are not selective. Among 21 defined PDI isoforms PDIA1, 3 and 17 (anterior gradient 2) seem to be the most relevant in the cancer progression as oncoprotein stimulating proliferation and promoting metastasis. To better characterize the 'PDIome' of various cancer cell lines, the aim of the studies was to investigate the repertoire of the PDI isoforms in breast, colon, urinary/bladder, prostate, lung and ovarian cancer.

Methods The studies were performed using breast (MDA-MB-231, 67NR, MCF-7, 4T1, T47D), colon (LOVO, HT-29, CaCO2), urinary/bladder (RT-112, 5637, UMUC-3), prostate (LNCaP, PC-3, Du-145, TRAMP C2, TRAMP C1), lung (LLC, NCI-H1703, A549, A427, NCI-H358, NCI-H1299) and ovarian cancer (A2780) with the aid of non-targeted, label-free LC-MS/MS analysis.

Results PDI repertoire in investigated cell line lysates was rich and included the following PDI isoforms: PDIA1, 3, 4, 5, 6, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and was specific for the cell type. PDIs were also detected in medium from cancer cell lines (PDIA1, 3, 4, 6, 10, 17). The majority of the investigated cell lines highly expressed the following PDI isoforms: 1, 3, 4, 6 and 9 with PDIA1 and 3 as the most abundant both in the cell and in the medium from cancer cells. Interestingly, cancer cell lines characterized by high invasiveness and metastatic potential (e.g. MCF-7, PC-3, HT-29) expressed high amounts of PDIA17, which was not present in non-malignant cell lines. The estradiol/testosterone stimulation greatly upregulated its expression however only in PDIA17 highly expressing cell lines, barely in none or low-expressing cell lines (e.g. MDA-MB-231, CaCO2, LNCaP).

Conclusions PDIA1 and PDIA3 represent the most abundant isoform of PDI in multiple cancer cells lines and they are also released extracellularly. PDIA17 expression seem to be more cell-specific and related to cancer cell malignancy. Accordingly, extracellular targeting of PDIA1 and PDIA3 may represent a non-specific to cancer type approach to target cancer, while targeting intracellular PDIA17 may represent a cancer-cell specific approach. Further studies are needed to test this hypothesis.

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TAILS based proteomic analysis of Zika virus infection.

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Introduction ZIKV is an arbovirus transmitted by *Aedes* sp. mosquito. In recent years, significant and rapid spread of this virus has been observed in areas where previously was not endemic. Based on the global research results, which proved that the viral NS2B-NS3 protease plays crucial role in the viral infection cycle, activity of viral protease NS3 has been analyzed using proteomic approach.

Methods In our research, 293T cell model was applied. 293T cells with transfected active NS3 protease were compared to control 293T cells with inactive form of this protease (point mutation: S135 → A). The cell lysates were suitably prepared for further proteomic study. NS3 protease study is still a challenging task which is often hampered by the lack of efficient analysis methods. The approach combining application of TAILS with an amine-reactive high molecular weight polymer (HPG-ALD) allows for analyzing labeled N-termini peptides by tandem mass spectrometry to discriminate the products of the NS3 protease cleavage. Bioinformatics tools allowed for identification of proteins and enabled the comparison of protein expression between the samples.

Results TAILS approach was applied to identify the potential targets for NS3 protease. Among all the identified proteins, the most important are proteins responsible for infection and inflammation processes, involved in transcriptional regulation and apoptosis.

Conclusions The presented results might be useful for the explanation of action of the NS3 protease in 293T cells, might have important meaning in viral infection study.

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Identification of protein changes in the blood plasma of lung cancer patients subjected to chemotherapy using a 2D-DIGE approach

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Introduction Lung cancer is the most common cancer in the world and a leading disease responsible for most cancer-related mortality worldwide. Late diagnosis is a significant factor contributing to poor lung cancer prognosis. For this reason, the development of biomarkers for effective prognosis is of utmost importance.

Methods In this study, we performed a comparative analysis of blood plasma samples obtained from non-small cell lung cancer (NSCLC) patients before and after second cycle of chemotherapy. The control group was selected from patients with non-cancerous lung diseases. Moreover, we sought to determine whether particular lung cancers (adenocarcinoma - ADC or squamous cell carcinoma - SCC) are related to differences in blood plasma protein abundance. Methods: Informed consent was obtained from eight control patients and eight NSCLC patients (four diagnosed with SCC and four with ADC) before and after chemotherapy. Blood plasma was depleted using albumin and IgG depletion spin traps (GE Healthcare). Proteins of the depleted blood plasma were precipitated using a 2-D Clean-up Kit, labelled with CyDye DIGE Fluor minimal dyes, loaded on 24-cm DryStrips, pH 3-10 NL separated by isoelectric focusing and SDS-PAGE (GE Healthcare). The gels were scanned and analysed with DeCyder software (GE Healthcare). Protein spots indicated by statistical analysis were identified by mass spectrometry (Autoflex Speed; Bruker Daltonics).

Results Analyses of gels revealed significant changes in proteins and/or their proteoforms between control patients and lung cancer patients, both before and after chemotherapy Most of identified proteins were related to inflammation, especially acute phase proteins (APPs). Control patients vs lung cancer patients before chemotherapy: Control plasma was characterized by a higher abundance of complement C3, coagulation factor XII, fibrinogen β chain, prothrombin, gelsolin, proapolipoprotein, inter-α-globulin inhibitor C4, α-2-HS-glycoprotein, α-2-macroglobulin and protein SP40,40. The plasma of lung cancer patients before chemotherapy was characterized by a higher abundance of fibrinogen α chain, zinc-α-glycoprotein, haptoglobin and orsomucoid 1. Control patients vs lung cancer patients after chemotherapy: The control plasma contained higher abundances of proapolipoprotein, coagulation factor XIII, clusterin, fibrinogen α chain, haemoglobin beta chain, inter-α-trypsin inhibitor, α-2-macroglobulin, protein SP40,40, transferrin and serotransferrin X1. The plasma of lung cancer patients after chemotherapy was characterised by a higher abundance of apolipoprotein A-IV, fibrinogen α chain, hemopexin, haptoglobin, α-2-macroglobulin b, immunoglobulin heavy chain and leucine-rich α-2 glycoprotein. Comparative analysis of ADC and SCC patients: The plasma of SCC patients before chemotherapy was characterized by a higher abundance of vitronectin, coagulation factor XIII, plasminogen and gelsolin compared to ADC patients.

Conclusions Our results emphasize the potential role of inflammatory proteins as biomarkers of lung cancer. The presence of numerous proteoforms for several biomarkers warrants an investigation of the relationship between PTMs and cancer. The variable expression of APPs can be potentially used for profiling lung cancer. Chemotherapy was accompanied by changes in proteins likely reflect disturbances in iron turnover (iron deficiency) after chemotherapy-induced anaemia. Significant changes in plasma between ADC and SCC patients were also revealed, suggesting a possible role of plasma vitronectin as a potential marker of SCC.

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Acquiring potential for motility is accompanied by profound changes in sperm proteome

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Introduction Testicular spermatozoa of salmonid fish are immature and characterized by no or low potential for motility. Such potential can be acquired through the incubation of sperm suspensions in solutions mimicking seminal plasma with high pH (artificial seminal plasma-ASP). It is unknown at present if proteins are involved in rainbow trout sperm maturation and acquiring the sperm motility. The objective of this study was to investigate the effect of 2 h incubation of sperm in ASP on the sperm motility parameters and viability, and the identification of proteins and phosphoproteins involved in the maturation of rainbow trout sperm by 2D difference gel electrophoresis (2D-DIGE).

Methods Semen collected from each individual (n=6) was divided into two parts: 1) fresh semen and 2) semen diluted at a ratio of 1:9 in ASP (100 mM NaCl, 40 mM KCl, 3 mM CaCl2, 1.5 mM MgCl2 and 50 mM Tris, pH 8.5) and incubated 2 hours. Sperm motility parameters was measured by Computer-Assisted Sperm analysis, the viability were measured using a Muse Cell Analyzer (Millipore, USA).Samples were precipitated using a 2-D Clean-up Kit, labelled with CyDye DIGE Fluor minimal dyes, separated by isoelectric focusing and SDS-PAGE. The gels were scanned and analysed with DeCyder software (GE Healthcare). Protein spots indicated by statistical analysis were identified by mass spectrometry (Bruker Daltonics). Phosphoprotein staining using Pro-Q Diamond Phosphoprotein (Invitrogen, USA).

Results The 2 h incubation of sperm in ASP increased percentage of sperm motility (from 28 to 56%), curvilinear velocity (from 115 to 145 μm s-1), straight-line velocity (from 16 to 25 μm s-1), the amplitude of lateral head displacement (from 13 to 19 μm) and average path velocity (from 50 to 72 μm s-1), however did not affect sperm viability. The incubation of sperm in ASP influenced the expression of 126 sperm protein spots in comparison to fresh sperm ($p < 0.05$ with FDR correction), out of which 120 spots were identified by MALDI-TOF/ TOF. For 120 proteins differentially expressed in male sperm, the small molecule metabolic process, amino acid metabolism and tricarboxylic acid (TCA) metabolic process as biological process were found (http://bioinformatics.sdstate.edu/go/). Additionally, microtubule-based process was assigned by STRING (https://string-db.org/). A total of 29 phosphoproteins that changed in response to maturation were classified as structural and calcium ion binding proteins as well as proteins involved in metabolism and protein folding.

Conclusions Our results indicated that maturation process of male spermatozoa involves the changes mainly in proteins involved in TCA cycle, amino acids metabolism, flagella, cytoskeleton organization and are putatively related to sperm energy metabolism and sperm motility. Our results clearly indicate the changes in sperm protein phosphorylation pattern in relation to maturation. The mechanisms of described protein changes likely involve post-translational modifications of their structure. The identified proteins could be biomarkers of mature spermatozoa and could be strictly evaluated in subsequent studies to verify their specific functions in spermatozoa. Such information provides new contributions to understanding the maturation process of the salmonid fish male gametes involved in the development of male fertility.

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Comparative proteomic analysis of high quality eggs obtained from wild-caught and domesticated (*Sander lucioperca*)

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Introduction Domestication process is indispensable element of successful expansion of intensive aquaculture of pikeperch, Sander lucioperca, highly valued freshwater fish species. Fish cultured in intensive indoor aquaculture systems are exposed throughout their life to the artificial conditions being highly different from the natural environment. This is the most probable cause of lowered and/or highly variable egg quality observed in intensively cultured fish. However, it until now little is known on molecular profile of eggs of domesticated fish and their comparison with the ones obtained from wild-caught specimens. Information on such a molecular differences would allow us to understand the processes involved in domestication and selection. In this study fluorescent two-dimensional electrophoresis (2-DIGE) followed by MALDI-TOF/TOF identification was applied to compare the protein profile of high quality eggs collected from wild and domesticated pikeperch populations during the controlled reproduction.

Methods Eggs from domesticated (n=6) and wild pikeperch females (n=6) were homogenized on ice in ND-RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% TritonX-100, 1% CHAPS, 2 mM NaF, 2 mM Na3VO4), sonicated and precipitated using 2-D Clean-up kit (GE Healthcare). Proteins were labelled with CyDye DIGE Fluor minimal dyes, separated by isoelectric focusing and SDS-PAGE. The gels were scanned and analysed with DeCyder software (GE Healthcare). Protein spots indicated by statistical analysis were identified by mass spectrometry (Bruker Daltonics).

Results Comparative 2D-DIGE analysis revealed 89 spots with a significant difference ($p \leq 0.05$) in their relative abundance between eggs of wild-caught and domesticated pikeperch, out of which 66 were identified using mass spectrometry MALDI-TOF/TOF. The 27 spots were more abundant in wild-caught pikeperch eggs and 39 spots were enriched in eggs collected from domesticated individuals. The identified proteins with a change in abundance were classified according to the "molecular function", which revealed that the largest percentage of proteins (50%) are implicated in catalytic activity (36% transferases, 26% oxidoreductases, 16% lyases) followed by binding (36%) (mostly 35% protein, 19% lipid and 19% small molecule binding). The differentially expressed proteins were also analyzed using the STRING database. Metabolic pathways, carbon metabolism, glycolysis/gluconeogenesis and biosynthesis of amino acids were indicated by STRING as the top KEEG pathways.

Conclusions Our results demonstrate the influence of indoor aquaculture on pikeperch egg proteome. This study indicates that eggs collected from wild-caught population of pikeperch differ from that of a domesticated population in terms of abundance of metabolic and binding proteins. Those proteins are likely involved in the formation and metabolism of egg yolk providing the energy reserve for nourishment of the developing embryos. The identification of specific proteins exhibiting a differential abundance in eggs of varying quality will contribute to the identification of the mechanisms determining egg quality defects.

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Study of cysteine proteases using fluorescent probes

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Introduction High sensitivity, versatility and quantitative capabilities of fluorescent probes caused the extensive usage of these compounds in biological research. By attaching such probes to a target molecule, they can serve as a marker in biological experiments. For instance, use of tagged inhibitors allows for identification of enzymes, selectively blocked by these compounds. In the present work, novel fluorescent inhibitors of cysteine proteases and their potential use in quantitation of the active level of the enzyme, have been described.

Methods Fluorescent inhibitors were obrained in the standard Fmoc solid-phase synthesis. The structure of a synthesized compounds was confirmed by MALDI-TOF/TOF MS. To purify the obtained peptides, C18 RP-HPLC was used. Staphopain c was applied as a model cysteine proteases in the enzymatic tests.

Results Using several separation methods, including PAGE and western blotting, we confirmed usefulness of the synthesized inhibitors in quantitative analysis of the enzyme activity level. It has been also checked, that slight changes of inhibitor's structure can significantly influence on the ability to quantitatively measure of selected enzyme activity.

Conclusions Owing to the high interest of our group in investigations of proteases that convert neuropeptides, this research is a part of our long-term studies aimed at identification and isolation of a family of endopeptidases converting dynorphins to shorter, but still bioactive fragments.

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Comparative transcriptome analysis of the interaction between *E.coli* and IPEC-1 treated with exopolysaccharides isolated from *Lactobacillus reuteri* DSM17938

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Introduction Exopolysaccharides (EPS) produced by lactobacilli possess modulatory effects on the gut mucosal immune system. The objective of this study was to investigate comprehensive picture of immunomodulatory effect of EPS on IPEC-1 cell during the enterotoxigenic E.coli (ETEC) infection.

Methods EPS was isolated and purified from L. reuteri DSM179L26 according to Kšonžeková et al. (2016). IPEC-1 cell line was grown in IPEC-1 medium at 37°C in 95% humidity and 5% CO2. At 70% confluency, cells were washed with sterile PBS, EPS was added at 0.1 mg/ml concentrations and incubated for 4 h. Cells were then challenged with ETEC (multiplicity of infection 50:1) without refreshing of medium for 45 min. Subsequently, the monolayers were washed with sterile PBS and stored at -20 °C until quantification. As a control cells were kept without any pre-treatment and infection or no EPS pretreatment was performed before ETEC infection.

Results In total 495 genes were differentially expressed in IPEC-1 challenged with ETEC, among them, 348 (70%) genes were up-regulated and 147 (30%) were down-regulated. In total 293 genes were found to be differentially expressed in EPS treated cells, among them, 233 (79.5%) genes were up-regulated and 60 (20.5%) were down-regulated. ETEC challenge affected expression of 64 genes related to the innate immune system, among them 20 were related to the Toll-like receptor cascades. 18 genes associated with Toll-like receptor 4 were evoked and 12 were related to C-type lectin receptors. EPS treatment of cells induced change in expression of 39 genes but ETEC challenge of pretreated cells affected only 28 genes categorized in these GO biological processes. ETEC challenge induced up-regulation of genes categorized in the GO biological process "cell-cell communication" (8 DEGs). Products of the genes involved other biological process like "cell junction organization" (6 DEGs), "cell-cell junction organization" (5 DEGs), "adherens junction interactions" (1 DEG) and "tight junction interactions" (6 DEGs) were also evoked. EPS-treatment of cells induced up-regulation of 4 genes but ETEC challenge of pretreated cells induced up-regulation of only 2 genes categorized in these GO biological processes. ETEC challenge affected expression of genes categorized in the GO biological process "extracellular matrix organization" (17 DEGs). These genes include those that are categorized in the GO biological process "collagen formation" (3 DEGs), "elastic fibre formation" (3 DEGs), "laminin interactions" (1 DEG), "non-integrin membrane-ECM interactions" (5 DEGs), " ECM proteoglycans" (6 DEGs), " degradation of ECM" (9 DEGs), and "integrin cell surface interactions" (5 DEGs). EPS treatment as well as ETEC challenge of pretreated cells affected expression of 7 genes.

Conclusions Protective effect of EPS was observed against ETEC infection. ETEC infection triggers TLR4 dependent pro-inflammatory response, whereas EPS pretreatment reduced this response by inducing TLR4-negative regulators like SIGGIR, SOCS1 and TNFAIP3. Moreover, genes expression involved in the reorganization of the cellular junctional proteins (hallmark of ETEC enterotoxins) were also suppressed by EPS pretreatment. For example CD151 and PARD6B genes which participate in the formation of hemidesmosomes and on formation of TJs, respectively were upregulate in EPS pretreated cells. Lastly, genes involved in degradation of extracellular matrix (mainly ADAM10, ADAM17, MMP9, MMP14, CAPN7 and CAST) were suppressed by EPS pretreatment, mainly.

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Ionizing radiation activates the atypical NF-κB pathway in RKO cells derived from human colorectal cancer.

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Introduction The cellular response to DNA damage imposed by ionizing radiation (IR) includes activation of the so-called atypical NF-κB pathway. The aim of the work was to analyze activation of NF-κB pathway in irradiated RKO human colorectal carcinoma cell line. The parental line (RKO-wt) and its strain expressing E6 protein from HPV16 virus (RKO-E6), which reduces the level and activity of the p53 protein (along with the corresponding RKO-neo control) were used.

Methods Cells were irradiated with a single ionizing radiation dose (in the range of 0.5-10 Gy), or incubated with the TNFα cytokine that activates the classical pro-inflammatory NF-κB pathway. At various time intervals after either type of stimulation levels of selected proteins associated with the activation of the NF-κB pathway were analyzed by Western blot.

Results We observed that high doses of radiation (4 Gy and above) activated the NF-κB pathway in the dose-dependent pattern. Moreover, the kinetics of NF-κB activation was slower in irradiated cells than in cytokine-stimulated cells. We analyzed the kinetics of activation of proteins such as RelA/p65 (phosphorylated on Ser536) and IκBα (phosphorylated on Ser32). The level of phosphorylated forms of these proteins is significantly higher in the RKO E6 than in the RKO neo (control line).

Conclusions We concluded that the presence of p53 affected activation of the atypical NF-κB pathway in cells exposed to IR.

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Quantitative proteomic analysis of serum-derived exosomes from polycythemia vera patients

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Introduction Cell-to-cell communication is a fundamental component of both normal physiology and pathophysiology. One of the mechanisms, by which cells communicate in healthy and diseased tissues, is the generation and processing of extracellular vesicles (EVs). Polycythemia Vera (PV) is a heterogeneous disease of stem cells with a preferential increase in the number of erythrocytes which, through resulting increased viscosity in combination with other pathogenic factors, may lead to thromboebolic complications. Although it is known that extracellular vesicles released from cell membranes and circulating in the blood can directly contribute to thrombosis through their procoagulant activity, the proteomic composition of these vesicles in PV patients has not been investigated before. The aim of this study was to conduct a proteomic analysis of serum-derived exosomes isolated from polycythemia vera patients and healthy donors.

Methods We processed exosome-enriched serum samples using bottom-up analysis according to Multiple Enzyme Filter Aided Sample Preparation approach (MED-FASP). To analyze samples, we used LC-MS/MS measurements on a Q-Exactive HF-X mass spectrometer. We quantitatively analyzed the absolute concentrations of identified proteins by Total Protein Approach (TPA).

Results 38 proteins were present at statistically significant different concentrations between PV patients' study group and healthy controls' group. The main protein components deregulated in PV were primarily related with excessive amounts of cells, increased platelet activation, elevated immune and inflammatory response, and high concentrations of procoagulant and angiogenic agents.

Conclusions This study provides the first quantitative proteomic analysis of the serum-derived exosomes in PV patients. This new knowledge may contribute to a better understanding of the mechanism of PV disease and further development therapeutic procedures.

Study of serum metabolom in canine babesiosis by mass spectrometry

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Introduction Canine babesiosis is an important worldwide tick-borne disease caused by the intra-erythrocyte protozoal parasites *Babesia canis* or *Babesia gibsoni* (Beck et al., 2009). Although the disease process primarily affects erythrocytes, it may also have multisystemic consequences (Barić Rafaj et al., 2013). The main complications are the development of an excessive inflammatory response called "systemic inflammatory response syndrome" or SIRS (Bone et al., 1992) and also a multiple organ dysfunction syndrome or MODS (Jacobson and Clark, 1994). Specific metabolites are being discovered as biomarkers to improve disease diagnosis, prognosis, and treatment outcomes. The emergence of innovative, post-genomic technologies, has led to the development of strategies aimed at identifying specific and sensitive biomarkers among thousands of molecules present in biological fluids and tissues (Moore et al., 2007). Nowadays, according to its great potential for biomarkers evaluation, metabolomics is one of the most frequently applied approaches in the field of systems biology (Robinson et al., 2014). Blood and urine contains a multitude of unstudied and unknown biomarkers that may reflect physiological and pathological states of tissues and organs. Particularly low-molecular-weight region of metabolome from blood samples is an important source of diagnostic biomarkers. The goal was to examine the difference of serum metabolome between dogs naturally infected with *Babesia canis* (*B. canis*) and healthy dogs using liquid chromatography coupled to mass spectrometry (LC-MS).

Methods Serum was collected from 12 dogs naturally infected with *B. canis* and 12 healthy dogs. Briefly, 25 µL serum aliquots were prepared, and 1000 µL of 1:3:1 chloroform:methanol:water was added to precipitate the proteins. The samples were allowed to cool on ice for 30 minutes before centrifugation to pellet the proteins. The Eppendorf tubes vortexed on 4˚C for 5 minutes, and then centrifuge for 3 minutes at 13.000 g at 4˚C. The supernatant (200 µL) was transferred to a screw-top vial and stored at 80˚C until liquid chromatography-mass spectrometry (LC-MS) analysis. Samples were analysed on an Orbitrap Q-Exactive mass spectrometer (Thermo Fisher Scientific) operating in alternating positive and negative negative modes with mass resolution 70.000 at m/z range 70 – 1050. Analyses were performed using Polyomics integrated Metabolomics Pipeline (PiMP) program specifically designed for metabolomics.

Results The metabolomics analysis resulted in the annotation of 1802 peaks, 158 of which showed statistically significant differences ($p < 0.05$) between dogs with B. canis infection and healthy control. The peaks represent metabolites in positive and negative modes. 22 identified metabolites were significantly changed. The most significant metabolites are Inosine, Hypoxanthine, Choline phosphate, Hypoxanthine, L-Kynurenine, and L-Cystine. Biological functions of differently abundant metabolites indicate the involvement of various pathways in canine babesiosis including aminobenzoate degradation, benzoate degradation, bile secretion, calcium signalling pathway, D-glutamine and D-glutamate metabolism, dioxin degradation, phenylalanine metabolism, and purine metabolism.

Conclusions The study confirmed that host pathogen interactions (Dog – B. canis) can be studied by metabolomics to assess chemical changes in the host, respectively that the differences in serum metabolome between dogs with *B. canis* infection and healthy dogs can be detected with LC-MS method. The non-targeted LC-MS metabolomic's approach profiled the metabolic change in serum from B. canis-infected dogs.

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Proteomic alterations revealed in the crosstalk of polymorphonuclear neutrophils and macrophages using dynamic SILAC methodology

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Introduction A finely-tuned balance of inflammation and repair orchestrates the efficient cardiac recovery following a myocardial infarction. An instrumental role in mediating these two processes is played by polymorphonuclear neutrophils (PMNs), which recruit and regulate the local differentiation of monocytes towards an inflammatory and\or reparatory macrophage (MAC) phenotype. The aim was to investigate the dynamic crosstalk between PMNs and MACs, identifying the alterations induced by the PMNs' secretome in the MACs proteome.

Methods Mice bone merrow-derived polymorphonuclear neutrophils were stimulated with either LPS (1000ng\ml) and IFNγ (20ng\ml) or IL4 (20ng\ml) to induce an inflammatory (N1) or reparatory (N2) phenotype. The secretome of the cells was collected after a 2h stimulation and put on top of naïve macrophages isolated from murine spleen monocytes. Dynamic stable isotope labeling of amino acids in cell culture (dSILAC) was applied in two steps using +6Da labeled lysine (for the first 3 days, concomitant with the stimulation with cell secretome) and +10Da labeled arginine-containing media (for the next 3 days). Macrophages with unstimulated neutrophil secretome (N0) were used for normalization. The cells were solubilized and suitably prepared for peptide nano-chromatographic separation and mass spectrometric analysis using the Easy nLC II coupled to the LTQ Orbitrap Velos Pro (Thermo Scientific) hybrid mass spectrometer. Proteome Discoverer 2.1 and Protein Center 3.16 (Thermo Scientific) were used for the bioinformatic analysis.

Results Proteins incorporating either one tag or the other were de novo synthesized as a result of a direct activation with N1 or N2 secretome, or following the removal of the stimuli. The global proteomic analysis demonstrated a significantly higher number (>25%) of identified macrophage proteins as a result of direct N2 secretome stimulation ($p<0,005$ when compared with either N0 or N1 activated macrophages). An average of 209 and 304 proteins carried the lysine tag the first 3 days, suggesting a differential synthesis rate under N1 and N2 stimulation, respectively, as opposed to the initial state (day 0). After stimuli removal and media change, the arginine tag was identified in 114 and 152 proteins in the two groups of macrophages, which continued to differentially synthesize proteins even after N1 and N2 secretome samples were discarded. The bioinformatic analysis demonstrated that the 275 proteins that were uniquely differentially abundant in the first 3 days as a result of the direct stimulation with N2 secretome were implicated in multiple signaling networks, such as phagosome, gap junctions or ECM-receptor interaction pathways. Furthermore, after N2 secretome removal, the proteins that uniquely carried the arginine tag were found to be associated with altered glycolysis/gluconeogenesis or biosynthesis of amino acids processes.

Conclusions Protein turnover was assessed using dSILAC and mass spectrometry. Novel protein mediators and functional networks have been identified with potential in the better understanding of the dynamics of PMNs and MACs crosstalk. The experiments will be further validated using immune cells extracted from animal models with induced acute myocardial infarction.

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Biological processes associated with mutations present in anaplastic and papillary thyroid cancers co-existing in the same thyroid gland

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Introduction Anaplastic thyroid cancer (ATC) is a rare but one of the fastest growing type of cancer with bad prognosis of 6 months survival after the diagnosis. The genetic background of ATC tumorigenesis is still a field of work for scientists. It is supposed that ATC can arise de novo or it derives from other differentiated tumours, like PTC or FTC. Here, we intend to find out the genes characteristic for ATC and corresponding biological processes involved in the carcinogenesis of ATC of both origins: de novo and derived from PTC.

Methods FFPE samples from 5 patients diagnosed with ATC only and 3 patients diagnosed with ATC and PTC together were collected. DNA extraction and exome sequencing were performed with 150 nucleotide long paired-end reads and depth of sequencing equal to 100. Data pre-processing consisted of mapping to the newest human reference genome GRChv.38 and detection of the mutations with MuTect2. Next, for each mutation Variant Effect Predictor tool was applied in order to find all possible effects that such mutation may have on certain transcript. Unique and common mutated genes were listed for every patient. Focus was put on mutations representing severe impact on organism. Functional analysis using Gene Ontology overrepresentation was performed on chosen genes in order to detect processes characteristic for ATC. Wang method for semantic similarity measure of GO terms and best-match average method for combining such result were applied.

Results Mutations were identified in 2398 genes characteristic for the group of ATC only and 810 genes for the group of ATC- PTC together, with 173 genes in common. Known target genes in ATC, including BRAF, PIK3CA, APC and TERT were identified. What is more, known oncogenes, like NOTCH2, IGF2R, EP400 or SMARCA4, were common in both groups of patients. Unique genes in ATC only and ATC-PTC groups were connected with 273 and 217 GO terms respectively, with 33 terms in common. Many of the terms are well known to be involved in carcinogenesis, including: negative regulation of JAK-STAT cascade, growth, T cell receptor signalling pathway or positive regulation of gene expression. Semantic similarity between lists of GO terms unique for each group was equal to 0.683, meaning that the characteristic and statistically significant GO terms for each group are not in the different branches of GO tree, but most of them are in close proximity.

Conclusions Findings concerning mutated genes in ATC with PTC are coherent with previous literature, where APC, TERT or BRAF were reported. Lists of mutated genes differentiating patients with ATC only and patients with ATC and PTC occurring together are highly variable. However semantic similarity ontology analysis with GO biological processes reveals coherent signatures. Lists of biological processes shared by all patients with ATC show high enrichment of processes well known to be involved in carcinogenesis.

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Determination of angiotensin levels in mouse and human serum samples with nanoLCMS method

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Introduction A family of angiotensins (Angs) consists of several peptide hormones participating in the regulation of blood pressure and organism water and electrolyte homeostasis. Those components of renin - angiotensin - aldosterone system (RAAS) are active in pico molar concentrations in blood, which, in combination with short half-lives and dynamic nature of the system render them challenging to assess. Growing body of evidence, presents Angs as a valuable diagnostic tool e.g. in adrenal insufficiency or a range of cardiovascular disorders and calls for a reliable analytical methods for their quantitation. Nano HPLC with mass detection characterizes with sensitivity and resolution necessary to detect such low abundant peptides in complex biological matrices. Our work included a preliminary development and validation of the method for several angiotensins analysis in serum samples.

Methods Blood collected to K2EDTA tubes was immediately mixed with protease and phosphatase inhibitors cocktail, and internal standard (AngII [Asn1, Val5]). Separated plasma underwent liquid-liquid extraction with cold acetonitrile (ACN) and resulting supernantant was lyophilized. Following reconstitution in phase A (1% acetic acid in water) samples were analyzed with nanoflow HPLC apparatus fitted with C18 column, in reverse phase mode. Initial chromatographic conditions involved 300 nl/ml flow of 98:2 ratio of A:B (1% acetic acid in ACN) and following 20 min method ramped this ratio to 25:75. NanoESI ion source was used to introduce sample into a triple quadrupole mass spectrometer. Quantitation was achieved with 9-point standard curve prepared from a mix of Angs standards added to pooled serum. Whenever possible standards and samples were measured in triplicates.

Results Among few tested preparation techniques, SPE columns, zip-tip microcolumns and repeated ACN extractions resulted in a noticeable analyte loss, without increased sensitivity. Standard curves for three analyzed angiotensins, i.e. AngI (1-10), AngII (1-8) and AngIV (3-8) showed good linearity with R2 values < 0,93. Linear regression for Ang (1-7), despite positive slope, resulted in R2 = 0,52. Retention times were stable across repetitive measurements, although %RSD in some cases was unacceptably high (over 100%). Preliminary trial to asses Angs levels in 8 patient samples resulted in inconsistent results. For AngI they ranged was 26.4 – 34.5 pg/ml, for AngII 9.4 – 574.0 pg/ml and 28.4 – 539.0 pg/ml in case of AngIV. Unexpectedly high values for Ang (1-7) concentrations were outside its standard curve range and considered unreliable.

Conclusions In relation to our interests in cardiovascular disorders mouse models, including apoE-KO and LDLR-KO strains, it's worth to mention reported higher sensitivity to Ang II-induced abdominal aortic aneurysm in apoE-KO. Cholesterol accumulation in podocytes, accompanying chronic kidney disease, was also connected to angiotensin II via its impact on increasing LDLR expression. Those findings suggest that a validated method of measuring different angiotensins levels is much needed research and possibly diagnostic tool. Our group earlier findings in ApoE -/- /LDLR -/- mice models also demonstrated significant differences in their Angs levels, in relation to WT animals and as some obstacles is still on the way to a robust analytical method our work develops it a few steps further.

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