The 12th Central and Eastern European Proteomic Conference

jointly with

The 39th Anniversary of the Institute of Cellular Biology and Pathology "Nicolae Simionescu"

ADVANCES IN PROTEOMICS AND PROGRESS IN PRECISION MEDICINE

October 24-26, 2018, Bucharest, Romania

BOOK OF ABSTRACTS

Organizers

Romanian Academy

Institute of Cellular Biology and Pathology "Nicolae Simionescu"

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The 12th Central and Eastern European Proteomic Conference

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The 39th Anniversary of the Institute of Cellular Biology and Pathology "Nicolae Simionescu"

October 24 – 26, 2018, Bucharest, Romania

Organized by:
The Institute of Cellular Biology and Pathology "Nicolae Simionescu" and Romanian Academy, Bucharest, Romania

ORGANIZING COMMITTEE

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Dr. Felicia Antohe, head of Proteomics Department
Drs. Luminița Ivan, Elena Uyy, Raluca Maria Boteanu, Viorel Iulian Şuică

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WELCOME ADDRESS

Dear Friends and Colleagues,

We, the Organizing committee and the CEEPC Board have the great pleasure to welcome you all to the 12th Central and Eastern European Proteomic Conference in Bucharest, initiated in the first meeting in Prague, Czech Republic in 2007 by Hana Kovarova and Suresh Jivan Gadher. The conference will be held together with the 39th Anniversary of the Institute of Cellular Biology and Pathology "N. Simionescu", the 2018 host of the event.

We salute the presence of eminent speakers and young researchers with whom to share novel ideas and fascinating research, for all to enjoy.

Promoting the European Union international cooperation principles, we are honored to welcome scientists from Europe and USA participating to our Conference, showing again that science is an international enterprise that need to be shared, continuously improved and finally to serve the human kind.

In keeping with the CEEPC philosophies, we have put together a multidisciplinary program focusing on a central theme *Advances in Proteomics and Progress in Precision Medicine*. We hope this topic will not only expand our knowledge in proteomics but also will open diverse new emerging research areas. We will discuss the diverse scientific, clinical and proteomic challenges and the means by which to speed up the translation of findings into viable solutions and /or therapies for diseases affecting mankind.

We hope you will enjoy the Romanian hospitality, the academic heritage of Bucharest, the productive interactions, networking and friendship.

We wish you a fruitful Conference and a pleasant stay in Romania!

The Organizing Committee

Conference program

Wednesday, October 24, 2018

09.00 – 09.30 Registration

Location: Institute of Cellular Biology and Pathology, "Nicolae Simionescu" (ICBP-NS), 8 B.P. Hasdeu Street, Bucharest

Session 1: 9.30 – 12.00
Chair: Maya Simionescu

Annual Report: on the road from the laboratory bench to precision medicine

Location: "George Palade" auditorium of ICBP-NS

09.30 – 10.00 **Maya Simionescu,** Director of ICBP-NS, Bucharest, Romania
ICBP-NS at 39 years

10.00 – 10.15 **Ileana Mânduțeanu,** Department of Biopathology and Therapy of Inflammation, ICBP-NS

10.15 – 10.30 **Anca Volumnia Sima,** Department of Lipidomics, ICBP-NS

10.30 – 10.45 **Felicia Antohe,** Department of Proteomics, ICBP-NS

10.45 – 11.00 **Alexandrina Burlacu,** Department of Regenerative Medicine, ICBP-NS

11.00 – 11.15 **Anca Violeta Gafencu,** Department of Genomics Transcriptomics and Molecular Therapies, ICBP-NS

11.15 – 11.30 **Adrian Manea,** Department of Genomics Transcriptomics and Molecular Therapies, ICBP-NS

11.30 – 11.45 **Adriana Georgescu,** Department of Pathophysiology and Pharmacology, ICBP-NS

11.45 – 12.00 **Irina Titorencu,** Department of Regenerative Medicine, Laboratory of Mesenchymal Stromal Cells Therapy, ICBP-NS

12:00 – 13:00 Lunch

Location: ICBP-NS, 8 B.P. Hasdeu Street

Session 2: 14.00 – 15.50

Co-Chairs: Maya Simionescu, Suresh Jivan Gadher

Opening ceremony of the 12th CEEPC

Location: The Romanian Academy, 125 Calea Victoriei

14.00 – 14.10 **Victor Voicu,** Member and Vice-president of the Romanian Academy, Bucharest, Romania

14.10 – 14.20 **Diana Loreta Păun,** Professor, State Adviser, Department of Public Health, Presidential Administration, Romania

14.20 – 14.40 **Maya Simionescu,** Director of ICBP-NS, Bucharest, Romania
The path to precision medicine: understanding diseases of cell organelles

14.40 – 15.00 **Suresh Jivan Gadher,** Founder Member of CEEPC, K.N. Oxford, United Kingdom
Credibility, cohesion and vision for Central and Eastern European Proteomic Conference

15.00 – 15.20 **Felicia Antohe,** ICBP-NS
Omics frontiers for personalized medicine

15.20 – 15.50 **Ales Svatos,** Max Planck Institute for Chemical Ecology, Jena, Germany
Solving the yellow mystery of Papaver nudicaule with an integrated – omics approach

15.50 – 16.10 Coffee break

Session 3: 16.10 – 17.30

Co-Chairs: Felicia Antohe, Ales Svatos

16.10 – 16.50 **Shlomo Sasson**, Institute for Drug Research, Faculty of Medicine, The Hebrew University, Jerusalem, Israel
Exploring glucolipotoxicity in pancreatic beta cells by combining advanced confocal analysis of the subcellular lipid map with proteomics

16.50 – 17.30 **Ingrid Miller,** Institute for Medical Biochemistry, University of Veterinary Medicine, Vienna, Austria
Challenges in Proteomics

18.00 – 20.00 **Welcome and get-together party** *(Location: House of Scientists, 9 Lahovary Plaza, Bucharest)*

Thursday, October 25, 2018
Location: ICBP-NS, 8 B.P. Hasdeu Street

08.30 – 09.00 Registration

Session 4: 9.00 – 10.30
Co-Chairs: Suresh Jivan Gadher, Anca Volumnia Sima

09.00 – 09.40 **Rainer Bischoff**, Groningen Research Institute of Pharmacy, University of Groningen, Groningen, Netherlands
Biomarker discovery and validation – from shotgun proteomics to targeted methods

09.40 – 09.55 **Mangesh Bhide**, University of Veterinary Medicine and Pharmacy, Kosice, Slovakia
Factor H binding proteins of Borrelia: immune evasion tools

09.55 – 10.10 **Jiri Petrak**, BIOCEV, First Faculty of Medicine, Charles University, Vestec, Czech Republic
The Pitchfork Strategy. A multi-pronged approach for membrane proteome profiling

10.10 – 10.30 **Linda Keller,** Application Specialist GE Healthcare Life Sciences, Munich, Germany
Efficient coverage analysis for HCP ELISA assay validation

10.30 – 11.00 Coffee break and poster session

Session 5: 11.00 – 12.35
Co-Chairs: Shlomo Sasson, Ileana Mânduțeanu

11.00 – 11.30 **Mădălina Oppermann**, Thermo Fisher Scientific, Stockholm, Sweden
Wide and Deep: New Reagents and Workflows for Multiplexed Quantification and Targeted Analysis

11.30– 11.45 **Brînduşa Alina Petre**, "Alexandru Ioan Cuza" University of Iaşi, Iaşi, Romania
Molecular identification of nitro-tyrosine modification in human eosinophil proteins by proteolytic affinity extraction - mass spectrometry (PROFINEX)

11.45 – 12.15 **Suresh Jivan Gadher,** Thermo Fisher Scientific, Carlsbad, USA.
Synergistic success of proteo - genomics in interrogating exotic biological fluids using a novel high sensitivity Immunoassay

12.15 – 12.35 **Matt Kennedy**, Waters HRMS Business Development Manager, Wilmslow, United Kingdom
The Next Generation of IMS Research Platform

12.35 – 13.45 **Lunch**

Session 6: 13.45 – 15.10
Co-Chairs: Juan Jivan Calvete, Mădălina Opperman

13.45 – 14.25 **Theo Marten Luider**, Erasmus University Medical Center, Rotterdam, Holland
Identification of Antibodies by integration of mass spectrometry and DNA sequencing

14.25 – 14.40 **Helena Kupcova Skalnikova,** Institute of Animal Physiology and Genetics, Czech Academy of Sciences, Libechov, Czech Republic
Cytokine profiling in melanoma patient serum for monitoring of cancer progression

14.40 – 14.55 **Cristiana Tănase**, National Institute of Pathology "Victor Babes", Bucharest, Romania
Proteomic approaches for the evaluation of natural products in cancer prevention and therapy

14.55 – 15.10 **Martina Macht**, Bruker Daltonik GmbH, Bremen, Germany
Unleashing the power of QTOF technology for proteomics with TIMS and PASEF

15.10 – 15.40 **Coffee break and poster session**

Session 7: 15.40 – 17.15
Co-Chairs: Goran Mitulović, Manuela Călin

15.40 – 16.10 **László Drahos**, Research Centre for Natural Sciences, Hungarian Academy of Sciences, Budapest, Hungary
Selection of Collision Energies in Tandem Mass Spectrometry Based Proteomics

16.10 – 16.25 **Tom Dennison**, Malvern Panalytical, UK
Characterising Extracellular Vesicles with Nanoparticle Tracking Analysis

16.25 – 16.45 **Manuela Călin,** ICBP-NS, Bucharest, Romania
Targeted nanocarriers to ameliorate vascular inflammation

16.45 – 17.00 **Tanja Panić-Janković**, Medical University of Vienna, Vienna, Austria
Background Proteins in Human Chorionic Gonadotropin Pharmaceutical Formulations of Different Origin

17.00 – 17.15 **Viorel Iulian Şuică,** ICBP-NS**,** Bucharest, Romania
Proteomic alterations induced by poly (2-ethyl butyl cyanoacrylate) nanoparticles

17.15 – 17.45 **CEEPC Board Meeting**

19.00 – 21.00 **Conference dinner** *(Location: Ramada Bucharest Parc, 3-5 Poligrafiei Ave, 1st District, Bucharest, Romania)*

Friday, October 26, 2018

Location: ICBP-NS, 8 B.P. Hasdeu Street

Session 8: 09.00 – 10.30

Co-Chairs: Jiri Petrak, Cornelia Bala

09.00 – 09.40 **Fernando J. Corrales,** National Centre for Biotechnolgy (CSIC), Madrid, Spain
One carbon metabolism and protein methylation. Implications in liver diseases

09.40 – 09.55 **Goran Mitulović,** Medical University of Vienna, Vienna, Austria
Micro-Pillar-Arrayed Column (µPAC) for Proteomics

09.55 – 10.10 **Cornelia Bala,** Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania
Branched-chain and aromatic amino acids in diabetes – application of metabolomics in clinical settings

10.10 – 10.30 **Filip Supljika,** Application Specialist of Malvern Panalytical, Zagreb, Croatia
Studying Proteomics with Microcalorimetry

10.30 – 11.00 **Coffee break and poster session**

Session 9: 11.00 – 12.30

Co-Chairs: Piotr Widlak, Mirela Sârbu

11.00 – 11.40 **Cristina Furdui**, Center for Redox Research in Biology and Medicine, Wake Forest School of Medicine, Winston-Salem, USA
Integrating Redox Effects in Analysis of Biological Systems

11.40 – 11.55 **Mirela Sârbu**, National Institute for Research and Development in Electrochemistry and Condensed Matter, Timişoara, Romania
Introducing ion mobility tandem mass spectrometry in glycoproteomics and glycolipidomics of human biopsies

11.55 – 12.10 **Xaveer Van Ostade**, University of Antwerp, Wilrijk, Belgium
Characterizing the molecular mechanism of the multifunctional antitumor compound withaferin A in a multiple myeloma model

12.10 – 12.30 **Adrian Manea, ICBP-NS,** Bucharest, Romania
Histone deacetylases as potential therapeutic targets in atherosclerosis

12.30– 13.45 **Lunch**

Session 10: 13.45 – 15.25
Co-Chairs: Cristina Furdui, Adrian Manea

13.45 – 14.25 **Juan Jose Calvete**, Evolutionary and Translational Venomics Laboratory, CSIC, Valencia, Spain
From low-resolution toxin-pattern recognition to toxin-resolved venom proteomes: New approaches in evolutionary and translational venomics

14.25 – 14.55 **Piotr Widlak**, Maria Sklodowska-Curie Institute - Oncology Center, Gliwice Branch, Gliwice, Poland
Discrimination of oral cancer from normal oral mucosa by mass spectrometry imaging of proteins and lipids

14.55 – 15.10 **Katarina Davalieva**, Research Centre for Genetic Engineering and Biotechnology "Georgi D Efremov", Macedonian Academy of Sciences and Arts, Skopje, Republic of Macedonia
Application of tissue proteomics for understanding the prostate cancer initiation and progression

15.10 – 15.25 **Oleksii Ivanov** – Promega GmbH, Mannheim, Germany
The Bioluminescent HiBiT Technology for CRISPR – Mediated Gene Tagging

15.25 – 16.00 **Coffee break and poster session**

Session 11: 16.00 – 16.30
Co-Chairs: László Drahos, Viorel Iulian Şuică

16.00 – 16.15 **Suresh Jivan Gadher**, Thermo Fisher Scientific, Carlsbad, USA.
Personalized cancer immunotherapy using GM-CSF to activate the body defense

16.15 – 16.30 **Raluca Boteanu**, ICBP-NS, Bucharest, Romania
Proteomic and bioinformatic analyses of bone healing using titanium implant with bioactive targeted surface in a rat tibial defect model

16.30 – 17.00 **Closing Remarks**

LIST OF POSTERS

Thursday, October 25, 2018

ThPo1 *Rebizak B., Mielczarek P., Bodzon-Kulakowska A.,* **Western Blotting – the devil is in the details**

ThPo2 *Skowronek A., Marczak L., Rutkowski T., Widlak P., Pietrowska M.,* **Profiling of serum metabolome of head and neck cancer patients undergoing radiotherapy**

ThPo3 *Zamfir A.D., Sârbu M., Vukelić Ž.,* **Analyses of glycolipid-peptide and glycolipid-protein interactions by chip-based mass spectrometry**

ThPo4 *Bazylak G., Pan T.L., Wang P.W., Leu Y.L., Wu T.H., Wu Y.C.,* **Proteomics discloses effect of saffron stigmata ethanolic extract on restoring viability in HepG2 cells under VCP gene silencing**

ThPo5 *Ionescu A.E., Menţel M., Leney A.C., Munteanu C.V.A., Heck A.J., Szedlacsek S.E.,* **EYA3 tyrosine phosphorylation by Src kinase from mass spectrometry to implications in proliferation**

ThPo6 *Marinescu G.C., Popescu R.G., Dinischiotu A.,* **Nicotinamide mononucleotide (NMN) effects on mitochondrial OXPHOS protein expression**

ThPo7 *Chiriţoiu G.N., Munteanu C.V.A., Jandus C., Ghenea S., Romero P., Petrescu S.M.,* **Mass spectrometry and T cell analysis reveals that N-glycosylation can impact antigen presentation in melanoma**

ThPo8 *Bielińska I., Sikora M., Jakubowski H.,* **Methionine-induced hyperhomocysteinemia causes changes in the mouse kidney proteome associated with blood coagulation**

ThPo9 *Jankovska E, Vit O., Svitek M., Holada K., Petrak J.,* **Two strategies for processing of human cerebrospinal fluid prior LC-MS/MS**

ThPo10 *Ner-Kluza J., Milewska A., Dąbrowska A., Mielczarek P., Pyrć K., Suder P.*, **iTRAQ based proteomic analysis of Zika virus infection based on 293T cells**

ThPo11 *Piechura K., Zingale G.A., Mielczarek P., Silberring J.,* **Activity of neuropeptides converting enzymes**

ThPo12 Smirnova L., Dmitrieva E., Seregin A., Letova A., Semke A., Zgoda V., **Search of peripheral markers associated with pathogenesis of schizophrenia**

ThPo13 *Gawin M., Wojakowska A., Pietrowska M., Marczak Ł., Chekan M., Widłak P.,* **Proteome profiles of different types of thyroid cancers**

ThPo14 *Abramowicz A., Marczak Ł., Smolarz M., Gładysińska M., Widłak P., Pietrowska M.,* **Ionizing radiation affects the composition of proteome of exosomes released by head and neck carcinoma in vitro**

ThPo15 *Behounek M., Chmel M., Havlenova T., Melenovsky V., Cervenka L., Petrak J.,* **Molecular changes in kidneys during chronic heart failure**

ThPo16 *Ner-Kluza J., Kosowicz K., Milewska A., Dąbrowska A., Pyrć K.A., Suder P.,* **ZIKA virus NS3 protease: substrate specificity investigations**

ThPo17 *Mielczarek P., Rozmus K., Silberring J.,* **Simulation of phase II metabolism to study interactions of metabolites with proteins**

ThPo18 *Pralea I.E, Buse M., Zimţa A., Morar-Bolba G., Berindan-Neagoe I., Iuga C.A.,* **Protein Extraction from Formalin-Fixed Paraffin-Embedded Tissue. A shotgun Proteomics application**

ThPo19 *Smoluch M., Mielczarek P., Kotlinska J.H., Silberring J.,* **I*n vivo* determination of the CYP2E1 expression in rat hepatic microsomes after drug administration**

ThPo20 *Sikora M., Marczak Ł., Perła-Kajan J., Jakubowski H.,* **Sex affects homocysteine modification at lysine residue 212 of albumin in mice**

ThPo21 *Kiprijanovska S., Stavridis S., Stankov O., Komina S., Petrusevska G., Davalieva K.,* **Potential urine biomarkers for prostate cancer identified by label-free nanoLC-MS/MS**

ThPo22 *Valekova I., Jarkovska K., Kotrcova E., Juhas S., Motlik J., Bucci J., Gadher S.J., Kovarova H.*, **Revelation of the IFNα, IL-10, IL-8 and IL-1β as promising biomarkers reflecting immuno-pathological mechanisms in porcine Huntington's disease model**

Friday, October 26, 2018

FrPo1 *Antolak A., Bodzon-Kulakowska A., Marszalek-Grabska M., Gibula-Bruzda E., Kotlinska J.H., Suder P.,* **Ethanol-induced alterations in ubiquitin-proteasome system**

FrPo2 *Albulescu R., Necula L.G., Neagu A.I., Dima S., Popescu I., Tănase C.,* **Evaluation of circulating angiogenic factors in hepatocellular carcinoma by proteomic technology multiplex array**

FrPo3 *Tofan V., Costache A., Țucureanu C., Onu A.,* **Expression and purification of stable uniform N^{15} labeled Shiga-like toxin 2 subunit B with application in mass spectrometry-mediated detection of hemolytic-uremic syndrome causing bacteria**

FrPo4 *Popa M.A., Mihai M.C., Constantin A., Şuică V., Costache R., Antohe F., Dubey R.K., Simionescu M.*, **Human mesenchymal stem cells migration proteins are upregulated by dihydrotestosterone treatment**

FrPo5 *Constantin A., Nemecz M., Dumitrescu M., Filippi A., Alexandru N., Smeu B., Petcu L., Georgescu A., Tanko G., C. Copaescu, Simionescu M.,* **Improved metabolic status in obese type 2 diabetic patients treated by sleeve gastrectomy is associated with increased circulating microRNA-126**

FrPo6 *Tanko G., Constantin A., Dumitrescu M., Nemecz M., Picu A., Smeu B., Guja C., Alexandru N., Georgescu A., Simionescu M.,* **Sera from obese type 2 diabetes patients undergoing metabolic surgery instead of conventional therapy exert beneficial effects on beta cell survival and function**

FrPo7 *Filippi A., Alexandru N., Voicu G., Constantinescu C.A., Rebleanu D., Fenyo M., Simionescu D., Simionescu A., Mânduțeanu I., Georgescu A.,* **Evaluation of the early and progressive changes in plasma, hemodynamic and cardiac parameters in an animal model of atherosclerosis-associated diabetes mellitus,**

FrPo8 *Butoi E., Cecoltan S., Ciortan L., Macarie R.D., Tucureanu M.M., Vadana M., Droc I., Simionescu A., Mânduțeanu I.,* **3D model to study human aortic valve disease**

FrPo9 *Iordache F., Alexandru D., Georgescu A., Airini R., Amuzescu B., Savu L., Maniu H.,* **Characterization of senescent versus early passages human amniotic fluid stem cells**

FrPo10 *Nemecz M. Tanko G., Constantin A., Dumitrescu M., Alexandru N., Fillipi A., Simionescu M., Georgescu A.,* **The mechanisms underlying protective effects of oleic acid against palmitic acid on pancreatic beta cell function**

FrPo11 *Niculescu L.S., Simionescu N., Fuior E.V., Stancu C.S., Carnuta M.G., Dulceanu M.D., Raileanu M., Dragan E., Sima A.V.,* **Inhibition of miR-486 and miR-92a decreases liver and plasma cholesterol levels by modulating lipid-related genes in hyperlipidemic hamsters**

FrPo12 *Toma L., Raileanu M., Deleanu M., Stancu C.S., Sima A.V.,* **Novel molecular mechanisms by which ginger extract reduces the inflammatory stress in TNFα – activated human endothelial cells; decrease of Ninjurin-1, TNFR1 and NADPH oxidase subunits expression**

FrPo13 *Truşcă V.G., Dumitrescu M., Fenyo I.M., Tudorache I.F., Gafencu A.V.,* **Bisphenol A down-regulates apolipoprotein A1 expression and exerts pro-atherogenic effects**

FrPo14 *Uyy E., Şuică V.I, Boteanu R.M., Ivan L., Antohe F., Simionescu M.,* **Mass Spectrometry evidence for modified protein composition of pulmonary lipid rafts in experimental diabetes**

FrPo15 *Roşca A.M., Pruna V., Tutuianu R., Neagu T.P., Lascar I., Simionescu M., Titorencu I.,* **Dermal Fibroblasts as new players in regenerative therapy**

FrPo16 *Ivan L., Uyy E., Boteanu R.M., Şuică V.I., Coman C., Berg S., Hansen R., Antohe F.,* **Exploration of mechanisms leading to plaque instability in a rabbit atherosclerotic model - preliminary data**

FrPo17 *Rebleanu D., Constantinescu C.A., Voicu G., Deleanu M., Gaidau C., Ignat M., Petica A., Călin M.,* **The effects of photocatalytic silver (Ag)-titanium dioxide (TiO2) nanoparticles on human lung epithelial cells**

FrPo18 *Constantinescu C.A., Fuior E.V., Rebleanu D., Voicu G., Deleanu M., Tucureanu M., Butoi E., Mânduțeanu I., Escriou V., Simionescu M., Călin M.,* **Downregulation of the receptor for advanced glycation end products (RAGE) in the aorta of APOE-deficient mice using P-selectin targeted RAGE-shRNA lipoplexes**

FrPo19 *Voicu G., Constantinescu C.A., Rebleanu D., Fuior E.V., Deleanu M., Tucureanu M., Butoi E., Mânduțeanu I., Escriou V., Simionescu M., Călin M.,* **P-selectin targeted lipoplexes carrying a shRNA plasmid to silence receptor for advanced glycation end products decrease monocyte adhesion to activated endothelial cells**

FrPo20 *Fuior E.V., Voicu G., Deleanu M., Rebleanu D., Constantinescu C.A., Safciuc F., Simionescu M., Călin M.,* **VCAM-1 targeted naringenin-loaded lipid nanoemulsions reduce monocyte adhesion to activated endothelial cells**

FrPo21 *Dumitrescu M., Truşcă V., Gafencu A., Burlacu A., Simionescu M., Askenasy N.,* **Adenoviral transduction of hepatocytes to induce Fas ligand expression**

FrPo22 *Vlad M.L., Lazar A.G., Manea S.A., Raicu M., Muresian H., Simionescu M., Manea A.***, Up-regulated NADPH oxidase-derived reactive oxygen species induce macrophage polarization towards M1 phenotype in vitro; potential implication in human atherosclerosis**

Wednesday Orals

Omics frontiers for personalized medicine

Felicia Antohe
Institute of Cellular Biology and Pathology "N. Simionescu" of Romanian Academy, Bucharest, Romania
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Background. The transfer of basic research data from discovery to clinical application requires smart strategies and high performant technologies. In the post genomic epoch many hopes were raised by the development of *Omics* projects with the promise to achieve personalized health care improvement, through prediction of disease risk, early diagnosis and adequate treatment. All the emerging *Omics* approaches: genomics, transcriptomics, proteomics, lipidomics, metabolomics and the recent meta-omics studies are based on the mass spectrometry key analytical technology. Thus, based on parameters as high sensitivity, mass accuracy and resolution it is possible to analyze complex probes containing a mixture of macromolecules in very small quantities operating in a global or targeted manner leading to new molecular biomarkers identification with positive impact on drug targeting and personalized therapies. Nowadays the challenge is to carefully validate the new molecular biomarkers and to thoroughly integrate all the *Omics* generated data using bioinformatics tools, to achieve a new horizon of precision medicine.

Aim. Diabetic kidney disease is the most common cause of chronic kidney disease all over the world. Novel precision medicine-based diagnostic biomarkers are desperately needed. Biochemical, histological and high performance mass spectrometry based proteomics were applied to identify potential molecular markers differentially expressed in kidney harvested from experimental diabetic mice.

Methodology. A diabetic type I experimental model was developed on double transgenic mice (dTg), Ins-HA+/-, TCR-HA+/- that were demonstrated to develop rapidly an aggressive diabetes and compared with the corresponding controls, single transgenic (sTg) Ins-HA+/-, TCR-HA-/-, and wild type (WT) mice.

Results. The preliminary results demonstrated significant ultrastructural changes in the phenotype of endothelial cells harvested from diabetic mice. The cells display a higher number of plasmalemmal vesicles and intracellular ribosomes indicating an active biosynthetic state. The LC-MS/MS bioinformatics analysis identified 1748 proteins in the detergent resistant membrane microdomains. The hiperglycemia has a regulatory consequence on the spectral abundance of alarmins, such as annexin A1, histone H4, HSPs and S100A6 and S100A9 proteins. Our novel data showed that HMGB1 presented a higher expression both at protein and gene level in diabetic animals, supporting the concept that HMGB1 is an active regulator of inflammation with significant impact on microvascular permeability.

Conclusion. Taken together the results support the concept of an inflammatory process maintained and amplified in diabetic mice, underling the associated dysfunction of microvascular endothelial cells in kidney.

Acknowledgment. The work was supported by The Romanian Academy and the Ministry of Research and Innovation UEFISCDI Grant no. 41/2018.

Solving the yellow mystery of *Papaver nudicaule* with an integrated -omics approach

B. Dudek, N. Wielsch, R.C. Menezes, A.C. Warskulat, Y. Hupfer, S. Lorenz, H. Vogel, C. Paetz, B. Schneider, A. Svatoš
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Background. *Papaver nudicaule* is a poppy plant originating from Siberia and widely distributed in Europe as a garden flower, commonly known as Iceland poppy.
Hypothesis. The pigments of the yellow variety are called nudicaulins[1] and have a unique structure, combining structural elements from indole and flavonoids[2]. Precursor-directed biosynthetic experiments and ^{13}C labeling experiments showed that the final precursors of nudicaulins are pelargonidin glucosides and indole[3]. Their biosynthetic pathways are well known, but the complete biosynthetic pathway for nudicaulins still remains obscure.
Aim. To assess the biosynthetic pathway for nudicaulins in flowers of yellow *Papaver nudicaule*.
Methodology. Buds in different developmental stages were harvested from plants in our greenhouse. RNA, proteins and metabolites were extracted using specific protocols. Metabolomics was performed by UPLC-HRMS methods. Transcriptomics was based on NGS Illumina RNAseq. DIGE-based quantitative proteomics was performed on 2-D PAGE and proteins from spots of interest were extracted. Peptides formed by trypsinolysis were analyzed on Synapt G2Si.
Results. The most important enzymes and corresponding transcripts involved in phenylpropanoid/polyketide as well as the indole pathway were characterized. Spontaneous formation of nudicaulins needs a very acidic pH value and does not happen during developmental stage 1-3 although the precursors are present. The search for a supporting enzyme (e.g. proton pump, transporter) is still in progress.
Conclusion. The molecular base of pelargonidin glucosides and indole in yellow poppy flowers was characterized in detail, but the final condensation step to nudicaulins is still not clear.
References. 1. Price et al. *J. Chem. Soc.* (1939) 1465–1468, 2. Tatsis et al. *Org. Lett.* (2013) 15, 156–159. 3. Warskulat et al. *ChemBioChem* (2016) 17, 318–327

Exploring glucolipotoxicity in pancreatic beta cells by combining advanced confocal analysis of the subcellular lipid map with proteomics

[1]S. Sasson and [2]G. Maulucci
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Background: Pancreatic beta cells are responsible for maintaining glucose homeostasis by secreting insulin in a regulated manner in response to alterations in blood glucose levels. Metabolic stressful conditions induced by high levels of glucose and saturated fatty acids alter beta cell functions in a process termed glucolipotoxicity. The complex biosynthetic pathways of proinsulin in beta cells and its processing into mature insulin molecules, followed by their packing in secretory granules under normal and glucolipotoxic conditions, have been extensively studies. This was accompanied with proteomic analyses of human and rodent beta cells. Currently, beta cell surface proteomic analysis further identified known and unknown proteins that are implicated in the adaptive response of beta cells to acute and chronic nutritional challenges.

Hypothesis and Aims: Despite recent advances in lipidomic analysis and lipid profiling of cells less is known about the distribution and roles of structural lipids and lipid mediators in beta cells under normal and nutritional overload conditions. We hypothesized that glucolipotoxic conditions could greatly impact lipid maps of beta cells and therefore aimed at monitoring lipid turnover in live beta cells (INS-1E cell line).

Methodology: We have employed advanced confocal microscopic imaging techniques and used lipid sensitive fluorescent probes to monitor lipid turnover in these cells under normal and glucolipotoxic conditions

Results: Our results on membrane structure and organization with the fluidity sensitive probe Laurdan identified preferential effects of hyperglycemia and saturated fatty acids (*i.e.*, palmitic acid) on the fluidity of insulin granule membranes, whereas the plasma membrane was less affected. Imaging of live cells under similar experimental conditions with the solvatochromic Nile Red probe allowed for real time quantitative analysis of lipid storage and lipolysis pathways by confocal spectral imaging of intracellular micropolarity. This approach enabled real-time detection of lipid turnover, triglyceride biosynthesis and generation of lipid droplets within live beta cells exposed to nutritional stresses.

Conclusions: The complex lipid maps that are generated by these analyses add to the proteomic analyses of beta cells the potential to decipher the continuous crosstalk between lipids and protein expression and function in normal and dysfunctional beta cells.

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Challenges in Proteomics

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The presentation will give an overview on developments and changes in methodology in proteomic investigations over the years. Improvements will be reviewed, making the approaches more sensitive and enlarging the spectrum of detectable molecules (pI, MW, hydrophobicity). Method refinements allowed today's higher sensitivity and throughput, as well as establishing public databases which are quite complete for the more common species, but need still further improvement for others. New software tools allow interpretation of larger datasets with pathway analyses, but only for well investigated species or sample types. Gel-based and gel-free methods will be compared, also in view of future challenges. Among those are the detection and further study of so-called proteoforms or protein species, i.e. protein modifications (isoforms, PTMs, size differences), as experience from recent years has pointed out their biological importance. Other challenges still lie in sample collection, reproducibility/statistical analysis, data validation or – due to increased sensitivity – detection of biological variability even under physiological conditions (e.g. development/aging, sex). Awareness of these challenges and avoidance of potential caveats will further improve data quality and interpretation.

Thursday Orals

Biomarker discovery and validation – from shotgun proteomics to targeted methods

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Background. In this keynote lecture, I will give an overview of methodological advances in the area of clinical biomarker discovery and validation. Examples will comprise the discovery of a biomarker for cervical cancer and the validation of a biomarker for chronic obstructive pulmonary disease (COPD).

Hypothesis. Proteomics biomarker research is moving from the discovery stage using shotgun proteomics to targeted protein analysis methods using selected reaction monitoring (SRM) mass spectrometry in combination with liquid chromatography.

Aim. To give an overview of biomarker research starting from data-intensive shotgun proteomics analyses to validated, high-sensitivity SRM-based methods.

Methodology. Biopsies from cervical cancer patients were prepared for laser capture microdissection (LCM). Samples were obtained from cancerous tissue, healthy epithelium and stroma. Differential, shotgun proteomics analysis resulted in detection of a number of upregulated proteins in cancerous tissue. Members of the mini-chromosome maintenancecomplex (MCM) showed strong discrimination providing leads for further validation.The levels of the soluble receptor of advanced glycation end products (sRAGE) werefollowed by validated LC-MS/MS methods in the SRM mode. Serum or plasma samples wereobtained from COPD patients in relation to the stage of disease, the development ofemphysema or acute smoking history. I will describe the development and validation of three LC-MS/MS methods that allow the accurate and precise quantitation of sRAGE at the ng/mL level and relate the results to various phenotypes of COPD.

Results. The MCM-3 protein allows discrimination of cancerous tissue from healthy tissue with high specificity and sensitivity. Recent results show that MCM-3 analysis may be combined with routine screening procedures using cytological samples to reduce the number of false positives. Our work on sRAGE shows that all three LC-MS/MS methods reach the relevant concentration range of 0.1 – 0.5 ng/mL. The methods fulfill all criteria of a validated bioanalytical method according to EMA and FDA guidelines. We show that sRAGE levels

correlate with disease severity (GOLD stage) and that they are affected by acute cigarette smoke exposure prior to sample collection. Initial data indicate that sRAGE may be a valuable biomarker for emphysema development.

Conclusion. The results show that a highly discriminatory protein biomarker for cervical cancer was discovered by shotgun proteomics in extremely small samples obtained by LCM. The results show further that highly sensitivity LC-MS/MS methods can be developed and fully validated according to international regulatory guidelines.

Acknowledgments. The work was supported by the Dutch Cancer Society (project RUG2011-5021) and the Dutch Technology Foundation (program P12-04)

Factor H binding proteins of *Borrelia: immune evasion tools*

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Background. Factor H binding by *Borrelia* has been correlated with pathogenesis as well as with host diversity. Here we present a novel factor H binding protein, an additional member in FHBP (Factor H binding protein) family. With the help of affinity legand binding assay (ALBA), 2-D electrophoresis, peptide mass figure-printing and *de novo* sequencing we confirmed this novel, ~ 19 kDa factor H binding protein of *Borrelia garinii* serotype 4. The protein was unique in this serotype and none of the other serotypes possess this protein.

Methodology. Seventeen *Borrelia* strains were grown in BSK-II medium, washed 5 times with ultra pure water and then resuspended in water containing 1% TFA (Sigma-Aldrich), 1% of nuclease mix and 1% of a protease inhibitor cocktail (GE Healthcare). Suspended cells were sonicated on ice and protein concentration was measured by the Bradford method.

Affinity ligand binding immunoblot (ALBI) was performed to detect factor H binding proteins of *Borrelia*. Unique 19 kDa protein was separated then with 2D gel electrophoresis and subjected for peptide mass fingerprinting (Bruker-Daltonics, Bremen, Germany) as per the protocol of Shevchenko. MALDI-MS data were obtained in an automated analysis loop using an Ultraflex time-of-flight (TOF) mass spectrometer (Bruker-Daltonics) equipped with a LIFT-MS/MS device. Subsequently, selected precursor ions were subject to fragment ion analysis in the tandem time-of-flight (TOF/TOF) mode to obtain the corresponding MALDI-MS/MS spectra. Automated analysis of mass data was performed using the flexAnalysis software (Bruker-Daltonics).

MASCOT peptide search was failed to assign a statistically significant peptide match with 19 kDa protein. Further manual *de novo* sequencing was attempted based on MALDI-MS/MS spectra. Peptide sequences obtained by *de novo* sequencing were submitted to the BLAST search algorithm at the NCBI (http://www.ncbi.nih-gov/BLAST/). As coiled-coil elements were demonstrated to be involved in the presentation of the fH binding sites, putative coiled-coil formation analysis for novel sequences was performed by using PepCoil software (http://bioweb.pasteur.fr/seqanal/interfaces/pepcoil.html).

Results. Using the ALBA, different fHBPs were observed (figure 1), however, a ~19 kDa protein of *B. garinii* serotype 4 was prominant (Lane 4) and was not observed previously by any researcher. When MALDI-MS and MALDI-MS/MS data from the ~19 kDa protein of *B. garinii* ST4 were used to search the NCBInr database, Mascot software failed to find any statistically significant protein hits; nevertheless, manual *de novo* sequencing using MS/MS fragmentation spectra from the peak showing at m/z = 2603.2 in the MALDI-MS spectrum from this sample generated the peptide sequence SNEKLEEDEENEAQQVNSLQNR. The short input BLAST search showed a complete sequence homology with a hypothetical protein of *B. garinii* PBi (Genbank AAU07257). Unfortunately, no information regarding the function and topology of hypothetical protein PBi was available in the public protein databases.

However, *in silico* analysis indicated that there was a high probability of two coiled-coil formations near the C-terminus (120 to 147 and 118 to 152 residues with a probability of 1.00), supporting the role of this novel protein in human fH binding.

Conclusion. The role of factor H binding proteins of *Borrelia* in immune evasion is emmense. We identified novel factor H binding proteins of *Borrelia* species.

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The Pitchfork Strategy. A multi-pronged approach for membrane proteome profiling

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Background and Aims. Roughly a quarter of human genes encode integral membrane proteins (IMPs). Their specific functions and localizations make IMPs attractive drug targets. However, their amphipathy, lack of trypsin cleavage sites and their relatively low expression levels complicate proteomic analyses of IMPs. Pheochromocytoma and paraganglioma (PHEO & PGL) are rare neuroendocrine tumors. Our goal is to gather data for a detailed description of membrane proteome of human PHEO & PGL that could help identify new drug targets and diagnostic markers.

Methodology. Among the most effective approaches for proteomic analysis of low-abundant IMPs are "divide and conquer" methods that selectively target either soluble (extra-membrane) or hydrophobic (transmembrane) segments of IMPs. In order to maximize the membrane proteome coverage, we combined both of these two strategies with a standard detergent-based tryptic digest into a multi-pronged "Pitchfork strategy". Specifically, we employed two well-established methods of glycopeptide enrichment (lectin-FASP and SPEG) along with our recently introduced hPTC method (high pH, trypsin, CNBr) which allows identification of IMPs based on their hydrophobic alpha-helices.

Results and conclusions. We apply the "Pitchfork startegy" to the membrane proteome profiling of human pheochromocytoma and paraganglioma (PHEO & PGL) samples. The Pitchfork strategy targets different features of IMPs and allows us to identify broader range of IMPs than the classical proteomic strategies. On average, we identify 800-1300 IMPs in each patient sample. It represents nearly 2000 unique IMPs in all PHEO & PGL samples analyzed to date. Among the identified proteins, we routinely observe numerous so-called "missing proteins".

Acknowledgments. The work was supported by the Ministry of Education, Youth and Sports of the Czech Republic (Inter-Action LTA USA 17148, PROGRESS Q26, NPU II. –LQ1604 and UNCE/MED/016).

Efficient coverage analysis for HCP ELISA assay validation

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The most widely used method to monitor the HCP levels in biologics is Enzyme-Linked ImmunoSorbent Assay (ELISA), which uses a set of polyclonal antibodies to detect HCPs as many as possible. These anti-HCP antibodies are critical reagents in the assay. The antibody coverage assay based on 2 Dimensional Electrophoresis (2DE) and subsequent Western Blot (WB) has been used to evaluate the performance of the anti-HCP antibodies. The result will be expressed as a % coverage of the antibodies to the total number of HCP spots separated by the 2DE (USP <1132> and Ph Eur 2.6.34).
The challenges in the coverage assay are:

1. Sensitive method to detect HCPs as much as possible
2. High resolution to separate the complex mixture of HCPs
3. Reliable comparison between total HCP & WB detection
4. Time consuming in both experimental preparation and output data analysis

We propose an enhanced anti-HCP antibody coverage analysis method, 2D Differential In Blot Electrophoresis (2D-DIBE). The solution is a fluorescent multiplexed methodology, based on proven 2D-DIGE & WB technologies which enable simultaneous detection of total HCP & WB with image acquisition hardware and analysis software. In this presentation we focus on the technical aspect of 2D-DIGE and DIBE technology and how these solve the current challenges of the coverage assay.

Wide and Deep: New Reagents and Workflows for Multiplexed Quantification and Targeted Analysis

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Analytical depth and quantitative precision are fundamental requirements in proteomics. Herewith we present methodologies and data supporting multiplexed approaches for proteome untargeted and targeted analysis.

Quantitative proteomics strategies using Tandem Mass Tag™ (TMT™) reagents enable sample multiplexing and precise measurement of protein abundance. However, successful execution of this workflow includes multiple steps that may require optimization including chromatography, mass spectrometry and data analysis. Therefore to be able to detect and diagnose co-isolation interference, enable MS method optimization and validation, we developed a yeast triple gene knockout (TKO) TMT11plex labeled peptide reference standard. The standard provides users a tool to measure the accuracy, precision and dynamic range of different mass spectrometry approaches, while also functioning as an excellent quality control assessment of the analytical platform performance.

Targeted proteomics analyses based on HRAM parallel reaction monitoring (PRM) measurements have delivered a significant increase in selectivity of measurements, allowing more sensitive endogenous peptide quantification in complex samples. Refined acquisition methods, such as dynamic retention time PRM (dRT-PRM), have enabled larger-scale experiments while still providing exquisite data quality. A new workflow was developed using PRM in combination with Multiplex Immunoprecipitation (mIP) and fast capillary-flow LC separation to perform high throughput monitoring of the mTOR/AKT signaling pathway.

Molecular identification of nitro-tyrosine modification in human eosinophil proteins by proteolytic affinity extraction-mass spectrometry (PROFINEX)

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Background. Tyrosine nitration is a posttranslational modification in proteins which may occur under physiological-conditions, but may be substantially enhanced in diseases associated with oxidative stress. The identification of 3-nitrotyrosine (3NT) residues is important for studies of *in vivo* mechanisms, and the characterization of functional consequences of nitration.

Hypothesis. While immuno-analytical methods suffer from low detection specificity of antibodies, mass spectrometric methods for identification of 3-NT are frequently hampered by low levels of modification, and by possible structural changes introduced by the nitration.

Aim. In this work, antibodies against 3NT have been employed in a new proteolytic affinity-mass spectrometry approach ("PROFINEX") for the direct, molecular identification of nitration sites in eosinophil proteins.

Methodology. Eosinophil proteins were isolated from patients with elevated eosinophils in blood due to chronic bacterial lung infection. For "PROFINEX"-MS approach a sepharose immobilized 3NT antibody column was prepared. The Eosinophil cationic protein and Eosinophil-derived neurotoxin were digested in solution, and the digestion mixtures applied on the affinity column. After 2 hrs incubation, several washing steps were performed to remove non-binding fragments, and then the bound peptides eluted and analyzed by nanoESI-FTICR- MS. In order to estimate the level of nitrated protein, the elution fraction was also subjected to Edman microsequencing.

Results. For ECP protein, the nano-ESI-FTICR mass spectrum of the elution fraction yielded a major doubly protonated molecular ion corresponding to the ECP: 23 - 34 peptide, nitrated at Tyr-33. The elution fragment was analyzed by nano-ESI-FTICR-MS providing most abundant multiply protonated molecular ions of EDN: 29 - 43, nitrated at Tyr-33. Using an additional "PROFINEX" experiment, the elution fraction was subjected to Edman microsequencing which showed a modification yield of approximately 10 % of Tyr-nitrated eosinophil proteins.

Conclusion. The PROFINEX-mass spectrometry approach is a high specific and sensitive tool for the molecular identification of Tyrosine nitration in proteins.

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Synergistic success of proteo-genomics in interrogating exotic biological fluids using a novel high sensitivity Immunoassay

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Keywords: ProQuantum™ Immunoassay, *antibodies, biological fluids*, biomarkers, tears

Quest for key biomarker(s) for diseases has become one of the biggest challenges of today and proteomics alone is not able to provide the answers as it does not have the luxury of amplification of the miniscule quantities that may be present in the biological fluid. Additionally, researchers are turning to exotic human biological fluids for specific indications and further interrogation of an organ or its biological fluid. One such example is the human eye and the tears with their comprehensive biomolecule repertoire which serve as a good source for biomarkers and offer an excellent opportunity for patient stratification in precision medicine. Tear collection is fast, noninvasive and offers a chance to determine ocular well as systemic diseases in patients.

Clinicians and translational research scientists studying the multifaceted etiology of various diseases require powerful investigational tools for targeted interrogation of gene activation, cellular interactions, receptor-mediated effects and intracellular signaling processes. Additionally, 'Proteo-genomics', or the integration of proteomics with genomics and encompassing transcriptomics, is a novel approach that promises to advance translational and clinical research. To this end, **ProQuantum™ High Sensitivity Immunoassay technology** incorporating analyte specificity of high affinity antibody-antigen binding with the signal detection and amplification of real-time PCR, offers a flexible and scalable assay platform enabling a multidimensional analyses of DNA, RNA, and now proteins. ProQuantum™ technology offers a greater sensitivity, better dynamic range than traditional methods, smaller sample consumption (1-2 μL per sample), a faster, simplified workflow and robust data analysis software. It is foreseeable that such a technology will have an integral role in clinical studies for establishing biomarker profiles of various disease status.

The Next Generation of IMS Research Platform

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Background. The Advanced MS Technologies (AMST) is a small group within Waters Global Products, formed in 2017 to develop research products directly with pioneering research groups.

Hypothesis. The Advanced MS Technologies team is dedicated to delivering customer benefit in the research market segment through the rapid evaluation, development and deployment of innovative MS capabilities.

Aim. To develop scientifically relevant research platforms utilising advanced technology

Methodology.The AMST team currently comprises 20 multifunctional specialists which is continually being expanded.

Results. An advanced ion mobility enabled research platform has been developed and applied to numerous application areas. The system will be delivered to collaboration partners in 2019.

Conclusion. The preliminary results illustrate the benefits of high resolution (>500) ion mobility as well as the benefits of the unique acquisition modes the unique geometry fascilitates.

Acknowledgments. This presentation was compiled with the assistance of the AMST Group & Financial Support from Waters Corporation.

Identification of Antibodies by integration of mass spectrometry and DNA sequencing

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Background. Mass spectrometry and whole genome sequencing give the possibility to identify antibodies specific for cancer by integration of techniques.
Hypothesis. By high resolution mass spectrometry and whole genome sequencing of antibody sequences it is possible to find antibodies specific for cancer.
Aim. To identify antibodies that correspond to multiple myeloma (Zajec et al., 17, 1326, 2018; J. Prot. Res.) and paraneoplastic syndrome (HuD) (VanDuijn et al., 8, 1286, 2017; Frontiers in Immunology).
Methodology. We developed a multi-omics technique to identify antibodies that can be used for detection of multiple myeloma during minimal residual disease.
Results. Proteotypic M-protein peptides as well as unique peptides derived from therapeutic monoclonal antibodies were targeted in serum of patients with multiple myeloma. We address the sensitivity in M-protein diagnostics and show that our mass-spectrometry assay is more than two orders of magnitude more sensitive than conventional M-protein diagnostics. The use of stable isotope-labeled peptides allows absolute quantification of the M-protein and increases the potential of assay standardization.
In another eexample the combination of NGS and proteomics analysis show that immune responses result in antibody sequence fragments that can be shared among subjects exposed to the same immunogen. NGS data provide a much deeper view on the immune repertoire, as well as an improved sequence accuracy. The proteomics data, however, still allows us to focus on an antigen-specific subset of immunoglobulin sequences in serum. This gives possibilities for diagnostics as well as potential therapeutic applications.
Conclusion. A simple (proteomics) assay for the presence of such an amino acid sequence could function as a proxy for a variety of immunological conditions, such as response to disease (multiple myeloma), vaccination, pathogen infection, auto-immune disease, or a response against tumor-associated antigens.

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Cytokine profiling in melanoma patient serum for monitoring of cancer progression

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Background. Malignant melanoma is a skin cancer with permanently increasing incidence and unfavourable prognosis in advanced stages. Interestingly, partial spontaneous regression has been observed in up to 50% of melanoma lesions suggesting a significant role of immune system in melanoma control.

Hypothesis. Cytokines are key factors regulating immune response and intercellular communication in tumour microenvironment. Blood serum cytokines may reflect the response of immune system to melanoma.

Aim. Proteomic profiling of cytokine levels in melanoma patient blood serum and identification of cytokines reflecting melanoma stage and generalization.

Methodology. Levels of 31 cytokines, chemokines and growth factors in blood serum of malignant melanoma patients and controls have been analysed using the Human Cytokine Magnetic 30-Plex Panel and CXCL1 Human ProcartaPlex™ Luminex xMAP® bead assays. Relation between levels of studied factors and Breslow index, generalization of tumours and clinical stage was evaluated. Expression of selected cytokines and their receptors in tumour sections, cultured malignant and stromal cells was evaluated by immunochemistry.

Results. Growth factors HGF, G-CSF and VEGFA, chemokines RANTES and IL-8 and cytokines IL-6, IFNα, IFNγ, and IL-1RA were discovered to reflect melanoma clinical stage and generalization. Both cancer cells and stromal elements participate in the production of such proteins.

Conclusion. This study demonstrates that malignant melanoma formation and generalization is reflected in serum samples. Secreted factors identified in this study are able to influence the tumour growth, its metastasising and anticancer activity of immune system.

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Proteomic approaches for the evaluation of natural products in cancer prevention and therapy

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Background. Research into natural products has experienced a certain resurgence recently. One reason for this interest is due to the growing evidence supporting the potential application of natural products as agents for cancer prevention and treatment.

Hypothesis. Natural products can regulate cellular signalling pathways, as well as down-regulate the expression of oncogenic miRNAs and up-regulate the expression of tumour suppressive miRNAs.

Results. By modulating these key processes, natural products can inhibit cancer cell growth and cancer stem cell (CSC) renewal, therefore deterring tumour progression and development. Furthermore, by targeting and inhibiting CSC, natural products could prevent the emergence of drug-resistant tumours. However, additional *in vitro* and *in vivo* studies and clinical trials are required to achieve the true value of natural products for the prevention and/or treatment of cancer.

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Unleashing the power of QTOF technology for proteomics with TIMS and PASEF

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Background. In data dependent acquisition experiments only around 20% of the eluting peptide features are targeted for MS/MS due to limitations in speed, sensitivity and resolution. Recently a novel approach was described, parallel accumulation – serial fragmentation (PASEF), that is able to increase the sequencing speed and sensitivity of MS/MS scans on a TIMS-QTOF setup significantly (Meier et al., JPR 2015, PMID: 26538118 and Meier et al. 2018, bioRxiv, submitted).

Hypothesis. Use of PASEF-TIMS-QTOF-MS/MS in shotgun proteomics experiments should lead to improved number of protein identifications combined with excellent reproducibility for quantification in whole proteome experiments.

Aim. Reducing the required sample amount and increasing the acquisition speed to improve the results in clinical studies.

Methodology. In a TIMS-QTOF instrument ions are accumulated for a user-defined time and released from the TIMS device depending on their mobility cross section. By applying the PASEF method multiple precursors per TIMS scan were selected by sub-millisecond switching of the quadrupole isolation window. Raw data were analyzed using DataAnalysis (Bruker Daltonics) and MaxQuant (MPI of Biochemistry). Different accumulation and release times (25-100 ms) corresponding to median ion mobility resolutions of up 78 were tested. The sensitivity was improved by targeting low abundant precursor ions several times.

Runtimes of only 5.6 min were performed by connecting with a novel nanoLC separation technology (Evosep, Odense, Denmark).

Results. As a reference test for the benefit of the high speed and sensitivity of PASEF, just 100 ng of a human cancer cell line (HeLa) protein digest were analyzed in a 90 min nanoLC gradient. From this run almost 40.000 peptides were identified resulting in more than 5000 protein identifications. With only 3 ng of HeLa digest still around. 1700 proteins could be detected.

Application of PASEF allows the selection of 12 precursors within 100 ms release time: this corresponds to approx. 120 MS/MS spectra per second and demonstrates the fast targeting of peptide features. With a novel nanoLC separation technology (Evosep) extremely short runtimes of only 5.6 min were performed, still resulting in the identification of 1400 proteins reproducibly.

Conclusion. The use of a dual Trapped Ion Mobility Spectrometry (TIMS) analyzer in a last generation UHR-QTOF architecture allows to reach MS/MS sequencing speed exceeding 100 Hz while improving the overall sensitivity and maintaining an ultra-high resolution at all MS/MS speed. Due to TIMS separation, the global peak capacity and hence selectivity of the system is also increased.

The low sample amount of the approach opens the method to real clinical applications with biopsies or laser-dissected material. Analysis after cell sorting and even single cell analysis are in reach.
This speed as well is suitable for the analysis of large sample cohorts, like in clinical studies.

Selection of Collision Energies in Tandem Mass Spectrometry Based Proteomics

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Background. The collision energy is the most important single parameter influencing the success of MS/MS-based peptide identification. In the present work we will study the energy dependence of peptide identification scores and determine the optimal collision energy.

Hypothesis. We believe that the collision energy in most proteomics experiments is not ideal. Conventional approaches to optimize the collision energy seem to be inadequate.

Aim. Our aim is to establish new criteria for optimizing the collision energy, and map the influence of collision energy on peptide identification and on sequence coverage.

Methodology. Tryptic digests of complex standards (*HeLa, E. Coli*) were subject to nano-HPLC-MS/MS experiments at various collision energies on a Bruker Maxis II ETD QTOF instrument. Spectra were matched against the SwissProt database using the Mascot search engine. MASCOT score vs collision energy curves were compiled and fitted using an in-house developed software.

Results. Cluster analysis of Mascot score vs. energy curves revealed two different curve shapes; one characterized by one maximum, the other by two separate maxima (unimodal and bimodal curves). For improved accuracy, optimum collision energy was determined by approximating the curve shape by one or two Gaussian curves. The lower energy maxima can be associated with the highest abundance of b ions, while the higher energy maxima are related to the optimum for y ions in the MS/MS spectra.

Conclusion. An unprecedented bimodal behavior of the score vs. energy curves, linked to the different behavior of y- and b-type ions, was identified for about half of the peptides. For these, collision energy optimization can increase the average score by ~15%, improving both sequence coverage, peptide identification scores and protein identification.

Acknowledgments. The work was supported by National Research, Development and Innovation Office (NKFIH K-109006). Á.R. and L.T. were supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences.

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Characterising Extracellular Vesicles with Nanoparticle Tracking Analysis

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The study of Extracellular Vesicles (EVs) has attracted significant amount of interest over the past few years. EVs from different sources may be useful biomarkers for a range of different diseases such as pre-eclampsia and cancer.

Nanoparticle Tracking Analysis (NTA) is a valuable technology within the field of EVs research, providing both high resolution size data and a number concentration. There is a growing interest in using fluorescence labelling to gain a better insight into the samples (F-NTA). F-NTA has been successfully employed to identify the presence of membrane containing particles which provides confidence in the purity of the sample. The use of antibodies and Quantum Dots allowed EVs exhibiting specific epitopes to be measured. Using this approach EVs of interest were able to be characterised from the total population. This presentation will cover the principles of NTA and how it can be used in the characterisation of EVs. We will also discuss how NTA can be used conjunction with other particle sizing technology to provide a comprehensive and powerful measurement suite.

Malvern Panalytical is a leading provider of scientific instrumentation for the measurement of elemental concentrations, crystallographic structure, molecular structure, remote sensing, rheology, particle size, particle shape, particle concentration and more.

Targeted nanocarriers to ameliorate vascular inflammation

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Background. Inflammation is a common process associated with numerous vascular pathologies. Cell adhesion molecules (CAM), such as VCAM-1 and P-selectin, that are specifically expressed by activated endothelial cells (EC) are promising targets for nanotherapeutic intervention.

Hypothesis. Targeting the inflamed endothelium by coupling peptides with high affinity for CAM to the surface of nanocarriers entrapping anti-inflammatory agents will highly increase their specific binding to activated EC and reduce the cell activation.

Aim. Development of suitable nanocarriers to perform specific and effective delivery of therapeutic agents to dysfunctional EC.

Methodology. We developed and characterized lipid-based nanoparticles directed towards CAM and used them as vectors for specific delivery of small molecule drugs (such as dexamethasone, chemokine receptors antagonists) or small interfering (si) RNA and monitored their anti-inflammatory effects *in vitro* using cultured endothelial cells and *in vivo* using mouse models.

Results. Nanocarriers directed to CAM bind specifically to activated endothelium and are functional in delivering anti-inflammatory compounds to EC, reducing the expression of proinflammatory genes and preventing the monocyte adhesion and transmigration to/through activated EC.

Conclusion. Nanocarriers which transport anti-inflammatory drugs at specific pathological sites in the vasculature are efficient in reducing the inflammatory processes.

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Background Proteins in Human Chorionic Gonadotropin Pharmaceutical Formulations of Different Origin

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Background. Gonadotrophins, including Human chorionic gonadotrophin (hCG), have been used since and for several decades to treat infertility by ovarian stimulation. The hCG is being extracted from urine of pregnant women and it does inevitably contains other proteins secreted into urine. The presence of other proteins varies from batch to batch and it can be significantly high

Hypothesis. Current study investigated the presence of proteins other than hCG in different batches of commercial formulations produced by extraction from urine and by using the recombinant approach. The total protein content varied from batch to batch and a large number of contaminant urinary proteins were identified in all analyzed samples except for the recombinant product.

Aim. Identifying proteins other than hCG in formulations used for in-vitro fertilization (IVF) treatment.

Methodology. The nano HPLC system used in all experiments was an UltiMate nanoRSLC 3000 HPLC system (Thermo Fisher, Germering, Germany), coupled to an QExactive Plus mass spectrometer (Thermo Fisher, Bremen, Germany). Separation column: µPAC™ column, 200cm length, with pillar array backbone at interpillar distance of 2.5µm, C18 (Pharma Fluidics, Zwijnaarde, Belgium), trapping column (trap column) was a PepMap C18; 100 Å pore size; 5 µm particle size; 0.3 mm ID x 5 mm length (ThermoFisher Scientific, Bremen, Germany). Database search was performed using Mascot and the SwissProt Protein database.

Results. For all samples analyzed, active substances were identified as major compounds. However, a number of other proteins were also identified in all samples. The majority of identified proteins were urinary proteins that were not removed during the process of hCG extraction from the raw material.

Conclusion. Products originating from the production process employing recombinant method have significantly less contaminant proteins of human origin than those produced from urine.

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Proteomic alterations induced by poly (2-ethyl butyl cyanoacrylate) nanoparticles

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Background. Microbubbles are well-established contrast agents in bio-medical area, but current formulations are fragile, exhibit a short blood circulation time and have limited targeting capabilities.

Hypothesis. Novel nanoparticle-based microbubbles have been proposed to overcome these limitations. These can both target biomarkers and are drug-loaded, with multimodal imaging properties, improving thus the current state of diagnosis and therapy of atherosclerosis or cancer.

Aim. To assess the action of poly (2-ethyl butyl cyanoacrylate) nanoparticles (NP) on the proteomes of different cell lines and murine hepatic tissue.

Methodology. Cultured EAhy926 human endothelial, RAW 264.7 murine macrophages and BxPC-3 pancreatic adenocarcinoma cells were incubated for 2 h in the absence or presence of 20 μg/ml NP. The cells were solubilized and suitably processed for mass spectrometric (MS) analysis using the EASY–nLC II - LTQ Orbitrap Velos PRO MS (Thermo Scientific). C57Bl mice were injected retro-orbitally with 40.8mg/kg body NP containing modified Nile Red fluorophore and subjected to the IVIS Spectrum *in vivo* Imaging System (Perkin Elmer). MS analysis was also performed on hepatic tissue harvested 6 hours post injection.

Results. Protein inference, label-free relative quantification and data mining proteomic *in vitro* analysis revealed the significant over-representation of spliceosome, ribosome, and protein processing in endoplasmic reticulum signaling pathways. The *in vivo* fluorescence detection revealed a maximum intensity of the signal in the liver at 6h post-injection. Also, the hepatic proteomic profile demonstrated a spectral abundance alteration of proteins implicated in response to stimulus, regulation of biological processes and metabolic processes.

Conclusion. The results suggest an altered protein biosynthetic machinery of cells exposed to NP, while the *in vivo* imaging experiments demonstrated a temporal bio-distribution and clearance of NP. Also, protein pre- and post-adsorption studies revealed the importance of NP protein corona composition for their *in vitro* and *in vivo* dynamic action.

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Friday Orals

One carbon metabolism and protein methylation. Implications in liver diseases

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Background. Primary liver cancer (HCC) is recognized as the fifth most common neoplasm and the second leading cause of cancer death worldwide. Although most risk factors are known, and the molecular pathogenesis has been widely studied the underlying molecular mechanisms remain to be unveiled. This is a central issue to facilitate the definition of novel biomarkers and clinical targets for more effective patient management.

Hypothesis. One carbón metabolism (1CM) is central for the maintenance of differentiated hepatocytes as it is considered the connection between intermediate metabolism and epigenetic regulation.

Aim. To assess the role of 1CM impairment in liver disorders and HCC and to investigate the underlying mechanisms.

Methodology. iTRAQ isobaric labeling was used to investigate the differential proteome associated to a methylthioadenosine phosphorylase deficiency (MTAP, SkHep1 cells). Differential methylproteome was studied by R-methyl peptide enrichment followed by LC-MS/MS analysis; measurements were done in a 5600 triple TOF. Targeted SRM assays were designed for 12 1CM enzymes; measurements were don in a 5500 QTRAP; data analysis was done with Skyline.

Results. iTRAQ analysis of SkHep1 deficient and non-deficient cells resulted in the identification of 216 differential proteins ($p<0.05$) that suggest deregulation of cellular pathways as those mediated by ERK or NFκB. R-methyl proteome analysis lead to the identification of 74 differentially methylated, including 116 new methylation sites. Inhibition of RNA binding proteins methylation is especially relevant upon accumulation of MTA in MTAP deficient cells. The phenotype associated with a MTAP deficiency was further verified in the liver of MTAP+/- mice. Measurement of MAT1A, MAT2A, MAT2B, GNMT, SAHH, CBS, CGL, BHMT, CHDH, MTAP, SHMT and DYR by SRM revealed a tissue specific expression profile. Pathway remodelling was detected in the liver of mice fed with high fat and choline and methionine deficient diets, upon CCl4 induced liver injury and in liver tumours.

Conclusion. In light of our data, it is tempting to suggest that the lack of MTAP is a bad prognostic hallmark in cancer. The systematic monitorization of one carbon metabolism in the liver may probe its usefulness for the assessment of liver parenchymal cells homeostasis.

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Micro-Pillar-Arrayed Column (µPAC) for Proteomics

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Background. Recently, a new type of commercially available separation columns manufactured from a silica wafer using lithographic micromachining techniques. According to the manufacturer: "The lay-out of the stationary phase support structure is carefully designed. The perfectly ordered backbone of the separation bed is formed by etching interstitial volumes out of a silicon wafer".

Hypothesis. As opposed to conventionally packed separation columns, the micro Pillar Array Column (PAC) is being produced using micromachining of designed chromatographic beds into silicon. The use of this manufacturing approach enables an almost perfect order of the pillars as opposed to packed columns where the column packing never can be perfect.

Aim. Increasing the peak capacity of the separation system and improving the number of protein identifications.

Methodology. The nano HPLC system used in all experiments was an UltiMate nanoRSLC 3000 HPLC system (Thermo Fisher, Germering, Germany), coupled to an QExactive Plus mass spectrometer (Thermo Fisher, Bremen, Germany). Separation column: µPAC™ column, 200cm length, with pillar array backbone at interpillar distance of 2.5µm, C18 (Pharma Fluidics, Zwijnaarde, Belgium). PepMap C18 nano separation column (75µmID x 50 cm length, 3µm particle size, 100Å pore size, ThermoFisher Scientific, Bremen, Germany) and the trapping column (trap column) was a PepMap C18; 100 Å pore size; 5 µm particle size; 0.3 mm ID x 5 mm length (ThermoFisher Scientific, Bremen, Germany).

Results. Highly reproducible separation of tryptic peptides was achieved at higher flow rates but significantly lower pressure as compared to packed columns. The number of identified proteins is comparable to the 75 µm x 50 cm nano column and surpasses it. The overall system's stability is higher in terms of reproducible retention times and stable column pressure.

Conclusion. The results show that µPACs can be successfully applied for analysis of proteomic samples.

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Branched-chain and aromatic amino acids in diabetes-application of metabolomics in clinical settings

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Background. The metabolome is the terminal downstream product of the genome and consists of the total complement of all metabolites in a biological specimen. Branched chain amino acids (BCAA): leucine, isoleucine, valine and Aromatic amino acids (AA): phenylalanine, tryptophan, tyrosine, histidine were shown to be associated with insulin-resistance in pre-diabetes and to decrease following treatment with insulin-sparing agents or bariatric surgery in patients with type 2 diabetes (T2D).

Hypothesis. In patients with long-standing T2D already treated with insulin, BCAA and AA could be associated with higher insulin requirements due to higher insulin-resistance.

Aim. To compare the concentration of BCAA and AA between tertiles of insulin dose/kg body weight and to establish possible correlations between BCAA and AA and clinical parameters in a group of patients with insulin-treated T2D.

Methodology. Patients with T2D treated with insulin for >6 months and with stable insulin doses (±10%) within 3 months before inclusion were enrolled in this study. Medical history and routine biochemical parameters including C-peptide were collected for each patient. Concentrations of BCAA and AA were measured in fasting conditions by advanced technique ultra-high performance liquid chromatography coupled with mass spectrometry UHPLC-QTOF-(ESI+)-MS.

Results. The study group consisted of 80 patients with insulin-treated T2D, of whom 58.8% were women, mean age 63.8±9.0 years and diabetes duration 12.9±7.0 years, median C-peptide 0.3 ng/ml. No correlations were found between individual concentrations of BCAA and AA or between sum of BCAA and AA expressed as Z-scores and insulin dose/kg of body weight, respectively.

Conclusion. The preliminary results suggest that in patients with long duration of diabetes and low beta-cell residual function evaluated by levels of C-peptide, BCAA and AA are not associated with insulin requirements.

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Studying Proteomics with Microcalorimetry

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Essentially all biological processes involve inter- and intramolecular interactions. The forces involved in these interactions are quantitated by thermodynamic parameters. Beyond thermodynamics, binding kinetics also provide a measure of the strength and nature of these interactions, which is critical to understanding the complete structure-function description of biomolecular interactions.

Microcalorimetry is an ultrasensitive technique that provides direct observations of thermal changes in a sample during these interactions. Differential scanning calorimetry (DSC) is used to study intramolecular interactions, such as protein unfolding and stability. Isothermal Titration Calorimetry (ITC) measures the enthalpy change, binding affinity, and stoichiometry of an intermolecular binding reaction, such as a protein-ligand or antibody-antigen interaction. This presentation will outline how these techniques are used in basic research, and drug discovery and development. We will also discuss how the data from these techniques can be combined with other biophysical characterization tools to better understand the implications of these properties on your research.

Integrating Redox Effects in Analysis of Biological Systems

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Background. Redox reactions govern all life on Earth and underlie development of many diseases.
Hypothesis. Development of reagents and methods for tracking redox processes in biological systems is critically needed to advance biomedical research and clinical care.
Aim. We aim to develop chemical tools for labeling sulfenylated proteins (-SOH) compatible with imaging (including PET imaging), mass spectrometry, flow cytometry, and Western blot workflows.
Methodology and Results. Multiple methods will be presented with associated results for select cases where these chemical tools were applied in biomedical and clinical research.
Conclusion. The results will highlight the value of combined chemical and high-end technologies in redox biology and medicine.

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Introducing ion mobility tandem mass spectrometry in glycoproteomics and glycolipidomics of human biopsies

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Background. Ion mobility separation mass spectrometry (IMS MS) opened new directions for structural analysis of biomarkers, due to its fundamental characteristic to separate isomers, isobars and conformers.

Hypothesis. Several studies have shown that the modified expression and also the elevated concentrations of glycopeptides and glycolipids in human patient biopsies as compared to healthy controls, serves for a disease diagnosis.

Aim. Implementation of a superior bioanalytical platform based on electrospray ionization (ESI) ion mobility mass spectrometry (IMS MS) in combination with collision-induced dissociation (CID) for mapping and structural analysis of glycopeptides and glycolipids (gangliosides, GGs) expressed in various human biopsies.

Methodology. The samples, dissolved in pure methanol, were infused into a Waters Synapt G2s at 2µL/min flow rate and 2 kV ESI voltage. To enhance the separation, IMS wave velocity was set at 650 m/s, IMS wave height at 40 V and IMS gas flow at 90 L/min. TOF analyzer was operated in the V-mode with an average resolution of 20,000. CID was performed after mobility separation in the transfer cell, using energies between 10 and 45 eV.

Results. The 2D plot of the GG mixture revealed not just their separation based on the charge state, but also on the carbohydrate chain length and the degree of sialylation. IMS MS technique in combination with collision induced dissociation (CID) MS/MS provided new insights into the ganglioside and glycopeptide composition and structure in the studied matrices. Moreover, the discovery of species modified by CH_3COO^- raises a series of issues regarding the cholinergic activity of GGs. Ion mobility separation following glycopeptides precursor ion sequencing by CID in the transfer cell was able to provide the nature and detailed structure of the isomers from a series of possible candidates.

Conclusion. The results demonstrate that IMS MS is a powerful and highly efficient technique able to unequivocally detect and characterize complex glycopeptides and glycolipids with potential biomarker role and discover new structures.

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Characterizing the molecular mechanism of the multifunctional antitumor compound withaferin A in a multiple myeloma model

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Despite progress and success in chemotherapy, many types of cancer remain largely incurable. Most often this is due to the development of (multi)drug resistance, a phenomenon characterized by cancer cells who manage to withstand the most powerful chemotherapies available during an acute anticancer response. In this respect, interest has recently focused on multifunctional chemotherapeutic compounds that simultaneously target a series of pathways, and by extend networks, peculiar to cancer development. Withaferin A (WA), a steroidal lactone from the plant Withania Somnifera, is such a multifunctional compound with promising antitumor responses.

To examine the pathways on which WA is acting, we used a multiple myeloma (MM) disease model for proteomics experiments, followed by pathway analysis with IPA.

A MM1R cell line was SILAC labeled and treated with- or left without WA to examine the WA effects on the cancer proteome. Two main approaches consisted of a differential expression experiment resulting in 121 upregulated and 77 downregulated (≤ 2 fold difference) proteins and a chemoproteomics approach resulting in 131 direct protein-WA interacting partners. Combining these data with the Ingenuity Pathway Analysis (IPA) database resulted in many influenced canonical pathways, molecular functions and diseases including protein ubiquitination, NRF2 mediated oxidative stress response and Waldenstrom's macroglobulinemia.

Further validation of the high throughput data was based on these IPA output and confirmed that WA causes an accumulation of ubiquitinated proteins, most likely as a result of targeting the proteasome. Also, the upregulation of the NRF2 stress response gene Heme Oxygenase 1 (HMOX1) after WA treatment was confirmed, suggesting that WA probably puts the already stressed cancer cells under additional (fatal?) stress. Interestingly, 12 proteins were assigned to involve Waldenstrom's macroglobulinemia and 9 out of those 12 were influenced by WA in a way that would be beneficial for the disease outcome. For example, we showed by Western blot that annexin A4 (ANXA4), a calcium/phospholipid-binding protein which promotes membrane fusion, is downregulated after WA treatment, whereas it is seen upregulated in the disease. Other proteins within this Waldenstrom's macroglobulinemia "network" will be validated in the near future.

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Histone deacetylases as potential therapeutic targets in atherosclerosis

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Aim: Acetylation of histone and non-histone proteins, controlled by histone acetyltransferases and histone deacetylases (HDAC), plays a major in regulation of gene expression. Evidence exists that HDAC inhibitors display beneficial effects in several experimental models of cardiovascular diseases. The role of HDAC isoforms in atherogenesis is not completely understood. The aim of this study was to investigate the expression profile of representative HDAC isoforms in human and experimental atherosclerosis and to evaluate the potential anti-atherosclerotic effects of HDAC inhibition in ApoE-/- mice.

Methods: Human non-atherosclerotic and atherosclerotic samples and ApoE-/- mice were used. The mice, maintained on normal (ND) or high-fat cholesterol-rich diet (HD), were randomized to receive vehicle/suberoylanilide hydroxamic acid (SAHA). Oil red O was employed to assess lipid depositions. The expression of HDAC subtypes, oxidative stress and inflammatory markers were determined by Western blot /microscopy.

Results: The protein expression levels of HDAC1, HDAC2, HDAC3, HDAC4, HDAC6, and HDAC11 isoforms were significantly induced in human atherosclerotic samples. Immunohistochemical staining of atherosclerotic lesions revealed that HDAC proteins are up-regulated within media and macrophage-rich areas. The increased expression of HDAC proteins, correlated with the severity of atherosclerotic lesions, was found in the aortas of ApoE-/- mice. Treatment of ApoE-/- (HD) mice with SAHA down-regulated the markers of oxidative stress (Nox1, Nox4, 4-HNE), vascular inflammation (NOS2, MMP9, CD68, CD45), matrix deposition and remodeling (fibronectin, MMP9), cell proliferation (PCNA), and reduced the extent of atherosclerotic lesions.

Conclusion. Our study provides evidence that pharmacological inhibition of HDAC may be an important therapeutic strategy in atherosclerosis.

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From low-resolution toxin-pattern recognition to toxin-resolved venom proteomes: New approaches in evolutionary and translational venomics

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Venoms are integrated phenotypes used by a wide range of organisms for predatory and defensive purposes. The study of venoms is of great interest in diverse fields, such as evolutionary ecology and biotechnology. On the other hand, legitimate snake bites caused to humans in their share natural environment is a serious neglected public health issue that disproportionaly affects the most impoverished and geopolitically disadvantaged rural communities of tropical and subtropical countries. This occupational and environmental "disease of poverty" affects 1.8-2.7 million people worldwide each year, causing 85-138.000 deaths, maiming 400.000 victims, and leaving an indeterminate number of survivors with psychological sequelae and other posttraumatic stress disorders. Toxins bearing the highest prey incapacitation activity are often also the most medically important molecules in the context of a human envenoming. Ecological and translational venomics are two sides of the same coin. Thus, understanding the complexity of venoms and their locus-resolved toxicological profiles can shed light on the mutually enlightening relationship between evolutionary and clinical toxinology to identify those toxins that should be neutralized to reverse the psathological effects of venom. Omics technologies have contributed to understanding the evolutionary mechanisms that molded snake venoms to their present-day structural and functional variability lanscape. The recent implementation of top-down MS and absolute quantification of intact proteins by elemental mass spectrometry promise to represent a quantitative leap in the transition to achieve toxin-resolved venom proteomes. The quantitative analysis of an antivenom's ability to recognize and neutralize the toxins present in medically relevant snake venoms represents the cornerstone on which its preclinical qualification should be based. The recently developed immunoaffinity chromatography-based third-generation antivenomics (3GA) platform allows the toxin-resolved determination of the maximal toxin-binding capacity of an antivenom and to quantify the percentage of clinically effective antibodies present in a given antivenom. The conceptual and operational principles of 3GA will be discussed and illustrated with recent representative examples.

Discrimination of oral cancer from normal oral mucosa by MALDI mass spectrometry imaging of proteins and lipids

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Background. Identification of molecular biomarkers for classification of cancer and differentiation between cancerous and normal epithelium remains a vital issue in the field of head and neck cancer.

Aim. To compare the ability of proteome and lipidome components to discriminate oral cancer from normal mucosa.

Methodology. Tissue specimens including squamous cell cancer and normal epithelium were analyzed by MALDI mass spectrometry imaging (MALDI-MSI). Two molecular domains of tissue components were imaged in serial sections – peptides (resulting from trypsin-processed proteins) and lipids (primarily zwitterionic phospholipids), then regions of interest corresponding to cancer and normal epithelium were compared.

Results. Heterogeneity of cancer regions was generally higher than heterogeneity of normal epithelium, and distribution of peptide components was more heterogenous than the distribution of lipid components. Moreover, there were more peptide components than lipid components that showed significantly different abundance between cancer and normal epithelium (median of the Cohen's size effect was 0.49 and 0.31 in case of peptide and lipid components, respectively). Multicomponent cancer classifier was tested (vs. normal epithelium) using tissue specimens from three patients and then validated with tissue specimen from the fourth patient. Peptide-based signature and lipid-based signature allowed cancer classification with weighted accuracy 0.85 and 0.69, respectively. Moreover, both classifiers had very high precision (0.98 and 0.94, respectively).

Conclusion. We concluded that though molecular differences between cancerous and normal mucosa were higher in proteome domain than in lipidome domain, imaging of lipidome components also enabled discrimination of oral cancer and normal epithelium. Therefore, both cancer proteome and lipidome are promising sources of biomarkers of oral malignancies.

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Application of tissue proteomics for understanding the prostate cancer initiation and progression

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Background. Prostate cancer (PCa) is the second most frequently diagnosed cancer in men and the fifth leading cause of cancer death worldwide. Despite the use of prostate-specific antigen test and number of tests that appeared in recent years there are major concerns in the clinical management of PCa such as lack of efficient test for disease detection, lack of efficient test for disease progression and urgent need for development of effective treatments against lethal castration-resistant prostate cancer (CRPC).

Hypothesis. Proteomics analysis of prostate tissue can point out valuable biomarkers for early diagnosis, cancer progression and new drug targets for management of CRPC.

Aim. In this study we focused on molecular characterization of proteins associated with development and progression of PCa.

Methodology. Proteomics profiling of PCa tissue samples ranging from Gleason 6-9 obtained with radical prostatectomy was done on HDMS QTOF using label-free data independent acquisition.

Results. The proteins with altered abundance among groups were identified based on 2 or more peptides with fold change of 1.5 or more and statistical significance ($p \leq 0.05$) was corrected for multiple testing. The altered proteome was analyzed in terms of functional annotation, pathway analysis using IPA and correlation with clinical data. Proteins with altered abundance in PCa showed the highest significant correlation with immune response, lipid metabolism and oxidative stress response. Subset of these proteins exhibited consistent regulation trend (up- or down-) with cancer progression (Gleason from 6-9). These proteins are intracellular, ubiquitously expressed, and involved mostly in cell communication/signal transduction, cell growth and energy pathways. Majority of them have association and/or represent prognostic markers for different cancers, but up to our knowledge none has been associated with PCa so far.

Conclusion. The identified biomarkers are worthy of future pursuit as potential indicators of PCa initiation and/or progression towards aggressive forms.

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The Bioluminescent HiBiT Technology for CRISPR-Mediated Gene Tagging

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Background. A common approach for investigating signaling mechanisms and the effects induced by synthetic compounds is through overexpression of recombinant tagged proteins. Nevertheless, it is well known that overexpression can cause artificial effects.
Hypothesis. Novel new method based on the combination of a new bioluminescent protein tagging system and CRISPR/Cas9 was developed to overcome these difficulties. This enables the cloning-free generation of endogenous protein assays and allows the simple quantification of intra- and/or extracellular protein levels after experimental manipulation under physiological conditions.
Aim. Study the proteins within their native environment by tagging of endogenous genes through CRISPR.
Methodology. A new protein tagging and detection method – the HiBiT system was developed. The technology is based on a split version of NanoLuc luciferase. The small tag size of HiBiT and the ability to measure protein expression at endogenous levels makes HiBiT well suited for CRISPR-mediated genome editing. We therefore, established a straightforward, cloning-free and rapid CRISPR/Cas9 protocol for the specific insertion of HiBiT into the genome. The applicability of this newly develop method is shown for hypoxia inducible factor 1A (HIF1α). The importance of low endogenous levels of expression in assay response is demonstrated.
Results. HiBiT is a tiny, bioluminescent, peptide tag that can be either expressed from a traditional expression vector or inserted into endogenous genes with a cloning-free CRISPR/Cas9 protocol. We were able to quantify proteins even at low endogenous expression levels in an antibody-free fashion with an exceptionally broad dynamic detection range. Here we present various applications of the HiBiT system such as the setup of protein stabilization/degradation assays (i.e. transcription factors, PROTACS, autophagy…) and GPCR internalization assays.
Conclusion. HiBiT can be rapidly and efficiently integrated into the genome to serve as a reporter tag for endogenous proteins within their native environment under the physiological conditions.

Personalized cancer immunotherapy using GM-CSF to activate the body defense

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Despite the excitement of novel cancer therapies, many fall short of effectiveness against solid tumours in the human body including the CAR-T (chimeric antigen receptor -T cell) therapy, various combination therapies and cancer vaccines. With majority of the tumour antigens being self-antigens, the circulating patient tumour cell repertoire has developed sophisticated mechanisms to avoid detection and elimination as well as tolerance and an ability to grow, divide and spread without control in their tumour microenvironment. In addition to this, many tumours such as pancreatic cancer, ovarian cancer, lung cancer, and head and neck cancers are often detected at a very advanced stage, adding a further challenge to an effective treatment. Sad fact remains that the majority of cancer patients with metastatic or advanced cancer will die.

The ultimate “immunogen” remains the entire cancer cell itself. It comprises the entire set of targets. This together with sustained production of the immune-boosting white blood cell growth factor GM-CSF at the site of vaccination with appropriate antigens could triggers an immune response against the patient’s cancer cells, facilitating the destruction of the tumour as well as ‘firing-up’ the patient’s immune system, possibly leading to a cure rather than just prolonging life expectancy.

A cancer immunotherapy approach will be presented which facilitates the patient's own immune system to recognize parts of the tumour cells as targets to be destroyed. As opposed to other anti-cancer therapies, such an approach may help destroy the tumour and allow the patient to heal in a more natural way with a positive outcome.

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Proteomic and bioinformatic analyses of bone healing using titanium implant with bioactive targeted surface in a rat tibial defect model

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Background. Titanium (Ti) based implants are successfully used as biomedical devices due to their excellent mechanical and biocompatibility properties. However, impaired fracture healing may occur for different causes, leading to delayed unions or non-union fractures.

Hypothesis. The stratified co-incorporation of FGF2, VEGF and BMP4 in a polymer matrix applied on the Ti surface using dip coating can induce an improved healing process than the individual action of the biomolecules.

Aim. To test the regenerative potential of titanium implant with bioactive surface and to identify downstream protein effectors involved in bone healing in a tibial defect in rat.

Methodology. Eighteen adult male Wistar rats were randomly divided into 6 groups. The control (C) group received Ti implants without growth factors, three groups had implants individually coated with FGF2, VEGF and BMP4 and the multi-component implants containing FGF2/ VEGF and FGF2/ VEGF/ BMP4 were used for the last two groups. The bone tissues were suitably processed for histology and mass spectrometric analysis.

Results. Histological and histomorphometric analysis showed that the incorporation of the three growth factors in titanium adsorbed polymer matrix induced an improved healing process when compared with the other experimental groups. The shotgun proteomic approach allowed the high-confidence identification of 1614 in total out of which 113 were differentially expressed. Additionally, GO Slim Biological Processes analysis of differentially expressed proteins indicated a large number of proteins clustered in the *response to stimulus* class. Further, collinearity between these proteins was performed using Pearson correlation matrices. Next, the STRING database version 10.5 was used to reveal the possible protein networks of the correlated proteins. Based on confidence prediction of the interaction between proteins, we built protein networks visualized using Cytoscape 3.5.1. For the titanium implant designed with a PEG surface containing optimized proportions of FGF2, VEGF and BMP4, the general interaction was very well organized in hub-based networks related with *response to chemical*, *wound healing* and *response to stress* pathways.

Conclusion. The proteomics results of this study allow for a new and in depth insight of the complex healing process, but further investigations are required to truly understand the roles and how the interactions of differentially expressed proteins exert their impact on the repair process of bone fracture.

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Thursday Posters

Western Blotting – the devil is in the details

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Background. Western Blotting is a well-established technique for measuring the amount of particular protein in the sample and is considered as a quantitative technique. Nevertheless, sometimes we forgot about the proper optimization of protein concentration in the sample, and we choose this value by judging the bands intensity "more or less" – with eyes, just looking for the "nice" bands.
Aim. Our aim was to show how measuring in a different protein concentration range may influence the final results.
Methodology. The microsomal fraction from rat liver (protein concentration: 20 mg/ml) was used during the experiments. The sample was prepared by acetone precipitation of proteins, then by centrifugation and mixing obtained protein pellet with the sample buffer. Defined protein concentrations were separated on 10% polyacrylamide gel, and electro-transferred from the gel onto the Immuno-Blot® PVDF membrane. Cytochrome 2C11 was detected using Anti-2C11 antibody, with TMB colorimetric visualization (GelDoc XR+, Bio-Rad).
Results. The curve for the visualization of the relationship between protein concentration and colorimetric results has been plotted. Then, protein concentrations (ratio 2:1) from two different ranges of the mentioned curve were taken, and the procedure was repeated to obtain the colorimetric results for these particular protein concentrations.
Conclusion. Obtained data suggest that the results of Western Blotting analysis should be considered with caution if proper optimization of the procedure was not performed. In our case, in the high protein concentration range, we obtained the value of colorimetric response equal to 1.1 (whereas it should be 2.0). It was expected since the curve of concentration/colorimetric response was not linear here. Nevertheless, in the linear range, we have obtained the value 3.1. Thus, our results stress the necessity of protein concentration optimization before performing a Western Blot analysis and interpretation of the results with caution.

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Profiling of serum metabolome of head and neck cancer patients undergoing radiotherapy

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Background. Cancer radiotherapy (RT) induces the response of the whole patient's body that could be detected at the blood level. Radiation-induced changes in proteome and transcriptome of serum have been widely described and discussed, however, metabolites present in blood have not been given as much attention. Metabolomics analysis of serum of cancer patients could also provide a valuable insight into the response of both tumor and whole organism to the treatment.

Hypothesis. Local irradiation during cancer radiotherapy induces whole-body responses that can be observed at the blood metabolome levels.

Aim. Comparison of serum metabolome profiles in head and neck cancer patients before and after radiotherapy.

Methodology. Serum samples from head and neck cancer patients (10 patients) were taken before (A) and after (B) radiotherapy. Healthy volunteers (10 individuals) were used as a control group. A mixture of MeOH/H_2O was used for extraction of low-molecular-weight metabolites. Samples were analyzed by gas chromatography-mass spectrometry (GC-MS).

Results. Changes in the level of several compounds were observed between samples collected before and after irradiation. There were 8 compounds, whose levels significantly decreased in post-treatment samples: Creatinine, L-Tryptophan, Ethanedioic acid, L-Alanine, L-Isoleucine, L-Proline, L-Leucine, Uric acid. There were 10 compounds, whose levels significantly increased in post-treatment samples: (R,S)-3,4-Dihydroxybutanoic acid, 3-Hydroxybutyric acid, 2,3-Butanediol monoacetate, Glucuronic acid, Galactonic acid, Octadecanoic acid methyl ester, 3-Methyl-2-ketobutyric acid, 3-Methyl-2-ketovaleric acid, 2-Ketobutyric acid, 2-Hydroxybutyric acid.

Conclusion. Radiotherapy caused significant changes in levels of several serum metabolites. This preliminary study will be followed by an analysis of metabolites present in extracellular vesicles (EVs) isolated from corresponding serum samples.

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Analyses of glycolipid-peptide and glycolipid-protein interactions by chip-based mass spectrometry

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Background. Gangliosides are localized in the plasma membrane, with the ceramide (Cer) portion rooted in the lipid bilayer and the oligosaccharide chain protruding freely outside the cell, acting as a receptor. Cholera toxin action is initiated by binding to the target cell surface via interaction between homopentameric B subunit (Ctb5) and the receptor molecules in the microdomains.

Hypothesis. Several studies have indicated that during the binding process, the molecule that serves as the cell-surface receptor of Ctb5 is preferentially GM1.

Aim. To study the noncovalent interaction between the subunit B of Cholera toxin (Ctb) and a native mixture of gangliosides extracted from adult human cerebellum by fully automated chip-nanoelectrospray ionization (nanoESI) on a NanoMate robot coupled to a high capacity ion trap (HCT) mass spectrometer (MS).

Methodology. The interaction assay involved the incubation of Ctb5 and gangliosides dissolved in 10mM ammonium acetate buffer at pH 5.8. This pH value enhanced the dissociation of the monomers constituting Ctb5 and allowed the direct interaction between the monomers and gangliosides. Aliquots of the reaction products were collected directly after 10, 30, and 60min of incubation in the 96-well plate of the NanoMate robot and immediately submitted to MS analysis.

Results. The noncovalent interaction assay followed by chip-based nanoESI MS results suggested for the first time that, exposed to a highly complex mixture of gangliosides under conditions facilitating the interaction, Ctb preferentially binds G1 class, irrespectively of the number of sialic acid residues or other modifications. Moreover, certain species of high sialylation degree or modified by fucosylation were for the first time discovered in human cerebellum.

Conclusion. This platform represents a method of choice not only for assessing the noncovalent interactions but also for the detection and identification of species of low expression in complex mixtures.

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Proteomics discloses effect of saffron stigmata ethanolic extract on restoring viability in HepG2 cells under VCP gene silencing

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Background. Disruption of valosin-containing protein (VCP) is sufficient to cause endoplasmaic reticulum (ER) stress in hepatocytes during ischemia-reperfusion (IR).
Hypothesis. Novel ethanolic extract KAREX [1], obtained from dry stigmata of *C. sativus* L. (saffron) with kaempferol content up to 20% (w/w), have been proposed to overcome the VCP related control of ubiquitination status of recruited substrates initiated by IR in hepatocytes.
Aim. To assess the proteomic action of KAREX on pro-apoptotic cell cycle after siRNA mediated silencing of VCP gene in transfected epithelial-like human hepatocellular carcinoma cells ($HepG_2$).
Methodology. Transient transfection of $HepG_2$ cells by VCP-gene targeting by three smart pool siRNA, incubation with KAREX (100 μg/mL) and viability quantitation by MTT assay. Flow cytometry after staining of DNA fragmentation for assay of apopototic $HepG_2$ cells. Global proteomic analysis using 2-DE coupled with MALDI-TOF-MS. Western immunobloting to confirm protein expression.
Results. Depletion of VCP in $HepG_2$ cells induced the *S*-phase cell cycle accompanied by higher proportion of induced sub-G1 phase peak as the reliable marker of apoptosis. KAREX treatment of VCP depleted $HepG_2$ cells significantly decreased the *S*-phase cell cycle arrest. Proteomic data indicated that VCP modification cause abnormal peroxiredoxins accumulation and ultimately trigger ER-dependend $HepG_2$ cell death.
Conclusion. The preliminary results suggest that KAREX could significantly protect from initiation of early apoptotic cell death in hepatocytes triggered by the VCP abolishment under IR injury condition, thus giving a novel hypothesis on the *in vivo* molecular mechanisms of KAREX in range of common ischemic diseases.

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EYA3 tyrosine phosphorylation by Src kinase. From mass spectrometry to implications in proliferation

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Eyes absent (EYA) are both non-thiol based protein tyrosine phosphatases (PTP) and transcriptional co-activators. They are members of a conserved regulatory network with functions in the development and homeostasis of various tissues and organs. Although the overexpression of EYAs has been reported in several types of cancers and its PTP activity proven to be involved in generating and maintaining these pathologies, their molecular mechanisms need further elucidation.

We hypothesize that EYA's molecular tools for signaling may be represented by both tyrosine phosphorylation and its capacity of autodephosphorylation. To verify these, we used mass spectrometry to study both Src-phosphorylation of human EYA3 and its capacity of autodephosphorylation, and proliferation assays to identify physiological implications for some of the phospho-tyrosine residues detected.

Initially, we identified the tyrosine residues on EYA3 WT and EYA3 D311N that are phosphorylated after *in vitro* incubation with Src at several time points. Successive tyrosine to phenylalanine mutations were made on EYA3 inactive mutant in order to verify the phosphorylation status of the detected sites in mammalian cells. The results obtained, after the quantification of the phosphorylation signal on western blots, revealed that some sites have a major impact and others minor contribution to the total phosphorylation. A set of tyrosine residues were most intensely phosphorylated by Src and they also could not be completely autodephosphorylated. For these tyrosines residues, we further investigated the implication of Src-induced phosphorylation on the proliferation of HEK 293T, MCF-7 and MDA-MB-231cells. The results obtained indicated that the impact on proliferation differs depending on the cell type in which the EYA constructs are expressed.

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Nicotinamide mononucleotide (NMN) effects on mitochondrial OXPHOS protein expression

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Background. Type 2 diabetes, a chronic and progressive disease with continuously increasing prevalence, drains more than 10% of global health budget. Although the molecular mechanism causing insulin resistance is not clear, recent studies show correlations with mitochondrial dysfunctions that may result from impaired mitochondrial biogenesis, deficiencies in mitochondrial electron transport chain (mtETC) or increased production of reactive oxygen species (ROS) in mitochondrial intermembrane space. Some studies suggest that a recent proposed mitochondrial biogenesis pathway (NAD^+/SIRT1/VHL/HIF1α/c-Myc/TFAM) is lost with aging and hypercaloric diet causing insulin resistance and other aging related disease, but can be restored by a short period treatment with nicotinamide mononucleotide (NMN), a NAD^+ precursor.
Hypothesis. NMN restores mitochondrial encoded OXPHOS proteins expression in muscle cells.
Aim. To assess the effect of NMN on mitochondrial OXPHOS proteins expression.
Methodology. Cultured C2C12 myotubes were maintained for two days in normo- respective hyper-glycemic conditions. In the third day, the myotubes were incubated for 24 h in the absence or presence of 100 μM NMN. The myotubes were then harvested and lysed. Proteins form mitochondria enriched fractions extracted by differential centrifugation were purified by chloroform-methanol precipitation. The peptides resulted by trypsin digestion of these proteins were resolved by 2D chromatography (SCX-RP), detected by ESI+ MS/MS Velos Pro linear Ion trap using a Top5 method, identified with Proteome Discoverer and relatively quantified by peptide-spectrum match (PSM).
Results. Most of the components of OXPHOS complex I where up-regulated in normo-glycemic NMN treated myotubes compared to untreated control. In hyper-glycemic conditions NMN treated cells OXPHOS subunits IV and V were over-expressed. Mitochondrial encoded COX2a4 subunit of complex IV is under expressed while all the other subunits (nuclear encoded) were over-expressed.
Conclusion. NMN modulates mitochondrial OXPHOS proteins expression but this study shows results contrary to the NAD^+/SIRT1/VHL/HIF1α/c-Myc/TFAM mitochondrial biogenesis pathway hypothesis.

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Mass spectrometry and T cell analysis reveals that N-glycosylation can impact antigen presentation in melanoma

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Background. Recent epidemiologic studies have revealed that the incidence and mortality of melanoma has globally increased in the past years. Immunotherapy has provided a good alternative to the conventional treatment of melanoma, mainly due to its low toxicity.
Hypothesis. Understanding the factors that can modulate epitope presentation in melanoma is a key-step in the development of new antigens capable of eliciting an increased immune response, in order to eradicate the tumor cells.
Aim. Assess the exact contribution of N-glycosylation located within or outside an epitope to antigen presentation.
Methodology. Tyrosinase N-glycosylation status in melanoma was analyzed using mass spectrometry. A375 melanoma cells expressing different tyrosinase glycosylation mutants were designed and characterized using biochemical methods. The cells were subject to cell surface acid elution for mass spectrometry analysis of a human tyrosinase derived epitope. The peptide was relative quantified in each condition and the results were validated in tumor recognitions assays using human CD8+ T cell clones directed against this epitope.
Results. In agreement with the prediction analysis, we show that human tyrosinase is glycosylated to all seven sites, in A375 melanoma cells. Moreover, the C-terminus glycans appears more important for peptide presentation, especially considering the localization of one these N-glycosylation sites within the epitope.
Conclusion. Tyrosinase is glycosylated in A375 melanoma cells and the position of the glycosylation sites can alter epitope presentation in A375 melanoma cells.

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Methionine-induced hyperhomocysteinemia causes changes in the mouse kidney proteome associated with blood coagulation

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Background. Homocysteine (Hcy) arises from the metabolism of methionine (Met). Inordinate consumption of Met causes hyperhomocysteinemia (HHcy), which is linked to pathologies in the cardiovascular system. However, underlying mechanisms are not fully understood.
Hypothesis. We hypothesize that HHcy induces changes in gene expression that impair kidney homeostasis, which in turn can lead to increased blood coagulation and thrombotic complications.
Methodology. We induced dietary HHcy by providing 3-month old C57BL/6J mice (n=8) with 1% Met in drinking water for 3-months. Control mice (n=8) received a plain water. Kidney proteomes were analyzed using label-free relative quantitative mass spectrometry. Proteins identified by at least 2 peptides and p values <0.05 for Met vs. control mice were considered as differentiating. Bioinformatic analyses were carried out using DAVID resources.
Results. We identified 36 kidney proteins with expression significantly altered by HHcy: 19 up- (e.g., Ctsa, Acat1) and 17 down-regulated (e.g., Fga, Ces1c). One of the KEGG pathways overrepresented in kidneys of HHcy mice is complement and coagulation cascades (12, 7-fold). The GO biological processes analysis revealed that the affected proteins participate in fibrin clot formation and fibrynolysis (e.g., Fga, Fgb, Fgg) .
Conclusion. These findings show that Met-induced HHcy dysregulates kidney proteostasis and induces pro-thrombotic changes that are associated with cardiovascular disease.

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Two strategies for processing of human cerebrospinal fluid prior LC-MS/MS

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Background. Cerebrospinal fluid (CSF) is a complex body fluid which is in direct contact with the central nervous system. This makes CSF an attractive source of potential biomarkers for neurologic diseases. Similarly to blood plasma, proteomic analysis of CSF is complicated by high dynamic range of individual protein concentrations and by the presence of several highly abundant proteins.
Aim. Since there is no specific method for relative depletion of the abundant proteins in CSF, we evaluated the effect of two different methods developed for human plasma.
Methodology. Triplicate samples of pooled human CSF were either immunodepleted using MARS 14 cartridge (Agilent) or subjected to the relative enrichment of low abundance proteins using hexapeptide ligand library known as ProteoMiner (Bio-Rad). All resulting fractions were digested with trypsin and analyzed by standard LC-MS/MS.
Results. LC-MS/MS analysis of crude CSF provided roughly 500 identified protein groups. Immunodepletion of CSF with MARS 14 cartridge increased the number of identifications to nearly 800, while treatment of CSF using ProteoMiner ligand library to only 600. To maximize the number of identified proteins we analyzed also the "waste" fractions generated in both methods: The fraction of proteins retained by the MARS 14 cartridge-contained more than 100 additional proteins not observed in the depleted CSF. Similarly, analysis of the ProteoMiner "waste" flow-through fraction provided 70 unique proteins.
Conclusion. Both strategies significantly increased the number of identified proteins compared to crude CSF. The immunodepletion strategy using MARS 14 enabled us to identify a higher number (800+100) of proteins in the depleted CSF and "waste" fraction compared to ProteoMiner treatment (600+70 proteins). Due to limited availability of CSF samples, it is advisable to prefer MARS 14 technology over ProteoMiner. Additional analysis of the "waste" fraction can significantly increase the number of identified CSF proteins.

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iTRAQ based proteomic analysis of Zika virus infection based on 293T cells.

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Background. ZIKV is a single stranded, positive sense RNA virus transmitted by *Aedes* mosquito. ZIKV infection is connected with various complications, including Guillain–Barré syndrome and congenital microcephaly, if pregnant women are infected. In recent years this virus has been assigned as a public health emergency, because of fast and easy spread into world areas where ZIKV is not endemic.
Hypothesis. 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 have been proposed to study action allowed for providing the proteomic results of great importance in viral infection investigations.
Aim. To find the expression changes in the proteome between samples with active NS3 protease and the control ones, using iTRAQ MS-based analysis.
Methodology. In these studies, a 293T cell model was used in three biological replicates. 293T cells with transfected active NS3 protease (cloned in the *E. coli* model) were compared to control 293T cells with inactive form of this protease (point mutation: $S^{135} \rightarrow A$). The cells were suitably prepared for further proteomic quantitative analyses. Protein labelling was performed according to the manufacturer protocol tor the iTRAQ 4-plex (AB Sciex). For off-line *nano*LC analysis Proxeon EASY-nLC II coupled with Proteineer fc II (Bruker-Daltonics) was applied. Mass spectrometry analysis of peptides in positive ion mode was done using UltrafleXtreme™ MALDI-TOF/TOF mass spectrometer (Bruker-Daltonics). Protein identification was performed using the MASCOT software (Matrix Science) with the aid of UniProt KB database.
Results. iTRAQ proteomic approach was performed to identify the potential targets for NS3 protease or proteins with changed expression evoked by cells proteome interaction with NS3 protease. Among all the identified proteins, the most promising tagets are proteins involved in infection and inflammation processes, responsible for regulation of apoptosis, transcriptional regulation.
Conclusion. Designing the quantitative proteomic approach to study influence of action of the NS3 protease in 293T cells, allowed for providing the interesting results which might have important meaning in viral infection investigations. This results will be base for further research in this field.

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Activity of neuropeptides converting enzymes

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Background. Neuropeptides occur in both central and peripheral nervous systems, where they play important, mediatory role in many processes. Biological activity of various neuropeptides is regulated, among other factors, by converting enzymes, that transform native neuropeptides into products with retained or changed biological activity.
Hypothesis. Because of the function of neuropeptides in neurotransmission and neuromodulation, mechanism for conversion of these compounds have received considerable attention. Identification of the released products is important to understand the pharmacological activity of these peptides and their mechanisms of action.
Aim. This work is focused on measuring proteolytic activities of the converting enzymes specifically acting on the opioid peptides, such as dynorphin B and nociceptin, that allow to investigate, which potential cleavage sites of that neuropeptides are crucial as primary targets of enzyme action. This may also provide a knowledge on the function of these enzymes in the regulation on neuropeptides levels.
Methodology. The spinal cord homogenates was fractionated by ion exchange chromatography. Fractions were collected and incubations of each fraction with dynorphin B and nociceptin were performed. Enzyme activity was monitored by measuring the formation of cleavage fragments by MALDI mass spectrometry.
Results. The activities of enzymes that convert dynorphin B and nociceptin were observed. In case of both dynorphin B and nociceptin converting enzymes, the optimal pH of enzyme activity was defined. Identification of cleavage fragments was performed by MALDI mass spectrometry. Moreover, classification of these peptidases based on selective inhibitors was performed.
Conclusion. In the present work, the fragments that are released from neuropeptides such as dynorphin B and nociceptin as a result of neuropeptides convertases activity, have been identified.

Search of peripheral markers associated with pathogenesis of schizophrenia

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Background. Schizophrenia is the most important mental disorders for social life. Since diagnostics of mental disorders is based only on clinical symptoms, there is a necessity in development of additional methods of biochemical/paraclinical diagnostics.
Hypothesis. However, no protein markers typical only for distinct disease are found still.
The proteomics approach enables to specify distinct minor proteins which can help to decipher molecular mechanisms/pathways involved in endogenous mental disorders.
Aim. Serum proteomes of patients with schizophrenia and healthy subjects are compared in the present study.
Methodology. For the research we used the serum of healthy and patients with schizophrenia. Preparation of samples included: purification from serum major proteins by affinity chromatography, separation of proteins by one-dimensional electrophoresis, in-gel tryptic hydrolysis of the separated proteins. LC-MS/MS analysis of the resulting peptides using mass spectrometer with an ion trap XCT Ultra. The protein was quantified by the ELISA method. Identification of proteins was carried out using Mascot software Ver. 2.1 («Matrix Science», USA).
Results. When comparing proteome profiles, different unique protein sets were revealed: 23 proteins typical for schizophrenia. Protein set in schizophrenia was mostly associated with nucleic acid and protein metabolism, immune response, cell communication, and cell growth and maintenance. Significant difference between groups was revealed in ANKRD12 concentration ($p = 0.02$), with maximum elevation of ANKRD12 concentration in schizophrenia.
Conclusion. Our results are presumably useful for discovering the new pathways involved in schizophrenia.

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Proteome profiles of different types of thyroid cancers

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Background. The majority of patients with thyroid cancer are diagnosed based on fine needle aspiration cytology (FNAC) of thyroid nodules. Further diagnosis is performed based on histopathological intra- or post-operative examination of the resected thyroid tissue. However, in some cases, cytological and histological patterns are ambiguous and proper classification is problematic.

Hypothesis. Classification of thyroid cancers might be improved markedly if new biomarkers identified with the use of high-throughput „omics" approaches could support diagnosis based on histopathological patterns.

Aim. To perform proteomics profiling of tissue specimens representative for major types of thyroid cancers: papillary (classical and follicular variant), follicular, anaplastic and medullary, as well as benign follicular adenoma using shotgun LC-MS/MS approaches.

Methodology. Protein extracts from archival FFPE cancer tissue samples were analyzed using a combination of Orbitrap and MALDI-TOF approach.

Results. Protein products of 5700 unique genes were identified. In general, a large difference between medullary, anaplastic and epithelium-derived differentiated cancers (papillary and follicular) was detected. Proteins characteristic for medullary and anaplastic cancers included factors associated with neuroendocrine functions and factors typically associated with advanced malignancies, respectively. A comparable overall similarity of follicular cancers to both variants of papillary cancers was found. Moreover, follicular adenoma showed higher overall similarity to follicular cancer than to either variant of papillary cancer. Proteins discriminating differentiated thyroid neoplasms included factors associated with lipid and hormone metabolism, regulation of gene expression and maintenance of DNA structure.

Conclusion. Several novel biomarker candidates of thyroid cancer subtypes were detected. Moreover, proteome data matched several features of transcriptome and metabolome profiles of thyroid cancers contributing to systems biology of this malignancy.

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Ionizing radiation affects the composition of proteome of exosomes released by head and neck carcinoma in vitro

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Background. Exosomes and other extracellular vesicles are key players in cell-to-cell communication. They are proposed to be involved in different aspects of the response to ionizing radiation, including transmitting radiation-induced bystander effect and mediating radio-resistance.

Hypothesis. The functional role of exosomes depends on their molecular cargo, including proteome content.

Aim. To establish the proteome profile of exosomes released in vitro by irradiated cells derived from oral cancer and to identify processes associated with radiation-affected proteins.

Methodology. Exosomes and other small extracellular vesicles were purified by size-exclusion chromatography from cell culture media collected 24 hours after irradiation of cells with a single 2, 4, and 8 Gy dose, and then proteins were identified using a shotgun LC-MS/MS approach.

Results. In general, exosome-specific proteins encoded by 1217 unique genes were identified. There were 472 proteins whose abundances in exosomes were significantly affected by radiation (at any dose), including 425 upregulated and 47 downregulated species. The most numerous group of proteins affected by radiation (369 species) included those with increased abundance at all radiation doses (≥2 Gy). Several functional GO terms were associated with radiation-affected exosome proteins. Among over-represented processes were those involved in response to radiation, metabolism of reactive oxygen species, DNA repair, chromatin packaging and protein folding.

Conclusion. The protein content of exosomes released by irradiated cells indicates their actual role in mediating the response to ionizing radiation.

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Molecular changes in kidneys during chronic heart failure

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Background/Aims: Chronic heart failure (CHF) influences normal kidney function and leads to cardiorenal syndrome that further promotes HF progression. To describe the molecular changes in kidneys during CHF, we performed comprehensive proteomic analysis of kidneys from an established rat model of HF.
Methodology. CHF in rats was induced by volume overload of heart due to surgically created aorto-caval fistula (ACF). After 21 weeks, cardiac and renal functions were studied and quantitative proteomic analysis of kidney samples was performed.
Results. CHF rats had cardiac hypertrophy, increased left ventricular end-diastolic pressure and showed signs of systemic and pulmonary congestion. Kidneys of HF rats had reduced renal blood flow, decreased sodium excretion and urine production. We performed three independent iTRAQ analyses including sample extraction, labeling, fractionation and LC-MS/MS. We identified almost 4000 proteins with FDR 0.01. HF rat kidneys showed 67 significantly differentially expressed proteins (>1.5-fold) compared to control kidney samples. The most upregulated protein was angiotensin-converting enzyme (ACE, >20-fold), which is the rate-limiting enzyme responsible for conversion of angiotensin-I into vasoactive angiotensin-II (ANG-II). The second most strongly upregulated protein was advanced glycosylation end product-specific receptor (RAGE, 14-fold). RAGE activation triggers proinflammatory response and promotes nephropathy. Among other markedly upregulated proteins were periostin (6.8-fold), caveolin-1 (4.5-fold) and other proteins involved in endothelial function (vWF, cavins 1-3, T-kininogen-2), proteins of ECM activation (MFAP4, collagen-VI, galectin-3, FHL-1, calponin), molecules involved in glomerular filtration integrity (CLIC5, ZO-1) and carboxylesterase-1D (CES1D) – the enzyme which converts most ACE inhibitors into active drugs.
Conclusion. Chronic heart failure is associated with kidney dysfunction and with marked changes in kidney proteome. Enhanced ANG-II signaling appears to be the dominant molecular feature of HF-induced kidney injury. Other proteins upregulated in HF kidneys, such as RAGE, periostin or caveolins show potential mechanistic links with enhanced ANG-II action. This study is the first that provides evidence supporting the involvement of RAGE in pathogenesis of HF-related kidney injury. Some of the differentially expressed proteins may serve as future markers and/or therapeutic targets for cardiorenal syndrome.

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ZIKA virus NS3 protease: substrate specificity investigations

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Background: ZIKA virus is a human pathogen, spreading with mosquito *Aedes africanus*. It spreads from eastern Africa to Asia, then to Europe and finally to both Americas. During infections of adults, progression of symptoms is usually not severe. Patients have elevated temperature, rash, influenza-like symptoms, except responsibility for Guillain-Barre syndrome. Unfortunately, during infection of pregnant women, ZIKA promotes development of microcephaly in fetuses. Up to date there is no effective therapy of infection available.
Hypothesis: ZIKA virus protease NS3 is the key enzyme responsible for infection of the human cells. There is limited knowledge about the activity of this enzyme. Like similar enzymes from the *flaviviridae* group, enzyme is serine protease with pseudotrypsin activity. Its specificity was estmated by peptide library, but we decided to test its specificity in the assay better reflecting biological environment.
Aim: Estimation of ZIKA NS3 serine protease substrate specificity.
Methodology: We prepared a mix of proteins at MW-s from 15 to 70 kDa, which are routinely analyzed in our laboratory as standards veryfing proper work of our nanoLC-MS/MS systems and added appropriate quantity of recombinant NS3 protease.
Results: We were able to find cleavage sites find after detailed analysis of nanoLC-MS/MS derived results. Our results partially validate previous results received after interaction between peptide library and protease NS3.
Conclusion: Our approach is useful during estimation of the substrate specificity of proteases. The model based on the real protein sequences seems to be better reflecting biological context of the investiated enzyme activity.

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Simulation of phase II metabolism to study interactions of metabolites with proteins

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Background. Currently the phase II metabolism is mainly studied using liver cells homogenates or liver microsomes. This techniques require advanced separation techniques to be able to identify products of phase II metabolism in complex biological mixtures. Here we present connection of electrochemical oxidation with mass spectrometry detection as an alternative tool to study phase II metabolism and interaction of metabolites with proteins.
Hypothesis. Paracetamol can be electrochemically oxidized into its metabolite NAPQI (*N*-acetyl-*p*-benzoquinone imine). This metabolite reacts with protein β-lactoglobulin and based on the mass spectrometry the product of this reaction can be specified.
Aim. The aim of the study was to confirm that electrochemical oxidation with mass spectrometry can be a powerful tool to simulate phase II metabolism to study interactions of pharmaceuticals metabolites with proteins.
Methodology. Paracetamol was oxidized by ROXY™ system (Antec, The Nederlands) on glassy carbon working electrode. The electrochemical oxidation product was mixed with β-lactoglobulin as a model protein. The conjugation process was controlled by MALDI mass spectrometry on ultrafleXtreme (Druker Daltonics, Germany). The protein with conjugated metabolite of paracetamol was then reduced, alkylated and digested by trypsin. Finally, the fragment mass spectra were obtained to confirm which amino acid was modified by paracetamol metabolite.
Results. Paracetamol was successfully electrochemically oxidized into its natural metabolite NAPQI with hepatotoxic properties. It was shown that the metabolite of paracetamol reacts with β-lactoglobulin leading into a conjugation product. The cysteine present in the protein sequence responsible for the conjugation process was determined based on fragment mass spectra.
Conclusion. It was shown that electrochemical oxidation with mass spectrometry detection can be an effective tool to study phase II metabolism of pharmaceuticals and interaction of metabolites with proteins.

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Protein Extraction from Formalin-Fixed Paraffin-Embedded Tissue. A shotgun Proteomics application

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Background. Formalin-fixed paraffin embedded (FFPE) tissue archives represent an abundant resource of clinically relevant material for biomarker discovery. Moreover, a few standard procedures are applied worldwide for the preparation and storage of these tissues. This offers an advantages in terms of availability, stability for preservation and clinical-pathological information of patient outcome- attributes that makes them a valuable alternative to fresh frozen tissue for biomarker analysis.

Hypothesis. FFPE tissues present a particular challenge for proteomic analysis due to the formalin-induced cross-linking of proteins. Protocols for analyzing FFPE tissues by LC-MS/MS exist, but there is need of standardization of procedures and quality data.

Aim. We performed and optimized an urea based protein extraction of formalin-fixed paraffin-embedded (FFPE) breast tissues due to buffer compatibility with liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI MS/MS).

Methodology. Whole slide mounted FFPE tissue sections were deparaffinized and protein extraction was conducted on a randomised pair of serial sections. Optimization trial consisted in using temperature and buffer composition to improve protein extraction and introducing an additional extraction step by bead mill protocol. Analysis of raw data and results was performed with Progenesis QI for proteomics (®Waters).

Results. Results were compared with regard to yield, total protein and peptide identification, missed cleavages and specific modifications. PANTHER (Protein ANalysis THrough Evolutionary Relationships) classification system was used to classify the identified proteins based on molecular function, biological process and pathway.

Protein extraction protocol consisted of both temperature and bead mill extraction, which provided a better total protein yield. However, the overall results indicate that the latter step does not provide a significant improvement of number, coverage or type of proteins identified.

Conclusion. Change in composition of the urea based buffer and protein extraction at 95°C for 20 minutes followed by 60°C for 2 hours provided a good protein yield and best protein and peptide detection for the tissue analysed when compared with the original urea/thiourea

protocol extraction. The introduction of the bead mill extraction reduced the overall performance of the extraction method. Dissection of TFFE should be taken into consideration when performing protein extraction.

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In vivo determination of the CYP2E1 expression in rat hepatic microsomes after drug administration

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Background. Drug dependence influences cell functions and their biological pathways, but the processes do not seem to be clear and understood. Global analysis of liver proteome can contribute to the explanation of drugs' influence on the metabolic processes and may help to explain the whole problem.
Hypothesis. The project presents application of Western Blotting in drug addiction with the focus on cytochromes P450 (CYP450), mainly responsible for the metabolic processes in liver. The results will present comparison of an influence of different drugs (amphetamine, morphine and cocaine) on proteins present in liver microsomes.
Aim. The rat liver microsomes were taken from control and drugs-addicted rats, and up- and down regulated proteins were evaluated.
Methodology. Adult male Wistar rats were treated s.c. with either saline (control) or amphetamine, morphine, or cocaine in saline, for 15 days. Liver microsomes were prepared according to a standard protocol. The proteins were divided into up- and down regulated groups, based on LC-MS^n analyses, followed by the Mascot server (Matrix Science) identification, after SDS-PAGE separation. The expression of the selected proteins was determined by Western Blotting and the catalytic activity of cytochromes P450 was also determined.
Results. CYP2E1 protein was chosen from up- and down regulated proteins identified by LC-MS^n and verified by Western Blotting for cytochrome P450 4A10 (CYP4A).
Conclusion. After administration of various drugs (amphetamine, morphine, and cocaine) to the rats, significant changes in liver proteome were observed, also including cytochromes P450 responsible for oxidative metabolism. The expression of CYP2E1 was determined.

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Sex affects homocysteine modification at lysine residue 212 of albumin in mice

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Introduction: Homocysteine (Hcy) modification of protein lysine residues (KHcy) is linked to heart and brain diseases. However, factors affecting KHcy modifications are not fully understood.

Objectives: Our objective was to examine effects of sex, age, and cystathionine β-synthase (*Cbs*) genotype on K525Hcy and K212Hcy modifications in mouse albumin.

Methods: We developed a LC/MS targeted assay, based on MRM, for quantification of K525Hcy and K212Hcy sites in serum albumin. We studied 1 to 9-months-old *Tg-I278T* $Cbs^{-/-}$ mice (tHcy 272±50 μM) and their *Tg-I278T* $Cbs^{+/-}$ siblings (tHcy 5.0±2.6 μM).

Results: Female (n=20) and male (n=13) $Cbs^{-/-}$ mice had significantly elevated albumin K525Hcy and K212Hcy modifications relative to their $Cbs^{+/-}$ female (n=19) and male (n=17) siblings. Age and tHcy explained 1.8-4.6% and 3.8-7.5% of the variance in K525Hcy and K212Hcy, respectively. Male mice had more K212Hcy in albumin than females ($Cbs^{-/-}$ mice: 5.8±4.2 *vs*. 3.2±1.4 units, P=0.023; $Cbs^{+/-}$ mice: 2.7±0.8 *vs*. 1.9±1.1 units, P=0.008). In contrast, albumin K525Hcy level was similar in males and females, both in $Cbs^{-/-}$ (1.6±1.0 *vs*. 1.4±0.7 units, P=0.54) and $Cbs^{+/-}$ (0.92±0.41 *vs*. 0.87±0.50 units, P=0.514) mice.

Conclusion: These results suggest that the sex-specific K212Hcy modification in albumin plays an important biological function in mice. Other factors that affect KHcy modifications remain to be identified.

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Potential urine biomarkers for prostate cancer identified by label-free nanoLC-MS/MS

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Background. Detecting prostate cancer (PCa) still remains a challenge due to the lack of specificity of the currently available tests, especially in patients with less than 10 ng/ml serum PSA.

Hypothesis. The identification of new, preferably noninvasive biomarkers that would detect PCa early and with high sensitivity is quite feasible with the advent of new high-throughput proteomic techniques.

Aim. In this study, we focused on the identification of new biomarkers for PCa in urine with higher specificity and sensitivity from the currently available tests.

Methodology. We compared urine samples from PCa patients (n=5) with Gleason score 7.0±1.2 and 7.5±2.1 (ng/ml) PSA with samples from BPH patients (n=5) with 6.9±1.9 (ng/ml) PSA. A label-free LC-MS/MS was performed on HDMS QTOF (Waters Corp) while comparative statistical analysis was performed with Progenesis QI for Proteomics (V 3.0.3) (Waters Corp).

Results. Statistically significant difference in abundance (BH FDR ≤ 0.05) and fold change of ≥ 1.5, showed 48 proteins (17 up-regulated and 31 down-regulated in PCa). The highest significantly affected biological processes were protein activation cascade (6.23e-5), inflammatory response (2.72e-4) and vesicle-mediated transport (2.72e-4). The protein set also showed significant association with complement and coagulation cascades pathway (8.75e-5). Twenty one proteins are expressed in at least normal prostate or prostate cancer tissue while 8 proteins (AZU1, IGHG1, RNASE2, PZP, REG1A, AMY1A, AMY2A, COL18A1) have not been associated with PCa so far.

Conclusion. We have identified a set of putative diagnostic biomarkers for PCa in urine using high resolution LC-MS. This study represents the starting point for future diagnostic accuracy evaluation of the identified biomarkers.

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Revelation of the IFNα, IL-10, IL-8 and IL-1β as promising biomarkers reflecting immuno-pathological mechanisms in porcine Huntington's disease model

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Huntington's disease (HD) is an inherited neurodegenerative disease with impairment of motor and cognitive functions. Studies in HD gene carriers expressing mutant huntingtin protein (mHTT) demonstrated altered immune response indicating that cytokines may have a significant role in disease development.

We utilized the novel transgenic HD model bearing N-fragment of human mHTT in minipig (Baxa M et al., J Huntington's Dis. 2013, 2:47-68), to investigate central nervous and peripheral cytokine levels. Using Luminex® xMAP™ multiplexing technology and Thermo Fisher Scientific Inc., Swine Cytokine Magnetic 7-plex Panel (InvitrogenTM LSC0001M), the biological fluids were interrogated to identify cytokine alterations related to HD progression. The modelling of interaction networks between such cytokines and HTT facilitated study of intracellular signaling pathways associated with immune response dysregulation.

The most pronounced change was the decline of IFNα in cerebrospinal fluid and secretome of microglial cells (MG). In addition, IL-10 was also lower in the same biological fluids. Elevated levels of pro-inflammatory IL-1 and IL-8 were secreted by MG whilst IL-8 was also increased in serum of transgenic minipigs. The high proportion of mHTT in MG may have a causative impact on cytokine production. A novel role for CREB-binding protein in HD pathogenesis was highlighted.

These findings underlined the roles of IFNα, IL-10, IL-1 and IL-8 in central nervous system inflammation and immune response imbalance in HD progression warrants further investigation into their usefulness in human patients.

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Friday Posters

Ethanol-induced alterations in ubiquitin-proteasome system

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Background. Ethanol is a widely abused substance triggering damage to several body organs, primarily the brain and liver. Chronic exposure to alcohol results in dependence and tolerance development especially in the case of youth substance abuse. Identification of molecular pathways that are affected by this substance could enhance understanding of ethanol action at the cellular level. The ubiquitin-proteasome system (UPS) is a proteolytic pathway, which is involved in the regulation of major cellular processes through degradation of abnormal or damaged proteins and also normal proteins of short half-live. Compelling evidence indicates that the UPS plays a role in the molecular changes induced by addictive substances. Proteasome dysfunction has been repeatedly reported in alcoholic liver disease.

Hypothesis. Ethanol evokes long-lasting changes in neuronal circuits and synaptic transmission through the regulation of enzyme activity and gene expression therefore chronic ethanol consumption may directly influence the process responsible for controlled protein degradation in the central nervous system (CNS).

Aim. The objective of these studies was to evaluate protein ubiquitination in brain cortex and spinal cord during alcohol addiction.

Methodology. Male Wistar rats were used for the experiments. Chronic ethanol addiction was evoked by *i.g*. injection of ethanol (5.0 g/kg) between 30^{th} and 50^{th} day of life. Control group received saline (0, 9% NaCl) in the same scheme. Tissue homogenates were separated using 1D electrophoresis and electrotransferred onto the Immuno-Blot® PVDF membrane. Ubiquitinated proteins were detected using Anti-Ubiquitin antibody with colorimetric visualisation.

Results. The study revealed differences in protein ubiquitination in CNS during chronic ethanol exposure.

Conclusion. Global changes in ubiquitination of proteins have wide-ranging consequences for the functioning of the organism. Understanding these regulations will ultimately provide new insight into the pathophysiology of alcoholism. In this work, general ubiquitination profiles of proteins were identified to investigate potential alterations regarding UPS. The obtained data may contribute to explaining the role of altered ubiquitination in organisms exposed to ethanol and the better understanding of addiction mechanism.

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Evaluation of circulating angiogenic factors in hepatocellular carcinoma by proteomic technology multiplex array

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Background. Hepatocellular carcinoma (HCC) represents now the third leading cause of cancer deaths worldwide and the incidence and mortality of HCC are increasing; each year, more than half a million people worldwide receive this diagnosis. HCC is an aggressive primary malignancy of the liver predominantly in patients with chronic liver disease and cirrhosis, characterized by inflammation, necrosis and hepatocytes proliferation. This tumor evolves with local expansion, intrahepatic spread, and distant metastases.

Hypothesis. Angiogenesis process has an essential contribution to metastasis and poor prognosis in HCC. Several angiogenic factors have been reported, and some of them are studied as prognostic factors or target molecules of chemotherapeutic drugs. Therefore, identification of new biomarkers of angiogenesis in HCC could contribute to the improvement of HCC patient outcome.

Aim. Our study aims to analyze the expression profile of the main circulating proteins involved in angiogenesis processes in HCC and to established correlation with the disease stage and progression.

Methodology. The study was performed on plasma samples obtained from HCC patients operated in Clinical Fundeni Institute compared to control subjects. Circulating plasma levels of cytokines and growth factors: IFN-gamma, IL-10, IL-12p70, PDGF-AA, IL1-beta, IL-2, IL-4, IL-6, IL-7, IL-8, TNFalfa, VEGF-A has been measured by xMAP array technique.

Results. The results obtained from xMAP array showed the overexpression of several plasma growth factors and cytokines involved in angiogenesis processes; this overexpression was positively correlated with advanced stages of disease.

Conclusion. Our findings suggested that selected proteins might be considered valuable candidate biomarkers in cancer staging with potential clinical value for HCC patients.

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Expression and purification of stable uniform N^{15} labeled Shiga-like toxin 2 subunit B with application in mass spectrometry-mediated detection of hemolytic-uremic syndrome causing bacteria

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Background. Hemolytic-uremic syndrome is a serious illness, mainly affecting children, frequently caused by Shiga-like toxin 2 producing bacteria. Diagnosis is cumbersome, therefore there is use for more rapid and simple approaches.
Hypothesis. It is proposed that use of tryptic digest of stable uniform N^{15} labeled Shiga-like toxin B subunit 2 (SLT2B) as an internal standard for lysates of suspicious bacterial isolates coupled with detection via MS-MALDI could quickly and confidently provide information concerning diagnosis.
Aim. To optimize the production of different versions SLT2B (N^{14} isotope, N^{15} isotope, polyHis-tagged) by means of bacterial expression and to further purify it. Also, to assess pattern of its tryptic digest.
Methodology. BL21 (DE3) bacterial strain was transformed with SLT2B-containing vectors. For N^{14} variants an autoinducible lactose-based culture medium was used, while for N^{15} variation, the culture medium included $N^{15}H_4Cl$ as singular source of nitrogen. After protein expression, cells were harvested and lysates were processed through multiple purification steps to give yield to purified SLT2B protein. Tryptic digests of SLT2B were analyzed by means Tricine SDS-PAGE and UHPLC-MS-MALDI (Bruker).
Results. SLT2B protein was successfully expressed in both N^{14} and N^{15} forms with or without a polyhistidine tag. Ni-NTA affinity chromatography provided purification for the His-TAG protein, while a combination of ion exchange chromatography and size exclusion chromatography allowed purification when no tag was attached. Trypsinization of the purified proteins was optimized and revealed multiple SLT2B-derived peptides that were subsequently identified through mass spectrometry.
Conclusion. Different versions of SLT2B were produced and multiple purification strategies were tackled in order to evaluate optimal workflows. While tests involving actual bacterial lysates mixed with SLT2B peptides are mandatory, the successful identification of peptides promises a feasible outcome.

Human mesenchymal stem cells migration proteins are upregulated by dihydrotestosterone treatment

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Background. Human mesenchymal stem cells (MSC) have been established as valuable candidates for cardiovascular remodeling.

Hypothesis. Based on the facts that adult MSC have the potential to repair/regenerate tissue following cardiac injury, and that androgens (dihydrotestosterone – DHT) influence cells growth and function. We hypothesize that DHT may play a role in the regenerative capability of adult stem /progenitor cells

Aim. To find out whether exposure of MSC to androgens have a beneficial effect on the cell's characteristic functions such as proliferation, viability and migration hence we employed qRT-PCR, Western Blot and mass spectrometry analyses.

Methodology. MSC were isolated from Wharton's jelly. Morphological and functional characterization was made according to "gold standard" protocols. DHT-stimulated cells were analyzed for genic and proteic upregulation expression of factors involved in migration activity. To a more enhanced proteic evaluation we performed also a mass spectrometry analysis for treated cells compared with the non-treated ones.Using specific human primers, we performed a qRT-PCR on ARNm isolated from MSC stimulated for more than 90 hours with 30 nM. Interest genes were normalized against GAPDH. We used specific labeled human antibody to perform a Western Blot analyze of proteins isolated from MSC stimulated in the same condition as above. Target signals were normalized against beta-actin. Following the same pattern of stimulation, we performed a mass spectrometry analysis carried out using the EASY n-LC II system coupled to the LTQ Orbitrap Velos Pro mass spectrometer (Thermo Scientific, California, USA). The bioinformatics analysis comprised database protein inference, relative quantification and Kyoto Encyclopaedia of Genes and Genomes (KEGG) signaling pathways over-representation and revealed proteins that participate in actin cytoskeleton rearrangement processes.

Results. The following novel findings were revealed by our experiments with MSCs: (a) DHT modulated *EMMPRIN*, *MMP2* and *MMP9* gene expression; to find out whether DHT affects the gene expression of ARs and of other molecules implicated in cell migration, cultured MSC were exposed to the hormone (30 nM) and then subjected to qRT-PCR assay. The experiments revealed that DHT induced a significant up-regulation of the mRNA for AR and of the molecules involved in cell migration, EMMPRIN and MMP-9; DHT had no effect on MMP-2 gene expression.

(b) DHT modulated *EMMPRIN*, *MMP2* and *MMP9* proteic level; we found that the secretion of MMPs is increased significantly in DHT-treated MSC, and this effect was specifically

enhanced in the presence of heart slices. These results suggest the possibility that factors generated by ischemic tissue stimulates MSC to increase the MMPs production, and this response supports the proteolytic activity, which is essential for clearing damaged scar tissue so as to facilitate MSC integration and tissue repair. Taken together the above findings suggest that DHT potentiates the integration of MSC into cardiac tissue and thus, may positively influence the repair process.

(c) Up-regulation of actin cytoskeleton in DHT-treated MSC; the relative quantification experiments were based on precursor ion alignment and intensity comparison between the DHT-treated and the control MSC. A coefficient of variation of <30% was allowed between technical replicates. The total ion chromatogram normalization method resulted in 869 proteins that are differentially expressed following the treatment with DHT. A supplementary filtration, allowing only the proteins quantified with at least 2 peptides/protein was chosen for further bioinformatics analysis. This resulted in a list of 258 proteins with spectral abundance alteration of at least 1.5-fold (DHT treatment/control samples) and statistical significance. Amongst them, *four* proteins which presented very high identification confidence (Mascot score>9000) and significant spectral alteration levels were found to be part of a key cell motility-signaling map, the regulation of *actin cytoskeleton* (KEGG entry hsa04810). This pathway was found to be statistically over-represented, after the implementation of a further significance correction, which was based on the false discovery rate algorithm (FDR p-value: 1.96E-2).

Conclusions The presence of DHT primed MSC to a probable migration effect. Therefore, all the data obtained by stimulating the stem cells with DHT can be used further on migration studies.

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Improved metabolic status in obese type 2 diabetic patients treated by sleeve gastrectomy is associated with increased circulating microRNA-126

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Background. Diabetes-related stimuli were reported to alter the expression of circulating microRNAs (miRNAs). Although metabolic surgery is commonly considered effective for obese type 2 diabetes (O-T2D) patients, little is known about the underlying mechanisms.

Objectives. To determine whether the O-T2D patients undergoing metabolic surgery exhibit changes in the level of circulating miRNAs, and to examine the modifications associated with their improved metabolic status.

Methodology. Blood samples were taken from 14 fasting O-T2D patients before and 1 year after sleeve gastrectomy (SG). Markers indicative of surgical response (glycaemic parameters, lipid profile, adipokine and gastrointestinal hormones) were evaluated. Circulating miR-126, miR-132, and miR-204 levels were measured by quantitative real-time polymerase chain reaction and normalized to synthetic cel-miR-39.

Results. One year after SG, the patients exhibited a substantial amelioration of the metabolic parameters: a significant decrease in plasma glucose, insulin, triglycerides, and leptin, and an increase in circulating adiponectin. Circulating miR-132 and miR-204 had similar expression levels before and after SG. Conversely, circulating miR-126 increased significantly (p=0.001) and this change correlated negatively with the metabolic parameters, and with the improvement of homeostasis model assessment of β-cell function (HOMA-β).

Conclusion. One year after sleeve gastrectomy the obese T2D patients exhibited a significant metabolic improvement associated with an increase in circulating miR-126. Since the latter reportedly participates in the maintenance of vascular homeostasis, our data propose miR-126 as a reliable biomarker and an indicator of improved vascular function after metabolic surgery.

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Sera from obese type 2 diabetes patients undergoing metabolic surgery instead of conventional therapy exert beneficial effects on beta cell survival and function

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Background. Pancreatic beta cells are highly sensitive to oxidative and endoplasmic reticulum (ER) stress, commonly occurring in type 2 diabetes (T2D) and obesity.

Objectives. We aimed at investigating the cellular responses of human beta cells exposed to sera from obese T2D patients treated differently, namely by conventional therapy or laparoscopic sleeve gastrectomy (LSG).

Methodology. Serum samples from obese T2D men randomized to conventional treatment or LSG were taken at baseline and 6 months later. After exposing 1.1B4 cells to study patients' sera, the following were assessed: cellular viability and proliferation (by MTT and xCELLigence assays), reactive oxygen species (ROS) production (with DCFH-DA), and expression of ER stress markers, oxidative- or autophagy-related proteins and insulin (by real-time PCR and Western blot).

Results. At 6-month follow-up, the participants undergoing LSG achieved an adequate glycaemic control, whereas conventionally-treated patients did not. As compared to 1.1B4 cells incubated with baseline sera (set as controls), cells exposed to sera from LSG-treated patients exhibited: (i) increased viability and proliferation ($p < 0.05$); (ii) diminished levels of ROS and p53 ($p < 0.05$); (iii) enhanced protein expression of autophagy-related SIRT1 and p62/SQSTM1 ($p < 0.05$); (iv) significantly decreased transcript levels of ER stress markers; and (v) augmented insulin expression ($p < 0.05$). Conversely, the 6-month conventional therapy appeared not to impact on circulating redox status. Moreover, 1.1B4 cells exposed to sera from conventionally-treated patients experienced mild ER stress.

Conclusion. Circulating factors in patients with improved diabetes after metabolic surgery exerted favourable effects on beta cell function and survival. These results may facilitate the design of future strategies targeted to protect beta cells and optimize the treatment of type 2 diabetes.

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Evaluation of the early and progressive changes in plasma, hemodynamic and cardiac parameters in an animal model of atherosclerosis-associated diabetes mellitus

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Background. Diabetes appears to contribute directly to the development of cardiovascular disorders including heart valve disease.

Hypothesis. Identification of distinct changes associated with diabetes occurring in the valve may lead to the targeting of these major events using appropriate therapies as a treatment for valvular diseases.

Aim. To evaluate the early and progressive changes in plasma, hemodynamic and cardiac parameters in vivo, and to establish causal associations among them in atherosclerosis-associated diabetes mellitus.

Methodology. The streptozotocin-induced diabetic apolipoprotein E-deficient mouse model maintained on a high-fat cholesterol-rich diet was used. The blood was collected at 1, 2, 4 and 8 weeks from the last streptozotocin injection. To assess the structural and functional aspects of aortic valve, aortic arch and left ventricle, echocardiography-based in vivo imaging was applied.

Results. Important increases of plasma glucose, cholesterol, LDL/HDL cholesterol, triglyceride and fetuin-A concentrations occurred just from the first week of diabetes. These caused early impairments in structure and function of: (1) aortic valve: thickness, calcification, increases of velocity time integral and transvalvular velocity and reduction of aortic cusp separation; (2) aortic arch: arterial wall thickness, greater aortic pulse wave velocity and lower distensibility; (3) left ventricle: shortening fraction and mass increases. Longer diet and treatment times did not induce more spectacular changes in the cardio-biochemical parameters.

Conclusion. Our study demonstrates abnormalities of cardiac structure and function despite the short duration of diabetes, highlights the potential high cardiovascular risk occurring in diabetes and provides new possible biomarkers and targets for cardiac-valvular disease.

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3D model to study human aortic valve disease

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Background. Calcific aortic valve disease (CAVD) is the third leading cause of cardiovascular disease being a progressive process, with initial valvular endothelium inflammation, followed by fibrotic thickening and extensive calcification of the valve leaflets. Currently, pharmacological interventions for slowing down the disease progression are unavailable and there are no physiologically suitable models for human CAVD.

Hypothesis. 3D models of human aortic valve may help to advance the understanding of the cellular and molecular mechanisms of human CAVD.

Aim. To obtain a 3D platform with structure similar with the human aortic valve leaflet to study valvular cells phenotypic changes relevant for human CAVD.

Methodology. Human valvular interstitial cells (VIC) were encapsulated in a Gel-MA hydrogel at a density of $2x10^6$/ml. 3D-constructs resulted from 100µl cell-laden hydrogel were crosslinked by exposure to UV light in a bioprinter (RegenHu). Valvular endothelial cells (VEC) were seeded on the construct surface. The constructs were maintained in culture for 7-21 days. Cell viability was determined using Live/Dead and cell phenotype was analyzed by Real Time-PCR and immunohistochemistry using specific primers and antibodies.

Results. In the current study, we obtained a 3D construct with human VIC inside and a VEC layer on the top. Encapsulated VIC developed a well-defined cell network within construct and express low levels of alpha-smooth muscle actin (α-SMA) compared with VIC in bi-dimensional culture. The Real-Time PCR data indicated that encapsulated VICs have a less activated phenotype than those grown in bi-dimensional culture. VEC seeded on the constructs adhere, proliferate and form a monolayer at surface of the construct exhibiting endothelial specific markers: PECAM-1 and von Willebrand factor.

Conclusion. We have developed 3D constructs with structure similar to human valve leaflet, with viable VIC encapsulated in constructs and VEC on the surface. The 3D model will help for further investigations of the dynamic interaction between valve interstitial and endothelial cells, in order to study the valvular disease progression and to develop potential therapeutic interventions.

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Characterization of senescent versus early passages human amniotic fluid stem cells

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Background. Senescence represents an adaptive cellular response to potentially stress. Although to date there is no unique marker to distinguish senescent cells from non-proliferating ones, a combination of markers can lead us to a senescent phenotype called senescence-associated secretory phenotype (SASP), consisting in a number of metabolic changes that try to remove stress factors.

Aim. The present study aims to explore activation of specific signaling molecules associated with SASP in our *in vitro* senescence model.

Methodology. Human amniotic fluid stem cells (AFSC) at passages 2 or 3 and senescent cells (maintained 40 days in culture without any passage) were analyzed for modification of stem cell markers by flow cytometry, for inflammatory markers by qRT-PCR and for ion currents by patch-clamp.

Results. Senescent AFSC were positively stained for SA-β-Gal as compared with early passages AFSC. The expression of surface markers decreased in senescent AFSC by 4 to 65% compared to early passages AFSC, as follows: CD29 by 43.6%, CD31 by 49.2%, CD44 by 75.5%, CD49e by 65.3%, CD54 by 1.8%, CD56 by 62.5%, CD73 by 82.3%, CD90 by 60.7%, CD105 by 65.5%, CD146 by 30.4%, respectively. Senescent AFSC featured increased expression of IL-1α, IL-6, IL-8, TGFβ, NF-βB (p65), suggesting a SASP. Via patch-clamp we identified 5 ion current components: K+ outward rectifier current, big conductance K+ channels (BK) current fluctuations, Na+ current, inward rectifier K+ current, and transient receptor potential (TRP)M7-like current.

Conclusion. In conclusion, in our model we evidenced specific changes in senescent vs. early passages AFSC, suggesting a shift from an anti-inflammatory phenotype to a proinflammatory one, thus limiting their therapeutic potential.

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The mechanisms underlying protective effects of oleic acid against palmitic acid on pancreatic beta cell function

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Background. Glucose and free fatty acids, the two main nutrients in energy metabolism in most organisms, are of particular interest in beta cell equilibrium.
Hypothesis. Palmitic acid, one of the most common saturated fatty acids has been shown to induce lipotoxicity in the pancreatic beta cells; however, the potential signaling intermediates have not been fully detected.
Aim. In this study, we aimed at identifying the mechanisms underlying the different effects of palmitic acid and oleic acid on human pancreatic beta cell function. To address this issue, the oxidative stress, endoplasmic reticulum stress, inflammation, apoptosis and their mediator molecules were investigated in the insulin releasing 1.1B4 beta cells exposed to palmitic and/or oleic acid.
Methodology. Cultured 1.1B4 beta cells were incubated for 24 h in the absence/ presence of 250μM oleic acid, palmitic acid or the combination of both free fatty acids and physiologically glucose concentrations.
Results. We found that, oleic acid promoted neutral lipid accumulation and insulin secretion, whereas palmitic acid was poorly incorporated into triglyceride and it did not stimulate insulin secretion from human pancreatic islets. In addition, palmitic acid caused **(1)** oxidative stress through a mechanism involving increases in ROS production and MMP-2 protein expression/gelatinolytic activity; **(2)** endoplasmic reticulum stress by up-regulation of chaperone BiP and unfolded protein response (UPR) transcription factors (ATF6, XBP-1) and by PTP1B down-regulation in both mRNA and protein levels; **(3)** inflammation through enhanced synthesis of proinflammatory cytokines (IL-6, IL-8); and **(4)** apoptosis by enforced expression of CHOP multifunctional transcription factor. Oleic acid alone had opposite effects due to its different capacity of controlling these pathways, in particular by SOD2 and PTP1B overexpression and reduction of the MMP-2 expression/activity, UPR transcription factors, and proinflammatory cytokines.
Conclusion. Our findings show the protective action of oleic acid against palmitic acid on beta cell lipotoxicity through promotion of triglyceride accumulation and insulin secretion and regulation of effector molecules involved in oxidative stress, endoplasmic reticulum stress, inflammation and apoptosis.

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Inhibition of miR-486 and miR-92a decreases liver and plasma cholesterol levels by modulating lipid-related genes in hyperlipidemic hamsters

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Background. MicroRNAs (miRNAs) are potent post-transcriptional gene regulators, known to modulate genes involved in lipid metabolism. Therefore, identifying the target genes of miRNAs involved in dyslipidemia and cellular dysfunction is important.

Hypothesis. Modulation of miRNA expression might ameliorate hyperlipidemia *in vivo*.

Aim. To evaluate the potential of *in vivo* inhibition of miR-486 and miR-92a to reverse hyperlipidemia, then to identify and validate their lipid metabolism-related target genes.

Methodology. Male Golden-Syrian hamsters fed a hyperlipidemic (HL) diet (standard chow plus 3% cholesterol and 15% butter, 10 weeks) were injected subcutaneously with lock-nucleic acid (LNA) inhibitors for miR-486 or miR-92a. Lipids and miRNAs levels in liver and plasma, and hepatic expression of miRNAs target genes were assessed in all HL hamsters.

Results. MiR-486 and miR-92a target genes were identified by miRWalk analysis and validated by 3′UTR cloning in pmirGLO vectors. HL hamsters had increased liver (2.8-fold) and plasma (2-fold) miR-486 levels, and increased miR-92a (2.8-fold and 1.8-fold, respectively) compared to normolipidemic (NL) hamsters. After 2 weeks treatment, liver and plasma cholesterol levels decreased (23% and 17.5% for anti-miR-486, 16% and 22% for miR-92a inhibition). Hepatic triglycerides and non-esterified fatty acids content decreased also significantly. Bioinformatics analysis and 3′UTR cloning in pmirGLO vector showed that Sterol O-Acyltransferase-2 (SOAT2) and Sterol-Regulatory Element Binding Transcription Factor-1 (SREBF1) are targeted by miR-486, while ATP-binding cassette G4 (ABCG4) and Niemann-Pick C1 (NPC1) by miR-92a. In HL livers and in cultured HepG2 cells, miR-486 inhibition restored the levels of SOAT2 and SREBF1 expression, while anti-miR-92a restored ABCG4, NPC1 and SOAT2 expression compared to scrambled-treated HL hamsters or cultured cells.

Conclusion. *In vivo* inhibition of miR-486 and miR-92a could be a useful and valuable new approach to correct lipid metabolism dysregulation.

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Novel molecular mechanisms by which ginger extract reduces the inflammatory stress in TNFα – activated human endothelial cells; decrease of Ninjurin-1, TNFR1 and NADPH oxidase subunits expression

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Background. Atherosclerosis is an inflammatory disease characterized by the formation of atherosclerotic plaques in the vessel wall of the large and medium arteries. A pivotal event in atherogenesis is the activation of endothelial cells (EC) which express pro-inflammatory cytokines and cell adhesion molecules that mediate the recruitment of monocytes from circulation in the sub-endothelial space. Published data provide strong evidence that many phytochemicals found in plants may be used to prevent or treat chronic diseases.
Hypothesis. Ginger extract (GEx) can attenuate atherosclerosis progression by ameliorating the dysfunction of human endothelial cells (HEC).
Aim. To investigate the potential of GEx and its' main active components 6-gingerol (6-G) and 6-shogaol (6-Sh) to decrease inflammatory stress in HEC and to identify new mechanisms of action of ginger against atherosclerosis.
Methodology. Cultured endothelial cells (EA.hy925) were exposed for 18h to 15ng/ml TNFα in the presence/absence of 10μM GEx, 6-gingerol (6-G) or 6-shogaol (6-Sh). The inflammatory stress was evaluated by measuring the expression of monocyte chemoattractant protein 1 (MCP-1) and vascular cell adhesion molecule 1(VCAM-1), and the rate of adhesion of monocytes to HEC. To identify the mechanisms of action of GEx, 6-G or 6-Sh, the expression of Ninj-1, TNFα receptor 1 (TNFR-1), receptor for advanced glycation end-products (RAGE), soluble RAGE (sRAGE), NOX4, p22phox, HO-1, intracellular ROS levels, NF-kB activation and Nrf2 translocation were evaluated.
Results. GEx decreases MCP-1 and VCAM-1 expression and monocyte adhesion to HEC. This decrease was associated with the: (1) decrease of ninjurin-1 expression; (2) reduction of TNFR-1 and of RAGE, in parallel with the increase of sRAGE; (3) decrease of intracellular ROS levels and of NADPH oxidase subunits expression; (4) activation of antioxidant Nrf2 and HO-1, and (5) inhibition of NF-kB. The benefic effects of 6-G and 6-Sh were weaker than those of GEx (GEx>6-Sh>6-G).
Conclusion. GEx might be a promising alternative to ameliorate disorders in which oxidative stress and inflammation are important.

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Bisphenol A down-regulates apolipoprotein A1 expression and exerts pro-atherogenic effects

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Background. Bisphenol A (BPA) is found in polycarbonate plastics, which are often used in containers that store food and beverages. The mechanism of pro-atherogenic effects of BPA is unclear. Apolipoprotein A1 (apoA1) is the main component of high-density lipoprotein (HDL), the lipoprotein with anti-atherosclerotic properties.
Hypothesis. Bisphenol A exposure having effects on apoAI gene expression may accelerate atherosclerosis.
Aim. Here we investigated whether BPA affects apoAI gene expression in hepatocytes, and thus disturbing the structure and functionality of HDL.
Methodology. ApoA1 gene expression was determined by RT-PCR. The capacity of BPA to modulate apoA1 promoter activity was assessed by transient transfections. NF-kB binding to apoA1 promoter was tested by chromatin immunoprecipitation and protein-DNA interaction assays. The effect of BPA on atheromatous plaque development was assessed in LDLR-/- (LDL Receptor deficient) mice fed a high-fat diet and treated with 50 μg BPA/kg body weight by gavage for two months. Aortic atherosclerotic lesions were evaluated by Oil red O staining and quantified by ImageJ software.
Results. Our data showed that BPA decreases apoA1 expression in hepatocytes. Chromatin immunoprecipitation and protein-DNA interaction assays demonstrated that BPA induces NF-kB binding to apoA1 promoter in hepatocytes. Analysis of apoA1 promoter revealed the presence of six NF-kB binding sites in the region -1223/-252 of apoA1 promoter. Plasma cholesterol and triglyceride levels were significantly higher compared to those in the control group, in which only the vehicle was administered. Convergently, HDL level was decreased by BPA treatment of LDLR-/- mice. Moreover, atherosclerotic lesions in the aortas of the BPA-treated LDLR-/- mice were more developed in BPA treated mice as compared to the controls.
Conclusion. BPA exerts its pro-atherogenic effects through NF-kB activation and down-regulation of apoA1 expression in hepatocytes. Identifying the mechanism of pro-atherogenic effects of BPA is critical for developing novel treatment strategies for atherosclerosis.

Acknowledgments. This work was supported by CNCS – UEFISCDI (PN-II-RU-TE-2014-4-2660, PN-III-P1-1.1-PD-2016, P4 PN-III-P1-1.2-PCCDI-2017-0697 within PNCD III) and Romanian Academy.

Mass Spectrometry evidence for modified protein composition of pulmonary lipid rafts in experimental diabetes

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Background. Diabetes and the associated hyperglycemia affect pulmonary physiology and biochemistry inducing endothelial impairment, as the first step in lung vascular dysfunction. Lipid rafts are claimed to be actively involved in signal transduction, cholesterol homeostasis, and vesicular trafficking.

Hypothesis. The present study aimed to determine whether lipid raft proteome could be involved in the onset of diabetic state in the pulmonary tissue.

Aim. To document the effect of hyperglycemia on lung endothelial cells, we designed experiments on streptozotocin-induced diabetes and on double transgenic diabetic mice to investigate: (1) the early morphological changes occurring in endothelial cells, (2) the ACE activity and cholesterol content of lipid rafts, and (3) the relative quantification of the significantly changed lipid raft protein composition.

Methodology. Biochemical assays, electron microscopy and LC-MS/MS analysis were performed on detergent resistant membrane (lipid rafts) microdomains isolated from the lungs of diabetic and non-diabetic mice models.

Results. Electron microscopy results showed a well-developed synthesis apparatus in diabetes, demonstrating a high metabolic activity. LC-MS/MS analysis revealed that hyperglycemia has a modulatory effect on the expression of 178 proteins that co-fractionated with lipid rafts. These proteins were found to be implicated in 13 statistically significant over-represented signaling pathways.

Conclusion. In diabetic lung, the endothelial cells exhibit both structural and biochemical modifications accompanied by enhanced cholesterol content and ACE enzymatic activity. In adition the mass spectrometry showed a modified expression of caveolin-1, alarmins, ribosomal and junctions proteins associated with lipid rafts. This may point out toward a possible novel regulatory pathways in the mechanism of microangiopathy installation modulated by the lipid rafts composition in the diabetic lung.

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Dermal fibroblasts as new players in regenerative therapy

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Background. Mesenchymal stromal cells (MSCs) represent one of the pillars of regenerative medicine. Stromal cells meeting the minimal requested characteristics of stem cells have been isolated from various sources, from which bone-marrow (BM) and adipose tissue (AD) remaining the most studied. However these sources provide little starting material, and require invasive extraction means, while fibroblasts are easily harvested in large numbers from various biological wastes.

Hypothesis. Fibroblasts could be an alternative to MSCs in regenerative therapies and dermal fibroblasts (DFs) are relative easy to isolate procedure and highly available.

Aim. To determine the phenotypic profile and trilineage differentiation potential of dermal fibroblasts comparative to BM and AD derived MSCs (BM-MSCs and ADSCs).

Methodology. BM-MSCs were isolated from BM aspirate, DFs and ADSCs were isolated by the explant method, and enzymatic digestion respectively. Cells were analyzed by flow citometry for CD73, CD90, CD105, CD45, CD34, CD11b, CD79a and HLA-DR. The trilineage differentiation (adipogenic, osteogenic and chondrogenic) was induced by cultivating the cells in specific media for 3 weeks, followed by fixation and corresponding staining: Oil Red, alkaline phosphatase/von Kossa and Alcian Blue.

Results. All the tested cells were positive for CD73, CD90, and CD105; negative for CD45, CD34, CD11b, CD79a and HLA-DR. Thus 97.7 ± 3.3 % of BM-MSCs, 98.8 ± 0.11% of ADSC and 99.5 ± 0.47% of DFs were positive for CD73, 98.8 ± 0.7 % of BM-MSCs, 98 ± 1.14% of ADSCs and 97.6 ± 3.2 % of DFs were positive for CD90 and 88.95 ± 2.2 % of BM-MSCs, 94.2 ± 6.9 % of ADSC and 92.4 ± 7.1 % of DFs were positive for CD105. The results showed that all tested cells types were able to differentiate towards adipogenic and chondrogenic. Regarding the osteogenic differentiation only BM-MSCs and ADSCs produced the calcification nodules, while the FDs were only positive for alkaline phosphatase.

Conclusion. Here, we demonstrate that FDs display the identical phenotype as classical progenitor cells (BM-MSCs and ADSCs). Moreover, FDs had a multilineage differentiation potential similar to BM-MSCs and ADSCs**.** Taken together, these data indicate FDs as promising players in regenerative medicine.

Acknowledgments. The work was supported by PN-III-P1-1.2-PCCDI-2017-0749, PN-III-P1-1.1-TE-2016-1592 and the Romanian Academy.

Exploration of mechanisms leading to plaque instability in a rabbit atherosclerotic model – preliminary data

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Background. Atherosclerosis is a lipoprotein-driven disease that leads to plaque formation at specific sites of the arterial tree through intimal inflammation, fibrosis, necrosis, and calcification. Sometimes such plaques may suddenly cause life-threatening coronary thrombosis clinically described as acute coronary syndrome.

Hypothesis. An atherosclerotic rabbit model, with a lipoprotein metabolism similar to the human patient, have been proposed to explore the mechanisms leading to plaque instability. We expect to identify an unambiguous alarmin expression pattern, associated to specific atherogenic signalling pathways that may explain the residual risk of cardiovascular-related events and associated mortality.

Aim. The project will explore the plaque instability and the early signs of risks and/or protective factors in coronary disease by using an experimental design comprising both the classical pharmacology (statin administration) and the newest generation of molecular biology approaches (RNA silencing or biological active agents such as PCSK9 antibodies). In preliminary studies the hypercholesterolemic food concentration and treatment to be used was evaluated.

Methodology. Adult male New Zeeland White rabbits (16 weeks-old) were randomly split into 5 groups: two animals were fed a diet containing either 0.5% or 1% high cholesterol diet and three animals were fed a standard diet together with inhibition of PCSK9 for 4 weeks. The inhibition of PCSK9 were obtained either with siRNA (molecule I or II) or using monoclonal antibodies against PCSK9 (Evolocumab, Repatha, AMGEN). Total cholesterol (TC), low-density lipoprotein cholesterol (LDL), triglyceride levels were measured by using Dialab kits. Levels of PCSK9 protein in plasma samples were detected by ELISA kit. The presence and the quantification of atherosclerotic lesions were assessed by Oil Red O and hematoxylin and eosin staining.

Results. The hypercholesterolemic diet induces a significant increase in lipid parameters in the plasma. In the present study, we found that irrespective of the cholesterol concentration diet the atherosclerotic plaques had more macrophage accumulation in the aortic lesion area and higher expression of α-smooth muscle actin and CD31 endothelial specific antigen were detected than in the animals fed a standard diet. The administration of anti PCSK9 antibody showed a decrease of the plasma PCSK9 protein in the treated animals.

Conclusion. The preliminary results suggest that hypercholesterolemic diet induces atherosclerotic plaque progression in rabbit model and for more advanced lesions, the 0.5% cholesterol diet will be extended to 12 weeks. The inhibition with anti PCSK9 demonstrated

to be functioning in the decreasing of LDL plasma and could be successfully used in the proposed study.

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The effects of photocatalytic silver (Ag)-titanium dioxide (TiO2) nanoparticles on human lung epithelial cells

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Background. The potential human health risks following the exposure to inorganic nanoparticles (NPs) is a very important issue for their application in leather finishing industry.
Aim. To investigate the effect of Ag/TiO_2 and $Ag/N\text{-}TiO_2$ NPs on human lung epithelial cells.
Methodology. Three formulations of photocatalytic NPs containing 0.53% Ag (Ag/TiO2: NP1), 1.14% Ag (Ag/TiO2: NP2) and 1.62% Ag (Ag/N-TiO2: NP3) were prepared by electrochemical deposition of Ag on the surface of TiO2 and N-TiO2 NPs and further characterized for size and zeta potential. Epithelial lung cells (A549 cells) were exposed for 24 hours to various Ag/TiO2 and Ag/N-TiO2 concentrations and cytotoxicity, cell death mechanisms, production of reactive oxygen species (ROS), the changes in activation/inactivation of p38 MAPK, JNK and ERK signaling pathways and the profile of inflammatory cytokine/chemokine were determined.
Results. A549 cells show differential sensitivity to various concentrations of each NPs formulations. Thus, NP1 and NP3 are more cyto-friendly as compared to NP2. A significant increase (up to 5 folds) in ROS production by A549 cells, that was dependent on dose and NPs formulation, was determined. In addition, no significative effect on total or phosphorylated level of protein p38 MAPK and JNK was observed, irrespective of NPs' formulation. NP2 formulation induces an increase in total and phosphorylated level of ERK1/2 in lung epithelial cells at a concentration of 0.3 mg/ml. NP3 formulation does not induce the expression of highly pro-inflammatory cytokines IL-1 and TNF-α in A549 cells.
Conclusion. The results suggest that NP3 is appropriate to be selected for coating the leather surface due to its properties that are more cyto-friendly as compared to other NPs formulations.

Acknowledgments. The study was funded by UEFISCDI, PNIII_15/2015 under SIINN-ERA-NET Programme.

Downregulation of the receptor for advanced glycation end products (RAGE) in the aorta of APOE-deficient mice using P-selectin targeted RAGE-shRNA lipoplexes

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Background. During the vascular inflammation process, endothelial cells (EC) express both RAGE and the cell adhesion molecule, P-selectin that initiate and perpetuate inflammation by promoting leukocyte infiltration into the vascular wall.

Hypothesis. We hypothesize that we could use P-selectin as a target for nanotherapy to efficiently silence RAGE exposed on the vascular activated endothelial cell membrane.

Aim. To target the P-selectin overexpressed in the aorta and to downregulate RAGE expression specifically.

Methodology. P-selectin targeted cationic liposomes (Psel-lipo) were prepared and used to form lipoplexes with different RAGE-shRNA plasmid sequences (Psel-lipo/RAGE-shRNA). The distribution and ability of lipoplexes to transfect the vascular endothelium was assessed by IVIS Imaging System. To down-regulate RAGE expression, Psel-lipo/RAGE-shRNA lipoplexes were i.v. injected 2 times/week for 4 weeks in ApoE-/-mice. After sacrifice, blood and tissue samples were collected and further processed into homogenates. The RAGE expression and the pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, MCP-1) and chemokine (MCP-1) in tissue homogenates were determined by Western-blot and ELISA assay, respectively.

Results. 1) Psel-lipo/RAGE-shRNA lipoplexes bind specifically to the aorta of ApoE-/- mice; 2) Psel-lipo/pEYFP lipoplexes induce the expression of yellow fluorescent protein in the aorta of ApoE-/- mice at 48 hours after administration; 3) the treatment with Psel-lipo/RAGE-shRNA specifically downregulated RAGE in the aorta and liver of ApoE-/- mice; 4) treatment with lipoplexes did not significantly alter the weight of the mice, the liver and kidney function, the plasmatic parameters and the production of inflammatory cytokines.

Conclusion. P-selectin targeted RAGE-shRNA lipoplexes bind specifically to the aorta and downregulate RAGE expression in aorta and liver of ApoE-deficient mice.

Acknowledgments. Study supported by UEFISCDI, PN-II-RU-TE-2014-4-1837 project.

P-selectin targeted lipoplexes carrying a shRNA plasmid to silence receptor for advanced glycation end products decrease monocyte adhesion to activated endothelial cells

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Background. Receptor for advanced glycation end products (RAGE) is expressed on endothelial cells (EC) and plays an essential role in vascular wall inflammation in atherosclerosis.

Hypothesis. P-selectin can be used as a target for nanotherapy of atherosclerosis because it is specifically expressed on the surface of EC in inflammatory processes.

Aim. To develop P-selectin targeted cationic liposomes to function as nanocarriers for specific delivery of shRNA for down-regulation of RAGE in EC.

Methodology. P-selectin targeted cationic liposomes containing the lipid 2-{3-[Bis-(3-amino-propyl)-amino]-propylamino}-N-ditetradecyl carbamoyl methyl-acetamide (DMAPAP) combined with 1,2-Dioleoyl-sn-glycero- 3-phosphoethanolamine (DOPE) were obtained by coupling a peptide with high affinity for P-selectin to a functionalized PEGylated phospholipid inserted in the liposomes' bilayer (Psel-lipo). Then, Psel-lipo were complexed with a mix of five plasmids containing shRNA RAGE sequences at different charge ratios (R). The lipoplexes were characterized for size and zeta potential. The cytotoxicity studies were performed by XTT assay after exposing EC (bEnd.3 cells) to targeted (Psel-lipo/shRAGE) and non-targeted (Scr-lipo/shRAGE) lipoplexes for 48 hours. The efficiency of RAGE down-regulation was analyzed by Western Blotting assay at 48, 72 and 96 hours of incubation. Next, we studied the functional role of silencing the RAGE expression on monocytes adhesion to EC, activated or not with LPS.

Results. The mean diameter of lipoplexes was around 150 nm with a zeta potential of -24mV (R=6). For all charge ratios investigated the reduction of cellular viability was about 20%. The treatment of EC with Psel-lipo/shRAGE determines a significative reduction in protein expression of RAGE at 72 hours after transfection and reduces by 50% the adhesion of monocytes to LPS-activated EC.

Conclusion. P-selectin targeted lipoplexes reduce specifically and efficiently the expression of RAGE in EC and decrease the adhesion of monocytes to activated EC.

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VCAM-1 targeted naringenin-loaded lipid nanoemulsions reduce monocyte adhesion to activated endothelial cells

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Background. Naringenin is a citrus flavonoid with well documented anti-atherosclerotic effects. However, the poor water solubility and reduced bioavailability are two major drawbacks for its therapeutic use.

Hypothesis. The encapsulation of naringenin into targeted lipid nanoemulsions (LN) will overcome its limitations and will enable a specific delivery to endothelial cells (EC).

Aim. To reduce TNF-α induced EC-activation with naringenin-loaded nanoemulsions (NAR-LN) directed to the cell adhesion molecule VCAM-1, expressed on activated endothelial cells (EC).

Methodology. NAR-LN, either non-targeted or targeted to endothelium by coupling on the surface a VCAM-1 recognition peptide, were prepared by the ultrasonication method and further characterized. The cytotoxicity studies were performed by XTT assay after exposing the human endothelial cell (EC) line EA.hy926 to various concentrations of NAR-LN for 24 hours. The uptake of Rhodamine-PE labeled LN by EC was assessed by flow cytometry and fluorescence microscopy. Next, we studied the functional role of EC treatment with NAR-LN on monocyte adhesion to EC, activated or not with TNF-α.

Results. Both non-targeted and VCAM-1- targeted LN were taken up by the EC in a dose-dependent manner. A significant increase was noted in the uptake of endothelium-targeted LN upon exposure to TNF-α, unlike the uptake of non-targeted LN, which was independent of endothelial activation by TNF-α. This differential behavior suggests distinct mechanisms of internalization, with a specific pathway for the targeted LN. No deleterious effect of NAR-LN on cell viability at doses corresponding up to 100 μM naringenin/0.5 mM lipid were detected in our experimental set-up. NAR-LN exerted a two times higher percentage of inhibition of monocyte adhesion to the TNF-α activated endothelium as compared to free naringenin at the same concentration.

Conclusion. Poorly water-soluble naringenin, can be successfully encapsulated in LN and targeted to activate EC. Their functional capacity to reduce monocyte adhesion to activated cells guides to further studies to search for their potential benefits in experimental atherosclerosis and diabetes.

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Adenoviral transduction of hepatocytes to induce Fas ligand expression

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Background. Fas ligand (FasL) is a member of TNF family which plays a key role in the immune response modulation. The interaction of FasL with its receptor (Fas/CD95) induces apoptosis in activated lymphocytes. The failure of FasL-Fas system contributes to the development of the autoimmune diseases.

Hypothesis. Ectopic expression of functional FasL would be able to induce apoptosis of the auto reactive lymphocytes, limiting their clonal expansion. This pathway could contributes to the restoration of self-tolerance in autoimmune diseases.

Aim. Our goal was to induce ectopic expression of functional FasL in murine hepatocytes, by adenoviral transduction.

Methodology. FasL adenovirus preparation included the following steps: (1) cloning of a FasL mini-gene (the gene without the second intron) in pAdTrack-CMV; (2) recombination of pAdTrack-FasL with AdEasy-1 in chemically transformed BJ5193 bacteria; (3) selection of recombinant clones by PCR; (4) adenoviral packaging in AD293 by transfection with PacI-digested recombinant plasmid using K2 reagent; (5) viral amplification; (6) viral purification by one-step ultracentrifugation on CsCl gradient; (7) adenoviral titration by flow cytometry. Hepa1-6 murine hepatocytes were transduced with 1-10 MOI and tested for FasL expression by RT-PCR, Western blot and flow cytometry. Apoptosis of A20 lymphoma cells after co-culture with adenoviral-transduced Hepa 1-6 cells was evaluated to test FasL function.

Results. FasL mini-gene was cloned into pAdTrack-CMV adenoviral vector. Sanger sequencing showed that the sequence was correct and contained two gene polymorphisms. The adenovirus was packed, amplified and titrated in AD293 cells. From ~$7x10^7$ cells, 10^8-$5x10^9$ transduction units (TU) were obtained for FasL-adenovirus (as compared with ~10^{10} TU for the control virus). For 1 TU/cell, more than 60% Hepa 1-6 cells were infected, while for 10 TU/cell, 100% of the cells were transduced. The infected cells expressed GFP, the reporter gene. Transduced Hepa 1-6 cells expressed FasL mRNA, as determined by RT-PCR, and FasL protein, as revealed by Western Blot and flow cytometry. FasL expressed by transduced hepatocytes was able to induce apoptosis of A20 lymphoma cells.

Conclusion. The adenovirus bearing FasL mini-gene is able to transduce hepatocytes with a very good yield. FasL expressed by transduced hepatocytes is properly folded and is functional in inducing cell apoptosis.

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Up-regulated NADPH oxidase-derived reactive oxygen species induce macrophage polarization towards M1 phenotype *in vitro*; potential implication in human atherosclerosis

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Background. Oxidative stress microenvironment shapes the phenotype of monocyte (Mon)-derived macrophage (Mac) in atherosclerosis. Two major Mac populations with different major phenotypes have been described: the pro-inflammatory (M1) and anti-inflammatory (M2).

Hypothesis. Down-regulation of proinflammatory molecules expression in M1-Mac and/or active induction of a specific M2-Mac subset may be therapeutically relevant for the outcome of atherosclerosis. NADPH oxidases (Nox) are major sources of reactive oxygen species (ROS) in Mac contributing to atheroma formation. The role of Nox-derived ROS in Mac polarization via redox-sensitive mechanisms is not known.

Aim. This study aimed at investigating the implication of Nox enzymes in mediating Mac polarization in atherosclerosis.

Methodology. Non-atherosclerotic (superior thyroid artery) and atherosclerotic (carotid artery) samples obtained as discarded tissues from patients undergoing carotid endarterectomy were used. Human THP-1 Mac were polarized into M1 and M2-Mac. The cells were further exposed (24 h) to M1/M2 polarization factors in absence/presence of vehicle or GKT137831, a clinically approved Nox1/4 pharmacological inhibitor.

Results. We found that Nox1, Nox2, Nox4, and Nox5 were significantly up-regulated in human atherosclerotic plaques compared to non-atherosclerotic samples. Immunohistochemical staining of human atherosclerotic lesions revealed that Nox proteins are up-regulated within $CD68^+/CD45^+$ Mac-rich areas. Significant increases in Nox1, Nox2, Nox4, and Nox5 expression levels were detected in M1-Mac as compared to resting Mac (M0) and M2-Mac in vitro. Pharmacological inhibition of Nox greatly reduced the expression markers defining the M1-Mac phenotype (TNFα, MCP-1, TLR2, TLR4, NOS2) and produced a significant up-regulation of CD163, a M2-Mac marker, in both M1-Mac and M2-Mac.

Conclusion. Our data indicate the existence of a new redox-sensitive mechanism whereby ROS generated by activated Nox contribute to Mac polarization. Pharmacological inhibition of Nox may be an attractive therapeutic strategy to reduce the overproduction ROS and inflammatory mediators derived from M1-activated Mac in atherosclerosis.

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BOOK OF ABSTRACTS

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