THE CROATIAN ACADEMY OF SCIENCES AND ARTS
The Department of Biomedical Sciences in Rijeka
UNIVERSITY OF RIJEKA
Department of Biotechnology
CROATIAN PROTEOMICS SOCIETY
THE CROATIAN MEDICAL ASSOCIATION – Branch office Rijeka

25th Symposium

MODERN MASS SPECTROMETRY IN MEDICINE

Endorsed by:

CroProt

2nd October 2018
10,00 am
University Campus Rijeka, University Departments, Lecture hall O-030,
Radmile Matejčić 2, Rijeka, Croatia

Organizers

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Scientific Committee

Daniel Rukavina, president
Jasna Peter Katalinić, Ron Hereen, Sandra Kraljević Pavelić

Organizing Committee

Jasna Peter Katalinić, president
Dina Rešetar Maslov, Marta Žuvić, Srđan Novak

Registration: 9,30 – 10,00 am

Free admission. Participants who want a certificate from the Croatian Medical Chamber need to register.
Refreshments during breaks and lunch are with no charge.
Parking is free and provided in the building of Student Centar Rijeka (Radmile Matejčić 5)

Information

Željana Mikovčić, Department of Biomedical Sciences in Rijeka
Radmile Matejčić 2, Rijeka
Phone: 051 584 826, e-mail: rimed@hazu.hr

P R O G R A M
OPENING
(10,00 – 10,15)

Introduction

Daniel Rukavina, M.D., PhD, Professor Emeritus, Head of the Department of Biomedical Sciences in Rijeka, Croatian Academy of Sciences and Arts
Jasna Peter Katalinić, PhD., Professor, Department of Biotechnology, University of Rijeka, Rijeka and Croatian Proteomics Society, Rijeka, Croatia

Welcome addresses

Marta Žuvić, PhD., Associate Professor, Acting Head of the Department of Biotechnology, University of Rijeka, Rijeka, Croatia

10,15 – 12,00 h

I. INTRODUCTORY LECTURES

Chairmen: Jasna Peter Katalinić and Ron Hereen

Jasna Peter Katalinić, PhD, Professor, Department of Biotechnology, University of Rijeka, Rijeka and Croatian Proteomics Society, Rijeka, Croatia
Revealing molecular signature in health and disease by modern mass spectrometry

Ron Hereen, PhD., Professor, Maastricht MultiModal Molecular Imaging Institute M4I, Maastricht University, Maastricht, Netherlands
Molecular patterns of health and disease: Translational imaging mass spectrometry

Coffee break: 12,00 – 12,30

12,30 – 14,15 h

II. MOLECULAR SCREENING FOR DISCOVERY

Chairmen: Sandra Kraljević Pavelić and Đuro Josić

Sergey Vakhrushev PhD., Associate Professor, University of Copenhagen, Copenhagen, Denmark
Cell engineering in current glycome discovery

Sandra Kraljević Pavelić, PhD., Associate Professor, Department of Biotechnology, University of Rijeka, Rijeka, Croatia
Profiling of complex matrices in drug development by mass-spectrometry-based methods

Lovorka Grgurević, MD., PhD., Professor, School of Medicine, University of Zagreb, Zagreb, Croatia
Mass spectrometry as a tool for biopharmaceutical drug development

Đuro Josić, PhD., Professor, Department of Biotechnology, University of Rijeka, Rijeka, Croatia
Use of foodomics methods as a tool in research and assurance of food quality and safety

Lunch with a panel of speakers: 14,30 – 15,00

15,00 – 17,30 h

III. METHODOLOGIES FOR QUALITY AND VALIDITY OF SCIENTIFIC EVIDENCE

Chairmen: Sergey Vakhrushev and Dina Rešetar Maslov

Anita Horvatić, PhD., Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia
Insights from quantitative proteome profiling of serum in canine leishmaniosis using different proteomic strategies

Tomislav Čaval, MSc., Utrecht University, Utrecht, Netherlands
Direct Quality Control of Glycoengineered Erythropoietin Variants

Dina Rešetar Maslov, MSc., Department of Biotechnology, University of Rijeka, Rijeka, Croatia
Data-Independent and Label-Free Approaches for Absolute Quantitative Mass Spectrometry Proteomics

ABSTRACTS

Revealing molecular signature in health and disease by modern mass spectrometry

Jasna Peter Katalinić
University of Rijeka, Department of Biotechnologyy and Croatian Proteomics Society

Fundamental research in lifesciences on structure and function of biomolecules stays in a continuous support of modern medicine. The knowledge about molecular structure of complex biomolecules as key players in biological processes was shown to be invaluable for understanding of health and disease, whereas its analytical strategies are undergoing a transformation into an integrated „omics" discipline. Experimental evidence previously collected by mass spectrometry during last decades is currently being put together with well established workflows using advanced new technology in mass spectrometers of ever increasing mass accuracy and speed of acquisition.
In this introductory lecture of the symposium an overview on mass spectrometric strategies to reveal molecular signature in health and disease will be presented. Concepts for integration of new big sets of data obtained by targeted experiments at high mass accuracy and high acquisition rates serve toward better understanding of cellular processes regulating translational and personalized medicine and development of synthetic vaccines for malignant and infectious diseases. Discovery of clinically relevant disease biomarkers using mass spectrometry-based proteomics has proven difficult, primarily because of the enormous dynamic range of bloodderived protein concentrations calling to broader view on other types of molecules and their distribution in liquids and tissues. Invited speakers will present this broader view in context of their exciting recent experimental results obtained by novel techniques.

Molecular patterns of health and disease: Translational imaging mass spectrometry

Ron M.A.Heeren
The Maastricht MultiModal Molecular Imaging institute M4I, Maastricht University, Maastricht, Netherlands

A comprehensive understanding of molecular patterns of health and disease is needed to pave the way for personalized medicine and tissue regeneration [1]. One barrier to predictive, personalized medicine is the lack of a comprehensive molecular understanding at the tissue level. As we grasp the astonishing complexity of biological systems (whether single cells or whole organisms), it becomes more and more evident that within this complexity lies the information needed to provide insight in the origin, progression and treatment of various diseases. The best way to capture disease complexity is to chart and connect multilevel molecular information within a tissue using mass spectrometry and data algorithms. Charting this territory through the generation of molecular maps from cells and tissue has become reality through the clinical implementation of imaging mass spectrometry [2] complemented with high throughput "omics" approaches. We have demonstrated how new MS based chemical microscopes target biomedical tissue analysis in various diseases as well as other chemically complex surfaces. In concert they elucidate the way in which local environments can influ-

ence molecular signaling pathways on various scales. The integration of this pathway information in a surgical setting is imminent, and innovations that push the boundaries of the technology and its application are coming to fruition.
State-of-the-Art molecular imaging with mass spectrometry now enables high resolution tissue screening that provides direct insight into tissue metabolism. Applications have penetrated various research domains from drug metabolism to the visualization of molecular signaling pathways in cancer. This lecture will highlight how mass spectrometry based multimodal molecular imaging can be used to reveal the cellular phenotypes. The development and application of new MS based chemical microscopes that target biomedical tissue analysis in various diseases as well as other chemically complex surfaces will be discussed. There is a clear need to add analytical structural separation utilizing ion mobility of gas phase ion chemistry. It will be demonstrated how to elucidate the way in which local environments can influence molecular signaling pathways on various scales.

Acknowledgements:
The Province of Limburg is acknowledged for their financial support through the LINK program. The M4I imaging MS team is acknowledged for their relentless efforts in progressing imaging science.

References:
1.Vaysse PM, Heeren RMA, Porta T, Balluff B (2017) *Analyst* 142:2690-2712
2. Chughtai K, Heeren RMA (2010) *Chem. Reviews* 110:3237-3277

Cell engineering in current glycome discovery

Sergey Y. Vakhrushev
Center for Glycomics, Departments of Cellular and Molecular Medicine and School of Dentistry, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark

Site-specific O-glycosylation is emerging as an important concept for regulating protein processing and functions. However, full understanding of the nature and functions of this abundant type of protein glycosylation is severely hampered by lack of tools for proteome-wide characterization of O-glycan structures at specific sites in O-glycoproteins. Current analytic strategies involve analyses of released O-glycans and/or enriched glycopeptides derived from total digests, which results in loss of important information of occupancy and glycan structures at specific sites. Use of different lectins or click chemistry methods for enrichment of glycopeptides have provided insight into Core1 O-glycan structures on a limited number of O-glycoproteins, but with the introduction of the gene engineered SimpleCell O-glycoproteomics strategy combined with sensitive lectin-enrichment and LC-MS/MS in data-dependent acquisition (DDA) mode and spectrum-centric strategy using database searching for data analysis [1-2], it became clear that the O-glycoproteome is vast with over 80% of all proteins trafficking the secretory pathway being O-glycosylated. It is thus evident that further developments in analytic strategies are required to be able to analyze O-glycan sites and structures and explore the important roles in complex biological samples.
Here we discuss different aspects of quantitative O-glycoproteomics applied to the analysis of human cell lines, body fluids and tissues.

[1] Steentoft C.; Vakhrushev S.Y.; Bennett E.P.; etc. Mining the O-glycoproteome using zinc-finger nuclease-glycoengineered SimpleCell lines. *Nat.* Methods **2011**, 8(11), 977-982.

[2] Steentoft C.; Vakhrushev S.Y.; Joshi H.J.; etc. Precision mapping of the human O-GalNAc glycoproteome through SimpleCell technology. EMBO J. **2013**, 32(10), 1478-88.

Profiling of complex matrices in drug development by mass-spectrometry-based methods

Sandra Kraljević Pavelić
Department of Biotechnology, University of Rijeka, Rijeka, Croatia

Developments in mass spectrometry-based analytical approaches have greatly facilitated implementation of proteomics and metabolomics technologies in drug research and development. Detailed information on protein or metabolite abundance and composition of complex natural matrices such as tissue, body fluids or raw material in biomedical production may be obtained due to high sensitivity and resolution of mass spectrometry which allows for identification and quantification of large number of molecules in a single measurement. This is highly relevant for the drug development process as such information may improve understanding of the mechanisms underlying pharmacological or toxic effects of drugs as well as improve medical products quality control and production process. In the field of cancer research, mass spectrometry-based proteomics has proven useful in identification of novel diagnostic and prognostic biomarkers and in determining the impact of genetic alterations on protein abundance and networks. The latter is important as it allows elucidation of mechanisms that shape cancer heterogeneity and determine response to therapy. Knowing which protein networks are deregulated by specific mutant oncogene could be particularly useful in designing novel treatment strategies to overcome cancer drug resistance. Findings from our on-going study demonstrate the ability of proteomics to identify subtle differences in protein expression driven by mutations in specific oncogenes such as BRAF and KRAS in colon cancer cells. By knowing which protein networks are aberrantly regulated as a consequence of specific gene mutation, it would be possible to develop novel treatment strategies tailored to specific genetic traits of an individual cancer patient. In addition, mass spectrometry holds great potential in discovery and characterization of novel bioactive compounds from natural sources that could prove efficient in different medical applications. The latter will be illustrated by data from our on-going study on liquid chromatography-mass spectrometry analysis of propolis to identify compounds with potential pharmacological activities. This and similar studies support the application of mass spectrometry in the development of novel medical products.

Mass spectrometry as a tool for biopharmaceutical drug development

Ruđer Novak[1], Slobodan Vukičević[1] i Lovorka Grgurević[1]
[1]Laboratory for Mineralized Tissues, Center for Translational and Clinical Research, School of Medicine, University of Zagreb, Croatia

The molecular processes involved in bone repair hold a key to development of new biological procedures for stimulation of bone healing. Currently, there is no adequate therapy available that can accelerate long bone fractures and promote its healing. Hence, there is a need for development of a new osteogenic device that will offer safe and cost-effective healing in these patients. The Osteogrow project has developed an entirely new, safe and cost-effective therapy that will decrease the need for secondary surgical interventions (unlike the current standard of care). The Osteogrow device contains of a biologically compatible autologous carrier made from peripheral blood to which recombinant protein BMP6 is added. The blood coagulum is placed at the site of bone fracture and within several months, a new bone piece is created, taking only millilitres of the patient's blood. With support of the EU FP7 grant we have completed the Osteogrow preclinical development and clinical studies in two indications (regeneration of the metaphyseal bone).
The rhBMP6 is produced in a mammalian cell culture using a lentiviral vector. In preclinical studies the secreted protein was extensively purified in several steps in order to clean the product from the majority of cell-culture derived contaminants. This clean-up process was fine-tuned by mass spectrometry, using a proteomics approach to detect any potential unwanted protein in the final product. Detection of rhBMP6 involved electrophoretic separation of the protein, followed by in-gel digestion, chromatographic separation of the peptides, and their validation analysis. RhBMP6 was enzymatically cleaved into peptides by trypsin or chymotrypsin, followed by liquid chromatography-mass spectrometric (LC-MS) analysis of the obtained peptides. Raw experimental data was analysed by MaxQuant software, and used for sequence confirmation of the produced rhBMP6. The method was employed for lot-to-lot identity testing in support of bioprocess development and clinical trials of the Osteogrow project.

Key words: biopharmaceutical drugs, BMP6

Use of foodomics methods as a tool in research and assurance of food quality and safety

Đuro Josić
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According to definition of Cifuentes, foodomics is "a discipline that studies the food and nutrition domains through the application and integration of advanced –omics technologies to improve consumer's well-being, health and knowledge" [1]. Consequently, foodomics requires reliable qualitative and quantitative information about food components by use of genomics, transcriptomics, proteomics and metabolomics methods, as well as already well-established methods for food analysis. This integrative approach enables much higher level of understanding of changes during food processing and storage, as well as detection of markers of food quality and safety. Together with newly developed high-throughput sample preparation procedures, mass

spectrometry based foodomics approaches became increasingly important. They are utilized in different foodomic studies, mainly for abovementioned investigations of food quality and safety, but also for detection and documentation of counterfeit foods and beverages. Mass spectrometry based foodomcs analyses enable studies of different food components as well as reliable quantification of individual high- and low-molecular food components in highly complex samples such as different food matrices. Our investigations of changes of food pathogens, namely Gram positive and Gram negative bacteria after treatment with different disinfectants illustrates the use of proteomics methods for the virulence and pathogenicity of food borne bacteria, their adaptation and survival under stress conditions and detection of possible biomarker candidates for food poisoning.

References:

1. A. Cifuentes, J. Chromatogr. 1216 (2009) 7109.

Insights from quantitative proteome profiling of serum in canine leishmaniosis using different proteomic strategies

Anita Horvatić
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Proteomic investigations in veterinary medicine are slowly gaining importance but are still lagging behind investigations in human medicine, as well as in their clinical research application. In most of the shotgun proteomic studies in veterinary medicine, mass spectrometers have been operated in data-dependent acquisition (DDA) mode addressing new biomarkers discovery in biological fluids, especially in serum. Recently, data-independent acquisition (DIA) has been introduced into this field, providing insights into physiology and pathophysiology in animals. Our interest is focused to canine leishmaniosis, a vector-borne zoonotic disease, which is caused by *Leishmania infantum*. Phlebotomine sand flies are the biological vectors and dogs are the main reservoir for human infection. Due to its zoonotic potential, it is of a great importance to diagnose this disease and treat infected animals. The aim of our study was to apply both proteomic strategies, *e.g.* DDA and DIA, for proteome profiling of serum from *leishmania* infected dogs in order to understand the pathophysiology of the diseases, as well as find proteins with diagnostic potential and for monitoring the success of treatment.
Serum samples of healhy dogs and Leishmania-seropositive dogs, as well as serum of clinically diseased dogs before and one month after the treatment were collected and analysed using Ultimate 3000 RSLCnano system combined with Q Exactive Plus and Orbitrap Elite mass spectrometers, respectively. DDA-based quantitative proteomic approach using tandem mass tags (TMT) followed by statistical analysis showed that from 117 identified serum proteins, 27 were significantly deregulated ($p<0.05$) after the treatment. Bioinformatic analysis using Cytoscape revealed their functions in immune response (*e.g.* IgA, ITIH4, fibronectin) and coagulation cascade (*e.g.* kininogen-1 isoform X1 and X2). Unlike DDA-based approach, DIA strategy using Spectronaut software and sample specific spectral libraries built from DDA runs enabled significant increase in number of identified proteins in shorter LC gradient time. A number of 15 statistically significant deregulated proteins ($p<0.05$) were detected in serum of Leishmania-seropositive dogs compared to healthy dogs.

In conclusion, DIA-based strategy showed greater potential for detailed proteomic profiling of serum in canine leishmaniosis compared to DDA-based strategy. Deregulated proteins, some of which were reported for the first time in canine leishmaniosis, were involved in inflammatory, coagulation or defence mechanisms and could be potentially suitable for the disease progression and treatment monitoring of this parasitic disease.

Direct quality control of glycoengineered erythropoietin variants

Tomislav Čaval
University of Utrecht, Utrecht, Netherlands

Recombinant production of Erythropoietin (EPO) in CHO cells is limited by the cells' capacity for N-glycosylation, and production of glycoproteins with robust, desirable homogeneous glycoforms remains a challenge. Recently, a wide-ranging knockout system targeting glycosyltransferase genes in CHO cells was reported. However, as EPO can exhibit hundreds of glycoproteoforms, thorough means are needed to assess the glycosylation profile in each engineered variant. Here we use high-resolution native mass spectrometry to measure the glycoproteoform profiles of 24 glycoengineered EPO variants. The observed glycosylation profiles revealed co-occurrence of hundreds of variants ranging from EPOs exhibiting heterogeneous, tetra-antennary, polyLAcNAc elongated N-glycans all the way to EPOs with homogeneous, bi-antennary N-glycans. As a first step we characterized wild-type EPO where in the native mass spectrum we observed EPO glycoproteoforms ranging from 29 000 Da up to 34 000 Da. This 5000 Da spread is caused by differential glycosylation. In total we observed around 200 mass-resolved glycoproteoforms. Next, we studied two glycoengineered EPO variants, mgat5 KO and *mgat 4A/4B* KO, which result in tri-antennary N-glycans stemming from the loss of β6- and β4-branch from tetra-antennary N-glycans, respectively. Although one would expect that such mirrored N-glycans would produce very similary glycoproteoform profiles, observed native MS spectra showed great variation between these 2 EPOs. In short, EPO from *mgat5* KO resulted in a more simplified spectrum due to a substantial decrease in polyLacNAc content demonstrating that the main acceptor site for polyLacNAc elongation on N-glycans is specifically the β6-branch and not the β4-branch. Finally, based on a unique mass, charge and intensity glycoproteoform profiles of each EPO variant, we classify them according to similarities in glycosylation profiles. This automated classification distinguieshes EPO variants based on glycan branching, elongation, and sialylation, which are all crucial for biotherapeutic efficacy.

Data-independent and label-free approaches for absolute quantitative mass spectrometry proteomics

Dina Rešetar Maslov
Department of Biotechnology, University of Rijeka, Rijeka, Croatia

For years much effort was put into constructing comprehensive protein catalogs, i.e. proteomes. Such in-depth proteomic studies, supported by Human Protein Atlas discoveries, unexpectedly demonstrated the extent to which diverse cellular systems have similar proteomes. Although fundamental, proteomes don't provide necessary data for describing and explaining complex biological mechanisms and dynamic processes. Therefore, tissue or cellular identity as well as dynamic nature of the proteome under physiological and experimentally manipulated conditions are primarily determined by the abundance at which constituent proteins are expressed.

The maturation of mass spectrometry (MS) – based quantitative proteomics in recent years has brought a variety of technologies, each with their own strengths and weaknesses. For those unfamiliar with the evolving landscape it can be difficult to match the experiment at hand with the best tool for the job.

This presentation will briefly introduce you to quantitative proteomic MS-based techniques that can easily be grouped into (1) label-free and (2) metabolic or chemical labeling approaches. Concisely, the most commonly used MS data acquisition strategies namely, data-dependent analysis (DDA) and data-independent analysis (DIA) as well as main differences between them, will be mentioned. In the main part of this presentation benefits and weaknesses of the label-free approach and data-independent acquisition strategies for absolute quantitative MS proteomics of complex biological systems and large-scale studies will be addressed in more detail. Method application examples on both *in vivo* models and clinical specimens will be presented. Special attention will be given to bottom-up sample preparation strategies and their importance in assessing reproducible and comparable label-free absolute quantitative proteomic MS data.