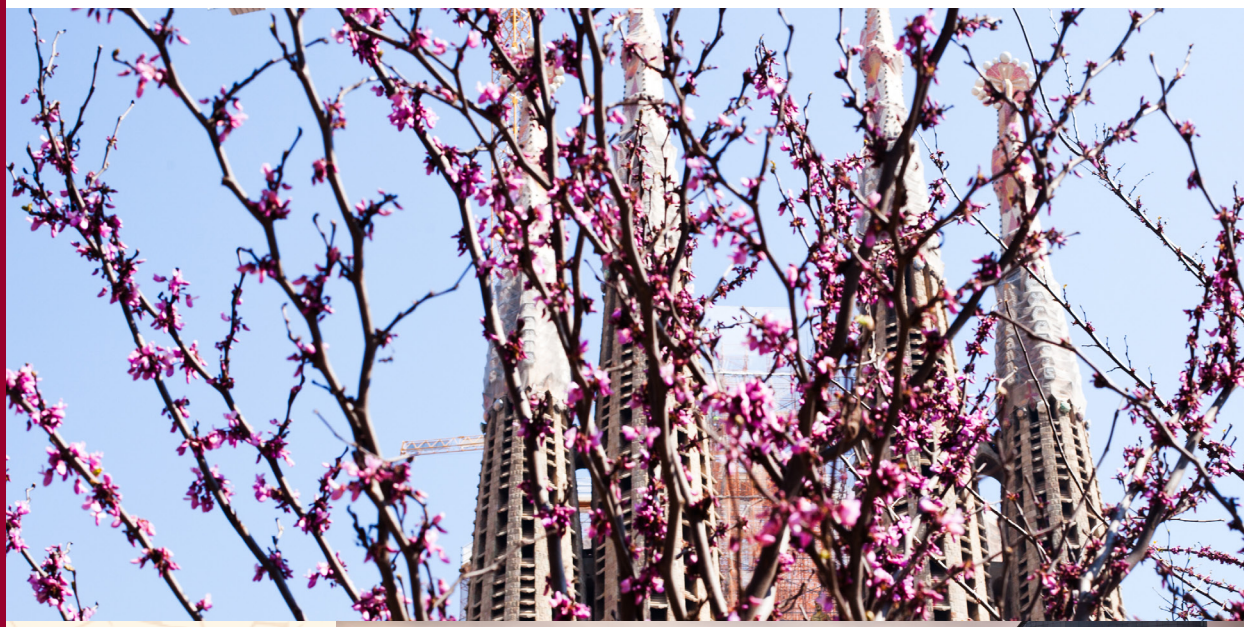
5th Congress of the Spanish Proteomics Society.
Time to Imagine

Barcelona, 5-8 February, 2013



www.seprot2013.org

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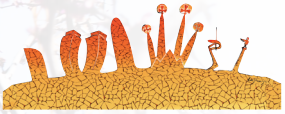
ABSTRACT BOOK of the

5th Congress of the Spanish Proteomics Society. *Time to Imagine*

Chairs

Montserrat Carrascal, CSIC/UAB Proteomics Laboratory, IIBB-CSIC, Barcelona
Eliandre de Oliveira, Proteomics Platform, PCB, Barcelona
Marina Gay, Mass Spectrometry Core Facility, IRB Barcelona



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COMMITTEES

Chairs

Montserrat Carrascal, CSIC/UAB Proteomics Laboratory, IIBB-CSIC, Barcelona
Eliandre de Oliveira, Proteomics Platform, PCB, Barcelona
Marina Gay, Mass Spectrometry Core Facility, IRB Barcelona

Scientific Committee

Fernando Corrales, CIMA, Universidad de Navarra, Pamplona
Fernando Vivanco, UCM, Fundación Jiménez Díaz, Madrid
Manuel M. Sánchez del Pino, Universidad de Valencia, Valencia
Ignacio Casal, CIB, CSIC, Madrid
Concha Gil, UCM, Madrid
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Jesús Vázquez, CNIC, Madrid
Juanjo Calvete, IBV-CSIC, Valencia
Ángel García, Universidad de Santiago de Compostela
Lucía Monteoliva, UCM, Madrid

Most welcome to Barcelona 2013

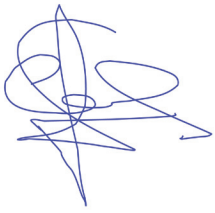
It is my great pleasure to welcome you all to the Spanish Proteomics Society, the 5th SEProt Scientific Meeting that is hosted this time in the cosmopolitan city of Barcelona.

Under the title of "Time to Imagine", the main topic areas chosen for the meeting are: Quantitative Proteomics, PTMs, Disease Proteomics, Networks/Protein-Protein Interactions, Bioinformatics, and Emerging Technologies. The organizers headed by the three chair ladies Monserrat Carrascal, Eliandre de Oliveira and Marina Gay have made a superb job putting together an excellent program including the most relevant issues in proteomics that will be presented and discussed by international experts in the field. The educational program as a pre-Congress event is also remarkable. Hot spots on sample proteomics research, from sample preparation to applications in biomedicine will be illustrated.

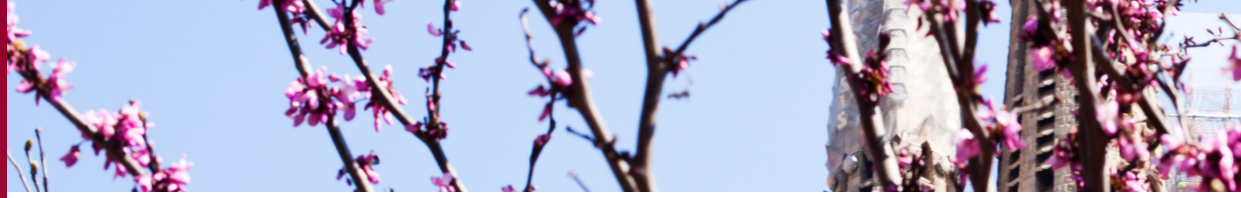
As in previous editions, I'm pleased to announce the SEProt awards to the best poster presentation and to the best publication in proteomics by an investigator working in a Spanish laboratory. Proteomics has experienced great progress in our country in the last few years and is a rewarding work for the SEProt to contribute recognizing this merit to scientists who make this possible. The support from Bruker Daltonics is deeply acknowledged.

The principal honour of the SEProt is its panel of most relevant scientists in proteomics that agreed to join us as Honorary Members. In Barcelona, the SEProt wants to pay tribute to Professor Richard Caprioli for his seminal work in imaging mass spectrometry. It is a great privilege having Professor Caprioli among us as Honorary Member.

I am certain that the 5th SEProt Congress will offer you the opportunity to meet with your colleagues for scientific interactions, discussions and debates in an exciting educational and professional environment. It is time now to start imagining the future based on the reality we imagined just a while ago.



Fernando J. Corrales
SEProt President



Welcome Address of the Organizing Committee

Dear colleagues, dear friends,

The Organizing Committee welcomes you to Barcelona for the 5th biannual SEProt congress.

This congress comprises an outstanding scientific programme, featuring sessions in state-of-the-art proteomics, plenary lectures, posters, oral communications, which along with the presence of very relevant researchers, will allow for thought-provoking, sharing and discussion of the latest scientific advances in the field.

Barcelona was chosen to host this congress because of the very important role it plays in proteomics research, having 10 different proteomics laboratories and a very diverse scientific community that benefits from private and public research clusters and facilities. Also, Barcelona has long been associated with cultural and industrial innovation, the most renowned example being Gaudi, but also with other very prominent geniuses such as Salvador Dalí, Narcís Monturiol, Pau Casals or Ferran Adrià. This edition's title, "Time to Imagine", aims to acknowledge that legacy and uses it to inspire new ways of building in our field.

The Organizing and Scientific Committees of the 5th SEProt Congress have worked with great enthusiasm in the preparation of this event. We would like to thank you for your participation as presenter, sponsor or attendee and we invite you to get involved in the scientific program and to enjoy the warm and vibrant city of Barcelona.

Kind regards,

Montserrat Carrascal **Eliandre de Oliveira** **Marina Gay**

Professor Richard Caprioli

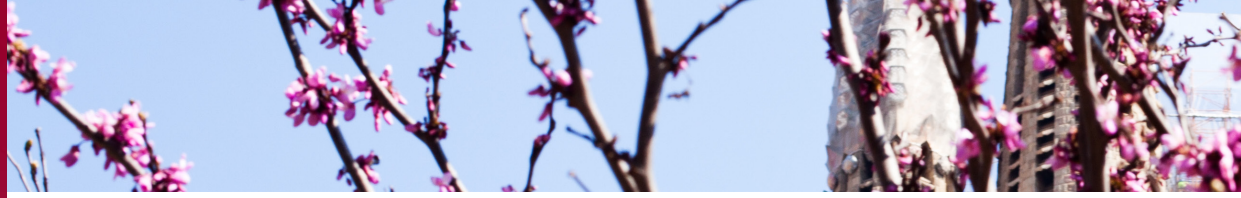
Professor Richard Caprioli Honorary Member of the SEProt

The Spanish Proteomics Society is proud to announce the appointment as Honorary Member of Professor Richard Caprioli for his eminent contributions in the field of proteomics.

Professor Caprioli received his B.S. in 1965 from Columbia University in New York, where he got his Ph.D. in Biochemistry in 1969 with Professor David Rittenberg. After a one-year postdoctoral fellowship he was appointed as Assistant Professor of Biochemistry at Purdue in 1970. Then he moved to the University of Texas Medical School in Houston in 1975 where he was Professor of Biochemistry and Molecular Biology and Director of the Analytical Chemistry Center. He moved to Vanderbilt University in 1998 where he is currently Professor in the Departments of Biochemistry, Chemistry, Pharmacology and Internal Medicine and is the Stanley Cohen Professor of Biochemistry and Director of the Mass Spectrometry Research Center at the School of Medicine.

Professor Caprioli has pioneered techniques such as imaging mass spectrometry to study the location and movement of proteins that are potential drug targets or biomarkers for disease. His general research interests lie in discovery of temporal and spatial processes in biological systems using mass spectrometry. This work has included technology developments in the areas of electrospray and laser desorption ionization mass spectrometry and their applications to intact tissues and in live animal systems, achieving ultra-high sensitivity detection of endogenous compounds (e.g., neuropeptides). Recent work involves the development of Imaging Mass Spectrometry, a technology whereby molecular images of peptides, proteins, drugs and other compounds are localized in tissue sections with molecular weight specificity. Richard made notorious contributions to specific research areas deciphering the spatial distributions of molecules in both health and disease. Of special interest are the molecular distributions, spatial rearrangement, and abundance of biological molecules in cancer tissues including human glioblastomas, breast cancer, colorectal cancer, prostate cancer and lung cancer. He has published over 300 scientific papers, including three books and holds 12 US patents involving mass spectrometry technologies.

Prof. Caprioli has been a member of the American Society for Mass Spectrometry since 1975; he served two years each as President of the Society and Vice-President for Programs. He is a member of the American Society for Biochemistry and Molecular Biology, the American Association for Cancer Research, and the American Chemical Society. Professor Caprioli has been the Editor-in-Chief of the *Journal* of *Mass Spectrometry* since 1990. He is currently Series Editor of *The Encyclopedia of Mass Spectrometry* and additionally has edited several volumes in this encyclopedia. Prof. Caprioli is currently serving a 3-year term on the Board of Directors of the HUPO and has been a member of the Board of Directors of the US HUPO since its inception.



In 2003, Dr. Caprioli received the Thomson Medal Award from the International Mass Spectrometry Society for “for outstanding achievements in mass spectrometry and for distinguished service to international mass spectrometry.” He was named one of the “Pioneers in Proteomics” by the National Institutes of Health and received the Donald H. Coffey Award from the Society for Basic Urologic Research in 2005. He received the Field and Franklin Award from the American Chemical Society in April, 2006 for Outstanding Achievement in Mass Spectrometry, the Eastern Analytical Society 2010 Award for Achievements in Mass Spectrometry and the HUPO Distinguished Achievement Award in Proteomic Sciences for 2010.

It is a privilege and a great honour for the Spanish Proteomics Society Professor Richard Caprioli having accepted the nomination to become Honorary Member and to deliver a Plenary Lecture at its 5th Congress.

Fernando J. Corrales
SEProt President

Convocatoria del quinto premio de la Sociedad Española de Proteómica

Barcelona, Enero del 2013

La Sociedad Española de Proteómica (SEProt) convoca la quinta edición del PREMIO SOCIEDAD ESPAÑOLA DE PROTEÓMICA destinado a reconocer la labor en el campo de la Proteómica de un científico que desarrolle su actividad en España. El premio, patrocinado por **Bruker BioSciences Española S.A.** (http://www.bruker.es), está dotado con 2000 € y será entregado por un representante de Bruker Española durante el 5º Congreso de la SEProt, Time to Imagine, que se celebrará en el Auditorio Axa en Barcelona entre los días 5- 8 de Febrero de 2013 (http://www.seprot2013.org/). En la presente convocatoria se otorgarán **dos galardones** que en ningún caso podrán ser compartidos. Una mitad del premio (1000 € y diploma acreditativo) será para **una publicación científica** relacionada con cualquier desarrollo o aplicación de la Proteómica. La otra mitad del premio (1000 € y diploma acreditativo) será para una contribución en forma de **panel** al 5º Congreso de la SEProt. Las decisiones de los jurados serán inapelables. Los Premios no podrán concederse al mismo científico dos veces y podrán quedar desiertos si así lo decidiese el jurado.

La elección de la publicación científica merecedora del galardón será realizada por un jurado de expertos designado a tal efecto por el Presidente de la SEProt. Solo podrán optar al Premio científicos españoles que sean o no socios de la SEProt. La labor investigadora deberá haber sido realizada en España y haber sido publicada entre Enero de 2011 y Diciembre de 2012. Los candidatos deberán remitir 1 copia del trabajo **en formato electrónico** al Secretario de la SEProt (Manuel Sánchez del Pino, sandelpi@uv.es). Deberán, asimismo, adjuntarse los datos personales y profesionales del candidato, y un breve resumen de las razones que, a juicio del candidato, debieran ser consideradas por el jurado. **La fecha límite para la recepción de los trabajos será el 25 de Enero 2013**. La resolución se dará a conocer el día 01 de Febrero de 2013 a través del portal electrónico de la SEProt y del Congreso. El galardonado será invitado a participar en el 5º Congreso de la SEProt exento del pago de las tasas de inscripción y a publicar un artículo en la revista Proteómica.

La elección del ganador(a) del Premio SEProt a la contribución en formato panel presentada al 5º Congreso de la SEProt se efectuará entre los primeros firmantes del estudio por un jurado designado a tal efecto por el Presidente de la Sociedad Española de Proteómica. Solo podrán optar al Premio científicos españoles sean o no socios de la SEProt. La labor investigadora considerada deberá haber sido realizada en España y ser inédita o, en todo caso, no haber sido publicada con anterioridad a Diciembre de 2012.

Fernando J. Corrales
Presidente de la SEProt

Programme Overview

	Tuesday 5	Wednesday 6	Thursday 7	Friday 8
09.00h	**Pre-Congress Educational Day** (9.00 - 17.10h) **Workshop COST-FAP** (9.00-16.30h)	**S1** (9.00 - 11.00h) **Quantitative Proteomics** *Tamar Geiger*	**S3** (9.00 - 11.00h) **Disease Proteomics** *Concha Gil*	**S6** (9.45 - 12.05h) **Emerging Technologies** *Roman Zubarev* *Yury Tsybin*
10.00h				
11.00h		**Coffee Break**		**Coffee Break**
12.00h		**S2** (11.30 - 13.30h) **PTMs** *Martin R. Larsen* *Judit Villén*	**S4** (11.30 - 13.50h) **Networks/Protein-Protein Interactions** *Alison E. Aschroft* *Xavier Avilés*	**Closing Lecture** (12.30 - 13.30h) *Juan J. Calvete*
13.00h		**Lunch**	**Lunch**	**Farewell**
14.00h		**Poster Session** (14.30 - 15.30h)	**Poster Session** (15.00 - 16.00h)	
15.00h				
16.00h	**Registration Opens**	**HPP** (15.30 - 17.30h) *Juan Pablo Albar* *Amos Bairoch* *György Marko-Varga*	**S5** (16.00 - 18.00h) **Bioinformatics** *Jürgen Cox* *S. Martínez-Bartolomé*	
17.00h				
18.00h	**Welcome and Opening Lecture** (18.00 - 19.30h) *Richard M. Caprioli*		**Coffee Break**	
19.00h			**SEProt Awards and General Meeting** (18.30 - 19.30h)	
20.00h	**Welcome Reception** (19.30 - 21.00h)			
21.00h			**Congress Dinner** (21.30h)	
22.00h				

Scientific Programme

Tuesday, February 5

Proteomics: basics and applications. Educational Day

Chairs: Joaquin Abian, Anabel Marina

09.00-09.50h Introduction to the course. Basic concepts, sample preparation and separation technologies
Joaquin Abián *(LP CSIC/UAB, Barcelona)*

09.50-10.40h Label-free quantitative proteomics: advantages and disadvantages
Miren Josu Omaetxebarria *(UPV-EHU, Bizkaia)*

10.40-11.00h **Coffee break**

11.00-11.50h Quantitative proteomics using isotopic labeling
Francesc Canals *(IRVH, Barcelona)*

11.50-12.40h Targeted proteomics using selected reaction monitoring (SRM)
Eduard Sabidó *(CRG, Barcelona)*

12.40-13.30h The phosphoproteome analysis
Anabel Marina *(CBMSO, Madrid)*

13.30-14.30h **Lunch time**

14.30-15.20h Study of protein structure by mass spectrometry
Ignasi Forné *(Ludwig-Maximilians-University, München)*

15.20-16.10h Clincal Proteomics
Fernando Corrales *(CIMA, Pamplona)*

16.10-17.00h Bioinformatics
Salvador Martínez-Bartolomé *(CNB-CSIC, Madrid)*

17.00-17.10h Concluding remarks
Joaquin Abian *(LP CSIC/UAB, Barcelona)****, Anabel Marina*** *(CBMSO, Madrid)*

16.00h Registration opens

Welcome and Opening Lecture

Chair: Fernando Corrales

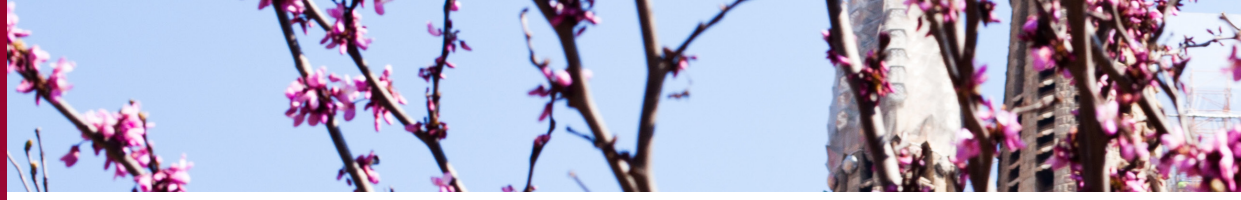
Welcome

18.00-18.30h ***Fernando Corrales, Montserrat Carrascal, Eliandre de Oliveira, Marina Gay***

Opening Lecture

18.30-19.30h MALDI Imaging Mass Spectrometry: molecular mapping beyond the microscope
Richard M. Caprioli *(Vanderbilt University School of Medicine, Nashville, TN, USA)*

19.30-21.00h Welcome Reception



Wednesday, February 6

Session 1: Quantitative Proteomics

Sponsored by: ABSCIEX

Chairs: Carme Quero and Eduard Sabidó

Plenary Lecture

09.00-09.40h Breast cancer proteomics using high resolution mass spectrometry and super-SILAC
Tamar Geiger *(Tel Aviv University, Tel Aviv, Israel)*

Oral Communications

09.40-10.00h SILAC-based quantitative analysis of human bone marrow mesenchymal stem cells secretome during chondrogenesis
Beatriz Rocha *(INIBIC-CHUAC, A Coruña, Spain)*

10.00-10.20h Determination of protein turnover in primary culture of fish myocytes by dynamic SILAC and shotgun proteomics
Miguel Martin-Perez *(Fisiologia i Immunologia, UB, Barcelona, Spain)*

10.20-10.40h Enabling the next generation of Mass Spectrometry-based Quantitation Workflows
Antonio Serna Sanz *(ABSCIEX)*

10.40-11.00h Development of a new proteomic tool with SILAC combined with hydrazide glycoprotein isolation to characterize degradome in breast cancer cells
Gemma Reverter-Branchat *(Laboratori de Proteòmica, VHIO, Barcelona, Spain)*

11.00-11.30h **Coffee break offered by:** BRUKER

Session 2: PTMs

Sponsored by: LI-COR Biosciences

Chairs: Joaquin Abian, Miren Josu Omaetxebarria

Plenary Lectures

11.30-12.10h Proteomics strategies for the characterization of Post-Translational Modifications of proteins
Martin R. Larsen *(University of Southern Denmark, Odense, Denmark)*

12.10-12.50h Phosphorylation and ubiquitylation crosstalk in protein degradation
Judit Villen *(Dept. Genome Sciences, University of Washington, Seattle, WA, USA)*

Oral Communications

12.50-13.10h High-throughput mitochondrial redox proteomics analysis of the effects of oxidative stress in cellular and animal models of cardiovascular disease
Jesús Vázquez *(CNIC, Madrid, Spain)*

13.10-13.30h Thiol redox proteomics in hypoxia cell signalling
Antonio Martínez-Ruiz *(Hospital Universitario de La Princesa, Instituto de Investigación Sanitaria Princesa, Madrid, Spain)*

13.30-14.30h **Lunch time**

14.30-15.30h Poster Session

HPP

Chair: Fernando Corrales

15.30-16.10h The human proteome project interconnected with large scale biobanking
György Marko-Varga *(Lund University, Lund, Sweden)*

16.10-16.50h Nextprot: the human protein-centric knowledge platform, status and future developments
Amos Bairoch *(SIB and University of Geneva, Geneva, Switzerland)*

16.50-17.30h Human Proteome Project: Characterization of the proteins encoded by the Chromosome-16 protein coding genes
Juan Pablo Albar *(ProteoRed-ISCIII, CNB-CSIC, Madrid, Spain)*

Thursday, February 7

Session 3: Disease Proteomics

Sponsored by: Thermo SCIENTIFIC

Chairs: Dolores Jaraquemada and Jaime Sancho

Plenary Lecture

09.00-09.40h Host-pathogen interaction: insights from proteomics
Concepción Gil *(Facultad de Farmacia, Universidad Complutense de Madrid, Madrid, Spain)*

Oral Communications

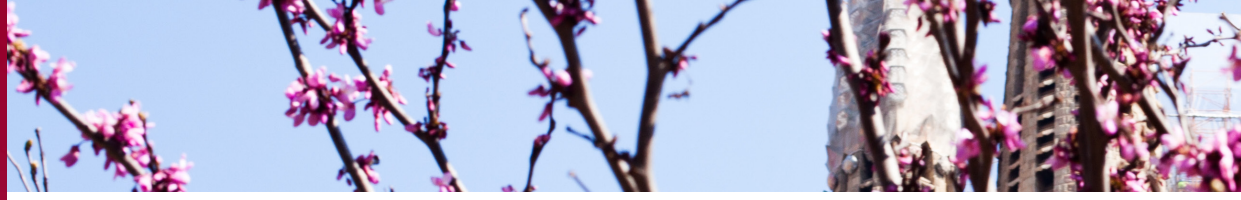
09.40-10.00h High resolution, accurate mass approaches for the quantification of target peptides
Sigrid Baumgarten *(Thermo Fisher Scientific, Paris, France)*

10.00-10.20h A new fast method for peptide biomarker discovery in human serum by mass spectrometry
Carolina Fernández-Costa *(INIBIC-CHUAC, A Coruña, Spain)*

10.20-10.40h Discovery, accurate inclusion mass screening and label-free quantification of potential biomarkers for colorectal cancer in serum of human patients
Marta Mendes *(CIB-CSIC, Madrid. Spain)*

10.40-11.00h Functional Proteomics approaches for high-throughput determination of small molecules interactions on cKIT
Manuel Fuentes *(CIC, Universidad de Salamanca-CSIC, Salamanca, Spain)*

11.00-11.30h **Coffee break offered by:** ISCIII ProteoRed



Session 4: Networks/Protein-Protein Interactions

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Chairs: Marta Vilaseca and Ignacio Casal

Plenary Lectures

11.30-12.10h Protein structure and dynamics: what can ion mobility spectrometry-mass spectrometry tell us?
Alison E Ashcroft *(Astbury Centre for Structural Molecular Biology University of Leeds, Leeds, UK)*

12.10-12.50h Proteomics and interactomics of carboxypeptidases and their proteinaceous substrates
Francesc Xavier Aviles *(IBB, UAB, Bellaterra, Barcelona, Spain)*

Oral Communications

12.50-13.10h Investigation of protein monomerization by capillary electrophoresis and ion mobility mass spectrometry. Application to superoxide dismutase 1 and lateral amyotrophic sclerosis
V. Sanz-Nebot *(Department of Analytical Chemistry, UB, Barcelona, Spain)*

13.10-13.30h Hydrogen-deuterium exchange for protein structure analysis
Robert Tonge *(Waters Corporation, Manchester, UK)*

13.30-13.50h Cyclodextrin- and methyl jasmonate-mediated resveratrol accumulation in grapevine cell cultures: an omics approach
Roque Bru-Martínez *(Faculty of Science, University of Alicante, Alicante, Spain)*

13.50-15.00h **Lunch time**

15.00-16.00h Poster Session

Session 5: Bioinformatics

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Chairs: Manuel Sanchez del Pino and Felix Elortza

Plenary Lectures

16.00-16.40h MaxQuant: computational mass spectrometry based proteomics for the masses
Jürgen Cox *(Max Planck Institute of Biochemistry, Munich, Germany)*

16.40-17.20h Complete proteomics data workflow using the proteored MIAPE-extractor tool
Salvador Martínez-Bartolomé *(ProteoRed-ISCIII, CNB - CSIC, Madrid, Spain)*

Oral Communications

17.20-17.40h A new approach for systems biology analysis of high-throughput quantitative proteomics experiments
Marco Trevisan-Herraz *(CNIC, Madrid, Spain)*

17.40-18.00h On the feasibility of 2D high pH / low pH liquid chromatography coupled to mass spectrometry in label-free intensity-based quantification
Alex Campos *(PCB, Barcelona, Spain & Integromics, Madrid, Spain)*

18.00-18.30h **Coffee break**

18.30-19.30h SEProt Awards and General Meeting

21.30h **Congress Dinner**

Friday, February 8

Session 6: Emerging Technologies

Chairs: Francesc Canals and Lucia Monteoliva

Plenary Lectures

09.45-10.25h Identifying and quantifying 10,000 proteins in 10 hours – feasible, possible, done?...
Roman A. Zubarev *(Karolinska Institutet, Stockholm, Sweden & Science for Life Laboratory, Stockholm, Sweden)*

10.25-11.05h Advances in middle-down and top-down proteomics
Yury O. Tsybin *(École Polytechnique Fédérale de Lausanne, Switzerland)*

Oral Communications

11.05-11.25h Antibody colocalization microarray: a scalable technology for multiplex protein analysis in complex samples
Mateu Pla-Roca *(Institute for Bioengineering of Catalonia, Barcelona, Spain)*

11.25-11.45h Conservation of peptide extracts from trypsin digestion of proteins in a hospital biobank facility
Jesús Mateos *(INIBIC- CHUAC, A Coruña-Spain)*

11.45-12.05h Proteogenomics of bacteria and archaea: a romper la piñata!
Jean Armengaud *(CEA, DSV, iBEB, Lab Biochim System Perturb, Bagnols-sur-Cèze, France)*

12.05-12.30h **Coffee break**

Closing Lecture

Chair: Montserrat Carrascal, Eliandre de Oliveira and Marina Gay

12.30-13.30h Hypothesis-driven omics to understand snake venoms and fight a neglected disease
Juan J. Calvete *(Instituto de Biomedicina de Valencia, CSIC, Valencia, Spain)*

13.30-14.00h Farewell

Opening & Closing Lectures

Opening Lecture

MALDI IMAGING MASS SPECTROMETRY: MOLECULAR MAPPING BEYOND THE MICROSCOPE

Richard M. Caprioli

Departments of Biochemistry, Chemistry, Pharmacology and Medicine
Vanderbilt University School of Medicine, Nashville, TN, U.S.A.

MALDI Imaging MS produces molecular maps of peptides, proteins, lipids and metabolites present in intact tissue sections. It employs desorption of molecules by direct laser irradiation to map the location of specific molecules from fresh frozen and formalin fixed tissue sections without the need of target specific reagents such as antibodies. Molecular images of this nature are produced in specific m/z (mass-to-charge) values, or ranges of values, typically covering the MW range 200-100,000. We have also developed a similar approach for the analysis of targeted areas of tissues by integrating mass spectrometry and microscopy, termed histology-directed molecular analysis, whereby only selected areas of cells in the tissue are ablated and analyzed.

We have employed Imaging MS in studies of a variety of biologically and medically relevant research projects. One area of special interest is the molecular mapping of changes observed in diabetes in both a mouse model and in the human disease. Major molecular alterations have been recorded in advanced diabetic nephropathy. Other applications include developmental studies of embryo implantation is mouse, renal cancers as well as that in other organs, and neurodegenerative disease. Molecular signatures have been identified that are differentially expressed in diseased tissue compared to normal tissue and also in differentiating different stages of disease. These signatures typically consist of 10-20 or more different proteins and peptides, each identified using classical proteomics methods. In addition, Imaging MS has been applied to drug targeting and metabolic studies both in specific organs and also in intact whole animal sections following drug administration.

This presentation will focus on recent technological advances both in sample preparation and instrumental performance to achieve images at high spatial resolution (1-10 microns) and at high speeds so that a typical sample tissue once prepared can be imaged in just a few minutes. Finally, new biocomputational approaches will be discussed that deals with the high data dimensionality of Imaging MS and our implementation of 'image fusion' in terms of predictive integration of MS images with microscopy and other imaging modalities.

Closing Lecture

HYPOTHESIS-DRIVEN OMICS TO UNDERSTAND SNAKE VENOMS AND FIGHT A NEGLECTED DISEASE

Juan J. Calvete

Instituto de Biomedicina de Valencia, CSIC, Valencia (Spain)

Research on venoms has been continuously enhanced by advances in technology. The emergence of "omic" technologies in the field of toxinology at the turn of the 21st century offered the unprecedented possibility to explore global biological trends and expand our understanding of the clinical correlation of the global toxin composition of venoms. The strength of these technologies (genomics, transcriptomics, and proteomics) rely in their complementarity, and thus the combined application to explore venoms generates novel perspectives in molecular toxinology. Accurate venomic data provide clues for correlating venom composition and the pathological effects of envenoming. In addition, the identification of evolutionary and immunological trends among venoms may aid replacing the traditional phylogenetic-driven hypothesis by a more rationale approach based on proteome phenotype and immunological profile similarities to produce effective polyvalent antivenoms, and to expand the clinical range of currently existing antidotes. These statements will be illustrated with recent studies on different snake taxa.

The physicist Freeman Dyson is reported to have remarked that 'scientific revolutions are more often driven by new tools than new concepts'. High-throughput "omic" technologies have changed the face of biological research, and many high-tech laboratories in industrialized countries have changed their paradigm of doing research from a "hypothesis-driven" to a "technology-driven" approach, the "hunters" and the "gatherers", respectively, in Sydney Brenner's words. The 'gatherers', with no immediate questions in mind, are the Lords of many superbly organized "omic" technology-driven facilities across the world. The gatherers' biology has been described as (patentable) 'discovery science'. In comparison, the hypothesis-driven science of the "hunters" seems to represent a curiously inefficient academic exercise. None of these approaches seem appropriate for systems biology venomics. A new paradigm has to be adopted. The new paradigm must rely on a strong biological ground elaborated on a deep knowledge of the system gained through the combined use of expensive and sophisticated "omic" technologies.

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Plenary Lectures

BREAST CANCER PROTEOMICS USING HIGH RESOLUTION MASS SPECTROMETRY AND SUPER-SILAC

Stefka Tyanova[1], Yair Pozniak[2], Juergen Cox[1] Matthias Mann[1] and Tamar Geiger[2]

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Application of proteomics technologies to basic cancer research as well as clinical research is a long lasting goal of the community. While cancer cell lines share multiple features with their tumor of origin, they don't represent the complexity of the tumor micro-environment and the heterogeneity that is associated with the tissue and whole body environment. Application of proteomics to tissues poses additional challenges related to the quantification methods, the small amounts of material, tissue availability and the internal tumor heterogeneity. For accurate quantification we previously developed the super-SILAC technology, which is mixture of five breast cancer cell lines that represents the tumor variability. It is used as a spike-in standard and enables accurate protein quantification. We combine the super-SILAC technology with the FFPE-FASP procedure that allows protein extraction and digesion from formalin-fixed paraffin embedded tumor samples with high efficiency. Last, to eliminate the effects of various cell types in the tissue we dissect the tissues to highly enrich the content of the cancer cells in the tissue.

We applied these methods to find a proteomic signature that discriminates between the three main breast cancer subtypes: Estrogen receptor or progesterone receptor positive (ER+/PR+) tumors, Her2/ErbB2 positive (Her2+) tumors or triple negative tumors. Deep quantitative proteomic analysis identified in total more than 12,000 proteins, and more than 8,000 proteins in each tumor sample. Bioinformatic analysis revealed a signature that discriminates between the subtypes and highlights the protein networks that distinguish between them.

This work shows the power of super-SILAC based proteomics combined with advanced bioinformatics to highlight key regulatory mechanisms in cancer. In the future these can be translated into improved diagnosis and therapeutics.

PROTEOMICS STRATEGIES FOR THE CHARACTERIZATION OF POST-TRANSLATIONAL MODIFICATIONS OF PROTEINS

Martin R. Larsen

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Virtually all cellular processes are tightly regulated by proteins with distinct functions. One of the fastest ways to alter the function of a protein is by post-translational modifications (PTMs). These can modulate many properties: conformational state, stability, activity, interaction partners and subcellular distribution. Many of the control mechanisms involved in the dynamic regulation of cellular expression patterns in health and disease are dependent on specific PTMs within key proteins. Therefore, the determination and quantitative assessment of PTMs on proteins is fundamentally important for elucidation of the complex processes that govern cellular events such as cell growth, division, differentiation and migration. As PTMs are usually mediated by tightly regulated, low abundance proteins, a small change in their expression or activity will result in a significant change in PTMs. This will likely be associated with a change in activity, partners, or cell location of the affected proteins and consequently a change in the dynamics and stability of the cell.

In the recent years methods, which allow researchers to specifically isolate and characterize proteins and peptides carrying several kinds of PTMs, such as phosphorylation, glycosylation and acetylation, have been developed. With these methodological development researches now have the tools to elucidate complex cellular signalling events, the effect of various PTMs on the dynamic of cells and complex combinatorial PTM cross talk in cellular signalling.

Here novel comprehensive multidimensional proteomics and PTMomics strategies, which allow quantitative assessment of non-modified peptides, phosphopeptides, sialylated glycopeptides and acetylated peptides, will be presented. Examples of the application of these strategies to study depolarization dependent signalling in isolated nerve-endings (synaptosomes), ischemic hearts and mouse brain development will be given.

PHOSPHORYLATION AND UBIQUITYLATION CROSSTALK IN PROTEIN DEGRADATION

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Protein post-translational modifications (PTMs) function as highly versatile signaling units capable of modulating protein activity, concentration, sub-cellular localization, and are critical in maintaining homeostasis. Aberrant regulation of PTMs, such as phosphorylation, has been implicated in the onset and progression of numerous diseases, most notably in cancer. Over two hundred different protein PTMs have been described. These PTMs do not exist in isolation -crosstalk between different types of modifications on the same protein molecule is utilized to add specificity and combinatorial logic to signal processing. The principles underlying PTM crosstalk -how does the presence of one modification influence the appearance of others, and how widespread is this crosstalk between modifications- remain unknown.

While proteomic technologies are now available to globally study individual PTMs, the study of PTM crosstalk has been a major challenge because methods are lacking to determine whether two PTMs co-occur on the same protein. We have develop two novel proteomic approaches to identify co-occurring PTMs, and applied them on a proteome-wide basis to interrogate the effects of protein phosphorylation on ubiquitylation.

Our results highlight the widespread usage of PTM crosstalk throughout the proteome, greatly expanding our knowledge of protein regulation. We find that phosphorylation events that co-occur with ubiquitin play unique roles, are more highly conserved, and are likely more functional than average phosphorylation events. We find a significant enrichment of ubiquitylation events in conserved regions of kinases, revealing a novel mechanism of how ubiquitylation can act to mediate signaling. The methods developed will find widespread applications in the study of crosstalk between any PTM pair. Furthermore, the conclusions derived from our work should influence the way we think about protein regulation and become the basis for future studies of protein post-translational modifications.

HOST-PATHOGEN INTERACTION: INSIGHTS FROM PROTEOMICS

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Host-pathogen interaction studies open interesting opportunities in the search of new virulence determinants and new targets for antimicrobial therapies. We are studying the *Candida albicans*-macrophages interaction, because together with neutrophils are the first line of host immune defenses.
We have analyzed protein differential expression of the ingested yeast using gel (DIGE- Differential In-Gel Electrophoresis) and non-gel proteomics (ITRAQ - Isobaric Tagging for Relative and Absolute Quantification). We provide evidence of a rapid protein response of the fungus to adapt to the new environment inside the phagosome by changing the expression of proteins belonging to different pathways. Network analyses allowed us generate a hypothetical model of *Candida* cell death after macrophage interaction, highlighting the interconnection between actin cytoskeleton, mitochondria and autophagy in the regulation of apoptosis. Different apoptotic markers like DNA fragmentation, ROS production, and caspase activity were assayed and our hypothesis was confirmed.
In addition we are analyzing the macrophage protein expression in response to *C. albicans*. We have employed SILAC (Stable Isotope Labeling by Amino acids in cell Culture) to study changes in macrophage proteins and phosphopeptides expression in response to the yeast. For the phosphoproteomic enrichment we used SIMAC (IMAC and Ti02) and CPP (Calcium Phosphate Precipitation and Ti02) as complementary techniques. 71 macrophage proteins showed differential expression in response to the yeast. These included proteins involved in the immune response, phagocytosis, anti-apoptosis and cytoskeletal rearrangement. Using SIMAC and CPP, we report the identification of 841 nonredundant phosphorylation sites from 522 proteins. The response of the macrophage phosphoproteome to *C. albicans* reflects changes in 191 phosphopeptides related with innate and adaptive immunity, protein kinases, cytoskeletal rearrangement as well as other cellular processes. Some proteins and phosphorylation sites were validated using Western Blotting. Moreover, apoptotic status of macrophages during the response to *Candida* was measured using different apoptotic markers. Our results suggest that C. albicans has an anti-apoptotic effect in macrophages, anti-apoptotic signals prevails over pro-apoptotic, this idea is also reinforced by the fact that Caspase-3 is not cleaved, TUNEL assays are negative, chromatin condensation after 24h of interaction is lower than 15%.

PROTEIN STRUCTURE AND DYNAMICS: WHAT CAN ION MOBILITY SPECTROMETRY-MASS SPECTROMETRY TELL US?

Alison E Ashcroft

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The term “structural proteomics” has been used to describe research aimed at determining how proteins carry out their unique functions. This area of proteomics involves the study of the 3D structures of proteins in order to uncover clues about a protein’s function that are not detectable from sequence analysis alone. Such structural analyses can also indicate how drug candidates bind to proteins and provide information about protein-protein interactions.

The recent commercialisation of ion mobility spectrometry – mass spectrometry (IMS-MS) instrumentation has provided the means of measuring the mass-to-charge ratio and cross-sectional area of biomolecules in a single, rapid experiment. This technology has proved successful for separating and characterising individual protein conformers and oligomers when co-populated within heterogeneous solutions, and for studying the assembly and architecture of non-covalently bound protein complexes. The application of IMS-MS to study the tertiary and quaternary structure of proteins and biomolecular complexes is the focus of this paper.

Monitoring protein folding, and unfolding is particularly important in the study of amyloid-related diseases whereby unfolded or misfolded proteins undergo self-aggregation into insoluble fibrillar structures. Data showing the separation of protein conformers arising from amyloidogenic proteins will be presented. The use of IMS-MS as a screening test for small molecule inhibitors, in which the mode of binding of different inhibitors with individual protein conformers can be discerned, is illustrated. Comparing the assembly pathways of different amyloidogenic proteins by monitoring protein aggregation into amyloid fibrils in real-time is discussed.

30 - 50% of proteins in eukaryotic cells are thought to lack a unique 3D structure but can undergo a folding event to form well-defined tertiary structure on binding to their physiological targets. An example of this is the intrinsically disordered protein bovine osteocalcin. Using IMS-MS to monitor changes in the charge state distribution and cross-sectional area of the protein during metal ion binding events is shown to provide information on the method of function of the protein.

The assembly pathways of biomolecular complexes of fixed stoichiometry and architecture are of intrigue. To illustrate this, the use of IMS-MS to unravel viral baseplate assembly is presented.

PROTEOMICS AND INTERACTOMICS OF CARBOXYPEPTIDASES AND THEIR PROTEINACEOUS SUBSTRATES

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The increase in the number of metallocarboxypeptidases (MCPs) evidenced in the last decade that their not fully understood simultaneous action on substrates as well their restrictions by natural modulators and inhibitors probably require, in regulated cases, a high complementation and tuning in their binding interactions. The fact that such substrates, as well the efectors, generally are of proteinaceous nature, add extra degrees of sophistication in these interactions (i.e. at the chemo-physical, discrimination capability and kinetic levels). Not to forget that, to achieve full specificity, the help of other factors (compartimentation, temporal expression and localization, degradation ...etc) from living organisms is required [(1-3)].

Nowadays we could count between 25 and 30 the number of variants of such MCP enzymes, classified between the M14A, B and C forms, to which we should add the recently emerging cytosolic ones (CCPs or Nna-likes) for the M14D subfamily in the MEROPS database [(4,5)]. Giving that all of them seems to keep the "canonical" metallo-carboxypeptidase domain and equivalent recognition sites, it is a real challenge to understand the complex interplay between them, their discriminative interaction with natural substrates (peptidic or proteic, since are proteases), and with the environmental proteinaceous inhibitors. It is also a challenge for drug-designers to generate synthetic ligands that specifically control them [(2)].

Coincidentally, MCPs (as other proteases, in general) have another characteristic interactomics feature: they act transiently on proteinaceous substrates, promoting cleavages on them which severely effects their conformation and functionality. How to detect such transient interactions is not always an easy task by using standard proteomics-interactomics approaches or addapted variants of them [(6-8)], in spite that frequently the promoted changes are very large (i.e. in size and other properties). We shall discuss several recent study cases of MCPs to clarify their interactomics with natural substrates and inhibitors and the strategies followed for their characterization.

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MAXQUANT: COMPUTATIONAL MASS SPECTROMETRY BASED PROTEOMICS FOR THE MASSES

Jürgen Cox

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Systems biology requires comprehensive data at all molecular levels. Until recently the determination of cellular protein levels was lagging behind the corresponding mRNA measurements. Now it is becoming feasible to extract system-wide quantitative data for nearly the complete proteome and for ten-thousands of posttranslational modifications. Underlying the recent success of the field are developments in computational proteomics, which now allow highly sophisticated and completely automatic analysis of raw MS data and streamlined bioinformatic and systems-level interpretation of the results. MaxQuant is an all-encompassing end-to-end computational workflow solution for quantitative proteomics. In this presentation the structure of MaxQuant and its crucial algorithmic parts are described as well as a perspective for future developments will be given and challenges will be discussed.

COMPLETE PROTEOMICS DATA WORKFLOW USING THE PROTEORED MIAPE-EXTRACTOR TOOL

***Martínez-Bartolomé S*[1], Medina-Aunon JA[1], López-García MA[1], González-Tejedo C[1], Gharbi SI[1], Navajas R[1], Albar JP[1]**

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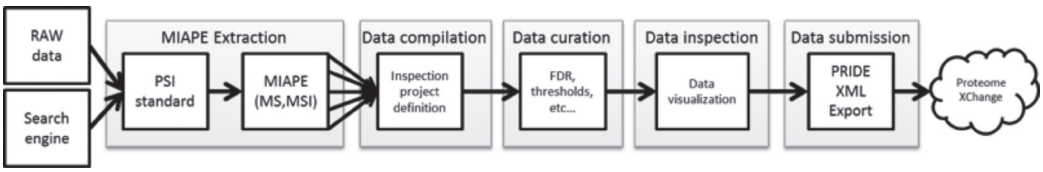
Increased sensibility in large-scale proteomics experiments is reached when using sample fractionation techniques, which results in the generation of multiple raw data files. Each one is usually independently analyzed and the results from these analyses are generally merged by simply appending excel tables in a single one. In this way, global analyses of the data are commonly performed manually, which emphasizes on the current lack of tools for complex proteomics datasets analysis.
Additionally, the complexity of the analyses increases enormously in a multi-laboratory environment in which large scale experiments are performed from different technological platforms, and where comparison between different results becomes of great interest.
The ProteoRed Miape Extractor tool has been developed based on proteomics data representation standards and the minimal reporting requirements (MIAPE: Minimum Information About a Proteomics Experiment) developed by the Proteomics Standard Initiative (PSI) from the Human Proteome Organization (HUPO). Taking advantage of these standards, the MIAPE Extractor tool is able to compile and curate huge amount of protein and peptide identification data, also allowing a visual inspection and comparison of the data in an easy and intuitive way.
The last step of the workflow consists in the generation of compatible files needed for data submission to a proteomics data repository such as the ProteomeXchange consortium, which will be required for publication in specialized proteomics journals.



The ProteoRed MIAPE Extractor is currently being used by all the participants of the Spanish consortium of the Human Proteome Project (spHPP), compiling, analyzing and sharing shot-gun identification data.
More than 160 experiments, containing almost 10 million PSMs, 750.000 different peptide sequences and 38.000 different proteins have been currently stored in the ProteoRed MIAPE repository using this tool (data from Dec 2012).

The tool is freely available at: http://www.proteored.org/MIAPEExtractor

S6. Emerging Technologies PL09

IDENTIFYING AND QUANTIFYING 10,000 PROTEINS IN 10 HOURS – FEASIBLE, POSSIBLE, DONE?..

Konstantin Chingin[1], Juan Astorga-Wells[1,2], Mohammad Pirmoradian Najafabadi[1,2], Harshavardhan Budamgunta[1], Thorleif Lavold[2], and Roman A. Zubarev[1,3]*

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It took proteomics more than 15 years from its inception to reach the bottom of the proteome – that is, to observe whole or almost whole expressed proteome (≥10,000 proteins). The very few reports on this achievement published so far have exercised almost no restrain on the amount of used material, labor or instrumental cost. Despite the recent advances in mass spectrometric instrumentation, it is clear that routine application of the whole-proteome analysis will require development of instruments and methods going far beyond the current state of the art. In particular, the very large dynamic range of cellular proteome – some seven orders of magnitude – is by at least 100 times exceeding the dynamic range of the most advanced proteomics instruments.

Traditionally, dealing with the dynamic range problem required extensive separation of proteins and peptides before the LC/MS analysis, which is one of the limitations of proteomics as it is currently practiced. Analysis of recent publications where detection of >5000 proteins is claimed revealed that, while half-proteome (ca. 5000 proteins) can be analyzed "in no time" (4-6 h), deeper analysis takes on average one hour of LC gradient for every 15-20 new detected proteins [1]. Therefore, useful data on the second half-proteome are accumulated at a rate comparable to that that of false positive rate. The task is therefore to reduce the pre-LC/MS separation to a bare minimum. In principle, a well-designed LC/MS experiment can analyze over 1000 unique proteins per hour of LC gradient. Therefore, aiming at detecting and quantifying 10,000 proteins in 10 h is a realistic (although at the moment of writing this abstract not yet reached) goal.

In our laboratory, we pursue different approaches that separately or in combination should in principle be capable of delivering the desired number of proteins within the given time limit. One of them is using a novel device for fractionation of peptides in ESI-friendly solution by their pI values [2]. The other approaches will be described; their relative advantages will be discussed, and the most recent results presented.

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ADVANCES IN MIDDLE-DOWN AND TOP-DOWN PROTEOMICS

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Traditional bottom-up proteomics (BUP) relies on analysis of short, ~10 amino acid long, peptides. Modern mass spectrometers allow efficient structural analysis of significantly larger peptides on the LC time-scale. Middle-down and top-down proteomics (MDP and TDP, respectively) approaches are considered as the near future alternative and complementary emerging technologies that shall allow addressing BUP limitations. We will present selected recent attempts to advance MDP and TDP approaches.

In regard to the MDP development, we will discuss the applicability of novel proteases and chemical methods of in-solution peptide backbone fragmentation to yield mixtures of peptides and proteins with rationally designed distinct molecular weight distributions, defined as: 0.6-3 kDa range for BUP, 3-7 kDa for BUP+, 7-15 kDa for MDP, and >15 kDa for TDP. Specifically, we will present the use of dibasic site-specific proteases that cleave at adjacent K and/or R sites resulting in longer peptides carrying multiple charges, including charges at one or both termini. To increase the efficiency of LC-MS/MS experiments we generate ion supercharging post-column to reduce the interference of supercharging reagents with the chromatographic separation. Furthermore, structural analysis of larger peptides demands a substantial increase in the acquisition speed of high-resolution and high mass accuracy MS and MS/MS data. We advocate for the use of super-resolution signal processing methods for FTMS, e.g., filter diagonalization method (FDM). We will present the envisioned benefits of both FT and FDM MS for the MDP development and for the targeted TDP structure analysis of large intact proteins, including the monoclonal antibodies.

Oral Communications

SILAC-BASED QUANTITATIVE ANALYSIS OF HUMAN BONE MARROW MESENCHYMAL STEM CELLS SECRETOME DURING CHONDROGENESIS

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Purpose: A major limiting factor for the application of human bone marrow mesenchymal stem cells (hBMSCs) in cartilage tissue engineering is the understanding of the multi-step process by which cartilage is developed, termed chondrogenesis. It is regulated by a variety of growth factors and modulated by extracellular matrix (ECM) proteins. Considering that the functional role for the majority of the proteins secreted during chondrogenesis is not fully established, we have applied the SILAC (Isotope Labeling by Amino Acids in Cell Culture) technique to analyze the extracellular protein expression profile of hBMSCs undergoing chondrogenic differentiation. **Methods:** hBMSCs isolated from 3 ostearthritic (OA) patients were grown in SILAC DMEM with two different isotope variants (medium and heavy) of lysine and arginine between 4-6 weeks. The fully labelled cell populations were then subjected to chondrogenesis using a home-made chondrogenic medium for 14 days. 24 h before collecting the secretome, the micromasses were carefully washed and changed to serum-free labeling media containing also chondrogenic inducers. Expression of cartilage-specific genes such as type I and II collagen and proteoglycans were used to verify the chondrogenicity of hBMSCs. Proteins in the CM harvested on day 2 and 14 of differentiation were precipitated and combined at a 1:1 ratio. Each mixture was then separated by 1D-SDS-PAGE and subjected to in-gel trypsin digestion using an automatic digester. The resulting peptide mixtures were analyzed by nanoLC coupled on-line to an LTQ-Orbitrap XL mass spectrometer and quantified using the MaxQuant software. **Results:** A progressive increase of type II collagen, agreccan and chondroitin-6-sulfate during the course of 14 days of differentiation was detected by inmunohistochemical assays, suggesting that hMSCs maintain their chondrogenic capacity in SILAC medium. Using the metabolic labeling strategy, we compared the secretomes at two different time points of chondrogenesis. More than 1000 proteins were identified with high confidence parameters. 20% of them were previously known ECM-related proteins. Among these, 34 exhibited a significant modulation of their levels during the process of chondrogenesis, including cartilage ECM proteins such as COMP, lumican, prolargin, fibromodulin and matrix gla protein (MGP), which could be involved in the organization and/or stabilization of cartilage matrix. All these proteins were increased at day 14 of chondrogenesis. Finally, pentraxin-related protein (PTX3), which plays a role in the regulation of inflammatory response, resulted decreased at day 14. **Conclusions:** This study was focused on characterizing the specific modulations in the secretome of hBMSCs undergoing chondrogenesis using the SILAC method. The identification and quantification of these secreted proteins provide information about the changes in the ECM synthesis during the differentiation process. Moreover, these findings enhance our knowledge on the extracellular regulation of this process and allow the identification of extracellular markers of chondrogenesis, which might have potential value in cartilage regeneration strategies.

DETERMINATION OF PROTEIN TURNOVER IN PRIMARY CULTURE OF FISH MYOCYTES BY DYNAMIC SILAC AND SHOTGUN PROTEOMICS

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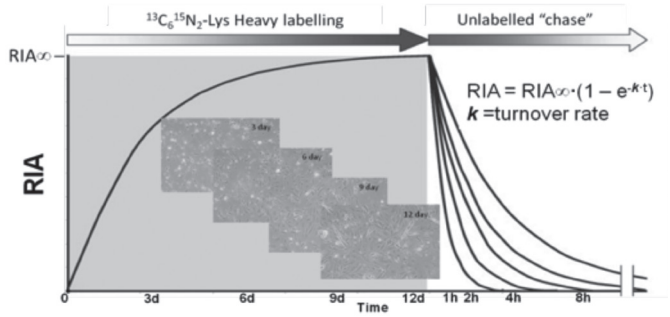
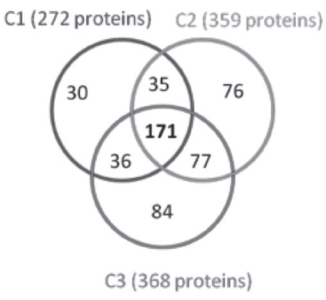
Understanding fish growth regulation at the molecular and cellular levels is especially needed for improving aquaculture. Bearing in mind that muscle growth will determine the final size of the animal and that normal cell growth and development requires a well-controlled balance between protein synthesis and degradation, the knowledge of protein turnover in muscle cells acquires special interest. We have studied protein dynamics in fish myocytes through a proteomics based strategy to determine the rate of turnover of individual proteins by "*dynamic SILAC*"1 (Stable Isotope Labeling with Amino acids in cell Culture). Primary cultures of muscle satellite cells of sea bream (Sparus aurata) fingerlings were incubated in triplicate with DMEM medium containing a stable isotope-labeled amino acid ([$^{13}C_6$ $^{15}N_2$] L-lysine) (heavy medium) until the stage of myotube (12 days)[2]. The cells were then "chased" with unlabelled light-Lys medium, taking samples at different time-points (0, 1, 2, 4 and 8 h) (Fig.1). Protein samples from each time-point were digested with trypsin using FASP (Filter Aided Sample Preparation) protocol, and then analyzed by LC/MS-MS. Raw data from MS were analyzed with MaxQuant software (v1.3.0.5) for protein identification and for determination of the ratio between 'heavy' and 'light' peptides (RIA = Relative Isotope Abundance). Preliminary results shows that incorporation of heavy-Lys in cells at day 12 was above 85% in average (RIA = 0.86 ± 0.02) and 171 proteins were shared among biological replicates (Fig 2). Ongoing analysis with the entire dataset will allow us to identify those proteins with greater differences in turnover rate. This work has addressed a number of technical challenges and represents the first study to use proteomic approaches to measure individual protein turnover in primary culture of fish tissues. Moreover, this strategy could be very useful to progress on identification of proteins that are major parts of protein turnover in myocytes under different nutritional or physiological conditions.





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ENABLING THE NEXT GENERATION OF MASS SPECTROMETRY-BASED QUANTITATION WORKFLOWS

Antonio Serna Sanz

ABSCIEX

Proteomics-based Mass Spectrometry has turned in the last years from untargeted to targeted analysis. Quantitation data have become more relevant to nowadays research as clearly reflected in the amount of projects that demand these kinds of workflows. Likewise technology did evolve to increase throughput in the analysis of complex proteomes, so must do to enable the quantitation of large numbers of protein sets in the best possible fashion. Here we will introduce a new workflow for Mass Spectrometry that is enabling this step forward. This workflow is based in a Data Independent Acquisition with discrete transmission windows across broad mass ranges. The type of information generated is facilitating the rapid generation of methods for quantitating numbers of proteins so far unreachable through classical targeted approaches.

DEVELOPMENT OF A NEW PROTEOMIC TOOL WITH SILAC COMBINED WITH HYDRAZIDE GLYCOPROTEIN ISOLATION TO CHARACTERIZE DEGRADOME IN BREAST CANCER CELLS

***Gemma Reverter-Branchat*, Joan-Josep Bech-Serra, Marta Monge, Núria Colomé, Francesc Canals**

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ADAM10 and ADAM17 (TACE) are enzymes with metalloprotease activity and a disintegrin domain in their structure, localized in the cellular membrane. Several of their substrates have been implicated in the pathogenesis or progression of breast cancer. For instance, it has been described that they promote cancer progression by releasing HER/EGFR ligands that culminates in increased cell proliferation, migration and survival. Therefore, a deeper knowledge of the substrate repertoire of these proteases will be helpful to better elucidate their role in tumor growth and metastasis and to evaluate their potential use as therapeutic targets.

The aim of this study was to develop a method to identify and characterize new substrates of proteases in breast cancer. MCF-7 cells were seeded in SILAC medium supplemented with normal or isotopically labeled amino acids. Cells were treated 48 hours with a non-specific metalloprotease inhibitor (BB-94). Then, extracellular media (ECM) was collected and compared with the opposite metabolic label without treatment. Sample was enriched in glycoproteins by specific oxidation of their carbohydrates followed by their covalent union to immobilized hydrazide. Glycosylated peptides were recovered by specific enzymatic digestion with a glycopeptidase. Finally, these peptides were analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

Our results show a wider substrate profile when compared with previous approaches. This protocol allows identification of more than one-hundred protease substrates in a single experiment. Moreover, it unravels potential new roles of those proteins in tumor development not deeply studied yet. Indeed, we find a relevant number of shed proteins involved in cell-to-cell signaling, cell adhesion and cell migration processes whose function most probably could be affected by ADAMs activity.

HIGH-THROUGHPUT MITOCHONDRIAL REDOX PROTEOMICS ANALYSIS OF THE EFFECTS OF OXIDATIVE STRESS IN CELLULAR AND ANIMAL MODELS OF CARDIOVASCULAR DISEASE

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Reactive nitrogen and oxygen species-mediated protein modifications are associated with patho-physiological states, but can also convey physiological signals. In spite of their biological relevance, little is known about the nature of specific oxidative modifications that are produced in situations of oxidative stress. We have recently developed GELSILOX, a new method for the simultaneous, high-throughput quantitative analysis of the total and thiol redox proteomes (Martínez-Acedo et al, Mol. Cell Proteomics, 2012). In this work, we describe results obtained from the application of GELSILOX to the analysis of oxidative thiol modifications that take place in the mitochondrial proteome in several cellular and animal models of cardiovascular disease.

We demonstrate that episodes of ischemia-reperfusion (IR) performed in isolated rat hearts produce an increase in oxidative damage in mitochondrial proteins, mainly in those belonging to oxidative phosphorylation complexes and to the Krebs cycle.

The majority of the Cys sites affected belong to iron-sulfur centers or are known to modulate protein functionality in a redox-dependent manner. Ischemic preconditioning (IP) of the heart, induced by previous short episodes of IR, inhibited the oxidative damage induced by full IR in subsarcolemmal (SSM) but not interfibrillar mitochondria (IFM), paralleling the effect of IR on oxidative phosphorylation activity. Both the effect of IR and the protection by IR in SSM could be reproduced in mitochondria isolated from normoxic hearts, demonstrating for the first time that preconditioning may take place in mitochondria in absence of cytosolic signals. The molecular mechanism of oxidative damage in mitochondria was further assessed in a mouse KO model of complex I degradation induced by the absence of complex IV. Complex I was stabilized by hypoxia and was degraded when mitochondria were subjected to reoxygenation. By combining GELSILOX with DiS, a novel, data-independent semitargeted quantitative proteomics strategy, we have been able to follow the time-course of protein abundance changes and oxidative modifications induced by reoxygenation. Structural analysis of protein changes and oxidative modifications showed that complex I was degraded as a consequence of oxidative alterations in critical Cys sites. We have also demonstrated the existence of oxidative mitochondrial modifications in a model of maladaptive cardiac hypertrophy produced in an EndoG KO mice model and also in aged animals, and are now establishing a detailed molecular model of the relation between oxidative state in mitochondria and pathology.

THIOL REDOX PROTEOMICS IN HYPOXIA CELL SIGNALLING

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Adaptation to decreased oxygen availability (hypoxia) is crucial for proper cell function and survival. In acute hypoxia, there is an increased production in reactive oxygen species (ROS), which can transduce signals by reversibly oxidizing cysteine thiols in certain proteins. We aimed to identify proteins that could be oxidized when subjected to acute hypoxia that could serve as early signals for adaptation to hypoxia.

We have developed novel thiol redox proteomic approaches, based on high-throughput LC-MS/MS peptide identification (GELSILOX) and fluorescence 2-DE ("redox fluorescence switch", RFS). Both techniques rely on specific derivatization of reversibly oxidized thiols and differential analysis without purifying modified peptides/proteins, which allow simultaneous analysis of variations in abundance at the single protein level.

With both techniques we have observed a general increase in cysteine oxidation in endothelial cells during acute hypoxia (2 hours, 0.5% O2). We have also identified specific proteins that are differentially oxidized in those conditions. By using complementary methods, we have studied the oxidation of individual proteins in different conditions, and identified the cysteine residues that are reversibly oxidized.

Most thiol redox proteomics techniques have been used to identify proteins that are differentially oxidized after strong oxidative treatments, related to oxidative stress. We show that the techniques we have developed can be useful for studying physiological treatments producing reversible oxidation in specific proteins, which can be involved in cell signalling. In addition, our results provide an important basis for future studies of the functional relevance of these oxidative modifications in the acute adaptive response to hypoxia.

HIGH RESOLUTION, ACCURATE MASS APPROACHES FOR THE QUANTIFICATION OF TARGET PEPTIDES

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Mass spectrometry has become an essential tool for understanding various aspects of biological systems. Putative biomarkers need to be quantitatively analyzed across large numbers of samples from multiple biological sources and conditions, either for understanding of signaling regulation or for verification and selection of final biomarkers. A fast, robust and cost-effective platform is required at this verification stage. A targeted MS approach, selected reaction monitoring (SRM), has been used as a gold standard methodology for quantitative analysis of peptide candidates improving performance over traditional ELISA assays. However, several intrinsic limitations remain, such as the extensive need for method development and difficulties associated with work in highly complex matrices, where signal interference is limiting performance. High resolution, accurate mass (HR/AM) methods provide an attractive alternative for targeted peptide analyses. High resolution on a Q Exactive benchtop mass spectrometer can resolve peptide species differing in mass by as little as 5 ppm, providing high selectivity. High Resolution methodology gives concrete benefits: more distinct features are measured in a given dataset, and their detection, in a combination of resolving LC-MS/MS strategies, leads to precise signal integration and confident peptide quantification, where interferences from matrix or from co-eluting species, are removed.
Here we describe a targeted HR/AM peptide quantitation workflow on the Q Exactive mass spectrometer, where peptide targets present in complex mixtures were quantified using multiplexing strategies.

A NEW FAST METHOD FOR PEPTIDE BIOMARKER DISCOVERY IN HUMAN SERUM BY MASS SPECTROMETRY

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The field of biomarker discovery is currently acquiring greater importance, especially with the recent emergence of new technologies, such as proteomics-based approaches. In proteomics, search for biomarkers in biological fluids such as human serum is a challenging issue, mainly due to the high dynamic range of proteins present in these types of samples. Therefore, removal of the most abundant proteins (depletion) is necessary. The basic scheme for proteomics relies on the depletion followed by the separation of a large number of proteins and their identification by mass spectrometry (MS). Prior to mass spectrometry, most common strategy used is depletion with inmunoaffinity columns followed by two-dimensional (2D) liquid chromatograpy (LC) separation. The drawbacks of these techniques are that they are expensive, laborious and spend much time.

In this work, we report a novel fast strategy based in a chemical sequential depletion, protein digestion accelerated with ultrasounds and a peptide sequential separation in a micro-solid phase extraction (SPE) cartridge of C18 media. Individual serum samples, 20 of each condition, were subjected to a chemical sequential depletion protocol involving two precipitation steps, first with DTT and then with ACN, previously described by our group1,2. Then, the samples were digested with trypsin and this process was accelerated with ultrasonic energy. The peptides were retained in a commercial tip with a C18 chromatographic media, and were eluted in four different amounts of acetonitrile (ACN), from low to high percentage of ACN. Each eluted fraction was analysed by MALDI-TOF/TOF spectrometry by quintuplicate. The reproducible peptides masses of each condition were used to create a bioinformatics software to discriminate between conditions.

This workflow was followed with 60 serum samples from patients suffering different rheumatic diseases (OA, AR and APS) and 20 healthy donors. We were able to differentiate between the four conditions by the peptides masses, and we created a new bioinformatics tool to classify the samples into these different conditions and to identify novel peptide biomarkers of these diseases.

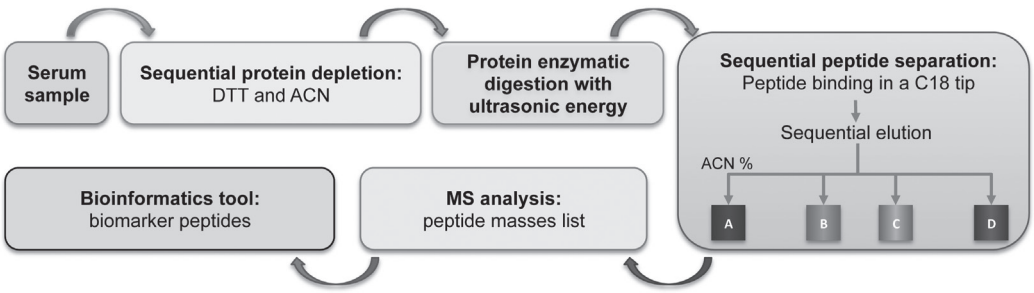

Figure: Comprehensive scheme of the workflow followed in this research.

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DISCOVERY, ACCURATE INCLUSION MASS SCREENING AND LABEL-FREE QUANTIFICATION OF POTENTIAL BIOMARKERS FOR COLORECTAL CANCER IN SERUM OF HUMAN PATIENTS

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Colorectal cancer (CRC) is the second leading cause of cancer related death in developed countries. Most of these deaths result of the appearance of metastasis as a consequence of the late diagnosis of the disease. Although there are some biomarkers like CEA (Carcinoembryonic antigen), mostly adequate for advance stages and for monitoring recurrence of the disease, the efforts to discover new biomarkers to detect metastasis and CRC recurrence more accurately have not been very successful to the date. Proteomics is a very powerful tool widely used in the discovery of biomarkers. Recently, using a SILAC based proteomic approach, we characterized the secretome of colorectal cancer metastatic cells identifying several proteins that might be potential CRC biomarkers. To confirm such possibility and furthermore identify new possible biomarkers for CRC, we performed a discovery proteomics analysis to characterize the serum from healthy patients and patients within all Duke's stages which will then allow us to perform accurate inclusion mass screening (AIMS) of some candidates and further quantify those candidates in the different stages using a label free approach. For that purpose, pools from serum of healthy and Dukes A, B, C and D stages patients were prepared. First, samples were depleted of the 12 most abundant proteins that would difficult the identification of the lower abundant proteins, *in-solution* digested with trypsin and fractionated by OffGel IEF in 24 fractions in order to decrease sample complexity of the peptide mixture and therefore maximizing the detection of lower abundant proteins. Finally samples were analyzed in a linear ion trap Orbitrap Velos. Peptides were eluted using a linear gradient of 120 min from 0-40% B (A: 0.1%FA/2%ACN; B:0.1% FA in ACN) at 200 nl/min. Mass spectra were acquired using a "top 15" method for the acquisition of a MS full scan followed by the fragmentation of the 15 most abundant precursors. Mass spectra were searched against the human human SwissProt/Uniprot database using both Sequest search engine through Proteome Discoverer and Andromeda search engine. Peptide identification was performed at a FDR of 1%.

In total, 1816 proteins were identified in patients' serum, from which 43 were coincident with proteins characterized in the secretome of KM12SM metastatic cells. Furthermore, 130 proteins were present only in serum from healthy patients while 289 proteins were present only in serum of patients with Dukes D. We are currently performing AIMS experiments to confirm the presence of these potential biomarkers in all Dukes stages and will further perform label free quantification to confirm diagnostic value.

FUNCTIONAL PROTEOMICS APPROACHES FOR HIGH-THROUGHPUT DETERMINATION OF SMALL MOLECULES INTERACTIONS ON CKIT

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cKIT is a trans-membrane receptor with tyrosin-kinase activity. Aberrant levels of cKIT activity have been associated with several hematopoietic disorders and gastrointestinal stromal tumors (GIST). Several point mutations of cKIT have been described in a cohort of 150 patients, mostly of them are related to tyrosin-kinase activity and interactions with Stem Cell Factor (SCF). As a consequence, more than 75% of the patients are resistant to conventional treatments, so new ones are needed. Here, we have designed and developed a Nucleic Acids Programmable Array (NAPPA), which content wild-type cKIT and all clinical point mutations, in order to discover new inhibitors of cKIT activity and deep into cKit-SCF interaction. By this approach, novel molecules have been discovered as useful for inhibition of aberrant cKIT activity.

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INVESTIGATION OF PROTEIN MONOMERIZATION BY CAPILLARY ELECTROPHORESIS AND ION MOBILITY MASS SPECTROMETRY. APPLICATION TO SUPEROXIDE DISMUTASE 1 AND LATERAL AMYOTROPHIC SCLEROSIS

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The native form of superoxide dismutase (SOD-1) is a homodimer that coordinates one Cu^{2+} and one Zn^{2+} per monomer (Cu_2,Zn_2-dimer SOD-1). SOD-1 aggregates are characteristic of amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disease in humans. As only a minor percentage of ALS cases can be explained by SOD-1 mutations, several authors have proposed that aggregation may be triggered by specific post-translational modifications (PTMs) or dimer dissociation. Here we describe capillary electrophoresis mass spectrometry (CE-MS) and nano-electrospray ionization-ion mobility mass spectrometry (IM-MS) for the analysis of SOD-1, as an alternative to the existing ESI-MS and LC-MS methods that do not allow dimer detection. Using the optimum methodologies to avoid metal release and to minimize dimer dissociation, both techniques allowed detecting monomeric and dimeric forms of bovine and human SOD-1 and several PTMs. IM spectrometry separates ions in the gas phase in function of their size-to-charge ratios in a similar way to charge-to-size based separations of ions in solution in CE. CE-MS and IM-MS yield three-way data (migration time-m/z-intensity and drift time-m/z-intensity, respectively), but while monomeric and dimeric SOD-1 solution forms comigrate in CE-MS, nano-ESI-IM-MS allowed separation of monomeric and dimeric gaseous ions with the same m/z values. This was crucial to obtain reliable information about monomer-to-dimer abundance ratios in blood samples from healthy control and ALS patients. A comparison between both groups of samples demonstrated that only IM-MS was able to confirm the presence of a higher relative abundance of Cu,Zn-monomer SOD-1 in patient samples. This novel analytical tool may be of great importance to elucidate the molecular mechanisms of aggregation related to PTMs or monomerization that may underlie ALS, and also other neurodegenerative diseases related to protein aggregation, such as Alzheimer's, Parkinson's, Huntington's and prion diseases.

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HYDROGEN-DEUTERIUM EXCHANGE FOR PROTEIN STRUCTURE ANALYSIS

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Hydrogen/deuterium exchange mass spectrometry (HDX MS) has proven to be a useful analytical method for the study of protein dynamics and changes to protein conformation. Typical uses include structural characterisation of recombinant proteins, mutation effects on protein structure and protein-protein and protein-ligand interaction mapping. Improvements in LC-MS such as UPLC and the incorporation of high peak capacity ion mobility separations have made HDX MS increasingly feasible to researchers, yet data interpretation remains a major challenge. Conventionally, the HDX MS data are interpreted either manually or processed with semi-automated tools to determine the deuterium uptake at peptide level. These are time-consuming processes due to the thousands of spectra involved for processing hundreds of peptides, multiple time-courses, replications in comparative analyses. In order to enhance the efficiency of data processing, a new HDX software tool, DynamX (1), is presented.

All samples were analysed using the Waters SYNAPT G2 HDMS in combination with the nanoACQUITY UPLC with HDX Technology. Analytical column was an ACQUITY UPLC BEH C18 1.7 µm 1.0 x 50 mm. The trap column was an ACQUITY VanGuard Pre-column, BEH C18 1.7 µm 2.1 x 5 mm. Online pepsin digestion was performed using a 2.1 x 30 mm immobilized pepsin column (Applied Biosystems). MSE data were collected for all analyses. Undeuterated analyses were processed using ProteinLynx Global Server (PLGS) 2.5 with IdentityE informatics. DynamX was used to measure the deuterium uptake of each peptide as a function of deuterium exposure time.

Experimental examples showing conformational changes of calmodulin upon calcium binding, and Fab paratope mapping will be presented. The automated software generated a list of reproducible peptic peptides based on retention time, intensity, fragment ions, and mass accuracy. The amount of deuteration of the identified peptides was automatically determined and displayed in processed spectra view. The deuterium uptake curves were automatically plotted in a function of time for comparative analysis using multiple batches of proteins. Finally the calculated results of relative deuteration and its difference were summarized in butterfly and difference charts for convenient data interpretation. In addition, DynamX was used to process the ion mobility data and successfully eliminated the interferences of co-eluting peptides separated by a mobility drift time. This process automation greatly improves the speed and accuracy of HDX MS experiments and is a step towards extending the application of this approach into new research areas.

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S4. Networks/Protein-Protein Interactions OC13

CYCLODEXTRIN- AND METHYL JASMONATE-MEDIATED RESVERATROL ACCUMULATION IN GRAPEVINE CELL CULTURES:AN OMICS APPROACH

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Grapevine (Vitis vinifera) cell suspension cultures accumulate large extracellular amounts of the stilbenoid resveratrol along several days, in response to elicitation with methylated cyclodextrins (MBCD) and methyl jasmonate (MeJA) [1,2,3]. The aim of this study was to investigate changes in both the transcriptome and cellular proteome potentially related to resveratrol production and accumulation in response to MeJA, MBCD and MBCD+MeJA treatments. The GrapeGenAffymetrixGeneChip® (23,096 probesets corresponding to 18,711 non-redundant grapevine transcripts [4]) and the quantitative proteomics gel-based DIGE technique were used to detect statistically significant changes in transcripts 24 hours, and proteins during an incubation time of up to 120 hours after elicitation, respectively. We focused on transcripts encoding metabolic enzymes from the primary metabolism precursors i.e. phosphoenol pyruvate and malonyl CoA to the final product, i.e. stilbenes. While CD or CD+MeJA induced accumulation of shikimate, general pheylpropanoid, stilbenoid and malonylCoA pathways transcripts, MeJA induced only general phenylpropanoid and stilbenoid pathways. In the proteome kinetics experiment sampling four data points (6, 24, 72 and 120 hours) from each experimental condition, 212 de-regulated unique spots were detected. Protein identification based in nLC-MS/MS analysis and NCBInr database search revealed a large complexity in the abundance pattern of several proteins relevant to resveratrol synthesis and accumulation. Stilbene synthases (STS), found in 29 unique spots, encoded by up to 40 paralogs, were classified into 6 phylogenetic groups. Such groups showed specific abundance patterns in response to the different elicitor treatments. The comparison of STS protein and stilbene metabolite profiles lead to detect potential STS groups specifically involved in the response to MBCD alone or combined with MeJA elicitors that lead to resveratrol accumulation. Likewise, glutathione-S-transferases (GST) were found in a high number of spots. These belonged to two tau phylogenetic subgroups, one phi and one lambda class. Interestingly, the five GST whose abundance profiles correlated with profiles of STS and metabolites composed one of the two tau class clusters, thus pointing to a potential role in resveratrol trafficking within the cell and across plasma membrane. Other findings will be presented and discussed in relation to the effect of MBCD and MeJA elicitation.

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A NEW APPROACH FOR SYSTEMS BIOLOGY ANALYSIS OF HIGH-THROUGHPUT QUANTITATIVE PROTEOMICS EXPERIMENTS

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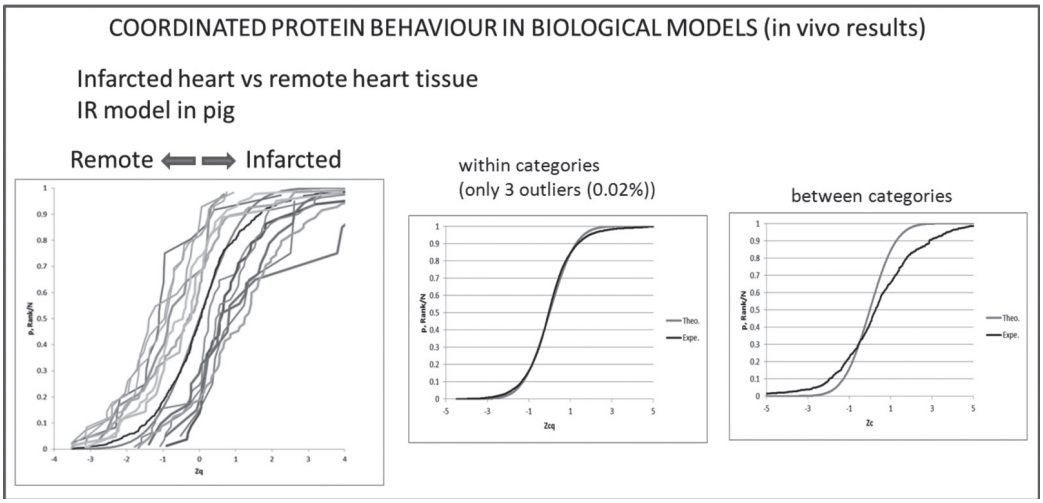
In a previous work we presented a statistical model for the analysis of quantitative proteomics experiments using stable isotope labelling approaches. Currently, the tools to perform systems biology analyses on these experiments are limited. In our laboratory we have further developed our algorithm to facilitate this kind of studies.
A large number of high-throughput quantitative proteomics experiments have been analysed, using samples from yeast, human, pig, rat and mouse models. Quantified proteins were integrated into ontological categories and the distributions describing the intra- and inter-category variability were analysed. Unexpectedly, we observed that the distribution of proteins within categories was undistinguishable from the expected null hypothesis behaviour for all proteomes studied to date, with a very low proportion of outliers. 90-98% of the proteins behaved in this coordinated manner. In contrast, 5-30% of categories were found to deviate in the treated samples compared to the null hypothesis. These category changes were not produced by individual protein deviations but were rather the result of a homogeneous increase or decrease of their protein components. To further study this behaviour, we have developed a novel algorithm to integrate quantitative protein information into ontological categories. With this algorithm we have been able to separate successfully the technical variance (intra-category protein variability) from the "*category variance*" (a novel concept that quantifies the global effect of treatments on cell functionality). In contrast, when proteins were randomly classified into the same categories, in none of the cases the category variance was significantly different from zero, demonstrating that the coordinated behaviour was not a consequence of protein grouping.
This new threshold-free approach for systems biology analysis of quantitative proteomics experiments allows a straightforward and global interpretation of biological results in terms of ontological alterations as well as the identification of specific proteins that show a separated, non-coordinated behaviour.



ON THE FEASIBILITY OF 2D HIGH pH / LOW pH LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY IN LABEL-FREE INTENSITY-BASED QUANTIFICATION

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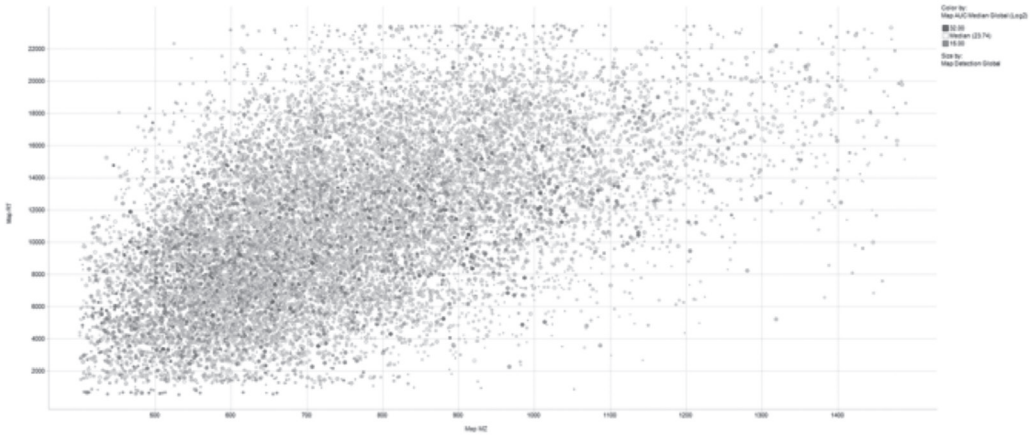
Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has emerged as a powerful analytical method for systematically profiling biological samples. With the advent of a new generation of LC-MS/MS systems, label-free intensity-based methods for comparative proteomics analysis has gained great popularity in the past years. Despite the major advancements in this field, the proteomics community still face important challenges in terms of reproducibility and sensitivity of label-free proteomics analysis. The problem of variability may significantly impact comparative studies aimed to profile biological states or phenotypes. Often label-free intensity-based quantitative proteomics is approached in an unfractionated fashion in order to achieve better reproducibility.
To make confident inferences about biological differences in quantitative analysis, proteomics experiments must be accurate and reliable. To this end, we implemented a series of performance metrics for benchmarking label-free intensity-based quantitative proteomics analysis of both fractionated (online high pH C_{18}) and unfractionated samples. As expected, the number of features, peptides and proteins detected was substantially increased following fractionation. Overall, we detected and consequently identified approximately 500,000 and 50,000 (figure 1) features across 8 samples after fractionation (8 fractions). Despite yielding more identifications, there is a general notion that fractionation deteriorates technical reproducibility. Therefore, we thoroughly evaluated the reproducibility of fractionated and unfractionated approaches. Overall, this study demonstrates how the performance metrics can be used to benchmark the quality of proteomics data generated in proteomics studies.

Figure 1. 2D feature map. Each feature detected and identified is shown in the map m/z (x-axis), elution time apex (y-axis) and colored according to its summed abundance across samples. The feature size in the map is represented by the feature detection frequency across the samples



ANTIBODY COLOCALIZATION MICROARRAY: A SCALABLE TECHNOLOGY FOR MULTIPLEX PROTEIN ANALYSIS IN COMPLEX SAMPLES

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DNA microarrays were rapidly scaled up from 256 to 6.5 million targets, and although antibody microarrays were proposed earlier, sensitive multiplex sandwich assays (MSA) (**Figure 1.A**) have only been scaled up to a few tens of targets. Cross-reactivity, arising because detection antibodies are mixed (usually known as "cocktail of detections"), is a known weakness of multiplex sandwich assays that is mitigated by lengthy optimization. In order to circumvent this limitation, here we propose a novel concept for multiplexing without mixing named antibody colocalization microarray (ACM) (**Figure 1.B**). In ACMs, both capture and detection antibodies are physically colocalized by spotting to the same two-dimensional coordinate. Following spotting of the capture antibodies, the chip is removed from the arrayer, incubated with the sample, placed back onto the arrayer and then spotted with the detection antibodies. ACM may be seen as an array of singleplex nano-sandwich assays, each requiring only 1 nL of dAb solutions. ACMs with up to 50 targets were produced, along with a binding curve for each protein (**Figure 2**).The ACM was validated by comparing it to ELISA and to a small-scale, conventional multiplex sandwich assay (MSA). Using ACMs, proteins in the serum of breast cancer patients and healthy controls were quantified, and six candidate biomarkers identified. Our results indicate that ACMs are sensitive, robust, and scalable (M. Pla-Roca *et al*. Molecular and Cellular Proteomics 2012).

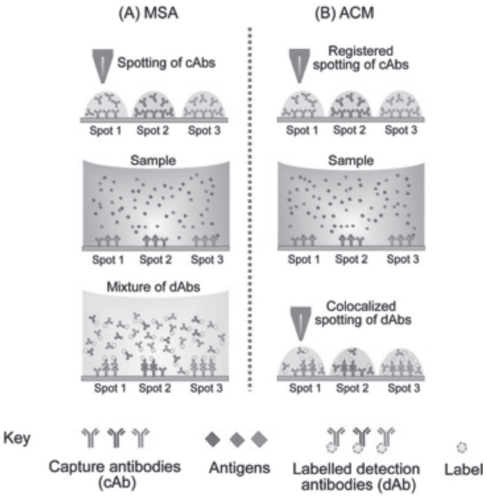

Figure 1. Schematic process flow for multiplexed sandwich assays (MSA) in microarray format, and the antibody colocalization microarray (ACM). In an ACM, the initial steps are identical to a MSA, with the difference that the first spotting is precisely registered and that a second round of spotting is used to deliver each dAb exactly on the spots with the matched cAb, physically colocalizing them. This strategy avoids mixing of reagents.

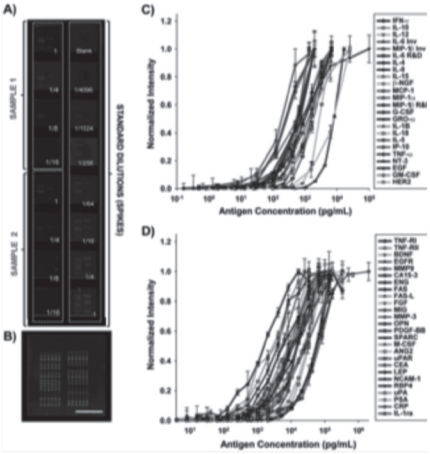

Figure 2. ACM layout and binding curves for 50 Ab pairs including. (A) Fluorescent micrograph of a representative slide with 16 replicate arrays incubated with two serum samples and corresponding dilutions (left), and buffer solution with recombinant proteins with seven dilutions and a negative control used to establish the standard curves (right). (B) Detail of a single array. Standard binding curves of Ab pairs with lower (C) and higher (D) sensitivity that extend into the low pg/ml range. Scale bar in (B): 2 mm.

CONSERVATION OF PEPTIDE EXTRACTS FROM TRYPSIN DIGESTION OF PROTEINS IN A HOSPITAL BIOBANK FACILITY

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Purpose: Protein fingerprinting and MSMS-based protein identification yields spare peptide extracts that, usually, are not conserved in the Proteomics Facilities due to lack of storage space. However, in most of the cases, a second analysis of the same sample is required some weeks or even months later by the user. If the original sample has not been properly conserved, that implies, for the user, to get enough amount of sample for a new analysis, which is time-consuming and, eventually, some problems in terms of reproducibility could arise. In the other hand, emerging Biobank Facilities, offer the solution for proper storage and conservation of samples in individually-coded collection tubes allowing automated pick-up of a specific sample without breaking the cold chain of the others. In this study we tested our Hospital Biobank as a facility for the proper storage for mid-term conservation of peptide extracts coming from samples with low and high protein complexity.
Methods: For PMF and MSMS analisis, purified proteins -Albumin, Holotransferrin and α-casein- were loaded in replicates in SDS-PAGE and subjected to standard *in-gel* trypsin digestion and peptide extraction. For LC-MALDI-TOF/TOF analysis, purified β-galactosidase and Human Serum Samples were *in-solution*-digested following standard procedures and desalted with C18 nu-tips. In both cases, peptides extracts were aliquoted in individually-coded Micronic ® tubes, dried in a speed-vac, bar-coded read and stored in a automated freezer in the Biobank facility. Samples were kept dried at -20 oC until the correspondent time-point of analysis, then reconstituted in the proper buffer and analyzed by either MALDI-TOF/TOF (Peptide fingerprinting and MSMS) or LC-MALDI-TOF/TOF following a highly reproducible pattern to assure the reproducibility of the results. Protein identification was done with either Mascot or ProteinPilot® as search engines using constant parameters.
Results and Conclusion: Over a period of three months, we checked four time points, at Days 0, 7, 30 and 90, although we plan to extend the study over a total period of one year. We compared MS and MSMS protein score, number of identified peptides and coverage of the identified proteins. In the low complexity samples the number of peptides detected decrease with the time affecting especially the MS score. However, two out of the three proteins –Albumin and Holotransferrin- are identified both by PMF and MSMS at day 90. By LC-MSMS, β -galactosidase and the most abundant Serum proteins are identified with good scores at day 90, although we found a decrease in the total number of detected peptides as well. Our results encourage us to use the Biobank Facility in the future for storage of peptide extracts.

PROTEOGENOMICS OF BACTERIA AND ARCHAEA: A ROMPER LA PIÑATA!

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High-quality genome annotation is today a crucial starting point for characterizing new microorganisms. Proteogenomics consists in better annotating genomes with the help of proteomic data. High-throughput identification and characterization of proteins by extra-large shotgun tandem mass spectrometry approaches allow identifying new genes and characterizing the exact initiation of the translation. We applied such strategy on various microorganisms, such as *Deinococcus deserti* VCD115, a bacterium from the *Thermus-Deinococcus* phylum that was isolated from surface sands from Sahara, the archaeon *Thermococcus gammatolerans*, and the marine bacterium *Ruegeria pomeroyi*. The D. *deserti* bacterium exhibits an extraordinary ability to withstand desiccation and ionizing radiations. We sequenced and annotated its genome by proteogenomics. We systematically investigated the existence of remaining unannotated genes, defined translational start sites, and listed signal peptide processing events and post-translational modifications. Surprisingly, experimental evidences were obtained indicating that translation is sometimes initiated in *Deinococcaceae* from non-canonical codons such as ATC and CTG. Based on our proteomic results we found that predicting translation initiation codons is still difficult for most bacteria. Proteomic-based refinement of genome annotations may be helpful in such cases. We are developing specific methods to enrich the peptides corresponding to the N-termini of proteins in the framework of a large program to better annotate various bacterial phyla.

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HPP (Human Proteome Project)

THE HUMAN PROTEOME PROJECT INTERCONNECTED WITH LARGE SCALE BIOBANKING

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The recent developments and announcement from the Human Proteome Organization (HUPO), on the Human Proteome Project (HPP) is a major undertaking, in some ways similar to the Human Genome Project (HUGO) that was announced by Bill Clinton and Tony Blair in June 2000. The major difference is that each of the approximate number of 20,300 proteins encoded by the human genome will be mapped to specific locations on individual chromosomes. Protein annotations will be linked to the human genome and to specific diseases by applying both mass spectrometry assays and antibody based assays [1-3]. This program is expected to play a central role in these developments, as a resourced facility where the basis of all the developments within HPP will be made publicly available [4]. Recently the initiation of the Chromosome initiative of HUPO, (C-HPP), the first step Toward completion of a Genome-Wide characterization of the human proteome was published (http://pubs.acs.org/toc/jprobs/current) with a total of 33 scientific papers.
Biobanks are a major resource for scientists to access unique patient samples for medical research. Many studies within clinical proteomics utilize stored samples contained in biobanks to measure specific end points. Today we have not yet achieved consensus on how to collect, manage, and build biobank archives in order to reach goals where these efforts are translated into value for the patient. Standardizations of quality control of samples being processed for storage as well as retrieval of stored samples are important goals in order to support the development of diagnostic tools such as biomarkers. Lack of available high quality sample collections with a wide range of disease cohorts has become a rate-limiting step for drug development, medical research and novel diagnostics. These developments will be of great value and importance to programs such as the Chromosome Human Protein Project (C-HPP) that will associate protein expression in healthy and disease states with genetic foci along of each of the human chromosomes. In this paper we review the technical and administrative aspects of biobanking and present examples of preferred best practice and potential future use [5].

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NEXTPROT: THE HUMAN PROTEIN-CENTRIC KNOWLEDGE PLATFORM, STATUS AND FUTURE DEVELOPMENTS

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In September 2008, the UniProt/Swiss-Prot group achieved a major milestone: the first complete manual annotation of the full set of human proteins (derived from about 20'000 genes). This corpus of data which is quite rich in information pertinent to modern biomolecular medical research clearly shows that there is a large gap in our knowledge of human proteins in terms of functional information as well as protein characterization (PTMs, protein/protein interactions, subcellular locations, etc.). This gap resides not only in the available experimental information, but also in the way this information has been stored, which is far from being sufficient to help researchers making sense of what all these human proteins do in our bodies! Therefore, in the framework of CALIPHO, an interdisciplinary group created jointly by the University of Geneva and the SIB, we are developing neXtProt, a new human-centric protein knowledge resource, with the aim to help researchers answer pertinent questions. To achieve this goal we need to build up on a corpus of both curated knowledge - originating mainly from the UniProtKB/Swiss-Prot knowledgebase - and carefully selected and filtered high-throughput data pertinent to human proteins. Such a data gathering and grading effort are complemented by the development of tools that allow such data to be analyzed.
neXtProt (www.nextprot.org) is a work in progress. Currently we are involved in supporting the efforts of HUPO Human Proteome Project (HPP). In this context we are integrating into neXtProt data originating from proteomics experiments, including peptides and PTMs identification. To allow our users to make the best use of the information that is available in the platform we have developed exports options (PEFF, XML) as well as programmatic access (API).
There are many things we plan to add to neXtProt in the near future especially in term of supporting information pertinent to protein networks. In this context we plan to integrate information originating from STRING as well as other resources.
There is a very long road in front of us and the challenge of integration the mass of heterogeneous knowledge on human proteins is a distant perspective and we hope you will enjoy working with neXtProt and will help us making it evolve by telling us of your specific needs.

HUMAN PROTEOME PROJECT: CHARACTERIZATION OF THE PROTEINS ENCODED BY THE CHROMOSOME-16 PROTEIN CODING GENES

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The Chromosome 16 Consortium is integrated in the global initiative Human Proteome Project that aims to map and annotate all proteins encoded by the genes on each human chromosome following a gene-centric strategy (C-HPP) to make progress in the understanding of human biology in health and disease (BD-CHPP). Overall, the B/D-HPP attempts to generate and disseminate the assays and resources to support the analysis of biological networks underlying biological processes and disease. The structure of the consortium involves 17 groups organized in five working sections, namely Protein Expresion and Peptide Standards, Selected Reaction Monitoring team, Protein Sequencing team, Bioinformatic Support team and Clinical healthcare and biobanking teams.
A description of chromosome 16 based on knowledge repositories has been analysed; the chromosome contains 866 protein coding genes including 595 "known" and 271 "unknown" 0r "missing" gene products (log(e)>-5 GPMDB), participating in 116 OMIM diseases. T-Lymphocytes, epithelial cells and fibroblasts were selected for further studies as transcriptomic evidences suggest that most chromosome 16 protein coding genes are expressed in these cell lines. For analytical purposes, the 866 proteins have been clustered in two groups according to the robustness of the evidences supporting their MS observation, It is expected to set SRM assays for the top rank third of chromosome 16 proteins in both protein groups.
The process is being developed on a multi-centric configuration, assuming the standards and integration procedures already available in ProteoRed-ISCIII, which are encompassed with HUPO-PSI initiatives. A biobanking initiative has been launched in collaboration with the Spanish National Biobanking Network to optimize methods for sample collection, management and storage under normalized conditions and to define QC standards.
As a proteomic pilot study, we are defining a comprehensive proteomic map based on high-resolution data dependent mass spectrometry to define the protein coverage of the Chr16. This study will permit to assist the development of MRM methods for the quantitative targeted strategy. We are dissecting the human T lymphoblast cell line (Jurkat T cells) using in a first instance a MUDPIT type approach, with in-solution digestion and off-line pre-fractionation by RP-HPLC-Basic. Then, to gain better insight

into integral membrane proteins, our analysis is combined with strong detergent protein extraction and 1D-gel-digestion. In all our workflows, data is acquired using a high resolution and accurate mass 5600 TripleTOF LC/MS/MS system (AB SCIEX) on long HPLC runs (4 hrs). This global proteome is then fed into our in-house MIAPE-Extractor tool that compiles all the Mascot-searched data and extracts specific MIAPE information relative to Chr16. This logistic permits the integration of the versatile formats and workflows that can all converge into providing a fast and complete understanding of the proteome of the cell model of interest. We anticipate that this rapid screen will greatly complement our efforts in mapping the Chr16.

Posters

PROTEIN EXPRESSION PATTERNS ASSOCIATED WITH INTRINSIC AEROBIC EXERCISE CAPACITY

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Exercise capacity is a complex attribute that involves different physiological systems under the influence of both genetic and environmental factors. Related to genetic influence, results have shown that more than 70% of aerobic exercise capacity is intrinsically determined. In this work, a comparative proteomic approach, two dimensional gel electrophoresis (2D) combined with MALDI-TOF/TOF tandem mass spectrometry, was used to investigate possible molecular differences at the protein expression level between rats heart (left ventricle - LV) with distinct intrinsic exercise capacity. Low running performance (LRP) and high running performance (HRP) rats were categorized by a maximal exercise test protocol performed on a motor-driven treadmill, according to total distance performed (TDP). The running capacity of HRPs was 3.5 fold greater than LRPs. Protein expression profiling revealed 29 statistically significant ($p<0,05$) differences between HRP and LRP, and 15 of these proteins were identified by MALDI-TOF/TOF (MS and MS/MS). Robust alterations were detected in components involved in antioxidant and stress response, miofibrillar and cytoskeletal proteins. Contractile proteins were found to have special expression modification: α-myosin heavy chain-6, myosin light chain-1 and creatine kinase up regulation in LV of HRP rats on patterns in HCR. In contrast, LV of LRP rats exhibited increase in abundance of protein associated with stress response. These animals exhibited enhanced expression of the antioxidant enzyme (aldehyde dehydrogenase 2), and heat shock proteins (α-crystallin B chain, heat shock protein β-2). In addition, the cytoskeletal proteins, desmin and α-actin, were upregulated in LCRs. In conclusion, our results suggest that the increased contractile proteins levels in HCRs rats may explain, in part, the improved exercise capacity. The increased stress protein expression in LCR suggests that the LV proteome of these animals are exposed to greater stress.

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DESIGN OF SRM BASED MASS SPECTROMETRY METHODS FOR QUANTITATIVE ANALYSES OF PORCINE ACUTE PHASE PROTEINS

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It is mandatory, after protein identification, to validate candidate markers in a larger amount of samples for biomarker discovery. The most used methods for validation of new markers are enzymatic or immunoassay-based commercial kits. However, these kits are not always available for farm animal species. SRM, a targeted quantitative proteomic technique, may be used as an alternative to commercial kits for the measurement and validation of target proteins.
Serum acute phase proteins (APPs) are widely recognized markers for inflammation, infection and welfare. In pigs the most important APPs include haptoglobin (Hp), C-reactive protein (CRP), the inter-α-inhibitor-heavy chain 4 (ITIH4, also called Pig major acute phase protein, or Pig-MAP), serum amyloid A (SAA) and apolipoprotein A-I (Apo A-I), but also other proteins are well known to mark the acute phase response, including fetuin A, albumin and transferrin.
We designed and optimized an SRM based method for the measurement of four APPs in porcine serum: Hp, ITIH4, Apo A-I and fetuin. The Skyline software (MacCoss Lab Software) was used to analyze the spectra of the targeted transitions corresponding to unique tryptic peptides of each APP. Serum samples were run on an AB SCIEX QTRAP® 5500 System.
Porcine serum samples with high APP levels were used for method optimization. Four proteotypic peptides were selected from Hp, ITIH4 and Apo A-I, and the 3 most intense transition signals from each of these were optimized, while for analysis of fetuin A, only a single peptide with high transition signals was available.
After method optimization, the method was used to measure the acute phase response of 15 sows, which were subjected to moderate stress after moving them from a pen to individual boxes for insemination and gestation. Samples were run as triplicate technical repetitions. The SRM data were compared to previous analyses based on commercial ELISA and colorimetric kits.
Changes in protein concentrations were observed by both SRM based and commercial kits, and a general agreement between these methods was clearly observed. SRM analyses confirmed previous observations that positive APPs (Hp and ITIH4) levels are generally elevated while negative APPs (Apo A-I and fetuin A) are decreased for a period of 2-4 days after the exposure to stress.
We conclude that SRM is an important alternative to commercial kits for the quantification of farm animal proteins.

FROM LIPIDOMICS TO TARGETED LIPID ANALYSIS: DIFFERENT STRATEGIES TO EVALUATE RELEVANT LIPID CONTENT OF HUMAN SERUM SAMPLES

Ferran Sanchez, Antonio Serna

AB SCIEX Barcelona, Spain

One common powerful mass spectrometry (MS) technique for direct analysis of global cellular lipidomes is the shotgun approach, which is basically infusion of the whole extract at one time using electrospray ionization. The idea is to generate high throughput data in conjunction with global analysis of cellular lipids directly from biological extracts. Although this easy-to-use and, thus, powerful approach is well established, alternative workflows can bring important (additional) information related to the nature of lipids, e.g. isomeric/isobaric lipid species, within the diversity of concentrations.
As electrospray ionization efficiency is depending on total amount of ions, infusion of concentrated solutions will highly be influenced by ion suppression. Hence, fastLC separation will avoid sensitivity issues of shotgun only approach and help to resolve complex lipid mixtures.
Last but not least, a reliable and straightforward way is usage of validated kit assay like AbsoluteIDQ™ p150 Kit (Biocrates, Innsbruck, Austria) to evaluate same kind of human plasma samples.
Within samples from human plasma distinct differences in the lipid classes and lipid species are found and evaluated using statistical and special lipid ID software for HRMS and HRMS/MS data and Biocrates' AbsoluteIDQ software solution to handle specific triple quad kit assay data.

PROTEOMIC CHARACTERIZATION OF THE ROLE OF SNAIL1 IN THE DIFFERENTIATION OF 3T3-L1 FIBROBLASTS

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Snail1 is a transcriptional repressor required for a correct embryonic development. Snail1 is required for specific processes, such as gastrulation. In cancer, Snail1 promotes the epithelial to mesenchymal transition (EMT). In this work, we have analyzed the control of Snail1 in the differentiation to adipocytes of the 3T3-L1 cell line derived from murine embryo cells. By retroviral infection, we generated 3T3-L1 cells over expressing Snail1 (3T3L1/Snail1) and stable control (3T3-L1/control) cells. We used cytokine antibody arrays and a SILAC quantitative approach to identify and characterize protein alterations induced by Snail1. Protein cell extracts were fractionated in 5 cellular fractions and separated by 10% SDS-PAGE. Gels with forward and reverse experiments were stained with Coomassie Blue and the whole lanes were horizontally cut into 18 slices prior to reduction, alkylation and digestion with trypsin. Tryptic peptides were scanned and fragmented with a linear ion trap-Orbitrap Velos (ThermoScientific). The Top-15 most intense ions were selected for collision induced dissociation (CID) fragmentation. We analyzed membrane, cytoplasmic and nuclear fractions for identifying a total of 6337 proteins, with 4764 quantified proteins, and 875 proteins deregulated >1.7-fold by Snail1 overexpression.
We performed *in silico* bioinformatics analysis using IPA, STRING and DAVID Functional Annotation with the differentially regulated proteins. The most significant altered functions were Cellular Movement, Gene Expression and Protein Synthesis. Furthermore, we analyzed alterations in the cytokine/chemokine profile in culture media by ectopic snail1 expression in 3T3-L1 by antibody microarrays using Ludesi software. After normalizing spot intensities and calculating ratios of expression, we observed that Snail1 induced the differential expression of 14 out of 62 cytokines/chemokines. Finally, we have validated the results by western blot, ELISA and RT-PCR using control and ectopic Snail1 expression 3T3-L1 cells to verify the differential expression of the proteins and their role in adipogenesis. We identified among the top deregulated proteins a large number belonging to the eIF complex, mTOR signaling, and the splicesome. We found interesting up-regulated proteins associated to early differentiation of adipogenesis (C/EBPβ) and down-regulated proteins implicated in the final stages of differentiation to adipocytes (Fatty acid-binding protein or Fatty acid synthase). On the other hand, we observed down-regulated in 3T3-L1/Snail1 important mediators of PPARγ pathway.
These results show that Snail1 ectopic expression is enough to inhibit the adipocyte differentiation signaling pathways (PPARγ or mTOR signaling). This work has provided many novel proteins with potential roles in the regulation of differentiation to adipocytes of the 3T3-L1 cells for future studies.

SHOTGUN PROTEOMIC ANALYSIS OF BANANA UNDER COLD STORAGE CONDITIONS

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Storage fruit at low temperatures has been widely used to extend its postharvest life. In this study, a shotgun proteomics approach was conducted on banana cv. Nanicão from a control group stored at 19 ºC compared with other group stored at 13 ºC for 15 days, and after that, transferred the temperature to 19 ºC for complete the maturation. Five pools of each group (control and cold) of "unripe" and "ripe" phase was prepared for analysis. The proteins were extracted with phenol and digested by trypsin. The resulting highly complex peptide mixtures were inject in an analytic column C18 and analyzed by RP-UPLC (nanoAcquity UPLC, Waters) coupled with nano-electrospray tandem mass spectrometry on a Q-Tof Premier API mass spectrometer. Databases searches for peptide identification were done with Mascot and proteins were identified by searching musa_pep database. Scaffold was used to validate MS/MS based peptide and protein identifications which identified 71 proteins (< 0.5% false discovery rate). Twenty-three significant proteins by Anova ($p < 0.05$) were submitted to Principal Component Analysis. The first component separated the samples by stage of maturation and the second component separated the control and cold groups, but there was a major discrimination in the cold group from ripe fruits, being farther than the control group in the graphic. The proteins superoxide dismutase [Cu-Zn], abscisic stress-ripening protein 3, putative horcolin, stromal 70 kDa heat shock-related protein and a chloroplastic probable steroid-binding protein were correlated with the unripe control samples and a putative stress responsive protein and cytochrome c oxidase subunit 5B mitochondrial precursor were strongly correlated with the unripe cold samples. Other proteins such as L-ascorbate peroxidase cytosolic and nascent polypeptide-associated complex subunit alpha-like protein were common for both groups. On the other hand, enolase, malate dehydrogenase, mitochondrial, metallothionein-like protein type 3, 1-aminocyclopropane-1-carboxylate oxidase, defensin-like protein 5, endochitinase, heat shock cognate 70 kDa protein and germin-like protein present a strong association with the ripe control samples while the cold group was characterized by the presence of thaumatin-like protein, isoflavone reductase homolog, granule-bound starch synthase 1, chloroplastic / amyloplastic, and a putative lichenase dirigent protein.

STAIN-FREE TECHNOLOGY AS NOVEL NORMALIZATION TOOL IN WESTERN BLOT ANALYSIS

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Proteomic technologies like two-dimensional electrophoresis (2-D PAGE) are valuable tools in semi-quantitative protein profiling studies in order to identify specific expression patterns enabling a better understanding of molecular events. The de-regulated protein spots on 2-D maps are usually identified and characterized by mass spectrometry. In addition, the quantitative protein profiling data need to be confirmed by a second, independent method like Western-blotting. Western blots are used to specifically measure the relative quantities of proteins of interest in complex biological samples. However, quantitative measurements can be subject to error due to process inconsistencies like uneven protein transfer to the membrane. These non-sample related variations need to be compensated for by an approach known as normalization. Two approaches to data normalization are commonly employed: housekeeping protein (HKP) or total protein normalization (TPN). In this study we evaluated the performance of Stain-Free (SF) technology as a novel TPN tool for western blotting experiments in comparison to GAPDH as a representative of the HKP normalization strategy. The target protein (TP) used for this study was MCM7, a DNA licensing replication factor, which was shown previously to be down-regulated by 20% in irradiated lymphoblastoid cells (LCL). We studied the relative expression level of MCM7 with a multiplex western blotting approach based on fluorescently labeled secondary antibodies and found that Stain-Free technology appears to be more reliable, robust and more sensitive to small effects of protein regulation when compared to HKP normalization with GAPDH. Stain-Free technology offers the additional advantages of providing check points throughout the western blotting process by allowing rapid visualization of gel separation and protein transfer.

QUANTITATIVE PROTEOMICS (iTRAQ) AS A TOOL TO UNDERSTAND THE PREMATURE AGING PROCESS IN PROGERIA

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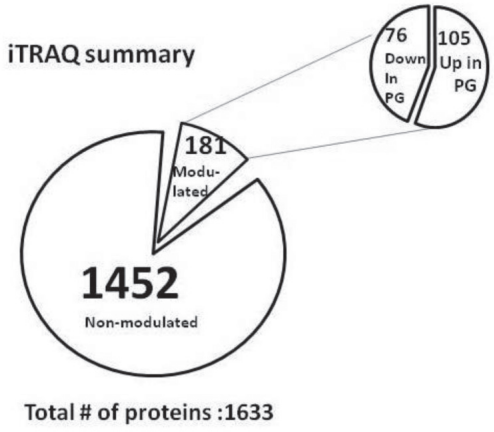
Purpose: Accumulation of a mutant form of the nuclear protein Lamin-A –called Progerin (PG) or Lamin AΔ50- is the causal agent of Hutchinson-Guilford Progeria Syndrome (HGPS) or Progeria, an accelerated aging disease. One of the main symptoms of this genetic disorder is a loss of sub-cutaneous fat due to a dramatic lipodystrophy. We stably induced the expression of human PG and GFP -Green Fluorescent Protein- as control in 3T3L1 cells using a lentiviral system in order to study the effect of Lamin A de-regulation in the differentiation capacity of this cell line, one of the most studied adipogenic models.

Methods: Efficient gene delivery of PG and GFP in the lentivirus-transduced cell lines were checked by immunoblotting. Proliferation rate was studied using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega). Adipogenic capacity was checked by immuno-histochemistry - Oil red Staining-. Quantitative proteomics analysis iTRAQ (ABSciex) was done in a 4800 MALDI-TOF/TOF system (ABSciex) using ProteinPilot software as search engine, String 9.0 software was used to establish altered pathways based on previously known protein interactions.

Results and conclusion: We found that over-expression PG by lentiviral gene delivery leads to a decrease in the proliferation rate (more than 50%) compared to the control. iTRAQ analysis showed 181 proteins significantly ($p<0.05$) modulated in PG-expressing pre-adipocytes, Several GO Biological Processes were altered including Structure of Cytoskeleton (**Vimentin, Ratio Control/PG=27.28**) Energy Generation (**ATP sinthase, Ratio Control/PG=0.34**), Lipid Metabolism (**Fatty Acid Synthase Ratio Control/PG =0.55**), Calcium Regulation (**Caldesmon Ratio Control/PG =2.29**) and Translation (**Elongation factor 1-beta, Ratio Control/PG =0.34**). Our results will be helpful for a better understanding of the aging process.



EVALUATION OF DIFFERENT DIGESTION TECHNIQUES TO IMPROVE DIGESTION REPRODUCIBILITY IN PROTEIN QUANTIFICATION

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Protein quantitation by mass spectrometry is commonly based on the generation of proteolytic protein fragments (peptides) that are later used to infer the corresponding protein abundances. During protein digestion there are several sources of potential variation such as incomplete protein digestion, unspecific cleavages, and different protease specificity[1], which might affect the reproducibility of protein quantitation[2].

In this study we evaluated the digestion efficiency of several in-solution and filter-aided digestion protocols[3] and assessed their influence in protein quantitation by mass spectrometry. We evaluated the the percentage of peptides with missed cleavages and the reproducibility of the different techniques at the peptide and the protein level both among technical replicas and among different techniques.

Our results clearly show that digestion reproducibility and protein quantitation results change significantly depending on the enzyme and the digestion technique used. These observations are of especial interest as they evidence potential important errors when estimating protein fold-changes and abundances in large-scale protein quantitation studies.

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BIOMARKERS OF TGFβ PATHWAY IN GLIOMAS

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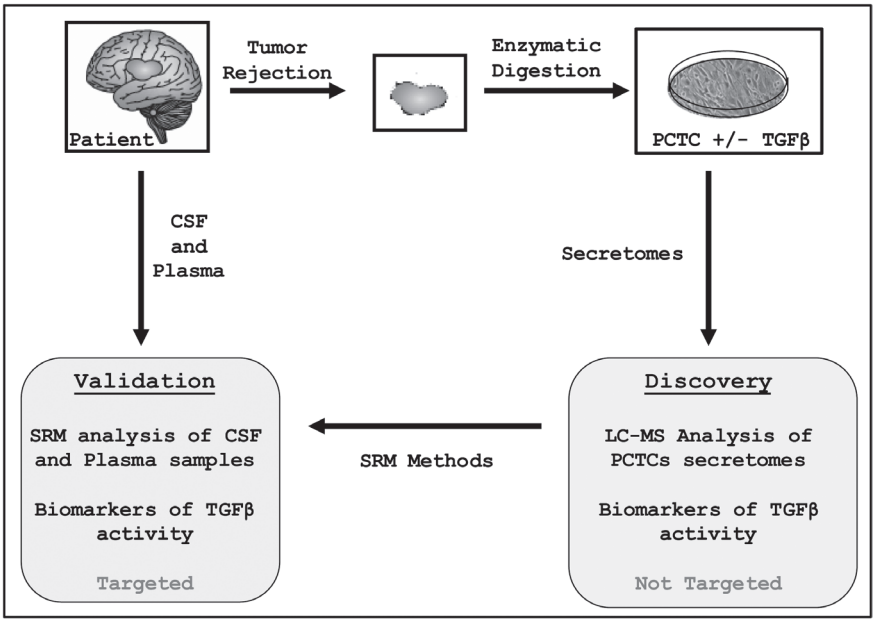
Gliomas are the most frequently primary tumors in the brain. Transforming growth factor β (TGFβ) pathway is related with a subgroup of them. Nowadays, TGFβ is being evaluated as a therapeutic target. The definition of a protein signature of the TGFβ activity in gliomas could help to select the population that could benefit from an anti-TGFβ therapy and monitor the treatment on the clinical assays. With the objective of identifying biomarkers of the TGFβ activity in gliomas, secreted proteins of primary cultured tumor cells (PCTCs), treated or not with TGFβ, were analyzed by label free and Isotope Code Protein Labeling (ICPL) quantitative proteomic experiments. Several candidate secreted proteins responding to TGFβ were identified. In order to validate these candidate proteins selected reaction monitoring (SRM) methods were developed. SRM methods were applied to cerebrospinal fluid (CSF) and plasma samples from glioma patients.

When CSF samples from glioma and other brain tumor patients were analyzed using the SRM methods developed, a clear correlation between the protein levels and the TGFβ concentrations was observed. Plasma samples from glioma patients under alternated treatment phases with a TGFβ inhibitor were also analyzed by the SRM method. The levels of some of the candidate proteins were found modulated along the treatment.

Globally, this group of proteins constitutes a possible protein signature useful for diagnosis and monitoring of the treatment of the glioma patients with TGFβ activity.



PROTEOMIC CHARACTERIZATION OF THE SARCOPLASMIC AND MYOFIBRILLAR PROTEINS IN LOCAL CHICKEN MUSCLE USING 2DE AND MASS SPECTROMETRY

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For a number of years, poultry selection has concentrated on growth velocity and muscle mass in meat lines. Proteomic methods are useful for the identification of proteins markers associated with meat quality traits in animal species. The information from proteome can be used to optimize the processing method and meat technologies and to assist in selection of markers by geneticists in poultry. The aim of this study was to apply a proteomic approach for the characterization of local chicken legs. We optimized methods for extraction and evaluated the reproducibility. Three different fractions were analyzed: total protein, sarcoplasmic protein and myofibrillar protein. The profiles of different fractions were analyzed by 2D SDS-PAGE and the most relevant spots were identified by MALDI-TOF/TOF.

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RICE PROTEOME UNDER SALT STRESS AT THE REPRODUCTIVE STAGE

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Salinity is one of the abiotic stresses with a highest impact on crop productivity, causing losses that can reach up to 50% of crop yield potential. Nearly half all irrigated land is affected by salinity with Portugal being no exception. It is known that rice pollen is particularly sensitive to abiotic stress at the early stages of development. Therefore we were interested in studying how salinity affects rice at the reproductive stage, focusing our analysis on the anthers, the male floral organ responsible for pollen formation. In fact, pollen development is controlled by complex mechanisms requiring coordinated gene expression between both somatic (from the anther's wall) and gametophytic cells (the pollen itself). In our study, salinity was applied immediately after panicle initiation and the anther material was collected 1 day before anthesis. In order to get a deeper knowledge on the salt stress response and on the possible mechanisms involved in salt tolerance, we further compared the protein expression patterns between tolerant and sensitive genotypes. Two of these varieties are advanced breeding lines included in the Portuguese rice breeding program.
A quantitative analysis of the rice anther proteome in response to salt stress was performed using two-dimensional difference in gel electrophoresis (2D-DIGE). Comparisons at proteome level were also established between two salt-tolerant rice genotypes and one salt-sensitive, together with physiological and agronomic measurements. As anthers are quite challenging to isolate (under stereomicroscope), we had to first test different protein extraction protocols in order to get simultaneously the highest protein yield and the 2D protein profile with the best resolution. Here we present the results concerning the optimisation steps on protein extraction and separation by 2-D DIGE. The rice anther proteins differentially expressed under salt stress were already validated by statistical analyses and will be further identified by mass spectrometry.

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PROTEOMIC PROFILES OF Pisum sativum INNER AND OUTER CHLOROPLAST ENVELOPE MEMBRANES

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The chloroplast envelope comprises a pair of membranes surrounding the chloroplast which control the communication between the chloroplast and the rest of the cell, and therefore is essential for the proper functioning of this semiautonomous organelle. Translocation of nuclear encoded precursor proteins across the envelope is achieved by the joint action of Toc and Tic translocons located at the outer and inner envelope membranes, respectively. The envelope participates in the translocation of intermediates of various metabolic pathways and inorganic ions, and also contains unique biosynthetic pathways for the synthesis of plastid membrane components, chlorophyll breakdown or synthesis of lipid-derived signaling molecules. Despite its importance, the proteomic characterization of this membrane system is challenging due to the difficulty of obtaining separate inner and outer membrane fractions with high purity and the inherent problems involving the separation and solubilization of highly hydrophobic membrane proteins. Therefore, the aim of this work was first to obtain inner and outer membranes from the envelope of *Pisum sativum* and second to characterize their protein profile by Shotgun proteomics which allows analyses of membrane proteins. Four independent biological replicates for both membrane systems were obtained and analyzed in a nHPLC-MS system. MS/MS spectra were processed using Proteome Discoverer software and identification was achieved by comparison with the NCBInr Green Plants database, using MASCOT as a search engine. Only proteins with two or more matched peptide sequences were considered as positively identified. Using this criterion, 546 proteins were identified in the envelope. Comparisons between the protein profiles of the inner and outer envelope membranes allowed identification of 22 and 96 proteins, respectively, which were assigned as specific for each membrane using ANOVA ($p<0.05$) and a relative abundance $\geq 85\%$ as selection criteria. A preliminary analysis of these protein species reveals that the outer membrane is mainly involved in transport processes, whereas the inner membrane participates in chloroplast functionality, including proteins related to protein metabolism, photosynthesis and energy.

A QUANTITATIVE PROTEOMIC APPROACH FOR THE DISCOVERY OF CHARACTERISTIC BIOMARKERS IN OSTEOARTHRITIS CARTILAGE

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PURPOSE: Osteoarthritis (OA) is characterized by the loss of structural components from the extracelullar matrix (ECM) of articular cartilage. The aim of this study was to perform a proteomics approach to identify and quantify those proteins released from normal (N) and OA human articular cartilage explants. Furthermore, we seeked to differentiate those proteomic signatures displayed from the wounded zones (WZ) from those corresponding to the area adjacent to the lesion, or unwounded zones (UZ), in the OA samples. These proteins released from cartilage might have a tissue-characteristic biomarker value for early diagnosis and/or therapy monitoring. **METHODS:** Macroscopically articular cartilage was obtained from the dissection of normal and OA femoral heads. The cartilage from normal samples, and from WZ and UZ of the same OA piece, was cut into five 6mm2 explants. Then, these explants were washed in PBS and harvested in 96-well plates with 200µL of serum-free DMEM. The conditioned media from each condition were collected at days 1, 3, 6 and replaced with fresh medium. Media from each condition were quantified, concentrated and precipitated with acetone. Finally, proteins were digested with trypsin and each peptide solution was labelled with different isobaric tags using the iTRAQ reagents. The labelled peptides of the different conditions were mixed, desalted and separated by HPLC. The resulting fractions were resolved by nanoLC-MS/MS and the relative quantification of the proteins was carried out with Protein Pilot 3.0 software. **RESULTS:** Protein concentrations were higher in the culture media from wounded zones than from unwounded zones of OA cartilage, due to the increased degradation that is taking place in the lesion. We first identified several ECM proteins that were differentially released between WZ and UZ in OA samples at two different times of culture. Most of them were increased in the wounded tissue, including collagens (I, II, VI and XV), proteoglycans (biglycan, lumican or perlecan) and proteins related with cell adhesion (tenascins, osteomodulin or vitronectin). In a second step, proteins released to the media up to day 6 of culture were compared between three conditions (WZ, UZ and normal tissue). This analysis led to the identification of 199 proteins, 110 of them quantitatively altered between the conditions of the study. Interestingly, several proteins were differentially released already in the unwounded zone of OA cartilage compared to the normal samples (osteopontin, matrilin-3, cartilage link protein or transforming growth factor-beta-induced protein), and their amounts do not seem to be increased in the WZ. Finally, a small set of proteins, including thrombospondin-1 or type XVIII collagen, was more abundant in the media from N cartilage than from OA. **CONCLUSIONS:** We have identified a profile of proteins released from diseased cartilage in OA. Our work describes a panel of cartilage-characteristic proteins with potential biomarker value of the tissue degradation process that takes place in OA. This panel will be further explored in biological fluids (synovial fluid and serum) for the development of early diagnosis and/or therapy monitoring strategies.

A NOVEL PLATFORM FOR THE ABSOLUTE QUANTIFICATION OF PROTEINS BASED ON ISOTOPE PATTERN DECONVOLUTION AND MINIMALLY ^{13}C-LABELLED PEPTIDES

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Enriched stable isotopes play a crucial role on the development of new MS-based techniques for protein quantification. First, using isotopic labelling, different methodologies have been developed to explore the dynamics of whole proteomes and provide relative comparisons of protein abundances between samples (relative quantification). Secondly, Isotope Dilution Mass spectrometry (IDMS) has been employed to develop methods capable of providing the highest level of accuracy and precision in the determination of proteins (absolute quantification). Only using absolute quantification approaches we can ensure that the measurement of the protein mass fraction in a given sample is traceable to the International System of units (SI).

The use of isotopically labelled "proteotypic" peptides can be considered as the best alternative for the absolute quantification of proteins. However, the main metrological drawback of protein determinations using labelled peptides is the initial certification of peptide standards (both natural abundance and isotopically labelled) on a mass basis. Additionally, the stability of the internal standard during enzymatic digestion and the accurate measurement of the molar ratio between natural and labelled peptides need to be addressed. Most of the current methodologies employed in this field make use of multiply labelled peptides to avoid spectral overlap and this can cause isotopic effects. Under those conditions, the ratio of intensities for the natural and labelled analogues is assumed to be the same as the ratio of moles. However, this assumption needs to be demonstrated for a large range of concentration ratios between the natural abundance and labelled peptide.

We propose here a novel platform for the absolute quantification of proteins which combines: a) the certification of the concentration of labelled peptides by reverse IDMS using certified natural abundance peptide standards. In this case, the certification process for the natural abundance standards follows a proprietary procedure based on post-column ^{13}C-IDMS after online chemical oxidation of the peptide to CO2; b) The use of minimally labelled peptides (only two ^{13}C atoms) added to the sample before the enzymatic digestion of the protein and, c) the measurement of the molar ratio between natural and labelled peptides by Isotope Pattern Deconvolution from the experimental isotopic abundances of the mixture obtained by MRM using the whole isotopic envelope. The performance of the procedure will be exemplified with the determination of the renal biomarker Cystatin C in human serum using a proteotyptic peptide labelled with two ^{13}C atoms.

CHARACTERIZATION OF PROTEOMIC PROFILES PREDICTIVE OF HEPATOTOXICITY ASSOCIATED WITH ANTI-TUBERCULOSIS DRUGS: A PILOT STUDY

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Nowadays, tuberculosis represents a major problem in public health both in non-developed and in developed countries. The increase of the incidence of tuberculosis during the last years has been related to an increase in complications derived from its treatment, as the appearance of resistance, therapeutic failure or hepatotoxicity. In particular, hepatotoxicity due to anti-tuberculous drugs is a relevant problem as it results in the modification of the treatment and in liver transplantation or death in the most extreme situations.

We have studied a large cohort of patients under treatment or profilaxis with antituberculous drugs, selecting from them a group (n=8) that developed hepatotoxicity, and a group of matched controls not developing hepatotoxicity (n=8). We have analyzed the plasma proteomic profiles of these patients, with the aim of identifying biomarkers which would be predictive of hepatotoxicity associated with anti-tuberculous drugs. For each patient, plasma samples were collected at the beginning of the treatment (basal conditions), and at the moment of appearance of hepatotoxicity or the end of the treatment, respectively. The comparative analysis of these 32 samples was performed using 8-plex iTRAQ reagents. Samples were first depleted of the 20 top abundant proteins in plasma using a Proteoprep-20 affinity column (Sigma). After tryptic digestion and labeling, iTRAQ-labeled samples were combined in five sets of labeled samples, each one including an internal standard, consisting on a pool of the 32 samples, labeled with iTRAQ-121. This workflow allowed thus the quantitative comparison of the 32 samples. Each labeled peptide mixture was first fractionated by "off-gel" isoelectric focusing, and the resulting fractions were subsequently analyzed by LC-MSMS on an Orbitrap Velos system. Quantification was based on the ratios between the intensity of each reporter ion and the internal standard. Approximately 350 proteins could be identified and quantified in each set, with an overlap of 194 proteins on the five iTRAQ 8plex sets. The results obtained in this analysis led to the identification of some candidate biomarkers predictive of the development of hepatotoxicity.

DECIPHERING THE CATABOLITE REPRESSION PHENOMENON BY QUANTITATIVE PROTEOMICS

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Sphingomonas macrogolitabida strain TFA is a Gram-negative bacterium which capacity to grow very efficiently in the aromatic compound tetralin (1,2,3,4-tetrahydronaphthalene) has already been well characterized and described in our laboratory. We are currently working on a more global understanding of the overall cellular processes applying a Systems Biology approach. Therefore, our aim is to combine the knowledge yielded by our studies in Genomics, Transcriptomics, and Proteomics in order to describe the transcriptomic and metabolic networks that operates in this bacterium to improve its response to environmental signals.

Carbon Catabolite Repression (CCR) is a global regulatory phenomenon that results in the establishment of a hierarchical use of the different carbon and energy sources. In TFA, tetralin metabolism is abolished in the presence of the better carbon source β-hydroxybutirate, which exerts carbon catabolite repression on the tetralin genes. In order to understand the global changes that occurs in TFA when growing making use of this compound, we have applied two different proteomic techniques:

On one hand, DIGE (Differential In Gel Electrophoresis) assays allowed us to identify specific proteins (labelled with fluorophores) that are over-expressed or repressed during CCR conditions at a specific time point during bacterial growth. This resulted in "static" global information of the TFA proteome in CCR conditions.

Secondly, we have detected newly synthesized or repressed proteins soon after the CCR stimulus was applied using radioactive labelling with 35S-methionine. Employing dual channel imaging and warping of two-dimensional protein gels[1] we have visualized global changes on gene expression patterns over time during TFA growth on CCR-conditions. By using a false colouring plus the overlap of those images taken at different times, it became possible to follow the fate of each single protein and visualize whether its expression was reduced or increased; therefore yielding a more "dynamic" information about the changes on the TFA proteome in CCR conditions.

This last approach allowed us to identify groups of proteins responding in the same way in TFA. These proteins could be part of the same regulon and this information could be used to search for common DNA sequences within the intergenic regions of the corresponding genes. These DNA sequences could represent binding motifs for global regulatory proteins involved in CCR in TFA. Finally, the total data yielded by the Proteomics assays will help us to perform an *in-silico* metabolic reconstruction of *Sphingomonas macrogolitabida* strain TFA.

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CHARACTERIZATION OF PROTEIN CHANGES ON THE STATOCYST ENDOLYMPH FOR THE ASSESSMENT OF ACOUSTIC TRAUMA ON COMMON CUTTLEFISH (Sepia officinalis)

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Artificial sound sources are increasingly contributing to the general noise budget of the oceans disturbing the natural balance of the marine ecosystems. Study of the effects of the anthropogenic sound sources has become a crucial question to environmentally regulate human activities on the oceans. Despite its importance, little information is currently available about how cephalopods, and marine organisms in general, process and analyze sound.

In a previous work we have described how some cephalopod species subjected to low-frequency sounds suffer important morphological and ultrastructural acoustic trauma, not compatible with life. Exposure to low-frequency sounds resulted in permanent and substantial alterations of the sensory hair cells of the statocysts, the structures responsible for the animals' sense of balance and position [1, 2].

With the aim of identifying changes in protein expression in the statocyst endolymph after low-frequency sound exposition, endolymph from exposed and non-exposed individuals sacrificed at different times after sound exposure was analyzed using two-dimensional differential gel electrophoresis (2D-DIGE) and the most relevant spots were identified by mass spectrometry. Some differential proteins with a significant change in abundance were identified, most of them being structural or stress-related proteins.

In summary, our experiments confirm that protein content of endolymph changes after been exposed to low frequency sounds. These changes can be expected to affect physiology and functioning of S. officinalis statocyst and alter the sensory information of this species.

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DETECTION OF POST-TRANSLATIONAL MODIFICATIONS IN HUMAN TRANSTHYRETIN ASSOCIATED WITH FAMILIAL AMYLOIDOTIC POLYNEUROPATHY BY A SRM MASS SPECTROMETRY METHOD

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Transthyretin (TTR) is an amyloidogenic tetrameric protein (55kDa) synthesized in liver and choroid plexus in brain. TTR is present in human plasma, transporting T4 hormone and retinol through the retinol binding protein (RBP). TTR is associated with several amyloidosis namely familial amyloidotic polyneuropathy (FAP), familial amyloidotic cardiomyopathy (FAC) and senile systemic amyloidosis (SSA), the first two classified as rare diseases[1-2]. Whereas FAP and FAC are caused by single point mutants, SSA is associated to the wild-type TTR. Variability of TTR is not only due to point mutations in the encoding gene but also to post-translational modifications (PTMs) at Cys10. Only around the 10-15% of the circulating TTR in plasma remains unmodified at this residue, finding as the most common PTMs the S-sulfocysteinylation, S-glycinylcysteinylation, S-cystinylation and S-gluthationcysteinylation[3]. It is thought that PTMs at Cys10 may play an important biological role in the onset and pathological process of the amyloidoses related to TTR.

The objective of our study is to establish the most significant Cys10 PTMs present in TTR human serum samples of patients with TTR-related amyloidosis carrying the most common V30M mutation and to establish a relationship between those PTMs and the clinical profile of the patients. We here will report the development of the methodology for detection and quantification of PTMs in serum samples. It involves a first enrichment step by TTR-immunoprecipitation using a polyclonal antibody, followed by protease digestion of the protein, and finally, the design of a Selected Reaction Monitoring (SRM) method to detect and quantify those PTMs (Fig.1). The enrichment step is shown to be highly efficient and reproducible. Different proteases were tested for TTR digestion to release the peptide of interest for quantification. Using properly designed labelled peptide standards, an SRM method has been developed for PTMs quantification.

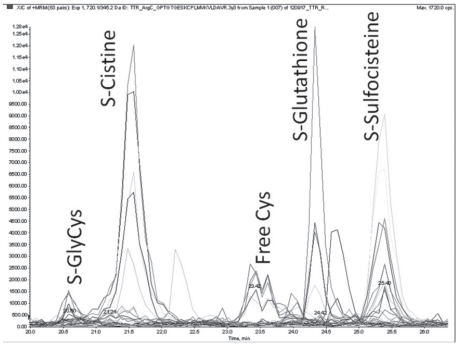

Fig.1 PTMs detection by an SRM method in TTR from human serum samples

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COMPREHENSIVE CHARACTERIZATION OF THE PEPTIDOME AND PHOSPHOPEPTIDOME DISPLAYED BY THE HUMAN LEUKOCYTE ANTIGEN (HLA)-B*40:02

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HLA class I molecules bind peptides derived from proteolytic degradation of endogenous proteins and present them to cytotoxic T lymphocytes, allowing the immune system to detect transformed or virally infected cells. The peptide pool presented by a particular HLA allotype consists on up to 10000 different peptides which share specific structural motives that determine its binding to the class I molecule. Posttranslational modifications are known to be present in some class I ligands. In this regard, phosphorylated peptides have raised much interest as potential targets for cancer immunotherapy since aberrant protein phosphorylation is a hallmark of tumor cells.
Using HLA-B*40:02 as a model molecule and combining affinity purification and high resolution mass spectrometry we have identified more than 2000 different ligands (FDR < 5%). Sequence analysis of this set of ligands revealed two strong anchor motives: Acidic residues at peptide position 2 and methionine, phenylalanine or aliphatic residues at the peptide C term. After IMAC and TiO2 enrichment, about 100 phosphorylated ligands were identified. To avoid false identifications every peptide sequence of this subset was further confirmed by comparing its experimental MS/MS spectrum with that obtained upon fragmentation of the corresponding synthetic phosphopeptide.
We suggest that high throughput identification of HLA class I bound ligands combined with phosphopeptide enrichment may be a useful approach to identify targets for T cell based immunotherapy.

DEPOLARIZATION DEPENDENT SPATIAL LOCALIZATION OF PROTEINS IN NERVE TERMINALS

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Phosphorylation is among the most widespread post-translational modifications in nature, and regulates a great variety of biological processes. On depolarized nerve terminals, phosphorylation and dephosphorylation play a great role on the release of neurotransmitters by exocytosis and the recycling of the synaptic vesicles involved on this process (endocytosis). Depolarization of the neuron membrane produces a collapse on the membrane potential leading to an influx of calcium ions into the nerve terminals. The calcium influx leads to extensive activation of phosphatases (e.i. calcineurin) and protein kinases (e.i. PKC and calcium/calmodulin dependent kinases) which control exocytosis and endocytosis and localization of proteins. Especially the localization of proteins is of great importance in nerve terminals as the specialized processes are relying on proteins being transported from the cytoplasm to the membranes or the opposite way to participate in various protein complexes. Phosphorylation could be important as a regulator of protein localization in nerve terminals.
The aim of this study is to identify proteins that travel between the cytoplasm and membrane upon chemical depolarization of nerve terminals and determine the influence of phosphorylation on localization of proteins in nerve terminals. This is a preliminary study to observe the transfer of proteins between the cytoplasm and membrane of the main proteins involved on the release of neurotransmitters from nerve terminals.
Nerve terminals are isolated from rat brains as synaptosomes, keeping most of their functions intact. After chemical depolarization using KCl, proteins were separated into membrane and cytoplasmic fractions using sodium carbonate combined with ultracentrifugation. The changes in the protein levels and protein phosphorylation after stimulation on the membrane and the cytoplasmic fractions were assessed using isobaric tags for relative and absolute quantification (iTRAQ). Mono-phosphopeptides, multi-phosphopeptides and glycopeptides were analysed with accurate liquid chromatography coupled with tandem mass spectrometry (nano LC-MS/MS) after enrichment and fractionation using the TiSH procedure (TiO2 SIMAC-HILIC).

CHARACTERIZATION OF S. SHIBATAE AND S. ACIDOCALDARIUS DNA-DIRECTED RNA POLYMERASE METHYLATION BY CID AND ETD

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Lysine methylation is a thoroughly analyzed post translational modification (PTM) whereby a hydrogen atom is replaced by a methyl group. This modification process is closely linked to regulation of transcription, protein stability, elongation, and protein interaction, among others.
Although methylation has been profusely analyzed in eukaryotes, little is known about this process in archaea. Lysine methylation is known to increase the pKa of the side chain, changing the hydrophobicity and solubility of the protein. Therefore, it may play an important role in the heat stability of proteins from hyperthermophilic crenarchaea. A recent MS-based analysis identified several methylated lysines in *Sulfolobus solfataricus* RNA polymerase, pinpointing that methylation occurs in crenarchaea.
Collision-induced dissociation (CID) is one of the most commonly used dissociation methods for characterizing peptides. CID, however, may fail to identify large, highly charged, modified peptides. Electron-transfer dissociation (ETD) has been shown to generate more complete series of ions, thereby leading to more extensive sequence information, and is of special interest for the analysis of PTMs. The use of ETD in conjunction with CID has been shown to be a very valuable approach because of their differential dissociation nature.
In this work we characterize the methylation pattern of the DNA-directed RNA polymerase from *Sulfolobus acidocaldarius* and *Sulfolobus shibatae*. Protein was both¬¬ in-gel and in-solution digested prior to analysis, and peptides were analyzed by both CID and ETD in an LTQ Orbitrap XL ETD mass spectrometer.
Our findings extend previous proteomic studies on this enzyme and indeed reveal that DNA-directed RNA polymerase is extensively methylated in both organisms.

N-GLYCOSYLATION ANALYSIS OF TRASTUZUMAB BIOSIMILAR CANDIDATES BY LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY STRATEGIES

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The biotechnological production of protein-based therapeutics is one of the fastest growing sectors of the pharmaceutical industry[1]. Biosimilars, also known as follow-on biologics, constitute subsequent "copies" of innovator biopharmaceutical products made by a different sponsor following patent and exclusivity expiry on the innovator product. Differences in impurities and/or breakdown products can have serious health implications and therefore it´s of high importance to deliver biosimilars with the same or the nearest structural properties than the patented product.

Trastuzumab (Herceptin), a commercially available monoclonal antibody (IgG1) employed to block the ErbB2 overexpression in breast cancer, it´s comprised of a tetramer of two heavy and two light chains with one N-glycosylation in each heavy chain. Several factors, such as growing conditions or cell types can determine the final structure of the glycans, significantly affecting the properties of the generated antibodies. Therefore, the production of antibodies with the appropriate N-Glycosylation is a critical step and one of the most time consuming parts of the developing process for those companies actually producing Herceptin biosimilars (or any other glycosylated drug).

In the present study, we describe two different but complementary strategies to characterize the N-glycosylation of Trastuzumab biosimilars currently in process. Both methodologies include a first step of enzymatic N-glycan releasing from the protein and a second step of glycan characterization. In the first case, N-glycans are fluorescently labeled with 2-aminobenzamide (2-AB) and separated by Normal phase HPLC (NP-HPLC). Different sugars will elute at different times and can be detected and identified employing specific sugar standards. In the second approach, released glycans are permethylated and analysed by MALDI-TOF/TOF, being able to determine the structure because of the differential sugar masses. Herein, the N-glycosylation structures of two different Trastuzumab biosimilars will be compared, and both approaches, HPLC and MALDI analysis, will be described.

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COMPARATIVE PROTEOMIC ANALYSIS OF MILTEFOSINE-SENSITIVE AND MILTEFOSINE-RESISTANT *Leishmania chagasi* CLINICAL ISOLATES

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Visceral leishmaniasis is a serious disease caused by protozoan parasites called Leishmania. The treatment for this disease is difficult, as it requires prolonged and painful applications of toxic drugs that are poorly tolerated. Therefore, one of the most significant advances in the leishmaniasis therapy was the identification of an effective and safe oral drug, the miltefosine. However, different sensitivities to miltefosine by *Leishmania* have been observed in clinically relevant species and the biological mechanism whereby *Leishmania* clinical isolates acquire drug resistance is poorly understood. In this context, comparative two-dimensional gel electrophoresis and mass spectrometry methodologies were applied to highlight and identify proteins that are differentially expressed between miltefosine-sensitive and miltefosine-resistant *L. chagasi* clinical isolates. It is described here a high-resolution proteome for *L. chagasi* promastigotes comprising an average of 459 spots (fig 1A), which corresponds to 5,7% of gene products predicted for *Leishmania* spp. Following comparison of the whole proteome profiles between sensitive and resistant strains, 67 differentially expressed spots were detected. Eighteen spots were found to be specific of a sensitive group and only one of a resistant group, while 48 spots changed in intensity between these groups. MALDI/TOF-TOF mass spectrometry allowed the identification of 48 spots (71,6%), corresponding to 39 distinct proteins of which seven are hypothetical proteins. Among the proteins identified, the comparative proteomics screen highlighted the peroxidoxin, a serine/threonine protein phosphatase, a calpain-like cysteine peptidase and a 4-3-3 protein-like protein that have been reported to play a role in the drug-induced programmed cell death in parasites (fig 1B), suggesting that resistance mechanisms to miltefosine are possibly associated with a lesser susceptibility to apoptosis. Our findings demonstrate that proteomic screen has great potential to identify protein differences between closely related parasite strains and can be used to distinguish parasite phenotypes of clinical relevance. Supported by: CNPq, CAPES and FAPES.

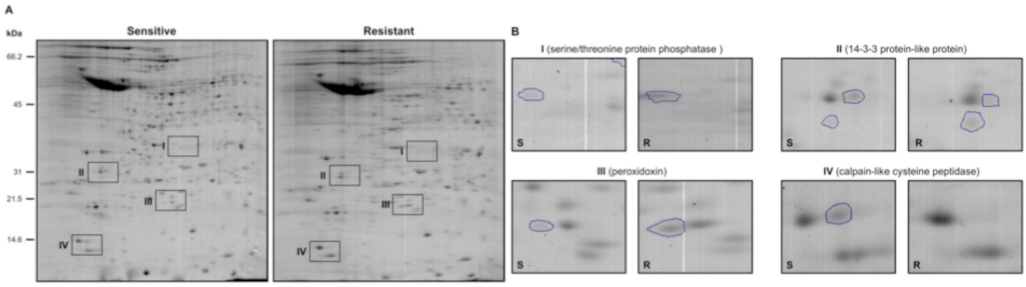

Figure 1. Comparative proteomics analysis between sensitive and resistant *Leishmania chagasi* clinical isolates. (A) Representative 2D gels comparing *L. chagasi* miltefosine-sensitive and miltefosine-resistant strains. Gel obtained after separation of 800 µg of soluble protein in IPG strip covering pH range 4-7. (B) Insets (I-IV) corresponding to zoomed gel areas with spots showing difference in protein expression between the miltefosine-sensitive and miltefosine-resistant group. S, miltefosine-sensitive group; R, miltefosine-resistant group.

IDENTIFICATION OF *Leishmania infantum* VIRULENCE FACTORS BY PROTEOMIC APPROACH

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Leishmania infantum chagasi is the visceral leishmaniasis (VL) agent in Brazil, a zoonotic disease with high morbidity and mortality with the dogs being the main reservoir. VL canine is a severe disease which the symptoms are present in less than 50% of infected dogs. Clinical variability suggests that parasite factors are involved in virulence. We used two *L. infantum chagasi* strains: MHOM/BR/1972/BH46 (BH46) and MCAN/BR/2000/BH400 (BH400) with high and low virulence, respectively. We realized in vitro and in vivo virulence characterization these strains by infecting macrophages and hamsters, respectively. These assays had shown a statistically significant bigger parasitism in cells and animals infected with BH400 than BH46 strain. Furthermore, we used proteomic approach to identify proteins that may be involved in virulence difference. The protein extracts were obtained from promastigotes forms and fractionated by electrophoresis two dimensional DIGE (*Differential Gel Electrophoresis*) using 18 cm pH 4-7 strips and 12% SDS-PAGE. The gel images were analyzed by DeCyder® software (GE Healthcare, USA). The analysis showed 13 spots with higher expression in BH400 than in BH46. No overexpressed spots were identified in BH46 when compared to BH400. The differential expressed spots were identified by Mass Spectrometry (MALDI/ToF-ToF) (Bruker Daltonics, Billerica, USA). Among them are α and β-tubulins, heat shock protein 83-1, paraflagellar rod protein 2C, putative cysteine peptidase, enolase, putative adenosine kinase, translation elongation factor 1-β, putative NADP-dependent alcohol dehydrogenase, putative proteasome activator protein pa26, kinetoplastid membrane protein-11, glutaredoxin-like protein and conserved hypothetical protein. Some the identified proteins are described as virulence factor in literature and their role in virulence phenotype will be evaluated by transgenic parasites. The virulence factor studies may contribute to the discovery of new targets with therapeutic potential against leishmaniasis and in parasite-host interaction.

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A STRUCTURE-BASED STRATEGY FOR EPITOPE DISCOVERY IN BURKHOLDERIA PSEUDOMALLEI OPPA ANTIGEN

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We present an approach integrating mass spectrometry, structural and computational biology with immunological tests to identify epitopes in the OppA antigen from the Gram-negative pathogen Burkholderia pseudomallei, the etiological agent of melioidosis. The crystal structure of OppABp, reported here at 2.1 A° resolution, was the basis for a computational analysis that identified three potential epitopes. In parallel, antigen proteolysis and immunocapturing allowed us to identify by mass spectrometry three additional peptides. All six potential epitopes were synthesized as free peptides and tested for their immunoreactivity against sera from healthy seronegative, healthy seropositive, and recovered melioidosis patients. Three synthetic peptides allowed the different patient groups to be distinguished, underlining the potential of this approach. Extension of the computational analysis, including energy-based decomposition methods, allowed rationalizing results of the predictive analyses and the immunocapture epitope mapping. Our results illustrate a structure-based epitope discovery process, whose application may expand our perspectives in the diagnostic and vaccine design fields.

Reference: Lassaux P, Peri C, Ferrer-Navarro M, et al. A Structure-based strategy for epitope discovery in *Burkholderia pseudomallei* OppA antigen. Structure (2013), 21, 1-9.

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ORNITHODOROS MOUBATA SOFT TICK SALIVA PROTEOME: A COMPARISON BETWEEN FEMALE AND MALE SALIVA

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Ticks are ectoparasites that feed on the blood of their hosts. To do this, ticks suppress the host haemostatic and immune systems by using anti-haemostatic, anti-inflammatory and immunomodulatory proteins that are synthesized in the tick salivary glands and inoculated with tick saliva into the host during the feeding process. The host immunosuppression caused by ticks can further facilitate the transmission of tick-borne pathogens. Therefore, these salivary proteins could be adequate antigenic targets for development of anti-tick vaccines that would have the dual effect of blocking the tick feeding and the pathogen transmission.

The objective of the present work was to identify the salivary proteins of *Ornithodoros moubata*, an African argasid tick that transmits Human relapsing fever and African swine fever. With this aim, we have used pilocarpine to induce salivation of *O. moubata* adult ticks and collected saliva samples from females and males separately. Saliva has been analyzed for protein identification in several ways: (i) samples of crude native saliva have been subjected to LC-MS/MS and database searching (EST_acari and NCBInr_metazoa); (ii) to make it possible the identification of the low abundant proteins, additional samples of saliva of both sexes have been equalized (ProteoMiner, BIO-RAD) and then subjected to LC-MS/MS and database searching; and (iii), female and male crude native saliva samples have been resolved by monodimensional SDS-PAGE and the stained gel bands sliced and analyzed by LC-MS/MS.

Here, we report the array of more than 200 proteins identified in the saliva of *O. moubata* showing: (i) the broad and complex composition of the saliva of this tick, in good agreement with the complexity of the argasid and ixodid sialomes described previously; (ii) a remarkable difference in the saliva proteomes of females and males, since hardly 6% of the proteins identified appeared to be shared by both sexes; and (iii) the presence in the salivary fluid of a wide range of proteins known to be housekeeping/intracellular, which lack classical secretion signals and could have been secreted in unconventional ways, including exosome secretion.

This is the first description of the proteome of the saliva of an argasid tick, and besides providing interesting information, it also opens new questions such as what the biological meaning of the observed differences in the saliva composition between females and males could be.

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PROTEOMIC IDENTIFICATION OF VASCULAR SURFACE PROTEINS ISOLATED FROM THE LIVER OF MICE INFECTED WITH SCHISTOSOMA BOVIS

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Schistosomiasis is a parasitic disease affecting man and domestic and wild animals that represents an important health and veterinary problem in many tropical and subtropical areas of the world. Schistosoma bovis is an intravascular parasite that lives within the venous portal mesenteric system of ruminants. Schistosome cercariae infect their vertebrate hosts through the skin. Then, they enter the blood circulation, performing an intra-organic migration along which they mature and reach their final localization, where they transform into adults and live for years. Eggs laid by *S. bovis* adult females in the mesenteric veins pass through the intestinal wall and then exit the host through the faeces, or they are swept into the liver and trapped in the sinusoids, where they induce granulomatous lesions.
The purpose of this work was to analyze the changes induced by *S. bovis* eggs and adult worms in the proteome expressed by infected mice on the endothelial cell surface of blood vessels of the liver. With this aim, we have utilized a methodology that allows the purification, identification and relative quantification of endothelial cell surface proteins after their selective *in vivo* biotin labeling. Trypsin digestion of the biotinylated proteins and subsequent liquid chromatography and tandem mass spectrometry analysis (LC-MS/MS) resulted in the identification of a total 167 non redundant proteins. Of these proteins, 47 were identified only in the liver of non-infected mice; 64 only in infected with *S. bovis* mice and 56 in both groups of mice. The efficiency of vascular biotinylation after in vivo perfusion was assessed in liver samples taken from the mice infected and non-infected with *S. bovis* by immunohistochemistry of paraffin sections and by western blotting with streptavidin-HRP.
Here we report the identified proteins classified according to their function and cellular location, and the differences between *S. bovis*-infected and non-infected mice in their vascular proteomes of the liver. This work provides the first data on the vascular surface proteome of the liver after *S. bovis* infection.

This work has been financed by project AGL2010-18163 from the Spanish Ministry of Science and Innovation.

PROTEOME MAPPING OF THE LUNG ENDOTHELIAL CELL SURFACE IN SCHISTOSOMA BOVIS-INFECTED HAMSTERS

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Schistosoma bovis is a blood-dwelling fluke worm affecting ruminants that lives for years inside its hosts vasculature, in direct contact with the vascular endothelium and exposed to the blood components. As do other species of *Schistosoma, S. bovis* penetrates into the host body through the host skin until reaching a skin capillary vessel. After that, it migrates through the blood stream towards the lungs, where it stays several days as schistosomulum larva, and later to the portal vein and the mesenteric vessels where it finally develops into the adult stage. Since this parasite is adapted to such an intravascular habitat, it is expected him to modulate the endothelial cell function to favor its own survival. The objective of this work was to investigate the proteome of the lung vascular endothelial cell surface expressed as a result of the presence of schistosmulae larvae. To accomplish this, we have subjected the lungs of S. bovis-infected and non-infected hamsters to vascular perfusion with a biotin ester reactant. In this way, the proteins from the endothelial cell surface showing free amino groups would become biotinylated allowing their efficient purification by streptavidin affinity chromatography. Trypsin digestion of the biotinylated proteins and subsequent liquid chromatography and tandem mass spectrometry analysis (LC-MS/MS) resulted in the identification of 185 and 143 non-redundant proteins in infected and non infected hamsters, respectively. The efficiency of vascular biotinylation after this *in vivo* perfusion procedure was assessed by immunohistochemistry in paraffin sections of lung, and by western blotting with streptavidin-HRP.
Here we report the identified proteins classified according to their function and cellular location, and the differences between *S. bovis*-infected and non-infected hamsters of their lung vascular proteomes were analyzed. This work provides the first data on the vascular surface proteome of the lung after 10 days *S. bovis* infection.

This work has been financed by project AGL2010-18163 from the Spanish Ministry of Science and Innovation.

EXAMINING DIFFRENTIAL PROTEIN EXPRESSION IN COLORECTAL CANCER USING MASS SPECTROMETRY-BASED PROTEOMICS APPROACH

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AIM. The need of new markers for evaluation of colorectal cancer is continuously raised by different authors. Recent mass spectrometry- based proteomics studies provide not only the identification and quantification of highly abundant proteins but also systematic insights into organization of cancer cells- changes in cellular signaling pathways, alterations in the cell-surface proteome or biomarkers abundance.
In this study we analyze proteomes of normal mucosal tissue, adenocarcinoma, and its nodal metastasis from laser microdissected patient matched archival FFPE clinical samples to identify the proteins that are key players in the development and progression of the colorectal cancer.
Material and methods. The workflow involved lysis of cancerous and noncancerous tissue from 8 patients in SDS-containing buffer. Detergent removal and consecutive digestion of the proteins with trypsin were prepared according filter aided sample preparation protocole modified for fomaline fixed paraffine embedded tissue samples (FFPE-FASP). Resulting peptides were fractionated by pipette-tip based strong anion exchange into 6 fractions and analyzed by LC-MS/MS on a linear ion trap Orbitrap mass spectrometer. The upregulation of 10 potential biomarkers in colorectal adenocarcinoma samples was confirmed by immunohistochemistry.
Results. Analysis of the data using the MaxQuant software version 1.2.2.8 resulted in the identification of > 8100 proteins from normal mucosal tissue, adenocarcinoma, and its nodal metastasis, among which more than 7500 are common for three stages. We found >1800 proteins significantly changed between normal colonic mucosa and cancer tissue, while there was observed minimal proteomic remodeling between primary cancer and metastases. Moreover, we reported that general transcription factors, nuclear transporters and chromatin activators are upregulated in the cancer samples, whereas the integral plasma membrane channel and transporter proteins were found downregulated in the same sample.
Conclusions. Present work enables systemic insights into the organization of the colorectal cancer cells and an estimation of the abundances of known biomarkers. Furthermore, the data obtained in our study allows to identifying a number of proteins that weren't known before to be significantly overexpressed in colorectal cancer.
Mass spectrometry-based clinical proteomics may be essential for the further identification and validation of new protein biomarkers that have shown vast promise for the early colorectal cancer detection and prediction.

2D-DIGE ANALYSIS OF FLUORESCENTLY LABELED SURFACE PROTEINS OF Xanthomonas citri CELLS INTERACTING WITH CITRUS-HOST

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The causative agents of citrus canker are bacteria from the genus *Xanthomonas*, of which *Xanthomonas citri* subsp. *citri* (XAC) is more virulent and attacks a greater number of hosts than other citrus canker pathogens. The genome of this bacterium has been totally sequenced. In this work it was performed a differential proteomic analysis of XAC proteins exposed in the cellular surface using fluorescent dyes. The bacteria were grown *in vitro* (Nutrient Broth medium) or *in vivo* (citrus-host leaves) conditions. Intact cells were labeled with CyDyes (GE Healthcare), washed to eliminate non-incorporated dyes, and finally disrupted. The protein extracts were analyzed by Two-Dimensional Difference Gel Electrophoresis (2D-DIGE) and a statistical analysis (ANOVA) was performed using DeCyder 7.0 software (GE Healthcare). Approximately 100 spots showed significant ($p<0.05$) differential expression and most of them were digested using trypsin and analyzed by mass spectrometry (ESI-QUAD-TOF) (LNBio, CNPEM-ABTLus, Campinas-SP, Brazil). The Mascot tool was employed to compare the results with annotated proteins of the respective NCBI genome database. Among the forty-six proteins identified, twenty-two were induced in the *in vivo* condition, including receptors, other known outer membrane proteins and several conserved hypothetical proteins. We concluded that surface proteins are involved in the pathogen response in citrus canker and could be important targets to drugs.

Keywords: citrus canker, *Xanthomonas citri* subsp. *citri*., pathogenicity, differential proteomic analysis, 2D-DIGE.

PROTEOMICS APPROACH TO STUDY EARLY BIOCHEMICAL EVENTS IN CARNATION (DIANTHUS CARYOPHYLLUS L.)-FUSARIUM OXYSPORUM F. SP. DIANTHI INTERACTION

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Vascular wilting caused by *Fusarium oxysporum* f. sp. *dianthi* race 2 is the worldwide most important disease affecting carnation (*Dianthus caryophyllus* L). Several strategies have been used to control this pathogen, but none has provided full protection. At present, no markers have been developed for resistant cultivar selection in breading programs, because of the poorly biochemical and molecular information about resistance to this pathogen. In order to know on this plant-pathogen interaction in terms of plant responses, a comparative proteomics approach has been used. In a preliminary experiment, we evaluated the differential protein accumulation during 6, 24 and 96 hours post-infection (hpi) with the pathogen, in two cultivars differing in resistance, using 1D electrophoresis. The protein extraction from stems and roots of carnation were carried by using protein precipitation protocols (TCA/acetone/phenol). After gel analysis using Quantity-one software (Bio-Rad) bands of interest were subjected to MS (MALDI-TOF-TOF) analysis after tryptic digestion of the contained proteins. In a second step, 2-DE analysis of extracts from roots and stems, at 6 and 96 hpi respectively, was performed, with gel images analyzed with the PDQuest software (Bio-Rad). Out of the 798 consistent spots detected and well resolved, 187 were variable among cultivars, tissue or treatments. Protein identification after MALDI/TOF/TOF analysis were done by searching at the NCBInr database using MASCOT (MatrixScience) as search engine, and limiting the taxonomic category to plants (*Viridiplantae*). We got confident matches for 25 out 78 selected spots (30% success). This low rate of identification is mainly due to the lack of reported sequences for carnation in public databases. Even so, we have identified some constitutive proteins associated with the resistant phenotype, such as a class III peroxidase and a NB-ARC resistance protein (Nucleotide Binding domain shared by Apaf-1, certain R gene products, and CED-4 fused to C-terminal leucine-rich repeats). Likewise, a differential protein accumulation associated to carbohydrate metabolism reorganization was found in the resistant cultivar during pathogen infection at the root level. The next step will be focused to develop a specific database for identifying those non-identified remaining proteins which are differentially accumulated in the resistant cultivar, and the use of 2-DE complementary shot-gun approaches to deep in the carnation proteome coverage.

SUBCELLULAR FRACTIONATION AS A STRATEGY TO IDENTIFY KEY PROTEINS RELATED WITH ONCOGENESIS

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It is becoming clear that the phenotypes that are characteristic of cancer cells are the result of numerous and heterogeneous mutations that affect many signalling pathways and a vast array of key cytoplasmic proteins. Increasing research is now devoted to the identification of the crucial oncogenic signalling molecules that contribute to the process of tumorogenesis. Proteomics has emerged as a promising tool for unraveling signalling proteins that are associated with oncogenesis, and in particular, to allow comparative studies between different types and states of cancer. An important body of knowledge has shown the similarities between the proliferative and invasive properties of trophoblastic and cancer cells. The aim of the present study was to compare the cytoplasmic sub-proteome of the human trophoblast HTR8/SVneo cell line with that of the malignant choriocarcinoma JEG-3 cell line in order to get insight into the molecular mechanisms responsible of oncogenesis. We obtained the cytoplasmic sub-proteomes free of membrane proteins by differential detergent fractionation (DDF), their enrichment and quality were evaluated by Western Blot, using antibodies against IGF-IIR membrane receptor, FAK, ERK and p53 as cytoplasmic signal transducers. The proteomes were resolved by 2D electrophoresis and analyzed on three different groups: unique protein spots from JEG-3 sub-proteome, unique protein spots from HTR8/SVneo sub-proteome and differential protein spots present in both samples. Selected protein spots were identified, initially by MALDI MS/MS, and afterwards by HPLC-ESI-MS/MS, and analyzed using the DAVID 6.7 and STRING 9.0 softwares. Initially, 4 spots in each group were analyzed by MALDI TOF/TOF, identifying 6 common proteins, 4 proteins expressed only by JEG-3 cells and 7 proteins expressed only by HTR8/SVneo cells. All of them presented annotation to intracellular part and 35.7% mutagenesis site annotation, which represent a suitable strategy to study cytoplasm proteins related to oncogenesis. Later, with the aim to find possible oncogenic proteins, a deeper study was developed on the unique protein spots obtained for JEG-3 sub-proteome, identifying 54 unique proteins by HPLC ESI-Q/TOF. Of these, 79.2% were protein binding, 50.9% had catalytic activity annotation, 13.2% oxidoreductase activity and 5.7% peroxiredoxin activity. In addition, proteins linked with β-alanine, cysteine and methionine metabolism, proteasome, glycolisis/gluconeogenesis, and expressed on response to ERK signaling pathway were detected. Furthermore, 22.6% had mutagenesis site annotation as Programmed cell death protein 6, Peroxiredoxin-2 and 6, Proliferation-associated protein 2G4 and Thioredoxin, among others. From these results, it is possible to deduce a rise in metabolic, antioxidant and protease activities on choriocarcinoma cells compared to trophoblast cells, as a tumorogenesis strategy. In conclusion, these analytical approach allowed to resolve and compare proteomes to find key cytoplasmic proteins possibly involved in altered networks and oncogenesis.

DIFFERENTIAL PROTEOMIC ANALYSIS OF THE HUMAN PLATELET RELEASATE FOLLOWING ACTIVATION WITH DIFFERENT AGONISTS

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Platelets are cytoplasmic fragments without nuclei whose main function is to keep primary hemostasis by forming thrombi after an injury of the endothelium. Undesired activation and formation of arterial thrombi are implicated in many diseases, such as myocardial infarction and stroke [1]. Once activated, platelets release a high number of proteins and other biomolecules, which is known as releasate. There are clear indications that proteins secreted by platelets are found in the atherosclerotic plaque contributing to its pathogenesis [2]. In recent years there have been several groups focusing on the study of the releasate, analyzing the effect of different agonists and antiplatelet agents such as *aspirin* [2,3]. In our study we tested the hypothesis that the platelet releasate varies depending on the platelet stimulus used to activate them. We compared the releasate of thrombin-stimulated platelets versus collagen-stimulated. Proteome analysis was based on 2D-DIGE and MALDI-TOF MS or LC-MS/MS. We run four technical replicates per group. We detected 1742 spots per gel, 131 of which appeared differentially regulated between both conditions. Spots were filtered based on two parameters: fold change ≥ 2 and $p < 0.05$. We identified 72 different regulated spots, most of which corresponded to α, β and γ chains of fibrinogen overexpressed in the releasate of collagen-activated platelets, which was found to be due to the ability of thrombin to transform fibrinogen into fibrin. We identified another 25 unique proteins (13 overexpressed in collagen, 11 in thrombin and one in both conditions). The identified proteins were secreted in a similar proportion by both secretory pathways (classical and non-classical), and 7 of them had not been reported previously in platelet releasate studies. Those include *nucleosome assembly protein 1-like* 1 (NP1L1), *glutathione peroxidase 1* (GPX1), *myosin regulatory light polypeptide 9* (MYL9), *caspase-3* (CASP3), ML12B, and *cardiotrophin-like cytokine factor 1* (CLCF1). The latter is a cytokine with B-cell stimulating capability. Validation studies were by western blotting. The interest of the present study was to determine which proteins are secreted differentially depending on the platelet receptor that is activated. This could have pathological implications; an important example is our study published in 2011, which shows a higher activation state of GPVI signaling (collagen receptor) in platelets from patients with acute myocardial infarction [4].
We are actually carrying out a study to compare releasates of collagen-activated platelets in presence / absence of inhibitors of secondary mediators (as the ADP inhibitor apyrase and the thromboxane inhibitor indomethacin) and integrilin (to inhibit aggregation by αIIbβ3). In this way we can study the effect of these drugs on the platelet releasate and identify proteins altered between both conditions, which would be interesting from a pharmacological point of view.

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MODIFICATION OF THE PROTEIN PROFILE OF BLADDER TUMOURS INDUCED BY THE INHIBITION OF NITRIC OXIDE PRODUCTION

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Bladder cancer (BC) is the second cause of death from male urogenital tract tumours. It is classified as non invasive (NMI) or muscle invasive, when it reaches the detrusor muscle. NMI tumours represent 80% of diagnosed BC and the transurethral resection can be useful to eliminate the tumour when there is only one injury and the surrounding tissue is histologically normal. However, recurrences appear in 50% of patients, and 30% of them present a more invasive pattern, requiring more aggressive therapies. The identification of new players involved in tumour progression can provide new therapeutic targets.

Nitric oxide (NO) is a free radical produced by NO synthase (NOS). We previously described that 50% of human BC express the inducible isoform iNOS. We determined that 80% of iNOS+ patients show tumoral recurrence in the first year after the treatment while only 20% of iNOS- patients show recurrence during that time. We also demonstrated that mouse MB49 BC growth is reduced with L-NAME (inhibitor of NO production) therapy. These results led us to propose that modulation of NO production in iNOS+ tumour could be a useful therapeutic strategy.

The aim of this work was to evaluate mouse MB49 BC protein expression and the modification of its protein profile upon L-NAME treatment.

MB49 bladder tumours derived from mice treated or not with L-NAME were compared by 2D-DIGE technology. A total of 52 proteins were identified as differentially expressed. L-NAME induces the over-expression of Ras suppressor protein1 (fold change 1.66; $p<0.04$) and reduces the expression of Vimentin (fold change 1.81, $p<0.01$), Hemoglobin (fold change 3.0, $p<0,001$) and S100-A9 (fold change 5.6, $p<0,008$). We analyzed by western blot the MAPK proliferation network to validate over-expression of Ras suppressor protein1. Both the cell line and MB49 derived tumours show up-regulation of pERK which is reduced in MB49+L-NAME. The reduction in Vimentin expression upon L-NAME treatment was validated by immunohistochemistry (IHC). Hemoglobin under-expression was validated by in vivo tumoral angiogenesis assays which demonstrated that blood vessel density ($p<0.05$) was reduced in mice treated with L-NAME. S100-A9 is a protein involved in immunosupression, related with appearance of myeloid suppressor cells. Down-regulation of its expression after L-NAME treatment was validated by IHC. We analysed 31 human BC samples and we observed S100-A9 expression in 87% of the samples, located in the tumoral cells and/or in the myeloid cells.

In conclusion, L-NAME inhibits BC growth by the inhibition of proliferation, angiogenesis and epithelial mesenquimal transition pathways. Given the large decrease in S100-A9 expression and its relevance in human BC, we propose to study in more detail its role in tumour progression. Taken together, these results suggest us that the inhibition of NO production would be a good therapeutic target in those patients with tumours expressing iNOS.

DIFFERENTIAL PROTEOMIC CHARACTERIZATION OF SEVERAL FAMILIES FROM BRACHYSPIRA HYODYSENTERIAE AND BRACHYSPIRA PILOSICOLI

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Pathogenic spirochetes that inhabit the large intestine have an intimate association with the colonic mucosa. In the case of the agent of swine dysentery, *Brachyspira hyodysenteriae*, the spirochetes localize within colonic crypts, penetrate the protective mucus barrier and invade the epithelium and the lamina propia. In contrast, the weakly B-hemolytic intestinal spirochete *Brachispira pilosicoli* (the agent of intestinal spirochetosis) attaches by one cell end to the colonic epithelium but it also may localize within the colonic crypts and invade beyond the epithelium.

By analogy with other pathogenic organisms, it is probable that proteins expressed on the surface of *B. hyodysenteriae* and *B. pilosicoli* play an important role in colonization and disease expression. This interaction may be direct (for example by mediating attachment to host cells) or indirect (for example by being the targets for the host immune response). It is interesting to note that relatively few Brachyspira outer membrane proteins have been characterized. To date only eleven proteins are available in Uniprot at evidence level for Brachyspira. All of them have been identified in *Brachyspira hyodysenteriae*.

The aim of this study is the characterization of the sufarce proteins of *Brachyspira Hyodysenteriae* and *Brachyspira Pilosicoli*.

To isolate the proteins located in the outer membrane of Brachyspira, a short incubation with trypsin was carried out adding the enzyme directly to the bacteria. After this incubation the supernatant protein were digested for 7 hours. Control samples were running in parallel to discard secreted proteins. Digested samples were analyzed by LC-μESI-MS/MS (Orbitrap).

Using this methodology more than 500 new proteins were identified, most of them located on membrane.

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2D-DIGE STUDY IN SEMINAL PLASMA FROM DIFFERENT INFERTILE GROUPS WITH A CHARACTERISTIC PROFILE OF SPERM DNA FRAGMENTATION

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Seminogram is the method of routine analysis in the laboratory of andrology. It gives a descriptive evaluation of semen, but these parameters are not always sufficient for accurate diagnosis, treatment or to explain the molecular basis of male infertility; in this sense, the identification and characterization of protein biomarkers are required. Human seminal plasma is loaded with proteins that are essential both for the sperm and for the fertilization process. For these reasons, two-dimensional gel electrophoresis (2D DIGE) was performed on seminal plasma from 24 males. The samples were selected according to three characteristics: the pathology, the rate of sperm with completely degraded DNA relative to total sperm with fragmented DNA (DDS) measured by Sperm Chromatin Dispersion (SCD) and the characteristic profile of Sperm DNA Fragmentation (SDF) measured by alkaline and neutral Comet assay. The selected patients were: six fertile donors (FD), six asthenoteratozoospermic (ATZ) patients, six asthenoteratozoospermic patients with varicocele (ATZ-VAR) and six patients of recurrent miscarriage with normal seminogram (RM). After silver staining, the differential expression proteins were analyzed by Nonlinear Dynamics Progenesis SameSpots analysis software. A total of 72 spots showed statistically significant differential protein expression ($p<0.05$) with a fold>2. Forty-three (60%) were differentially expressed in FD group compared to the other groups, 16 (22%) in ATZ patients, 9 (12.5%) in ATZ-VAR patients and 4 (5.5%) in patients with MR. These results could allow distinguishing between fertile and infertile individuals and also between fertile individuals and RM although these groups have both normal seminogram. ATZ and ATZ-VAR patients have similar seminogram but only six spots had similar protein expression and were different from the other groups suggesting that asthenoteratozoospermia might be caused by different molecular mechanisms. Thirty spots of interest were identified by mass spectrometry to verify these findings.

EFFECT OF PROTEIN SOLID SUPPORT AND EXPRESSION PLATFORM ON BIOMARKER DISCOVERY AND VALIDATION

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Colorectal Cancer is one of the deadliest cancers in developed countries; its early diagnosis would improve survival rates. Detection of antibodies directed to Tumor Associated Antigens (TAAs) through ELISA provide a reliable alternative to Fecal Occult Blood Testing (FOBT), colonoscopy or CEA analysis, characterized by lower costs and higher specificity. In previous studies we have reported the diagnostic value of 6 TAA's identified in ProtoArray v4.0, and 6 TAAs in phage display arrays.
By testing ProtoArray version 4.1, characterized by a different and thinner nitrocellulose support, we identified 3 new TAAs: GTF2B, HCK and EDIL3. To compare their diagnostic performance to previously identified TAAs, we expressed full-length human TAA's in E.coli or Sf9 cells and tested their diagnostic value by indirect ELISA using 153 patients' sera. In order to assess the effect of the recombinant expression platform on TAA discrimination power, we compared the performance of selected TAA's expressed in both E.coli and Sf9 cells using smaller patient cohorts. The validation cohort was distributed through colorectal cancer patients, healthy individuals and patients suffering from other cancers. Differences among groups were detected through one-way parametric analysis. Predictive ability was determined by logistic regression and ROC curves. TAA redundancy was examined through Principal Component Analysis.
In total, we expressed and purified to homogeneity 13 human recombinant TAAs, and amplified 6 phages displaying human peptide TAAs. By adding Edil3, HCK and p53 to previous panel composed of GTF2i, SREBF2, MST1/STK4, SRC and multiplexed PIM1, MAPKAPK3 and FGFR4, we have improved the diagnostic panel previously described. With an AUC=0.94 and 86% specificity at a 90% fixed sensitivity (Fig1), we achieved an optimal value for cancer screening. Adding GTF2B to GRN, GTF2i, MST1/STK4 and SULF1, we were able to discriminate late stages C and D from early stages A and B with an AUC of 0.90 and 87% sensitivity for a fixed 90% specificity.
Regarding comparison of protein expression systems, we found generally lower false positive rates when using Sf9 produced proteins, leading to higher specificities, although serum dilution and quantity of protein adsorbed needs to be optimized case by case.
By using different protein array technology as well as different expression platforms we have improved diagnostic performance and added prognostic abilities to previously described panel.

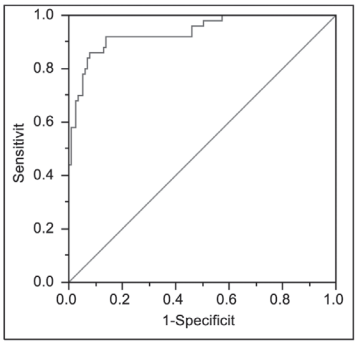

Figure 1: Combined ROC Curve for described diagnostic algorithm.

ANALYSIS OF CONIDIAL GERMINATION OF *Colletotrichum acutatum*

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One of the main problems in the actual agriculture is the appearance of fungal plant diseases that produces critical losses in the fields. *Colletotrichum acutatum* is an important phytopathogenic fungus causing anthracnose in commercially important fruit crops, especially in strawberry. The fungus uses a set of infection strategies to complete its infection cycle and avoid the activity of commercially available fungicides. IN this sense, conidia produced by the fungus are survival structures which play a key role in host infection and fungal propagation. Despite its relevance to the fungal life cycle, conidial biology has not been extensively investigated.

We had developed a proteomic approach to provide the first description of the conidial proteome during germination process in *C. acutatum*. We had compared the proteomic profile of ungerminated and germinated conidia by using a two-dimensional electrophoresis combined with MALDI-TOF/TOF mass spectrometry. We have identified 365 proteins in 354 spots, which represent 245 unique proteins. We had identified some proteins with key functions in pathogenesis, while the role of most of them remains undilucidated.

All identified proteins have been classified according to their molecular function and their involvement in biological processes, including cellular energy production, oxidative metabolism, stress, fatty acid synthesis, protein synthesis and folding. This report constitutes the first comprehensive study of protein expression during the early stage of the *C. acutatum* conidial germination. It advances our understanding of the molecular mechanisms involved in the conidial germination process, and provides a useful basis for the further characterization of proteins involved in fungal biology and fungus life cycles.

PROTEOMIC ANALYSIS (2D-DIGE and MS/MS) OF SERA AND LYMPHOYD ORGANS FROM COLLAGEN-INDUCED ARTHRITIC MICE

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Introduction. Collagen-induced arthritis (CIA) in C57BL/6 (B6 WT) mice closely resembles human rheumatoid arthritis (RA) in terms of its disease course, histological findings and response to drugs treatment. We have recently reported that CD38 deficient mice (CD38ko) develop an attenuated form of CIA as compared with WT mice (Postigo et al., *Mice Deficient in CD38 Develop an Attenuated Form of Collagen Type II-Induced Arthritis*, PlosOne , 2012).
Objetive. In this work we have performed a proteomic approach to identify CD38-dependent changes in protein abundance in CIA+ mice. To this end, proteomic analysis using 2D DIGE in serum and lymphoid organs such spleen and lymph nodes to differentiate between WT and CD38ko mice in CIA model.
Materials and Methods. Blood serum samples from WT and CD38 KO mice, which have immunized with chicken collagen type II, were treated with ProteoMiner beads to equalize protein concentrations and subjected to 2D-DIGE and MS-MALDI-TOF analysis. Differential protein expression was validated by ELISA, or Western-blotting. Spleen and lymph nodes samples were disaggregated using MicroRotofor Lysis kit (Bio-Rad) and subjected to 2D-DIGE and MS/MS analysis. First dimension were run on Protein IEF Cell and 2nd dimension on Criterion Dodeca Cell (BioRad). Gels were scanned with the Amersham Typhoon Imager 9410. Two variables were determined: affectation (affected vs unaffected) and mouse type (WT vs CD38ko) for two-way ANOVA analysis using DeCyder 7.0 software (GE). The EDA module 1.0 was used for multivariate analysis.
Results. Using multivariate analyses, 25 differentially expressed spleen proteins were able to discriminate between WT and CD38ko mice, while in lymph nodes 8 differentially expressed proteins could be used to differentiate whether mice were affected or not affected. In serum some proteins such as serum amyloid A, transthyretin, IgG, ficolin, complement C4b were overexpressed in affected WT as compared with affected CD38ko mice, while in the latter proteins related to apolipoproteins were found to be overexpressed; ELISA or Western Blot validated some of these differences.
Conclusions. Multivariate analyses showed that there were some proteins differentially expressed in spleen and lymph nodes that could be used to distinguish whether mice were WT or CD38ko, or whether mice were affected or not, respectively. In contrast, multivariate analysis of serum proteins was not powerful enough to discriminate between these variables. However, most of the down-regulated proteins identified in the sera of collagen-immunized CD38 KO mice are involved in inflammatory processes. Some of them could be indicative of the relatively milder pathological process found in CIA+ CD38 KO than in WT mice.

SUBPROTEOME ANALYSIS REVEALS REMARKABLE DIFFERENCES IN PERIPLASMIC PROFILES OF *Xanthomonas* spp. DIFFERING IN VIRULENCE AND CITRUS-HOST RANGE

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Citrus canker is a disease caused by *Xanthomonas* spp, a Gram-negative bacterium, and its control could have an important impact in the economy of Brazil and other citrus producers around the world. The aim of this work was to perform differential proteomic analysis of the periplasmic protein profiles from the types A, B and C of *Xanthomonas* spp, which had their genome previously sequenced. Whereas *Xanthomonas* citri subsp. citri (XAC) is the most virulent and infects all types of citrus, *Xanthomonas* fuscans subsp. aurantifolii (Xau-B) is less virulent and *Xanthomonas* fuscans subsp. aurantifolii (Xau-C) has a unique citrus-host. The bacteria were grown in XAM-1, which is known to be a pathogenicity-inducing medium for XAC, and in a non-inducing pathogenicity medium (Nutrient Broth, NB). Periplasmic proteins were separated by two- dimensional electrophoresis (2D-PAGE) and the gels were stained using Coomassie Brilliant Blue R-250 and documented. A comparative analysis of the protein profiles between XAC and Xau-B, and between XAC and Xau-C, grown in the same culture medium, was performed using ImageMaster Platinum software (GE Healthcare). Spots that showed a significant differential expression by statistical analysis (ANOVA, $p < 0.05$) were isolated from gels and identified by ESI-QUAD-TOF mass spectrometry (LNBio, CNPEM-ABTLus, Campinas-SP, Brazil), using the respective databases. Differential expression for superoxide dismutase was confirmed by Western blot. The results showed that XAC, Xau-B, and Xau-C have remarkable differences between the periplasm protein profiles in both conditions of growth, even though most genes related to the differential proteins are present in the genome of all of them. This work showed that the subproteomic analysis of the periplasm is an important tool to differentiate the species of *Xanthomonas* spp (supported by FAPESP and Fundecitrus, Brazil).

Keywords: Citrus canker, *Xanthomonas* citri subsp. citri, *Xanthomonas* fuscans subsp. aurantifolii type B; *Xanthomonas* fuscans subsp. aurantifolii type C; pathogenicity; differential proteomic analysis, two-dimensional electrophoresis.

SUBPROTEOME ANALYSIS IN Xanthomonas citri subsp citri PERIPLASM FOR HETEROLOGOUS EXPRESSION OF GENE PRODUCTS INVOLVED WITH PHYTOPATHOGENICITY

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Citrus canker is an economically important disease that affects all citrus growing areas. There is no effective control or cure for such disease. The citrus canker causative agents are bacteria from the genus Xanthomonas. The most virulent strain is X. *citri* subsp. *citri* (XAC) which infects all types of citrus. Differential proteomic analysis of periplasmatic proteins from XAC was performed by comparison of protein profiles after growth in pathogenicity- inducing medium (XAM1) or non-inducing medium (Nutrient Broth) analyzed by two dimensional electrophoresis (2D-PAGE). Several spots showed significant differences on expression levels between the two conditions, which were digested with trypsin and analyzed by ESI-QUAD-TOF mass spectrometry (LNBio, CNPEM-ABTLus, Campinas-SP, Brazil). Data mining was performed based on annotations from XAC genome databases. Among XAC proteins identified as higher expressed in XAM1, two were classified as “Proteins involved with Pathogenicity, Virulence, and Adaptation” (Genome annotation), namely superoxide dismutase (SOD, code XAC2386) and phosphoglucomutase (PGM, XAC3579). Such genes and proteins could be interesting targets for inhibition studies, as molecular markers in the development of canker control tools and for future functional studies, including gene knockouts. Furthermore, functional and structural studies on the proteins could provide additional knowledge about citrus canker infective process. For cloning purposes, oligonucleotides were designed to amplify both structural genes from XAC genomic DNA and engender recombinant expression systems. Molecular cloning and protein production were successfully accomplished in E. coli. Both plasmidial clones were confirmed by complete DNA sequencing and provide soluble homogeneous purified proteins.
Native SOD expression profiles were analyzed in pathogenicity- inducing medium or non-inducing medium by Western blot, confirming the SOD differential expression in XAC. Recombinant PGM is being used aiming production of antibodies.
Work is in progress and initial structural studies on both SOD and PGM are being conducted. Other differently expressed genes in Xanthomonas shall be cloned and their product studied. Defining protein targets in comparative proteomics and using recombinant expression for the study of such targets provided a valuable approach on understanding and hopefully helping on controlling the citrus canker.

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CHROMOSOME 16 CONSORTIUM: CURRENT DEVELOPMENTS ON THE CHARACTERIZATION OF THE PROTEINS ENCODED BY THE CHROMOSOME 16"

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The Chromosome 16 Consortium is integrated in the global initiative *Human Proteome Project* that aims to develop an entire map of the proteins encoded following a chromosome-centric strategy (C-HPP) to make progress in the understanding of human biology in health and disease (BD-CHPP). After several preliminary meetings, the kick-off workshop was held in Madrid on April 2012. The structure of the consortium involves 17 groups organized in four working sections, namely SRM and protein sequencing, antibody and peptide standard, clinical healthcare and biobanking and bioinformatics. During this meeting the general objectives, working plan and timeline of the project were defined.
A description of chromosome 16 based on knowledge repositories was presented; the chromosome contains 870 protein coding genes including 599 "known" and 271 "missing" gene products, participating in 110 OMIM diseases. Lymphocytes B, epithelial cells and fibroblasts were selected for further studies as transcriptomic evidences suggest that most chromosome 16 protein coding genes are expressed in these cell lines. For analytical purposes, the 870 proteins have been clustered in two groups according to the robustness of the evidences supporting their MS observation, using a cut-off log(e) value of -15 (562 proteins with log(e)<-15 and 300 with log(e)>-15). It is expected to set SRM assays for the top rank third of chromosome 16 proteins in both protein groups.
The process will be developed on a multicentric configuration, assuming the standards and integration procedures already available in ProteoRed-ISCIII, which are encompassed with HUPO initiatives. A biobanking initiative has been launched in collaboration with the Spanish National Biobanking Network to optimize methods for sample collection, management and storage under normalized conditions, and to define QC standards.

CHROMOSOME 16. TRANSCRIPTOMIC AND SG PROTEOMIC PROFILING OF LYMPHOCYTE T JURKAT CELLS, MCF-7 EPITHELIAL CELLS AND CCD18 FIBROBLASTS

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To uncover the chromosome 16 associated proteome and to take advantage of the generated knowledge to make progress in human biology in health and disease, a consortium of 15 groups was organized in four working groups: SRM and protein sequencing, antibody and peptide standard, clinical healthcare and biobanking and bioinformatics. According to a preliminary in silico study integrating knowledge from Ensembl, UniProtKB and GPMDB, Jurkat T lymphocyte cells, MCF-7 epitelial cells and CCD18 fibroblast are theoretically expected to express more than 70% chromosome 16 protein coding genes. To define in detail the molecular profile of the abovementioned cell lines, Affymetrix microarray based analyses and SG proteomics profiling were performed. a total of 19340 genes were expressed in at least one of the three cell lines, 82% were common, 1% were specifically detected in MCF7 cells, 2.6% in CCD18 and 0.9% in Jurkat cells. Up to 1533 genes from Chr16 were not detected, most of these being non-protein coding genes. However, 84.6 % (736) of the Chr16 protein coding genes were detected among a total number of 18465 protein coding genes homogenously distributed across chromosomes with roughly 75% coverage in all cases with the only exception of chromosome Y. Assuming a FDR below 1% at the protein level, 9488 proteins were identified, 6092, 5547 and 7197 in MCF7, CCD18 and Jurkat cells respectively, 40% commonly found in the three cell lines. The distribution of identified proteins across chromosomes is very dissimilar with chromosome coverage that was about 30%, in clear contrast with the transcriptomic results that showed coverage. Among the identified proteins, 388 are encoded by Chr16 genes, 155 were common to the three cell lines while 44 were only found in MCF7, 24 in CCD18 and 71 in Jurkat cells. Noteworthy, 9 of the identified proteins are among the group of missing proteins. The identified proteins represent a 44.6% coverage of Chr16 protein coding genes and correspond in all cell lines to proteins encoded by high expression genes with some exceptions, as might be expected.

PROFILING THE CHROMOSOME 16 BY HIGH-RESOLUTION DATA-DEPENDENT MASS SPECTROMETRY

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The Spanish Chromosome 16 Consortium forms part of the global initiative Chromosome-based Human Proteome Project (C-HPP), which aims to map the gene-coded proteins on each human chromosome to complement our understanding of the human biology in health and disease (B/D-HPP). The project is based on a multicenter configuration, adopting the general rules established for the HPP.

To characterize the proteome of chromosome 16 (Chr16), we are defining a comprehensive proteomic map based on high-resolution data dependent mass spectrometry. This study will permit to assist the development of MRM methods for quantitative targeted analyses.

An initial in *silico* study showed that lymphoid tissues expressed at least 75% of the total (n=870) protein coding genes of Chr16. We are dissecting the human T lymphoblast cell line (Jurkat T cells) using in a first instance a MUDPIT type approach, with in-solution digestion and off-line pre-fractionation by basic-RP-HPLC. Then, to gain better insight into integral membrane proteins, our analysis is combined with strong detergent protein extraction and 1D-gel-digestion. In all our workflows, data is acquired using a 5600 TripleTOF LC-MS/MS system (AB SCIEX) on long HPLC runs. Data generated from these experiments is analyzed using our in-house ProteoRed MIAPE-Extractor tool, http://www.proteored.org/miape, which not only allows data calculations, data set comparisons and general analyses in an efficient way; but also extracts specific MIAPE information relative to Chr16.

The different proteomic approaches conducted and their biological replicates have led to the identification of more than 12,000 proteins in Jurkat T cells, assuming a FDR below 1% at protein level. Among the identified proteins, 447 are encoded by Chr16 genes, reaching near 50% coverage of Chr16 protein coding genes. Optimization of sample preparation to better extract membrane proteins, fractionation procedures to enrich low abundance proteins and definition of additional cell lines and environmental conditions are key aspects to take into account to extend our Chr16 proteome coverage.

Finally, we show that these ongoing shotgun experiments are providing important hints to establish and optimize S/MRM methods for all proteins coded by Chr16 genes.

UNRAVELING SEROLOGIC RESPONSE TO THE *CANDIDA ALBICANS* CELL SURFACE-ASSOCIATED IMMUNOME DURING YEAST-TO-HYPHA TRANSITION IN INVASIVE CANDIDIASIS PATIENTS

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Invasive candidiasis (IC) is a leading infectious cause of disease and death in cancer, post-surgical and intensive care patients because its early diagnosis is extremely difficult, resulting in delayed therapy and ensuing fatal outcomes. Better diagnostic biomarkers for invasive candidiasis (IC) are therefore needed to tailor and individualize therapeutic decision making and minimize its high morbidity and mortality. We investigated whether profiling of IgG antibody response to the Candida albicans cell surface-associated immunome during yeast-to-hypha transition (an important virulence factor) in IC might uncover molecular fingerprints of invasive infection and *C. albicans* morphogenesis. To address this goal, we performed 2-DE followed by Western blotting with human sera, mass spectrometry and bioinformatic analyses to examine serum IgG antibody-reactivity patterns to *C. albicans* yeast and hyphal cell surface-associated proteins (Y-CSPs and H-CSPs, respectively) in IC and non-IC patients as well as capture ELISAs to validate immunoproteomics-based results. A total of 28 immunogenic CSPs were differentially detected in yeast and hyphal forms by serum IgG antibodies from IC patients. Unsupervised clustering methods revealed two IgG antibody-reactivity signatures that segregated IC specimens hybridized with Y-CSPs and H-CSPs into two distinct groups. Pairwise correlation mapping across serum samples further highlighted that distinct biologic changes underlay commensal-to-pathogen and yeast-to-hypha transitions. These 28-IgG antibody-reactivity profiles could also accurately discriminate IC from non-IC patients. Supervised classification analyses with leave-one-out cross-validation identified a 3-IgG antibody-reactivity signature as the best diagnostic predictor of IC. By using capture ELISAs, we validated its discriminatory power in an independent group of IC and non-IC patients. Our findings provide a molecular signature of IC that may early and accurately discriminate IC from non-IC patients. These further offer new insights into pathogenic processes triggered during dimorphic transition and IC pathogenesis.

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MOLECULAR PROFILING OF SEROLOGIC RESPONSE TO CANDIDA ALBICANS SOLUBLE PROTEINS IN NON-NEUTROPENIC PATIENTS WITH INVASIVE CANDIDIASIS

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Early diagnosis of invasive candidiasis (IC) in non-neutropenic patients is crucial for a successful clinical outcome in such patients. However, it still remains a challenging task for physicians. In order to identify and validate useful serological biomarkers for early IC detection in this group of patients, serological proteome analysis and computational biology tools were applied to profile serum IgG-antibody reactivity to *Candida albicans* soluble proteins in non-neutropenic patients with and without IC. A total of 18 immunogenic *C. albicans* soluble proteins were differentially recognized by serum IgG antibodies from non-neutropenic IC patients as compared to controls. Unsupervised two-way hierarchical clustering and principal-component analyses of these 18-IgG antibody-reactivity patterns discriminated IC patients from controls reliably and independently of baseline characteristics of the study population. Selected biomarker candidates were then expressed in a heterologous system and validated using established prototype immunoassays in an independent non-neutropenic patient cohort. Receiver-operating-characteristic curve analyses highlighted that this panel of selected biomarkers showed a good ability to discriminate IC from non-IC patients. Multivariate logistic-regression models further revealed a positive association between the IC risk and this panel that was unbiased by traditional clinical IC-risk factors and other patients-related variables. We conclude that if confirmed in prospective cohort studies, this subset of clinical biomarkers may be valuable for early and accurate diagnosis of IC in non-neutropenic patients. In addition, this panel may also be useful in complement blood cultures for early IC detection in these high-risk patients because these selected serological biomarkers were identified in the absence of candidemia. Our findings further uncover new biologic perspectives into antibody response to IC.

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PROTEIN PROFILES DERIVED FROM DIET AND EXERCISE: CAN WE TAKE THEM AS MOLECULAR SIGNATURES OF VASCULAR WELLNESS?

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Diet and exercise constitute two of the main pillars in preventing and treating cardiovascular diseases. Despite of this assumption, the molecular mechanisms ultimately responsible behind the assigned beneficial effects remain unclear. The aim of the present study was to detect protein changes reflecting an improved physical condition in response to diet/exercise and to identify those proteins that could participate in maintaining vascular wellness.

In the present study, 44 rugby players (all healthy, with not cardiovascular risk) enrolled in a 6 month diet intervention program, following either a hyperproteic diet with low glucemic index (PD), or a standard Mediterranean diet (MD) in parallel to their training season. Blood and urine samples were collected on day 0 and 6 months later. Plasma was obtained by centrifugation and both, plasma and urine were aliquoted and stored at -80C. Anthropometrical data and exercise intensity were also recorded. Only 26 rugby players completed the study: 10 following PM, 12 following the MD and 4 players that didn´t follow any specific diet.

Protein profiles in both plasma and urine were analysed using a Surface-Enhanced Laser Desorption and Ionization Time of Flight (SELDI-TOF) mass spectrometer. Strong anion exchange (Q10) affinity array was selected for the study, allowing the detection of a high number of protein peaks. For protein identification, a complementary approach using same anion exchange chromatography (Q1) was employed, and fractions were separated by SDS-PAGE gels for protein identification by mass spectrometry.

Anthropometrical data didn´t show significant differences between diets or exercise intensity. Nevertheless, independently of diet, a low body mass gain was seen, related with increase of muscle and body fat decreasing, with a general better outcome for those following the MD.

SELDI data confirmed the lack of statistical significant differences between diets or due to exercise intensity. However, individuals were clearly clustered when comparing protein profiles regarding body changes, distinguishing 2 groups: players gaining or losing weight, with those maintaining weight (<1.5 kg difference) distributed in both groups. Protein peaks with p-values <0.05 showed six main behaviors regarding weight changes. Interestingly, individuals maintaining weight behaved in a particular manner versus gain/loss of weight. A tentative identification of such proteins have been made using TagIdent tool with some of the proteins being involved in glucose/fat metabolism, appetite regulation or hearth functioning. Further confirmation of the identity by using liquid chromatography and MALDI-TOF/TOF is in progress.

HLA-DR-ASSOCIATED HUMAN THYMUS PEPTIDOME IS MAINLY COMPOSED OF HIGH-AFFINITY PEPTIDES
RUNNING TITLE: HLA-DR PEPTIDE REPERTOIRE IN HUMAN THYMUS

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Major histocompatibility complex class II (MHC-II) molecules associate to and display antigenic peptides on the surface of antigen-presenting cells (APC). In thymus, self-peptides associate to MHC-II molecules expressed by defined populations of APCs, specialized in different steps of T cell selection. Cortical epithelial cells show a distinct processing machinery that may allow the generation of unique peptides for positive selection. Medullary epithelial cells transcribe genes that are restricted to peripheral tissue, presumably generating proteins and making their peptides available for negative selection. Dendritic cells can present material from the thymus environment and from the periphery. Very few data are however available on the peptides being presented by MHC molecules in the thymus. Here we report the application of mass spectrometry to analyze and identify MHC-II-associated peptides in five fresh human thymus samples. The data show a diverse self peptide repertoire, mostly constituted by predicted MHC-II high binders.

These data constitute a first direct assessment to HLA-II bound peptidoma and provides an insight into how it is generated and how it drives T cell repertoire formation.

COMPARATIVE PROTEOMIC ANALYSIS OF SECRETED PROTEINS AND EXTRACELLULAR VESICLES FROM CANDIDA ALBICANS ECM33Δ MUTANT AND SC5314 WILD TYPE STRAIN

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Candida albicans is a dimorphic fungus member of the human microbiota that causes a range of opportunistic infections from superficial to systemic. *C. albicans* Ecm33p is a glycosylphosphatidylinositol-linked cell wall protein that is important for cell wall integrity and critical for normal virulence in the mouse model of hematogenously disseminated candidiasis.[1,2] The ecm33Δ mutant (RML2U) presents an altered cell wall and aberrant morphology. Furthermore, it was shown that vaccination of BALB/c mice with this mutant protected them from a subsequent lethal infection with the virulent strain SC5314 in a systemic candidiasis model. As the cell wall proteins of the mutant strain are involved in host immune response a comparative study between mutant and parental strain surfomes was carried out revealing a completely different protein profile: ecm33Δ presented a larger number of proteins that the wild type what could elicit the high protective ability shown[3]. Interestingly, proteins involved in cell wall organization and biogenesis are the only ones that are less abundant in the mutant. This might be due to the relevance of Ecm33p function for the anchoring of covalently cell wall proteins. To validate this hypothesis we are currently doing a comparative analysis of *C. albicans* wild type and mutant secretome. Cells grown in minimal medium to exponential phase were harvested by centrifugation and the resulting supernatants were concentrated and analyzed by LC-mass spectrometric.
On the other hand, in recent years extracellular vesicles have been describe as a general mechanism in the molecular traffic across the cell wall to the extracellular space in fungi, being important contributors in the pathogenic process[4]. Transmission electron microscopy (TEM) of *C. albicans* cultures revealed the presence of intact vesicles that could be important in the transport of proteins related with virulence and host immune response. In this work, we also purified extracellular vesicles from ecm33Δ mutant and wild type cultures by ultracentrifugation of supernatants. The subproteome of these vesicles is analyzed by MS after their purification.

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COMPREHENSIVE CHARACTERIZATION OF THE PEPTIDOME AND PHOSPHOPEPTIDOME DISPLAYED BY THE HUMAN LEUKOCYTE ANTIGEN (HLA)-B*40:02

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HLA class I molecules bind peptides derived from proteolytic degradation of endogenous proteins and present them to cytotoxic T lymphocytes, allowing the immune system to detect transformed or virally infected cells. The peptide pool presented by a particular HLA allotype consists on up to 10000 different peptides which share specific structural motives that determine its binding to the class I molecule. Posttranslational modifications are known to be present in some class I ligands. In this regard, phosphorylated peptides have raised much interest as potential targets for cancer immunotherapy since aberrant protein phosphorylation is a hallmark of tumor cells.

Using HLA-B*40:02 as a model molecule and combining affinity purification and high resolution mass spectrometry we have identified more than 2000 different ligands (FDR < 5%). Sequence analysis of this set of ligands revealed two strong anchor motives: Acidic residues at peptide position 2 and methionine, phenylalanine or aliphatic residues at the peptide C term. After IMAC and TiO2 enrichment, about 100 phosphorylated ligands were identified. To avoid false identifications every peptide sequence of this subset was further confirmed by comparing its experimental MS/MS spectrum with that obtained upon fragmentation of the corresponding synthetic phosphopeptide.

We suggest that high throughput identification of HLA class I bound ligands combined with phosphopeptide enrichment may be a useful approach to identify targets for T cell based immunotherapy.

SELF-ASSEMBLED PROTEIN ARRAYS FROM AN ORNITHODOROS MOUBATA SALIVARY GLAND EXPRESSION LIBRARY AND ITS APPLICATION TO STUDY THE TICK-HOST INTERPLAY

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Protein interactions play a critical role in the regulation of many biological events and their study in a high-throughput format has become a key area of proteomic research. Nucleid Acid Programmable Protein Arrays (NAPPA) technology allows the construction of protein arrays from cDNA expression libraries in high-throughput cell-free systems to study protein interaction and functions.
Tick saliva contains anti-haemostatic, anti-inflammatory, and immunosuppressive proteins that counteract the host haemostatic, immune, and inflammatory responses allowing the ingestion of host blood and facilitating host infection by the tick-borne pathogens. Identification of such proteins and their functions could help in the selection of antigenic targets for the development of anti-tick and transmission-blocking vaccines. With that aim, we have prepared a cDNA expression library from the salivary glands of *Ornithodoros moubata*, which is a vector of Human relapsing fever and African swine fever, and subsequently produced a self-assembled protein microarray using 480 randomly selected clones from that library. The reproducibility of the array, its representativeness of the tick salivary protein repertoire, and the functionality of the *in situ* expressed proteins have been checked, demonstrating that it is a suitable tool for the identification and functional characterization of soft tick salivary molecules that interact with host proteins.
The array was screened for P-selectin ligands and several clones in the array were shown to bind to human recombinant P-selectin. One of them was a likely secreted tick phospholipase A2, which may represent a potential new ligand for P-selectin. As these salivary molecules are likely involved in blood meal acquisition through the modulation of the host immune and haemostatic responses, this new high-throughput tool could open new avenues for development of new therapeutic agents and control strategies against ticks and tick-borne pathogens.

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HIGH RESOLUTION HYDROGEN/DEUTERIUM EXCHANGE MASS SPECTROMETRY MAPS CONTACT SURFACES OF VEGF AND A NOVEL RECOMBINANT MONOCLONAL ANTIBODY FRAGMENT

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Vascular endothelial growth factor (VEGF) is an endothelial cell-specific angiogenic and vasculogenic mitogen. VEGF also plays a role in pathogenic vascularization which is associated with various clinical disorders, including cancer and rheumatoid arthritis.
A novel recombinant monoclonal antibody has been designed as a VEGF antagonist, preventing its interaction with its receptors and therefore acting as a potential drug for cancer treatment. The antibody fragment recognizes the VEGF dimer exclusively but its binding site to VEGF is not yet known. Mapping the antigen epitope is a key step in defining antibody specificity, predicting cross-reactivity, in assay development, rational vaccine design, and in understanding the fundamental aspects of protein-protein interactions.
Amide hydrogen/deuterium exchange (HDX) coupled with proteolysis and high resolution MS has become a powerful method to study protein dynamics, protein-ligand interactions, and protein-protein interactions. In general, when performing antigen-antibody experiments by HDX-MS, regions of the antigen with different deuterium content in the presence or absence of the antibody are defined as the epitope.
In this study, we have designed two complementary labeling strategies to get insight into the VEGF interaction sites with the antibody fragment. Strategy 1 performs the deuterium labeling of VEGF and Mab separately, before inducing their interaction. The complex is then incubated in aqueous buffer, therefore interchanging all deuterium except those in the binding regions and in other protected regions due to the tridimensional structure of the complex. Strategy 2 induces the complex first and than performs deuteration. In this case, non labeled regions would indicate VEGF-Mab sites of interaction and other protected regions, including the surface contact regions between monomers. Deuterated and non-deuterated control experiments of VEGF were also performed in the absence of the antibody. Here we performed a semi simultaneous reduction and digestion after H/D exchange and under quench conditions (0 ºC, pH 2.5). On-line desalting was then carried with an in-house double valve system, servicing a trapping and analytical column for sample resolution. All system was maintained at 0 ºC and column eluent was coupled to an LTQ-FT Ultra mass spectrometer. Data processing was done with HDX Sierra Analytics software. Percentage of deuteration for each region of the protein was calculated by comparing the results of the HDX experiments and putative regions of interaction VEGF-Mab were determined.
It is the first time that VEGF has been studied by HDX-MS and that this methodology has been applied to determine the epitope of VEGF against a monoclonal antibody fragment.

COMPLEMENTARY ACTION OF CASPASES 3 AND 7 IN THE BREAKDOWN OF BOVINE AND PORCINE MYOFIBRILAR MUSCLE PROTEINS

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The conversion of muscle into meat is a complex process in which numerous biochemical reactions are involved. Among them, the hydrolysis of structural proteins by the action of endogenous muscle enzymes is in direct relation to the development of meat tenderness. Recent investigations have demonstrated the onset of apoptosis in post-mortem muscle cells and how this can influence the final quality of meat. Programmed cell death or apoptosis is mediated by the action of caspases, a particular group of enzymes that are in charge of cell dismantling.
In this work, we report the selective and complementary action of effectors caspases 3 and 7 on the hydrolysis of myofibrilar muscle proteins. Thus, bovine and porcine muscle protein extracts were incubated in the presence of recombinant purified caspases 3 or 7 for a period of 48 h. Changes in the protein profiles after incubation were monitored by SDS-PAGE. Identification of both intact proteins and peptide fragments generated by the proteolytic action of caspases was carried out by in-gel trypsin digestion of bands, followed by peptide analysis on a LC-ESI-Ion Trap MS equipment and further identification using the mascot search engine.
Between the main observed actions of caspases, it is worth highlighting the selective action on myosin heavy chain, which was effectively degraded by caspase 7 but not by caspase 3. However, the main hallmark of caspase-mediated proteolysis was the generation of a polypeptide of around 30 kDa, generated from both bovine and porcine muscle extracts in the presence of either caspase 3 or 7. Proteomic analysis revealed that this characteristic fragment was the product of actin degradation in all cases. On the other hand, a protein band of around 22 kDa was observed to be highly hydrolysed by caspase 3 in both bovine and porcine muscle extracts, which was not the case for caspase 7.
The present work represents an important advance in exploring the potential consequences that apoptosis can have in post-mortem muscle in relation to caspases proteolytic action, selective degradation of the muscle protein structure, development of meat tenderness and final meat quality.

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A CANDIDA ALBICANS PEPTIDEATLAS

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Candida albicans is an organism of great clinical importance since, despite asymptomatically colonizing mucoses of 30 to 50% of the population [1], it may cause opportunistic infections in specific cases such as weakened host immune defenses, a common circumstance in cancer and AIDS patients as well as in post-chirurgical and IC units stays. However, this clinical relevance is not reflected in the number of large-scale publicly available proteomics resources which is currently still very limited. We announce here the creation of a *C. albicans* PeptideAtlas [2] comprising 21938 distinct peptides at a 0.24 % false discovery rate (FDR) that account for 2562 canonical proteins at a 1.2% FDR distributed across 16 experiments, attaining a coverage of 41.17% of the 6223 *C. albicans* open reading frame sequences in the database used for the searches. This *C. albicans* PeptideAtlas will provide a number of useful features, like comprehensive protein and peptide-centered data exploration tools, and it will stand as a valuable resource to assist SRM experiments aiding in the selection of candidate proteotypic peptides.

To construct the *C. albicans* PeptideAtlas, a target fasta database was first built using sequences from the Candida Genome Database and appending them with cRAP contaminants and corresponding reversed decoy sequences. MS raw data were processed with the Trans-Proteomic Pipeline (TPP)[3], a suite of software tools for processing shotgun proteomics datasets. Briefly, X! Tandem with the k-score algorithm identifies the highest scoring peptide for each spectrum, PeptideProphet calculates probabilities of correct identification for each spectrum, iProphet combines evidence from multiple identifications of the same peptide across spectra, experiments and charge and modification states, ProteinProphet performs statistical validation at protein level. Finally MAYU estimates FDR at the different levels.

The PeptideAtlas build also provides the cumulative number of peptide and proteins across the experiments, information on protein to genome location mappings and the peptides empirical and predicted proteotypic scores ESS and PSS.

Additionally, and due to the particularly rich number of identifications in experiments aimed at detection of phosphorylated proteins, we have created a similarly processed version of the *C. albicans* PeptideAtlas including in this case PTMprophet results that provide, alongside each modified residue, the probability that the post-translational modification is truly detected at that site.

The current version of the *C. albicans* PeptideAtlas will be available at:
https://db.systemsbiology.net/sbeams/cgi/PeptideAtlas/buildDetails?atlas_build_id=323
and the *C. albicans* PeptideAtlas version including PTM results at:
https://db.systemsbiology.net/sbeams/cgi/PeptideAtlas/buildDetails?atlas_build_id=324

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DEVELOPMENT OF A SEMANTIC WIKI-BASED LIMS FOR PROTEOMICS LABORATORIES THAT SUPPORT EXPERIMENT AND SAMPLE TRACKING

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In the last years, the unprecedented throughput, the experimental complexity, and the rapid changes in the proteomics field led to an exponential increase on experimental data. It is crucial for researchers to store and track all these data, their derived processed files, and their associated experimental metadata, and to retrieve them anytime. For these reasons, a dedicated laboratory information management system (LIMS) that reflects the particularities of the proteomics experiments is highly required to facilitate the aforementioned data handling, and to accommodate the quality assurance requirements demanded for data publication.

ProteoWiki is a Semantic MediaWiki implementation to streamline generation, management, and analysis of proteomics data. In this system, users can enter information about their samples and the analyses to be performed by using a semantic-enabled form built on top of a wiki page. After submitting an on-line request, a workflow is created, and different experimental tasks are assigned to the lab operators. Users and operators, according to their profile and granted permissions, can track the state of the requests and the associated experiments at any time. The final output is the generation of a report that summarizes all the steps and parameters in analyzing proteomics samples, while keeping track of the original files.

User management, sample tracking, task assignment and lab scheduling, are all automated processes in ProteoWiki, thus highly improving laboratory efficiency, increasing throughput and ensuring data traceability for the final user.

SMART HYDROGEL PARTICLES TO DEPLETE PLASMA SAMPLES

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Plasma proteomics represents a challenge due to the extreme dynamic range of plasma, with high abundant proteins such as albumin accounting for more than 50 % of total protein content. Typically, depletion or fractionation steps are required prior LC-MS/MS analysis to have access to the low abundant sub-proteome.
N-isopropylacrylamide-based hydrogel particles have raised a high interest based on their dynamic characteristics, with a size and structure sensitive to external stimulus such as temperature or changes of pH. Mainly focused in drug release applications, in proteomics these have been employed as a cut-off filter with a pore size small enough to trap only the low MW proteins (Luchini et al, 2008).
Here, we report the use of pH-sensitive N-isopropylacrylamide hydrogel particles copolymerised with acid acrylic to effectively deplete albumin from human plasma samples. Once particles are incubated with the sample, most of proteins are effectively harvested within minutes into the acrylamide mesh. However, at concentrations higher than a fixed threshold, albumin gets irreversibly trapped inside particles. Within the elution step, the rest of the proteins are released from particles to be further analysed. Here, standard proteomic bottom-up analysis of the depleted mixtures has demonstrated the utility of this approach.

DIFFERENTIAL ANALYSIS OF THE PROTEOME OF Saccharomyces bayanus var. uvarum INDUCED BY TEMPERATURE, AND ITS INFLUENCE IN WINE QUALITY

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Nowadays, due to the increasing competitiveness in the international wine market, it is necessary a process of continuous improvement in the winemaking facilities and processes. These advances include, between others, the introduction of new varieties of white and red grapes to improve the fermentation process. However, to gain specificity and distinction in wines is essential to develop a broad knowledge of microbiological fermentation. Moreover, it is also necessary the development of criteria to select the most appropriate fermentation yeast and to study its fermentative behavior during the process.

Our group has been developing several studies to analyze the diversity of yeasts involved in the wine making process that are of responsible of wine taste and complexity. During one of these projects, we isolated *Saccharomyces bayanus var. uvarum* from autochthonous microbiome of red wines in "Ribera del Duero" (Spain) winery during several vintage samplings. This strain has shown a great oenological interest, due to posses the ability to grown at low temperatures fermenting the must. It provides to the obtained wine of high-valuable sets of aromatic compounds, increasing its organoleptic qualities.

To detect the proteins involved in this process, we prepared two different wine fermentations of synthetic must, at two different temperatures, 13 °C and 25 °C. After protein extraction, we develop two different analyses, qualitative and quantitative, both in collaboration with "Cambridge Centre for Proteomics" (University of Cambridge). For the study of the qualitative analysis was used Coomassie staining and MALDI TOF/TOF mass spectrometry, with the identification of 71 differential spots. The quantitative study was conducted using 2DE-DIGE, being 51 the number of identified spot (up or down-regulated). The relevance of this identification in the winemaking process and wine quality is now under molecular analysis.

The main aim of the present project is to detect those differential proteins at two different temperatures, 13 °C and 25 °C and study its influence in winemaking process. By determining these proteins we will develop new strategies to improve the winemaking process, i.e. as markers for select yeast with better qualities, or those proteins involved in the wine bouquet. As far as we know, there is not previous report about proteomic approaches of *S. bayanus var. uvarum*, that seems to direct the initials steps of must fermentation where the aromatic profile is forming.

EFFECT OF MATRIX DEPOSITION TECHNIQUES IN THE ACHIEVED LATERAL RESOLUTION IN MALDI IMAGING MASS SPECTROMETRY (MALDI-IMS)

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MALDI-MS imaging has recently emerged as a powerful technique for analyzing the spatial distribution of peptides and small proteins within biological tissues. Obtained signals can be correlated with underlying tissue architecture without any geometrical distortion, enabling the so-called Molecular Histology. This technique offers several advantages over other imaging methods like autoradiography, such as the high specificity of MS detection, minimal sample preparation, and applicability to a wide variety of analytes. One drawback, on the other hand, is that the matrix spray stage is a critical step determining the final quality of the obtained images. Recently, many advances in the practice of imaging mass spectrometry have taken place, like automated matrix sprayers, making the technique more sensitive and robust. In this work we have compared images obtained from different mussel gastrointestinal tissue preparations sprayed in manual (standard TLC sprayer) and automated form (Bruker´s ImagePrep automated sprayer) using standard sinapinic acid (SA) matrix. Although the overall quality of obtained spectra with both techniques is similar, preparations in manual matrix spray, achieve only lateral resolutions of up to 100 µm, whereas the automated spray procedure can achieve resolutions of up to 50 µm, near the current technical restrictions in resolution of our MALDI spectrometer, at around 30 µm.

Last Minute Abstracts

Poster P59

PLASMA BIOMARKERS FOR PATIENTS WITH HEPATOCELLULAR CARCINOMA IDENTIFIED BY SPECTRAL COUNT

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Biomarker discovery in human disease is a highlight to improve early diagnosis and predict response to treatment and prognosis. Mass spectrometry is a high throughput proteomic methodology that has been proved as a useful tool for biomarker identification. Hepatocellular carcinoma (HCC) is the main cause of death in cirrhotic patients. In spite of surveillance programs in cirrhotic patients, only about 40% of the HCC are diagnosed at early tumor stages when cure is still feasible. Therefore, the identification of biomarkers in cirrhotic patients is needed to use them as complementary tools for surveillance.

Plasma samples were depleted of abundant interfering proteins (ProteoPrep-20 kit, Sigma-Aldrich), fractionated by SDS-PAGE electrophoresis and enzymatically digested. The resulting peptides were analyzed by liquid chromatography coupled with mass spectrometry using SEQUEST as search engine and Trans-Proteomic Pipeline (v4.6.1) to generate probabilities for protein identifications. Quantitative analysis was done using Spectral Count algorithms.

RESULTS: Here we have analyzed the variability of 17 selected cirrhotic patients, 10 of them with HCC, to adequate this methodology to identify possible biomarkers and the ability to be coupled to high throughput analysis by mass spectrometry. In relation to the protocol, we observed that the few number of reutilization of enrichment columns is a critical point to eliminate high abundant proteins properly. However, we found a good correlation (75-97%) of protein profiles among the different samples that have a similar number of total spectra (spectra media: 446337 +/- 71663). Moreover, sixteen of the 17 samples share more than 85% of the proteins identified. Using spectral count, putative diagnostic markers were identified by comparing the plasma proteome of cirrhotic patients with HCC versus those without HCC (p value <0.01, number of peptides >1, 80% protein probability). In conclusion, we have been able to identify putative diagnostic biomarkers of HCC by performing a high throughput proteomic analysis of plasma samples.

PROTEORED MULTICENTER EXPERIMENT FOR LONG-TERM QUALITY CONTROL EVALUATION OF PROTEOMICS CORE FACILITIES

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Quality control (QC) is becoming increasingly important in proteomic experiments in order to guarantee the quality of research results. Deployment of QC metrics helps in monitoring stability, overall performance and reproducibility of analytical techniques. In an attempt to dispel some of the notions that LC-MS-based proteomics is poorly reproducible, the proteomics community has demonstrated increasingly concerns about the quality of proteomics data made publicly available. Here we describe the ProteoRed Multicenter Experiment for Quality Control (PMEQC), a longitudinal QC multicenter study involving 12 institutions, to assess the repeatability of LC-MS/MS proteomics data within a specific site, the reproducibility across multiple sites and across multiple platforms. Our experimental design also provided a unique opportunity to assess the repeatability of protein sample preparation within a specific site.

The main study was divided into 2 sub studies (Study A and B) that evaluate separately inter- and intra-laboratory variability. Each participant received two sample vials of trypsin-digested yeast proteins (Study A) and the same undigested protein sample (Study B). All participants were requested to follow a strict LC-MS/MS guideline for sample injection amounts and LC gradient. To enable inter-laboratory comparisons, data analysis was centralized and performed under standard procedures using a common workflow that includes well-known software tools for proteomics analysis such as msconvert.exe, X!Tandem, PeptideProphet, OpenMS and R programming language.

Here, we summarize the key findings of the PMEQC project and provide technical insights to better understand and pinpoint the main sources of variability and other issues faced by proteomics core facilities. Our study reveals that the overall performance regarding reproducibility, sensitivity, dynamic range, among other metrics, is directly related to laboratory staff expertise, and less dependent on instrumentation. Furthermore, the present study provides a rich data set of metrics against which other laboratories can benchmark their performance.

Poster P61

RELATIVE QUANTITATION OF TMT-LABELED PROTEOMES – FOCUS ON QUANTITATIVE PRECISION AND ACCURACY

***S. Baumgarten*, R. Viner, M. Scigelova, M. Zeller, M. Oppermann, T. Moehring and V. Zabrouskov**

Introduction. Isobaric tagging methods involving 'differential' isotope labeling using chemical tags are very popular and universally applicable approaches measuring relative amounts of proteins across two or more different samples. The main focus of the presented work was on assessing the percentage of quantifiable peptides, the quantitative precision, and the quantitative accuracy for a TMT sixplex-labeled complex proteome sample.
Methods. A digest of 9 proteins in equimolar amounts, which was aliquoted into six identical fractions labeled each with one of the TMT sixplex reagents, and mixed to obtain the final ratio 10 : 1 : 10 : 2 : 10 : 1.5, was added to the background of a TMT sixplex-labeled E. coli lysate digest.
Peptides were analyzed using nano-LC coupled to the Orbitrap Elite. Two different acquisition methods were evaluated.
Proteome Discoverer 1.3 was used for protein identification and quantification.
Quantitative precision was expressed as protein ratio variability. Quantitative accuracy was presented as a deviation of a measured ratio value from the expected value.
Results. The percentage of peptides whose fragmentation spectra contained all six reporter ions (quantifiable peptides) exceeded 90% for any sample load tested.
The variability was lower than 10% for approximately 90% of the quantified proteins at a 500 ng sample load, but only for about 70% at a 20 ng sample load.
The Orbitrap detection at resolution settings 15,000 corresponds to effective resolution >27,000 for m/z 126-130. Consequently, a very tight mass tolerance (+/- 10 ppm) could be used effectively filtering most reporter ion interferences.
For higher ratios (theoretical ratio 10:1) the median peptide ratio increased from 5.64 (Top10 method) to 7.97 (MS3 method).
Conclusions. An improved mass spectrometer design resulted in a high percentage of quantifiable peptides.
The quantitative precision is signal intensity-dependent.
Very high resolution effectively eliminated isobaric interferences in the reporter ion region.
The impact of peptide precursor co-isolation issue on quantitative accuracy was minimized with MS3-based acquisition method.

IODOACETYL TANDEM MASS TAGS FOR CYSTEINE PEPTIDE MODIFICATION, ENRICHMENT AND QUANTITATION

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Introduction. Thermo Scientific Tandem Mass Tag® (TMT®) Reagents enable concurrent identification and multiplexed quantitation of proteins in different samples using tandem mass spectrometry. Here, we report the development of an irreversible, cysteine-reactive TMT reagent containing an iodoacetyl reactive group (IodoTMT™). Due to the irreversible labeling of the IodoTMT reagent, it can be used for quantifying cysteine modifications such as S-nitrosylation, oxidation and di-sulfide bridges.
Methods. Cell lysates and purified proteins were denatured in 6M Urea/50 mM Hepes buffer, pH 8.0 and reduced with TCEP at 50°C for 60 min for total cysteine alkylation experiments. Samples were then labeled with excess IodoTMT, mixed and desalted prior to enzymatic digestion. For cysteine S-nitrosylation quantitation, samples were treated with nitrosylation donor agents (e.g. GSH-NO) and alkylated with MMTS to block unmodified sulfhydryls. After desalting, S-nitrosyl groups were then selectively reduced using ascorbate to generate free sulfhydryls for Iodo TMT reagent labeling. IodoTMT-labeled peptides were enriched using an immobilized anti-TMT antibody resin and analyzed using a Thermo Scientific LTQ™ Orbitrap XL or a Thermo Scientific Orbitrap Elite mass spectrometer. Data analysis was performed with Thermo Scientific Proteome
Discoverer™ 1.3 software.
Results. We have developed and used an IodoTMT reagent to irreversibly label sulfhydryls of cysteine-containing peptides for multiplex quantitation by LC-MS. IodoTMT reagents showed efficient and specific labeling of peptide cysteine residues with reactivity similar to iodoacetamide. We used the IodoTMT reagent as a probe for labeling S-nitrosylated cysteines in a modified S-nitro switch assay. IodoTMT reagents successfully labeled S-nitrosylated cysteines after selective reduction using ascorbate. In addition, we discovered that addition of 1mM copper sulfate to the switch reaction buffer inhibited IodoTMT reagent labeling. Finally, we characterized an anti-TMT antibody developed against the reporter region of the TMT reagent for Western blot detection of IodoTMT-labeled proteins and immuno-enrichment of IodoTMT-labeled proteins and peptides. Overall, using the combination of IodoTMT labeling with anti-TMT enrichment has several advantages over previously described cysteine-reactive workflows including: 1) more specific labeling of sulfhydryl groups; 2) a choice of six-plex isobaric multiplexing or two-plex isotopic quantitation using the same chemistry; 3) isobaric tagging for more efficient MS data analysis; and 4) a simpler workflow using antibody-based capture for labeled peptide enrichment.

Poster P63

A HIGH RESOLUTION/ACCURATE MASS TARGETED APPROACH FOR KINASE INHIBITION SCREENING USING A QUADRUPOLE ORBITRAP MASS SPECTROMETER

Ryan D. Bomgarden[1], Scott Peterman[2], Rosa I. Viner [2], Chris L. Etienne[1], John C. Rogers[1], Sigrid Baumgarten[3]

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Introduction. Development of novel protein kinase inhibitors remains a strong focus of the pharmaceutical industry. Performing multiplexed protein kinase detection/quantification presents many challenges. Recently, probe-based assays were introduced to increase kinase enrichment as well as provide a selective screening approach for kinase inhibitor analysis using tandem mass spectrometry. Here, we present a high resolution/accurate mass targeted approach for kinase inhibitor screening in A549 cells within a high-throughput workflow.

Methods. A549 cell lysates were treated with staurosporine(0-10 uM). Each sample was reacted with desthiobiotin ATP or ADP probes and then processed according to the manufacturing instructions to enrich labeled kinase active-site peptides. All mass spec experiments were performed on QExactive (Thermo Scientific, Bremen). Initial kinase identification and quantification was performed in data dependent acquisition mode followed by database searching and spectral library creation. Targeted quantification/verification was performed by narrow mass range multiplex SIM. Orthogonal experiments were performed by Western Blots.

Results. Desthiobiotin-ATP and -ADP are two nucleotide derivatives that have been shown to selectively label kinase active sites. Using these probes to enrich kinase active-site peptides, we identified 126 kinases from A549 cell extracts using high resolution, accurate mass (HR/AM) spectrometry. Targeted SIM multiplexing enables shorter acquisition cycle times and is fully compatible with LC constraints of targeted proteomics experiments. We also assessed specificity of kinase inhibitors - staurosporine by determining IC50 values for kinase active-site peptides after drug treatment. The results for the staurosporine treated A549 cell line showed over 70% kinases demonstrated strong inhibition by the drug as expected. In addition, we validated kinase inhibitor targets using a parallel Western blot workflow.

Conclusion. Kinase inhibition measured using targeted HR/AM spectrometry had strong correlation with Western blot data, previously published results, and enabled global profiling of kinase inhibitor target and off-targets.

COST Workshop: Farm animal & food proteomics

PROTEOMICS A NOVEL TOOL FOR ANIMAL SCIENCE: ADVANTAGES AND DRAWBACK

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Proteomics being the science that studies the proteome i.e. protein expression in a given tissue, fluid or organism has obtained several successes in numerous fields of application that include, for instance biomedicine, vaccine development or the study of processing of foods. Animal science, animal production and the processing and transformation of foods of animal origin is a key economic activity in the European Union and in the rest of the world and essential for the livelihood of numerous citizens. Animal products are essentially proteinaceous products and therefore the study of the protein changes in animal products obtained as a consequence of management and production/processing practices is of enormous importance to this area of activity. Albeit its usefulness, quite a limited number of studies have been performed using proteomics based approaches. In this presentation we will examine the major areas of applications of proteomics in the context of animal science, presenting study cases of specific successful application cases. We will also present concise considerations on the major drawbacks that have hindered a wider use of proteomics in the field of animal sciences. We will finally make proposals on how such limitations may be overcome leading to a larger use of proteomics in animal science.

THE PROTEOMIC TOOLBOX: 2-DE GELS AND MORE

Ingrid Miller

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Gel-based proteomics usually relies on two-dimensional electrophoresis, classically consisting of a first-dimensional charge-based separation (isoelectric focusing) followed by SDS-PAGE according to size, all under denaturing and reducing conditions. Protein spots are detected, spot volumes quantified, and potentially interesting candidates identified by mass spectrometric methods. High-quality analyses today also comprise pathway analysis and verification of the findings, e.g. by immunoblotting or targeted proteomics (SRM/MRMs) [1].

However, we should not forget that the panel of electrophoretic methods is much broader, including also separations under native or non-reducing conditions or combination of other separation criteria. This may allow the study of protein complexes or interactions, as well as application of other, specific detection methods, for instance zymography [2].

Verification of 2-DE results is not just a nuisance asked for by reviewers, but - with high-quality antibodies - an important tool to learn more about the detected spots and the proteins behind, especially when applied as 2-DE blots: most proteins consist of multiple spots or spot chains, but 2-DE quantification only picks single differentially regulated spots in comparisons of two different groups of samples. Other spots of the same protein may be regulated in a similar or diverse manner, and knowledge about that leads to different conclusions. In addition to detection of specific isoforms, 2-DE blots also give information on protein size and intactness / degree of fragmentation.

Besides sample selection and study design, appropriate sample collection and preparation are key issues. Prefractionation (based on subcellular fractionation, chromatography, preparative electrophoresis, affinity methods) helps to reduce the complexity of specimens and to focus on selected subproteomes whose variation may otherwise have escaped our notice [3].

For a given project, ideally, a variety of methods should be used to investigate different aspects: e.g. histology, metabolomics, enzymatic or immunological assays, additional specific tests characterizing health status, or different methodological approaches, in order to get a (more) global picture or to investigate mechanisms (e.g. [4]).

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DISCOVERY BASED AND TARGETED MASS SPECTROMETRY IN FARM ANIMAL PROTEOMICS

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Technological advances in mass spectrometry have greatly improved accuracy and speed of analyses of proteins and biochemical pathways. These proteome technologies have transformed research and diagnostic methods in the biomedical fields, and in food and farm animal sciences proteomics can be used to investigate and monitor specific marker proteins and peptides within complex food matrices, as for example, for guaranteeing safety and quality of processed and stored foods like cheese and cured meat. Likewise, specific diagnostic markers associated with compromised welfare, or with early infections can be monitored to improve welfare in large industrial settings of current livestock industry. The combination of discovery based LC-MS/MS methods and the more hypothesis-based targeted mass spectrometry method commonly referred to as selected reaction monitoring or SRM, provide a powerful approach for investigating farm animal biology. SRM is particularly important for validation biomarker candidates.

This talk will introduce the use of different mass spectrometry approaches through examples related to food quality and animal welfare, including studies of gut health in pigs, host pathogen interactions in the bovine mammary gland, and the monitoring meat and milk quality markers. Also work related to collecting farm animal proteome information within the PeptideAtlas frame (www.peptideatlas.org) will be discussed. The PeptideAtlas database currently includes data from shotgun proteomics experiments from tissues and body fluids from pig, cow and horse, and currently provides the primary public resource for designing SRM methods for farm animal applications.

PROTEOMICS AND INFLAMMATION: AN OPEN DOOR IN THE STUDY OF FARM ANIMAL DISEASES

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Acute phase proteins (APP) have been identified in whey and sera from healthy and mastitic cows through the proteomic analysis using two-dimensional electrophoresis (2-DE) coupled with Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). Although normal and mastitis serum samples show relatively similar protein composition, marked differences in expression levels and patterns can be observed. Conversely, normal and mastitis whey showed a very different composition, likely due to extravasation of blood proteins to the mammary gland. Different isoforms from the most abundant protein in milk, casein, were detected in both normal and mastitis whey. Other proteins, such as lactotransferrin, were only detected in the inflamed animal samples. Immunoglobulins showed different patterns but not increased levels in the inflamed whey. Also, many cellular proteins present in mastitis cow's whey were absent from healthy cow's milk. They are responsible for the great change in composition between normal and mastitis whey, especially those which exert a biological function related to immune defense. Data collected in this work are of interest for gaining information about physiological changes in protein patterns in different fluids as a result of an acute phase process in farm.

UNRAVELING THE MILK PROTEOME: PERSPECTIVE IN HUMAN NUTRITION AND ANIMAL PRODUCTION

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Milk composition in mammalian species is higly specific and it is design to the needs of the newborn. It contains proteins, and numerous biologically active substrates such as lipids, vitamins and mineral salts. Through their major proteins components, milk fat globule, caseins, and serum proteins, milk is able to cover the nutritional needs of the newborn in all mammalian species and it protect the baby against infection, inflammation and oxidative stress. The newborn is particularly immature: innate immunity, adaptive immunity and intestinal immaturity are needed to be enforce and milk proteins rapresents exogenous protective help and a strong immunomodulating source. Moreover, the development of the composition of the intestinal microflora of the neonate is modulated ad selected by characteristic milk proteins. Several works highlighted interdependency of different milk components, as well as ontogeny of the intestinal function, development of the mucosal intestinal immune system, colonization by the intestinal microbiota and protection against pathogens. Milk proteins influence medium and long term heath status, for this reason is strongly mandatory to assure a quality and deep knowledge about proteomics of milk of both human and mammalians use for nutrition. Nowadays, it is clear that besides the immunoglobulins, milk also contains a wide range of minor immune-related proteins that represents the real first line of defence against pathogens. We have used proteomics science to characterise the repertoire of milk proteins in detail, in human, in formula, in ruminants, to better understanding complexity and different biological functions between species. Milk can represent in animal production a simple and accessible sample to monitor health status of animals. This study is a contribute to a better exploring of health properties of milk, as well as to build the basis to stress and to promote high-value ingredients from milk useful also for dairy products and nutrition industry.

Roncada P, Piras C, Soggiu A, Turk R, Urbani A, Bonizzi L. Farm animal milk proteomics. J Proteomics. 2012 75: 4259-74. doi: 10.1016/j.jprot.2012.05.028.

BIOMARKERS OF BEEF SENSORY QUALITIES: FROM PROTEOMICS TO PHENOTYPING TOOLS

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For beef producers, it is of interest for breeding or finishing purposes to predict the ability of live animals to produce good meat, with specific attention towards tenderness. Today tenderness can be estimated only after slaughter, by sensory analysis tests and/or mechanical measurements. Thus, the Beef sector is looking for biological or molecular indicators that would identify live animals with desirable quality attributes, in order to direct them towards the most appropriate production system. Tenderness is a complex trait with high and uncontrolled variability at the origin of a dissatisfaction of the beef consumers. For several years, various genomics programs have been conducted at the national and international level in order to reveal genomic markers of tenderness (Cassar-Malek et al., 2008). Comparative transcriptomic (Bernard et al., 2007) and proteomic analysis, conducted on bovine muscles with low or high tenderness scores estimated by sensory analysis and/or mechanical measurements, bring up a list of potential biological markers which may be used as phenotypic biomarkers to predict the "tenderness potential" of an animal or a carcass (for review see: Picard et al., 2010, 2012).
The strategy developed in our team for few years is: (1) to validate the relationship between meat tenderness and the protein biomarkers on a large number of cattle types as the most representative of the French production systems and beef consumption (male and female, beef and dairy breeds); (2) to determine the effects of management factors (age and diet) on the expression of these biomarkers; (3) to develop an antibody micro-array tool for molecular phenotyping of beef tenderness from the list of validated biomarkers. We have developed a prototype which is internationally the first phenotyping tool for meat tenderness. We are completing it with biomarkers of other sensory qualities.
This antibody micro-array will be available for the Beef industry for evaluation of meat quality and for optimization of cattle breeding for meat production. This tool could also be used for sensory quality phenotyping in genomic breeding schemes.

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List of Authors

Bad Time for Science
A S.L.

In my song a rhyme
Would seem to me almost like insolence.

Fighting within me are
Enthusiasm about the blooming apple tree
And the horror of the house painter's speeches.
But only the second thing
Drives me to my desk.

Bertolt Brecht
(Schlechte Zeit für Lyrik, "Malos tiempos para la lírica")

Now that I write
I cannot ignore the evidence,
I cannot deny
That we feel the moan of defeat,
That roads are closing and hopes shut down
And a thick despondency clouds us.

It is bitter to face that harsh reality,
That woodworm
That undermines decades of effort,
The building
We built with the cement of enthusiasm,
The stone of imagination,
And that devastates
Our fresh sap, the flower of our youth.

All around I find nothing
But autumn's traces,
A misty horizon, a leafless wind.
And the slow, insidious,
Damp of discouragement leaks in.
Where is the dawn, the air? The light flees,
Cracks, unravels. And the haunting
Shadows of doubt lurk.

I wonder
If there is any use
To this stubborn struggle
Against all the obstacles and all
The impossibilities,
These long detours that hardly advance us,
This endless movement toward a goal
That is never within sight,
This tenacity that steadies us,
Keeps us trustworthy
Guardians of an idea,
This captive and measureless time,
This crucible where we forge
In our image
The new face of nature,
This slow walk toward the origin,
Discovering a step in a cloud
And creating a past in the sand,
This learning from hidden evidence
And symmetry's silent edge,
This fight against errors that rise up,
Against a wave of chance, the liquid noise,
I wonder if it is any use,
If it has any worth.

And a breath rises,
A sudden impulse,
A resentment that smolders, a cry still
Suppressed in its birth, the seed of rage,
The desperation that struggles and sparks,
The lash of anger.

A certainty emerges,
Dark and torrential, in the marrow,
With a jolt,
With the crack of a branch. I'm sure now,
Convinced.
I affirm. I place myself. Everything
Becomes solid, takes shape, has a place, makes sense.
We have not come here to hunker down.
The mountain is there, but clear in contour.
And with it, the evidence,
The clear inertia of our own weight,
The passion of our hands. Yes, surely
And emphatically, I trust.
And so, strengthened and free,
I rejoin the effort
On the common front of fortitude,
Following
Our nearest
And decisive example: the river of life
Moving ever forward.

Jesús Vázquez
English version by Jess Jackson



Bad Time for Science
A S.L.

In meinem Lied ein Reim
Käme mir fast vor wie Übermut.

In mir streiten sich
Die Begeisterung über den blühenden Apfelbaum
Und das Entsetzen über die Reden des Anstreichers.
Aber nur das zweite
Drängt mich, zum Schreibtisch.

[En mi canción una rima
casi me resultaría una insolencia.

En mí luchan
el entusiasmo por el manzano en flor
y el espanto ante los discursos del pintor de brocha gorda.
Pero sólo lo segundo
me impulsa a escribir.]

Bertolt Brecht
(Schlechte Zeit für Lyrik, "Malos tiempos para la lírica")

Ahora que escribo
No puedo ignorar la evidencia,
No puedo negar
Que sentimos el clamor de una derrota,
Que se ciegan caminos y se truncan esperanzas
Y nos nubla un espeso desaliento.

Es amargo enfrentarse a esa cruda realidad,
A esa carcoma
Que desmorona un esfuerzo de décadas,
El edificio
Que construimos con el cemento del entusiasmo,
La piedra de la imaginación,
Y que también desola
Nuestra savia más joven, nuestro mejor retoño.

Alrededor no encuentro
Sino huellas de otoño,
Un horizonte en brumas, un viento deshojado.
Y se filtran, insidiosas,
Las lentas humedades del desánimo.
¿Dónde está la claridad, el aire? La luz huye,
Se agrieta, se deshila. Y amenazan
Las inquietantes sombras de la duda.

Me pregunto
Si sirve de algo esta obstinada lucha
Contra todas las barreras y todos
Los imposibles,
Este largo rodeo para avanzar apenas,
Este constante progresar hacia una meta
Que no se avista nunca,
Este tesón que nos mantiene
Enteros, fidedignos
Guardianes de una idea,
Este tiempo cautivo y sin medida,
Este crisol donde forjamos
A nuestra imagen
El nuevo rostro de la naturaleza,
Este lento caminar hacia el origen
Descubriendo un peldaño en una nube
Y creando un pasado entre la arena,
Este aprender de la evidencia oculta
Y el filo callado de la simetría,
Este luchar contra el error que aflora,
La ola de un azar, el ruido líquido,
Me pregunto si vale para algo,
Si vale
De algo.

Y surge un hálito,
Un repentino impulso,
Un resquemor que prende, un grito todavía
Callado en su nacer, el germen de la ira,
La desesperación que puja y que crepita,
El latigazo de la rabia.

Una certeza emerge,
Torrencial y oscura, entre la médula
Con una sacudida,
Con un crujir de rama. Estoy seguro
Ahora, convencido.
Me afirmo. Me sitúo. Todo adquiere
Opacidad, contorno, rol, cordura.
No hemos llegado aquí para agacharnos.
La montaña está ahí, mas despejada.
Y con ella, la evidencia,
La clara inercia de nuestro propio peso,
La pasión de nuestras manos. Sí, seguro
Y rotundo, ya confío.
Y así, fortalecido y libre,
Me sumo de nuevo a nuestro esfuerzo
En el frente común de la entereza,
Siguiendo
Nuestro más cercano
Y decisivo ejemplo: el río de la vida,
Avanzando siempre hacia adelante.

Jesús Vázquez

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