Italian Proteomics Association

4th ANNUAL NATIONAL CONFERENCE

June 22 – 25, 2009

UNIVERSITY MILANO-BICOCCA, ITALY
Aula Magna, Piazza dell'Ateneo Nuovo, 1
Milano

ABSTRACTS VOLUME

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PROGRAMME

MONDAY 22 JUNE

14.00 – 16.30	Workshop Thermofisher
15.00 - 17.00	Registration
17.00	Conference opening
17.30	Opening Lecture by 2004 Chemistry Nobel Laureate *Prof. Aaron Ciechanover.* Cellular protein degradation: From the bench through human disease and onto drug targeting
19.15	Welcome Cocktail

TUESDAY 23 JUNE

I SESSION: Clinical proteomics

9.00	Proteomics of heart failure *M. Dunn (Dublin, Ireland)*
9.45	Acute pancreatitis and malignant biliary structures: two ways of practicing clinical proteomics *P. Lescuyer (Geneve, Switzerland)*
10.20	Coffee break
10.55	Liver and gut microbiome: a systems biology perspective on human ageing and longevity *C. Franceschi (Bologna, Italy)*
11.30	Lectures from abstracts ANALYSIS OF SERUM PROTEOME FROM PATIENTS WITH DIABETIC NEPHROPATHY BY CLINPROT/MALDI-TOF-MS FOR DISCOVERY OF POTENTIAL BIOMARKERS *E. Gianazza (Monza, MI)* MALDI-TOF-MS PROTEIN PROFILING REVEAL DIFFERENTIAL POST-TRANSLATIONAL MODIFICATIONS OF TRANSTHYRETIN IN MULTIPLE SCLEROSIS *D. Pieragostino (Chieti)* GENE ONTOLOGY-BASED ANNOTATION AND COMPARATIVE ANALYSIS OF PROTEINS EXTRACTED FROM PROTEOMICS OF 100 BREAST CANCER PATIENTS. *I. Pucci-Minafra (Palermo)* BIOMARKER DISCOVERY IN THE CEREBROSPINAL FLUID OF CREUTZFELDT-JAKOB DISEASE PATIENTS: A MALDI-TOF MS PROTEIN PROFILING STUDY *E. Urso (Cosenza)*

12.30 Workshop Applied Biosystems

12.30 Lunch

13.30 Poster session

II SESSION: From cellular proteomics to systems biology

14.30 Understanding the phenotypic variability of Osteogenesis Imperfecta and developing new therapeutic approaches using the knock in murine model BrtIIV
A. Forlino (Pavia, Italy)

15.05 New concepts in glycomics: how to encompass carbohydrate structure diversity
J. Peter-Katalinic (Muenster, Germany)

15.40 Quantitative targeted Proteomics for the analysis of cellular networks
P. Picotti (Zurich, Switzerland)

16.15 Lectures from abstracts
IDENTIFICATION OF A NUCLEAR ISOFORM OF THE DOUBLE-STRANDED RNA DEPENDENT PROTEIN KINASE PKR IN ACUTE LEUKEMIA CELLS
W. Blalock (Bologna)
COMPARATIVE PROTEOMIC ANALYSIS OF CISPLATIN SENSITIVE SHSY5Y NEUROBLASTOMA CELL LINE AND ITS RESISTANT COUNTEPART BY 2DE AND LABEL-FREE LC-MSE
S. D'Aguanno (Roma)
AN INTEGRATED APPROACH OF PROTEOMICS AND GENOME-WIDE ANALYSIS STUDIES REVEALS CRITICAL RESIDUES FOR APE1/REF-1 FUNCTIONS
C. Vascotto (Udine)
SILAC-BASED QUANTITATIVE PROTEOMICS FOR FUNCTIONAL ANALYSIS OF MIR17-92 TARGETS
E. Vitale (Milano)

17.15 Members meeting

WEDNESDAY 24 JUNE

III SESSION: Microbiology

9.00 Mycobacterial and Helicobacter proteomics
P. Jungblut (Berlin, Germany)

9.45 Proteomic analysis of human bacterial pathogens
P. Cash (Aberdeen, Scotland)

10.20 Coffee break

10.55 Strategies for quantitative proteomics: to label or not to label?
R.J. Beynon (Liverpool, UK)

11.30 Lectures from abstracts
PROTEOMIC ANALYSIS OF HUMAN U937 CELL LINE ACTIVATION MEDIATED BY Haemophilus influenzae TYPE b P2 PORIN AND ITS SURFACE-EXPOSED LOOP 7
A. Chambery (Caserta)
PROTEOMIC INSIGHT ONTO PATHOGENETIC ROLE OF CAMPYLOBACTER JEJUNI S LPS ON HUMAN GUILLAIN BARRE SYNDROME INDUCTION
A. D'Alessandro (Roma)
INVESTIGATION OF ANTIBIOTIC RESISTANCE IN ESCHERICHIA COLI ENTERO-PATHOGENS
F. Deriu (Milano)
INVESTIGATION OF RIBOSOMAL PROTEOMIC PROFILES BY MALDI TOF-MS BIOTYPER FOR RAPID IDENTIFICATION AND CLASSIFICATION OF ASPERGILLUS SPP. FROM CLINICAL SAMPLES.
L. Mancinelli (Roma)

12.30 Workshop

12.30 Lunch

13.30 Poster session

IV SESSION: Plant proteomics

14.30 Proteomic approaches to deepen the biochemical and physiological events involved in fruit ripening
L. Espen (Milano, Italy)

15.05 System biology in plants
W. Weckwerth (Wien, Austria)

15.40 Poster prizes

16.15 Lectures from abstracts
VERNALIZATION IN WINTER WHEAT AND BASIC DIFFERENCES WITH SPRING COUNTERPART: A PROTEOMIC INVESTIGATION
M.G. Egidi (Viterbo)
PROTEOMICS OF LEMON (CITRUS LIMON) PEEL REVEALS THAT THIS TISSUE IS PRODUCING HIGH QUANTITY OF ALLERGEN MOLECULES
S. Mazzuca (Rende, CS)
PROTEOME ANALYSIS OF CITRUS SINENSIS L. (OSBECK) FLESH AT RIPENING TIME

V. Muccilli (Catania)
THE APPLICATION OF A MULTIVARIATE STATISTICAL APPROACH TO ISOLATE PROTEINS USEFUL IN DEFINING GRAPE SKIN RIPENING
A.S. Negri (Milano)

17.15 Working groups meeting

20.00 SOCIAL DINNER at the Acquario Civico di Milano

THURSDAY 25 JUNE

V SESSION: Advances in quali-quantitative proteomics

9.00 Molecular imaging by mass spectrometry: assessing spatial and temporal factors in biological and medical research
R. Caprioli (Nashville, USA)

9.45 An integrated LC/MS strategy for high definition biomarker discovery and validation
M.A. McDowall (Manchester, UK)

10.20 Coffee break

11.00 Chemical proteomics for the discovery of vascular markers of pathology: from the bench to the clinic
D. Neri (Zurich, Switzerland)

11.30 Lectures from abstracts
TOP DOWN PROTEOMICS WITH A QTOF INSTRUMENT AND COLLISION INDUCED DISSOCIATION
A. Armirotti (Genova)
CHEMICALLY MODIFIED MESOPOROUS SILICA BEADS:A NEW EFFICIENT AND RELIABLE PLATFORM FOR BODY FLUIDS PROTEOMICS
F. Casadonte (Catanzaro)
GLOBAL COMPARISON OF SECRETOMES FROM MULTIPLE PANCREATIC CANCER CELL LINES BY A LABEL-FREE MASS SPECTROMETRY-BASED PROTEOMIC WORKFLOW
S. Schiarea (Milano)
TITANIUM DIOXIDE COATED MALDI PLATE FOR ON TARGET ANALYSIS OF PHOSPHOPEPTIDES
F. Trota (Milano)

12.45 Closing remarks

LECTURES' ABSTRACTS

R001

THE UBIQUITIN PROTEOLYTIC SYSTEM: FROM BASIC MECHANISMS THROUGH HUMAN DISEASES AND ONTO DRUG TARGETING

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Between the sixties and eighties, most life scientists focused their attention on studies of nucleic acids and the translation of the coded information. Protein degradation was a neglected area, considered to be a non-specific, dead-end process. While it was known that proteins do turn over, the large extent and high specificity of the process - whereby distinct proteins have half-lives that range from a few minutes to several days - was not appreciated. The discovery of the lysosome by Christian de Duve did not significantly change this view, as it was clear that this organelle is involved mostly in the degradation of extracellular proteins, and their proteases cannot be substrate-specific. The discovery of the complex cascade of the ubiquitin pathway revolutionized the field. It is clear now that degradation of cellular proteins is a highly complex, temporally controlled, and tightly regulated process that plays major roles in a variety of basic pathways during cell life and death, and in health and disease. With the multitude of substrates targeted, and the myriad processes involved, it is not surprising that aberrations in the pathway are implicated in the pathogenesis of many diseases, certain malignancies and neurodegeneration among them. Degradation of a protein via the ubiquitin/proteasome pathway involves two successive steps: (a) conjugation of multiple ubiquitin moieties to the substrate, and (b) degradation of the tagged protein by the downstream 26S proteasome complex. Despite intensive research, the unknown still exceeds what we currently know on intracellular protein degradation, and major key questions remain unsolved. Among these are the modes of specific and timed recognition for the degradation of the many substrates, and the mechanisms that underlie aberrations in the system that lead to pathogenesis of diseases. The recent discovery of modification by ubiquitin-like proteins along with identification of “non-canonical” polyubiquitin chains that serve non-proteolytic functions, have broadened the scope of the system beyond proteolysis and set new challenges in for biologists and proteomic experts. Major challenges in the field are clearly (i) identification of the cellular proteins tagged by ubiquitin and ubiquitin-like proteins, (ii) identification of the downstream elements recognized by these chains, and (iii) deciphering the structure of the different ubiquitin and ubiquitin-like chains that tag the different proteins.

R002

PROTEOMICS OF HEART FAILURE

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Background.
Diseases resulting in heart failure cause severe myocardial dysfunction, either through enlargement of the heart as a result of hypertrophy and dilatation of cardiac muscle (dilated cardiomyopathy, DCM), or through restricted blood flow and oxygen supply caused by atherosclerosis of the coronary arteries (ischemic heart disease, IHD). Although the leading cause of death in the Western world, the only effective long-term treatment for end-stage heart failure is cardiac transplantation. Proteomics is playing an important role in the characterisation of global alterations in protein expression in heart disease. It is providing new insights into cellular mechanisms involved in cardiac dysfunction and has the potential to result in the development of new diagnostic/prognostic markers, and to identify new therapeutic targets. Previous proteomic investigations in heart disease, whether using 2D gel- or MS-based workflows, have focused on the analysis of total protein extracts from whole cardiac tissue samples. However, due to their limited dynamic range, current proteomic platforms are unable to reveal the complete proteomic complexity of whole tissue samples. This problem can be addressed by sub-proteome analysis, which through reduction in sample complexity has the potential to reveal biomarkers of heart failure. Here we have investigated the cardiac hydrophobic (membrane) sub-proteome in heart failure. Membrane proteins are critical components of cellular structure and function and are, moreover, important targets for drug development.
Methods.
Using samples of control human heart tissue, we have validated a Triton X-114 (TX-114) phase extraction method for the analysis of cardiac hydrophobic and membrane-associated proteins. This method was then used to enrich for hydrophobic proteins in control, DCM and IHD samples. We then identified, annotated and quantified disease associated protein expression changes in this sub-proteome through label-free LC/MS^E analysis.
Results.
The advantage of the TX-114 phase extraction method, compared with more conventional methods of membrane protein enrichment (e.g. density gradient centrifugation) is that it can be applied to the relatively small samples of cardiac tissue that are generally available in studies of human heart disease. Based on sub-cellular location and GRAVY score analysis, the study of control cardiac tissue (n=3 samples) showed a 60% enrichment of hydrophobic and membrane bound cardiac proteins in the detergent phase following TX-114 extraction. This method had a high degree of technical quantitative reproducibility (mean %RSD 11.8%). In the subsequent disease study, we analysed 3 pooled samples (n=5 individuals in each pool) from each of 3 disease groups (Control, DCM, IHD). Again we were able to demonstrate a low degree of technical variability, and the biological variability within each group was also low. From a total of more than 1,000 proteins identified and quantified in the hydrophobic cardiac sub-proteome, some 57 proteins were found to be differentially expressed between the three sample groups, with 6 proteins being unique to DCM, while 15 proteins were unique to IHD.
Conclusions.
We have shown that the TX-114 phase extraction method successfully enriches for the hydrophobic and membrane-associated sub-proteome of the human heart. The label-free LC/MS^E method that we have used for quantitative expression analysis of this cardiac sub-proteome has a high degree of technical reproducibility, enabling meaningful studies of differential protein expression in human heart disease (DCM and IHD). We have identified 57 proteins that are differentially expressed in this sub-proteome in heart failure, with a small number being unique to DCM and IHD. The identities of some of the proteins that are differentially expressed in DCM and IHD indicate marked impairment of Ca^{2+}-handling in heart failure. The disease-associated differentially expressed proteins are currently being validated on individual patient extracts by Western blotting and Multiple Reaction Monitoring (MRM).

R003

ACUTE PANCREATITIS AND MALIGNANT BILIARY STRICTURES: TWO WAYS OF PRACTICING CLINICAL PROTEOMICS

Lescuyer P [1, 2], Farina A [1], Fétaud V [1], Lassout O [1,2], Pastor C [3], Dumonceau JM [4], Frossard JL [3, 4], Hochstrasser DH [1,2]

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Proteomics has been for many years presented as a powerful tool for the discovery of new disease biomarkers that could open new perspectives in laboratory medicine. In this context, we would like to focus on the importance of accurately weighing up the value of data generated by proteomic analysis and the implications that this has for an efficient integration of this technology in the biomarker discovery pipeline. Indeed, proteomics is not a direct provider of disease biomarkers but rather a screening tool that allows selecting a reasonable number of candidate proteins from a complex sample. Therefore, it is crucial to integrate the proteomic discovery tools into a pipeline that will maximize the likelihood of selecting relevant candidates for further validation studies. In particular, this implies, from the beginning of the project, a close collaboration with the end-users of disease biomarkers, i.e. clinical biologists and physicians. Accordingly, we are trying in our laboratory to implement such workflows for the discovery and validation of disease biomarkers. However, to reach the same goal, different strategies can be used. The probably most popular strategy in clinical proteomics is based on the descriptive and comparative analysis of body fluids to search for tissue leakage biomarkers. We applied this type of approach on bile to search for biomarkers of malignant biliary strictures. Biliary strictures are pathological conditions involving the abnormal narrowing of the common bile duct and impeding bile outflow from the liver to the small intestine. Malignant and benign etiologies of biliary strictures usually share similar symptoms resulting in difficulties for differential diagnosis. In this project, we combined in-depth analysis of bile proteome and quantitative proteomic analysis of this fluid using samples from patients with malignant and non-malignant strictures. We identified several cancer-related proteins and preliminary validation studies are currently in progress to evaluate their potential interest for malignant biliary strictures diagnosis. A second strategy for clinical proteomics is to search for disease-associated proteins directly in affected tissue collected either from animal-models or patients. We used such an approach to investigate the pathobiologic processes involved in acute pancreatitis. Acute pancreatitis is an inflammatory disease of the pancreas characterized by a dramatic clinical presentation and variable severity. In most patients, the pathology is self-limited and regresses spontaneously without any complications but, in approximately 20% of the patients, the disease is severe and associated with a high mortality rate (10-15%). Therefore, it would be of great interest for clinicians to have new prognosis biomarkers allowing early detection of severe forms of the disease. We performed proteomic analysis on pancreatic tissue extracts from rats with experimental pancreatitis. We also analyzed an experimental model in which the severity of the pathology was reduced by prior exposure of animals to thermal stress. The aims of these different analyses were to better understand processes leading to pancreatic tissue injury and to discover factors modulating the course of the disease. In particular, we identified proteins that could be involved in heat-induced protection against pancreatic tissue injury. We also identified changes in protein expression between disease models of different severity that could help understanding molecular events determining the course of the disease.

R004

LIVER AND GUT MICROBIOME: A SYSTEMS BIOLOGY PERSPECTIVE ON HUMAN AGING AND LONGEVITY.

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Proteomics is one of the most important approach to identify the basic mechanisms of underpinning aging and longevity. Indeed, proteomics, together with genetics, genomics, metabolomics, and other "-omics" is capable of generate high dimensionality data which can be analyzed within the framework of a systems biology approach. Our laboratory is focused on the biological bases of inflammaging, i.e the systemic, low grade, chronic inflammatory process that accompany the aging process and constitute the pathogenetic background of the major age-related diseases. After a brief review of the major characteristic of inflammaging from an immunological and evolutionary point of view , we will present data concerning the proteomics of aging liver and a new project on gut microbiota in centenarians. Within the frame of the UE project "PROTEOMAGE", we have performed a preliminary analysis of the proteomics of human liver, using biopsies from organs to be transplanted derived from donors from different age (age range 23-86 years). Always within the proteomage project we will present data regarding the interactome of two isoforms of p53 (at codon 72), which , according to previous results from our laboratory, play a complex role in human aging and longevity. Finally, we will present a project aimed to ascertain the role changes of gut microbiome composition can play in immunosenescence and inflammaging.

R005

UNDERSTANDING THE PHENOTYPIC VARIABILITY OF OSTEOGENESIS IMPERFECTA AND DEVELOPING NEW THERAPEUTIC APPROACHES USING THE KNOCK IN MURINE MODEL BRTLIV.

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Background. Osteogenesis imperfecta (OI) is an heterogeneous group of skeletal disorders characterized by bone deformity and fragility. The Classical form of OI has a dominant negative transmission and it is caused by mutations in COL1A1 or COL1A2 genes, coding for the alpha chains of type I collagen, that constitutes over 90% of the bone organic matrix. The most frequent molecular defects in OI patients are point mutations changing the amino acid Glycine, that is present every three position in both alpha chains and it is important for the proper triple helical folding of the type I collagen molecules. We generated some years ago a knock-in murine model for OI, BrtlIV. BrtlIV carries a Gly349Cys substitution in the alpha1 chain of the endogenous type I collagen and shows a moderate or a lethal OI outcome reproducing the phenotypic variability reported for human OI patients. Taking advantage of that we investigated the molecular basis of this variability by evaluating bone gene expression by microarray and bone protein profile by 2-DE and mass spectrometry in the OI murine model BrtlIV. We then used our murine model to develop a cell therapy treatment which employs in *utero* transplantation to avoid marrow ablation for this metabolic inborn disorder.
Methods. Total RNA was extracted from calvarial bone of newborn surviving and lethal BrtlIV and wild-type mice. Custum array EC Matrix (MEMOREC) containing 188 relevant extracellular matrix genes was used. Validation was performed by competitive and Real Time RT-PCR. Protein from calvarial bone of mutant BrtlIV and wild-type mice were extracted and separated by 2D gels. We used mass spectrometry for spots identification, PDQuest software (BioRad) for data analysis and western blotting for validation.
The bone marrow cells were isolated from long bones of eGFP-CD1 mice and were injected into the liver of E14.5 WT and BrtlIV embryos. Mice were analyzed at 2 m, the age corresponding to the more severe BrtlIV bone phenotype, compared to WT. Engraftment was evaluated by fluorescence microscopy, FACS and Real time PCR.
pQCT and MicroCT were used to evaluate bone properties. Collagen analysis at the bone trabecular and diaphysial region was evaluated by SDS-PAGE. Matrix mineralization was evaluated by InfraRed spectroscopy.
Results. Four transcripts resulted consistently more expressed in the lethal with respect the surviving BrtlIV mice: GADD153, Bmp6, Bmp7 and PRELP, whereas three genes resulted down regulated: Matrilin 4, Microfibril Associated glycoprotein 2 and Thrombospondin 3. GADD153, a transcription factor activated by ER stress, resulted increased also at the protein level.
We generated the first reference 2-DE map for calvarial tissue, identifying 164 spots corresponding to 97 distinct proteins and the comparison between protein pattern of lethal, surviving and wild type mice showed in mutant mice an increase of □ and □ fibrinogen and in non lethal BrtlIV an increase of □B crystallin.
Adult bone marrow donor cells from eGFP transgenic mice engrafted in haematopoietic and non haematopoietic tissues, differentiated to trabecular and cortical bone cells, and synthesized up to 20% of all type I collagen in the host bone. The transplantation eliminated the perinatal lethality of heterozygous Brtl IV mice. At 2 months of age, femora of treated Brtl mice had significant improvement in geometric parameters ($p<0.05$) versus untreated Brtl mice, and their mechanical properties attained WT values.
Conclusion. The increased expression in lethal animals of the transcription factor GADD153 indicated that a different response to ER stress could be involved in different OI outcome. AlphaBCrystallin is an intracellular chaperone and its increment in non lethal mutant mice again pointed out in the effect of intracellular environment for the phenotype modulation. The higher expression in lethal BrtlIV of the extracellular matrix proteins Prelp, Bmp6 and Bmp7 and the reduced expression of Matrilin 4, Microfibril-associated glycoprotein 2 and Thrombospondin 3, revealed that the extracellular matrix composition also modulates OI phenotype.
Our IUT results suggested that the engrafted cells form bone with higher efficiency than the endogenous cells, supporting *in utero* transplantation as a promising approach for the treatment of genetic bone diseases.

ABSTRACT NOT AVAILABLE

R007
QUANTITATIVE TARGETED PROTEOMICS FOR THE ANALYSIS OF CELLULAR NETWORKS

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Background. To comprehensively understand organisms and their physiological or pathological behaviours it is important to model how their constituting networks –e.g. metabolic or signalling networks- operate and are controlled. To study these properties, it is crucial to measure all the elements that constitute the system, which are often associated to a wide range of molecular properties and cellular abundances. However, comprehensive measurements are technically difficult, even in a simple eukaryotic organism such as yeast, especially at the proteome level. Shotgun proteomic methods are capable to identify, several thousand proteins in a sample. However, these methods are non-targeted, *i.e.* in each analysis they stochastically sample a proteome fraction. Each repeated analysis required for comparing different states, will sample only a subset of the proteins in the network of interest, thus precluding the generation of complete datasets. An additional limitation is the difficulty in detecting low abundant proteins. To overcome these limitations we proposed a targeted proteomic strategy based on selected reaction monitoring (SRM).
Methods. The method consists of the following steps: (i) a list containing all proteins in the network is created. (ii) For each protein proteotypic peptides (PTPs, peptides that are unique to a protein and preferentially detectable by MS) are selected based on previous evidence or by bioinformatic prediction. (iii) Precursor/fragment ion relationships are established, that identify each PTP. These are pairs of mass-to-charge values that are selected with two analyzers of a triple quadrupole mass spectrometer to isolate the peptide and corresponding fragment ion(s). The relationships (termed SRM transitions) are effectively MS assays that monitor the peptide and thus the corresponding protein in a complex digest. (iv) The assays are used to quantitatively monitor the set of proteins in cells grown under conditions inducing different modes of operation in the network under study.
Results. First, we tested the depth and sensitivity of the SRM-based proteomic approach. We demonstrated that proteins spanning the whole range of abundance in yeast, between 1.3E6 copies/cell and <50 copies/ cell could be detected by SRM in total, unfractionated yeast proteome digests. These results showed that a solid basis has been established to attempt a comprehensive analysis of a yeast protein network. As an example, we applied the approach to the analysis of the yeast metabolic network, composed by proteins spanning all levels of abundance. For each protein in the network we developed SRM assays and we used them to quantify the set of metabolic proteins in yeast grown under conditions inducing radically different metabolic setups (glucose, ethanol, galactose-based, complex medium, anaerobic conditions) and in a growth time-course of yeast cells transiting through a series of metabolic phases. Protein abundance data were also combined to a reference transcript dataset. Results from all metabolic pathways associated to central carbon metabolism will be presented and discussed.
The generation of SRM assays for yeast proteins prompted us to develop a database that supports the collection, organization and dissemination of the SRM assays. This resulted in the construction of the MRMAtlas, a web-based resource to store the coordinates of the developed SRM assays and share them between different laboratories. The MRMAtlas, currently contains assays for > 1500 yeast proteins, including complete pathways, and efforts are underway to bring the atlas to completion for *S. cerevisiae*.
Conclusions. The data generated set us on a path towards a quantitative modeling of the dynamics of the yeast metabolism. In addition the assay generated will allow other researchers to monitor the responses of sets of yeast proteins of interest to any stimulus at high-throughput. These advances open a new avenue in the quantitative and qualitative analysis of proteins in the context of systems biology research.

R008

MYCOBACTERIAL AND HELICOBACTER PROTEOMICS

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Background:Thirteen years of bacterial proteomics work in MPIIB focused on *Mycobacterium tuberculosis* and *Helicobacter pylori* resulted in lists of diagnostically, therapeutically and vaccine-relevant candidates, a proteomics data base system (http://www.mpiib-berlin.mpg.de/2D-PAGE/) and basic insight into the complexity and structure of proteomes.
Methods: Several prefractionation methods, immunoproteomics, 2-DE/MALDI-TOFTOF-MS, LC-ESI-MS, SDS-PAGE-LC-MS were applied to analyse as many as possible of the bacterial proteins.
Results: *M. tuberculosis*: 2-DE/MALDI-MS and/or LC/ESI-MS were applied to compare cellular proteins, secreted proteins and proteins within the phagosome between the attenuated strain BCG and the virulent strain H37Rv. Proteins occurring only within the virulent strain were postulated as vaccine candidates. After applying additional criteria a small set of proteins were tested in the mouse model as a potential vaccine with promising results. Clinical investigations did not completely fulfil the expectations for a powerful vaccine. Here by 2-DE/MALDI-MS 379 ORFs were identified, which was complemented by an LC/MS approach with additional proteins of 461 ORFs. The total of 840 ORFs represents 21 % of the complete predicted proteome. *H. pylori*: Vaccine and diagnostically relevant proteins were revealed by immunoproteomics and the detection of the most common proteins of the fractions cellular proteins, secreted proteins, outer-membrane proteins, structural proteins, water-solubilizable proteins, urea-solubilizable proteins, and SDS-solubilizable proteins. Combined with further criteria three candidates were determined with a multiparameter search and these three candidates tested in a mouse model with promising results. Again the clinical tests did not fulfil the expectations. In total 567 proteins were detected, which represents 36.6% of the complete predicted proteome. 2D-PAGE database system: A relational database system based on MYSQL was built up, which allows the storage and data mining of the accumulated 2-DE and MS data. Many questions can be answered by working with the database, e.g.: By which method can a certain protein be investigated? Are certain protein classes within a compartment overrepresented? Is there a bias of LC-MS against low Mr proteins? Together with the program PROMPT the fate of the membrane proteins during sample preparation for 2-DE was elucidated. Posttranslational modifications can be searched with the peak lists stored in the database.
Conclusion: Protein species concept: In both bacteria each of the most common proteins occurred in several spots on 2-DE gels suggesting the presence of several protein species per protein even in bacteria. Each protein species is chemically defined by its amino acids and their posttranslational modifications. The chemical structure defines the function of a protein species. For functional investigations it is necessary to separate and identify the protein species to correlate them with a change of the biological situation such as control and disease. For functional correlations a 100% sequence coverage is necessary as was shown to be reachable in the example of ESAT6 proteins of *M. tuberculosis*. The increase of throughput at the protein species level is one of the major challenges of proteomics in the next years. The search for biomarkers should be focussed on the protein species level instead of the protein level.

R009

ANALYSING THE PROTEOMES OF BACTERIAL PATHOGENS USING 2D ELECTROPHORESIS

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Background. Proteomic technologies are widely used in microbiology to analyse global changes in protein synthesis to monitor both bacterial gene expression and cellular physiology. In the area of Medical Microbiology the proteomes of human bacterial pathogens have been extensively characterised to either type isolates for epidemiological studies or identify and characterise pathogenic determinants. We are currently using proteomics to analyses a range of bacterial pathogens, including *E. coli* and members of the *Helicobacter* genus. *E. coli* (specifically Uropathogenic *E. coli* [UPEC]), is a major cause of Urinary Tract Infections (UTIs) with the infecting bacteria originating probably from the intestine. Questions remain on the degree of uniformity of UPEC isolates and the extent of their adaptation to the urinary tract as they initiate infection. We are looking at the diversity among UPEC isolates at the level of their proteomes. Similar approaches to those employed to characterise UPEC isolates are also used to define the proteomes of non-pylori *Helicobacters*. The non-pylori *Helicobacters* typically infect sites throughout the gastrointestinal tract and are thus exposed to a variety of environmental stresses during infection of their host. Although many non-pylori *Helicobacters* primarily infect non-human hosts, a small number of species have been implicated in human infections.
Methods. UPEC isolates were grown on nutrient agar plates incubated in an aerobic atmosphere. Non-pylori *Helicobacter* species were grown on blood agar plates in a microaerophilic atmosphere. Total cellular protein extracts were prepared by detergent disruption of the bacteria combined with sonication. Hydrophobic proteins were selected using Triton-X114 phase separation. The proteins were analysed using 2-Dimensional Gel Electrophoresis (2DGE) on a small-format system and the protein profiles compared using Progenesis SameSpots software and Principal Component Analysis (PCA). Selected protein spots were identified by peptide mass fingerprinting and LC-MS.
Results. The proteomes of 12 randomly collected UPEC isolates and 4 faecal *E. coli* isolates were compared using 2DGE; replicate preparations were included in the analysis for each isolate. The 2D protein profiles of the cellular proteins for the UPEC isolates were similar overall. However, PCA readily discriminated the UPEC isolates from each other and put them into 5 major clusters (Figure 1). One UPEC isolate formed a unique group (Cluster I) whereas the remaining four clusters (II to V) contained between 2 and 4 UPEC isolates. Differential expression of membrane proteins has been proposed as one of the adaptations associated with UPEC during a UTI. Hydrophobic proteins were analysed to act as an initial approximation of the bacterial membrane proteins. Isolate-specific differences in the hydrophobic proteins were observed between UPEC and faecal *E. coli* isolates. As might be expected there was extensive heterogeneity in the proteomes of the representative non-pylori *Helicobacters* analysed as part of our work. In order to focus the analyses to key proteins expressed by these bacteria comparative proteomic studies (2DGE and PCA) were carried out against *H. pylori* as a reference. Based on these comparisons abundant proteins were selected from the profiles of the non-pylori *Helicobacter* species for identification by MS. Since there is limited genome sequence data available for most of the non-pylori *Helicobacters* many of the protein identifications remain tentative, since they are based on similarities to gene/protein sequences from either other *Helicobacters* or related bacteria. In addition to looking at the total cellular proteins, parallel studies are in progress to locate those proteins that may be involved in the host-bacterium interaction by defining the hydrophobic protein sub-proteome as well as proteomic responses to acidic conditions.
Conclusions. 2DGE combined with computer analysis of the protein profiles is a sensitive method to discriminate among closely related UPEC isolates to identify key proteomic differences to be targeted for further analysis. The diversity observed among UPEC isolates may be due to a number of factors including genetic variation, varying antibiotic sensitivities and pathogenic variation. Even where limited genomic sequence data are available, as for the non-pylori *Helicobacters*, the classic 2DGE-MS approach can assist in the targeting of specific proteins for future analysis to provide further insights into the pathogenesis of these bacteria.

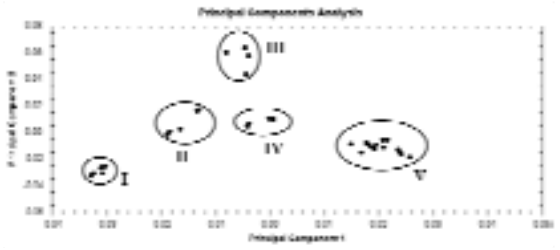

Figure 1: PCA of 2D protein profiles of UPEC isolates.

R010

TO LABEL OR NOT TO LABEL: THAT IS THE QUANTIFICATION

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When the discovery phase of proteomics is complete, the recovery of quantitative data becomes increasingly important. Many strategies emphasise relative quantification, but there are a number of approaches that can now deliver absolute quantification, and thus, exact copy numbers of proteins in the cell or analytical system. Such quantitative strategies fall naturally into two discrete categories; those that use the well established precepts of stable isotope labelled internal standards (AQUA, QconCAT, PSAQ) and those that use the intrinsic properties of peptides or their fragmentation products (MS^E, APEX, spectral counting). Each of the two approaches have both advantages and disadvantages, and it is increasing clear that the two strategies are best converged into a complementary workflow that capitalises on both approaches, ideally within a single analytical sample stream.
In this presentation, I will discuss the relationship between quantification strategies, and address the merits of a convergent approach in application areas including proteome normalisation methods, metabolic pathway analysis and deconvolution of quantification for highly isomeric proteins. I will compare MS^E data with QconCAT data and give examples of the application of both quantitative workflows. I will finally address the generic issue of the “grand challenge” of the complete quantification of a proteome.

Acknowledgements
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R011

PROTEOMIC APPROACHES TO DEEPEN THE BIOCHEMICAL AND PHYSIOLOGICAL EVENTS INVOLVED IN FRUIT RIPENING

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Fruit ripening is generally characterized by many biochemical events such as chlorophyll degradation, biosynthesis of carotenoids, anthocyanins, essential oils, flavour and aroma components, changes in organic acid and sugar contents and modifications in cell-wall architecture. Anatomically, fruits are formed by different tissues (*i.e.* skin, flesh, seeds and vascular bundles) that have peculiar functions. Although processes activated during ripening strictly depend on the genetic background of the plant, environmental conditions strongly affect the ripening process. The resulting changes in colour, texture, flavour, aroma and nutritional content have the physiological function to make fruits attractive to a variety of seed-dispersing organisms. Moreover, these changes are responsible of the nutritional properties that are extremely important for human diet, since fruits provide fibre, minerals, vitamins and other beneficial compounds such as antioxidants.

On the basis of the respiratory trend and ethylene evolution fruits can be further classified as climacteric and non-climacteric. In climacteric fruits, such as tomato, peach, banana and apple, this hormone plays a central role in the regulation of ripening which is characterized by a typical peak of respiration and a burst of ethylene production. Differently, in non-climacteric fruits, such as grape, strawberry and citrus, ethylene is not essential for the ripening process. Nevertheless, recent studies revealed that some processes in climacteric fruit are ethylene-independent and that this hormone plays a role also in non-climacteric ones.

In the last years, together with a lot of transcriptomic analyses, some proteomic studies have been conducted to shed light on the molecular bases of ripening. Although the widening of genomic information for many plants has paved the way to study fruit proteomes, an effort was required to define satisfactory methods for protein extraction and purification from these samples. In fact fruits are typical recalcitrant plant material because of the high concentration of interfering compounds such as phenolics, terpenes, organic acids, ions, carbohydrates and proteolytic and oxidative enzymes. In this view, different protein extraction procedures have been tested, showing that phenol extraction followed by methanol/ammonium acetate precipitation has higher efficiency in removing interfering compounds. Preliminary experiments conducted in our laboratory showed that the introduction of washing steps with organic solvents considerably improved the efficiency of this method. Nevertheless, it can be concluded that every extraction procedure is not generalizable, but it must be fitted to the intrinsic fruit characteristics.

Among different possible technical approaches, 2-DE is the most adopted procedure to study fruit proteomes. Many of these studies have been performed on fruits of large interest for the human diet such as tomato, grape, citrus and strawberry. These researches were essentially based on the comparison of the proteomes extracted from whole fruits at different ripening stages. According to the transcriptomic analyses, the results provide a description of the cascade of the biochemical and physiological events typical of fruit ripening. Besides the expression changes in proteins involved in carbon metabolism, energy production, redox status control and cellular signaling, these proteomic analyses revealed that many proteins are linked to stress responses and senescence. Moreover, some peculiar traits in different species were found.

Particular attention has been addressed to grape berry, a fruit with remarkable economical relevance for both fresh consumption and processed products, such as wine, juice and dried fruit. Some studies focused on specific tissues (*i.e.* mesocarp or skin) and allowed to obtain information about their physiological roles. In particular, primary metabolism, that was down-regulated in the mesocarp, rises in the skin, according to the metabolic requests, being this tissue the site of important biosynthetic pathways (*e.g.* anthocyanin). Moreover, the comparative analysis among genotypes characterized by different abilities to accumulate anthocyanins has led to a deeper knowledge on the relationships between primary and secondary metabolisms. Finally, some proteomic data emphasized the relevance of the skin as a physical barrier playing an important role in berry protection. Taken together, these studies underline the importance of focusing on isolated tissues, since each of them presents unique features in the evolution of the proteome during ripening.

Although further efforts are still necessary to fully elucidate the biochemical and physiological events involved in fruit ripening, these first proteomic analyses improved the knowledge about the role of specific pathways relating to different fruit tissues functions. Further important information, which up to now are quite completely lacking, will emerge from studies on post-translational modifications as well as at sub-cellular level.

R012

MASS WESTERN, MAPA, PROTMAX AND PROMEX: STRATEGIES FOR QUANTITATIVE PROTEOMICS DATA INTEGRATION IN SYSTEMS BIOLOGY

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In many approaches of modern biology proteins need to be accurately identified and quantified to determine their involvement in a specific metabolic, developmental or environmental process. Techniques addressing this are especially important in the post-genome area. Here we can design genome-wide strategies to investigate the plant system as a whole, however, high sample throughput is needed to analyze dynamic systems. Typical strategies for targeting specific proteins in complex samples are antibodies and immunochemical methods. However, besides the time-intensive and laborious task to generate protein specific antibodies, in many cases it is problematic to yield selective antibodies able to distinguish proteins with high homology or homologous epitope regions. A targeted mass spectrometric technique called ***Mass Western*** is able to distinguish highly homologous proteins and, at the same time, quantify these proteins in e.g. pmol per cell or freshweight or total protein. Furthermore it is possible to establish a sample throughput not achievable with classical antibody approaches by analyzing several dozens of proteins in one sample. We will present applications for the accurate analysis of low abundant proteins and whole protein isoform families in *Arabidopsis thaliana* [1, 2] and enzyme pathways in metabolic processes like photosynthesis in *Chlamydomonas reinhardtii* [3], and drought stress processes during symbiotic nitrogen fixation in *Medicago truncatula* [4].
A non-targeted approach for protein quantification and database independent identification of cSNP/protein polymorphism and other protein modifications using ***MAPA*** (mass accuracy precursor alignment) will be presented as well. This approach is based on spectral count of m/z ratios and subsequent protein identification of statistically significant changes performed with a novel algorithm called ***Protmax*** [5]. Strategies are proposed how to **integrate quantitative proteomics data as well as metabolomics data** – a second research field in our group - into typical systems biology strategies like genome annotation, metabolic modeling approaches and investigations in stress physiology in plants [6]. All the proteomic data are stored in ***ProMEX*** [7], a mass spectral reference library, which iteratively links the approaches described above. Recently, ***ProMEX*** was cross-referenced with UNIPROT.

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R013

ASSESSING SPATIAL AND TEMPORAL PROTEOMICS: MOLECULAR PROFILING AND IMAGING OF TISSUES BY MASS SPECTROMETRY

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The spatial and temporal aspects of molecular processes in cells and tissues play an enormous part in the biology that defines living systems. Profiling and Imaging MALDI MS provides an effective means to measure and assess those dimensions on a molecular basis, including peptides, proteins, lipids, metabolite as well as others. It provides the capability of mapping the location of specific molecules such as drugs, lipids, peptides and proteins directly from tissue sections. Molecular patterns can be directly correlated to known histological regions within the tissue, allowing for the direct monitoring of proteins specific to these regions within a tissue sample. Profiling and imaging MS have been used to characterize many tissue types, including human gliomas and lung cancers, as well as tumor response to specific therapeutics, suggesting the use of proteomic information in assessing disease progression as well as predicting patient response to specific treatments.

In the profiling mode, frozen tissue specimens are sectioned (~10 μm thick), and are analyzed in a histology-directed manner in collaboration with pathologists to obtain molecular profiles from discrete areas of tissue. In the imaging mode, high density laser ablation of an ordered array of spots over the entire tissue gives rise to a 2-dimensional ion density map (or image) with 30-80 μm lateral resolution in which location and relative abundance of a given analyte is displayed. From the analysis of a single section, images at virtually any molecular weight may be obtained. In addition to MALDI ToF MS and MS/MS instrumentation, the capabilities of ion mobility MS and FTICR MS for profiling and imaging of tissues will be discussed.

This presentation will describe several biological applications of this technology, including examples of discovery in mouse developmental models and the profiling of human tumors, characterizing protein differences between tumor grades, and monitoring protein changes due to drug therapy. We have applied this technology for the creation of 3-D protein images of substructures of mouse brain, to drug targeting and metabolic studies and the measurement of concomitant protein changes in specific tissues after systemic drug administration. Finally, we explore the correlation of lipid and protein changes in several systems in both health and disease.

R014

AN INTEGRATED LC/MS STRATEGY FOR HIGH DEFINITION BIOMARKER DISCOVERY AND VERIFICATION

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Background

Biomarker R&D is one of the major applications of proteomics. The workflow conventionally begins with a 'global discovery' study (*e.g.* high resolution LC/MS/MS) of a limited number of samples to identify a range of candidate proteins. 'Targeted proteomics' techniques (*e.g.* tandem quadrupole LC/MRM) are subsequently employed to efficiently verify these candidates within a biologically diverse cohort.

In global discovery studies there is a growing consensus that exact mass MS/MS aids the unambiguous matching of tryptic peptide spectra to databanks of known protein structure. Where exact mass measurement of both precursor and product ions minimize false discovery (protein identification). Exact mass, electrospray, LC/MS of complex protein digests (*e.g. E. Coli*) can detect *Ca* 150,000 unique ions. Deconvolution of such data may reveal *Ca* 20,000 non-redundant precursor ions for MS/MS interrogation. It is advantageous to capture information from as many tryptic peptides (precursors) as possible to maximize sequence coverage and thus visualize proteins in 'high definition'. High sequence coverage facilitates the mapping of modifications, the quantification of protein isoforms and the experimentally justified selection of proteotypic peptides (with optimal MRM transitions) for targeted analyses. Currently the low duty cycle of serial LC/MS/MS techniques require multiple injections to additively compile an exhaustive inventory of peptides when analyzing complex protein digests. In addition commonly used two dimensional chromatographic strategies (SCX and RP HPLC) cause a significant number of peptides within a digest mixture to be 'split' (*i.e.* diluted) across multiple fractions. Consequently low abundance peptides often fall below the LC/MS detection limit – further restricting the total number of peptides reported in a discovery study.

Target compound analyses, from small molecule drugs to proteotypic peptides, are ubiquitously performed by 'tandem quadrupole' (TQ) LC/MRM. TQ instruments have high sensitivity and excellent quantitative dynamic range in the targeted (MRM) mode of operation. However, they have a very low duty cycle (sensitivity) for the acquisition of full MS/MS spectra. This precludes real-time verification of proteotypic peptide sequences targeted for quantification by LC/MRM.

Methods

We have developed a novel Q-ToF mass spectrometer incorporating a compact ToF mass analyzer optimized for discovery proteomics at high resolution (>40,000 FWHM), high mass accuracy (<2ppm) and high spectral acquisition rate (20 spectra/sec). Moreover this system enables high bandwidth (UPLC/MS^E) data acquisition allowing multiple peptide precursor ions to be interrogated (fragmented) in parallel. Moreover we have developed a 2D (RPxRP) UPLC separations strategy that minimizes peptide splitting across fractions - further increasing the total number of peptides detected from a single injection. A novel TQ instrument incorporating a combined traveling-wave collision cell and ion trap (with mass selective ejection) has been employed to enable targeted proteotypic peptides to be quantified by LC/MRM with real-time sequence confirmation at high sensitivity.

Results

Discovery analysis of model systems (*e.g. E. Coli*) by 2D UPLC/MS^E demonstrate a consistent increase (2-3x) in the total number of replicating (n=3) peptides observed - facilitating the detection and quantification of peptide modifications, protein isoforms and the experimentally justified selection of proteotypic peptides (with optimal MRM transitions) for targeted analyses.

R015

CHEMICAL PROTEOMICS FOR THE DISCOVERY OF VASCULAR MARKERS OF PATHOLOGY: FROM THE BENCH TO THE CLINIC

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Background:One avenue towards the development of more selective anti-cancer drugs consists in the targeted delivery of bioactive molecules (drugs, cytokines, procoagulant factors, photosensitizers, radionuclides, etc.) to the tumor environment by means of binding molecules (e.g., human antibodies) specific to tumor-associated markers. In this context, the targeted delivery of therapeutic agents to newly-formed blood vessels ("vascular targeting") is particularly attractive, because of the dependence of tumors on new blood vessels to sustain growth and invasion, and because of the accessibility of neo-vascular structures for therapeutic agents injected intravenously.
Methods: My laboratory has developed a novel chemical proteomic methodology for the identification and relative quantification of vascular markers in normal organs and a sites of disease. The technology is based on the biotinylation of vascular structures (e.g., by terminal perfusion of tumor-bearing mice or by *ex vivo* perfusion of surgically-resected organs with cancer), followed by protein purification and comparative mass spectrometric analysis.
Results: In the lecture, I will present the findings of chemical proteomic investigations performed on rodent tumor models and in surgically resected human kidney and colon with cancer. More recently, we have extended the application of this methodology to the study of diabetic nephropathy and of vascular alteration in Alzheimer disease. Seven derivatives of human monoclonal antibodies, developed in my lab in collaboration with Philogen SpA, are currently being investigated in numerous multicenter clinical trials in Ital, Germany and UK.
Conclusions:
Target discovery activities based on chemical proteomic methodologies support the development of innovative antibody-based therapeutic agents.

ORAL COMMUNICATIONS' ABSTRACTS

O 001

ANALYSIS OF SERUM PROTEOME FROM PATIENTS WITH DIABETIC NEPHROPATHY BY CLINPROT/MALDI-TOF-MS FOR DISCOVERY OF POTENTIAL BIOMARKERS

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Background: Diabetic nephropathy (DN) is the major vascular complication of diabetes mellitus (DM) and it is the leading cause of end stage renal disease (ESRD) worldwide. DN accounts for 40-50% of Type 1 diabetic patients (T1D) that require renal replacement therapy and it is associated with increased cardiovascular risk. Despite significant progress in understanding DN, the cellular mechanisms leading to the renal damage are still incompletely understood. The earliest evidence of possible nephropathy in diabetic subjects is an increase in the albumin excretion rate. However this alteration is not a sufficiently accurate predictor of DN risk. Currently there are no specific biomarkers for an early detection of DN. The characterization of serum protein profile is an important tool to discover and identify disease-correlated biomarkers and for the deepening of the knowledge on pathophysiological aspects of DN. The aim of this study is the evaluation of proteome in serum by ClinProt-MALDI-ToF approach, in order to identify possible markers able to discriminate healthy subjects from type 1 diabetic patients with (T1DN) and without nephropathy (T1D).
Methods: Serum protein profiles of control subjects (n = 9), T1D patients (n = 10) and T1DN patients (n = 4) were obtained by ClinProt/MALDI-ToF technique. Serum proteome was prefractionated using C8 coated magnetic beads, which mainly enrich proteins in range of 1-10 KDa, based on an automated procedure using a ClinProt Robot. Spectra were acquired in positive linear mode with a Reflex IV MALDI-TOF. Data analysis was performed using the statistical software ClinProTools 2.1 for the elaboration of diagnostic models. Eluted fractions from normal subjects and diabetic patients not used for MALDI-ToF analysis were pooled for μHPLC-ESI MS/MS analysis with an Esquire 3000 Plus™ and Ultimate 3000 system. A capillary column C18 was used for HPLC and the flow rate at the end of the column was set at 300 μL/min. The obtained chromatograms were analyzed with the software DataAnalysis and the resulting mass lists were used for database search using Mascot® on a local server.
Results: Statistical analysis with ClinProTools 2.1 software showed several ions differently regulated in serum samples among the three classes. The high complexity of spectra profiles required the use of different algorithms supplied by the software for cluster analysis. A group of five statistically different (p-value < 0.05) signals (A= m/z 1014 ± 8 Da, B = m/z 1201 ± 8 Da, C = m/z 1445 ± 8 Da, D = m/z 1972 ± 8 Da and E = m/z 1532 ± 8 Da) able to discriminate the three populations with specificity of 100% and sensitivity values of 80% for T1D patients and 100% for T1DN patients was built. The simultaneously combination of five signals makes the discriminant cluster very robust, but the three classes could be distinguished also using only two ions of these. Through LC-ESI-MS/MS analysis, three of these signals differently expressed in serum of controls and T1D patients were identified as the intact form of the Fibrinopeptide A and its fragments. Two of these peptides (at m/z 1019.50 and 1205.57 Da) were down regulated in T1D, while the third one (m/z 1535.69 Da) showed a tendency to the up expression.
Conclusions: Our results suggest that a proteomic approach based on magnetic beads represents an useful method to discover possible clinical biomarkers. Moreover we demonstrate the capability of selected signals to differentiate T1D patients from T1DN and from normal subjects. Fibrinopeptide A was found to be enhanced, as expected from literature, in diabetic nephropathic patients probably caused by intrarenal coagulation. A lower expression of the two fibrinopeptide A fragments, not yet described in literature, was also noticed. These data suggest a possible different catabolic pathway of Fibrinopeptide A in diabetes which needs further studies. These results show the possibility to identify a pattern of peptides allowing to distinguish the three populations and to provide novel information about development of diabetic nephropathy.

The present work has been supported by FIRB: Rete Nazionale per lo studio del proteoma umano (n. RBRN07BMCT).

O 002

MALDI-TOF-MS PROTEIN PROFILING REVEAL DIFFERENTIAL POST-TRANSLATIONAL MODIFICATIONS OF TRANSTHYRETIN IN MULTIPLE SCLEROSIS

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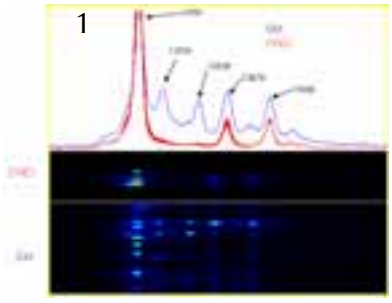
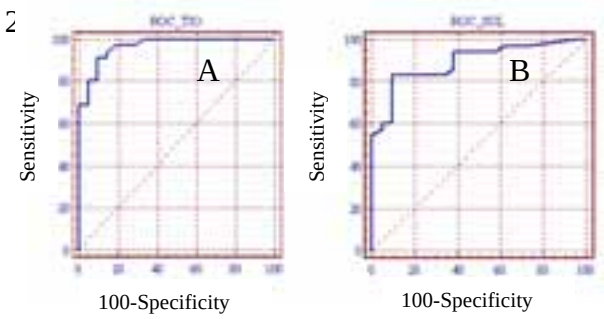
Background: Multiple Sclerosis (MS) is an often disabling demyelinating disease of the central nervous system. The cause of MS has not been fully elucidated, although genetic, environmental, and immunological factors play a pathogenetic role. A clinical proteomics investigation on biofluids is currently largely pursued to tentatively identify protein profiles that are diagnostic or prognostic indicators of disease course. MALDI-TOF-MS is a powerful tool to investigate large number of clinically relevant molecules in different tissues. However, protein profilingmay not be quite reproducible, because a number of clinical and pre-analytical chemistry factors constitute major sources of variability and bias. After pre-analytical and analytical validation procedure, we investigated low molecular weight protein patterns in biological fluids highlighting atypical oxidative isoforms of CSF Transthyretin in MS patients.
Methods: 36 patients with definite MS and 22 patients with Other Neurological Diseases (OND) were included in this study. The diagnosis in each patient was defined according to individual disease diagnostic criteria. Biological Sample (10 μl) preparation was performed by microSPE (Millipore tip size P10). Protein profiling analysis were performed with a Reflex IV MALDI-TOF mass spectrometer (Bruker Daltonics, Germany). The selected m/z range was of 5-20 kDa. Data normalization and processing were performed using optimise peak detection routine (LIMPIC) able to describe each group under investigation. Protein signals were further identified after preparative HPLC separation by peptide sequencing on a nanoLC-Q-TOF TANDEM mass spectrometer.
Result: Analytical assessment has been pursued returning high performances: CV%≤10.7 (n=12) for inter-day experiments, mass accuracy of 100 ppm. Pre-analytical data showed critical conditions for biofluids proteome depending on storage times and temperatures. Once validate the method was applied to investigate the polypeptide profile in different physio-pathological condition. Our results highlight a differential distribution of protein patterns in the CSF of patients affected by MS in respect to OND group. Fig. 1 shows the average m/z profile of the Transthyretin (TTR) in MS patients (blu) and in OND group (red). The canonical covalent adducts of TTR with cysteine (TTR-Cys) and cysteinglycine (TTR-CysGly) are formed by the production of mixed disulphides involving the thiol of Cys^{10} showed at m/z 13879 Th and 13936 Th respectively.





Our experiments univocally assign the signals at 13791 Th and 13839 Th, highlighted only in MS samples, as TTR. Redox experiments return the evidence that this isoforms are mixed disulfide of Cys^{10} forming thiocystein (TTR-SH) and sulphocystein (TTR-SO_3H) respectively. Kolmogorov-Smirnov test shows a $p<0.0001$ in discriminating the MS group vs the OND group for both signals. In Fig. 2A we report a receiver operating characteristic (ROC) curve analysis based on the TTR-SH adducts. The accuracy of this parameter in classifying the two groups is outlined by a sensitivity of 91.4% and a specificity of 90.5%. When the total investigated population is discriminated according to a relative ratio of TTR-SH over free TTR less with a cut-off of 0.13 (A). The ROC curve based on the TTR-SO_3H form is reported in Fig. 2B. A TTR-SO_3H ratio lower or equal to 0.13 discriminate the groups with a sensitivity of 82.9% and a specificity of 90.3%. Plasma TTR of the same MS patients not shows the differential isoforms indicating that the redox imbalance is focused in central nervous system.
Conclusion: We have found a differential TTR oxidative isoforms in CSF of MS patient by using a MALDI-TOF-MS method. The method was underwent to pre-analytical and analytical validation. Statistical analysis reveal that both signals of TTR adducts have a strong diagnostic relevance and, in fact, it is possible to

segregate the investigated population of the MS-affected subjects from the control group. Functional experiments are ongoing to evaluate the biological consequence of these post-traslactional modification.
This work has been supported by the “Rete Nazionale di Proteomica”, Progetto FIRB RBRN07BMCT

O 003

GENE ONTOLOGY-BASED ANNOTATION AND COMPARATIVE ANALYSIS OF PROTEINS EXTRACTED FROM PROTEOMICS OF 100 BREAST CANCER PATIENTS

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Background: Current clinical parameters for breast cancer diagnosis and therapy are: tumour size, axillary lymph node status, histological grading and presence or absence of metastases. Prognostic/predictive properties, such as oestrogen and progesterone receptor status, and human epidermal growth factor receptor (HER-2/neu) status are currently used for therapeutic decision. Conversely, it is now emerging that the number of genetic mutations and epigenetic deregulations in cancer is far more higher than previously thought. Therefore, proteomic screening for differential protein expression in subsets of tumor samples is an essential tool for generating data bases and biomarker discovery. The aim of present study was to extend to a significant number of proteins and patients our previous data, contributing to the knowledge of biological pathways involved in breast cancer and to the molecular-based classification of breast cancer patients.
Methods: Aliquots of breast cancer tissues and their adjacent non-tumoral tissues were obtained during surgical intervention from patients who did not receive any neoadiuvant therapy. Diagnosis of ductal infiltrating breast cancer (DIC) was confirmed by histopathology. Sample preparation for proteomics was performed as described (Pucci-Minafra et al. Proteomics Clinical Applications, 1, 118-129, 2007; Pucci-Minafra et al. Journal of Proteome Research 7, 1412-18, 2008). Quantitative determination of protein spots was normalized for actin.
Results: We have collected for the present study 100 proteomic maps from G2/G3-DIC patients, and 13 from non-tumoral mammary tissues. All detected proteins were identified, or confirmed, by mass spectrometry. Collectively we have annotated 209 protein spots, corresponding to 122 genes. Genes were analyzed by the instruments of DAVID Bioinformatics Resources (Dennis et al. 2003; Huang et al. 2009). The Gene Accession Conversion tool recognized 112 unambiguous Gene IDs, over the 122 ones present in our list. The gene list was correlated to 136 functional terms, but only 36 correlations were highly significant (Benjamini values from $1x10^{-8}$ to $5.6 x10^{-3}$). Nine terms over the 36 corresponded to the function of apoptotic processes and in particular 24 genes were related at the highest significant value ($1x10^{-8}$) with the regulation of programmed cell death; 50% of the genes belonging to this category, codify for proteins with anti-apoptotic functions. The cluster of anti-apoptotic proteins, corresponding to 12 genes and 22 protein isoforms was compared among the 100 proteomics maps and the 13 reference non-tumoral tissue maps. The comparative proteomic profiling showed: 1) a highly significant overexpression of several members of the anti-apoptotic protein cluster in the cancer tissues *vs* non tumoral counterparts; 2) a relative variability of the expression levels of the normalized proteins within patients. Among proteins of this category reaching the high levels of expression, we observed the nucleophosmin (NPM), a crucial regulator of p53; the translationally-controlled tumor protein (TCTP1), a protein involved in calcium binding and microtubule stabilization; cofilin (COF1), an actin-binding protein responsible also for the signal translocation from cytoplasm to nucleus; annexin A1 (ANXA1) a calcium/phospholipid-binding protein which promotes membrane fusion and ruffling, and the glutathione s-transferase (GSTP1), which plays important roles in detoxification but having also a role in susceptibility to cancer.
Conclusions: The application of the powerful Bioinformatics Resources for gene/protein classification provided by DAVID knowledgebase, while confirming our previous protein classification, introduced new terms for further remodulation of protein clusters on the basis of the multiple functions for individual proteins. In particular we found a high number of proteins with specific biochemical functions, converging towards common pathways. Overall, a predominant pathway was the programmed cell-death, which resulted to be the most robust among patients, both as number of proteins involved and as level of significativity. We believe that the present collection of human breast cancer proteomics represents a valid contribution for clinical applications to breast cancer.
Figura 1: Overview of the miniaturized proteomic maps of the 100 patients utilized for present investigation. Numbers on the maps correspond to individual patients.

O 004

BIOMARKER DISCOVERY IN THE CEREBROSPINAL FLUID OF CREUTZFELDT-JAKOB DISEASE PATIENTS: A MALDI-TOF MS PROTEIN PROFILING STUDY

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Background: Creutzfeldt–Jakob disease (CJD) is a progressive neurodegenerative disorder of the human central nervous system (CNS) caused by prion infections that invariably leads to a fatal event. MALDI-TOF protein profiling analysis permits the detection of peptides and small proteins in complex protein mixtures with great accuracy. We applied this analysis to cerebrospinal fluid (CSF) from 15 patients affected by Creutzfeldt-Jakob disease (CJD).
Methods: We compared the levels of the normalized ion signals of 11 sporadic and 4 genetic CJD forms with those from 10 healthy control subjects and 8 non-CJD relapsing-remitting multiple sclerosis patients.
An aliquot of each sample was subjected to a desalting/concentration step over a ZipTip C18 .The eluted samples were typically mixed at a 1:1 vol/vol ratio with a CHCA matrix solution and 1 µl of this solution was deposited onto stainless steel target surfaces and allowed to dry at room temperature. Peptide/protein profiles were analyzed using a Voyager DE PRO MALDI-TOF mass spectrometer . The acquired raw spectra were then processed for automated advanced baseline correction and noise filter with Voyager Data Explorer software, version 4.1. Finally the peak lists were imported for data transformation and statistical analysis.Ions with a signal equal or superior to 1% were collected. The signal intensities were normalized presenting the values as area percent of the total area of all the signals. The obtained dataset was subjected to a hierarchical cluster analysis and to the Mann-Whitney U-test. Peptides/ small proteins of interest were partially purified by HPLC and characterized by the MALDI-TOF peptide mass fingerprinting and in some cases by post source decay (PSD) analysis. A quantitative determination of thymosin β4 was also performed using an independent enzyme immunoassay (EIA) analysis.
Results: Statistical analysis of the data set using the Student's t-test demonstrated 61 differentially expressed ion signals in the CJD group compared to the control group; in particular, 21 ion signals were up-regulated and 40 ion signals were down-regulated. Among the ions/proteins differentially expressed, three with m/z of 8565.8, 4963.5 and, 3238.4, showed an increased area percent and were of high statistical significance ($p<0.0001$). By applying the hierarchical cluster analysis to the data set of the differentially expressed ion signals, 3 well-separated groups without overlay were generated corresponding to the controls, CJD patients, and MS patients. The first two proteins correspond to free Ubiquitin and Thymosin beta 4 n-acetylated (TB4) respectively, while the third one was uncharacterized. The EIA data showed that thymosin β4 levels had an increased trend in the CSF of CJD patients compared to the controls and multiple sclerosis subjects, validating the mass spectrometry data.
Discussion. Diagnosing ante-mortem CJD is a challenging task. We have applied proteomic analysis on CSF in searching for molecular markers useful for diagnostic and physiophatological purposes. Elevated levels of Ubiquitin were already found in CJD patients while this report appear to be the first showing a Thymosin beta4 involvement in a neurodegenerative disease. There are not definitive data concerning the expression of TB4 in the central nervous system but recent works have shown its distribution in glial cells. The elevated level detected by us in CJD might reflect than the well known glial reaction found in the brain of the CJD patients.
Conclusions: our results let us to propose TB4 as a new candidate marker for CJD disease.

O 005

IDENTIFICATION OF A NUCLEAR ISOFORM OF THE DOUBLE-STRANDED RNA DEPENDENT PROTEIN KINASE PKR IN ACUTE LEUKEMIA CELLS

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Background: The double-stranded RNA dependent kinase PKR is a member of the eIF2α kinase family. This innate immune kinase plays a central role in inflammatory cytokine signaling, angiogenesis and apoptosis. Phosphorylation of eIF2α by PKR results in inhibition of general translation, but still allows for the translation of select mRNAs encoding proteins involved in innate immune and cell stress responses. In addition to translation regulation, PKR is also required for proper NF-κB signaling in response diverse stimuli including TNFα and Toll-like receptor signaling. Recent reports have indicated that PKR is overexpressed and its kinase activity is elevated in several tumors including breast and colon carcinomas as well as melanoma. We recently reported that acute leukemia cell lines contain elevated PKR activity as compared to normal controls, which is required for leukemic cell growth. Following examination of myelodysplastic syndrome (MDS) patient samples, we found that the number of cells positive for activated PKR (as determined by immunocytochemistry using a phospho-specific antibody to T451; p-T451 PKR) increased with increasing risk of progression to acute myelogenous leukemia (AML). Additionally, activated PKR in high-risk MDS patient samples was localized to the nucleus. As the presence of activated PKR (p-T451 PKR) in the nucleus has not been previously reported nor is its significance known, we examined the expression, T451 phosphorylation status, activity, and composition of nuclear PKR.
Methods: Acute leukemia cell lines (HL60, THP-1, CCRF-CEM and Jurkat) were fractionated and the proteins separated by SDS-PAGE. Separated proteins were immunoblotted using antibody against PKR or p-T451 PKR. Both total lysate and nuclear lysate from CCRF-CEM cells were analyzed by 2-D gel electrophoresis. The separated proteins were again blotted using either PKR or p-T451 PKR to determine the number of PKR forms present. Nuclear PKR was purified by liquid phase separation of the nuclear lysate on an ion-exchange column. Purified nuclear PKR was then analyzed for activity in a standard PKR kinase assay. Purified nuclear PKR was also analyzed and sequenced by mass spectrometry.
Results: PKR from each of the acute leukemia cell lines was recognized as two bands between 58-68 kDa following SDS-PAGE while p-T451 PKR was recognized as a doublet between 70-72 kDa in HL60 and THP-1 cells and as a single band of 72 kDa in CCRF-CEM and Jurkat cells. Fractionation analysis revealed that the slower migrating PKR band was predominantly localized to the cytoplasm while the faster migrating PKR band predominantly localized to the nucleus. In contrast to analysis of total cell lysates, fractionation reveal two forms of p-T451 PKR. The slower migrating form which ran as a 72 kDa protein in SDS-PAGE, localized mainly to the cytoplasm while the novel faster migrating band recognized by the p-T451 PKR antibody ran as a 56-58 kDa protein. Analysis of CCRF-CEM lysate by 2-D gel electrophoresis indicated that nuclear PKR existed in greater than 5 forms and activated PKR existed in greater than 3 forms based on pI. Each of these forms had an approximate molecular weight of 56-62 kDa based on their migration in the second phase. Kinase assays using purified nuclear PKR revealed that it is an active kinase. Mass spectrometry identified nuclear PKR as a novel PKR isoform.
Conclusions: We have identified a novel isoform of PKR which localizes to the nucleus and is an active kinase. As PKR expression and activity are associated with progression and invasiveness of certain cancers and nuclear localization of activated PKR is associated with progression in MDS, identification of this isoform may further explain the significance of PKR activity in human cancers. Although there are no known nuclear-specific proteins that are substrates for PKR, PKR is associated with DNA damage repair and cell cycle progression. As nuclear PKR is an active kinase, nuclear targets are likely to exist. The identification of nuclear PKR is likely to facilitate discovery of a nuclear signaling network required for leukemic cell maintenance.

O 006

COMPARATIVE PROTEOMIC ANALYSIS OF CISPLATIN SENSITIVE SHSY5Y NEUROBLASTOMA CELL LINE AND ITS RESISTANT COUNTEPART BY 2DE AND LABEL-FREE LC-MS^E

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Background: Platinum based drugs (such as cisplatin) are widely used in chemotherapy of neuroblastoma but patients easily develop cisplatin resistance that confers them poor prognosis. Several mechanisms could be implicated in cisplatin resistance but they are not sufficient to exhaustively explain this resistance emergence. A global quantitative analysis by shutgun proteomics of protein changes induced by cisplatin in a resistant human neuroblastoma cell line should offer an excellent opportunity for better understand the mechanisms that can contribute to this clinically relevant phenomenon.

Methods: Total proteins from sensitive and resistant human neuroblastoma cell line SHSY5Y were separated by 2DE and after gel image analysis differentially expressed proteins were identified by MALDI-TOF-TOF (Bruker, Germany) experiment. Comparative analysis was also performed by loading protein digests on a nanoACQUITY UPLC^{TM} System coupled with a Q-Tof Premier^{TM} Instrument (Water Co.) Peptides were trapped on a 5 µm Symmetry C18 trapping column and separated using a C18 NanoEase^{TM} 1.7 µm BEH, 75 µm X25 cm nanoscale column. Sample was initially transferred with an aqueous 0.1% formic acid solution to the precolumn with a flow rate of 15µl/min for 5 min. Mobile phase A was water with 0.1% formic acid, and mobile phase B was 0.1% formic acid in acetonitrile. Peptides were separated with a gradient of 3-40% mobile phase B over 120 min at flow rate of 250nl/min, followed by a gradient of 40-90% mobile phase B over 5 min and a 15 min rinse with 90% mobile phase B.
The Q-Tof Premier^{TM} mass spectrometer was operating in Expression mode. Samples were run in triplicate. Protein identification and relative quantification were performed by PLGS software (Water, Micromass Co).

Results: In this study we applied both a classical proteomic approach based on 2DE gels differential analysis coupled to mass spectrometry protein identification and a more innovative label-free LC-MS^E quantification method to identify proteins associated with cisplatin resistance by comparing proteome from sensitive and resistant human neuroblastoma cell lines. 14 and 89 differentially expressed protein were identified by the two distinct approaches respectively. Changes in the expression level of some proteins were validated by western blotting experiments.
The differentially expressed proteins are involved in multiple cellular functions such as DNA repair, stress response, cytoskeletal interactions and degradation. Datasets acquired by the two different approaches were used together for a further bioinformatics analysis to estimate protein networks involved in cisplatin resistance. This analysis suggested the involvement of Nrf2 , a bZip transcription factor that moves into the nucleus in response to oxidative stress binding to ARE. Nrf2 translocation into nucleus in resistant cell line was confirmed by immunofluorescence experiment.

Conclusions: Label-free LC-MS^E quantification method, based on high peptide retention time reproducibility and exact peptide mass, allows a large number of proteins to be screened simultaneously. Combining of 2DE followed by mass spectrometry identification and qualitative and quantitative proteome profiling by LC-MS^E and bioinformatics tools is a useful strategy to obtain an extensive system biology overview of different cellular aspects in response to drug resistance. We propose that the Nrf2 pathway, which plays a protective role in normal cells, can be a potential target to control cisplatin resistance in neuroblastoma.

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O 007

AN INTEGRATED APPROACH OF PROTEOMICS AND GENOME-WIDE ANALYSIS STUDIES REVEALS CRITICAL RESIDUES FOR APE1/REF-1 FUNCTIONS

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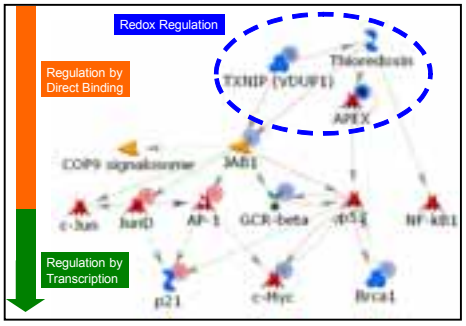
Background: Apurinic apyrimidinic endonuclease/redox effector factor 1 (APE1/Ref-1) protects cells from oxidative stress by acting as a central enzyme in base excision repair pathways of DNA lesions and as a redox transcriptional co-activator. Its dysregulation has been associated with cancer development and contrasting data have been published on the biological relevance of the two functions and on the molecular mechanisms involved. We combined both mRNA expression profiling and proteomics analysis to determine the molecular changes associated with APE1 loss-of-expression induced by siRNA technology. Moreover, to dissect between redox- and DNA-repair-mediated effects, we performed knock-in experiments using wild type (WT) and various APE1 mutants which were characterized through gene expression and functional proteomics studies.

Methods: HeLa cells have been used for the development of APE1's conditional knock-down system by using RNA interference (RNAi) technology. For generation of APE1 knock-in cell lines, an APE1 expression vector was created by cloning the cDNA coding sequence into p3XFLAG-CMV-14 vector. To avoid the degradation of the ectopic APE1 mRNA by the specific siRNA sequence, two nucleotides of the APE1-cDNA coding sequence were mutated leaving the APE1 amino acid sequence unaffected. The APE1 siRNA clone was stably transfected with p3XFLAG-CMV/APE1, WT APE1, and several mutants (C65S, C310S, H309N, K6K7/R6R7, 31-34A). Individual clones were isolated and analysed for their ability to express the ectopic recombinant protein. Expression proteomic analysis has been performed on total, nuclear and cytoplasmic cellular extracts. Functional proteomic analysis was performed to identify the interacting partners of C65S mutant with respect to the WT protein. For gene expression analysis, human genome HG U133 PLUS2 microarrays on Affymetrix platform was used. Biological data have been obtained through clonogenic assay, Annexin V, Caspase 3/7 activation and cleavage assay.

Results: mRNA expression profiling and proteomics analysis on APE1's knocked-down cells delineated the APE1 involvement in cell growth, apoptosis, intracellular redox state, mitochondrial function, and cytoskeletal structure. The knock-in experiments using the WT and various APE1 mutants clearly confirmed the essentiality of the DNA-repair function and their molecular effects were analyzed by expression profiling through microarray analysis. We concentrated on C65S and 31-34A mutations because of their opposite effects, C65S acting as a loss-of-function and 31-34A being a gain-of-function. The non-cleavable mutant 31-34A did not show a significantly different gene expression profile compared to the WT protein, suggesting that the proteolytic removal of the N-terminal part of APE1 leads to its functional inactivation *in vivo* without affecting protein half-life. The C65S mutant affected the global gene expression profile and, in order to better understand the loss-of-function of the C65S mutant, we analysed the effect of this mutation on the endonuclease activity of APE1 over abasic dsDNA by cleavage assay. Interesting, the enzymatic activity of C65S mutant was significantly lower than the WT protein. Thus, by a functional proteomic approach, we found that in the interactome network of APE1, as a consequence of C65 to S mutation, the presence of two protein species increased with respect to that of the WTAPE1. These protein species were further identified as peroxiredoxin-6 (PRDX6) and the protein disulfide-isomerase A3 (PDIA3). Interestingly, both of them are involved in redox-assisted protein folding processes. The possible effect on the structural folding of APE1 *in vivo* of residue C65 was evaluated by limited proteolysis studies on immunoprecipitated WT and C65S APE1 proteins. Time-course analysis using trypsin and chimotrypsin proteases clearly demonstrated that, while the pattern of proteolytic fragments generated upon digestion was similar for APE1 WT and C65S mutant, their kinetics showed quite different behaviour suggesting a role for C65 in controlling the globular folding of APE1 *in vivo*.



Conclusions: In this work we have identified changes at the proteome and at the gene expression level associated with the loss of APE1 expression in HeLa cells by using RNAi technology and an unbiased

comparative approach of proteomics and microarrays analysis. Furthermore, we employed a knock-in strategy to reintroduce mutant APE1 proteins in APE1-silenced cell clones to determine the role of crucial amino acid residues in APE1's redox and DNA-repair activity. Overall, our data show that APE1 acts as a hub in coordinating different and vital functions in mammalian cells, highlighting the molecular determinants of the multifunctional nature of APE1 protein. Finally, we propose a new role for C65 residue in mediating APE1's redox folding through the interaction with PDIA3 and PRDX6 proteins.

O 008

SILAC-BASED QUANTITATIVE PROTEOMICS FOR FUNCTIONAL ANALYSIS OF MIR17-92 TARGETS

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Background MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression by targeting transcripts and leading to their translation repression and/or their destabilization. Computational studies suggest that each miRNA can have hundreds of targets, although the complete mechanism of action remains unclear. The evidence that aberrant expression of certain miRNAs is observed in several tumours, suggests a link between miRNAs and cancer. One example is the miR-17-92 cluster that is amplified in several lymphomas as well as in B-cells chronic lymphocytic leukaemia, CLL, where it acts synergistically with the oncogene c-myc. Interestingly, some of the identified miR17-92 targets are cancer-related genes. As such, a comprehensive screening for miRNA targets is essential to elucidate the role of this cluster in oncogenesis. Regulation by miRNAs occurs at the post-transcriptional level, therefore a screening technology allowing global analysis of targets at the protein level is required. Stable Isotope Labelling by Amino acid in Cell culture (SILAC) allows an accurate and quantitative analysis of proteome and comparative measurement of protein levels from different functional states.
Here we describe the usage of SILAC-based strategy to profile protein changes upon over-expression of miR17-92 in cultured B-lymphoma cells from lamda-myc mice. In parallel, transcriptomics analysis will be acquired by microarrays in order to discriminate between the two levels of gene expression silencing mediated by the 17-92 cluster: mRNA degradation or inhibition of translation.
Methods: The experimental set-up consists in a combination of SILAC labeling with over-expression of miR17-92 in B-cell lymphoma. SILAC is a metabolic labeling: cells, growing in media enriched in stable isotope-containing amino acids, incorporate them in newly synthesized proteins. When light and heavy cell populations are mixed, they remain distinguishable by Mass Spectrometry and protein abundances can be determined from the relative MS signal intensities. To acquire the reference B cells SILAC H/L proteome, cells were grown in parallel in “light” and “heavy” (Arg10 and Lys8) DMEM. After 18 replication cycles, cells were collected, mixed 1:1 and total extracts were prepared by RIPA lysis. Protein were separated by SDS-PAGE, subjected to “in-gel” Trypsin digestion and analyzed by Liquid Chromatography- Mass Spectrometry (LC-MS/MS) on to a LTQ-FT. We used a 140 minutes gradient from 2% to 60% acetonitrile. Data analysis was carried out by means of MaxQuant software that allows high accuracy protein quantification. For miR17-92 over-expression in B-cells lymphocytes, the miR17-92 DNA chromosomal locus was cloned into the bicistronic retroviral vector pMIG, a retroviral vector optimized for expression in our cell line. A retroviral infection was performed: Phoenix ecotropic packaging cells were transfected with miR17-92 construct and the supernatant produced, containing viruses, was used to infect B-cells lymphocytes.
Results: By analyzing the reference one-to-one SILAC proteome, we identified with high confidence 2962 proteins, identified with two peptides, with at least one unique. The precision of the experiment and the uniform incorporation of the labeled amino acids were clearly demonstrated by the distribution of the log2 ratios Heavy/Light, fitting a normal distribution with standard deviation of 0.108. The functional analysis based on the Gene Ontology (GO) of our experimental proteome confirmed a good correlation with both the genome-predicted proteome and the reference transcriptome publicly available in the Gene Expression Omnibus (GEO) repository. Moreover, the comparison of the biophysical properties (pI and molecular weight) of the proteins identified with the theoretical proteome calculated from the genome sequence, showed very good proteome coverage. In our “high confidence proteome” we could reconstruct different physiological and metabolic pathways, for instance we could identify more than 90% of the proteins involved in the B-cell receptor signaling pathway, one of the molecular processes that regulate B lymphocytes immune function.
Having established the SILAC in this cellular model system, we are now working on the optimization of the miR17-92 over-expression by using the retroviral construct already designed, and also by testing alternative more potent lentiviral vectors in preparation.
Conclusions: In this work we use a quantitative proteomics methodology applied to the analysis of a complex biological system. The miR17-92 cluster represents a very interesting model system, due to its role in cancer. We have established for the first time the SILAC approach in lymphoma model cells and we have acquired the proteome with the identification of 2962 proteins in unambiguous way. This is the most extensive quantitative and high confidence proteome obtained to date in B-lymphocytes and it can be taken as a reference dataset that represent a source of information for immunologists working on this model system. The immediate application is the over-expression of miR17-92 in a SILAC-based experimental

setup. Profiling the level of thousands proteins in lymphoma cells lines upon over-expression of this cluster, will lead to the definition of the complex network of the target genes regulated by the miR17-92 with a focus on those with potential role in cancer. As a future perspective, a complementary validation of our results will be offered by a “reverse” experiment, where we will “deplete” the cluster by knocked-down strategies and we will profile changes in proteins: in this set up the specific targets will be identified as proteins up-regulated upon miR silencing.

O 009

PROTEOMIC ANALYSIS OF HUMAN U937 CELL LINE ACTIVATION MEDIATED BY *Haemophilus influenzae* TYPE *b* P2 PORIN AND ITS SURFACE-EXPOSED LOOP 7

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Background. The outer membrane of Gram-negative bacteria, embedded with diverse proteic and lipopolysaccharidic molecules, functions as a complex permeability barrier protecting the bacteria against harmful agents. Being a relevant component of the Gram-negative bacterial outer membrane, porins play an important role in pathogenesis of bacterial infections and stimulate immunological responses inducing the release of several cytokines. Among the pathogenic Gram-negative bacteria, *Haemophilus influenzae* type *b* (Hib) is responsible for a variety of infections, ranging from bronchitis to meningitis, in both children and adults. The invasive bacterial infection is characterised by inflammation mainly mediated by cytokines and chemokines. One of the most abundant components of the Hib outer membrane is the P2 porin, which has been shown to induce the release of several inflammatory cytokines. Synthetic peptides corresponding to loops L5, L6 and L7 activate JNK and p38 mitogen-activated protein kinase (MAPK) pathways, being the L7 loop the most active peptide. It is becoming increasingly clear that the virulence of any particular microrganism is the result of complex interactions between the organism and its host. Signals leading to cellular activation are often generated at the cell surface after binding of receptors to their appropriate ligands.
In the present study the differential protein expression of Hib P2- and L7-exposed U937 cell line was analyzed by a bottom-up mass-spectrometry proteomic approach. Both untreated U937 cells and lipopolysaccharide (LPS)-activated U937 were used for comparative purposes.
Methods. Total cell proteins were separated by 2D-electrophoresis and resulting gels were analysed for comparison. Selected differential protein spots with qualitative and significantly quantitative ($P < 0.05$ based on ANOVA analysis) protein expression were identified by peptide mass fingerprint (PMF) based on MALDI-TOF mass spectrometry and database search.
Results. Proteomic analysis yielded a list of qualitative and quantitative changes occurring upon Hib P2- and L7 activation in U937 cells. Amongst the proteins differentially expressed many are described as directly or indirectly involved in different signaling cascades and host immune and inflammatory responses. Interestingly, for several differentially expressed proteins a similar behaviour was observed following whole P2 porin and L7 activation. Furthermore, a common response was also observed for several proteins upon LPS activation (e.g. alpha enolase, nucleophosmin and stathmin). Most of the differentially expressed proteins are involved in metabolic processes, remodelling of cytoskeleton, stress response and signal transduction pathways. In particular, several differentially expressed proteins were found to be involved in nucleotide metabolic pathways (e.g. deoxyuridine 5 triphosphate nucleotidohydrolase, nucleoside diphosphate kinase A, Inosine 5 monophosphate dehydrogenase) and modulation of splice site selection, the latter category including several splicing factor arginine serine rich and heterogeneous nuclear ribonucleoproteins. In addition, many molecular chaperones, folding catalysts, and proteases, some of which classified as heat shock proteins were found to be differentially expressed upon Hib P2- and L7 activation.
Conclusions. This study represents the first attempt to identify by a proteomics approach, the post-transcriptional alterations in the Hib P2 and L7-exposed U937 cells. The proteomic analysis provided a candidate list with potential relevance in the host immune and inflammatory response upon the Hib P2 and L7 activation. The present work provide the basis for dissecting signal transduction cascades activated upon porin stimulation with the aim to understand the molecular events involved in the modulation of pathogen-host cell interactions.

O 010

PROTEOMIC INSIGHT ONTO PATHOGENETIC ROLE OF CAMPYLOBACTER JEJUNI' S LPS ON HUMAN GUILLAIN BARRE' SYNDROME INDUCTION

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Background: Guillain-Barré syndrome (GBS) is an autoimmune disease affecting the peripheral nervous system, often associated with antecedent events, particularly gastroenteritis and respiratory infectious diseases. Serological investigation on patients with GBS show, in some cases, high titres of antibodies against *Cytomegalovirus* (CMV), *Epstain Barr Virus* (EBV) and most frequently *Campylobacter Jejuni* (CJ). Although the scientific pathophysiologic connection between pathogens and the destruction of peripheral nervous system (PNS) neurons (antigenic mimicry) are widely known, the clinical outline of GBS is still blurry. Since studies on GBS animal models have often provided not riproducibile data, several hypothesis based on the concomitance of additional risk factors (i.e., flagellin or other proteins) linked to the pathogen and host sensitiveness are suggested. Our main perspective is to characterize, by proteomics, glycomics and lipidomics approaches, the different antigenic shares of C. jejuni 's LPS embroiled in immune response activation.

Methods: After phenolic extraction LPS of *C. jejuni* is subjected to high-resolution separation, by using different approaches (chromatographic and electrophoretic separations). In particular the typified LPS (Penner serotype) extracted from biological samples of a patient affected by GBS has been analyzed and compared with a reference one (ATCC 43446, Penner serotype). Peptide Mass Fingerprinting of peptide mixtures, obtained from proteins associated to LPS is performed by SDS-PAGE coupled with MALDI-TOF/TOF mass spectrometry. We have also analyzed protein differential profiles of the two samples by mass spectrometry in linear mode (MALDI-TOF). According the protocol described by Phillips N.J. and colleagues we have obtained the O-deacylated LPS, preserving the sialic acid bond, and analyzed it by MALDI-TOF in reflectron negative modality. In the same conditions we have performed the evaluation of differential signals from saccharidic fractions of LPS. Lipidomics analysis is carried out by liquid chromatography on a gradient of tetrahydrofuran coupled to mass spectrometry by nanoHPLC-ESI Q-TOF.

Results: Interestingly in LPS deriving from a patient affected by GBS we have identified several proteins (Invasion phenotype protein, Major Outer Membrane Protein, TonB-d colicin receptor, Capsular Polysaccharide Transporter and Flagellin A) involved in mechanisms of "immune escape" and host sensitiveness neutralizations. In particular in an electroeluted saccharidic fraction we have found the flagellin A, known as the main risk factor linked to GBS. Moreover from MALDI-TOF analysis, carried out in linear mode, we have observed a distinct and intensive signal in both saccharidic and lipidic fraction of LPS, probably reconducible to flaA or proteins belonging to flagellin family. The analysis of saccharidic fractions give us a differential pattern of specific signals that will be subjected to deconvolution for structural characterizations. In addition PCA and PLS-DA analysis on lipidomics underline a relevant Variable Important for the Projection of the model (VIP), trough which we have estimated the molecular formula of specific lipids.

Conclusions: The aim of our study is the identification of earlier and sensitive GBS biomarkers. Our purpose for the future is to correlate the structural informations obtained by "omics" approaches with experimental evidences of immune activations, by flow-cytometric assay on dendritic cells.

O 011

INVESTIGATION OF ANTIBIOTIC RESISTANCE IN ESCHERICHIA COLI ENTERO-PATHOGENS

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Background: The worldwide emergence of antibiotic-resistant bacteria poses a serious threat to human health. In the past decade, the spread and inappropriate use of antibiotics caused a considerable increase in the number of antibiotics to which bacteria have developed resistance. Understanding molecular mechanisms of resistance is extremely important to develop new therapeutic strategies for the control of these bacteria.
In the current study, proteomics were used to characterize multi-resistant Escherichia coli entero-pathogens and to identify putative markers of antibiotic resistance.
Methods: Faecal samples obtained from cattle were sampled on agar plates and *Escherichia coli* were recovered from them obtaining a total of 50 E.coli isolates. Susceptibility to 13 different antibiotics was performed by the agar disk diffusion method and samples were classified on the basis of different resistance profiles and included in this study. Quantitative two dimensional electrophoresis was performed on 12 different samples classified in two groups: multi-resistant and sensible. Experiments were done in triplicate using different pH ranges to optimize spot resolution and to visualize different isoforms on gels. After image and statistical analysis performed with Progenesis SameSpots software (Nonlinear Dynamics), proteins were processed with MALDI MS for identification.
Results: Image analysis highlighted significantly up and down regulated proteins in multi-resistant group versus sensible group (fig.1). 17 of these spots were identified with MALDI-MS (fig.2). In particular, enzymes involved in sugar metabolism (spots 5, 3, 12, 15) are up regulated in antibiotic resistant bacteria, while lipid and amino acid synthesis are down regulated (spots 9, 13, 14, 16). Tricarboxylic acid cycle, which is an alternative route to Krebs cycle is down regulated (spot 1, 17) in resistant bacteria. Other proteins more expressed in resistant bacteria are involved in different mechanisms as stress response, transport, and signalling (spots 2, 4, 11, 6, 7, 10).

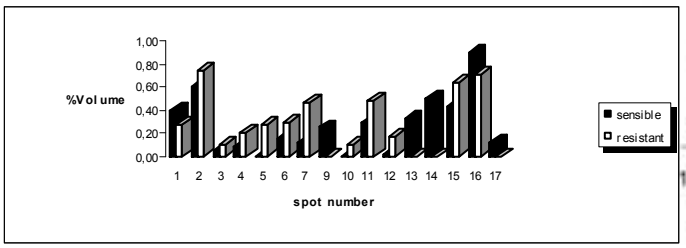
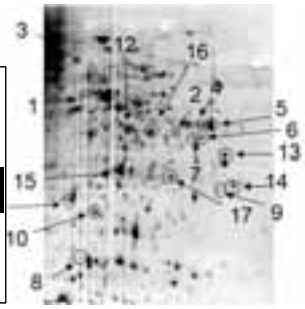


Fig.1: up and down regulated proteins.



1	*isocitrate dehydrogenase*
2	*outer membrane protease ompP*
3	*phosphoglucomutase*
4	*terminase, ATPase subunit*
5	*glyceraldehyde-3-phosphate dehydrogenase*
6	*curved DNA-binding protein*
7	*Thiosulfate-binding protein*
8	*Major exported protein*
9	*3-oxoacyl-[acyl-carrier-protein]reductase*
10	*S-ribosylhomocysteine lyase*
11	*Enoyl-[acyl-carrier-protein] reductase*
12	*Malate dehydrogenase*
13	*FKBP-type peptidyl-prolyl cis-trans isomerase FKPA*
14	*glutamine-binding periplasmic protein*
15	*triosephophate*
16	*cysteine synthase A*
17	*Fumarate reductase iron-sulfur subunit*

Fig.2: 2D map (pH 4-8), proteins identified with MALDI MS

Conclusions: This study shows a global view of cellular process changes related to antibiotic resistance in E. Coli. In particular the main changes linked to sugar metabolism pathways may suggest an highest energy request by resistance bacteria.
Enoyl-[acyl-carrier-protein] reductase (Spot 11), that increase in resistant bacteria, is reported as part of proteins systems activated in antibiotic resistance, as well as thiosulphate-binding protein (spot 7) and outer membrane protease ompP (spot 2), that are part of most studied complexes for resistance mechanisms and pathogenesis. An interesting mechanism of modulation of bacteria cells during antibiotic resistance may be explained with the increased of S-ribosylhomocysteine lyase (spot 10), a protein involved in synthesis of autoinducer 2 (AI2) which is a universal signal for bacteria. Such molecule is involved in 'quorum sensing'

mechanism, which may be used by a variety of bacteria for communication among and between species and may be responsible for regulation of virulence genes in E.coli.

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O 012

INVESTIGATION OF RIBOSOMAL PROTEOMIC PROFILES BY MALDI TOF-MS BIOTYPER FOR RAPID IDENTIFICATION AND CLASSIFICATION OF *ASPERGILLUS* SPP. FROM CLINICAL SAMPLES.

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Background. Fungal infections represent a growing diagnostic and therapeutic challenge of increasing concern due to the frequency and severity of disseminated mycoses in diversified patients, resulting in significant morbidity and mortality. Patients are currently at high risk for fungal opportunistic infections due to the elevated impact of changes in medical practice. Culture-based methods are characteristically slow and have poor sensitivity; thus, other methods, based on the detection of fungus-specific, antigenic and metabolic components are being developed to enable early diagnosis. In our study we exploited different *Aspergillus* spp. ribosomal protein profiling from reference and clinical specimens by the MALDI-ToF MS (Matrix-assisted laser description ionization- time of flight-mass spectrometry) Biotyper with the aim to provide a standardized and reliable method for fungal identification.
Methods. We established a rapid and reproducible protein extraction protocol from mould culture to produce suitable template for MALDI ToF analysis. Ribosomal protein profiles were collected both from reference and clinical strains of ten *Aspergillus* species purchased from the culture collection of Centraalbureau voor Schimmelcultures (CBS) and collected at the Microbiology Unit of the Bambino Gesù Hospital. Generated spectra were processed and analysed to estimate reproducibility of multiple replicates. Finally, eight overlapping spectra for each *Aspergillus* species were selected and evaluated for variance by principal component (PCA) and clustering analysis. Selected spectra were uploaded into a specific *Aspergillus* MALDI TOF-MS library to perform identification of fungal clinical specimens. MS outcomes of 50 *Aspergilllus* clinical isolates were hence corroborated by comparing identification data to the 28S rDNA D2-LSU (large subunit region) sequencing speciation.
Results. On visual inspection, the similarity of spectra produced by different *Aspergillus* species were recognized and, furthermore, differences amongst spectra were easily detected and confirmed by dendrogram analysis. The *Aspergillus* spectra showed typical MALDI-ToF-MS profiling with distinguishing peaks ranging from m/z 2,000 up to 16,000, strongly species-correlated. The reproducibility of the MALDI-ToF method was tested by similarity and PCA outcome of spectra belonging to the same species. The enlarged datasets provided high matching scores (2.3-3.0 range) for *Aspergillus* spp. identification from clinical samples. MALDI-ToF-MS identifications provided 100% of concordance with 28S rDNA D2-LSU sequence analysis for all the analysed clinical isolates.
Conclusions. New proteome profiling-based assays for detection of mould fungi may be an optimal diagnostic approach to overcome current culture-based methods, encompass multiple fungal genera, and for being applied to a variety of specimen types. In our experience, MALDI-ToF is currently under setting and may represent a new frontier for the rapid diagnoses of mould infections (within 1 hour) from culture to identification hit.

O 013

VERNALIZATION IN WINTER WHEAT AND BASIC DIFFERENCES WITH SPRING COUNTERPART: A PROTEOMIC INVESTIGATION

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Background: Vernalization is a natural strategy worked out by winter wheat and other plant species planted in autumn to allow winter survival: once vernalization reaches a saturation point, flowering takes place in spring, when long-day photoperiod standards are met. The aim of the present study was to clarify interrelationship between vernalization saturation (transition from the vegetative to the reproductive phase) and expression of cold-induced genes at the molecular level in common wheat (*Triticum aestivum*) by using proteomic analysis.
Methods: Plants were grown at optimal temperature for 14 days (zero point), then transferred to conditioned chambers kept at 4°C; leaves of winter and spring ecotypes have been harvested after 63 and 42 days of cold treatment, respectively. Proteins were extracted and subjected to 2DE.
The first 2-DE dimension was performed using 17 cm pH 4-7 IPG strips with the Amersham Ettan™ IPGphor II™ isoelectric focusing system. For the second dimension, 16% polyacrylamide large gels were used. Differentially expressed spots were excised and digested with trypsin, peptide mixtures were separated using a nanoflow-HPLC system. Peptides were eluted directly into a High Capacity ion Trap (model HCTplus, Bruker-Daltonik, Germany). The scan range used was from 300 to 1800 m/z. Protein identification was performed by searching in the National Center for Biotechnology Information nonredundant database (NCBInr) using the Mascot program. Physiological parameters, like LT50, chlorophyll fluorescence, chlorophyll concentration, proline and soluble carbohydrates have also been determined. Moreover, three among the most representative cold-regulated proteins identified in proteomic analyses were confirmed at the gene expression level using RT-PCR assays.
Results: Proteomes of a winter wheat ecotype (Cheyenne) versus its cold sensitive counterpart (Koohdasht) have been examined and compared through classical two-dimensional electrophoresis maps. For both ecotypes, more than 100 differentially expressed spots have been detected. The major cold-induced identified proteins could be linked to specific metabolic pathways (carbon fixation, photosynthesis, sugar and nitrogen metabolism).
Conclusions: Isolation and characterization of differentially expressed proteins between the two groups confirmed that winter wheat does not suffer cold stress, but activates both cold acclimation and vernalization processes that herald spring flowering, whereas spring ecotype increases expression of stress-related protein products to face adverse conditions. In particular, plant exposure to low temperatures increases soluble sugar levels in leaf cells, either caused by a new synthesis either due to a failure in plant vascular conductance. High sugar levels could explain down-regulation of photosynthetic machinery both in winter and spring ecotypes, according to the so called "sugar sensing" mechanism, previously described by many authors. Our findings demonstrated an increase in winter wheat of Calvin cycle enzymes and those devoted to nitrogen metabolism, suggesting the existence of a programmed and precise cellular response made by winter ecotype during cold season for getting ready to subsequent flowering time.

O 014

PROTEOMICS OF LEMON (CITRUS LIMON) PEEL REVEALS THAT THIS TISSUE IS PRODUCING HIGH QUANTITY OF ALLERGEN MOLECULES

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Background: Lemon is a traditional crop of the Mediterranean area. Its fruit juice is used to make refreshing drinks, while essential oils are extracted from lemon peel and used as scents. The effects of lemon fruit products on human health have been also described, mainly due to their high content of antioxidant molecules, such as vitamin C, monoterpenes, carotenoids and flavonoids. In Italy (Campania, Calabria and Sicily) are the most important regions devoted to lemon crops, where a large number of genotypes is present. We have started to characterize the fruits of lemon trees grown in Rocca Imperiale, a nice locality in Calabria (Southern Italy), where the selected genotype (var. Femminello) is cultivated under optimal conditions of climate, thus producing some high quality fruits. In particular the fruit epicarp is characterized by a large number of oil glands producing a high amount of monoterpens, among which lemonen is the major component (up to 70%) in respect to other genotypes of the same grove. These observations suggest that not only the genetic trait might be responsible of the organolectic qualities of var. Femminello fruits, both also environmental condition that allow the specific gene expression during ripenin; thus we have applied a proteomic approach in the epicarp tissue (flavedo) with the aim of obtaining a genomic characterization at protein level.
Methods: Fruit peel was collected from a selected limon crop var. Femminello. Excised tissues were immediately frozen in liquid N_2 and processd for protein extraction. We used different protein extraction methods (Spadafora *et al.*, 2008; Wang *et al.*, 2006; Saravanan and Rose, 2004), two- dimensional electrophoresis (2-DE) of protein extracts, mass spectroscopy analyses and bio-informatic tools for protein identification. Adapting the extraction method based on TCA (trichloroacetic-acid) proteins precipitation with subsequent purification in a phenol phase, we obtained 2-DE protein maps. Each protein was characterized in terms of molecular weight (MW), isoelectrics point (IE), relative abundance (NV) in protein extracts.
Results: For the last few years a *Citrus* genome-wide ESTs project has been initiated and consists of 222,911 clones corresponding to 19,854 contigs and 37,138 singletons. We analyzed the lemon flavedo proteome taking advantage of the *Citrus* database, with the aim to obtain the metabolic characterization at protein level of our selected crop. Identification of the spots obtained from the wole sample by 2-DE separation, analysis in LC/MS and bioinformatic supports (GPM, BLAST and MASCOT softwares) allowed us to determin more then 60 proteins. We individuated different biosynthetic pathways involving the identified proteins and enzymes. In particular many proteins are involved in the primary metabolisms, such as photophosforylation and oxidative phosphorylation, while other ones belongs to the oxidative stress and secondary metabolisms. A group of proteins was classified as “sweet orange proteins” whose functions are still unknown. Finally a large number of spots were found to correspond to the peptide fragments of the glycoprotein Cit s1, an allergen among *Citrus* species (Poltl et al.,2007).
Conclusions: As main results we found that four spots reaching the 15 % of expressed proteins, among them we found a great represantion of peptides fragments from Cit s1 glycoprotein. This protein was previous found in the orange juice, suggesting that its synthesis occurred in the fruit endocarp (fruit pulp); conversely, our finding indicated that the synthesis of Cit s1 could take place in the peel, migrating in the juice following the crushing of the fruit. Suggestions how obtained juice with low allergen concentration will discuss.

O 015

PROTEOME ANALYSIS OF CITRUS SINENSIS L. (OSBECK) FLESH AT RIPENING TIME

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Background: Citrus has a non-climacteric fruit ripening behavior and an original anatomical fruit structure. The pericarp consist of the external part, the epicarp or flavedo, and the clear inner portion, which is the mesocarp or albedo, constituting with the flavedo the 'peel'. The flesh juice sacs accumulate sugars and organic acids; most of the tissue is rich of water, causing difficulties in the extraction of nucleic acids and proteins. At the ripening time –the flesh of blood oranges (Moro, Sanguinello and Tarocco) is characterized by the synthesis and the accumulation of anthocyanins. Generally, the ripening process includes combinations of changes in cell-wall composition, in acidity and soluble solid concentrations in flesh colour, thanks to accumulation of anthocyanins (in blood cultivars) and in volatile aroma compounds. These changes make fruits soft and sensitive to pathogens attack. Even if the characterization of proteins isolated from Citrus flesh tissue would represent a powerful tool to deepen the omics view on citrus fruit metabolism, only few proteomics data are available.
Methods: Fruits of Moro, a blood cultivar and Cadenera, a common orange, were sampled at ripening time. Protein extraction was performed following a phenol extraction protocol. To define the protein expression patterns, we performed 2-DE associated to quantitative image analysis. Three replicate gels for each of the three different extract were processed concurrently with the PDQuest software (Biorad). More than 800 spots were detected in both proteomic samples. Statistical analysis allowed the identification of significant differences in the proteomic profile: proteins with a minimum of 2-fold differential expression were subjected to further analyses. Each differentially expressed spot was excised from polyacrylamide gels after Coomassie Blue-stained from gel. Spots were digested with trypsin and analyzed by capillary RP-HPLC/nESI-MSMS (Ultimate 3000 LC system, Dionex Corporation; LTQ, Thermo Fischer Scientific). Protein identification was performed by searching peptide sequences obtained by MSMS data against NCBI non-redundant protein databases on all available higher plant proteins (*Viridiplantae*) and EST database (dbESTs). All identifications obtained by means of MASCOT software were confirmed by manual interpretation of the tandem mass spectra.
Results: Among the 64 spots differentially expressed (39 in Cadenera and 25 in Moro), 55 spots, corresponding to 49 different proteins, were identified by searching both protein and EST database. Proteins were manually annotated using published references. It seems that the most important difference between blood and common oranges relates to the anthocyanins content. More than one third of the varying protein spots (Sugar metabolism, Secondary metabolism, Oxidative process) characterize blood oranges, thanks to anthocyanin pathway. The most abundant spots isolated in Moro concerns Sugar metabolism. These data confirmed the sugar accumulation, that is more abundant in blood oranges than in common ones. In relation to Secondary metabolism, the chalcone synthase (CHS) and the flavanone 3-hydroxylase (F3H) were included in the early steps of the anthocyanins pathway. Among Oxidative process, the isolation of the APX (Ascorbate peroxidase) in Moro justify the effect of anthocyanins, that as antioxidants, interrupt the cascade of uncontrolled oxidation in each organelle. Proteins related to defence and stress response are correlated to common oranges.
Conclusions: A proteomic approach was conducted to analyze the most abundant proteins over-expressed in Moro and Cadenera flesh at ripening time. This is the conclusive work of a complementary study in which differential ESTs isolated in Moro and Cadenera flesh at ripening time, using a SSH library, were subsequently spotted in a custom microarray and hybridized with RNA of the same samplings during three maturity stages. Among the 82 differential genes isolated through the SSH library, only 7 were identified as proteins: pectinesterase, F3H, CHS, succinyl CoA beta subunit, valencene synthase and PEP carboxylase. The remaining proteins over-expressed in Moro and in Cadenera were not isolated as transcripts, and *vice versa*.
Studies indicate that a notable change in protein expression occurs in Moro and Cadenera suggesting that anthocyanins, and all correlated pathway, differ between blood and common oranges. When comparing our results with those of previously published transcriptomic studies, some discrepancies were noted, confirming the necessity to compare these two different sets of data to obtain a full view of a biological pathway.

O 016

THE APPLICATION OF A MULTIVARIATE STATISTICAL APPROACH TO ISOLATE PROTEINS USEFUL IN DEFINING GRAPE SKIN RIPENING

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Background: Ripening is the last period of berry development and starts at the end of a lag phase in which there were no modifications of berry volume and weight. During this period, several changes take place: berries soften because of structural modifications of the cell walls and there is an upsurge in the levels of many metabolites, moving from the accumulation of glucose and fructose and the decline of malic acid to those compounds of the secondary metabolism that are present in really low quantities.
The exocarp, simply called “skin”, the outer component of the berry, represents a physical barrier between the external environment and the inner tissues and is the site of synthesis of exclusive compounds, such as anthocyanins in red cultivars. Moreover, its importance is underlined by a previous work that compared the main mature berry tissues at the transcriptomic level and underlined the high number of genes expressed in the exocarp and that the profile of the skin is the most peculiar fingerprint of the global set.
In order to plan an adequate winemaking and to improve the overall wine quality, suitable devices useful to precisely describe grape composition are needed. Parameters such as sugars, organic acids and potassium, became useful and cheap devices for the definition of the ripening degree but more accurate and reliable markers are required, especially to pinpoint other crucial aspects for winemaking such as aromatic and phenolic potentials. In the last few years, proteomics turned out to be a suitable tool in the search for candidate biomarkers, especially in the clinical field, employing in a growing number of cases multivariate statistical techniques that can clearly represent the overall structure of the dataset achieving at the meantime the elimination of the contribution of the experimental error. In order to isolate a panel of proteins that could be tested as ripening markers, we carried out a comparative 2-DE analysis among skins of *cv* Pinot Noir grapes collected in three different moments from véraison to full maturation.
Methods: The study was conducted considering skin samples collected at véraison, at middle maturity and at full ripening. After extraction, proteins were separated by two-dimensional electrophoresis (2-DE), using a pH 3-10 linear electrofocusing gradient in the first dimension and 12.5% polyacrylamide homogeneous gels in the second dimension. The analysis of the gels stained with cCBB detected about 1000 spots/gel. After ImageMaster® Platinum 6.0-assisted gel alignment, the spot volumes dataset was analyzed by Principal Component Analysis (PCA) coupled to a multivariate classification tool called Forward Selection LDA (FS-LDA). The robustness of the discrimination models was verified by leave-one-out cross-validation. The characterization of the most significant spots was carried out by means of LC-ESI-MS/MS.
Results: The application of FS-LDA performed on PCA scores allowed to discriminate the samples and to arrange the spots according to their significance in the derived models. In particular a clear separation does exist between the patterns relating to the first two moments of ripening and the one relating to mature berries.
Thirty-six spots among the most significant ones in the three models were identified. Many of the proteins with higher discriminating power are known to be involved in biotic and abiotic stress responses (e.g. chitinases, beta-1,3-glucanases, PR-10, PPO). Along with these classes of proteins, some spots relating to processes of the primary metabolism, such as glycolysis (e.g. enolases, glyceraldheide-3 phosphate dehydrogenase) and Krebs cycle (e.g. aconitase and fumarate hydrolase), were identified.
Conclusions: Though the employment of FS-LDA it was possible to correctly distinguish the three samples and it was remarkable the fact that the proteome of mature berry skins was clearly different respect to those collected in the previous moments, suggesting that also in a continuous process such as ripening it could be possible to isolate protein markers for the last phases of grape development. The fact that among the most significant spots there were proteins involved in the response to biotic and abiotic stresses fit with previous observations indicating that the expression of these enzymes is often developmentally regulated and that they are also present in healthy samples. In order to understand if they can be tested as candidate biomarkers, it will be necessary to perform further analyses that should take into account berries collected during several vintages. On the other hand, the characterization of enzymes related to the primary metabolism suggested that their expression during ripening in the skin could be required in order to sustain several biosynthetic activities of this tissue.

O 017

TOP DOWN PROTEOMICS WITH A QTOF INSTRUMENT AND COLLISION INDUCED DISSOCIATION

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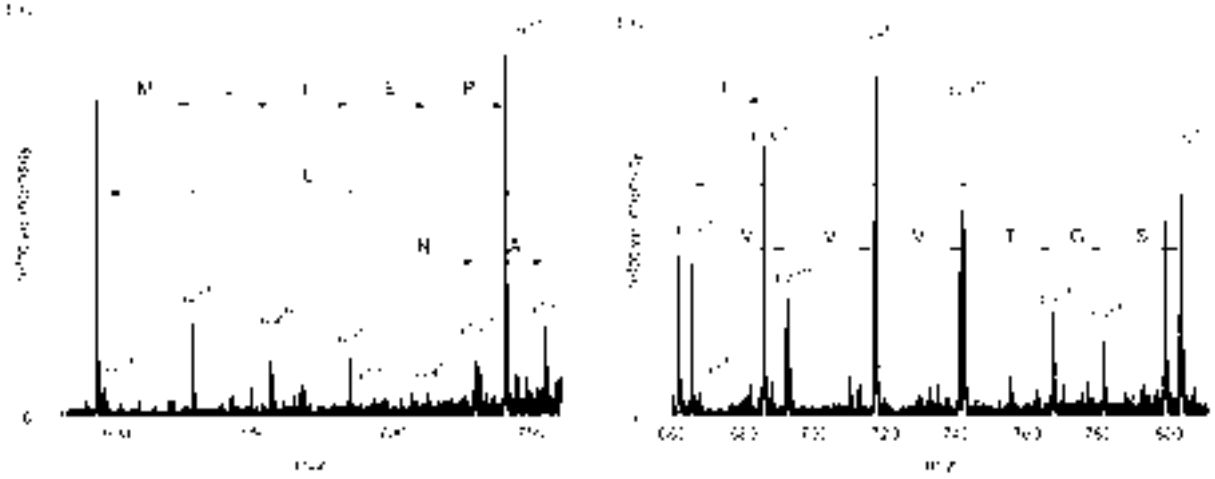
INTRODUCTION Top-down mass spectrometry has become, over the last years, more than just a promising technique for protein analysis and it is now a routine approach in proteomics. This technique does not need the protein to be purified and does not require digestion. Unfortunately, top down proteomics is very demanding in terms of instrumental setup, because only high-end instruments (FTICR or Orbitraps) allow resolution of spectral peaks of very high charge states. In addition, routine top-down analysis is performed using ETD or ECD techniques. With slight modifications of the instrumental parameters, we demonstrate nevertheless that satisfactory top-down data can be obtained with CID tandem MS on a QTOF instrument not originally designed for this purpose.
INSTRUMENTAL AND **METHODS** Experiments were carried out on a QSTAR quadrupole-TOF mass spectrometer equipped with a Nanospray ion source. Proteins were dissolved at a final concentration of 100 fmoles per microliter. Spectra were recorded in positive ion mode.
Results Protein identification is achieved with both N and C terminal sequence tags, obtained as y or b type ions (see Figures 1 and 2 respectively) and BLAST database searches. The accurate mass measurement of multiply charged fragment ions (mostly y and b-type), in combination with the FindPept tool hosted on the Expasy server, supplements the limited set of cleavage sites and provides an high degree of sequence coverage (90-100%). Post translational modification issues can be addressed too.



Conclusions This approach might help those MS core facilities that are not able to afford very high resolution instruments, thus expanding the benefits of top down protein analysis over the worldwide MS community. The effective operative resolution of the instrument used for this work rarely exceeds 8000 (against 100000 of a FTICR-MS) and charge states higher than six/ seven are often impossible to assign. On the other hand, the cost of this instrument is by far lower than that of a FTICR-MS or an Orbitrap. Our method allows TD proteomics in a pure MS/MS philosophy, with intact proteins, not fragments, used as precursor ions. Our results were indeed obtained avoiding the well known preliminary “in-source” fragmentation of the protein followed by MS/MS analysis of a fragment ion. We believe therefore that the critical advantage of our method resides in giving the opportunity to extend the benefits of TD mass spectrometry to potentially hundreds of MS core facilities all over the world, perhaps opening new opportunities for biomedical scientists.

O 018

CHEMICALLY MODIFIED MESOPOROUS SILICA BEADS: A NEW EFFICIENT AND RELIABLE PLATFORM FOR BODY FLUIDS PROTEOMICS

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Background: Promising profiling techniques based on new material/solid phase extraction for capturing "molecular signatures" from body fluids are being coupled to matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/MS). Sample preparation significantly influences spectrum quality in this ionization method. We have recently developed a platform based on mesoporous silica beads (MSB) for plasma high abundant proteins depletion. Tuning the pore size distribution and surface adsorptive properties these substrates selectively capture peptides and small size proteins excluding large size proteins such as Human Serum Albumin. Plasma samples are exposed to beads, captured molecular species are extracted and then profiled by MALDI-TOF MS. Given the high surface area, MSB offer the desired adsorptive capacity for binding and enrichment of low abundance peptides present in body fluids. The absorption properties of mesoporous silica can be modified since the pore walls exhibit a high concentration of silanol groups on the surface, which can be functionalized with different chemical species.
Methods: Aminopropyl, N-(2-Aminoethyl)-3-aminopropyl and N, N, N' tris-carboxymethyl ethylene diamine (TED), have been introduced onto mesoporous silica surfaces in order to selectively modulate peptide enrichment. Different conditions for adsorption/binding and elution of plasma and urine peptides have been accurately evaluated for each functionality to determine the best performances in terms of peptide extraction and MALDI data generations.
Results: The use of MSB appears to provide sufficiently rich fingerprints of plasma and urine, as revealed by comparing mass spectra of unprocessed plasma and urine to mass spectra generated after MSB processing.
All MALDI spectra were generated using the same settings in the range from 800 to 10000 Da, imposing the stringent condition of a signal-to-noise ratio of higher than 10 for peak detection. Apart from some peak clusters related to the matrix CHCA used in MALDI analysis in the range between 800 and 1100 Da, the most of the detected signals for all the bead functionalities were in the range from 1100-5000 for plasma and 1100-6000 for urine, exhibiting some sporadic high intensity peaks outside this range. Aminopropyl derivatized MSB produced the highest number of peaks for plasma samples, whereas for urine the largest number of peaks were obtained with TED functionalized MSB. Derivatization of mesoporous silica materials with the aminopropyl group increases specific plasma peptides subset recognition in comparison to non functionalized mesoporous silica materials. At the same time, TED groups on the mesoporous silica surface significantly amplify peak detection for urine peptides. Moreover the reproducibility of sample preparation by different functionalized beads was evaluated *via* three replicate analyses of plasma and urine samples. Lower coefficients of variation in the mass values and peak intensities resulted for plasma in comparison to urine samples, nevertheless these where satisfactory for diagnostic purposes.
Conclusions: The data obtained strongly suggest that, besides functionalization, tuning the pore size, pore volume and the surface area of mesoporous silica may also be a viable approach to biomarker harvesting. Functionalized MSB can be readily prepared by a simple and low-cost procedure; the method described is fast and easy to perform for processing body fluids prior to MS analysis, thus minimizing the possible degradation of biological samples. Future development of our technology along this line will include surface functionalization with alkyl and aryl groups, which could increase the hydrophobicity of the mesoporous silica and allow the selective capture of hydrophobic peptides for mass spectrometric assay.

O 019

GLOBAL COMPARISON OF SECRETOMES FROM MULTIPLE PANCREATIC CANCER CELL LINES BY A LABEL-FREE MASS SPECTROMETRY-BASED PROTEOMIC WORKFLOW

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Background. The cancer *secretome* represents a rich repository where to mine potentially useful information for both cancer biology and clinical oncology. Proteins *abnormally* released by malignant cells can in fact influence tumor growth, invasion and metastasis. Exploring the cancer secretome with a hypothesis-free global proteomic approach should help identify patterns of secreted protein representing recurrent *signatures of malignancy,* or else patterns that are unique to cancer cell subsets. This may help 1) unravel the complex mechanisms of tumor growth and spread, 2) find new *therapeutic targets,* or 3) discover new candidate *prognostic or predictive cancer biomarkers*. The *early discovery phase* of secretome mining must rely on a simplified proteomic workflow. An ideal method should 1) be applicable to multiplexed sample sets, 2) avoid expensive reagents and complex procedures, 3) require a reasonable amount of laboratory work and instrument run-time, and 4) produce large amounts of proteomic information which is based on *high-quality protein identification* and offers semi-quantitative information. In this study a mass spectrometry based workflow has been set up and applied to the characterization and comparison of secretomes from multiple pancreatic adenocarcinoma cell lines. There is in fact an urgent need for early detection markers and novel therapeutic targets for pancreatic cancer, a rapidly lethal disease often escaping early diagnosis.
Methods. Four human *pancreatic ductal carcinoma* cell lines (PT45, Panc1, MiaPaCa2, Aspc1) and the immortalized human *normal pancreatic ductal epithelial* cell line HPDE6 were grown to 90% confluence in RPMI 1640 with 5% FBS. After 7 wash steps, cells were incubated in serum-free RPMI for 24 h and the conditioned medium (CM) collected and pooled from different flasks, filtered (0.2 µm), then concentrated and buffer-exchanged by ultrafiltration (5KDa MWCO). Proteins (20 µg per cell line) were separated by 1DE. Each gel lane was cut into 24 bands and in-gel digested with trypsin. An aliquot (1/10) of the digests was analysed by liquid chromatography-tandem mass spectrometry. MS spectra were acquired by an LTQ Orbitrap XL with resolution 60,000, in parallel with low-resolution MS/MS scans of the four most abundant precursor ions being acquired in the LTQ, excluding singly-charged ions. For each cell line, the MS/MS data from the 24 bands were analyzed —both separately and after merging— by Mascot against Swiss Prot database performing at the same time a decoy search. After results filtering (see Results), validated proteins were listed with their Mascot's emPAI value as an approximate index of the relative amount of each protein. Pathway and functional analysis with different bioinformatics tools (e.g. GeneGO's Metacore, DAVID, etc.) is ongoing.
Results. The workflow we have set up includes protein fractionation by 1DE gels, protein identification by in gel-digestion, analysis of tryptic peptides by LC-MS/MS, relative quantitation of proteins by the emPAI index, and pathway/functional analysis by bioinformatics tools. Proteome-scale experiments using MS allow to identify thousands of proteins but since the aim of this exploratory survey, more than to simply generate catalogs of proteins, was to highlight dysregulated processes/pathways, much effort was put into maximizing the confidence of protein identification. The MS/MS analysis gave a mass accuracy stably <2 ppm. A first Mascot search with stringent parameters lead to the identification of 1700-2200 proteins per line. These were reduced to 300-500 proteins by applying post-search filtering to all analyses using strict probability thresholds leading to a final False Discovery Rate <0.15%, and listing as valid only proteins with at least two high-confidence (99.9%) peptides. Only the proteins (about 90%) further validated by Spectrum Mill, another search engine based on a different algorithm, were left in the final list. The final high-confidence identification list comprised a total of 800 unique proteins. The emPAI values obtained showed a 3-order of magnitude range (range 0.01-70, with most proteins found between 0.1 and 10). The overall reproducibility of the procedure (from CM collection to MS/MS analysis) was warranted by the low emPAI variation across the 5 lines (CV=13%) observed for the residual bovine serum albumin (BSA). Among the 800 unique proteins, the 140 proteins common to both normal epithelial and all the four cancer cell lines, were for the majority comparably expressed (a CV ranging from 12 to 50% was observed for 60 of these proteins). A small subset (n=9) of proteins were *absent in HPDE6* but stably secreted by *all four* cancer cell lines. For example, emPAI (mean ±SD) for Vimentin, CD9 and Myosin-9 were 17±4; 0.4±0.1; 0.4±0.2. Another large protein group included the 90 proteins *exclusively secreted by HPDE6*, which may represent a functionally interesting set to investigate processes that are underrepresented in malignant cells. Each of the cancer cell lines also secreted unique proteins (a few dozen for each cell line).

Conclusions. The simplified proteomic workflow presented here allows a first-round comparison of different secretomes with bioinformatics tools for pathway and functional enrichment analysis. In this first application we indeed found not only expectedly dysregulated proteins, but also novel potential signatures of malignancy that appear to be worth a more in-depth analysis. Among other benefits, our approach allows to revisit — from a hypothesis-driven perspective— the large amount of data still "hidden" in the raw LC-MS/MS results, as well as in the single-band identifications. The sensitivity of our method also allows to re-analyze the remaining stored digests several times by using the mass-exclusion approach, which can allow to mine secretome at a deeper level.

O 020

TITANIUM DIOXIDE COATED MALDI PLATE FOR ON TARGET ANALYSIS OF PHOSPHOPEPTIDES

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Background: reversible phosphorylation of proteins is an important regulatory mechanism that occurs in all organisms. As a branch of proteomics, phospho-proteomics involves the analysis of protein phosphorylation and makes use of mass spectrometry (MS) and specific methods to purify phosphorylated proteins and peptides. The main goals of a phospho-proteomic experiment are the enrichment of phosphate-containing proteins or peptides and their characterization by MS. Recently it has been shown that titanium dioxide has a peculiar affinity for the phosphate group and this property has been exploited in phosphorylated proteins enrichment. Nanostructured titania could be an ideal candidate for phosphorylated protein enrichment before MS analysis. Pulsed Laser Deposition (PLD) is a versatile technique for the deposition of nanostructured films even with complex stoichiometry and with a fine control on the material properties. Our objective goes in the direction of simplifying phosphopeptides analysis procedures providing a unique tool, a Titanium dioxide coated MALDI plate, designed to permit enrichment and detection with the same platform, eliminating tedious washing and centrifugation steps, speeding up analysis and giving at the same time a high sensitivity due to the benefits given by the use of a nanoscaled technology.
Methods: α- and β-casein, RNase, BSA, and myoglobin were reduced, alkylated and digested using trypsin. Matrix-assisted laser desorption ionization (MALDI) mass spectra were acquired on a Voyager DE-STR time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA). A 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA) was used to perform MS/MS experiments were in reflectron mode with CID gas on. Standard stainless steel MALDI TOF and MALDI TOF/TOF plates were coated with titanium oxide thin films (200 nm thickness) by pulsed laser deposition. The stainless steel deposition chamber was equipped with a turbomolecular pump connected to a primary pump and with a gas inlet system with pressure gauges for fine pressure control from high vacuum (10-5 Pa) to the atmosphere. Here a Ti target was ablated in 20 Pa O_2 pressure exploiting UV laser pulses (duration ≈ 7 ns) from a quadrupled Nd:YAG laser (λ) 266 nm, 10 Hz repetition rate). Then 2600 laser pulses were focused on the target with an energy density (fluence) of about 3 J/cm2. Titanium oxide was deposited at room temperature and subsequently annealed in air at 400°C or 800°C for 1 h. A copper grid was applied to the MALDI plates for titanium dioxide deposition in specific wells.
Results: the tool we developed consists in a new MALDI plate which is selectively coated with a titanium dioxide film with a thickness of about 200 nanometers. This support (T-plate) works both as a device for the purification and for the analysis of phosphorylated and non-phosphorylated peptides. Performances of this coated MALDI plate in the enrichment of tryptic phosphopeptides were tested by using α- and β-casein (two standard phosphorylated proteins) in complex mixtures together with non-phosphorylated proteins. The experimental strategy consisted in depositing a small amount of this mixture containing both phosphorylated and non-phosphorylated peptides onto the T-plate. As only peptides containing phosphogroups interact with the Titanium dioxide coating, after washing away from the surface the non-specific bound peptides, only the species of interest are detected by MS. Part of the work consisted in the optimization of the enrichment and analysis conditions, to reach high selectivity and good sensitivity towards mono- and multi-phosphorylated peptides.
Conclusions: a new MALDI target, called T-plate, was produced by exploiting pulsed laser deposition of a nanostructured titanium dioxide thin film onto a stainless steel plate. The advantages of using the T-plate involve practical use, fast experimental steps and the possibility of using the same active surface many times. The compatibility with a MALDI-TOF/TOF instrument could then open the perspective of using it for the identification of phosphosites in complex biological samples, exploiting the high mass accuracy in the MS/MS mode and the possibility of coupling an LC device for the separation and automated deposition of sample fractions.

POSTERS' ABSTRACTS

001

DIFFERENTIAL PROTEIN EXPRESSION IN CYTOKINE-SENSITIVE AND -RESISTANT PANCREATIC BETA CELL LINES.

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Background: Type 1 diabetes (T1D) is a chronic disorder that results from the immune-mediated destruction of insulin-producing β-cells of the pancreatic islets. Cytokines, which are released by mononuclear cells infiltrating the islets, affect β-cells at several levels. In rodents, β-cell killing is due to the synergistic effects of proinflammatory cytokines (IL-1β, IFN- β, TNF-β and IL-6), which may induce cell necrosis or apoptosis. In human a major cytokine-mediated effect on pancreatic islets is β-cells apoptosis. It is well established that cytokine-induced β-cell destruction is mediated by a complex network of intracellular signalling cascades. However the exact mechanisms and pathways involved in the vulnerability of pancreatic beta cells are still unclear. The aim of our study was to discover cytokine-mediated changes in protein expression profiles in resistant *vs.* beta cell sensitive lines. To this purpose a well standardized mouse beta cell line (βTC3, kindly provided by S. Efrat, Tel Aviv University, Israel) was used.

Methods: βTC3 cells were cultured in high glucose DMEM, 15% FBS, L-glut and antibiotics. Cytokine resistance was induced after prolonged treatment (12 weeks) of βTC3 cells with a cytokine cocktail (100 IU/ml of IL-1β and 100 IU/ml of IFN-β). Cells were harvested when reached 80% confluence. Resistant βTC3 cells were characterized by higher viability and proliferative capability vs sensitive-βTC3 and were apoptosis-resistant when treated with high IL-1β concentrations (250-1000 IU/ml). Proteomics preparation was performed according to our standard protocols (Pucci-Minafra et al. Proteomics 2002/2004/2006). Silver stained gels were analyzed with ImageMaster 2D Platinum software. Quantitative variations in protein expression levels were calculated as the volume of the spots (i.e integration of optical density over the spot area). In order to correct for differences in gel staining, spot volumes relative to the sum of the volume of all spots on each gel (%Vol) were calculated by the software. Protein identification was performed by Mass spectrometry on a Voyager DE-PRO mass spectrometer (Applied-Biosystems) after in-gel digestion of protein spots, using sequencing-grade trypsin (20 μg/vial). The matrix was 2,5-Dihydroxybenzoic acid from Fluka. The mass spectra were recorded in the 500-5000 Da range, using a minimum of 100 shots of laser per spectrum. Delayed extraction source and reflector equipment allowed sufficient resolution to consider MH^+ of monoisotopic peptide masses. Internal calibration was done using trypsin autolysis fragments at m/z 842.5100, 1045.5642 and 2211.1046 Da. Peptide mass fingerprinting was compared to the theoretical masses from the SwissProt or NCBI sequence databases using Mascot (http://www.matrixscience.com/).

Results: Proteomic maps of duplicate experiments showed both qualitative and quantitative differences in protein profiles of cytokine-resistant βTC3 cells *vs* the sensitive ones, likely corresponding to a significant group of strategic genes. Differentially expressed protein spots, including isoforms, were 86 corresponding to 59 individual gene products. Among these, significant differences were observed in a subgroup of 42 proteins, 35 being upregulated and only 7 downregulated. Interestingly the majority of upregulated proteins included glycolytic enzymes (ALDOA, TPIS, G3P, PGK, KPYM, ENOA, LDHA and LDHB). Specific components of the catabolic protein machinery (UBIQ, CATD), a member of the annexin family, a galectin protein family member (LEG1), 2 members of the S100 calcium-binding protein family (S100A6, S100AB), 5 members of redox protein family (GSTP1, GSTO1, PRDX1, SODC), cell-cycle and biosynthesis regulators (NDKA, NDKB, EF1B, DDAH1), chaperone/heat shock proteins (PDIA3, HSP71, GRP78) resulted also up-regulated.

Conclusions: The proteomic approach to comprehend cytokine-mediated β-cell cytotoxicity presented here may be useful for identification of relevant mechanisms for understanding β-cell death mechanisms after cytokine exposure. Making the β-cell more resistant to mediators of the immune system may be used for prolonging the survival of transplanted islets or engineered β-cells in Type 1 diabetes, and potentially prevent the ongoing β-cell destruction in predisposed individuals.

002

IDENTIFICATION OF PROGNOSTIC MARKERS FOR NEPHROPATHY IN URINE FROM CHILDREN AFFECTED BY TYPE-1 DIABETES

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Background. Type-1 diabetes is one of the most common autoimmune diseases in the world, with a genetic and an environmental contribution. Frequently, this pathology is accompanied by renal, ocular and cardiovascular complications. This study aims at establishing early determinants of renal function decline in type-1 diabetes. Diabetic nephropathy affects approximately 30% of type-1 or type-2 diabetic patients and takes several years to become clinically evident. Microalbuminuria, the first sign of nephropathy, usually can be detected after 10-15 years of diabetes. Although it is reasonable to suppose that, in predisposed patients, the pathologic mechanisms leading to nephropathy start acting since the onset of diabetes, we have presently no tools allowing to identify in advance the patients predisposed to this diabetic complication.
Methods. Urine from children affected by type 1-diabetes, with altered renal volume but normal albumin excretion rate, are investigated to identify prognostic markers for diabetic nephropathy. Twenty-four patients (10 males and 5 females, years 0-10; 6 males and 3 females: 11-15 years) and thirty-five healthy volunteers (15 males and 10 females, years 0-10, mean 7 years ; 6 males and 4 females: 11-15 years, mean 13 years) have been enrolled. 20ml of urine are concentrated and depleted of the most abundant proteins using Genway IgY-12 column. The flow through proteins are precipitated with acetone/methanol, washed with acetone/methanol and then loaded on Immobiline Dry strip pH 4-7 NL, 13 cm for the first dimension and an SDS-PAGE gel 12.5% for the second dimension. Images of Silver stained gels are acquired using ProXPRESS instrument and Same Spots software for the statistical analysis. Each sample is run in triplicates and every single patient is compared to a pool of urine derived from donors matched per age and sex. Differentially expressed proteins (threshold= 1.5 fold, $p<0.05$) are identified by mass spectrometry analysis of the tryptic digests. Validation data are obtained by Western Blotting (WB) analysis.
Results. Preliminary data on the first analysed group of patients (males, 11-15 years) show that some proteins are commonly regulated in the urine from different patients compared to healthy controls. Among the others, we validate the identification of two proteins by WB: zinc alpha-2 glycoprotein (ZAG) and gelsolin and, by densitometry, we get a semi-quantitative evaluation of their increase in patient urine using creatinine value as normalizer. Considering what is known from literature, these two proteins are particularly interesting and promising. ZAG is a 33-kDa single polypeptide chain. It is synthesized in the epithelial cells of prostate gland and liver and subsequently secreted into various body fluids such as serum, seminal plasma, sweat, saliva, cerebrospinal fluid, milk, urine and amniotic fluid. One of the main functions of ZAG is in the depletion of fatty acids from adipose tissues, and hence, it is also known as a lipid-mobilizing factor. It has already been found upregulated in the vitreous fluid of patients with proliferative diabetic retinopathy and in mice with diabetic nephropathy. As regards gelsolin, our hypothesis is that its upregulation in urine from patients can be the result of podocyte injury, which is a characteristic defect of the glomerular filter in diabetes. Normally, podocytes, which are specialized epithelial cells that cover the outer layer of the glomerular basement membrane, form the final filtration barrier to protein loss, so that a podocyte defect may determine an increased glomerular permeability, leading to the excretion of proteins in amounts not found in normal urine.
Conclusions. Proteomic analysis can be a promising tool for the identification of early biomarkers involved in the pathogenesis of diabetic nephropathy. Further studies are needed to address the precise role of our candidate proteins and their potential as prognostic markers.

003

A PROTEOMIC APPROACH TO THE STUDY OF CORNELIA DE LANGE SYNDROME

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Background: Cornelia de Lange Syndrome (CdLS) is a rare multisystem congenital disorder with a prevalence estimated as high as once per 10000 births. The phenotype is clinically eterogeneous and characterized by facial dysmorphia, upper limb malformations, growth retardation and neurodevelopment delay, from moderate to severe. The disease can be caused by mutations in the evolutionarily conserved components of the cohesin pathway, that mediate cohesion between replicated sister chromatids in dividing cells, thereby enabling a proper chromosome segregation and post-replicative DNA damage repair. Current studies also revealed that changes in gene expression by transcription regulation in post-mitotic cells could account for variable deficits of Cornelia de Lange patients, but this mechanism remains to be elucidated.
About half of the patients with CdLS carry mutations in the NIPBL protein, a regulator of cohesin loading and unloading onto DNA; while mutations in two cohesin structural components SMC1A or SMC3 (Structural Maintenance of Chromosomes 1A and 3) contribute to the 5% of cases and result in a mild phenotype, with absence of the major abnormalities.
Up to date the etiopatology of CdLS has mostly been investigated at genomic level and, to our knowledge, this is the first attempt to characterize CdLS by a proteomic approach.
Methods: Here we compared lymphoblastoid cell lines from CdLS patients affected by different mutations in SMC1A and SMC3 genes with healthy control individual cells, using the quantitative 2D-DIGE analysis. In DIGE gels we combined seven CdLS patients and seven control individuals according to age and type of mutation. Moreover, to avoid staining bias, CdLS proband and control individual samples were alternatively labelled with Cy3 and Cy5 fluorophores. The results were obtained using the Decyder 2-D Software v 7.0.
Results: Several differences were visualised and identified using MALDI-TOF MS and ESI-IonTrap. Many interesting proteins with defective expression in CdLS patients were related to glycolysis pathway, nucleotide catabolism and salvage, cytoskeleton and chromatin organization.
Conclusions: Further analyses, such as immunoblotting and functional assays, will be applied to validate our data. The identified proteins may shead new light on the functional role of cohesin subunits and their implication in Cornelia de Lange Syndrome onset.

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004

DETECTION OF TRIM50 INTERACTING PROTEINS TROUGH PROTEOMIC APPROACHES

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We recently showed that the protein encoded by the *TRIM50* gene was part of the E3-ubiquitin ligase family, peptides that catalyze the transfer of ubiquitin moieties to specific substrates. *TRIM50* is hemizygous in the Williams Beuren Syndrome (WBS), a contiguous genetic disorder characterized by a number of physical and developmental disabilities. WBS is caused by a 1.5 Mb deletion at 7q11.23 that include at least 25 genes. The proven haploinsufficiency of *TRIM50* open the interesting hypothesis that the ubiquitin-mediated proteasome pathway might be involved in the WBS phenotype. The TRIpartite motif (TRIM) proteins harbour from their N- to their C-terminal end, a RING domain, one or two B-boxes (B) and a predicted Coiled coil domain (CC). Trim50 has a RING domain which is a zinc-binding motif located at the N-terminal portion of almost all TRIM proteins. This domain has now been shown to confer E3 ubiquitin ligase activity, which allows to mediate ubiquitylation events. The B-box domains are almost always followed by a coiled-coil domain, which is a motif found in many proteins families. This domain mediates homomeric and heteromeric interactions among TRIM family members and other proteins, in particular self-association.
To get insight on its role and to identify putative TRIM50-interacting peptides we performed fluorescence and electronic microscopy and proteomic approaches.
By using indirect fluorescence and electron microscopy, we founded that TRIM50 protein localizes in highly mobile, labile and dynamic cytoplasmic bodies. CLEM-microscopy showed that it localizes in the multi-vesicular protein structures resembling the autophagosome. Interestingly, preliminary results showed that TRIM50 is part of cellular structures, such as aggresomes, involved in stress cellular processes.
Next, we focused our efforts to purify and identify Trim50 protein partners by using two proteomic approaches combining immunoprecipitation assays of tagged Trim50 with advanced mass spectrometry technologies. For immunoprecipitation assay HEK293T cells were stable transfected with p3XFLAG expression vector encoding the 3XFLAG-TRIM50 cDNA. HEK293T cells transfected with FLAG-TRIM50 and the specific protein complexes formed *in vivo* were immunoprecipitated using anti-FLAG antibodies. The complex protein components were separated by SDS-PAGE and stained with Colloidal Coomassie. The bands of interest will be excised from the gel, *in situ* digested and the resulting peptide mixture analysed nanoLCMSMS methods. The partial sequence information obtained from just one peptide is often sufficient to identify a protein by searching protein and expressed sequence tag databases. These results should allowing us to clarify the various roles played by Trim50 in the WBS pathology.
Next step of the study will be the identification of TRIM50-substrates aimed to characterize multiprotein complexes and signaling pathways where the protein could be involved and ultimately the role of this gene in WBS.

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005

THE PROTEOME OF PLATELETS IN PATIENTS WITH CORONARY ARTERY DISEASE.

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Background: Coronary atherosclerosis (CAD), a chronic inflammatory disorder, arises from the interaction of conditions such as dyslipidemia, hypertension, diabetes, environmental factors and genetic susceptibility. Although CAD can remain stable for many years, occlusive coronary thrombus formation at the site of atherosclerotic plaque rupture may lead to unstable angina or myocardial infarction (acute coronary syndrome, ACS). Platelets can play a role in the initiation, development, and progression of CAD. However, it is still not known whether CAD causes specific changes in the expression/distribution or amount of platelet proteins. Thus, in this study we investigated the protein patterns of platelets isolated from patients with coronary atherosclerosis (CAD).

Methods: The study population consisted of twenty-six patients with CAD (n=14 with non-ST-elevation acute coronary syndrome with a final diagnosis of non-ST-elevation myocardial infarction, NSTEMI, and n=12 with stable angina patients). Proteomic studies of platelets were performed following the suggestions of the Platelet Physiology Subcommittee of the International Society on Thrombosis and Haemostasis Scientific Standardisation Committee, with every effort being made to avoid any errors that could compromise the data; in particular platelet activation was minimised during blood collection and subsequent cell isolation, as was contamination with other cells: the studied samples contained <5 leukocyte/10^5 platelets, as assessed by cell counting and flow cytometry.

Results: Two-dimensional electrophoresis coupled to mass spectrometry allowed the identification of seven proteins differentially expressed: two involved in energy metabolism (2-oxoglutarate dehydrogenase, OGDH, and lactate dehydrogenase, LDH), three associated with cytoskeleton-based processes (γ-actin, coronin 1B, and pleckstrin), one involved in the platelet aggregation (thrombospondin-1, TSP-1), and one in protein degradation process (proteasome subunit type 8, PSB8). OGDH and a cleaved form of γ-actin were significantly higher in platelets of patients than in controls, whereas LDH was higher only in platelets of patients with acute coronary disease. Coronin, TSP-1, and PSB8 were less expressed in platelets of patients, as was for the basic isoforms of pleckstrin. Measurements of the enzymatic activities of OGDH and LDH showed that the increases in protein expression levels are paralleled by changes in their functional activities.

Conclusion: This study revealed that platelets proteome is altered during CAD and allowed the identification of protein changes not previously connected with CAD: the increased expression and activity of enzymes involved in the glycolysis and oxidative phosphorylation and the alteration of proteins associated with cytoskeleton-based processes are indicative of platelet activation.

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006

PROTEIN PHOSPHORILATION IN RENAL CELL CARCINOMA STUDIED BY PROTEOMIC APPROACH

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Background: Phosphorylation is an heterogeneous reversible process that occurs in one-third of all proteins in eukaryotic cells and is one of the most abundant post-translational modifications (PTMs). This transient and highly dynamic event, regulated by kinases and phosphatases causing modifications in folding and functions of proteins, protein localization, complex formation, and degradation, can be studied by proteomics tools. In our previous study among several proteins identified in renal cell carcinoma (RCC) we found down-expression of mitofilin, a critical organiser of mitochondrial cristae morphology. This protein showed in two-dimensional gel electrophoresis (2-DE) a train of different isoforms at experimental pI of 5.50-5.95 and 65-82 kDa Mw ranges. The aim of the present work was to detect phosphorylated mitofilin isoforms to highlight the possible correlation between mitofilin expression and phosphorylation in RCC mitochondrial modifications.
Methods: We performed two methods to separate phosphorylated from un-phosphorylated proteins of RCC and normal renal cortex tissues: immunoprecipitation with a phospho-serine and threonine antibody mixture, and affinity chromatography purification (Phosphoprotein purification kit, Qiagen). Then, proteins were separated by 2-DE and transferred by western blot onto PVDF membranes. Finally, mitofilin was immunodetected with specific anti-mitofilin antibody.
Results: Either immunoprecipitation and purification of phosphorylated proteins showed no significant difference of phosphorylated mitofilin pattern between RCC and normal tissues, although its total isoform number (40) detected by 2-DE immunoblot, and abundance evaluated by 1-DE immunoblot (RCC/CORTEX =0.48, *p-value*=0.0011) were decreased in tumor samples.
Conclusions: Since no difference of phosphorylated mitofilin isoforms was pointed out comparing RCC to normal tissues, mitofilin phosphorylation does not seem to be involved in abundance changes of the protein in RCC. Therefore, our results suggest that other post-translational modifications have to be investigated.

007

DIFFERENTIAL EXPRESSION OF TWO FORMS OF ANNEXIN A3 IN WELL-CHARACTERIZED HUMAN NORMAL CORTEX AND RCC PRIMARY CULTURES

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Background: Renal-cell carcinomas (RCC) account for about 85% of renal cancers and are characterized by different subtypes. The clear-cell and papillary subtypes of sporadic RCC account for about 75% and 12% respectively. The molecular analysis of these tumors is complicated due to the mixture of neoplastic and normal cells which compose them. To overcome this problem we established and extensively characterized primary cell cultures of RCC and normal renal tissue. This in vitro model has been useful for molecular characterization of Annexin A3 (AnxA3) that was recently described as biomarker in different tumors. There are no data about AnxA3 in kidney and RCC, although it has been described that it enhances HIF-1 transactivation activity and consequent angiogenesis involved in the pathogenesis of RCC.

Methods: 60 tumor and 54 normal renal tissue samples were enzimatically dissected with collagenase, plated and cultured in DMEM with 10% FCS. For immunofluorescence and FACS analysis cells were probed by antibodies against cytokeratin, vimentin, CD13, CA9, calbindin-D28K, CD31, alpha-SMA, CD11b, CD14 and Wilm's Tumor (WT) protein and by Alexa Fluor 488, 555 or 594 conjugated secondary antibodies. Flow Cytometry was performed using a BD FACSCanto™ Flow Cytometer. Taqman real time PCR, performed on an ABI PRISM 7900HT sequence detection system, was carried out in a 20 µl mix containing 100 ng of cDNA sample. For 1-D Western blotting, 30 µg of different protein lysates were separated on NuPage 4%-12% Bis-Tris Gels and for 2-D Western blotting 50 µg of proteins loaded on 70 mm 3-10 IPG-strips were then separated on NuPage 4%-12% Bis-Tris Gels. Corresponding nitrocellulose membranes were probed with rabbit polyclonal anti-AnxA3 and anti-actin antibodies and secondary antibodies coupled to horseradish peroxidase and SuperSignal Detection System. For Mass Spectrometry analysis 200 µg of cortex lysate was separated on 2-DE and blotted on membrane as described above. Immunoreactivity for AnxA3 has been evidenced on membrane and image acquired with Image Scanner (Amersham) were matched with corresponding images of SYPRO-stained gel and membrane by Image Master 2D Platinum. Most intense protein spot evidenced by AnxA3 antibody was excised and submitted to MALDI-TOF analysis. Tissue microarray (TMA) analysis with anti-AnxA3 antibody was also performed.

Results: Primary cultures from 44 out 60 RCC tissues (34 RCCcc, 8 RCCpap and 2 RCC mixed) and from 50 out 54 normal cortex tissues were obtained. Immunofluorescence and FACS analysis evidenced that more than 90% of cells both in cortex and RCC cultures expressed epithelial markers. In particular cortex cultures are composed of ephitelial cells of both proximal ($CD13^+$) and distal ($calbindin^+$) tubule origin. In RCC cultures instead about 93% of cells were CD13-positive according with the proximal tubular origin of RCCcc and RCCpap. About 67% of cells in RCC cultures were positive for CA9, one of the potential biomarker of RCC. The endothelial, podocyte and monocytic/macrophagic contamination was quite low (less than 5%). Fibroblastic contamination, evaluated analyzing the expression of alpha-SMA, was about 9% only in RCC cultures. In this well characterized in vitro model the expression pattern of AnxA3 was studied. AnxA3 transcript was significantly more abundant in cortex than in corresponding RCC cultures. By 1-D Western blot and anti-AnxA3 antibodies two bands of 36 and 33 kDa in both normal cortex and RCC primary culture lysates were detected. Furthermore, in RCC primary culture lysates the 33 kDa protein band has been found significantly up-regulated and the 36 kDa protein band down-regulated respect to matched cortex cultures. By 2-D Western blot five-six spots corresponding to 36 kDa isoform and two additional spots likely corresponding to 33 kDa isoform of AnxA3 were detected. The identity of the 36 kDa major AnxA3 spot was confirmed by MALDI-TOF analysis. This two band expression pattern was also present in the corresponding tumor tissues. An heterogeneity in AnxA3 expression, without any correlation with clinico-pathological characteristics, was evidenced, by tissue microarray (TMA) analysis *performed* in 51 samples.

Conclusions: Primary cell cultures from RCC and normal renal cortex were established with high efficiency and reproducibility and were estensively characterized. We use this well-characterized model of primary cell cultures and different technical approaches to show that RCC and normal tubular cells express two isoforms of AnxA3 with a differentially expression pattern between tumor and normal cells. Moreover, this pattern is maintained also in corresponding neoplastic and normal renal tissues. The differential expression pattern of

AnxA3 has to be ascribed only to normal and tumor renal cells although its pathogenetic implications for RCC are still unknown.
The present work has been supported by FIRB: Rete Nazionale per lo studio del proteoma umano (n. RBRN07BMCT).

008

MOLECULAR AND PROTEOMIC STUDY OF EPITHELIAL-MESENCHYMAL TRANSITION OF RENAL TUBULAR CELLS INDUCED *IN VITRO* BY HIGH GLUCOSE CONCENTRATION

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Background. Diabetes is an important social health problem. One of the most serious complications of diabetes is diabetic nephropathy (DN) that is characterized by tubulointerstitial fibrosis (TIF). Over the past years, it has been shown that TIF arises not only from "activation" of renal fibroblasts, but also from tubular epithelial cells that become collagen producing cells, throughout the epithelial-mesenchymal transition (EMT) process. To understand the molecular mechanisms of EMT in DN and to evaluate the involvement of the non-receptor tyrosine kinase Arg in cytoskeleton reorganization occurring during EMT process, we used an *in vitro* model of diabetic tubular EMT establishing well-characterized primary cultures of human tubular epithelial cells (HUTECs) cultured with high glucose concentration.
Methods. Primary cultures of human tubular epithelial cells were established and extensively characterized by immunofluorescence and FACS analysis. These cultures were submitted to treatment with 450 mg/dl glucose and the EMT development has been proved by i) cellular phenotypic changes, evaluated by contrast phase microscopy; ii) immunocytofluorescence and FACS analysis, evaluating the expression of the epithelial markers E-cadherin and cytokeratin and of the mesenchymal marker alpha-SMA; iii) molecular analysis of E-cadherin and alpha-SMA expression, evaluated by Real-Time PCR and 1D-Western blot. Cell death assay using AnnexinV/propidium iodide double staining and flow cytometry analysis was also performed. This *in vitro* EMT model was used to analyze Arg expression at transcript and protein level by Real-time PCR and 1-D Western blotting, to evaluate Arg-related cytoskeletal proteins, like F-actin and paxillin, by immunofluorescence analysis, and to obtain, by a proteomic approach with two-dimensional difference gel electrophoresis (2D-DIGE), a preliminary profile of the differentially expressed proteins between high glucose treated and control tubular primary cultures. Statistic multivariate analysis (PCA, principal component analysis) of EDA software, was also applied to these proteiomic data.
Results. The phenotypic transition induced by high glucose treatment was demonstrated by the changes in cell morphology and by an upregulation of the mesenchymal marker alpha-SMA together with a significant downregulation in the expression of the epithelial markers E-cadherin and cytokeratin. Furthermore high glucose treatment does not induce an increase of epithelial cell death. In this well-characterized *in vitro* model of tubular EMT we evidenced a downregulation of Arg both at transcript and protein level. At 96h, when high glucose treated cultures show a mesenchymal phenotype, we evidenced also an increase of stress fibers and focal adhesions. Finally, we used our EMT model also to investigate by 2D-DIGE the impact of high glucose concentration on proteomic pattern in HUTECs. We analyzed 8 different pairs of samples and we found 18 proteins differentially expressed in high glucose treated HUTECs, compared with control cells at 96h of growth. In particular, ten proteins were statistically down-expressed and eight over-expressed. Although we do not know yet their identity, the Hierarchical Clustering Analysis performed on our data assembled them in two different class groups corresponding to high glucose treated and untreated cell cultures.
Conclusions. We performed a well-characterized EMT model by high glucose treatment of HUTECs. We studied Arg expression in these model evidencing a downregulation of Arg transcripts and proteins that correlates with cytoskeletric rearrangements occuring during EMT development. Based on documented role of Rho GTPase activation in EMT development and of Arg in Rho GTPase inhibition, we can hypothesize that the downregulation of Arg expression, induced by high glucose treatment, increases Rho activation that is responsible for stress fibers and focal adhesions formation and also for E-cadherin down-expression observed in high glucose treated HUTECs. These preliminary encouraging data induce us to further use this EMT model in order to better investigate the Arg role in modulation of tubular EMT by Arg silencing with siRNA technology. Moreover, this is the first overall proteomic approach to study EMT in human primary cultures treated with high glucose. The identification of the proteins differentially expressed will help to clarify the molecular pathways involved in the early steps of tubular EMT and will help the detection of predictive markers of diabetic nephropathy development.

009

INTRACELLULAR PROTEOME AND SECRETOME PROFILE OF CIGLITAZONE-DIFFERENTIATED HUMAN HEPATOCARCINOMA CELLS.

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Background: Hepatocellular carcinoma (HCC) is the most common primary cancer of the liver and is the third leading cause of cancer mortality worldwide. Unfortunately, the prognosis of HCC remains unfavorable because of its late diagnosis, especially in patients afflicted by chronic liver diseases. Curing HCC, usually through surgery, is possible in fewer than 5% of all patients. Median survival time from diagnosis is 4–8 months and is shorter for patients with cirrhosis. Diagnosis is generally determined by NMR and/or ultrasound data that showed the presence of neoplastic nodules generally greater than 2 cm. All these factors relating to the detection and diagnosis of HCC reinforce the assertion that the discovery of predictive tumor markers must be a priority of biomedical research and not only in liver cancers. Numerous proteomic studies on hepatocarcinoma have been conducted through comparative analysis of normal and neoplastic liver cells without obtaining definitive results because of wide biological variability of clinical samples. For these considerations, our approach tried to obtain information by modulating the differentiation grade of an experimental model of hepatocarcinoma by adopting a new differentiating agent. Drug induced cell differentiation represents a promising experimental model for proteomic analysis of cancer cells. In fact, by modulating and monitoring neoplastic cell differentiation it could be possible to identify cytodifferentiation related protein expression changes that can be subsequently utilized in vivo as potential cancer biomarkers. One main advantage of this approach with established cultured cell lines is the significant reduction of biological variability normally observed in clinical biomarker research, with important implications also in prognosis and therapy. Moreover, at this regard, it could be useful also to underline that hepatocellular carcinoma can represent an interesting experimental model to analyze a proteomic profile of undifferentiated vs. differentiated cancer cells in general. In fact, by using differentiating agents, it could be easy to monitor functional and structural differences during drug-induced cancer cell differentiation.
Methods: The human hepatocellular carcinoma cell line, HepG2, were grown under a humidified atmosphere of 5% CO2 in Dulbecco's Modified Eagle's Medium with 4.5 g/L glucose, supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Exponentially growing cells were incubated with different concentrations of ciglitazone, predissolved in 0.1% DMSO as a vehicle. Cell number and viability were assessed by trypan blue exclusion assay, and hepatocarcinoma differentiation markers (albumin, transferrin, and cholinesterase) were measured in culture supernatants. Total cell lysates were subjected to 2-DE, and significantly modified spots were identified by MALDI analysis. Differentially expressed proteins were validated by immunoblotting analysis also performed in clinical specimens of liver cancer tissue and matched normal liver tissue. For secretome analysis, cell were grown in serum-free culture medium and incubated with different concentrations of ciglitazone. After 96 hours of culture, supernatants were collected and precipitated for extraction of the secreted proteins and 2-DE analysis. Identified proteins were imported into IPA software to explore relevant biological relationships and interactions between genes and other important biological molecules.
Results: Ciglitazone is a strong differentiating agent for the HepG2 cell line, as showed by the antiproliferative effect calculated by monitoring cell growth after treatment with different concentrations of drug (- 30% with 1 µM, to almost 70% with 50 µM), by the re-activation of some typical functions of normal hepatocytes, such as albumin (+70% for 10 µM, +300% for 50 µM), transferrin (+130% for 10 µM, +400% for 50 µM), and cholinesterase (+2% for 10 µM, +300% for 50 µM) synthesis and the reduction of α-fetoprotein production (−34% for 10 µM, −75% for 50 µM). Moreover cell cycle analysis highlighted a significant cycle arrest of ciglitazone-treated cells in the G2/M phase which is a peculiar aspect of most cancer cell types treated with differentiating agents and a dose-dependent reduction of S phase cells. 2-DE analysis revealed that differentiation process is associated with significant (modifications of protein expression related to cell antioxidant systems, the cell cycle apparatus, signal transduction pathways, cellular stress and invasiveness, showing that differentiated HepG2 cells displayed a less invasive phenotype, reduced migration and invasive properties with respect to undifferentiated cells. Importantly, immunoblotting analysis showed that trends of changes in the same direction as those detected in the 2-DE analysis in undifferentiated/differentiated Hep-G2 cell line were also detected in lysates of human cancer liver/matched normal liver tissue. Moreover, IPA analysis displayed various direct and indirect interactions between our differentially expressed proteins and the oncosuppressor p53, a key protein of cell cycle regulation. Also the relative composition of secretome

profiles, as consequence of the differentiation process, was found also affected by several functional alterations as well as intracellular protein composition.
Conclusions: Analysis and characterization of intracellular proteome and secretome profiles of cancer cell lines represents a valid approach to provide valuable information about the cellular signaling pathways involved in the development and maintenance of the transformed phenotype. Importantly, these results seem to stress clinical relevance and the potential prognostic/diagnostic role of some oncoproteins in human hepatocarcinoma. Our results clearly confirm previous proteomic approaches examining hepatocarcinoma, in particular, and cancer, in general, regarding the pathogenetic roles of some structural and functional proteins in neoplastic transformation. In fact, analysis of these studies seems to show that some proteins and/or protein patterns typically recur during dedifferentiation/differentiation of various cancer cell lines and also in vivo situation. In addition, combining the information of both intracellular proteome profile and secreted proteins could offer new potential candidates for biomarkers useful to early detection of pathology. Moreover, this approach of investigating different dynamic aspects related to cancer differentiation could shed some light not only on the molecular mechanisms at the basis of this neoplasia, but also on potential new diagnostic, prognostic, and therapeutic markers.

010

FUNCTIONAL GENOMICS AND PROTEOMICS APPROACHES TO STUDY BREAST CANCER: DIFFERENCES BETWEEN PATIENTS NEGATIVE AND POSITIVE TO 18F-FDG PET SCREENING.

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Background: Breast cancer is not a single disease, but rather presents distinct subtypes associated with different clinical outcomes. Understanding this heterogeneity is a key factor for the development of targeted cancer-preventative and therapeutic interventions.18F-fluoro-2-deoxy-D-glucose Positron Emission Tomography (18F-FDG PET) has been evaluated in breast cancer for the characterisation of primary tumours, lymph node staging and the follow-up of patients after surgery, chemotherapy and/or external radiotherapy. PET is a molecular imaging technique sensitive to functional or metabolic changes in tissues. Since functional changes precede anatomical changes, FDG PET has the capability to detect viable tumour tissue early through its elevated glucose metabolism in comparison with the surrounding normal tissues A major advantage of FDG PET imaging compared with conventional imaging is that it screens the entire patient for local recurrence, lymph node metastases and distant metastases during a single whole-body examination using a single injection of tracer, with a reported average sensitivity and specificity of 96% and 77%, respectively. A general limitation of this new application of this technology are false negative results (not all breast cancer patients are positive to PET analysis) and interpretative pitfalls due to physiological tracer distributions. Proteomic and transcriptomic platforms both play important roles in cancer research, with differing strengths and limitations. Here, we describe a proteo-transcriptomic integrative strategy for understanding the differences and similarities between patients resulted positive and negative to PET analysis by combining the direct visualization of differentially expressed proteins with the high-throughput scale of gene expression profiling. In this study, 2D-Difference in Gel Electrophoresis (2D-DIGE), MALDI-ToF mass spectrometry and DNA-microarray analysis were utilized to compare breast cancer samples of patients positive and negative to 18F-FDG PET analysis. Our study illustrates how the systematic integration of proteomic and transcriptomic data could provide new suggestion for accelerating cancer biomarkers discovery.

Methods: Breast cancer patients were selected by Nuclear Medicine unit of S.Raffaele Hospital of Milan. Samples were divided into two groups: positive and negative to 18F-FDG PET analysis. Proteomic analysis was performed using 2D-DIGE and MALDI-ToF mass spectrometry. These were applied in order to identify the differentially expressed proteins between two groups. Each Protein extract was labelled with Cy5 dye (GE Healthcare), while the internal standard was labelled with Cy3 dye (GE Healthcare). The first dimension was performed on 24 cm 3-10 NL pH gradient IPG strips using an IPGphor electrophoresis unit (GE Healthcare), the second dimension was carried out in 20x25 cm 12%T-2.5%C PAGE gels using Ettan Dalt II system (GE Healthcare). Images were visualized using a Typhoon 9200 scanner (GE Healthcare) and analyzed with DeCyder software version 6.5. Total RNAs was extracted from breast cancer tissue with Trizol Reagent (Invitrogen) and treated with Quick Amp Labeling Kit-Two color (Agilent Technologies) to obtain complementary fluorescent RNA (cRNA). Each RNA extract sample was labelled with Cy5 dye (Agilent Technologies), while the internal standard was labelled with Cy3 dye The fluorescent cRNA samples were hybridized onto Whole Human Genome 4x 44K array (Agilent Technologies). Images were achieved by an Agilent's DNA Microarray Scanner with Sure Scan high-Resolution Technology (Agilent Technologies) and analyzed using Feature extraction expression software. Intensity signals were computed with the GeneSpring GX software (Agilent Technologies).

Results: Differential protein expression between positive and negative patients to 18F-FDG PET analysis, was evaluated using 2D-DIGE analysis. Differential analysis, using the Student's *t*-test provided by DeCyder software, revealed 61 spots differentially expressed ($p<0.05$): 41 spots up-regulated and 20 down-regulated ones. 29 spots were identified by MALDI-ToF. Most representative functional groups of proteins are involved in glucose metabolic pathway, chaperon proteins and cytoskeletal proteins. We then integrated the proteomic information with gene expression data. Microarray analysis identified a number of transcripts modulated in patients compared to Human Universal Reference Total RNA (Clontech) used as internal control for microarray expression profiling experiments. Up and down regulated transcripts were grouped

according to their biological function. The more represented GO classes were: glucose metabolism, processing, cell metabolism, signal transduction and protein metabolic processes.
Conclusions: Transcriptome and proteome analysis of breast cancer tissue of patients negative and positive to 18F-FDG PET analysis revealed quantitative differences between the two group of patients allowing patients clusterization. The large-scale generation and integration of genomic, proteomic, signalling and metabolomic data are rapidly increasing, allowing the construction of complex networks that can provide a new framework in the comprehension of molecular basis of physiological or pathophysiological states.

011

REDOX PROTEOMICS IDENTIFICATION OF OXIDATIVELY MODIFIED MYOCARDIAL PROTEINS IN HUMAN HEART FAILURE: IMPLICATIONS TO PROTEIN FUNCTION

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Background: Increasing experimental evidences support the concept that oxidative stress is increased in the failing heart and contributes to the pathogenesis of myocardial remodeling which leads to heart failure (HF). The effect of reactive oxygen species (ROS) can take place on various biological molecules, such as carbohydrates, lipids, nucleic acid and proteins. However, proteins are considered the major targets for ROS because of their abundance in biological systems and because they are primarily responsible for most functional processes within cells. Thus, this study is intended to identify the oxidized proteins in the myocardium of HF patients and to analyze the consequences of oxidation to protein function.
Methods: Cardiac levels of oxidized proteins were measured by immunoassay and analyzed by a proteomic approach in left ventricular tissue of both failing (n=14) and non-failing (n=13) human hearts. HL-1 cardiomyocytes were incubated in the presence of stimuli relevant for HF in order to assess the generation of ROS, the induction of protein carbonylation, and its consequences to protein functions.
Results: Myocardial levels of oxidized proteins were significantly higher in HF patients than in controls ($p<0.01$). We identified two proteins which underwent oxidation, creatine kinase M-type (M-CK) and, to a lesser extent, α-cardiac actin (ACTC). Exposure of cardiomyocytes to stimuli relevant to the physiopathology of HF resulted in ROS generation and M-CK carbonylation with loss of its enzymatic activity.
Conclusions: This study indicates that CK is the major protein target of ROS in the human failing heart. The oxidative modification of CK leading to the impairment of its enzymatic activity might aid in explaining the decrease of CK activity and consequently the defects in the energy metabolism in the failing heart.
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012

PROTEOMIC ANALYSIS OF URINE IN RENAL CELL CARCINOMA PATIENTS BY CLINPROT APPROACH

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Background: Renal cell carcinoma (RCC) is the most common malignancy of the kidney and accounts for 3% of all adult cancers. The most frequent histological subtype of this tumor is the clear cell RCC (ccRCC) which arises from the proximal renal tubular epithelium. Symptoms occur late in the course of tumor, so patients have poor prognosis because of its high resistance to radio- and chemio-therapies. Therefore biomarkers for early detection of RCC, for the differential diagnosis of RCC from benign renal lesions when imaging is not helpful, for prognosis and follow up are urgently needed. Protein profiling by mass spectrometry (MS) represents a promising tool for biomarkers discovery and for a better understanding of disease molecular mechanisms. The aim of this study is the characterization of urine protein profiles of control subjects, RCC patients and follow-up at 4 (FU4) and 8 (FU8) months by ClinProt technique linked with MS analysis. In particular one of the objective was to validate the cluster of signals able to discriminate RCC patients from normal subjects in urine published last year with new controls and patients. We analyzed also samples collected during the follow-up period in order to verify the capability of this approach to discover possible biomarkers predicting tumor relapses.

Methods: Urine samples of new control subjects (n = 37), ccRCC patients collected in the morning just before the surgery (n = 29), urine of patients collected the day before surgery (n = 23) and follow-up urine (n = 12 at 4 months and n = 21 at 8 months) have been purified by ClinProt techniques with C8 hydrophobic coated magnetic beads. Mass spectra from 1000 to 10000 Dalton were acquired using a Reflex IV MALDI-TOF instrument in positive linear mode by the optimization of an automated acquisition method. Statistical analysis of the obtained spectra profiles and discriminant pattern recognition was performed using the software ClinProTools 2.1 through the use of algorithms properly supplied. Receiver Operating Characteristic (ROC) curve analysis and Area Under the Curve (AUC) calculation were done directly with this software to determinate diagnostic efficacy of each single marker.

Results: The statistical comparison of average spectra from controls and first morning urine of ccRCC patients collected directly into bladder showed some altered protein profiles in several samples, which were to remove them manually. The previous published pattern of markers (A= m/z 1825 Da, B = 1912 Da and C = 1966 Da), applied to these new samples, still allowed to distinguish the patients from controls with fairly good sensibility and specificity values (specificity and sensitivity of 100% and 80%, respectively) compared with those previously obtained (100% specificity and 90% sensitivity). Within the protein profile of these new samples, two statistically different (p-value < 0.05) peptides (m/z 1656 and 3255 Da) showed high discriminant capability. Their combination with two of the previously identified signals allowed to improve cancer detection. Data processing of average protein profiles from controls and RCC collected the day before surgery also revealed several ions differently expressed. Both old and new clusters allowed to obtain good specificity and sensibility values (92% specificity and 86% sensitivity for the old cluster and 97% specificity and 81% sensitivity for the new one). Diagnostic capability was improved by combining the signal at m/z 1898 Da to the other ones giving 92% and 90% of specificity and sensitivity, respectively. External validation of this new cluster with the old data set showed 100% and about 90% of specificity and sensitivity, respectively. Diagnostic efficacy of these clusters to distinguish RCC patients grouped on tumour stage and the signal intensity of these potential markers versus disease progression were also evaluated. Moreover no statistical significant differences were observed between the average spectra from urine collected in the follow-up period at 4 and 8 months. Statistical analysis of urine of control subjects versus follow-up patients showed several ions with statistically different intensity that can be used to discriminate the two groups.

Conclusions: Diagnostic capability of the already published cluster of signals was confirmed with a new cohort of control subjects and RCC patients. Moreover a new group of signals were detected in urine which may be included in this cluster.

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013

EVALUATION OF THE OCHRATOXIN A TOXICITY IN KIDNEY BY PROTEOMIC TOOLS

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Background: Ochratoxin A (OTA) is a mycotoxin produced by the fungi *Aspergillus* and *Penicillum.* It is a secondary metabolite found as a contaminant in a variety of very common foods and beverages: mostly cereals and grain products but also beer, wine and meat derived from animals fed with contaminated fodders. Because of its very prolonged half life in blood the human exposure to this contaminant is of relevant importance, in particular because OTA was found to be cancerogenic, nephrotoxic, teratogenic neurotoxic and immunotoxic in several species of animal experiments and it is actually classified as "probable carcinogenic" from IARCC. Despite its toxicity, the mechanisms underlying these various toxic effects of OTA have not been still elucidated in detail. The aim of the present study was to gain more insight into the molecular changes that occur after oral exposure to OTA in kidney.
Methods: Kidney protein extracts were obtained from mice fed OTA (0.5 mg/Kg b.w.) for eight weeks to obtain a chronical intoxication effect and were resolved by 2DE. For IEF 120 μg of proteins were loaded on linear IPG strips (pH 3-10; 7cm); second dimension was performed on 12% SDS-PAGE. Comparative image analysis of five replicates, performed with PDQuest™ (BioRad), highlighted the differentially expressed proteins, which were identified by peptide mass fingerprint on Voyager-DE Pro MALDI-TOF or by MS/MS analysis on ESI-Q-TOF (Q-STAR).
Results: The proteomic analysis of kidney proteins showed that 22 proteins were differentially expressed after 8 weeks of exposure to OTA: in particular 13 proteins were down-regulated by the OTA treatment, while the expression of 9 proteins was up-regulated. Among the differentially expressed proteins 7 were involved in metabolic processes: the down-regulated enzymes bisphosphate-3'nucleotidase, V-ATPase H^+, malate dehydrogenase and isocitrate dehydrogenase. The OTA up-regulated enzymes were adenylate kinase, glyceraldehyde-3-phosphate dehydrogenase and electron transferring flavoprotein dehydrogenase. Moreover, we observed a modulation effect in the expression of two isoforms of ATP synthase. OTA caused also the down regulation of the cytoskeletal proteins vinculin and tropomyosin, and the up-regulation of spectrin 2. As regards the proteins with anti-oxidant functions, the toxin induced the down regulation of GSH synthetase, HSPD1 and the up regulation of the phosphotriesterase. Other proteins, whose expression levels were modulated by the treatment with OTA, were the molecular chaperon calreticulin (up), eukariotic translation elongation Factor 1 alpha 1 (down), both involved in the protein synthesis/folding processes and homogentisate 1,2-dioxygenase (down).
Conclusions:. The results show that the OTA toxicity is exploited by altering protein synthesis, cellular metabolism and cell cycle; by a general rearrangement of the cytoskeletal proteins and by depleting some of the proteins involved in the protection of the cell from oxidative damage.

014

SALIVARY PROTEOMIC PROFILE IN SJÖGREN'S SYNDROME BY SELDI-TOF/MS.

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Background: Sjögren's Syndrome (pSS) is a chronic autoimmune disease characterized by atrophy and malfunction of exocrine glands, which leads to dry mouth (xerostomia) and dry eyes (xerophthalmia) and is therefore expected to influence the composition of saliva and tear fluids. The etiopathogenesis of the disease is generally thought as multifactorial involving a genetic predisposition, ambiental and autoimmune factors. In a previous study we investigated saliva of patients affected by SS with two-dimensional electrophoresis (2-DE) combined with matrix-assisted laser desorption/ionization time-of-flight-mass spectrometry analysis (MALDI-TOF-MS). The aim of the present study was to perform a proteomic analysis of SS patients' saliva with surface-enhanced laser desorption/ionization time-of-flight/mass spectrometry (SELDI-TOF/MS). This technology utilises a combination of mass spectrometry and chromatography to facilitate protein profiling of complex biological mixtures and can resolve low-mass proteins (<of 25 KDa) providing a complementary visualization technique of the 2-DE.
Methods: Saliva samples were obtained from 40 patients with pSS and 30 healthy controls with similar demographic characteristics. Whole saliva samples were centrifuged to remove undissolved material. Aliquots of resulting supernatants (corresponding to 10µg) were analysed by SELDI-TOF/MS. Saliva was applied to the spots of Protein Chip Arrays, we used three different Protein Chip Arrays: CM10 (weak cation exchanger), Q10 (strong anion exchanger) and H50 (hydrophobic surface). After incubation period (1h), unbound proteins were washed off the spots using buffers as required by the array chemistry; finally matrix (sinapinic acid-SPA) was applied to each spot to facilitate desorption and ionization. The array is then placed into the ProteinChip SELDI reader for mass spectrometric analysis. The spectra obtained were analysed by ProteinChip data manager software.
Results: The analysis of the obtained spectra on three chips, allowed us to observe 29 peaks that were significantly decreased in pSS patients and 11 peaks that were significantly increased. The average intraclass variability was found to be in the normal experimental range (CV of 4.2 to 14.5%). A preliminary comparison against literature and protein databases of differentially expressed peaks suggested for some of these a potential identification. For example we observed an up-regulation of β2microglobulin (m/z=11735, $p<0.0001$) and a down regulation of transthyretin (m/z=14266, $p<0.0001$). Further studies could be useful to confirm these data and identify other significant peaks detected.
Conclusion: These preliminary results demonstrated the effectiveness of SELDI-TOF/MS as a tool for discovering potential biomarkers in whole saliva samples of pSS patients.

015

PROTEIN BIOMARKERS IN BRONCHOALVEOLAR LAVAGE FLUID OF PULMONARY LANGERHANS CELL HISTIOCYTOSIS (PLCH) PATIENTS

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Background: Langerhans Cell Histiocytosis (PLCH) is a rare smoking-related interstitial lung disease (ILD) with poorly defined pathogenetic mechanism. In order to improve the knowledge about this pathology we performed a study based on proteomic approach. This methodology previously has been employed for a wide spectrum of lung disease like lung cancer, interstitial lung disease, cystic fibrosis, acute lung injury and chronic lung rejection. Studies about protein composition of BAL in PLCH together to the other lung disease aim to build a data base to compare protein composition in different disease and control and to plot disease-specific protein maps, to identify potential markers useful for diagnosis, prognosis and drug effects.
Methods: With the intent to identify the different protein expression between PLCH patients and control subjects we performed a 2-DE separation after the definition of the different BAL cell components by differential cell count and lymphocyte phenotype studies. After images analysis, different expressed proteins were identified by MALDI-TOF MS.
Results and conclusion: Differentially expressed protein spots found by image analysis, have been identified by MALDI-TOF MS and ESI ion trap MS/MS. Among these proteins we can mention Complement C3 beta (p=0.009), Apo-A1 (p=0.001) and Ceruloplasmin (p=0.003) were up-regulated in PLCH patients and SP-A1 (p=0.001), calcyphosine (p=0.02), PIGR (p=0.02) were down-regulated. The results are very promising and will be validated to confirm the usefulness of these proteins as biomarkers.

Acknowledgment:
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016

DIFFERENTIAL PROTEOMIC ANALYSIS OF SUBFRACTIONED HUMAN HEPATOCELLULAR CARCINOMA TISSUES

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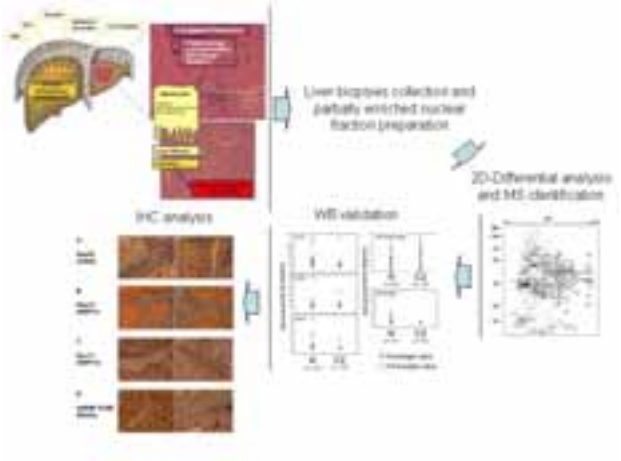
Background: Hepatocellular carcinoma (HCC) is the most relevant and common primary cancer of the liver and it is closely associated with chronic infection by the hepatitis B virus (HBV) or the hepatitis C virus (HCV). The proteomic approach is a valuable tool to detect and identify proteins that are associated with HCC and to discover new potential biomarkers of the disease. Development of HCC is in fact a complex process involving multiple changes in gene expression and proteome profile.

Methods: We used two-dimensional (2-DE) gel electrophoresis separation and MALDI-TOF-MS Peptide Mass Fingerprint (PMF) identification of proteins from nuclear enriched fractions of liver biopsies. Twenty pairs of specimens (tumoral and non-tumoral) were obtained from 20 HCC patients. To exclude the contribution of genetic variation, differential analysis was performed between the pair specimen of the same patient. Nuclear proteins were separated in the first dimension by isoelectric focusing on immobilized pH 3-10 gradient strips and by 12% SDS-PAGE in the second dimension. Image analysis was performed by the Image Master 2D software (GE Healthcare). Western blot analysis, used to confirm differentially expressed proteins, was extended to the whole casistic. Statistical analysis was performed to test the reliability of our data.

Results: First, we obtained a proteome map of the nuclear-enriched liver biopsies. This includes 83 spots corresponding to 52 different species. Then, we carried out a differential analysis of proteins from tumoral and non tumoural tissues of HCC. A significant change in expression level was found in 16 proteins. Of these, UTP-glucose-1-phosphate uridylyltransferase (UGPA), methylmalonate-semialdehyde dehydrogenase (MMSA), electron transfer flavoprotein subunit β (ETFB), hnRNP A2/B1 (Q9BWA9), the genomic scaffold hCG2001950, hydroxymethylglutaryl-CoA synthase (HMCS2) and actin-related protein 2 (ARP2) represent new targets being never associated to HCC before. Among previously reported HCC-associated proteins we have found Heat Shock Cognate 71 kDa protein (HSP7C), Heat shock 70 kDa protein 1 (HSP71), 60 kDa heat shock protein (CH60), Cathepsin D (CATD), Alpha-enolase (ENOA), 3-ketoacyl-CoA thiolase (THIM), Aldolase B, fructose biphosphate (ALDOB), Peptidyl-prolyl cis-trans isomerase A (PPIA) and ATP Synthase (ATPA). Further analysis by Western blot confirmed reliability of our analysis and to determine the expression level of Hsc 71, Hsp60, Hsp70, hnRNP A2/B1, Arp2, ETFB and ATP Synthase in the same samples. These results may provide useful insights for understanding the mechanism involved in the process of HCC carcinogenesis providing new candidate biomarkers.



Conclusions: Despite the rather limited number of patients, the comparative analysis reported in this study has revealed a significantly altered expression level for various proteins. Some of them have been already proposed as candidate biomarkers of HCC, other have been originally proposed here. Future experiments

will investigate the involvement of these proteins in HCC, further exploring the possibility that tumor development may be directly related to their deregulated expression.

017

CBX7 ROLE IN TUMOR PROGRESSION BY FUNCTIONAL PROTEOMICS APPROACHES

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Chromobox protein homolog 7 (CBX7) encodes a novel polycomb protein (Pc) of 28.4 kDa and 251 amino acids that contains a "chromodomain" between amino acid 10 and 46. CBX7 belongs to the chromobox family protein and it is a member of the Polycomb repressive complex 1 (PRC1), that together with the PRC2 mantains developmental regulatory genes in a silenced state. Mouse CBX7 associates with facultative heterochromatin and with the inactive X chromosome, suggesting a role of the CBX7 protein in the repression of the gene transcription. Previous studies have demonstrated that the CBX7 gene was drastically down-regulated in six thyroid carcinoma cell lines in comparison with normal thyroid cells. Furthermore, the analysis of CBX7 expression in a large number of thyroid carcinoma samples revealed a progressive reduction of CBX7 levels that is well related with the malignancy grade of the thyroid neoplasias. CBX7 expression decreased in an increasing percentage of cases going from benign adenomas to papillary, follicular and anaplastic thyroid carcinomas. More recent results confirmed a correlation between low CBX7 expression and a reduced survival in colon carcinoma. Restoration of CBX7 expression in thyroid cancer cells reduced growth rate with a block in G1 phase of the cell cycle, indicating that CBX7 plays a critical role in the regulation of the transformed thyroid cell proliferation.
In order to investigate the mechanism by which the loss of CBX7 expression correlates with a highly malignant phenotype, we decided to identify CBX7 interacting proteins by functional proteomic approaches.
Protein complexes were allowed to form in vivo within transiently trasfected HEK 293 expressing CBX7-V5. The cell extracts were immunoprecipitated with anti-V5 antibodies using suitable experimental conditions to avoid dissociation of the complexes. The immunoprecipitated material containing the protein bait and its interacting partners were then fractionated on 12% one-dimensional gel to enhance the resolution at high molecular weight. The resulting gel was stained with Colloidal Comassie, proteins were digested *in situ*, analysed by nanoLCMSMS and identified by database search using the MASCOT software.

RETE NAZIONALE DI PROTEOMICA PROGETTO FIRB RBRN07BMCT "ITALIAN HUMAN PROTEOMENET".

018

IDENTIFICATION OF NOVEL CFTR PROMOTER REGULATORY ELEMENTS

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Cystic fibrosis (CF) has a prevalence of 1:2,500 newborns and is the most frequent lethal genetic disorder among Caucasians. The disease depends on alterations of the CF transmembrane conductance regulator (CFTR) protein, a cAMP-activated chloride channel located in the apical membrane of most secretory cells, due to CFTR gene mutations.
More than 1600 CFTR mutations have been described so far, but about 10% CF alleles do not bear mutations in the coding regions of the gene; furthermore, a large phenotypic heterogeneity of the disease have been described so far also in affected sibpairs; finally, the regulatory mechanisms of gene expression are poorly known.
We studied the promoter region of CFTR in a large group of CF patients and in controls. In particular: i) we analyzed, by direct gene sequencing, the DNA sequence of about 6000 bp at the 5' of the CFTR gene; we identified some novel gene variants in CF patients ii) we cloned the promoter region containing these mutations upstream to the reporter system of luciferase gene to verify the effect of these mutations on gene expression levels; iii) we searched by a modified "fishing for partner" approach novel proteins that may interact with the CFTR promoter, with particular regard to two gene regions at a different distance from CFTR.
These results strongly suggest that the promoter regions of CFTR may have a relevant role in the regulation of gene expression (it will be useful, in future, to assess the activity in different organ and tissue from affected patients). Mutations in such area may act as disease causing, also impairing the interaction with potential regulatory elements.
The identification of the protein machinery binding to the promoter regions will be achieved by using functional proteomics approaches. The oligonucleotide sequence corresponding to a specific CFTR promoter region will be synthesised with a biotin tag and used as bait. The oligonucleotide will be immobilised on Streptavidin derivatised magnetic beads and incubated with a nuclear cell extract to isolate its interacting proteins by affinity purification. The same experiment will be carried out using randomised oligonucleotide as control. The proteins fished out by the baits will be separated by SDS-PAGE and identified by mass spectrometry methodologies and protein databases search. The identification of proteins normally involved in the binding of normal CFTR promoter will allow us to further elucidate the gene regulation mechanisms.

We gratefully acknowledge contributions from "Rete Nazionale di Proteomica", Progetto FIRB RBRN07BMCT, Italian Human Proteomenet; "Fondazione Italiana per la Ricerca sulla Fibrosi Cistica"; MIUR (PS 35-126/Ind); Regione Campania (DGRC2362/07).

019

IMPACT OF ORANGE JUICE INTAKE ON PLASMA PEPTIDOME PROFILING

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Background: The EU FLORA project (Flavonoids and related phenolics for healthy Living using Orally Recommended Antioxidants) aims at giving an important contribution to understand the beneficial influence of flavonoids and related phenolics could exert on human health. Orange juice is characterized by substantial accumulation of flavonoids and phenylpropanoide. Particularly, the presence of anthocyanins (72 mg/L) is typical of blood orange varieties.
We hypothesized that orange juice could cause changes in plasma protein profile.
Based on differential analysis and detection of plasma protein profile obtained by MALDI-TOF mass spectrometry, a valid method has been developed with the intent of investigating the changes of plasma proteins induced by a diet rich in anthocyanins.
Methods: *Sample collection:* 5 mL of citrated blood were collected from 18 volunteers who had received in a cross-over design blond or blood orange juice (1 liter/day) for four weeks.
Sample preparation: Plasma samples, obtained by centrifugation at 3000 rpm for 20 minutes (4°C), were diluted 1:4 with 10%ACN and centrifuged for 30min at 14000g in Centricon tubes at a 30000 Da cut -off; the filtrates were concentrated by speed vac and purified by ZipTip C18.
Sample acquisition: Spectra were acquired by Voyager-DE STR (Applied Biosystem) operating in reflectron positive ion mode; a mix of 4 calibrants was used as internal mass calibration; for each sample 3 spectra replicates were acquired and aligned by the software ALISPECTRA.
Statistical procedure: Analysis of variance (ANOVA) was performed in order to characterize the variables (peaks) with significant differences between means/groups; Principal Component Analysis (PCA) was performed using the selected variables from ANOVA in order to evaluate the natural grouping without any prior hypothesis.

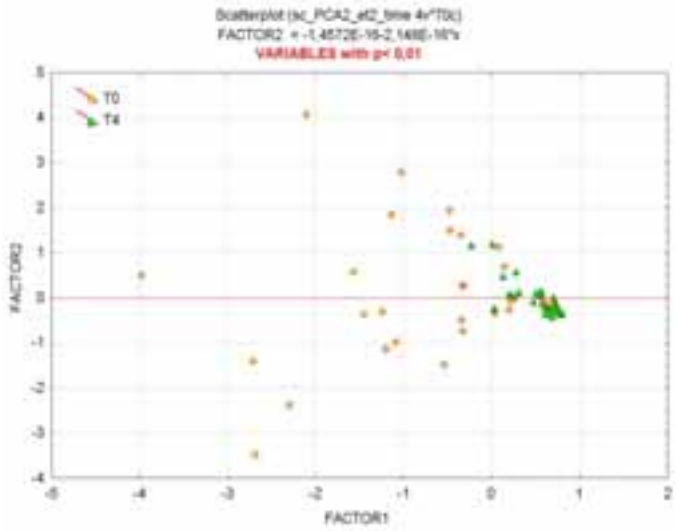

Figure 1. Principal component analysis performed on 18 volunteers, using 73 variables (peaks) previously selected by ANOVA.

Results: Data exploration was carried out on the complete MALDI-TOF spectra with PCA. The analysis, on the basis of plasma profile, shows an effect due to administration of either orange juice. Further, the dietary intervention has changed the response of each individual plasma profile. The juice intake appears to standardize the plasma profile versus a pattern well defined for all volunteers.
Conclusion: Mass spectrometry can generate a proteome/ peptidome fingerprint of plasma. Advances in proteomics lead to improve approaches in molecular diagnostics, capable of identifying, by statistical models, dietary components that could be protective against common diseases.

020

PROTEOMIC PROFILE OF FINE NEEDLE ASPIRATION FLUID OF THYROID FOLLICULAR CANCER

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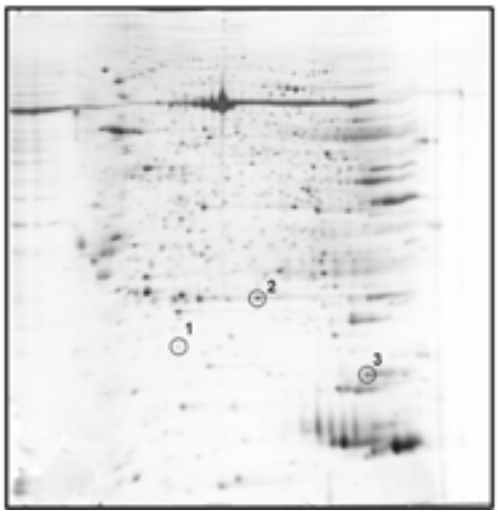
Background: In previous experiences we demonstrated fine needle aspiration fluid (FNA) usefulness in the comparative analysis of thyroid tumors suggesting new proteins as potential biomarkers to differentiate classical (cPTC) and tall cell (TCV) variants of papillary cancers with respect to control. Another variant of aggressive thyroid cancer difficult to diagnose is papillary follicular variant (FVC). In this study we extend our research to define the FNA protein pattern of follicular variant of papillary cancer and to compare it with that of control and other variants of papillary cancer.
Methods: FNA was made on thyroid nodule, ipsilateral normal tissue and controlateral lobe of patients undergoing thyroidectomy for follicular nodule. Samples from cPTC (20), TCV (6), FVC (6) were included. The fluids were immediately centrifuged at 2300 x g for 20 min at 4°C. Proteins from resulting supernatants were precipitated using thrichloroacetic acid (TCA). The proteins profile of FNA was obtained by two-dimensional electrophoresis and the difference of proteins expression in different variants of papillary cancer respect to control was evaluated using Image-Master 2D Platinum. Proteins of interest were identified by peptide mass fingerprinting via MALDI−TOF mass spectrometry.
Results: The proteins profile of FNA of FVC (Fig.1A) well overlaps with those obtained from previous other variants of papillary cancer. Similar to cPTC and TCV we observed a significant up-regulation of specific functional proteins as glycolitic enzymes, serum and structural proteins with respect to the control (Fig.1B). In particular, an increase of ferritin light chain (spot n°1; p=0.029), protein DJ-1 (spot n°2; p=0.0475) and cofilin-1 (spot n°3; p=0.0021) confirms the potential role of these proteins in the thyroid cancer progression.



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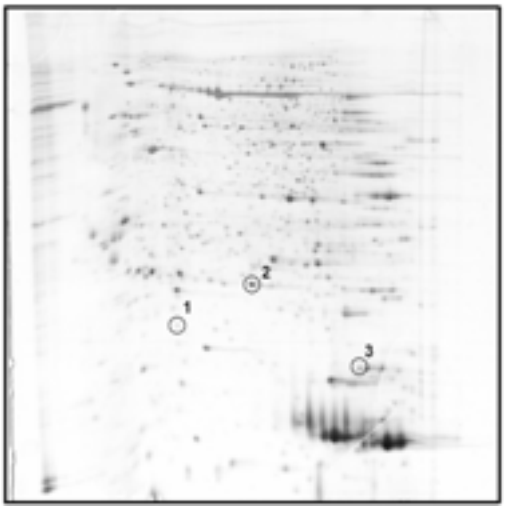

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Conclusions: These findings illustrate the potential use of FNA proteomics to identify protein changes associated with thyroid cancer and to advance potential protein biomarkers in the diagnostic classification of the disease.

021

COMPARATIVE PROTEOMIC PROFILE OF RAT SCIATIC NERVE AND GASTROCNEMIUS MUSCLE TISSUES IN AGEING BY 2-D DIGE

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Background: Ageing induces a progressive functional decline affecting the entire organism, therefore, the loss of muscle mass and function (sarcopenia) contributes significantly to a loss of functional autonomy, increased prevalence of falls, and greater morbidity. In muscles, the age-related degenerative changes produce alteration of morphology associated to muscle fiber atrophy, loss of satellite cells, and remodelling of neuronal structures. The mechanism of muscle wasting is poorly understood but it could be related to a protein synthesis/degradation imbalance, to the post mitotic nature of myofibers or to a dependency of myofibers on motoneurons innervation. Morphological changes induced by ageing indicate a decrement of muscle fiber size, a change in fiber type distribution and appearance of mitochondrial aggregates with an increment of lipofuscin. The functional and morphological changes in muscles are observed in a parallel manner in nerves. Actually, the ageing process in peripheral nerves induces important biochemical, morphological and functional variations both in myelin and in axons.
Methods: In the present study we characterized sciatic nerves and gastrocnemius muscles by light and electron microscopy. Samples were oriented for cross sectioning of the nerves and for longitudinal or cross sectioning of the muscles. Semi-thin section from each sample were stained with 0.5% toluidine blue in 1% sodium borate and examined by light microscopy (Zeiss Axiophot Photomicroscope). Ultrathin sections were examined under the electron microscope (Zeiss EM 10). The proteomic profile of gastrocnemius muscles and sciatic nerves from six 8- and six 22-month-old rats was examined by 2-D DIGE and mass spectrometry. 2-D DIGE was conducted on protein extracts from each animal, labelled with Cy5 dye, while the internal standards, generated by pooling together an aliquot of each nerve and muscle extracts respectively, were labelled with Cy3 dye. The minimal labelling was performed according to manufacturer's recommendations. CyDye labelled gels were visualized using a Typhoon 9200 laser scanner (GE Healthcare). Spot detection was performed using DeCyder difference in-gel analysis module V. 6.5. For protein identification, semipreparative gels containing 400 µg of total protein extract per strip, were loaded with unlabelled sample; electrophoretic conditions were the same as 2-D DIGE, except that gels were stained with a protein fluorescent stain. Tandem electrospray mass spectra were recorded using a HCT Ultra mass spectrometer (Bruker Daltonics) interfaced to a MDLC capillary chromatograph (GE Healthcare). Proteins were identified by correlation of uninterpreted tandem mass spectra to *Rattus* entries in NCBInr database, using MASCOT software.
Results: In young rats the muscle fibers present physiological morphology with regular spaces between fibers, while in old rats a number of abnormalities indicative of a degenerative, paraphysiological process were observed. The old muscular fibers showed signs of atrophy, with myofibrillar loss and degeneration, internal migration of nuclei and longitudinal fiber splittings, as previously observed in old and senescent muscles. The electron microscopy analysis clearly supported an increase in subsarcolemmal mitochondrial aggregates and an increased number of lipofuscin granules. Moreover, the myofibrillar degeneration seemed to be mainly related to the disappearance of sarcomeres and was accompanied by the dilatation of sarcoplasmic reticulum. According to alterations described in peripheral nerves of old rats, the sciatic nerve showed typical age-associated abnormalities of myelin, like enfoldings, invaginations and presence of "onion bulb" structures. In addition, the electron microscopy revealed some interesting details: the onion bulbs, a typical sign of nerve degeneration, were present at different stages. Some myelinated fibers appeared completely degenerated with myelin breakdown, side axonal atrophy and enfolded loops of myelin. Differential proteomics of aged gastrocnemius was achieved by analysing 3130 spots per gel. Among them, 319 were differentially expressed (t-test, $p < 0.01$) in old vs. young rat muscles indicating a large (10%) protein reassessment induced by the ageing process. Relevant changes were found in proteins involved in metabolism, contractile machinery and cytoskeletal organization, signal transduction, stress control and transport. Differential proteomics of aged sciatic nerve detected 2566 spots, 101 of them were differentially changed (t-test, $p < 0.01$) in young vs. old male animals, suggesting that rearrangement induced by ageing in sciatic nerve involves 4% of the total detected spots. Deregulated functional categories included anaerobic metabolism, transport, cell structure and calcium homeostasis.

Conclusions: In this study we confirmed that ageing induces several morphological changes, both in skeletal muscles and in peripheral nerves, which might affect the physiology of these tissues. These changes are accompanied by deregulation of specific proteins directly related to morphological and functional adaptation; some of them are specific to muscle function, while others are typical of the nerve ageing process. We can conclude that there are many proteins that could be monitored as indicators of nerve and muscle degeneration. Further investigation and validation in human samples will be required to demonstrate that muscle tissue can be a target for the diagnosis and follow up of both neurodegenerative and regenerative processes.

Acknowledgements: This work has been funded from: Italian Ministry of University and Scientific Research (Grant: FIRBRBRNO7BMCT to C.G.).

022

PROTEOMIC ANALYSIS OF *LOLIUM PERENNE* POLLEN EXTRACT: IDENTIFICATION OF NOVEL COMPONENTS RECOGNIZED BY IGE ANTIBODIES AND COMPARATIVE EVALUATION OF ALLERGIC PATIENTS SENSITIZATION PROFILES.

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Background: Allergic diseases affect more than 25% of the world's population and their prevalence increased expecially in developed countries. Pollens are the most frequent cause of seasonal allergic rhinitis. Their allergenicity is linked to a limited number of proteins which are readly released from pollen grains upon hydratation. In the last years, proteomic investigations in biochemical and immunological characterization of allergens allowed a more comprehensive knowledge of these allergy-eliciting molecules. In particular, 2-D Electrophoresis (2-DE) was successfully employed to resolve complex allergen sources, separating different allergen isoforms and studying post-translation modifications, at the same time mass spectrometry analysis provided a useful tool to identify novel allergens.
Methods: A sample of *Lolium perenne* (ryegrass) pollen extract was analyzed by nUPLC-MS^E. A triptic digestion of the extract was trapped on a Symmetry C18 5µm, 180 µm X 20 mm precolumn and the peptides were separated on a nanoEase^{TM} BEH C18 1.7µm, 75µm X 25cm nanoscale LC column (Waters Corp) with a gradient 3-40% of acetonitrile containing 0.1% formic acid at flow rate of 250nl/min over 120 min, followed by a gradient of 40-90% over 5 min. Eluted peptides were analyzed by Q-Tof Premier mass spectrometer (Waters Corp). Continuum LC-MS data were processed using ProteinLynx GlobalServer v2.3 and searched in both Uniprot and SwissProt Databases restricted to Poales taxonomy.
Ryegrass pollen extract was also analyzed by 2-DE. Gels were either stained with Colloidal Coomassie or transferred onto a nitrocellulose membranes for the subsequent immunoblot analysis with serum samples of 19 patients sensitized to ryegrass pollen. 2-DE immunoblot images were acquired and analysed by ImageMaster analysis software (Amersham Biosciences). Single immunoreactive spots were excised from Coomassie stained gel, subjected to "in gel" tryptic digestion and analyzed by Ultraflex III MALDI TOF/TOF spectrometer (Bruker-Daltonics). Database search was performed using MASCOT 2.2.03 algorithm against NCBInr database rescricted to Viridiplantae taxonomy.
Results: In a preliminary experiment ryegrass pollen extract quality was evaluated by shotgun proteomic analysis. All the 6 ryegrass allergens annotated in the Official List of Allergens of the International Union of Immunological Societies (IUIS) were recognized demonstrating the integrity of starting material. Further 7 proteins were recognized because they shared identical sequence peptides with homologous proteins of related grass species. A more detailed investigation of pollen protein components was performed by 2-DE. IgE-binding proteins visualized by immunoblot were excised from corresponding coomassie-stained gel and analyzed by peptide mass fingerprinting (PMF) and MS/MS, resulting in the identification of 27 spots. In addiction to the previously reported allergens, we recognized 3 putative ones: Fructosyltransferase (*Lolium perenne*), Cyclophilin (*Triticum aestivum*) and Legumin-like protein (*Zea mays*). Reactivity profiles of each patient were also investigated. Spot immunoreactivities were evaluated by image analysis and expressed as intensity percentage. Clustering serological profiles on a matrix based on presence/absence of allergen components we defined a cohort of patients characterized by a more complex 2-DE pattern. All these patients recognized profilin, considered the main cross-reactive allergen in grass pollen, and showed increased levels of IgE antibodies and a higher susceptibility to sensitization toward different allergen sources.
Conclusions: Allergic diseases evolve through repeated exposures to allergen sources inducing atopic subjects to develop IgE toward a growing number of allergens. At the same time the polyclonality of immune response increases producing a more diverse repertoire of IgE antibodies. Consequently cross-reactivity reactions might occur with higher probability. We observed that the cohort of patients with a more complex 2-DE immunoblot pattern present higher total IgE levels and higher disposition to co-sensitization. This behaviour is mainly associated to profilin IgE recognition but other minor components of grass pollen, such as IgE-binding proteins identified in this work, may be involved in cross-reactivity phenomena in virtue of homology with allergens from other sources.
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MUSCLE PROTEOME CHANGES IN PATIENTS AFFECTED BY POLYMYOSITIS AND DERMATOMYOSITIS

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Background: The inflammatory myopathies, described as idiopathic, represent the largest group of acquired and potentially treatable myopathies. On the basis of clinical, histopathological, immunological and demographic features, they can be grouped into three major subsets: dermatomyositis (DM), polymyositis (PM) and inclusion-body-myositis (IBM). The presence of moderate to severe muscle weakness and inflammation is a common feature. Dermatomyositis is a microangiopathy affecting skin and muscle; it's identified by a characteristic rash which follows or precedes muscle weakness. The skin manifestations include a heliotrope rash on the upper eyelids, often associated with oedema and erythematous rash on face, neck, anterior chest, shoulders, knees, elbows and malleoli. Polymyositis is defined as a subacute myopathy evolving over weeks or months and it's carachterized by weakness of the proximal muscles. Unlike dermatomyositis, in which the rash facilitate the diagnosis, the onset of polymyositis cannot be easily identified, actually, PM mimics many other myopathies and it's diagnosed by exclusion. Both these disorders induce an activation of the immunological system. In DM a complement-dependent humoral process, thought to be initiated by antibodies to endothelial cells, results in a microangiopathy with secondary ischemic changes in muscles. On the other hand, in both, PM and IBM, there is a T-cell response with invasion of muscle fibres by CD8+ lymphocytes and perforin-mediated cytotoxic necrosis. PM, DM and IBM represent a treatable group of disorders by immunotherapies but an accurate and early diagnosis would be essential due to the high toxicity of these therapies. To provide new markers and increase the comprehension of molecular basis of inflammatory miopathies a muscle proteomic profiles of patients affected by PM and DM *versus* control subjects was performed.
Methods: Frozen muscle biopsies from 8 patients affected by PM, 6 patients affected by DM and 7 control subjects, were homogenized in 1 ml of TRIZOL reagent. After total RNA extraction (used for gene expression profiling studies), the remaining phenol acid phase was processed to extract protein. After DNA isolation, proteins were precipitated from the phenol-ethanol supernatant with isopropyl alcohol (800 µl per 1 ml of TRIZOL) according to manufacturer's recommendations. The obtained pellets were dissolved in 200 µl of lysis buffer per samples (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, 1 mM PMSF). The pH of the protein extracts was adjusted to pH 8.5 and sample concentrations were determined using PlusOne 2D-Quant kit. Protein extracts (50 µg) from each set type were labelled with 400 pmol Cy5 dye (CyDye, GE healthcare), while an internal standard, generated by pooling together an aliquot of all muscle samples, was labeled with Cy3 dye. Minimal labeling was performed according to manufacturer's recommendations. Samples were separated on 24 cm, 3-10 non linear pH gradient IPGstrips (GE Healthcare). Each sample was run in triplicate. Second dimension was carried out in 12% polyacrylamide gels. CyDye-labeled gels were scanned on a Typhoon imager (GE Healthcare). Differential analysis was performed using ANOVA provided by DeCyder software (GE Healthcare). The spots differentially expressed were identified by MALDI-ToF/ToF and ESI MS/MS.
Results: Differential protein expression was evaluated using 2D-DIGE on 3-10 NL IPG Strips. 2D gels were scanned on a Typhoon 9200 scanner and the digital images were analysed. After automatic spot detection, background subtraction, and volume normalization approximately 4000 spots were detected. Differential analysis was performed by DeCyder software and spots differentially expressed (p<0.01) were identified by MS/MS. 99 spots changed between control subjects and patients affected by PM, among these 54 were identified (21 spots resulted down regulated in PM patients and 33 up regulated); 134 spots resulted differentially expressed between control subjects and patients affected by DM, 72 were identified (28 resulted down regulated in DM patients and 44 up regulated); and only 19 spots changed between PM and DM patients, 8 identified (all down regulated in PM patients). Patients affected by PM and DM shown a down regulation of some key enzymes involved in glycolysis and TCA cycle; and an up regulation of a series of proteins involved in the processing and presentation of antigens, in the response to stress and regulation of apoptosis.
Conclusions: The idiopathic inflammatory myopathies are an important and treatable group of disorders. However the potential toxicity associated with the immune therapeutic regimens used to treat these

disorders requests an accurate diagnosis. Data provided by our approach represents a starting point to find a new diagnostic strategy of these disorders.

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LIPIDOMIC INVESTIGATION FOR THE CHARACTERIZATION OF CIRCULATING POLAR LIPIDS IN MULTIPLE SCLEROSIS

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Introduction: Multiple Sclerosis (MS) is a neurodegenerative autoimmune demyelinating disease affecting young adults. The aetiology still remains a mystery and diagnosis is still impaired by the lack of defined molecular markers. The autoimmune response in multiple sclerosis remains incompletely characterized. Recent studies suggest additional non proteic mediators of autoimmune brain inflammation such as lipids. In order to investigate characteristic serum metabolic "fingerprint pattern" as the result of the onset of disease we developed a liquid chormatography/mass spectrometric method followed by statistical multivariate analysis investigation. Once improved the method was applied in a preliminary clinical cross-sectional investigation of multiple sclerosis affected patients vs healthy subjects.
Methods: Polar lipids were analyzed using a CapLC system (Micromass, Waters) coupled on-line with a Q-TOF-Ultima instrument (Micromass, Waters) equipped with a nano-Lock-Spray source operating in positive ion mode. Elution at 3μl/min was obtained using a column Jupiter (phenomenex) 5 μm, 300 Å 300 μm inner diameter × 150 mm, with a water/ tetrahydrofuran gradient in the presence of 0.2 % of formic acid. The method was developed and optimized using seven representative lipids (3 sphingolipids, 3 glycerophospholipids and 1 glycosphingolipid), Deuterated Sphingomielin (PSM d31) used as internal standard. A liquid-liquid extraction following the modified "Bligh and Dyer" method was used for the lipids purification before analysis.
Centroid data acquisition in the 300-2000 m/z range and GluFibrinoPeptide (GFP) 200 pg/μl as reference compound for on-line recalibration data (m/z = 785.8426 Th) were used. Data processing was performed using MarkerLynx software (Waters) obtaining a data format compatible with Simca-P (Umetrics AB, Umeå, Sweden) used for multivariate analysis.
Results: We developed an LC-MS method for lipid profile investigation in biological samples. This method allows to get a lipid profile containing thousands of mass signals for each analyzed sample deriving from metabolic fraction extracted. The analytical performances were tested using a surrogate of biological matrix (4% bovine albumin) spiked with lipid standards at different concentrations. Excellent percentage recoveries and linearity response were achieved (correlation coefficient $R^2 \geq 0.985$ for each analyte, RSD% ≤ 15%, accuracy ≤ 10%), as a confirmation of a good analytical reproducibility which is an essential feature in quantitative investigation (n=5). Mass accuracy was below 15 ppm for each analyte (n=18). The method was optimized using a representative class of phospholipids and applied for the screening of the total pool of lipids in a definite biological sample. The LC-MS method described was applied to study the circulating lipid pattern in 18 MS patients with defined relapsing remitting course, 17 patients with Other Neurological Diseases (OND) and16 Healthy Controls (HC). Data acquired were underwent to multivariate analysis using Simca-P software where each variable was the m/z associated to its own retention time. Principal Component Analysis (PCA) clearly separate the lipid profiles of Multiple Sclerosis patients from those of control subjects. Partial Least-Squares Discriminant Analysis (PLS-DA) for the first two components showed the separation between the three groups. In order to select the differential lipids, a parameter VIP (Variable Importance in the Projection) with a score ≥ 1.5 was employed to highlight the major discriminant variables. 43 signals resulted significant in discriminating the groups. Structural characterization of differential lipids was tentatively performed through molecular fragmentation experiments by tandem mass spectrometry (MS/MS, neutral loss scan, parent ion scan). As a support for the molecular fragmentation data, was possible to look up in a on-line resource of lipid data bank, mainly by the use of accurate mass experimental data (www.lipidbank.ip, www.lipidlibrary.co.uk, www.cyberlipid.org, www.lipidmaps.org, www.lipidat.chemistry.ohio.state.edu).
Conclusion: Here we describe a lipidomics based LC-MS method as a powerful way of potential biomarker discovery. Application on multiple sclerosis disease showed remarkable results highlighting differential serum lipids in MS patients. Validation and extension of this investigation will be necessary to confirm the potential biomarkers highlighted, giving an important functional insight, possibly shading a light on their implication in the pathological process.
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CHANGES IN HUMAN BLOOD PLATELET PROTEOME FOLLOWING CHRONIC INTAKE OF ORANGE JUICE

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Background: The EU FLORA project (Flavonoids and related phenolics for healthy Living using Orally Recommended Antioxidants) has been developed on the basis of growing evidences on the role of flavonoids and related phenolics as example of bioactives that have beneficial influences on a number of important risk factors associated with cardiovascular diseases, cancer and age-related diseases. Beside their main physiological function in hemostasis, platelets are also highly involved in pathological processes, such as atherothrombosis and inflammation and proteomics offers the opportunity to comprehensively explore the proteins involved in the various pathways of platelet function.
The health related properties of polyphenols contained in orange juice are mainly based on their antioxidant activity. We studied whether a chronic intake of orange juice affects platelet protein expression and, in particular, their carbonylation, which is an important protein modification elicited by oxidative stress.
Methods: Blond or blood orange juice were administered in a cross-over design to 16 volunteers (8 smokers and 8 non-smokers). Fresh human blood was collected from volunteers. Platelets were separated and resuspended in PBS, proteins were precipitated with 15% TCA and washed twice with ice-cold acetone. The pellet was resuspended in sample buffer, labeled with CyDye and subjected to IEF. The IPG gel strips were placed on top of SDS gel and all gels were runned at the same conditions. Labeled proteins were visualized using a Typhoon 9410 imager, gels were fixed and then visualized with silver staining.
Gel analysis was performed using DeCyder 2-D Differential Analysis Software v6.5 (GE Healthcare). All pooled standard/sample gel image pairs were processed by the Difference In-gel Analysis (DIA) software module to codetect and differentially quantify the protein spots in the images. Gel to gel matching of the spot maps was then performed using the Biological Variation Analysis (BVA) software module. Finally Extended Data Analysis (EDA) module carried out intra- and inter-gel statistical analyses.
The spots were digested with trypsin, peptides from mass spectra of in-gel digest samples were matched against databases such as Swiss-Prot, NCBInr, using the Mascot search engine (Matrix Sciences) for PMF (peptide mass fingerprint).
Results: In the non-smoker group, Transketolase and Pyruvate kinase increased after blond orange juice intake, while after blood orange juice intake Coronin, β-Fibrinogen precursor, ATP synthase (mitochondrial F1 complex), Nucleosome assembly protein1 and Actin significantly increased and Thrombospondin-1, Glutathione-S-transferase and Lactate dehydrogenase B significantly decreased.
In the smoker group, following blond orange juice intake, Talin and Miosin significantly increased while after blood orange juice intake Peroxiredoxin 6 and Filamin A increased, whereas HSP 70 kDa and Protein disulfide isomerase decreased.
The carbonylated platelet protein profile was changed after juice intake; in particular a dramatic decrease in the carbonylation degree of Actin was observed after blood orange juice intake.
Conclusions: Platelet proteins, in particular carbonylated proteins, were differentially expressed following a diet intervention with orange juice, as detected by proteomic approach. (EU, 6FP,contract n 007130)

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PROTEOMIC DISCOVERY OF NEW DIFFERENTIATION MARKERS IN THE U937 CELL LINE

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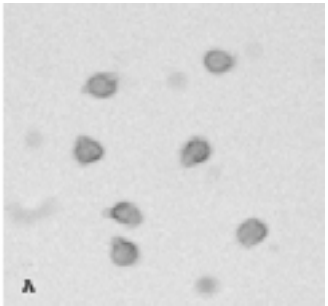
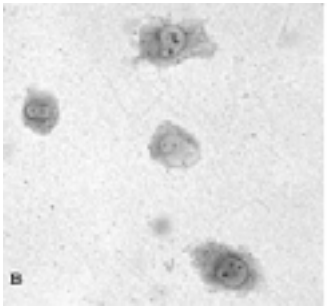
Background: The U937 cell line, originally established from a histiocytic lymphoma, has been widely used as a powerful in vitro model for haematological studies. These cells retain the immature cell phenotype and can be induced to differentiate by several factors, among which tetradecanoylphorbol acetate (TPA). The aim of this study was to identify new differentiation markers of U937, to implement the potentiality of this model for the study of myelomonocytic differentiation and human leukemia diseases.
Methods: U937 cells (provided by ATCC, American Type Culture Collection, Massas, VA.) were seeded at concentration of $5x10^5$/ml in RPMI-1640 medium supplemented with 10% foetal bovine serum, 1% penicillin and 1% streptomycin. Cell differentiation was induced by incubating cells with 10 ng/ml (16 nM) 12-O-tetradecanoylphorbol-13-acetate (TPA), for 72h, according to the protocol of Stöckbauer et al. (Neoplasma 30, 257-272, 1983). Proliferating control cells were cultured under the same conditions for 72h, in the absence of TPA. At 72h after seeding, proliferating cells were removed from flasks by pipetting, centrifuged at 1,000 rpm and washed with ice-cold phosphate buffered saline (PBS) to remove serum, while the TPA-treated adherent cells were scraped from the flask and processed as the other cultures. Both cells cultures were separately incubated on ice for 30 min with RIPA buffer and a mixture of protease inhibitors and processed for proteomic analyses, according to our published protocols (Pucci-Minafra et al. Connective Tissue Research. 49, 252-2562, 2008).
Results: Cell differentiation was monitored by morphological and immunological assays. Figure A shows a microscopic field of undifferentiated floating cells and the figure B the adherent differentiated counterpart. The differentiated cells were shown positive to two differentiation markers for the macrophagic line, i.e. CD206 and CD14. The proteomic results have highlighted that the transition from the proliferating-floating U937 cells to their non-proliferating-adherent counterparts, is correlated with the modulation of a set of protein spots, likely corresponding to a significant group of strategic genes. Among these, 4 appeared to be down-regulated, while 12 spots, corresponding to 11 individual proteins, were overexpressed. The first group contained the nucleoside diphosphate kinase A and B, the phosphoglycerate mutase1 and one isoform of the triosephosphate isomerase. Among the second group were 4 annexins, 3 members of S100 protein family, calreticulin, the glycoprotein C1qBP, the heat shock proteins GRP94 and HSP71, and the thioredoxin.





Conclusions: This study represents a contribution to the widespread effort for making progress in the collection and integration of proteomics with biomedical and clinical data, having as major goal the identification of proteins or genes with critical roles in relevant pathways involved in haematopoietic cell differentiation and blood cancer. The present results have highlighted interesting proteomics changes, not described before, occurring as a consequence of the TPA-induced differentiation of the myelomonocytic cells U937. A first set of modulations include gene products playing a key role in the control of cell maturation and metabolism. The second ones include proteins involved in biological pathways relevant for the macrophagic functions, such as surface and membrane traffic, phagocytosis and antigen-presenting pathways. We propose that this panel of differentiation markers may be of valuable utility as prognostic/predictive factors for haematological malignancies.

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PROTEOMIC ANALYSIS OF FROZEN AND FORMALIN-FIXED PARAFFIN-EMBEDDED HYPERPLASTIC PARATHYROID GLANDS

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Background: Proteomic techniques are widely applied to differentiate normal from cancer cells and to identify biomarkers for specific cancers. Even if a number of proteomic studies have been increasing in the field of cancer research, at this time not even one proteomic study has been performed regard biomarkers in parathyroid carcinoma. Parathyroid carcinoma is a rare cause of parathyroid hormone dependent hypercalcaemia (PTHP) with incidence value in PTHP patients less than 1% of cases. Diagnosis of a parathyroid malignancy is notoriously difficult and it is often impossible to distinguish benign from malignant disease without clear evidence that the tumor is invasive. Until now, no genetic or protein markers that reliably distinguish carcinoma from adenoma have been identified. Although fresh or frozen samples are more ideal samples for proteomic investigation, they are difficult to obtain in large numbers especially for this rare kind of tumour. Therefore, we performed a proteomic analysis both in frozen and formalin-fixed paraffin-embedded (FFPE) parathyroid tissue samples. In fact, formalin fixation and paraffin embedding of tissue represent the standard processing methodology routinely practiced in pathology laboratories resulting in stable form of tissue easily stored at room temperature. However, in these samples proteins are difficult to extract because formalin reaction with amino groups of basic amino acid residues causes the formation of covalent cross-linking of proteins.
Methods: Specimens of hyperplastic parathyroid glands were frozen at -80 °C immediately after the surgery from 16 patients classified in three groups depending on their calcaemia levels: control group; high iCa2+ (A); medium iCa2+ (B); low iCa2+ (C). Aliquots of samples were homogenized, centrifuged and the pellet was solubilized in rehydration solution. The insoluble material was centrifuged and the supernatant was subjected to two-dimensional electrophoresis (2DE), stained with silver and images were analyzed with Image-Master 2D Platinum software. Spots of interest were identified by mass spectrometry.
Specimens of FFPE hyperplastic parathyroid tissues were collected from 2 patients. 5 μm section pieces (7-15 slides) were deparaffinized in 2-5 changes of xylene for 10 min each. Then the tissue was rehydrated through a series of graded alcohols (100% ethanol two times, 85% ethanol, 70% ethanol) for 10 min each. Tissue was resuspended in 20 mM TrisHCl at pH 4, 6 or 9 with 2% SDS and 0.2 M glycine, sonicated 3 times for 10 sec each and proteins were extracted for 1 hour under agitation at 4°C. The homogenate was heated at 100°C for 20 min and at 60°C for 2 hours. The unsolubilized material was pelleted at 48000 g for 20 min at 4°C. Aliquots of protein fraction were precipitated with TCA-acetone or with methanol-chloroform prior to undergo to mono- and two- dimensional electrophoresis. Proteins were stained with Coomassie or silver and images were analyzed with Image-Master 2D Platinum software.
Results: About 1150 spots have been detected in frozen hyperplastic parathyroid glands and comparison of each group of pathological samples with respect to controls allowed us to identify qualitative and quantitative differences. In particular, a different expression of mitogen-activated protein kinase was observed among the three groups in accordance with the calcaemia increase, moreover a peculiar expression of tropomyosin alpha-4 chain in the B group and of aconitase hydratase in the C group was found.
These results encouraged us to search for a strategy to identify potential biomarkers also in parathyroid carcinoma available only as FFPE tissues. Then we preliminarily applied protein extraction protocols to samples of hyperplastic parathyroid glands. Among the different protein extraction protocols tested from FFPE tissues, a major yield of spot number is obtained at pH 4 or 6 followed by protein precipitation with methanol-chloroform (220 spots). Although the spot number is significantly lower than those obtained from the frozen tissue the yield is comparable to those reported for other FFPE tissues. After comparison of the gel images a matching of about 30% was found between FFPE gel and frozen tissue map. Some of these spots were identified as ATP syntase subunit beta, 40S ribosomal protein SA and cytochrome b-cl complex subunit 1.
Conclusions: In this study 2DE resulted a useful tool to investigate the presence of potential biomarkers in parathyroid diseases, moreover, our preliminary results obtained from FFPE tissues provide an initial detailed framework to carry out the further protocol refinement needed to investigate rare parathyroid cancer.

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EVALUATION OF DIFFERENT METHODS OF ALBUMIN PURIFICATION IN THE BIOMARKER DISCOVERY OF HEPATOCELLULAR CARCINOMA.

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Background: Hepatocellular Carcinoma (HCC) is among the major causes of cancer death worldwide. HCC develops in more than 80% of cases in patients with cirrhosis with an annual 2-5% incidence. Cirrhosis is the greatest risk factor for HCC in Western populations and the cirrhotic patients should be monitored for early detection of outbreaks of the HCC. However, current diagnostic approaches are not very reliable in early diagnosis. Abdominal ultrasound examination is currently the only recommended analysis for the monitoring of cirrhotic patients. Unfortunately, ultrasound techniques are not able to identify tumors smaller than 1/2 cm or the presence of early invasion. In addition the current circulating markers such as alpha fetoprotein, are affected by low sensitivity and specificity. To detect either the early onset of cancer or microvascular invasion it is necessary to identify new circulating markers with high sensitivity and significance. The identification of tumor markers in body fluids, however, presents considerable difficulties due to the complexity of the fluids. Particularly, a high protein content and highly different concentrations in the levels of circulating proteins (up to 12 orders of magnitude). In serum, the twenty most abundant proteins quantitatively represent 99% of the total, albumin alone is 50-60%. And this is a huge obstacle because the most abundant proteins mask the less abundant proteins that may contain important diagnostic information. Considering that it is possible to remove the most abundant proteins and analyze the remaining fraction, we focused on a single protein, human serum albumin (HSA). This protein acts as carrier forming stable complexes with peptides and small proteins. It was therefore suggested the possibility that some of the tumoral factors, once secreted, remain bound to albumin. All these data allowed to formulate the following approach: albumin purification from serum samples followed by the analysis of the profile of the albumin bound proteins and peptides.
Methods: We have assessed different approaches to search for a reliable method for the albumin depletion from human serum that is also suitable for large-scale screening of patients with HCC. Initially we used two immunomagnetic beads that have different chemical characteristics: Chemicell® and Dynabeads®. The first characterized by cyanide groups, is ideal for coupling reactions with ligands having primary amino groups, the second instead have tosylate groups able to bind to amino-or sulphydryl groups. We coated the beads with a monoclonal antibody anti-albumin, we incubated the coated beads with control serum and then we eluted albumin from beads using 0.1 M glycine. In addition to the immunomagnetic beads, we evaluated three commercially available different depletion kits characterized by a resin already packed with anti-albumin antibody: Calbiochem® ProteoExtract® Albumin Removal Kit, Vivapure® Anti-HSA Kit for Human Albumin Depletion and Enchant™ Life Science Kits Albumin Depletion. The experimental protocol, therefore, included control serum incubation followed by HSA elution as above. Eluted solutions were analysed by SDS-PAGE, mixed directly with sample buffer, and by MALDI-MS (Applied Biosystems® Voyager-DE PRO®), after desalting mixed with sinapinic acid and alpha-ciano-4 hydroxycinnamic acid matrices, respectively in the mass range of 2000-200000 m/z and 2000-20000 m/z.
Results: Using SDS-PAGE followed by tryptic digestion and MALDI-MS we found the presence of albumin and other human serum proteins in the solutions eluted from the two bead-based systems. The latter were also analyzed directly by MALDI-MS and subjected to repeated analysis to assess the *intraday* and *interday* reproducibility. The resulting spectra showed profiles that were not super imposable. The same analytical SDS-PAGE and MALDI-MS protocols were then applied to the solutions eluted from the three resin-based HSA depletion kits examined in the present study. The most satisfactory results were obtained with the Calbiochem kit that produced a high HSA signal associated with very low signals from unspecific proteins. Furthermore, the MALDI-MS spectra generated by this kit showed a satisfactory *intraday* and *interday* reproducibility.
Conclusions: Our data indicate that depletion systems based on immunomagnetic beads suffer of low selectivity and poor reproducibility, making them not ideal for biomarker discovery and protein profile after HSA depletion. Of the antibody prepacked resin based kits, the Calbiochem depletion kit produced high-quality HSA signals through SDS-PAGE and MALDI-MS and a lower abundance of non specific proteins.

Our future goal will be to study the proteome associated with eluted albumin and thus extend the use of the kit with sera of HCC patients.

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PROTEOME ANALYSIS AND CORRELATED SIGNALING NETWORK OF REVERSINE TREATED MURINE FIBROBLASTS

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Background: It has been shown that terminally differentiated fibroblasts can be induced to de-differentiate into progenitor cells, almost indistinguishable from *bona fide* embryonic stem cells, as they possess almost identical gene expression profiles and the same differentiation potential (including teratoma formation capability).The use of synthetic small molecules to induce the de-differentiation process seem to be a potentially safer approach. A synthetic purine, called "reversine", has been shown to induce the de-differentiation of myoblasts into mesenchymal progenitors. Moreover, reversine-treated human or murine fibroblasts could be induced to differentiate into skeletal muscle, smooth muscle and bone. Thus, it seems clear that the use of small molecules that increase cellular plasticity, *i.e.* allow terminally differentiated cells to trans-differentiate into a different cell type, could be an alternative to the "*genetic manipulation*" approach, and it would also provide a tool for investigating the complex mechanisms regulating cell differentiation. Altogether, these and other evidences seem to support the notion that reversine induces an initial growth arrest, which seems to be a key step for the reprogramming process. However, the full mechanism whereby reversine is able to increase the plasticity of adult cells has yet to be clarified. In this context it is of interest to define proteins involved in this induced growth arrest.
Methods: Primary mouse dermal fibroblasts were obtained from transgenic mice expressing GFP. Fibroblasts were treated with reversine, dissolved in DMSO, at concentration of 5 mM in 10% FBS DMEM, 18–24 h after seeding. Cell analysis was performed on at least 20000 events for each sample by FACSCalibur System (BD) and DNA profile was analyzed by MODFit 3.0 (Verity Software House). 2D-DIGE was conducted on protein extracts from each set type of three separated culture plates, labelled with Cy5 dye, while the internal standard, generated by pooling together an aliquot of each reversine-treated fibroblasts extracts and each control, was labelled with Cy3 dye. The minimal labelling was performed according to manufacturer's recommendations. CyDye labelled gels were visualized using a Typhoon 9200 laser scanner (GE Healthcare). Spot detection was performed using DeCyder DIA module V. 6.5 (difference in-gel analysis, GE Healthcare). For protein identification semipreparative gels, containing 400 μg of total protein extract per strip, were loaded with unlabelled sample; electrophoretic conditions were the same as 2D-DIGE, except that gels were stained with a protein fluorescent stain. Tandem electrospray mass spectra were recorded using a HCT Ultra mass spectrometer (Bruker Daltonics) interfaced to a MDLC capillary chromatograph (GE Healthcare). Proteins were identified by correlation of uninterpreted tandem mass spectra to *Mus Musculus* entries in NCBInr database, using MASCOT software. No mass and pI constraints were applied. One missed cleavage per peptide was allowed, and the fragment ion tolerance window was set to 0.3 Da. Differentially expressed proteins were evaluated by IPA (Ingenuity System, Montain View, CA).
Results: Approximately 2700 spots were matched per gel. Among them 75 were differentially expressed (t-test, $p<0.01$) in treated *vs* untreated cells. Protein load increment induces a loss of isoforms resolution causing a minor number of represented spots, only 66 of the differentially expressed spots were picked up and 53 of them identified by MALDI-ToF and ESI MS/MS. Murine reversine-treated fibroblasts in this study indicate changes in proteins involved in cytoskeletal and cell shape remodeling, RNA export, degradation machinery, folding, stress control and ATP production. The results provided by this differential study support data from previous studies, confirming the effects of reversine on cell cycle, cell proliferation and cell morphology. To gain better insights into biological significance of the quantitative differential results, deregulated proteins were analyzed by IPA to get statistical information about signalling pathways involved in cellular response to reversine treatment. 42 proteins were involved in 5 networks on the basis of IPA criteria. The networks with high score are associated with cellular assembly and organization, cancer, cell to cell signalling and interaction, cell death, molecular transport and cellular function and maintenance.
Conclusions: Proteomic investigation coupled with bioinformatic tools demonstrated to be an appropriate approach to study the effects induced by treatments of cell culture with new drugs. The main changes induced by reversine on fibroblasts indicate a primary role of reversine in cell cycle arrest, in inducing cytoskeletal remodelling, in promoting positive stress response, in modulating mRNA export, in regulating cell signaling and proliferation.

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030

COMPARISON BETWEEN EX-VIVO AND IN-VITRO AGING MODELS IN THE STUDY OF HUMAN DERMAL FIBROBLAST SENESCENCE

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Background: Aging is the outcome of interactions between genetic factors and the overtime accumulation of a variety of deleterius stochastic changes. Several theories have been proposed to explain this process. However, results may vary depending on the experimental model and on the environmental conditions. It has been suggested that, together with an increase of genome complexity, the impact of candidate genes on lifespan becomes smaller and perhaps complicated by the presence of genetic networks that, through multiple interactions, may further affect lifespan. Nevertheless, there are data supporting the crucial role of mitochondrial oxidative stress as a determinant of both healthspan and lifespan. It is believed that cultured human dermal fibroblast (HDF) may represent a suitable model for investigating how mesenchymal cells can modify their behaviour and consequently connective tissue homeostasis. We have already demonstrated that the protein profile of fibroblasts isolated from donors of different ages exhibited several differentially expressed proteins, supporting the concept that HDF cultured in vitro from young, adult and old subjects retained, in vitro, at least some of the characteristics they exhibit in vivo, not only regarding proliferative capabilities, but also as far as metabolic and degradative pathways as well as the capacity to cope with endogenous and/or exogenous stresses. These data are in agreement with the hypothesis that senescence is a progressive phenomenon being the result of both genetic and epigenetic factors. Never the less, the molecular consequences of in vitro aging, on cell phenotype, are still elusive and even more unclear is the relationship and/or the pathways that are differently affected during in vitro or ex-vivo aging. We have therefore examined the behaviour of HDF isolated from young and old subjects (ex-vivo aging model) and we have compared these cells at early and late population doubling level (PDL) (in-vitro aging model). Human dermal fibroblasts in culture undergo a finite number of divisions and progressively reach a state of irreversible growth arrest, a process known as replicative senescence (RS).
Methods: Fibroblasts were obtained from skin biopsies of young (< 15 years) and old (> 80 years) female donors. For all experiments, cells were cultured until confluence and, at different PDL, have been analyzed for oxidative stress parameters and protein expression. In particular, intracellular levels of reactive oxygen species (ROS) were estimated by flow cytometry using the dihydroethidium probe (DH2) for O2•- detection. Fibroblasts were treated with 1 μM DH2 for 60 minutes at 37 °C, trypsinized, collected in PBS and analyzed. To reveal hydrogen peroxide, cells were trypsinized, washed and stained with H2DCF-DA (2μM) for 30 min at 37°C. Before flow cytometric analysis, cells were centrifuged and resuspended in PBS. Data have been analyzed with GraphPad vers.4.0. Protein extracts were loaded (30μg proteins/lane) on 1-DE 10% polyacrylamide gel under reducing conditions and transferred to NC. Antibodies towards proteins involved in oxidative stress response as well as aging protein markers were used for WB [namely, protein disulphide isomerase (PDI), caveolin 1 (CAV1), calreticulin (CLR), 78kDa glucose regulated protein (GRP78), Hsp27 and Hsp60, annexin II (ANXII), FKBP52 and thioredoxin (TRX)]. Western blots were visualized using the ECL plus detection system according to the manufacturer's protocols. Densitometric analysis of protein bands was performed using ImageQuant TL v2005 software. The aging phenotype was evaluated by β-galactosidase assay at different PDL.
Results: At low PDL, all cell lines were characterized by a good proliferative index and fibroblasts showed a typical elongated shape. By increasing PDL, fibroblasts gradually enlarged and, after 30 PDL, reduced their proliferation capability. Morphological modifications were associated with changes in the expression of membrane proteins, as CAV1 and ANX II, that appeared markedly increased in in-vitro aged cell lines, similarly to what observed for FKBP52. Proteins of the endoplasmic reticulum (i.e. PDI, CLR and GRP78), as well as molecular chaperones (i.e. Hsp60 and Hsp27), were significantly down-regulated in in-vitro aged fibroblasts, independently from donor's age. By contrast, oxidative stress parameters, revealed by DH_2 and H_2-DCFDA fluorescent probes, appeared significantly increased in fibroblasts from old donors already at low PDL; values further increased when cells reached RS. Interestingly, age-dependent accumulation of ROS was associated, for instance, to down-regulation of TRX, which was particularly evident in fibroblasts from old donors. Beta-galactosidase was significantly increased only when fibroblasts reached RS.
Conclusions: Data clearly demonstrate that fibroblasts from aged donors (ex-vivo aging model) could represent a suitable model for investigating the relationships between aging and oxidative stress, highlighting the importance of oxidative stress as an early marker of aging, even in the presence of optimal environmental conditions. However, the great majority of parameters are modified only during RS (in-vitro

aging model), supporting once more the hypothesis that cells, at increasing PDL, could be considered a more reliable model of senescence, thus resembling more closely the in vivo situation.
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EVALUATION OF DIFFERENT FRACTIONATION STRATEGIES TO IDENTIFY SERUM MARKERS FOR PROSTATE CANCER.

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Background: Prostate cancer (PCa) is the most common solid organ malignancy affecting men in Western word. Currently, serum prostate specific antigen (PSA) is the screening test widely used in the diagnosis and management of patients with PCa. In relation to the poor sensitivity and specificity of PSA, there is an urgent need for additional markers. To improve the diagnostic accuracy, proteomic profiling of serum represents a valuable tool for biomarker discovery in prostate cancer. Our study aims describing and comparing serum proteome pattern in PCa patients and in matching control group. In order to enhance the detection of low abundance proteins fraction (LAP), the most probable source of biomarkers, the performance of three different strategies: ProteoMiner (Bio-Rad), MARS Human-7HPLC colum immunoaffinity depletion (Agilent) and Dynabeads SCX (Invitrogen), in combination with 2-D electrophoresis (2-DE) analysis, are evaluated. Advantages and disadvantages of these approaches are discussed and results are compared.
Methods: Donors: serum samples are from patients with A) PCa and PSA>4ng/ml, B) BPH (benign prostate hyperplasia) and PSA>4ng/ml, C) BPH and PSA<4ng/ml and D) healthy donors. Sera from each group (20 patients/group) are pooled. Sera from donors are matched for age and dietary and smoking habits. ProteoMiner: 1ml of each pooled serum sample is loaded on the ProteoMiner column. Two different elution methods are tested: 1) single step elution protocol (elution reagent: 4M urea, 1% CHAPS, 5% acetic acid), compatible with the following 2-DE; 2) sequential elution procedure (elution reagents: a) 1M sodium chloride, 20mM HEPES, pH7.4; b) 200mM glycine, pH2.4; c) 60% ethylene glycol; d) 33.3% 2-propanol, 16.7% acetonitrile, 0.1% trifluoroacetic acid). Protein precipitation step with acetone is set-up prior to 2-DE. MARS Human-7HPLC: the 7 most abundant proteins (HSA, IgG, IgA, transferrin, haptoglobin, α1-antitrypsin, fibrinogen) are depleted using a System Gold HPLC apparatus (Beckman). To remove salts from elution buffers and concentrate depleted sera, a protein precipitation is carried out using 7.5% TCA for 1h at -20°C, followed by 4 washes in 90% acetone. Dynabeads SCX: Sera pools are diluted 1:50 in 20mM citric acid pH3, 10mM sodium chloride, which represents the absorption buffer required for the prefractionation tool. Sequential elution by increasing pH is achieved. Gel images analysis: for spot detection, images of gels captured with ProXPRESS 2D System (Perkin-Elmer), are analyzed by Progenesis SameSpots software (Nonlinear Dynamics Ltd)
Results: ProteoMiner: single step elution protocol is chosen for further experiments, as it exhibits a better reproducibility of spots detected in 2-DE compared with the sequential elution procedure. Reproducibility of protein yield is evaluated for sera pool of group A and D: 1468±35 µg, CV%=2.4 and 1340±33 µg, CV%=2.6, (mean ± SD) (n=3), for A and D. MARS Human-7: referring yield, reproducibility of amount of proteins quantified in flow-through fractions are 195µg ± 35, CV% 4, (mean ± SD) (n=8). respectively The number of spots detected in 2-DE, evaluated for triplicate samples, always shows a good reproducibility with CV% lower than 10% both for ProteoMiner and MARS. Dynabeads: SCX fractionation is not satisfying, as high abundance proteins compete with LAP for absorption to solid phase on the beads, resulting in almost the same protein composition in the different fractions, as verified by nLC-MS/MS analysis. In addition this approach shows a high variability, so it is abandoned. Comparison of 2-DE images of serum of pool D evidenced that complementary results are provided by ProteoMiner and MARS techniques.
Conclusions: In this study the experimental procedures for ProteoMiner, MARS and Dynabeads SCX strategies were set up, including sample preparation and 2-DE protocols.
Our findings suggest that immunodepletion and ProteoMiner approaches are complementary and can be readily integrated into an analytical strategy for biomarker discovery. Future investigations will be focused to describe proteomic profile of different group of patients above-named utilizing both the procedures.

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BIOINFORMATICS ANALYSIS OF RCC PROTEIN PROFILE SIGNATURES

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Background: Renal cell carcinoma (RCC) is the most common neoplasm in the adult kidney and represents 2-3% of all cancers. Moreover RCC is one of the most therapy-resistant carcinomas, which respond very poorly or not at all to radiotherapy, hormonal therapy and chemotherapy. Patients' outcomes with surgery are primarily dependent on tumor characteristic. This suggest that a better understanding of changes in protein expression levels and their functional pathways is essential in order to improve current diagnosis, prognosis, and treatments of RCC. Therefore we have undertaken a differentially proteomic analysis and subsequently a computational analysis of pathway and network to identify possible biological processes involved in RCC.

Methods: Comparative proteomic analysis was performed using two-dimensional difference gel electrophoresis (DIGE) both on RCC and adjacent normal tissue counterparts (n=20). The protein spots were analyzed using the DeCyder software program (version 6.5 GE Healthcare) and the Extended Data Analysis (EDA) module allowed to measure the abundance of each of the protein spots in the experimental samples. Next, the Biological Variance Analysis (BVA) module was used to compare the expression of protein spots between the two groups. Spots with statistically significant variations were excided from the gels and proteins were identified by PMF technique. The analysis was leaded with automated procedures of gel spot digestion using the integrated workstation PROTEINEER DP. Proteins were identified by MALDI TOF MS Reflex IV in positive ion reflector mode. Biological processes and interaction networks among these differentially expressed proteins were generated using Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Mountain View, CA, www.ingenuity.com).

Results: 121 statistically differentially expressed spots ($p<0.05$) were excided from gel and analysed in MALDI-TOF spectrometry. Of these, 34 protein spots were more expressed in tumor and 87 more expressed in normal. Using IPA software we have undertaken a subdivision of the proteins in the most significant pathways according to the main functional categories including biological process, molecular functions and relevant interactions amongst themselves and other proteins. In particular, these proteins were mapped into three major networks: (a) Lipid Metabolism, Small Molecule Biochemistry, Metabolic Disease; (b) Small Molecule Biochemistry, Metabolic Disease, Cell-To-Cell Signaling and Interaction; (c) Nucleic Acid Metabolism, Small Molecule Biochemistry, DNA Replication, Recombination, and Repair. These pathways had scores of 41, 32 and 26 respectively. Canonical pathway analysis using IPA demonstrated that important signaling pathways are highly associated with mithocondrial dysfunction.

Conclusions: We have found a group of proteins which are dysregulated in RCC. Proteome analysis combined with bioinformatics tools performed on these proteins have confirmed the complex nature of this malignant tissue that involved different metabolic pathways. Therefore this approach could be a first step towards elucidation of the metabolic signatures of RCC and key biological process altered or disrupted in this tumor.

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CIRCULATING T-CELLS AS MOLECULAR BIOSENSORS: THE CASE OF A CUTANEOUS HODGKIN LYMPHOMA

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Background: A 69 years old man showing, from 5 years, an erythematous papulo-nodular lesion at the right lumbar region. The patient presented good physical conditions and declared no symptoms associated with the lesion. A biopsy was performed allowing diagnosis of a Hodgkin lymphoma. All clinical tests performed to assess the patient's general state led to a diagnosis of an Hodgkin lymphoma localized exclusively to the skin, a condition described rarely in medical literature. Since a proteomic study on circulating T-lymphocytes from control subjects and from patients affected by different pathologies (including lymphoproliferative disorders) had been carried out in our laboratories, we decided to compare the proteome profile of T-cells obtained in the described case with those previously acquired.
Methods: T-lymphocytes were purified by peripheral blood mononuclear cells using the Pan T Cell Isolation Kit II, human (Miltenyi Biotec Inc., USA). Purified T cells were lysed in 0.1% sodium dodecyl sulphate (SDS)/2.3% dithioerythrol (DTE), proteins were precipitated with 80% (v/v) cold acetone, resuspended in 8 M urea/4% CHAPS solution and quantified using the Bradford assay. Proteins were separated by two-dimensional gel electrophoresis and differential analysis was performed using the Bio-Rad PDQuest 7.1 software. Spots of interest were identified by mass spectrometry.
Results: Comparison of T-cells protein expression profiles shows some peculiarities in T-lymphocytes derived from the cutaneous Hodgkin lymphoma case. In detail, an altered ratio between different isoforms of Glyoxalase I (GI) was found. GI is involved in detoxification of cellular metabolites and its dysregulation has been described in many tumors. Furthermore, Calgranulin A and B, which form a heterodimer called Calprotectin, are considerably decreased. Calprotectin participates in several biological processes and is involved particularly in the pathogenesis of cutaneous diseases.
Conclusions: Cutaneous Hodgkin lymphoma is an atypical condition. In the reported case, the patient didn't develop in systemic lymphoma across a long period. Based on the hypothesis that the patient might harbour some peculiar characteristic leading to protection from cancer spreading, proteome profile of T-cells was acquired, revealing unusual traits. Their biological significance will be addressed by further studies focused on the identified proteins.

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IS THERE A COMMON PATHWAY RESPONSIBLE FOR ECTOPIC CALCIFICATION IN PXE AND PXE-LIKE DISORDERS?

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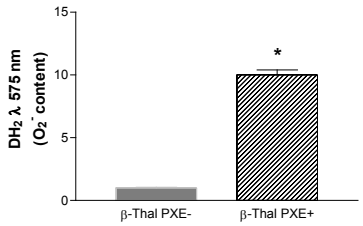
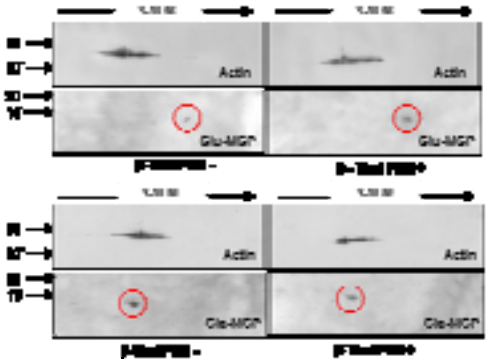
Background: In contrast to the biomineralization physiologically occurring in bones and teeth in vertebrates, calcification of soft tissues is a pathologic feature. Although similarities have been noted between the two processes, the molecular mechanisms regulating ectopic calcification are poorly understood. It is already known that in pseudoxanthoma elasticum (PXE), a genetic disorder due to ABCC6 gene mutations, clinical manifestations are due to progressive mineralization of elastic fibers. It has been recently demonstrated that dermal fibroblasts isolated from these patients show an oxidative stress condition and that alterations in the vitamin K-dependent γ-carboxylase system are involved in the uncontrolled maturation of Matrix Gla Protein (MGP), a molecule that acts as an inhibitor of ectopic calcification, when appropriately carboxylated. In order to understand if the same pathogenetic mechanisms are involved in other disorders, with PXE-like manifestations, we have investigated human dermal fibroblasts isolated from patients affected by β-thalassemia, a hereditary disorder of the β-globin chain, that is frequently (>20% of patients) associated to the occurrence of ectopic calcification.
Methods: Human dermal fibroblasts were isolated from patients affected by β-thalassemia with (β-Thal PXE+) and without PXE like manifestations (β-Thal PXE-). Cells were cultured until confluence and analysed for: i) ROS concentration by EPICS XL flow cytometer using the WINMDI 2.8 program; ii) activity of extracellular superoxide dismutase (EC-SOD) by spectrophotometric assay; iii) expression of protein disulfide isomerase (PDI), calumenin (CALU) and Matrix Gla Protein (MGP) by 1D and 2D-western blot and densitometric analysis of protein bands/spots intensity using ImageQuant TL v2005 software.
Results: The production of $O_2^{\bullet -}$, measured in β-Thal PXE- and β-Thal PXE+ fibroblasts by FACS using the DH_2 fluorescent probe, was significantly augmented in β-Thal PXE+ fibroblasts. Evaluation of EC-SOD in the culture medium showed an increased activity in β-Thal PXE+ cultures compared to β-Thal PXE-. Immunoblots experiments have shown that PDI and CALU were differentially expressed in β-Thal PXE+ and in β-Thal PXE-, since PDI significantly decreased, while CALU increased in fibroblasts from patients with PXE-like manifestations. MGP expression, using antibodies specific for the uncarboxylated (Glu) and carboxylated (Gla) forms of MGP, revealed that the amounts of Glu- and Gla-MGP were increased and decreased respectively, in β-Thal PXE+ vs β-Thal PXE- cells.

$O_2^{\bullet -}$ content measured by FACS



2D-WB for Glu- and Gla-MGP



Conclusions: Results demonstrate that a redox imbalance occurs in β-Thal PXE+ both at intracellular (i.e. $O_2^{\bullet -}$ production) and extracellular levels (i.e. EC-SOD activity), similarly to PXE fibroblasts. Moreover, we are providing further support to previous findings obtained in PXE on the role of under-carboxylated MGP in elastic fiber calcification, extending these observations to β-Thal PXE+ fibroblasts. The rearrangement of protein expression in PXE and in β-Thal PXE+ fibroblasts, possibly mediated by a condition of oxidative stress, leads to the downregulation of PDI, a protein necessary for the efficiency of the vitamin-k dependent carboxylation system, and to the upregulation of CALU, a protein that functions as a negative modulator of protein γ-carboxylation. The result is a deficient production of the mature carboxylated form of MGP, due to impairment in vitamin K recycling. These data underline the importance of monitoring this system, as a reliable marker for the occurrence of ectopic calcification and shed light on the presence of common

pathways for the occurrence of ectopic calcification in PXE and PXE-like disorders, independently from the gene-defect.

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SOLUBILIZATION AND SAMPLE LOADING METHODS OF HUMAN FOLLICULAR FLUID FOR 2DE ANALYSIS.

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Background: Human follicular fluid (HFF), which prevalently results from granulose cell secretion and the capillary diffusion, has been proven to contain biologically active proteins that may affect follicle growth and oocyte fertilization. Based on this concept, the biomolecular characterization of HFF has recently attracted considerable attention as a probable source of biomarkers for oocytes quality evaluation in *in vitro* fertilitazion programs.
Follicular fluid, like plasma, is characterized by a vast protein complexity and a broad dynamic range of protein abundances that hinder its analysis. The aim of this study was to determine a proper solubilisation and resolution method of HFF in 2DE, minimizing sample manipulation and experimental artefacts.
Methods: Follicular fluids from women undergoing *in vitro* fertilization were collected after centrifugation at 2500 x g for 10 minuts and stored until use. Equal protein amounts were subjected to two-dimensional electrophoresis. Here we compared two different protocols for sample preparation and three different modalities of sample loading in the first dimension.
Results: Protein denaturation/solubilization achieved heating samples in a SDS/DTE solution and anodic cup-loading gave the best results. According to this methodology also low-abundance proteins, in particular at low molecular weight, were detected, and high molecular weight proteins were well resolved at the acidic area. Albumin horizontal streaking was substantially reduced and the general resolution of proteins localizing near albumin was appreciably improved.
Conclusions: Although proteome technologies in reproductive medicine research are not as yet widely applied, characterization of the HFF proteome may have a significant impact on oocyte quality estimation and, maybe, on *in vitro* fertilization success rates.

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REPRODUCIBILITY OF THE HUMAN CEREBROSPINAL FLUID PROTEIN PROFILING ANALYZED BY MALDI-TOF MASS SPECTROMETRY

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Background:. Protein profiling of cerebrospinal fluid (CSF) using MALDI mass spectrometry seems to be a new diagnostic tool for early detection of neurological diseases. CSF is the only body fluid in direct contact with the brain, thus it is a potential source of biomarkers. However, the analytical reproducibility of MALDI profiling strategy is a significant challenge if applied to clinical purposes.
Methods: The experiments were conducted on CSF samples derived from 7 healthy subjects examined for idiopathic intracranial hypertension and shown to be neurologically normal without radiological abnormalities on MRI. An aliquot of each sample underwent a desalting/concentration step over ZipTip C18. After protein purification samples were typically mixed at a 1:1 vol/vol ratio with CHCA matrix solution and 1 µl of this solution was deposited onto stainless steel target surfaces and allowed to dry at room temperature. Peptide/protein profiles were analyzed using a Voyager DE PRO MALDI-TOF mass spectrometer (PerSeptiveBiosystems).Separate spectra were obtained for a restricted mass-to charge (*m/z*) range (1000-25000 Da) in linear mode geometry. The acquired raw spectra were processed with Voyager Data Explorer software, version 4.1. Ions with a signal equal or superior to 1% were collected and the signal intensities were normalized presenting the values as area percent of the total area of all the ion signals. To evaluate the intraexperiment reproducibility each sample was assayed in quadruplicate; while for assessment of interexperiment variation, the samples were analyzed two times on separate days. Finally the relative peak area for each sample was organized in a dataset and the mean value, standard deviation and coefficient of variation (CV) was calculated.
Results: We consider as number of reproducible peaks the total number of ion signals detected at least in three out of four spectra, for each sample, obtained using our sample preparation method. In so doing an average of 350 peaks with signal to noise ratio > 1 were taken in to account. Among them a significant number of signals, for each sample, showed a good CV value for the relative peak area. With our approach we achieved a mean intraexperiment CV of the peak area of 12% (range 2%-50%). For assessment of interexperiment variation 1 sample was measured on 2 different days and the mean CV calculated was 5% (range 1%-20%).
Discussion. A key issue in CSF profiling is the preparation of the protein sample for the mass spectrometry experiment. In order to reduce the possible causes of variations we focused our attention on several aspects: sample, preparation storage and handling. The final optimised protocol is currently being used for the preparation of CSF samples with the aim of finding mass spectral patterns and biomarkers that could be used for the early diagnosis of neurological diseases. The CVs values calculated ranged in the values used in clinical diagnosis that have been argued to be typically in the range of 1.5%-10% including inter laboratory variation.
Conclusions: Our results could be considered as preliminary data. In order to improve the analytical performance of MALDI TOF protein profiling we will extend the approach to a major number of CSF samples.

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STUDY OF HEMOCOMPATIBILITY OF DIALYSIS MEMBRANES BY A PROTEOMIC APPROACH

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Background: Hemodialysis treatment of patients suffering from end-stage renal disease shows shortcomings and it is still associated with considerable morbidity and mortality [1] because of the multitude of blood-membrane interactions that may occur during the extracorporeal procedure. For this reason it is important to improve biocompatibility of dialyzers. The biocompatibility of hemodialysis membrane depends on the physical and chemical properties of the material and surface, pore size/geometry, and water permeability [2].

In this study we assessed membrane compatibility of two different types of dialyzers, by correlating the absorbed proteome repertoire with chemical features of the membrane surface by using proteins retained onto dialysis membranes during hemodialysis session.

Methods: The membrane materials tested in the present study included Cellulose Triacetate (CTA; Sureflux) and Polysulfone Helixone (FX60).Three patients undergoing chronic hemodialysis were treated with either dialyzers, sequence of use being randomized. After the hemodialysis session, dialyzers were collected and absorbed proteins eluted by chaotropic buffer. Isoelectrofocusing was performed in IPGphor system (Amersham Biosciences-GE Healthcare) by using Immobiline Dry strips 18 cm, pH interval 3-10 non linear and second dimension was pursued in 12% acrylamyde gels. Protein spots were visualized by MS compatible silver staining and bidimensional maps were aligned and analyzed by PD-Quest 7.1. Only significant spots according to the Student's t-test (p value<0.05) with spot volume values > 3500 ppm and a fold change>3 were isolated by excision from the 2-DE gels, analyzed and identified by Peptide Mass-finger printing and MALDI-TOF-MS/MS sequencing.

Results: After statistical analysis, a total of 54 differentially expressed spots were identified: 22 proteins more concentrated in FX60 membrane and 32 in CTA. In FX60, 14 proteins are more retained: mannan-binding lectin serine protease 1 isoform 2 precursor, fibrin alpha C term fragment , amyloid related serum protein SAA (2 spots), beta-2 microglobulin, ficolin 2 isoform b precursor, ficolin 2 isoform a precursor (4 spots), chain A of human carbonic Anhydrase I, chain B of human hemoglobin (2 spot) and alpha globin. A variant of amyloid related serum protein SAA (3 spots), fibrin alpha C term fragment (2 spots), chain A of human hemoglobin and beta chain of globin (2 spots) seems to be present only in FX60 respect to CTA. Chain A of apolipoprotein C-III, apolipoprotein J (4 spots), apolipoprotein A-I (6 spots), apolipoprotein E precursor (3 spots), retinol-binding protein 4, alpha-1-antitrypsin (2 spots), apolipoprotein A-IV precursor (3 spots), chain A of serum albumin (5 spots), immunoglobulin light chain (2 spots) and complement factor D preproprotein, are more retained in CTA respect to FX60 while alpha-1-microglobulin/bikunin preproprotein, chain A of human apolipoprotein A-I, apolipoprotein A-IV, immunoglobulin light chain and IGHG1 protein seems to appear only in CTA respect to FX60.

Conclusion: It is interesting to observe that some of the identified proteins are relativity more abundant in a synthetic membrane such as FX60 respect to CTA, in particular ficolins. Recently a lot of reports showed that dysfunction or abnormal expressions of ficolins may play crucial roles in the pathogenesis of human diseases including infectious and inflammatory diseases, e.g., recurrent respiratory infections. Other characteristic identified proteins of FX material are amyloid related serum protein SAA, mannan-binding lectin-associated serine protease 1 and beta 2-Microglobulin.

Many proteins are retained onto CTA dialysis membranes, in particular Apo E, ApoA-I, Apo A-IV and ApoJ. Lipid and lipoprotein disorders in dialyzed patients are well known. In fact, changes in concentrations of ApoE, ApoB, ApoA-I and ApoC-III may occur during the dialysis treatments (3).

Thus, our study shows that dialyzer membranes have different adsorption characteristics. The clinical significance of these findings remains to be established in long-term clinical studies.

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038

PROTEOMIC CHARACTERIZATION OF NEUROMUSCULAR PATIENTS CARRYING LAMIN A/C MUTATIONS

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Background. Lamins A/C, encoded by the LMNA gene, are important components of the nuclear lamina, where they assemble in intermediate filaments in a very regulated manner, through post-translational modifications and protein associations. They play a fundamental role in nuclear integrity, chromatin organization and transcriptional control. Their multiple functions are believed to be the explanation for the growing number of diseases caused by genetic defects affecting lamin A/C structure or processing. Laminopathies include muscular dystrophy, lipodystrophies, peripheral nerve cell diseases as well as cardiomyopathies. To understand the molecular pathogenesis, we apply a proteomic approach to skin fibroblasts, aimed at identifying protein changes associated with LMNA genetic defects, in a wide cohort of patients with skeletal and cardiac muscle disease. Patients are classified according to their genotype and clinical phenotype, including evaluation of muscular weakness, electromyography, echocardiogram and family history acquisition. The importance of these clinical parameters is evident, as well as anamnesis, considering the enormous clinical variability that can be found even within the same family. Our choice to characterize protein profile of skin fibroblasts depends on what is known from literature: nuclear alterations due to LMNA mutations have been shown to be present in fibroblasts from Emery-Dreifuss muscular dystrophy and familial partial lipodystrophy patients. However, this remains to be verified. In addition, since it has been suggested that laminopathies can be considered as a result of defects in cell proliferation and since skin fibroblasts represent models for the proliferative capacity of adult stem cells, they may constitute a good model to evaluate the role of lamin A/C in cell proliferation.
Methods. 2D-electrophoresis, followed by image analysis and mass spectrometry, is employed to analyse skin fibroblasts from lamin A/C patients (11) *versus* controls [both healthy donors (3) and patients with muscular disorder not related to lamin defects (8)]. In order to define which proteins are differentially expressed in patients versus healthy controls, we use a double selection criterion: t-student's test p-value <0.05 and fold-change >1.5. Western blotting (WB) analysis is carried out to validate protein identifications.
Results. Preliminary results from *LMNA* patients show that gene mutations not only affect the expression of the mutated gene product itself, but also induce a multitude of other changes in proteins involved in endoplasmic reticulum folding, metabolism and cytoskeletal rearrangement. These findings are in agreement with previous results, where a role of lamin A/C in controlling cytoskeletal organization has been evoked to explain the compromised function of striated muscle. It is noteworthy that, even if the analysed samples are derived from patients with different *LMNA* mutations, some proteins are affected in a common way. Moreover, we can observe that some proteins are affected only in *LMNA* patients but not in patients wit a different neuromuscular defect. Actually, in the protein map for one patient, who presents only a silent mutation of lamin gene (L561L), we identify few spots absent uniquely in his skin fibroblasts. Among the others, one spot is interestingly assigned to alpha-Crystallin B chain both by mass spectrometry and WB analysis. It is normally expressed in the lens, skeletal and cardiac muscle, lung, kidney and nervous system and serves as chaperon protein for desmin. Mutated forms of this protein cause myofibrillary myopathy as mutated desmin. This finding can eventually provide the actual cause of the observed clinical phenotype in the patient, re-addressing the molecular analysis to alpha-Crystallin B chain gene, thus providing an additional diagnostic tool in the study of laminopathies.
Conclusions. Our approach will allow to elucidate complex pathways associated with *LMNA* variants, to identify other proteins involved in the pathogenesis and eventually confirm the previously reported lamin partners.

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URINARY PROTEOME MODULATION UNDER HIGH ALTITUDE HYPOBARIC HYPOXIA

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Background: Hypoxia is a fundamental mechanism of injury in several diseases. The study of human response to hypoxia occurring as consequence of hypobaric condition defines the fields of high-altitude medicine and physiology. In fact, as already reported and demonstrated, the physiological and pathophysiological responses to extreme environmental challenges like hypobaric hypoxia may be at least in part similar to responses to the hypoxia characterizing patients affected by diseases such as cardiac failure or obesity-related hypertension.
The physiological processes characterizing adaptation to acute and prolonged hypobaric hypoxia exposure at high altitude, include pulmonary, cardiac, renal and ematological changes, which are likely to reflect also reactive changes in protein synthesis, as previously shown by studies focusing on changes in proteomics at muscle cells level. No information is conversely available on changes in biological fluids proteins under these conditions.
MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry) is a powerful tool that can be used to provide biological fluids protein profiles and disease-related modifications. However body fluids are complex mixtures that need to be pre-fractionated before MS analysis. The ClinProt technique allows to obtain a selective enrichment of peptides and proteins in biological samples through the use of magnetic beads (MB) with activated surfaces. Peptides isolated by ClinProt can be analyzed by MALDI-TOF MS to investigate the peptidome/proteome profile of the studied groups and to identify differentially expressed proteins. Moreover part of the eluted sample can be stored for the characterization of peaks of interest by LC-ESI-MS/MS.
Aim of our study was to apply these technologies to the assessment of changes in urine proteins induced by high-altitude hypobaric hypoxia. In particular, the identification of peptides/proteins involved in human response to this condition has been carried out on urine samples through ClinProt approach to deepen molecular mechanisms of high altitude hypobaric hypoxia exposition.
Methods: Urine samples have been collected from 19 healthy subjects nearly at sea level (Milan - 122 m above sea level) and at 5400 m altitude (Everest base camp). Subjects were randomly and blindly assigned to placebo (n=9) or telmisartan (n=10) therapy (80mg/die) for 6 weeks before urine collection at sea level. Then, continuing the pharmacological treatment, they flew to Kathmandu and, after 3 days, ascended to 3500mt (Namche Bazar). After 3 days acclimatization at Namche Bazar they walked 5 days (with 1 day acclimatization at 4200 m) to Everest Base camp, at 5400 m. Urine samples were collected 2 days after arriving at base camp.
Urine purification was performed by ClinProt C8-Hydrophobic kit (Bruker Daltonics) using an optimised protocol. Briefly, 10 µL of urine were incubated with 5 µL of C8 MB and 20 µL of binding solution followed by 3 washing steps. Specifically bound peptides/proteins were eluted in 50% acetonitrile solution and spotted with HCCA (α-cyano-4-hidroxycinnamic acid) on an AnchorChip target for MALDI-TOF MS analysis.
Statistical analysis was performed through ClinProTools 2.1 software, to compare protein profiles obtained from the studied groups in the different experimental conditions.
Results: At sea level, urine protein profiles spectra obtained from patients randomized to placebo or telmisartan did not show statistically significant differences. This was the case also when considering urine samples collected at 5400 m. Conversely, the comparison by means of the ClinProTools software of urine samples collected at sea level and at high altitude allowed the identification of 6 differently abundant peptides ($p<0.01$). More in detail, peptides at m/z 1149, 1165, 1548 and 1564 were over-expressed in urines collected at 5400 m while peptides at m/z 2195 and 6183 were under-expressed at 5400 m than at sea level.
Conclusions: Our study allows for the first time to explore protein expression in biological fluids of healthy subjects exposed to high altitude hypobaric hypoxia. Preliminary analysis of data shows that the urinary proteome is not affected by blockade of the angiotensin II AT1 receptors neither at sea level nor at 5400 m altitude. On the other hand, the cluster of signals detected by comparing urines collected at sea level and at Everest base camp allowed to identify 6 peptides differently regulated in hypobaric hypoxia condition. The identification of these signals, currently in progress, could lead to define molecules involved in high altitude hypobaric hypoxia adaptation response. Their identification may also offer a deeper insight into the

molecular mechanisms of pathologic conditions often associated with hypoxemia, such as advanced heart failure obesity associated with sleep apnoea and hypertension.

040

A NEW POTENTIAL EARLY BIOMARKER OF PRETERM PREMATURE RUPTURE OF MEMBRANES IN AMNIOTIC FLUID

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Background: Preterm birth is responsible for 75% and 50% of infant mortality and infant morbidity, respectively. The leading cause is the premature spontaneous rupture of the amniotic membranes (PROM) with release of amniotic fluid (AF) more than 1 hour before the onset of labour. PROM can be subdivided into term PROM (tPROM), that occurs after the 34th gestational week, and preterm PROM (pPROM), that occurs before the 34th gestational week and it's strictly related to prematurity.
Premature infants are at high risk for morbidity and mortality because of bronchopulmonary dysplasia, necrotizing enterocolitis, intraventricular hemorhage and periventriculary leukomalacia. Moreover pPROM affects infant neuro-development.
Critical challenges about pPROM are: etiopathogenesis, early diagnosis of asymptomatic intrauterine inflammation, identification of women at risk of pPROM and predictors of pregnancy outcomes.
The identification of early predictive biomarkers and latency predictors discovery should lead to preventive strategies, targeted treatments and improve the outcomes.
Up to now there are different proteomic approaches to biomarker discovery. Among them, ClinProt technique, which is based on magnetic beads with chemically or biologically activated surfaces, is very promising. Through ClinProt approach prepurification of the biological fluids proteome can be carried out and results in enriched and simplified fractions for MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization -Time of Flight Mass Spectrometry) analysis.
Several examples showing the feasibility of proteomic approaches to AF investigation in preterm birth and pPROM have been reported in literature. Aimed to search possible pPROM biomarkers we have applied ClinProt technique to investigate AF.
Methods: Amniotic fluid were non-invasively collected from vaginal posterior fornix of women with pPROM (≤34 gestational week (GW) (group 1: n=11, mean GW 29.8±4.7) and tPROM (group 2: n=11, mean GW 39.1±1.2), and invasively collected from women submitted to genetic amniocentesis for advanced maternal age (group 3: n=16, mean GW 17.2±0.7).
Aliquots of AF were purified by the use of ClinProt purification reagents supplied with C8-Hydrophobic kit (Bruker Daltonics) with an optimised protocol and analyzed with MALDI-TOF MS in order to generate proteomic profiles. The identification of potential proteomic biomarkers of pPROM was obtained by HPLC-ESI-MS/MS (High Performance Liquid Chromatography-Electrospray tandem mass spectrometry) and Mascot search engine.
Results: Comparison between average spectra of groups 1 and 2 led to detect three signals (at m/z 1427, 3001, 3316) over-expressed in pPROM AF. The signals were able to discriminate the two populations with high accuracy (ROC curves: peak m/z 1427 AUC =1, peak m/z 3001 AUC =0.98, peak m/z 3316 AUC =1).
The biomarker at m/z 1427.7 was identified through HPLC-ESI-MS/MS analysis as a fragment of a protein encoded by a previously sequenced gene (KIAA1522) located on chromosome 1. The expression of this has been detected in fetal brain and liver. Moreover, this protein shows several phosphorilation sites but it is not yet characterized neither for structure nor for function.
The over-expression of this peptide was also found in a sample of AF collected from a women during genetic amniocentesis test. The woman was asymptomatic at the time of the test (17.2 GW) but later (at 33rd GW) she developed a preterm premature rupture of the membranes. This sample was correctly attributed to pPROM group by the cluster of the three selected signals.
Conclusions: Proteomic analysis led to identify new potential biomarkers of pPROM in amniotic fluid. The presence of one of these peptides in one patient in amniocentesis group that afterwards developed a preterm premature rupture of the membranes suggests its possible use as early predictive biomarker of pPROM. Moreover, the literature shows the relationship between the increase of another fetal brain expressed protein, the calcium-binding S100B, and the two leading causes of pPROM: intra-amniotic infection and inflammation.
These data strengthen the reliability of this clinical proteomic approach to biomarker discovery.

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041

CORRELATION BETWEEN MMPS AND PROTEOMIC PROFILES OF BREAST CANCER TISSUES.

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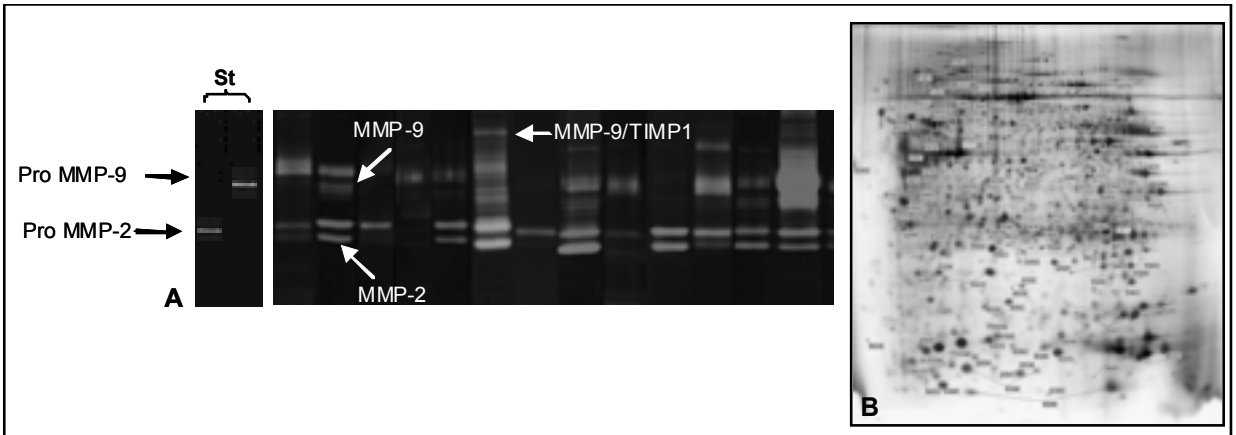
Background: The repertoire of proteases that cells and tissues coordinately regulate in order to modulate their local environment is the *degradome* which in humans is represented by at least five catalytic classes. Proteases act in the extracellular environment and have been related with tumorigenesis and metastasis by virtue of their ability to degrade the extracellular matrix. The roles of proteases in cancer are now known to be much broader than simply degradation of extracellular matrices during tumor invasion and metastasis. The matrix metalloproteinases (MMPs) constitute a family of about 25 secreted and cell surface enzymes that process or degrade numerous peri- and extracellular proteins. MMP's substrates include other proteinases, proteinase's inhibitors, chemotactic molecules, latent and active growth factors, growth factor binding proteins, cell surface receptors, cell-cell adhesion molecules and extracellular matrix molecules. MMPs thus have been proven to be essential in a variety of steps during carcinogenesis. Although increased expression of proteases at the level of transcripts and enzymatic activity has been observed in many tumors, especially in breast and colon cancer, their correlation with the different tumour phenotypes remain to be determined. The aim of the present work was to correlate the levels of MMP-2, MMP-9 and MT1-MMP with the corresponding proteomic profiles in surgical explants of patients diagnosed for ductal infiltrating breast cancer.
Methods: Tissue extracts were assayed by Gelatin Zymography (0,1%) to detected the MMP-2 and MMP-9 activity levels, by western blot for MT1-MMP expression levels and by 2D-IPG for proteomic expression profile. Quantification of gelatinolitic activities and expression levels of MT1-MMP was performed by the ImageQuant TL software (AMERSHAM). Quantitative analysis of proteomic maps, calculated as % of volume of the proteomic spots (V%) was performed by ImageMaster 2D Platinum.
Results: Breast cancer tissues showed variable levels of gelatinolytic activity corresponding to the pro-enzymatic and active forms of MMP2 and MMP9. Moreover the majority of the samples showed lytic bands with high molecular weight, identified as MMP9 dimers and as MMP9/TIMP1. Pro-MMP9 and the bands with high molecular weight displayed a higher variability levels among patients, and only sporadic correlation with the expression of MT1-MMP. On the other hand, an interesting correlation between the activity levels of the gelatinases and the expression of some proteins belonging to the category of programmed cell death (DAVID Bioinformatics Resources 2008) was also found.



A) Gelatin zymogram of breast cancer tissues and **B**) a prototype of proteomic map.

Conclusions: Our results suggest that the combination of these distinct proteomics/proteases-based methods may be of great utility to identify new patterns of protein/enzymes involved in cancer progression. Therefore, the multi-factorial adequate analysis of these molecules could provide new prognostic information contributing to the determination of more therapeutic strategies for each patient.

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MUSCLE PROTEIN DYSREGULATION DURING BED REST: THE EFFECT OF VIBRATION COUNTERMEASURES

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Background: skeletal muscle tissue is characterized by great plasticity which can be defined as the capacity to change its fiber type distribution and metabolism to adapt to external or internal stimuli. Actual and simulated microgravity (bed rest) are known to lead to muscle wasting and weakness. Several factors contribute to this phenomenon, disuse being a primary cause, though recent evidence suggests that microgravity itself has a direct effect on protein synthesis. The loss of muscle strength, however, is not only due to disuse-atrophy, but also to other muscular factors. In fact, the loss of muscle strength induced by these conditions is greater than the decrease in muscle size with a resulting decrease in force per unit cross-sectional area (F/CSA). The molecular mechanisms responsible for this phenomenon are not still understood. In a previous study, severe deregulation of transport, stress response, contractile and metabolic proteins was described in the human vastus lateralis (V) and soleus (S) muscles after 55 days of bed rest. In the present investigation the effects of resistive-like vibration countermeasures (RVE) was assessed in 4 subjects undergoing the same protocol. Differential protein analysis, carried out by fluorescence SDS, 2D-DIGE and mass spectrometry, was combined with quantitation at the RNA level by real time PCR of muscle markers of atrophy/hypertrophy pathways after bed rest without and with resistive exercise countermeasures (RES).
Methods: the study was performed on 4 healthy subjects (males, age range 24-43) involved in the "Berlin Bed Rest Study" before and after 55 day of bed rest with RVE. Muscle samples (13-15 mg) from vastus lateralis (a plantar extensor) and soleus were obtained by standard needle biopsy (Bergstrom's technique). After biopsy the samples were solubilised in a lysis buffer and were accurately quantitated; fifty micrograms of muscle lysates, adjusted to pH 8.5, were differentially labelled with CyDye fluors. Protein extracts from each subject were run in triplicate in a non-linear 3-10 pH gradient, 18 cm long (GE Healthcare) and separated on 12% constant concentration polyacrylamide gel. The 2DE gels were acquired by a laser scanner and the images analysed with the dedicated software DeCyder (GE Healthcare). Proteins of interest were robotically excised using Ettan Spot Picker (GE Healthcare); in-gel digestion was carried out with trypsin (Promega) and mass spectrometric analyses of tryptic digest were performed by using MALDI-ToF (GE Healthcare). Fast and slow MHC isoforms from vastus lateralis and soleus muscle were separated using 6% SDS-PAGE and the gels were stained using Sypro Orange (Invitrogen). Some cell signalling genes, AKT, FoxO3a, mTOR, PGC1α, were selected for real-time PCR experiments. Total RNA was extracted from 10 mg of vastus and soleus tissue using TRIZOL reagent and 1 µg RNA was reverse-transcribed using SuperScriptII RT and oligo(dT) according to the manufacturer's instructions (Invitrogen). Primers were designed with Primer Express software and tested for their specificity to human tissues using NCBI database. Real-time PCR reactions were performed in triplicate with Fast SYBR Green Master Mix and processed on 7500 Real-Time PCR System (Applied Biosystems). Quantitative data were analyzed by the average of triplicates Ct (cycle threshold) and normalized to housekeeping RNA 18S gene. Levels of mRNA expression were determined using the SDS software version according to the $2^{-\Delta\Delta ct}$ method.
Results and conclusions: the differential analysis on vastus lateralis shows both qualitative and quantitative differences after 55 days of bed rest with RVE; statistical analysis (Student T-test) revealed the presence of significant differences in 38 spots, 27 were found to be down regulated and 11 up regulated; 31 of them were identified by MALDI-ToF. As regard to soleus muscle: 59 spots were differentially changed, 43 were down regulated and 16 were up regulated; 48 of them were identified by mass spectrometry. The identified proteins were grouped in functional categories and the most interesting finding did concern: 1. structural proteins increased only in S; 2. contractile proteins were generally down regulated in both V and S; 3. metabolic proteins showed a different behaviour in the two investigated muscles: proteins involved in aerobic metabolism were up regulated in V whereas in S were partially down regulated. As far as anaerobic metabolism is concerned, glycolytic proteins were markedly increased in S whereas those of V where only slightly affected; 4. transport proteins increased only in S; 5. proteins involved in the control of oxidative stress were greatly increased in S and slightly up regulated in V. With regards to muscle fiber types, both V and S underwent a transition from I to IIA. The differential analysis of muscle proteome sheds light on the

changes induced by bed rest and vibration countermeasures on protein synthesis and catabolism. Independent of countermeasures, there appears to be a marked decrement of transcripts in all subjects indicating an absence of transcriptional activity at 55 days of bed rest in the vastus and soleus tissue. It were observed among the samples some differences in the magnitude of the transcript level changes that are attributable to individual variability. RES countermeasures are apparently ineffective.
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043

PROTEOMIC PROFILE OF RCC MICRODOMAIN SUBCELLULAR FRACTIONS

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Background: Renal cell carcinoma (RCC) is among the 10 leading causes of cancer-related deaths worldwide and its incidence is increasing steadily. At the time of diagnosis, 30% of patients have metastases and another third will develop metastatic disease within 10 years. Moreover metastatic RCC is radio- and chemotherapy resistant. Therefore, it is important to identify differentially expressed proteins to be used as potential diagnostic and/or prognostic markers in RCC. A promising strategy is subcellular comparative proteomics that allows to enrich specific cell compartments and assess differences in protein expression patterns. Thus, in the present study we investigated the proteomic profile of a peculiar subcellular compartment, plasma membrane microdomains, which are sphingolipid- and cholesterol-enriched assemblies within plasma membranes, involved in cell signalling, transport and proliferation. They were shown to be involved in many membrane related human diseases, such as cancer. In particular in a recent study we demonstrated the over-expression of two typical microdomain protein components, caveolin-1 and flotillin-1, in RCC compared to homologous normal renal tissue: therefore, defining the microdomain proteome is an attractive goal.
Methods: Membrane microdomains have been prepared from surgical samples of RCC and adjacent normal kidney (ANK), following nephrectomy. Plasma-membrane-enriched fractions were treated with Triton X-100, in which microdomain structures are insoluble, and after floatation in a discontinuous sucrose density gradient, 1 ml fractions were collected from the top of the gradient. Subcellular fractions, microdomain-enriched, displaying the highest caveolin-1 and flotillin-1 enrichment, were concentrated by ultracentrifugation and analyzed by monodimensional electrophoresis. For protein MS identification, MD derived from RCC and ANK tissues of 7 patients were pooled, and proteins separated both by 12% and 4-12% gel electrophoresis (NuPAGE electrophoresis system, Invitrogen). After Coomassie Blue staining, bands were excised from the inferior half of 12%, to investigate lower molecular weight proteins, and from the superior half of 4-12% gel, for larger ones. Then, microdomain proteins were identified by nanoLC-ESI/MS-MS.
Results: Here, we report 71 proteins identified from microdomains isolated from RCC tissue, and 87 proteins from ANK microdomains. About 68% of the identified proteins have been described previously as membrane-associated and about 50% as microdomain-associated. Well-known caveolar and non-caveolar microdomain markers such as caveolin-1 and flotillin-1/2 were identified. Cytoskeleton structural constituents and proteins related with the regulation of the interaction of cytoskeletal constituents with the cell membrane and particularly with microdomains were found (i.e.: actin, spectrin). Other identified proteins include signal transduction molecules, such as Ras-related protein Rab-1B, and several G proteins. Some proteins show putative post-translational modifications that favor their localization in the lipid-raft environment, such as GPI (alkaline phosphatase and renal dipeptidase).
Conclusions: In conclusion, this study indicates that the strategy of comparative subcellular proteomics can be applied also at the plasma membrane level, in spite of the known difficulties in efficiently recovering hydrophobic proteins after 2DE analysis. Since most of the tumour markers currently used, i.e., PSM, CEA, mucins and others, are actually membrane proteins, the recognition of differential amounts of plasma membrane proteins in RCC microdomains looks promising in view of new biological marker discovery. The present work has been supported by FIRB: Rete Nazionale per lo studio del proteoma umano (n. RBRN07BMCT).

044

DEVELOPMENT OF A MASS SPECTROMETRY-BASED METHOD FOR THE EVALUATION OF SERUM EXOPROTEASE ACTIVITY IN INFLAMMATORY BOWEL DISEASES PATIENTS.

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Background: The importance of inflammation, fibrinolysis and coagulation network in the pathogenesis of inflammatory bowel diseases (IBDs) has been widely described in the literature, but it is still unclear if it represents a consequence or a cause of the disease. Recently an activity of specific Crohn's disease (CD) serum exoproteases (enzymes responsible for the cleavage of biologically active peptides) acting on few proteins commonly involved in the inflammatory and the coagulation processes was identified and its use as diagnostic tool was supposed. We describe here a method for the evaluation of the exoprotease activity in serum samples from healthy (H) subjects and CD and Ulcerative colitis (UC) patients based on matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) followed by chemometric statistical analysis.
Methods: Serum samples from a total of 24 subjects, including 8 healthy donors volunteers (H; 5 M and 3 F), 10 Crohn's disease patients (CD; 6 M and 4 F) and 6 ulcerative colitis patients (UC; 3 M and 3 F), were provided by the Gastroenterology Unit at S. Orsola University Hospital of Bologna (Italy) following a strictly controlled sample collection protocol. The diagnosis of the CD and UC was achieved evaluating clinical symptoms, clinical chemistry data and conventional endoscopical procedures and only patients with active disease were included. Fibrinopeptide A (FPA) and Complement 3f (C3f) peptides were spiked as surrogate substrate into each serum samples and the small peptides degradation products obtained after the enzymatic cleavage were analyzed by MALDI MS at different incubation time (0, 1, 3 and 5 hours). Glu-Fibrinopeptide B was used as a standard for the normalization of the results. The exoprotease activity on the endogenous peptides was evaluated too.On the acquired spectra the ratio of the intensities of all the peaks derived from the degradation of C3f and FPA(-A) and of the intensities of internal standard GFP were calculated, and the normalized intensities of the degradation products of the endogenous C3f and FPA(-A) at the same incubation time were subtracted. The obtained data matrices were used for the following chemometric analysis.
Results: Multivariate statistical analysis strategies were performed on the CD, UC and H degradation peptides mass spectra. The cluster of C3f and of FPA (-A) degradation peptides obtained respectively after 1 hour and 3 hours of incubation time were identified as the most relevant for the discrimination among the three categories of samples. A 79% sensitivity and a 68% specificity were obtained by clustering analysis while a 89% and a 73% of respectively correct classification and prediction ability was achieved by Linear Discriminant Analysis (LDA). The ability to discriminate among healthy and diseased subjects, and in particular among CD and UC serum samples was evaluated also by Principal Component Analysis (PCA).
Conclusions: The developed mass spectrometry-based method for the evaluation of serum exoprotease activity in IBDs patients demonstrated its ability to discriminate among healthy and diseased subjects and among CD and UC patients. The ability of this screening and diagnostic serum assay to analyze a high number of samples in a short time will allow its application on a larger cohort of about 100 serum samples from H subjects and patients affected by CD, UC and other inflammatory diseases to further evaluate the specificity of these clusters. Moreover a comparison of the results achievable by this test and by the already available serum antibody tests has to be performed in order to develop a serum diagnostic assay able to reduce or delete the need of invasive procedures for the diagnosis of IBD.

045

DEVELOPMENT OF AN EFFICIENT AND VERSATILE METHOD FOR PROTEIN EXTRACTION FROM FORMALIN-FIXED, PARAFFIN-EMBEDDED TISSUES

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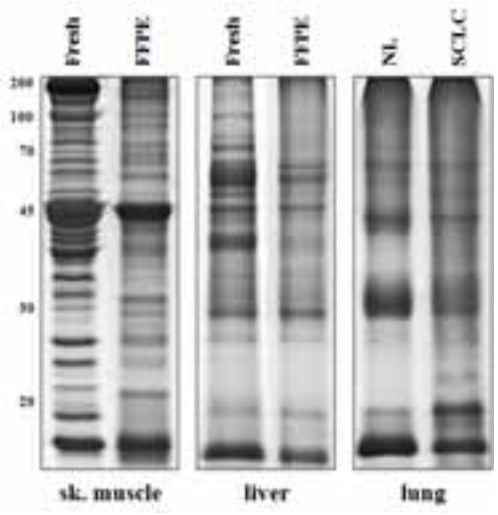
Background: Formalin-fixed, paraffin-embedded (FFPE) tissue banks generated for histopathological analysis and stored in hospitals worldwide can be considered as a "hidden treasure" which could open the way to retrospective studies for biomarker discovery and validation. Unfortunately, the fixation procedure causes an "entrapment" of macromolecules within the tissue matrix, due to intra- and intermolecular crosslinking, which dramatically impairs protein extraction efficiency and antigen immunoreactivity. Antigen retrieval (AR) is still considered as the only procedure able to induce a considerable improvement in antigenic reactivity of FFPE tissues, through a high-temperature treatment which probably induces cleavage of protein-protein bonds. Current methodologies that aim to extract proteins from FFPE tissues are actually based on the antigen retrieval concept. However, the procedures developed so far exhibit a poor suitability for downstream proteomic techniques. Thus, the aim of this research was to develop an extraction method with a high degree of efficiency and versatility.

Methods: Samples used for method optimisation were sheep skeletal muscle and liver. Fresh-frozen and FFPE replicates of both tissues were cut from the same animal. Human bioptic samples (normal lung and small cell lung carcinoma, respectively) were obtained from a hospital FFPE archive. FFPE microtome sections were deparaffinised with xylene and rehydrated with a graded series of ethanol. Protein extraction method was carried out as follows: tissue pellets were immersed at a 20% w/v ratio in a 20 mM Tris HCl pH 8.8, 2% SDS, 200 mM DTT, and then subjected to high-temperature extraction at 100°C for 20 min, and then at 80°C for 2 h with shaking. SDS-PAGE, colloidal coomassie protein staining, western immunoblots, reverse-phase protein arrays, and in-gel trypsin digestion were performed according to standard procedures. LC-MS/MS analyses of tryptic digests were performed on a nano-LC-nanoESI-Q-TOF hybrid mass spectrometer (Waters). ProteinLynx and Mascot softwares were used for spectra analysis and protein identification, respectively.

Results: In order to access to a large amount of tissues without any ethical problem, and to eliminate biological and experimental variability, we carried out method optimisation using an animal model, namely skeletal muscle and liver tissues of a single sheep individual. Proteins were extracted from fresh-frozen and FFPE replicates of both tissues for comparative aims. Optimisation experiments showed best extraction efficiency from FFPE tissue sections after performing deparaffinisation with xylene, rehydration with a graded series of alcohols, and serial incubation at 100°C and 80°C with a buffered detergent solution in a reducing environment. Our method produced average protein yields of 16.3 µg and 86.8 µg per 80 mm^2 FFPE muscle and liver tissue slice, respectively, whereas a commercial extraction method stated the value of 15 µg per 10 µm thick, 80 mm^2 wide, liver tissue slice. 1D-PAGE patterns of FFPE protein extracts appear comparable to those corresponding to fresh-frozen tissues, in terms of quality and relative protein abundance (see figure). Anti-GAPDH western blotting and reverse phase protein arrays showed a clear signal in all protein extracts, confirming that immunoreactivity and molecular weight were preserved. A comparative GeLC-MS/MS analysis was carried out by cutting corresponding areas from matched lanes, performing *in situ* digestion, and then subjecting tryptic peptides to MS/MS analysis. MS data obtained from selected gel-separated regions demonstrated that this approach allows to detect main protein species, with comparable results between fresh-frozen and FFPE extracts, concerning both statistical significance and peptides number, interestingly, also for basic proteins. The same method was used for extracting proteins from human FFPE tissues (normal lung, NL and small cell lung carcinoma, SCLC), in order to assess applicability of the procedure on routinely collected, FFPE hospital biopsies. Electrophoretic patterns and anti-GAPDH reactivity



were comparable and reproducible. GeLC-MS/MS technique on matched gel areas allowed to carry out a differential analysis, leading to the detection of several tumoral markers in small cell lung carcinoma, which were not present in the normal lung extract. The reactivity for one of these markers (chromogranin A) was confirmed in western blot and protein arrays as well.

Conclusions: The extraction method described above is simple, efficient and amenable to different analytical platforms, such as SDS-PAGE, western blotting, protein arrays, and GeLC-MS/MS. Future implementations will extend the application field to different proteomic and immunological techniques (such as 2D-PAGE and DIGE, and ELISA, respectively) and to widespread differential proteomics analysis of human FFPE biopsies.

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A PROTEOMIC APPROACH TO IDENTIFY PROTEINS TRAPPED IN ADSORBENT RESIN CARTRIDGES USED FOR COUPLED PLASMA FILTRATION-ADSORPTION TREATMENT OF HEALTHY AND SEPTIC PIGS

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Background. Proteomic techniques have become important in the study of diseases by providing an integrated view at the protein level. The identification of protein markers can be used for diagnosis and prognosis of disease as well as targets for the development of new drugs. Efficient proteomic methods for profiling the plasma proteins trapped in adsorbent resin cartridges are highly desirable for designing new bio-filters to be used for coupled plasma filtration-adsorption (CPFA) processes. Development of procedures to separate proteins from complex mixtures and to obtain structural information about proteins of interest are two key areas in proteome research. In this work a dedicated proteomic approach based on nanoHPLC–ESI-IT-MS/MS was proposed for the analysis of proteins trapped in adsorbent resin cartridges used for the coupled plasma filtration-adsorption treatment of healthy and septic pigs. The five main steps of the proteomics analysis, (i) protein extraction from resin cartridges, (ii) two-dimensional gel electrophoresis, for protein separation and profiling, (iii) in-gel proteolytic digestion, (iv) mass analysis by nanoHPLC-ESI-MS/MS of peptides resulting from the enzymatic cleavage and (v) bioinformatics, for protein identification and characterization, have been optimized. Prior to electrophoresis, the efficiency of different extraction solutions and steps were critically evaluated. The final purpose and the potential impact of the proposed proteomic approach is the identification of the possible proteins present in acute-phase septic plasma of pig.
Methods. Extraction of proteins from resin cartridges was performed by sonication, centrifugation and filtration, with an extraction solvent consisting of 8 M urea and 2% Triton X-100. After that, the separation of complex protein mixtures was carried out by two-Dimensional Gel Electrophoresis (2D-E) using an Isoelectric Focusing IPGphor™ and Hoefer SE 600 system (Amersham Pharmacia Biotech). Between-gel comparisons of different samples were carried out by scanning and image analysis of stained gels. Spots of interest were manually excised from gels and digested by using a proper optimized trypsin protocol. Chromatographic analysis was carried out by a nano-HPLC Dionex LC-Packings apparatus (Amsterdam, The Netherlands). The UV flow cell, set at 214 nm, was connected to an Esquire 3000 plus™ IT mass spectrometer Bruker Daltonics (Bremen, Germany) equipped with an online nano-ESI source. Separations of peptides resulting from the in-gel enzymatic cleavage were achieved on a PepMap™ C18 analytical column, following a PepMap™ C18 nano trap column. The sequence information containing MS/MS spectra have been compared against comprehensive protein sequence databases by using the search program Mascot-Matrix Science.
Results. The optimization of the chromatographic separation conditions were carried out on a standard solution of peptides originating from bovine cytochrome C. The gradient elution was combined with a single injection mode, taking into account the extremely small amount of sample obtained from each gel spot. The optimized gradient, which starts with an isocratic step with aqueous acidic solution, allowed an efficient sample pre-concentration and desalting, consequently no loading process onto the trap column and no multiple injections for each protein spot were required, prior to separation. In order to find the most suitable protein extraction protocol from the adsorbent resin cartridges, different solvent mixtures and extraction steps have been tested and compared. Three sequential steps consisting of sonication, centrifugation and filtration have been performed on the resin deriving from the cartridge, by using solvent extraction mixtures containing different detergent agents, organic contents and salt concentrations. The best results in terms of recovery, ease to use and reduced analysis time were achieved by using an aqueous solution composed of 8M urea and 2% Triton X-100. After the 2-DE separation and the enzymatic digestion of the excised spots from gel, the tryptic peptides have been analyzed by the nanoESI-IT-MS/MS for protein identification; MS/MS spectra have been recorded for all peptides showing a signal intensity higher than 10^5 arbitrary units and were searched against the full, nonredundant protein sequence database to find the peptide that most closely matches the observed spectrum. This approach is suitable for automation and a high sample throughput can be achieved. The described methodology, developed on healthy pigs, was subsequently applied to septic samples in an attempt to identify the nature of proteins trapped into the sorbent cartridges and to characterize the quality of the bio-filters to be used for CPFA. Good results were obtained in terms of coverage and score for the protein identification both for healthy and septic pigs.

Conclusions. In this work a powerful method for the proteomic analysis of proteins trapped into adsorbent resin cartridges used for coupled plasma filtration-adsorption has been developed. The nanoLC-ESI-ITMS/MS procedure was tailored for detecting and sequencing the highest number of peptides from a single analysis, by using few microlitres of the enzymatically hydrolyzed protein extract. The described procedure represents the first step for identifying protein markers present in plasma of septic pigs and for designing new bio-filters able to control the inflammatory imbalance and to improve the outcome of severe sepsis.

047

PROTEOMICS ANALYSIS OF SERUM PROTEINS EXPRESSION AND SPECIFIC OXIDATION IN CROHN'S DISEASE PATIENTS

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Background: Chronically inflamed intestine and/or colon is characterized by production of several proteins involved in inflammatory response and in oxidative stress. Sources of these oxidants are mainly phagocytic leukocytes. These cells are known to be present in large numbers in the inflamed mucosa and they produce significant amounts of reactive oxygen species (ROS) in response to inflammatory stimuli. In this way several inflammation related proteins are increased and their isoforms, if validated, can be used as biomarkers.
Methods: Serum samples from 7 healthy controls and 8 early stage Crohn's disease patients were available for comparative proteomic analysis and oxidative stress analysis.
IEF was performed loading (cup) 100 μg of serum proteins on a pH 3–10 NL 18cm long IPG strip (GE Healthcare) until 140000 VhT. Second dimension was executed using homemade 10% acrylamide vertical SDS-PAGE slab gels of dimension 200x200x1mm. Gels obtained were stained with silver nitrate and Image analysis was performed using Progenesis SameSpots (Nonlinear Dynamics) image analysis software.
Oxidative status was evaluated by labeling of free sulfhydryl groups with 5(Iodoacetamido)fluorescein (5-IAF) followed by a two step precipitation and a classical two dimensional electrophoresis. Images were acquired with Pharos FX Molecular Imager (Bio-Rad) with an excitation wavelength of 490-495 nm and an emission wavelength of 515-520 nm (green). Obtained images were analyzed with Progenesis SameSpots (Nonlinear Dynamics) image analysis software.
Results: □1antitrypsin, □1antichymotrypsin and complement 3 chain C (C3c) were overexpressed during early stage of Crohn disease and clusterin, retinol binding protein and transthyretin were underexpressed (Table 1). It has been found a transthyretin isoform at *pI* 4.7 and a C3c complement isoform at *pI* 4.9 expressed only in Crohn's disease patients.
Experiments for the evaluation of oxidative status showed that 5-IAF fluorescence levels of serum ApoA1 and Transthyretin were lower in pathological serum samples than in controls ($p \leq 0,05$). This evidence shows an increased oxidative stress dependent-damage of these proteins in the pathological status vs control (Fig.1).

N°	Protein description	Swiss Prot Accession Number	Protein level
1	α1antitrypsin	P01009	↑
2	α1antichymotrypsin	P01011	↑
3	Haptoglobin	P00738	↑
4	Complement 3 fragment C	P01024	↑
5	Retinol binding protein	P02753	↓
6	Clusterin	P10909	↓
7	Transthytretin	P02766	↓
8	Transthytretin isoform *pI* 4.7	P02766	↑
9	Complement 3 fragment C isoform *pI* 4.9	P01024	↑

Table 1

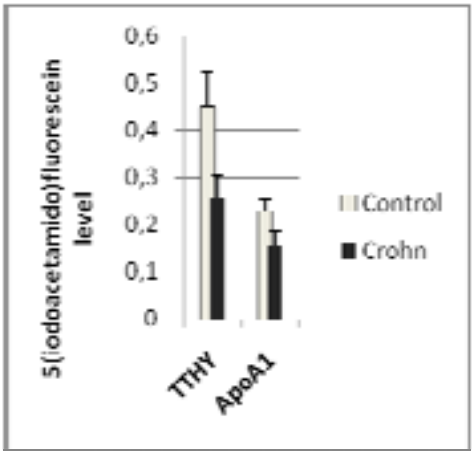

Fig.1

Conclusions: α1-antitrypsin and α1-antichimotrypsin also called serpina1 and serpina3 are protease inhibitors and both are positive acute-phase indicators, their plasma concentration increase as a result of inflammation. Overexpression during early stage of Crohn disease demonstrates that, since the early stage of the pathology, inflammation plays a crucial role.

C3c is a fragment derived from the proteolysis of C3b, overexpression during the early stage may explain that complement metabolism could be linked to the pathology. Furthermore image analysis revealed a C3c isoform at *pI* 4.9 expressed only in Crohn's patients. This isoform could be interesting as possible serum biomarker.
Moreover intestinal inflammation is accompanied by excessive production of reactive oxygen and nitrogen metabolites. The levels of the most important antioxidants are seriously imbalanced within the intestinal mucosa in Crohn's patients and are not able to contrast the negative effect of ROS. This data can explain why some serum proteins as transthyretin and apolipoprotein A1 show an increased oxidation of their sulfhydryl groups compared with control.

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DIFFERENTIAL PROTEOMICS OF RCC URINARY EXOSOMES

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Background: Exosomes are nanometer sized (50-100 nm) membrane vesicles released by most living cells, upon fusion of multivesicular bodies with the plasma membrane. Secretion of exosomes by tumor cells is enhanced, suggesting their involvement in cancer progression. Urinary exosomes have been shown to derive from every epithelial cell type facing the urinary space. For this reason they are considered an important source of urinary proteins and a promising starting material for biomarker discovery. Clear cell renal carcinomas (cRCC) is representing about 3% of all kidney cancers. No biomarkers for early diagnosis of cRCC in asymptomatic patients or for post-surgery monitoring are yet available. We addressed the proteomic analysis of the exosomes isolated from control and cRCC patients urines in order to look for differences to be exploited as potential tumor marker.
Methods: 30-50 ml of 2nd morning urines were collected from 8 cRCC patients and 7 matched controls and stored at -80°C. Exosomes were then isolated from total urines, by ultracentrifugation after cells and debris clearing. Protein composition was assessed by mono and 2D-EF, followed by CBB staining or immunoblotting with antibodies against specific membrane proteins. Quantification of ECL detected protein bands was accomplished by densitometry and band intensity normalization was achieved by Ponceau S staining to assess protein loading. Statistical analysis was performed by unpaired t-test using GraphPad Prism 4.0 software.
Results: Results show that the proteomic profile of urinary exosomes is different from total urine, since the most abundant soluble urinary proteins (i. e. albumin and uromodulin, of plasmatic and renal origin, respectively) are depleted. Accordingly, an enrichment of minor urinary proteins is accomplished. Several membrane proteins could be identified in control and cRCC patient urinary exosomes: preliminary results show that some of them display differential amount in cRCC patient exosomes. In particular Aquaporin-1, and P-Glycoprotein, whose expression has been reported to be downregulated in cRCC, are shown to have reduced content in patient exosomes, while Matrix metallo-protease 9 and Carbonic Anhydrase IX, which were reported as overexpressed in RCC, result more abundant. A different behavior is displayed by the Extracellular Matrix Metalloproteinase Inducer (EMMPRIN, CD147, basigin): although it is shown increased in the membrane fraction of RCC tissues, its content in patient exosomes is much lower than in control ones. Finally, both exosome populations contain similar amount of other membrane proteins such as Flotillin-2.
Conclusions: In conclusion our work suggests that proteomics of urinary exosomes show potential for the identification of RCC biomarkers. The present work has been supported by FIRB: Rete Nazionale per lo studio del proteoma umano (n. RBRN07BMCT).

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IMMUNOREACTIVITY IN RENAL CELL CARCINOMA STUDIED BY PROTEOMIC APPROACH

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Background: The immune system of cancer patients is known to be activated against tumor specific antigens including mutated gene products, and modified or over-expressed proteins. Therefore, the serological identification of patient's autoantibodies binding tumor antigens may provide diagnostic and prognostic information. Since renal cell carcinoma (RCC), the most common neoplasm affecting the adult kidney, is characterized by a variety and heterogeneity of histological subtypes, drug resistance, and absence of molecular markers, the identification of renal cancer associated antigens can promote development of new therapies or vaccine, and improve patient management.

Methods: We applied a serological analysis combined with proteomics-based approach (SERPA) to detect autoantibodies, associated with post-translational modifications, subcellular localization or other functional aspects of immunoreactive proteins, to investigate changes of humoral immune response in RCC. We utilized sera obtained 8 months after surgery of 8 RCC patients with sera already studied before surgery. We separated proteins of the same RCC tissues by two-dimensional gel electrophoresis (2-DE) and after transfer by western blot onto PVDF membranes we detected immunoreactivity using patient sera as primary antibodies.

Results: We identified 14 proteins that specifically elicited immunoresponse by autoantibodies of only RCC patient sera collected before surgery, 12 of these were no longer immunoreactive when sera collected 8 months after surgery were utilised as primary antibody.

Conclusions: This is the first time ever in which follow up sera of RCC patients were analysed to evaluate any likely persistence of immunoreactivity after surgical therapy.

The loss of autoantibodies against renal cancer proteins after surgery suggests that the removal of tumor specifically modified this response.

050

RECOMBINANT FACTOR VIII CONCENTRATES: A COMPARATIVE PROTEOMIC ANALYSIS

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Background: Haemophilia A is an inherited blood coagulation disorder which affects one in 10,000 males. It is an X-linked disease caused by defects in the expression or function of the plasma glycoprotein factor VIII (FVIII). FVIII is an essential protein cofactor in the intrinsic pathway of blood coagulation that functions as a cofactor for the serine protease factor IXa in the proteolytic activation of factor X to Xa. The clinical manifestations of haemophilia A are categorized with respect to the severity of FVIII deficiency. FVIII activity levels of 5–25% are associated with a mild disease state (DS), 1–5% with a moderate (DS), and less than 1% with a severe (DS). Bleeding into joints and muscles is frequently observed in haemophilia A patients and may eventually cause pain, swelling and severe limitations of motion. The most common and effective therapy for FVIII deficiency is replacement therapy with either plasma-derived or recombinant FVIII concentrates. Originally isolated from pooled human plasma, FVIII concentrates are also produced through techniques that employ genetic engineering. The introduction of recombinant FVIII concentrates greatly reduced many of the concerns associated with the use of plasma-derived products, including transmission of blood-borne infectious pathogens and acute allergic reactions related to pyrogens. The FVIII gene discovery and sequencing led to the development and production of the first generation of commercially available recombinant FVIII (rFVIII) products, heralding a new era of viral safety in the treatment of haemophilia A. After that several rFVIII concentrates have been developed. The objectives of this study were to compare the structural heterogeneity of high-purity rFVIII preparations of commercially available products used in the treatment of haemophilia A.
Methods: Three commercially available rFVIII concentrates were compared: Helixate NexGen [octocog alfa, CSL-Behring], ReFacto [moroctocog alfa, Wyeth] and Advate [octocog alfa, Baxter]. 4-12% mono-dimensional gel electrophoresis (1D SDS-PAGE) was performed on rFVIII preparations and rFVIII fragments generated by thrombin digestion. rFVIII preparations were analyzed also by two dimensional gel electrophoresis using for the first dimension 17 cm pH 4-7 IPG strips with the BioRad™ Protean IEF Cell isoelectric focusing system. For the second dimension, 11% polyacrylamide large gels were used. Differentially expressed spots were excised and digested with trypsin, peptide mixtures were separated using a nanoflow-HPLC system. Peptides were eluted directly into a High Capacity ion Trap (model HCTplus, Bruker-Daltonik, Germany). The scan range used was from 300 to 1800 m/z. Protein identification was performed by searching in the National Center for Biotechnology Information nonredundant database (NCBInr) using the Mascot program.
Results: Initial 1D SDS-PAGE comparison of rFVIII samples revealed similar banding patterns among recombinant factors, showing major species at approximately 90 kDa, 80 kDa and above 170 kDa. The rFVIII fragments generated by thrombin digestion show a different SDS-PAGE profile respect to those derived from pdFVIII. In particular rFVIII samples showed two different bands at 40 and 43 kDa identified as the A2 domain. Some impurities derived from the manufacturing process were found by 2DE analysis in the Helixate NexGen. On the contrary Refacto and Advate showed a very similar 2DE profile.
Conclusion: our preliminary data show that proteomic approach may allow the quality control and the molecular characterisation of different rFVIII concentrates, thus providing a tool for valuable insights into the mechanisms underlying their immunogenic potential.

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MOLECULAR STUDIES OF HYPOFRACTIONATED RADIOTHERAPY IN MCF-7 CELL LINE

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Background. Radiotherapy (RT) is widely used for the treatment of oncological patients. Cytotoxic effect of RT is a complex phenomenon involving DNA breaks, production of free oxygen radicals, alteration of functional proteins and of apoptotic mechanisms. RT dose is conventionally fractionated in multiple small fractions in order to minimize damages to normal tissues. Understanding the molecular mechanisms involved in the response of tumors to different exposure to ionizing radiation will improve radiotherapy and/or radiochemothrapy effects. Following this purpose a full and selected molecular investigation at proteomic and genomic level, respectively, were conducted on a human breast tumor cell line (MCF-7), in order to set up methodological strategies to approach patients analysis. As regard to proteomic study, differential in gel electrophoresis (DIGE) was adopted to elucidate changes in protein expression in MCF-7 cell line exposed to hypofractionated radiotherapy at different doses. In addition, molecular signalling on selected gene pathways involved in DNA repair, survival and/or apoptosis, were studied by Real Time PCR and western blot analysis within 26 hours of single dose.
Methods. MCF-7 cells purchased from American Type Cellular Collection (ATCC) were seeded in T-25 cell culture flasks using MEM 10% FCS and grown at 37°C under 5% CO_2-95% air athmosphere in a humidified incubator. Cells at sub-confluence were irradiated with 2 Gy or 8 Gy daily doses for 10 days by CLINAC 600 (Varian) instrument. 24 hours after each irradiation dose, cells were harvested counted and the pellet stored at -80°C. Protein extraction was carried out by 2D DIGE lysis buffer (7 M urea, 2M thiourea, 10 mM Tris, 4% CHAPS) containing a protease inhibitor cocktail. Proteins extracts (50 µg) from each set type were labelled with 400 pmol Cy5 dye (CyDye, GE healthcare), while an internal standard, generated by pooling together an aliquot of all cell extracts, were labeled with Cy3 dye. Minimal labeling was performed according to manufacturer's recommendations. Samples were separated on 18 cm IPG strips, pH 3-10 non linear gradient (GE Healthcare). Each sample was run in triplicate. Second dimension was carried out in 12% polyacrylamide gels. CyDye-labeled gels were scanned on a Typhoon imager (GE Healthcare). Differential analysis was performed using ANOVA provided by Progenesis SameSpot software (Nonlinear Dynamics). The spots differentially expressed were identified by MALDI-ToF/ToF and ESI MS/MS.
As regard to changes in mRNA transcriptional activity of specific genes, cells were harvested 2, 4, 6, 10 and 26 hours after single dose irradiation with 2 Gy or 8 Gy and the Time Course of the events monitored. RNA was extracted utilizing Trizol reagent and reverse-transcribed using SuperScriptII RT and oligo(dT) according to the manufacturer's instructions (Invitrogen). Primers were designed with Primer Express software and tested for their human specificity using NCBI database. Real-time PCR was performed for 40 cycles with Fast SYBR Green Master Mix and processed on 7500 Real-Time PCR System (Applied Biosystems). Reactions were conducted in triplicate for each sample, quantitative data were analyzed by average of triplicates Ct (cycle threshold) and normalized versus housekeeping RNA 18S gene. For immunoblot analysis, the Trizol proteic phase was solubilized into lysis buffer, electrophoresed through SDS-polyacrylamide gels and then transferred to PVDF membrane. Proteins corresponding to genes previously quantitated by Real Time PCR, together with their phosphorilated form were quantitated by antibody reaction, recognized and visualized by ECL Plus (GE Healthcare).
Results. MCF-7 cells treated with 2, 4 and 6 of 2 Gy and 8 Gy ionizing radiations doses, respectively, were selected on the basis of cell growing curve, and analysed by 2D-DIGE versus untreated cells. The comparative statistical analysis identified 130 spots differentially expressed in all different doses. Our attention was focused on the identification of these changed spots and was conducted by MALDI Tof/Tof analysis. Identified proteins were classified according to their functional properties.
As regard to mRNA transcripts, differences in gene expression of ATM, Chk2, p53, BAX, Bcl-2, Survivin, Rad 50 and Rad 51 versus untreated cells were screened by Real-Time PCR. Statistical analysis indicated that DNA repair and survival activity were induced in the early phase of irradiation at 2Gy dose. Interestingly with 8 Gy dose , the increased trascription of these genes was observed only after 10 hours. No correlations between differences in gene expression and protein quantification was detected.

Conclusions. These results could provide new insights to elucidate molecular mechanisms adopted by cancer cells to survive after an irradiation-induced damage opening new avenues in the selection of appropriated therapies to counteract cancer growth. They also could offer to clinicians the rationale for choosing hypofractionation radiation therapy.

052

CALGRANULIN A IS A POTENTIAL INDICATOR OF OVARIAN CANCER PROGRESSION

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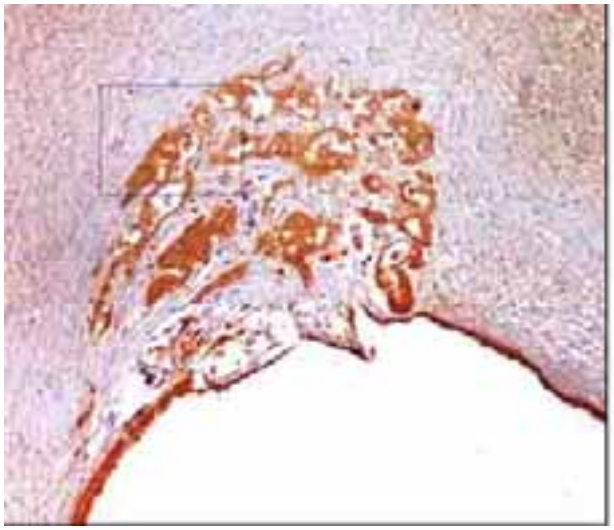
Background: The epithelial ovarian carcinoma is the most leading cause of death among gynaecological malignancies in industrialized countries and in Europe. The lack of adeguate screening tests and the relatively asymptomatic nature of early stage disease follow that most patients are diagnosed at the advanced stage of disease (III-IV), when the cancer has progressed beyond the pelvis and chances of long term survival are significantly decreased. There is an urgent need for the development of multiple diagnostic/prognostic markers to detect and monitor ovarian cancer. Aim of this study is to define a specific protein expression pattern associated to metastatic ovarian cancer.
Methods: Six patients with ovarian carcinomas (3 endometrioid and 3 serous) in advanced stage (III-IV) were enrolled and biopsies of tumoral and healthy tissue were performed. Peritoneal fluid was also collected. Samples analysed by 2DE, loading 120 µg of proteins on IPG strips (17cm pH3-10 nonlinear). Then, the IPG gels were transferred onto 13% polyacrylamide gels. The protein spots were visualized with a modified silver staining protocol. Spot detection, matching and quantification analysis were carried out with PDQuest 7.1.1 software package. The proteins differentially expressed were identified by mass spectrometry analysis (ESI-Q-TOF-MS/MS).
Results: Only one protein was found up-regulated in ovarian cancer tissue, while 5 proteins were down-regulated. The over-expressed protein was S100A8 (Calgranulin-A) protein; whereas ANXA 5 (Annexin5), Leg 3 (Galactose-Specific Lectin3), CRBP-1 (Cellular retinol binding protein 1), RKIP (phosphatidylethanolamina-binding protein), GSTA2 (Glutatione S-transferase A2) protein resulted down-regulated. The over-expression of S100A8 protein was confirmed by immunohistochemistry (see figure). This protein was also detected in peritoneal fluid of the patients.



The up regulation of S100A8 in pathological samples is confirmed by immunohistochemistry.

Conclusions: Using 2DE and mass spectrometry analysis of biopsies and interstitial fluids of six metastatic ovarian tumors (endometrioid and serous), we have identified six protein that are disregulated in advanced state, and that might be correlated to an aggressive behaviour of disease. Of interest the up-regulation of Calgranulin-A: although the precise role of S100 protein in carcinogenesis is not clear, it seems that formation of homo- and hetero-dimers, binding of Ca2+ and interaction with effector molecules are essential for the development and progression of many cancers. Strong S100A8 up-regulation has been found in skin, breast, lung, gastric, colorectal, pancreatic, and prostate cancer. In human bladder cancer, over-expression of S100A8 is associated with stage progression, invasion, metastasis and poor survival. Several studies have suggested that S100 proteins promote cancer progression and metastasis through cell survival and apoptosis pathways and significant alterations in the expression of the S100 members were found in different tumor types. Therefore changes in the expression and/or function of S100 proteins may represent a key step during cancer development and progression and enhanced S100A8 levels in pathological

conditions of chronic inflammation as well as in cancer argue for a possible role in inflammation-associated carcinogenesis.

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PROTEOMIC PROFILE OF RENAL CELL CARCINOMA BY 2-D DIGE

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Background: Renal cell carcinoma (RCC), the most common neoplasm affecting the adult kidney, is characterised by a variety and heterogeneity of histological subtypes, drug resistance, and absence of molecular markers, therefore the identification of diagnostic and prognostic markers is crucial. Two-dimensional difference gel electrophoresis (2-D DIGE) technology in combination with mass spectrometry (MS) can be applied to detect changes of protein expression in RCC makers research. 2-D DIGE is an electrophoretic technique able to separate with high resolution complex protein mixtures of two different samples labelled with diverse CyDyes on the same gel. Moreover, an internal standard is utilised to minimise experimental variability and to normalise protein abundance.

Methods: We applied 2-D DIGE combined with MALDI-TOF MS analysis to 20 pairs of RCC and autologous normal renal cortex. The images of gels were analysed by DeCyder 6.5 and EDA softwares, and differentially expressed protein spots were excised from Flamingo stained preparative 2-DE gel and analysed by MALDI-TOF MS.

Results: 121 spots out of 2500 displayed differential expression, 34 and 87 of these were respectively over- and down-expressed in RCC (*p-value* <0.05 in 75% of gels). The multivariate Hierarchical Clustering analysis applied to gels and protein spots exactly separated the two sample classes in two groups: the normal one more homogeneous in spot abundance, and the RCC one composed with three diverse subgroups not related to Grade or TNM stage. Moreover, four spots including two dehydrogenases, nocturnin fragment, and S100-A11 protein were able to split RCC from normal tissues with an accuracy of 100%.

Conclusions: Applying 2-D DIGE to RCC we point out a list of 121 proteins among which 4 accurately discriminated RCC from normal tissue.

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IMMUNOPROTEOMICS OF 5-ALPHA-REDUCTASE ISOFORMS IN SOCIALLY REARED AND ISOLATION REARED RATS

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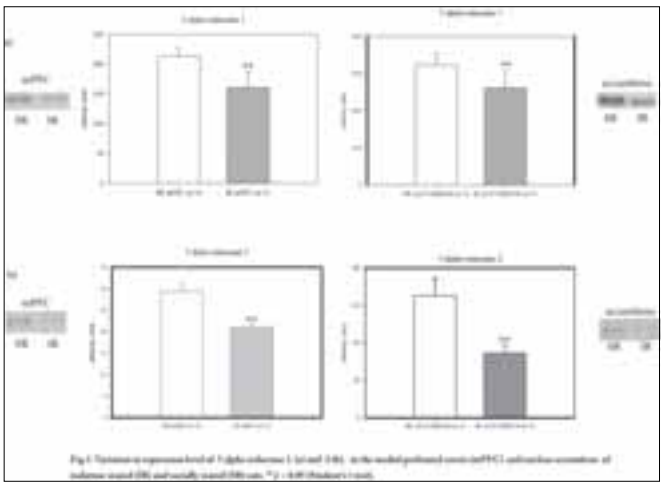
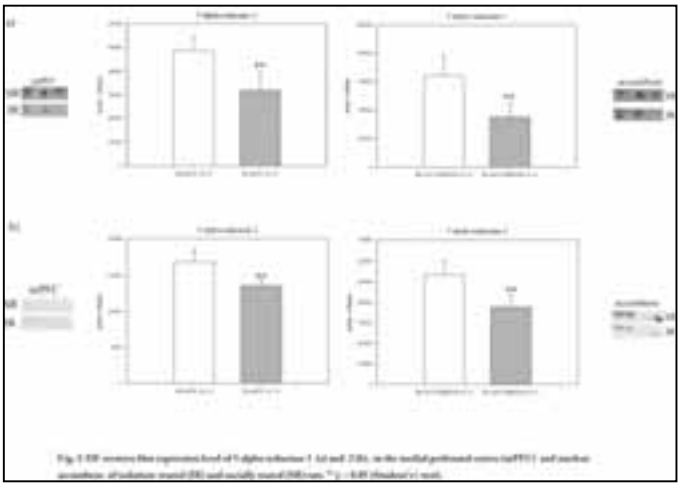
Background: Schizophrenia is a very serious and frequent psychiatric disorder, affecting 1% of world population. So far, schizophrenia treatment is mainly based on antipsychotics, which often have significant side effects. Recent experimental evidences indicate that the 5-alpha-reductase (5AR) inhibitor finasteride might be a valuable therapeutic alternative to antipsychotics, probably through its effect on neurosteroid synthesis. In the present study, immunoproteomics were used in an animal model of schizophrenia, to evaluate the expression level of brain 5AR-1 and -2, the rate-limiting enzymes of one of the major metabolic pathways in brain steroidogenesis.
Methods: Animal model of schizophrenia was induced by post-weaning isolation of rats (isolation reared, IR rats) for 6-9 weeks. Socially reared (SR) animals were used as control. Seven days after assessment of psychotic-like behavior, rats were sacrificed by decapitation, and the brain areas of interest removed. Experiments were conducted in triplicate using different pH ranges to optimize spot resolution and to visualize different isoforms on blots. Quantitative 1D and 2D western blotting with homemade antibodies were performed on two brain areas (medial prefrontal cortex and nucleus accumbens) of SR and IR rats. Image and statistical analysis were performed with Progenesis SameSpots software (Nonlinear Dynamics) and SigmaPlot 11 (Systat).
Results: Image and statistical analysis of 1D- (fig.1) and 2D (fig.2) western blot highlighted a significant downregulation of 5AR-1 and -2 proteins in IR- versus SR group.





Conclusions: The main outcome of this study is that isolation rearing, a validated animal model of schizophrenia, induced a down regulation of both 5AR isoforms in rat brain. Such deficit might be relevant to the well known role of neurosteroids in schizophrenia. In light of these findings, it might be proposed that the antipsychotic like effect of finasteride found in IR rats could arise from a pharmacological effect amplified because of down regulation of isoforms of 5AR in IR rats. These results may therefore contribute to understand the neurochemical alterations present in schizophrenia and to unravel the mechanism by which finasteride exerts its antipsychotic activity.

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COMPARATIVE PROTEOMIC ANALYSIS OF BALF FROM PATIENTS WITH DIFFERENT CONNECTIVE TISSUE DISEASES

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Background: Proteomic analysis of bronchoalveolar lavage fluid (BALF) can potentially provide important information about changes in protein expression and secretion in the course of airway or connective disorders. Previous studies have shown that the protein composition of BALF is altered in several diseases, including pulmonary fibrosis associated with systemic sclerosis (SscFib+), Polymyositis (PM) and Dermatomyositis (DM), connective tissue disorders in which an interstitial lung disease (ILD) can be frequently detected. In the course of PM/DM, the serologic finding of antibodies against tRNA-synthetases, whose most common is anti-histidyl t-RNA synthetase (Jo1), identifies a disease subset in which patients are more likely than other PM/DM patients to develop ILD and that is classified as Antisynthetase Syndrome (AS). Prognosis and response to treatment of ILD in AS patients seem different with respect to ILD in the course of classic PM/DM or in other CTDs as Systemic Sclerosis. Through bidimensional gel electrophoresis we compared the proteins expressed in BALf of SSCFib+ patients, PM/DM patients, AS patients with ILD and subjects with polymyositis and dermatomyositis in association with various autoimmune and connective tissue diseases, in particular with systemic sclerosis (Overlap syndrome).
Methods: BALf (brochoalveolar lagavage fluid) samples were collected from 5 DM patients, 4 AS patients anti-Jo-1 positive, 4 patients with myositis overlap syndrome, and 6 patients SScFib+.
BAL was performed following the standard procedure. To remove the cells and debris, all samples were filtered through gauze and centrifugated, and the supernatant (BALf) was then divided into aliquots and stored at –20°C for protein analysis. All samples were dialyzed in the presence of, protease inhibitor, lyophilized and the protein pellet was resuspended in a solubilization buffer. For each experiment (analytical) of 2-DE the same amount of protein (70 µg) was used; a higher (1 mg) was loaded for the subsequently mass spectrometry identification experiments. We compared the BALf protein profile obtained from 4 group; the total number of spots, non-matched spots and spot parameters were assessed by the PDQuest 7.1 software. The proteins differently expressed were analyzed by MALDI-TOF or LC-MS/MS.
Results: 2-DE analysis was performed in triplicate on each BALf to produce 57 gels in total. The mean spot numbers in coomassie stained gels were 365 ± 84 in SscFib+, 258 ± 48 in PM/DM, 284 ± 32 in AS and 323 ± 51 in Overlap Syndrome. The comparative analysis of profiles evidenced that certain protein spots were apparently differentially expressed among groups. 20 of these spots were present in SSCFib+ only, 9 were present in PM/DM patients only, 3 spots were exclusive of AS patients and 12 in Overlap patients. Most of these protein spots are still under identification by mass spectrometry.

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PROTEOMIC AND BIOCHEMICAL EVALUATION OF BICARBONATE DIALYSIS WITH HIGH FLUX MEMBRANES

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Background: The age of patients in maintenance hemodialytic treatment (MHD) is progressively increasing. Aged patients are commonly treated with standard bicarbonate hemodialysis (SHD) using low-flux (LF) membranes having any clearance for low-molecular weight proteins (LMWP), like beta-2-microglobulin (B2M).
The aim of this study was to evaluate the accuracy of biochemical and proteomic methods to assess the efficiency of high-flux (HF) in comparison with LF dialytic membrane in removal of small and middle molecules.
Methods: Nine patients (72-84 years) in MHD (6 months-6 years), treated by SHD with a LF polysulphone (Fresenius F8), in stable clinical conditions and adequate nutritional status. Dialytic efficiency, tolerability, and effects on inflammation, cardiovascular parameters and biocompatibility were assessed by means of standard laboratory techniques and using proteomic methods during dialytic treatment with using HF dialyzers (Triacetate: Nipro N190 FH; Helixone: Fresenius FX 80; Polyamid: Gambro Polyflux 210 H) in comparison with LF polysulphone (Fresenius F8). Patients were treated in randomized rotation with each HF dialyzer for 1 week, after an equilibration treatment period of 2 weeks with SHD with LF membrane, maintaining constant blood- and dialysate-flow during the study period.
Results: The removal of small molecules was very high (urea reduction ratio 72-75 %). and similar with all membranes. The removal of middle molecules was much higher with HF membranes (B2M reduction ratio 60-69 %) than with LF membrane. The removal of middle molecules was significantly higher with N190FH than with the other HF membranes, showing a very high RR of myoglobin, homocysteine and BNP. TNF concentrations decreased after HF dialysis. Serum C reactive protein was unmodified, while a slight increase in IL6 and IL10 was found with all membranes.
SDS PAGE performed on sera and, more clearly, those on ultrafiltrate fluid, demonstrated a high removal of LMWP, in the range 10-30 kDa, with N190 FH, higher than that obtained with FX80 and Polyflux 210 H. No removal of LMWP was observed with LF membrane. 2DE gels confirmed the removal into the dialysate fluid of a high number of proteic spots in the range 10-30 KDa with N190 FH. Few proteic spots with an estimated MW around 10-12 kDa, possibly B2M, were detected in 2DE gels from dialysate fluid also with FX80 and Polyflux 210 H. No proteic spots were found using LF membrane.
These data indicate that HF membranes, utilized for SHD in aged patients do not induce inflammation, keep a very high depuration for low molecules, while increase markedly the clearance of middle molecules. In particular, dialytic efficiency of triacetate membrane is very high and similar for small and middle molecules, like B2M and myoglobin. Proteomic analysis demonstrate a higher convection into the dialysate of LMWP in comparison with the other membranes.
Conclusions: Proteomic analysis of dialysate and biochemical tests allow a precise assessment of the kind of molecules removed through the different dialytic membranes. Furthermore, the mass spectrometric analysis of isolated spots, should precisely identify the proteins removed.

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HUMAN SKELETAL MUSCLE PROTEINS DATABASE

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Background: In last years mass spectrometry has been recognized as a powerful technology for identification and analysis of proteins particularly in proteomic field. For this reason different tools and protocols have been developed to determine molecular weight, primary structure, post-translational modifications and proteins localization in cells. To define a complex mixture of proteins, however, mass spectrometry must be coupled with an appropriate separative technique, such as, isoelectric focusing, bidimensional electrophoresis or liquid chromatography. Skeletal muscle tissue represents a good candidate for biomarkers detection due to its capability of modulate metabolic and contractile proteins composition in response to physiological and pathological stimuli often not directly related to muscle itself. For such a reason, the construction of a large data base of proteins expressed and modulated in the muscle tissue is an essential step for the identification of new biomarkers and for the comprehension of molecular mechanisms at the basis of physiological (i.e. ageing), paraphysiological, (i.e. bed rest and acute and chronic hypoxia) and pathological conditions (i.e. muscular and neuromuscular disorders).
Integration of these data within a single resource provides information in the context of individual proteins and enhances the use of proteomic data.
Methods: For proteins identification 400 ug of proteins extracted from human skeletal muscle were loaded in a semi-preparative gel. Sample was separated on 18 cm IPG strips, pH 6–11 and pH 4-7 gradients. Gels were stained with Deep Purple, (GE Healthcare). Images acquisition was performed using Typhoon 9200 laser scanner. Spots of interest were excised from gel using the Ettan spot picker robotic system (GE Healthcare), destained in 50% methanol/50 mM ammonium bicarbonate (AMBIC) and incubated with 30 uL of 4 ng/uL trypsin (Promega) dissolved in 10 mM AMBIC for 16 h at 37°C. Tandem electrospray mass spectra were recorded using a HCT Ultra PTM Discovery System mass spectrometer (Bruker Daltonics) interfaced to a Ettan MDLC chromatograph (GE Healthcare). Peptides were eluted with an ACN/0.1% formic acid gradient. For identification the spectra were searched in the NCBI nr database using BioTools (Bruker Daltonics) interfaced with MASCOT software. One missed cleavage per peptide was allowed, and the fragment ion mass tolerance window was set to 0.3 Da.
Results: In order to improve protein separation two gradients were utilized. After images aquisition 204 and 140 spots, respectevely, were selected by the software, excised and subjected to *in situ* protein digestion by trypsin. Peptides were analized by mass spectrometry coupled to reverse phase chromatography. About 250 proteins were identified; some of them were isoforms of the same protein. Tandem spectra generated by HCT Ultra were elaborated by BioTools software to identify 3500 unique peptides. Moreover, only proteins identified with two or more peptides were considered, in order to reduce false positive. Using this approach, we identified also low abundant proteins and proteins with extreme pI values.
Conclusions: Due to the high dynamic range, mass spectrometry by itself do not allow the identification of all components of a complex mixtures, such as an entire tissue extract. High abundant proteins, actually, hide low abundant species; for this reason an appropriate separative method prior to the identification step is needed. To overcome the limitations due to dynamic range, human skeletal muscle proteins were separated by two-dimensional electrophoresis utilizing two different pH ranges and identified by mass spectrometry. These ranges were able to separate the highly abundant contractile proteins and the low abundant metabolic and signalling proteins facilitating the data base construction. The application of off gel technique is currently in progress to include structural and costameric proteins which are excluded from 2D separation.
The list of identified proteins with mass data will represent a useful tool for the scientific community involved in muscle research.

Acknowledgements: This work has been funded from: Italian Ministry of University and Scientific Research (Grant: FIRBRBRNO7BMCT to C.G.)

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MOLECULAR ADAPTATION OF HUMAN SKELETAL MUSCLE TO ACUTE AND CHRONIC HYPOXIA: THE CONTRIBUTION OF PROTEOMICS

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Background. At the steady state under normoxia, 90% of available oxygen is utilized by mitochondria to generate ATP through oxidative phosphorylation, to maintain cellular functions. Hypoxic stress, which occurs when oxygen supply or demand are compromised, represents a severe condition for cells, tissues and organisms survival. In humans, hypoxic stress occurs in some physiological conditions such as fetal development, exercise and climbing at high altitude and in a wide range of clinically relevant disorders where the hypoxia may initiate, be maintained or be a result of a disease state. This is the case of lung diseases, ischemia, stroke and cancer. The exploration of cellular mechanisms underlying beneficial adaptive processes and detrimental responses to hypoxia represents the object of several current research studies leading to increase knowledge and possibly modulate or develop new treatments for human diseases. Therefore, studying these mechanisms in the context of critical illness is difficult due to patients heterogeneity. In addition, many pathological and physiological processes occur concurrently, and to separate cause and effect as a single feature of a disease (tissue hypoxia) is rather impossible. Hypoxic cellular responses are common to tissues independently from causes, for such a reason, the investigation realized on healthy individuals progressively exposed to hypoxia through ascent to high altitude may provide information about the nature of the hypoxic processes occurring in patients. This study is part of a large research project named Caudwell Xtreme Everest and coordinated by the Centre for Altitude, Space and Extreme Environment Medicine at the University College of London. The expedition was realized in April and May 2007 and it involved 200 individuals most of them were volunteers and the remainder were doctors and scientists. Participants were progressively exposed to hypobaric hypoxia on the trek to Everest Base Camp at 5300 m. Fifteen individuals ascended up to 8000 m. The measurements, performed at sea level, in four laboratories at 5300 m and in two laboratories high on Mount Everest, included cardiopulmonary exercise testing, neuropsychological assessment, near infra-red spectroscopy of brain and exercising muscle, blood markers and daily recording of simple physiological variables. Muscle biopsies were taken from some individuals at sea level and at high altitude after two weeks (early Everest) and after fifty days (late Everest) of exposure and they were utilized in the present study in order to investigate the alteration occurring in skeletal muscle proteome of humans exposed to hypobaric hypoxia. The aim of this study was to characterize the muscle proteome in the acute fase (early Everest) of acclimatization and in the long-term exposure (late Everest).
Methods. Proteome analysis was performed utilizing 2D-DIGE and MALDI-ToF mass spectrometry. Vastus lateralis muscle biopsies were obtained from five subjects before and after two weeks of progressive exposure to hypobaric hypoxia (5300 m) and from eight subjects before and after fifty days of exposure to hypobaric hypoxia (5300 m-8400m). Each protein extract was labelled with Cy5 dye (GE Healthcare), while the internal standard was labelled with Cy3 dye (GE Healthcare). The first dimension was performed on 24 cm 3-10 NL pH gradient IPG strips using an IPGphor electrophoresis unit (GE Healthcare), the second dimension was carried out in 20x25 cm 12%T-2.5%C PAGE gels using Ettan Dalt II system (GE Healthcare). Images were visualized using a Typhoon 9200 scanner (GE Healthcare) and analyzed with DeCyder software version 6.5. Statistically significant differences were computed by paired Student's t-test, the significance level was set at $p<0.05$. Differentially expressed proteins were identified with Ettan MALDI-ToF Pro (GE Healthcare).
Results. 2D-DIGE analysis revealed a significative difference in 74 spots (58 less abundant and 16 more abundant in high altitude versus sea level) for the early Everest and in 60 spots (41 less abundant and 19 more abundant in high altitude versus sea level) for the late Everest. Twenty one of the statistically changed spots were common to both conditions. MALDI-ToF mass spectrometry identified 51 spots for the early Everest and 46 spots for the late Everest. The majority of the modulated proteins is represented by metabolic enzymes belonging to glycolysis, tricarboxylic acid cycle, fatty acid beta oxidation and oxidative phosphorylation, and by proteins involved in the regulation of protein translation. All these proteins were considerably less abundant in high altitude versus sea level and they were more represented in the early Everest. A deregulation was found also for proteins involved in the stress response, particularly, we found an

increment of some antioxidant enzymes. A marked modulation was observed also in cytoskeletal and some isoforms of contractile proteins.
Conclusions. Proteomic analysis provides a clear picture of the events occurring in muscle tissue under early and long-term hypoxia exposure. Particularly, it allows to underline the more relevant changes at the metabolic, functional and structural level involved in the tissue adaptation at the limit of humans tolerance of O2 deprivation.

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INVESTIGATING THE BIOCHEMICAL PATHWAYS INFLUENCED BY DOPAMINE TOXICITY AND α-SYNUCLEIN OVEREXPRESSION TO ELUCIDATE PARKINSON'S DISEASE PATHOGENETIC MECHANISMS

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Background: The most distinctive sign of Parkinson's disease (PD) is the selective degeneration of dopaminergic neurons of the substantia nigra. This is due to the specific vulnerability of these cells, which in turn is related to dopamine itself. However, the precise biochemical mechanisms that lead to neurodegeneration are still not clear.
Alpha-synuclein is a protein variously connected with PD: first of all there are familial forms of the disease (PARK1 and PARK4) linked to mutations or duplication and triplication of the α-synuclein gene; moreover it is the predominant component of the characteristic inclusions of PD (Lewy bodies). To better understand the pathogenesis of PD we exploited a cellular model able to highlight the biochemical pathways influenced by dopamine toxicity, α-synuclein overexpression and their interconnections. Proteomics allowed us to characterize these pathways without any *a priori* hypothesis.
Methods: Human neuroblastoma SH-SY5Y cells were stably transfected for α-synuclein overexpression (tr) or for the expression of β-galactosidase as control (β-gal). Both cell lines were treated or not with dopamine (DA, 250 μM) in the presence of catalase (CAT, 700U/ml), in order to eliminate aspecific effects due to H_2O_2 in the extracellular space. Proteins were extracted with a buffer containing 7 M urea, 2 M thiourea, 4% CHAPS and loaded on 13 cm 3-10NL IPG DryStrips (GE Healthcare). IEF was performed at 18°C on IPGphor (GE Healthcare). SDS-PAGE was performed using 13% polyacrylamide gels. Gels were stained according to MS-compatible Sinha's silver staining method. 2-DE maps from 3 independent experiments were scanned and analyzed with ImageMaster 2D Platinum V5.0 software package (GE Healthcare). Spots were quantified on the basis of their volume (after intensity normalization) and analyzed according to the analysis of variance. Relationships were considered statistically significant when $p<0.05$. Selected spots were excised, destained and digested with modified porcine trypsin overnight at 37°C. Peptide mixtures (10 μl) were separated by using a nanoflow-HPLC system (LC Packings) at a flow rate of 200 nl min^{-1} and a linear gradient from 2% acetonitrile, 0.1% formic acid to 50% acetonitrile, 0.1% formic acid in 40 min. Peptides were eluted directly into an ion trap Esquire 3000 plus (Bruker-Daltonics). Database search with the peptide masses was performed against the NCBI non-redundant database using Mascot (http://www.matrixscience.com).

Results: Statistical analysis of silver-stained gel images revealed 28 spots to be significantly different in at least one of the four groups considered. 23 differentially expressed proteins were identified by LC-MS-MS. Proteins were therefore divided in 2 groups depending on the condition that affected protein levels (dopamine treatment or overexpression of α-synuclein). In the latter group 6 proteins were regulated in a more complex way, i.e. α-synuclein overexpression modulated the dopamine effect. Proteins were analyzed in terms of both network enrichment and functional classification using *PPI spider* (http://mips.helmholtz-muenchen.de/proj/ppispider/). The figure shows a significant ($P<0.05$) model for network enrichment based on known protein-protein interactions for proteins that displayed significant changes following dopamine treatment, regardless of α-synuclein

overexpression (top), and for proteins that displayed significant changes as a consequence of α-synuclein overexpression or as a result of the association of dopamine treatment with α-synuclein overexpression (bottom). Dopamine exerts its toxic effects affecting pathways of oxidative stress, energetic metabolism, apoptotic cell death, NF-κB cascade, and cytoskeleton maintenance. Proteins that displayed significant changes associated to α-synuclein overexpression are actors/regulators of the NF-κB pathway, the apoptosis, the cytoskeleton maintenance or the neuronal plasticity.
Conclusions: The body of the results presented here contributes to better characterize how dopamine and α-synuclein could be involved at the origin of the degeneration of dopaminergic neurons that typically occurs in PD. In particular, modulation of the NF-κB pathway appears to be a central event both in dopamine toxicity and in response to α-synuclein overexpression.

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IDENTIFICATION OF Y745 OF VAV1 AS A TYROSINE RESIDUE CRUCIAL IN MATURATION OF APL-DERIVED CELLS

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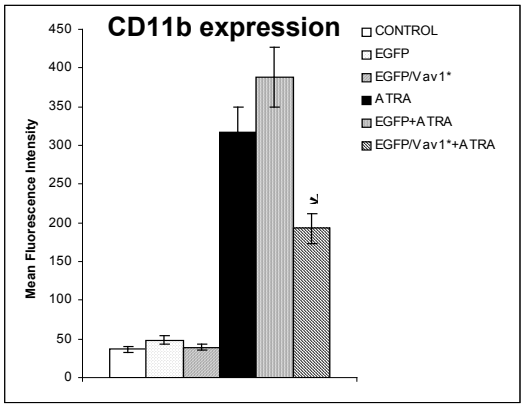
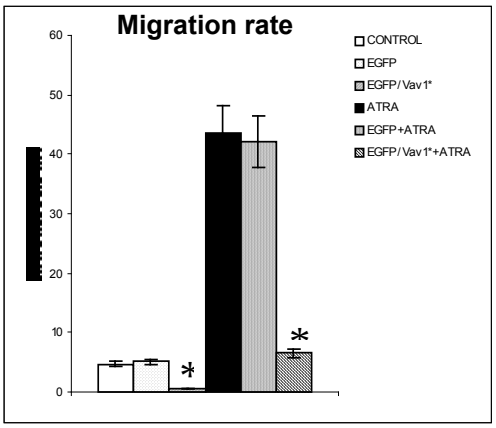
Background: Vav1, whose physiological expression is restricted to hematopoietic system, is one of the signalling proteins up-regulated by ATRA in APL-derived promyelocytes. At variance with maturation of normal myeloid precursors, Vav1 plays an important role in the agonist induced differentiation of APL-derived cells. In particular, the down-modulation of Vav1 during ATRA treatment prevents maturation and its over-expression in differentiating cells strengthens the effects of the drug. By means of array profiling obtained with differentiating APL-derived cells, we have demonstrated that the forced reduction of Vav1 during the treatment with the agonist reduces the number of ATRA-induced genes. By means of a proteomic approach, we have also demonstrated that Vav1 plays a role in modulating the expression level of the protein tool by means of which ATRA executes the maturation program of tumoral promyelocytes. Since high levels of tyrosine phosphorylated Vav1 accumulate in differentiating APL-derived cells and no data on tyrosine residue/s of Vav1 involved in maturation of myeloid precursors are available, we tried to identify the tyrosine residue/s of Vav1, whose phosphorylation plays a role in overcoming the differentiation blockade of APL-derived promyelocytes.
Methods: The APL-derived NB4 cell line was induced to differentiate with ATRA and subjected to isoelectric focusing (IEF) in free solution with MicroRotofor to improve the recovery of Vav1, since this protein is a relatively low abundant cell protein. Vav1 was then immunoprecipitated from the pull of the Vav1-containing fractions and, after mono-dimensional electrophoresis and staining of the polyacrilamide gel, the bands corresponding to Vav1 were excised and subjected to mass spectrometry analysis. A plasmid expressing a mutated form of Vav1, in which tyrosine 745 was replaced with a phenylalanine, was then originated by *in situ* mutagenesis. NB4 cells were then transiently transfected with the plasmid expressing the Y745F mutated form of Vav1 and the effects of this mutation on ATRA-induced differentiation were evaluated, in terms of CD11b expression and migration capability.
Results: The analysis of mass spectra acquired by MALDI-TOF/TOF showed a single peptide, including the tyrosine 745, corresponding to a putative phosphorylated Vav1 peptide, in both control and differentiating NB4 cells. As deduced by cytofluorimetrical analysis of the over-expressing cells, the mutation of the Y745 residue of Vav1 counteracted the ATRA-induced increase of CD11b expression. Since granulocytic differentiation is associated to the acquisition of a migratory capability, transfected NB4 cells were subjected to migration assays, showing that Y745F strongly reduces the ability of differentiating tumoral promyelocytes to cross the porous membrane in response to ATRA-treatment.





Conclusions: The above reported results allowed to identify a not yet described Vav1 tyrosine, Y745, as a crucial residue in regulating the acquisition of a differentiated phenotype by ATRA-treated tumoral myeloid precursors. Even though further analysis will be necessary to clearly establish the functional role of tyrosine phosphorylated Vav1 in this cell model, these data may contribute to better define the involvement of Vav1 in the maturation process of APL-derived cells and to identify new specific molecular targets for therapy of this myeloid leukemia.

061

A NON CONVENTIONAL PROTEOMIC STUDY OF VSMC ACTIVATION

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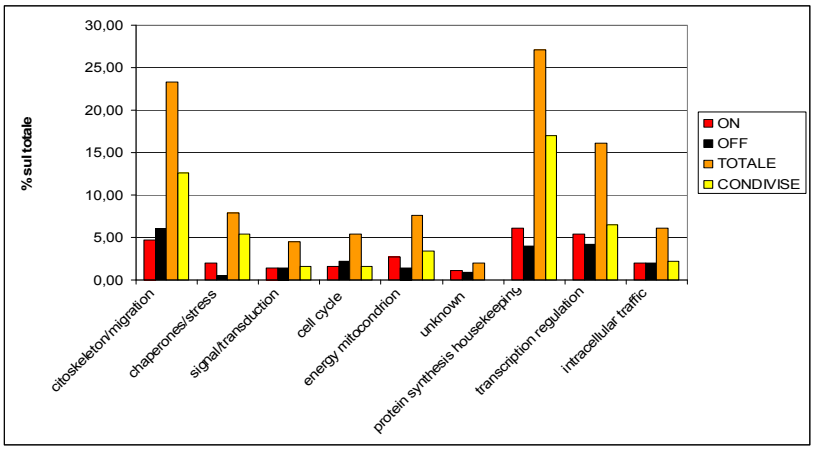
Background: : Vascular smooth muscle cells (VSMC) are mature cells that maintain great plasticity and are capable of switching from a contractile-quiescent to a proliferative-migratory phenotype. The switch to this last state, that can be named “activated”, is set up by plasma growth factors that trigger a signalling cascade able to modulate differential protein phosphorylation. The study of VSMC phenotypic change is of primary significance since this phenomenon is engaged in atheroma formation and intima hyperplasia onset.
Methods: Whole protein extracts from quiescent or activated VSMC were fractionated by ion chromatography (anion and cation exchange) and grouped in families with homogeneous chemical-physical properties (acidic and basic proteins). After enzymatic digestion of the two protein classes, the peptide mixture was fractionated by nano-HPLC, using a C18 column. The fractions were collected by a robotic system and spotted on the target for MALDI TOF/TOF analysis. A mass range from 900 to 4000 Da was considered for peak selection. The top 15 masses in each spot (12-s chromatography time) were then selected for MS/MS analysis. Searches were performed against the SwissProt protein database for Mammalia. A positive identification was accepted at the 97% confidence level.
Results: Protein extracts from contractile-quiescent and serum-activated VSMC were separated by ion chromatography. The overlapping between acidic and basic proteins was 23.5% and the evaluation of pI in the acidic and basic fractions showed a good discrimination of the stationary phases according to their ionic features. MS/MS analysis let the identification of 739 proteins that were grouped in homogeneous functional classes. Differential group distribution between VSMC phenotypes was examined and interestingly, in the activated cells we observed a major occurrence of cell factors responsible for protein synthesis, for folding and for cell distribution.



Conclusions: A chromatographic approach allowed the fractionation of the complex protein content of VSMC. The peptide analysis picked out by ion chromatography showed a low level of overlapping thus a good selectivity in relation to the ionic features. Thanks to the proteome simplification achieved, 739 proteins were identified and differences between the protein content of activated and quiescent VSMC were observed. This, though preliminary, study confirmed data already obtained in our lab on the chaperones involvement to cell activation. The techniques utilized resulted proper for a more methodical work aiming to the identification of differentially expressed or post-translationally modified markers.

062

IDENTIFICATION OF ANKRD2/ARPP AS AN AKT SUBSTRATE IN C_2C_{12} MUSCLE CELLS.

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Background: Akt/PKB is a serine/threonine kinase activated by multiple cellular signals. Three isoforms of Akt exist, namely Akt1/PKB□, Akt2/PKB□, and Akt3/PKB□ that might have redundant but also specific roles. In muscle cells, activation of Akt is crucial for survival, differentiation and regeneration. Recently, we demonstrated the involvement of Akt in the correct function of nuclear lamina of muscle cells by phosphorylation of Lamin A/C (1). Using the same phospho-proteomic approach, we decided to identify novel phospho-substrates of Akt in C_2C_{12} differentiating myotubes following the arrival of stress stimuli.
Methods: Differentiated C_2C_{12} myotubes were exposed (or left untreated) to H_2O_2 stimulation. Cells pellets were opportunely lysed and proteins were separated by 2D-electrophoresis using a 3-10 IEF strip for the first dimension on a Protean IEF cell (Bio-Rad). The experiment was performed in replicate: one gel was stained with MS-compatible Coomassie blue, while the other was blotted onto nitrocellulose and revealed with an anti phospho-Akt substrate antibody (Cell Signaling) specifically designed to detect proteins phosphorylated by Akt. The overlapping spots were excised from the Coomassie-blue stained gel and peptides were extracted by tryptic in-gel digestion for LC/MSMS analysis.
Results: Our attention was drawn by a train of spots which, according to MS analysis, corresponded to Ankyrin-Repeat protein with a PEST motif and a Proline-rich region (ARPP). ARPP, also known as Ankrd2 (ANKyrin Repeat containing Domain protein 2), is a member of the muscle ankyrin repeat proteins (MARPs), which are involved in muscle stress response pathways after eccentric contraction, acute exercise, or during work overload hypertrophy. These proteins are also involved in pathological disorders such as tumors and some muscular dystrophies (2). LC/MSMS identification was next confirmed by Western Blotting analysis using an anti ARPP antibody. In the untreated sample, the spots recognized by this antibody run both at the theoretical isoelectric point (5,95) and molecular weight of ARPP (40 kDa). In contrast, following H_2O_2 treatment these spots shift toward the acidic site of the gel, as expected after phosphorylation events.
Conclusions: In this work we describe the use of a phospho-proteomic approach for the identification of novel Akt substrates during the exposure of muscle cells to oxidative stress. Our results show that the muscular protein ARPP is a novel phospho-Akt substrate. Moreover, we demonstrate that already after five minutes of stress stimulation, Akt is activated and phosphorylates ARPP. In particular, after a transitory drop, at three hours of stress stimulation the intensity of Akt-dependent ARPP phosphorylation undergoes to a new and robust increase, indicating an active role of Akt-phosphorylated ARPP during stress stimulation. We also demonstrated that after five minutes from the arrival of ROS, ARPP and Akt physically associate. In particular, we observed that ARPP preferably binds to Akt2, which is the isoform mainly involved in the muscle differentiation program.
Roles for Akt-dependent ARPP phosphorylation are discussed.

1. Cenni et al., J Proteome Res. 2008, 7:4727-35.
2. Tsukamoto Y et al., Histochem Cell Biol. 2008, 129:55–64.

063

QUANTITATIVE PROTEOMICS ANALYSIS OF HISTONE ACETYLATION IN HUMAN ACUTE PROMYELOCYTIC LEUKEMIA

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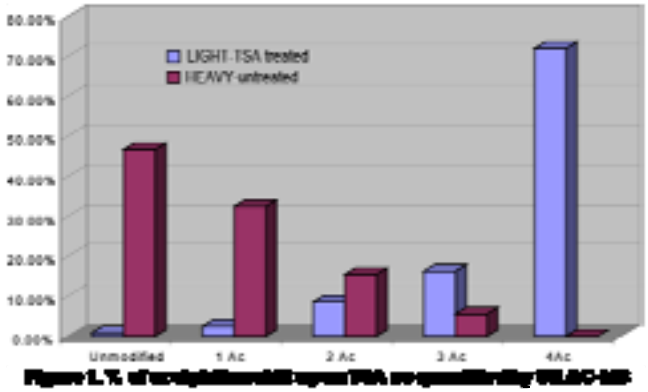
Background: Histone tails on the nucleosomes are subject to enzyme-mediated post-translational modifications (PTMs). Combinations of these modifications create a "Histone Code" that regulates various chromatin functions, such as gene transcription and silencing, and chromatin remodeling, etc. The enzymes which catalyze these modifications have been intensively studied over the past 10 years. This is the case of histone deacetylase (HDACs), whose aberrant expression and/or activity has been related to oncogenesis in several studies. Moreover, small-molecule with the capacity of interfering with HDACs has been extensively studied as potential anti-cancer drugs. Essential for fully decrypting this code is an efficient methodology for detection of site- localization, and quantitation of these PTMs. To this aim, we applied Stable Isotope Labeling by Amino acids in Cell culture (SILAC) coupled to high-resolution mass spectrometry technology to identify and quantitatively measure histone PTM patterns, with a special focus on acetylation. We chose human acute promyelocytic Leukemia (APL) as model system: APL is caused in 100% of cases by Retinoic Acid Receptors (RAR) translocations, and the promyelocytic leukemia fusion protein PML-RAR represents the most frequent rearrangement in this kind of leukemia. Although the molecular mechanism behind the disease is not fully clarified, many studies indicate that PML-RAR can recruit HDACs to chromatin, causing histone deacetylation and subsequent aberrant expression of target genes. In order to shed a light on this mechanism, we used the NB4 cell lines which carry t(15;17) PML-RAR fusion gene to study how the inhibition of various HDACs alter the acetylation pattern on core histones. As first experiment, we tested Trichostatin A (TSA), a potent and generic inhibitor of HDACs. In parallel, we are performing knock-down of different HDACs classes by RNA-interference (RNAi) and combining it with SILAC strategy, to profile Histone PTMs changes upon ablation of single enzymes.

Methods: In a SILAC set-up, stable isotope labeled amino acids are added to cell culture media and are therefore incorporated during proteins synthesis. ''Heavy'' and ''light'' media are prepared by adding $^{13}C_6{}^{15}N_4$ L-arginine (R10) and $^{13}C_6{}^{15}N_2$ L-lysine (K8) (Sigma Isotec) or the corresponding non-labeled amino acids (R0, K0), respectively. When light (L) and heavy (H) cell populations are mixed, proteins can be quantified with Mass Spectrometry (MS) analysis by using the relative MS signal intensities.

In the present study we combined SILAC metabolic labeling with RNA-interference of HDAC-1 in human acute promyelocytic Leukemia NB4 cell lines. In order to acquire a reference proteome, NB4 cells cultures were grown in light and heavy medium, in parallel. After seven replication cycles, cells were harvested, mixed 1:1, and total extracts were prepared. Proteins were separated on gradient SDS-PAGE and in-gel digested with trypsin. The peptides mixtures were analyzed by nano-flow liquid chromatography with an Agilent 1100 LC system coupled to LTQ-FT Ultra mass spectrometer (Thermo Electron). Peptides were separated on a C18-reversed phase column with a 140 min gradient of acetonitrile. The raw data files were analyzed with MaxQuant software, in combination with Mascot search engine. For the HDAC inhibitor experimental set-up the light cells were treated with different TSA concentration and mixed 1:1 with the untreated one (heavy). In this case heavy media were prepared by adding $^{13}C_6{}^{15}N_4$ L-arginine only. The nuclear extracts were prepared and core histones extracted in 0.4 N ice-cold HCl. Core histone were in solution digested with Arg-C, to generate an optimal length of heavy (R10) and light (R0) peptides for MS-quantitative analysis.

Results: In the NB4 reference proteome we quantified 2449 proteins, with a minimum of two peptides, at least one unique and an estimated false discovery rate of 1%. We also calculated the distribution of the log2 H/L ratios of all proteins identified: the histogram obtained fitted with a normal distribution with standard deviation of 0.130. These results prove the homogeneity of incorporation of the labeled amino acids and indicate a very accurate quantification for all classes of proteins. Moreover, the comparative SILAC-based analysis between the cells with and without TSA allowed measurement of acetylation changes upon drug treatment on several residues on both histone H4 and H3. Hyper-acetylation induced by TSA was calculated by comparing the relative intensity of the same



peptides in heavy (untreated) and light (treated) form. The Fig.1 shows the acetylation patterns profiles measured for the peptide GKGGKGLGKGGAKR (4-17) of Human H4.

Conclusions: We report here a robust and comprehensive SILAC- proteome acquired in a human NB4 cells, model for APL. The high confidence proteome of 2449 proteins can be used as a reference dataset for studies on leukaemia. We also reported a simple and accurate strategy to quantify acetylation changes after treatment with HDAC inhibitors. Furthermore, we plan to perform global protein expression profiles in the same conditions, in order to analyze the downstream consequences of altering chromatin acetylation patterns, at the level of gene expression.

064

CHARACTERZATION OF THE INTERACTOME NETWORK INVOLVING THE MULTIFUNCTIONAL PROTEIN APE1/REF-1 BY FUNCTIONAL PROTEOMICS APPROACHES ALLOWS DISCOVERY OF A NEW UNSUSPECTED ROLE IN RNA METABOLISM

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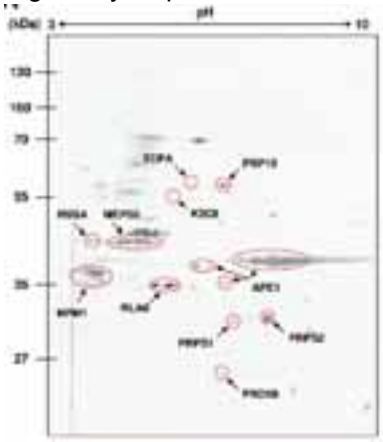
Background. APE1/Ref-1 (APE1) is a multifunctional protein, which is required for early embryonic development in mammals and exerts both DNA repair and transcriptional regulatory activities. As a DNA repair protein, APE1 is able to initiate repair of apurinic/apyrimidinic (AP) sites in DNA by catalyzing hydrolytic incision of the phosphodiester bond just upstream from the damage. Despite several functions were described for APE1 protein, molecular mechanisms underlying its behaviour are not fully understood yet. The 33-N-terminal sequence of APE1 is only present in mammals and is unstructured in solution, thus it is a good candidate for being considered a gain-of-function instrument during evolution and responsible for regulating the APE1 interactome network and its different functions, as well.
Methods. For the identification of molecular interacting partners of APE1, we used a HeLa cell model in which the expression of the endogenous APE1 protein was inducibly knocked-down by using a RNAi technology and replaced with the expression of an ectopic FLAG-tagged siRNA-resistant cDNA expressing either the full-length or the N-delta33 truncated APE1 protein devoid of the first 33 aminoacids. Total cell lysates underwent co-immunoprecipitation using a commercial anti-FLAG resin, followed by either RNA extraction, reverse-transcription and Real-Time PCR Amplification or 2-DE separation followed by spots identification by MALDI mass spectrometry. Levels of intracellular oxidized RNA were assessed by performing RNA extraction followed by Northwestern analysis and using a specific anti 8-OHG monoclonal antibody. Apoptosis was assessed by Annexin V staining; moreover cell growth ability was also measured by performing colony survival assays. Colocalization of the proteins was evaluated by immunofluorescence confocal analysis. To analyze whether NPM1 is able to exert an effect on endonuclease activity of APE1, AP Endonuclease activity assays on abasic DNA and RNA were performed with a recombinant purified APE1 protein in the presence of increasing amounts of recombinant purified NPM1 in either the wild-type or deletion mutant forms.
Results. We characterized an APE1 interactome map involving 10 different proteins, most of which interact with APE1 through its 33-N terminal sequence. Among these, there are several rRNA binding proteins suggesting a potential role of APE1 in ribosomal RNA metabolism. We further demonstrated that APE1 can bind to 18S and 28S rRNA in vivo and has an endonuclease activity over RNA molecules containing an abasic site. The in vivo biological relevance of these observations is demonstrated by the impaired cleansing ability to remove oxidized RNA shown by APE1 knocked-down cells, which parallels their increased apoptotic rate. We also investigated the effect of APE1-NPM1 interaction on APE1 function. By performing AP endonuclease Activity Assays, we demonstrated that NPM1 stimulates APE1 endonuclease activity on abasic dsDNA but decreases APE1 endonuclease activity on abasic ssRNA, thus representing a new regulatory loop.



Protein Identity	Swiss Prot Entry	Gene Name	MW (kDa)	pI	n° spots	n° peptides matched/sequenced	Interaction with N-del. ta33 APE1
T-complex protein 1 subunit alpha	P17987	CCT1	57	5.4	1	10/13	no
Pre-mRNA-processing factor 19	Q9UM54	PRPF19	53	6.0	1	6/11	no
Keratin type II cytoskeletal 8	P05787	KRT8	50	5.1	1	9/11	no
40S ribosomal protein SA	P08865	RPSA	38	4.5	1	7/13	reduced
Methylosome protein 50	Q9BQA1	WDR77	38	4.8	1	6/11	yes
Nucleophosmin	P06748	NPM1	35,34,34	4.6 4.5 4.7	3	9/13 8/8 7/7	no
60S acidic ribosomal protein P0	P05388	RPLP0	34,34,34	5.0 5.1 5.2	3	7/7 9/10 10/14	no
Ribose-phosphate pyrophosphokinase 1	P60891	PRPS1	31	7.0	1	8/8	yes
Ribose-phosphate pyrophosphokinase 2	P60891	PRPS2	31	6.4	1	6/7	yes
Peroxiredoxin 6	P30041	PRDX6	25	6.0	1	7/8	reduced

Conclusions. Our data demonstrate an unsuspected role of APE1 in the rRNA quality control process that may open new perspectives in the comprehension of the nuclear and extranuclear functions of this protein, with a significant impact on human pathological processes such as neurodegenerative diseases and cancer.

065

IMPACT OF CHANGEABLE PROTEIN ENVIRONMENT ON DISEASE-RELATED MUTATIONS

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Background: Many diseases are caused by disease-related mutations (DRMs) in the sequence of specific genes, altering several aspects of the gene/gene products cellular roles. The detailed knowledge of DRM-caused cellular mechanism disruptions is instrumental to better devise therapies, but elusive. An often neglected aspect is that a DRM is not only altering one single immutable product, but instead a spectrum of variants, subject to dynamic regulation that might temporarily or permanently impacting features and functions, both at cellular/tissue (through alternative splicing, post-translational modifications, transient interaction with binding partners, etc.) and individual level (through polymorphisms). We aimed at a systematic and detailed view of the protein and protein context in which DRMs are hosted. This often-neglected aspect can offer novel perspectives on the molecular mechanisms through which the DRM alters cellular processes, and can lead to new insights on potential therapies.
Methods: We performed a systematic analysis of how mutations can act on and be affected by their environment, taking into account the changeable and dynamic nature of the affected gene products. In particular, we analyzed, both at the primary and at the tertiary structure levels, disease-related mutations, DRM-hosting proteins and DRM protein environments in terms of polymorphisms, other DRMs, functional sites and alternative splicing events. We applied bioinformatics and statistical methods for the analysis of the sequence, structural and functional context in which a DRM is found. Variation data is obtained through public repositories: NCBI's OMIM provides disease-associated human variants; Cancer-related mutations are retrieved from Swiss-Prot annotation; SNP data are collected from public repositories such as dbSNP, HGVbase, HGMD, HapMap. Furthermore, several public resources provide information on functional site occurrences in the protein sequence, such as PROSITE, ELM, and Phospho.ELM. All the putative alternative splicing isoforms corresponding to a protein (a gene) query of the Ensembl database are collected using the Perl Application Programme Interfaces (APIs). Protein 3D models are built by comparative modeling. Threading methods are not used systematically, but only for cases of special interest and when more reliable methods fail. The in-house MAISTAS server is used to i) collect all putative isoforms of the input gene, ii) build, whenever possible, three-dimensional models for all of them using state of the art methodologies and iii) evaluates their structural feasibility.
Results: We identified several examples of significant context-dependent features of DRMs. Such relationships, such as between DRMs and protein regions corresponding to alternatively spliced exons, or with functional site environments, provide clues about the necessary context in which an occurring mutation might be deleterious. We included in the analysis the functions the hosting protein carries, and how the mutation can interact with them; which isoforms are mostly affected by the mutation; how an individual variation plays a role in disease onset/progression, and therapies. Furthermore, we are setting up a platform for automatically annotating genetic disease-associated mutations, transferring it to the human protein sequences and structures and inferring the context in which the mutation lies, assisting the user in the interpretation of how specific genetic profiles translate into observed phenotypes. Our results are being organized in a coordinated platform consisting of a database for efficient data storage and retrieval and of computational tools to integrate, display and annotate data. This platform is tailor-made for being of easy usage for clinicians and biologists to interpret DRMs in a well-annotated context and is aimed both at automatically retrieving DRM information and analyzing user-submitted data, by transferring it to the human protein sequences and structures and inferring the context in which a DRM lies. We expect that this analysis and the related platform will provide novel insights into the mechanisms leading to the disease, and to novel ways to tackle it.
Conclusions: We expect our analysis to represent a new perspective on the effects of gene products changeability on DRM, by the dissemination of not only the results of our research but also a new paradigm in which DRMs are analysed and annotated. Our analysis represents the first bioinformatics high-throughput approach for the contextualization of variations causing diseases, focused on both the sequence and the 3D structure of proteins and their features, and on how the mutations can interact with - alter and be altered - by them.

066

SHOTGUN PROTEOMICS ANALYSIS OF INCREASED TACE ACTIVITY IN MICE FED A HIGH FAT DIET

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Background. TACE (TNFα converting enzyme) critically regulates the inflammatory processes as it releases from the cell surface several transmembrane proteins, including TNFα and its receptors TNFR1 and TNFR2. TACE activation is consequent to both concomitant actions of several intracellular signals as well as reduction of its major inhibitor Timp3. TNFα is believed to play a role in the progression of Non Alcoholic Fatty Liver Disease (NAFLD) due to its ability to increase inflammatory signals via NF-κB activation. Epidemiological studies suggest among the metabolic complications of obesity NAFLD is sometimes evolving to steatohepatitis, cirrhosis or hepatocellular carcinoma. We conducted a label free quantitative shotgun proteomic approach, based on nano ultra performance liquid chromatography (nUPLC) coupled to MS^E, to find out the cellular and physiological effects of liver specific-TACE activation under metabolic stress and its role in the onset of hepatic steatosis.

Methods. *Timp3*$^{-/-}$ mice on a C57/BL6 background and their WT littermates were fed a High Fat Diet (HFD, 60% of calories from fat) for 20 weeks after weaning. TACE activity was determined using the SensoLyte 520 TACE Activity Assay Kit. Protein samples from liver homogenates were in-solution digested with trypsin and peptides were analyzed by nUPLC-ESI-MS^E. Prior loading, Enolase *Saccharomyces cerevisiae* digestion was added to samples as internal standard in order to perform quantitative analysis. Peptides were trapped on a 5 µm Symmetry C18 column 180 µm X 20 mm and analyzed using a 180 min RP gradient at 250nL/min (3 to 40% acetonitrile over 120 min) and an 1.7 µm BEH 130 C18 NanoEase™ 75 µm X 25 cm LC column on a nanoACQUITY UPLC™ System coupled to a Q-Tof Premier™ mass spectrometer (Waters). Data was acquired in parallel parent and fragment ion analysis MS^E: step between low (4 eV) and high (15-40 eV) collision energies on the gas cell, using a scan time of 1.5 per function over 50-1990 m/z. Processing of continuum LC-MS data, protein identifications and analysis of quantitative change in protein abundance were performed using ProteinLynx GlobalServer v2.3 (PLGS, Waters). Proteomic data were analyzed through the use of Ingenuity Pathways Analysis (IPA, Ingenuity Systems®, www.ingenuity.com), a web-based application that enables mining, visualization and exploration of relevant biological functional associations significant to the experimental results. Adenoviruses expressing GFP only or GFP and TACE were used to infect SV40-tranformed hepatocytes. 5-µm-thick paraffin sections of formalin-fixed liver tissue were stained with hematoxylin and eosin and Masson's trichrome for histological analysis in order to evaluated the severity of NAFLD.

Results. A HFD prolonged for 20 weeks is associated to increased TACE activity in *Timp3*$^{-/-}$ mice. Liver lysates from WT and *Timp3*$^{-/-}$ mice fed a HFD were analyzed by shotgun proteomics. This analysis led us to identify 38 proteins showed significant alteration in levels between WT and *Timp3*$^{-/-}$ mice. An unbiased systems biology approach through IPA software identified that Timp3 knockout carried significantly different signals involving liver fibrosis, steatosis, cholestasis and hyperbilirubinemia. Moreover, this bioinformatic approach identifies several proteins associated to hepatic system disease, amino acid and lipid metabolism and especially Adenosine Kinase (ADK), S-adenosylmethionine synthetase isoform type-1 (MAT1A), Glycine N-methyltransferase (GNMT) and Fatty acid-binding protein, liver (FABP1). Liver lysates from WT and *Timp3*$^{-/-}$ mice were immunoblotted confirming that ADK, MAT1A, GNMT were significantly decreased while FABP1 was significantly increased in liver of *Timp3*$^{-/-}$ mice compared with WT littermates. To directly link these proteins to TACE activation and lipotoxicity, we adenovirally over expressed TACE in hepatocytes in the presence or absence of increasing concentrations of palmitic acid. Immunoblot analysis confirmed that ADK, MAT1A, GNMT and FABP1 were modulated *in vitro* in a manner similar to that found *in vivo*. Analysis of liver histology revealed that after 20 weeks of HFD *Timp3*$^{-/-}$ mice manifest macrovesicular steatosis with features of ballooning degeneration as in grade 2 human steatohepatitis, compared to microvesicular steatosis in WT mice.

Conclusions. We used a shotgun proteomic approach linked to bioinformatic analysis to identify proteins differentially expressed in *Timp3*$^{-/-}$ mice fed a HFD and to unravel the physiological effects of TACE activation after a prolonged metabolic stress. The use of chromatographic columns with smaller particle size as well as LC pumps with higher pressure limits and nano-flow deliver capacity allowed improved chromatography performance (high reproducibility). Moreover, the high-speed MS-MS/MS switching capabilities and the parallel parent and fragment ion analysis MS^E permitted the collection of precursor ions

and fragmentation data in the same chromatographic run, due to the sequential low and high collision energy data acquisition. The analysis of this kind of data by a specific software (PLGS) revealed quantitative assessment of changes between samples with higher precision, without using stable isotope techniques. A bioinformatic analysis of biological processes most significantly perturbed by the analysed dataset, suggests that sustained TACE activation contributes to a steatosis phenotype in the presence of lipid overload. This hypothesis was confirmed by liver histological analysis and by literature findings of a protective role of ADK, MAT1A and GMT against hepatic steatosis and an opposite FABP1 role. Lastly, our data support the concept that TACE is a novel regulator of hepatic metabolism that is activated in the course of metabolic toxicity induced by western diet, contributing to the development of NAFLD through multiple mechanisms.
This work has been supported by the “Rete Nazionale di Proteomica”, Progetto FIRB RBRN07BMCT

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A COMBINATION OF CHROMATIN IMMUNOPRECIPITATION AND MASS SPECTROMETRY ANALYSIS TO DISSECT THE HISTONE CODE AND THE ASSOCIATED PROTEOME, AT SPECIFIC GENOMIC LOCI

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Background: *N*-terminal "tails" of Histones, the proteinaceous core of nucleosomes, are subjected to different post-translation modifications (PTMs), that significantly extend the information potential of the genetic code. The histone code hypothesis suggests that the cell uses a combinatorial system of modifications on the tails to "tag" precise chromatin regions for specific functional states. PTMs on histones are thought to function as "docking sites" for regulatory proteins that "translate" these modifications into physiological response on chromatin. In the past years many studies demonstrated that Mass Spectrometry (MS) is a very successful tool to detect histone PTMs and to reveal interplays between them. However, a limitation of these studies rely on the fact that analysis is carried out on bulk preparations of histones, which reduce the characterization of PTMs patterns on specific loci. An independent, widespread approach, the Chromatin Immunoprecipitation technique (ChIP), allows mapping modifications at the resolution of a few nucleosomes within the genome. However, ChIP is based on the use of antibodies against single PTM, so it can not dissect the combinatorial aspect of the code.
We describe the establishment and optimization of a ChIP~MS approach, an analytical strategy that combines the two approaches, profiting from their strengths and overcoming their limitations. ChIP~MS will allow dissecting the code of histone PTMs and the proteome associated to specific genomic loci. To establish this strategy, we chose human HeLa S3 as model system, due to the possibility to easily scale up the cell growth and therefore to start form large amount of material. Furthermore, we focused on chromatin regions enriched for tri-methylation at lysine 4 on histone H3 (H3K4me3). The choice of this PTM is strategic, because it is considered as a hallmark of promoters regions of actively transcribed human genes.
Methods: Technical approach: Native ChIP comprises three basic steps: 1) DNA isolation and digestion in small oligonucleosomal stretches (mono- to tetra-nucleosomes), 2) co-immunoprecipitation of chromatin with an antibody specific to the modification of interest, 3) separation and recovery of the immunoprecipitate DNA and proteins.
Both DNA and proteins are subsequently subjected to specific analysis to evaluate the enrichment of loci of interest. The DNA fragment enriched by ChIP is isolated and analyzed by real time PCR (qPCR) to quantify DNA levels of loci localized at the transcription start site (TSS). For the investigation of the enriched proteins (core histones and other chromatin interactors), a specific MS-based analysis is described in the following paragraph.
Experimental set-up: Human HeLa S3 cells were processed as following: i) isolation of nuclei; ii) micrococcal nuclease digestion of chromatin to obtain oligonucleosomes; iii) pre-clearing and immunoprecipitation of nucleosomes with anti-H3K4me3 monoclonal antibody and with anti-HA as negative control; iv) SDS PAGE (Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis) separation of enriched proteins; v) "in gel" trypsin digestion of band corresponding to histones after chemical modification of lysine by D6-acetic anhydride; vi) mass spectrometry analysis by Matrix Assisted Laser Desorption Ionization/Time-of-Flight (MALDI-TOF) and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS).
Results: qPCR on the immunoprecipitated DNA indicated an enrichment of H3K4me3 at TSS in the 1.4-29 fold range for 5 highly transcribed genes, suggesting that the protocol is working successfully.
The analysis at the proteins level of the material immunoprecipitated with anti-HeK4me3 reveals a slight but significant enrichment of to the histone octamer, when compared to the mock control. We next performed MS analysis on the histone H3, to verify the enrichment of tri-methylated H3K4 and to thoroughly analyse the other PTMs associated, both on H3 and on other core histones. Histone are rich in Lysines and Arginines therefore Trypsin is a sub-optimal protease. We followed an alternative strategy based on the derivatization of Lysines by a D6-acetic anhydride, as previously described. The preliminary results from LC-MS/MS analysis indicated an about 10 fold enrichment of the peptide (3-8) from H3 containing tri-methylated K4, in the immunoprecipitate H3, when compared the amount of tri-methylation in the input. This is extremely encouraging because a reasonable start material permits to obtain an effective enrichment. However, the initial results reveal also the remaining of significant background, asking for protocol optimization with the implementation of wash conditions and/or "pre-clearing" steps. Furthermore, we plan a scale up to obtain higher amounts of material for MS, which will give stronger signal and higher confidence in peptide identification and quantification.

Conclusions: The protein output of a chromatin immunoprecipitation is a source of largely unexploited information and MS analysis of this material is the elective tool to fill this gap in knowledge. Our preliminary results confirm the feasibility of the ChIP~MS strategy. Following steps will involve: the analysis of cross linked material and the combination with Stable Isotope Labelling by Amino acids in Cell culture (SILAC) for the quantitative evaluation of proteins and PTMs that synergize with H3K4me3.

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MOLECULAR RECOGNITION OF HUMAN PROTEINS BY *STAPHYLOCOCCAL* ADHESINS: A COMPUTATIONAL BIOLOGY APPROACH

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Background: Bacterial pathogenicity often relies on the efficient binding of proteins/receptors of the host organism. Unveiling the molecular/structural basis of the mechanisms that bacterial proteins use to recognize and bind host proteins is essential for the development of new molecules of therapeutic interest. Extensive crystallographic investigations have suggested that staphylococcal adhesins adopt an interesting mechanism to attach human proteins. In particular, the analysis of the crystal structure of the complex between the *Staphylococcus epidermidis* SrdG adhesin and a synthetic model peptide of fibrinogen has led to the hypothesis that recognition and binding occurs by a complicated process denoted as "Dock, Lock, and Latch" (DDL). The same mechanism has been proposed to operate for the binding of *Staphylococcus aureus* adhesin CNA to human collagen. In order to obtain further information on the mechanism of staphylococcal adhesins binding to human proteins, we have undertaken molecular dynamics (MD) simulations of the proteins CNA and SrdG. These investigations have been conducted on adhesion-ligand complexes as well as on intermediate states along the recognition process. **Methods:** The starting coordinates of SdrG and CNA complexes were derived from the Protein Data Bank. Simulations were conducted on the full-length proteins as well as on individual domains. The simulations were run with periodic boundary conditions by using the GROMACS software package 3.3. In all simulations the model was immersed in a rectangular box filled with water molecules. The OPLS force field and the Tip4 water model were used in the simulations. Systems were simulated in NPT ensemble by keeping constant temperature (300 K) and pressure (1 atm). Bond lengths were constrained by the LINCS procedure. Lennard-Jones interactions were calculated with a 12Å twin-range cutoff. Electrostatic interactions were treated using the Particle Mesh Ewald (PME) method with a cutoff of 10.0 Å. Results: Simulations performed on the complexes of SdrG with fibrinogen and on CNA with collagen provide clear indications on the nature of the interactions that favour the formation of these assemblies. In line with previous experimental reports, the SdrG-fibrinogen interaction essentially relies on an extended network of H-bonds. On the other hand, our analysis reveals the presence of firmly bound water molecules at the interface of CNA-collagen complex. The analysis of the molecular properties of the individual domain of the protein indicates that they are stable in the simulation timescale. A remarkable stability is also exhibited by the domains that present an incomplete Ig-fold. **Conclusions:** The MD analysis here presented provides new insights into the recognition process of human proteins by *Staphylococcal* adhesins. In particular, the extensive calculations conducted on the domains of the proteins that exhibit an incomplete β-structure indicate that they are stable in the simulation timescale. This suggests that they preserve a conformational state which is fully compatible with the DDL mechanism. These findings are rather different from those observed in isolated pilin subunits despite the close structural analogies of these two protein classes (incomplete Ig-fold). These observations may be interpreted by taking into account the different biological processes in which pilin and adhesin proteins are involved. The analysis of the complex of CNA with a collagen-like model peptide demonstrated that water molecules play an important role in mediating the interactions between the adhesin and the triple helix. Since the extensive hydration is a universal property of polypeptides/proteins assembled in triple helix, these findings may be directly extended to the interactions of collagen with other biological partners.

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SURFACE-ACTIVATED CHEMICAL IONIZATION AND CATION EXCHANGE CHROMATOGRAPHY FOR THE ANALYSIS OF ENTEROTOXIN A

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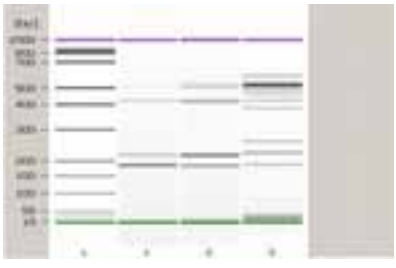
Background: Surface-activated chemical ionization (SACI) has been widely used in recent years for the analysis of different compounds (e.g., peptides, street drugs, amino acids). The main benefits of this technology are its high sensitivity and its effectiveness under different chromatographic conditions (i.e., ion exchange chromatography and reversed-phase chromatography). Here we used SACI in conjunction with quadrupole time-of-flight mass spectrometry to analyze enterotoxin A (Figure 1), which is produced by Staphylococcus aureus, in milk matrix using both ion exchange chromatography (LC-CEC-SACI-MS/MS). SACI had increased sensitivity as compared with electrospray ionization. Moreover, the higher quantitation efficiency of this technique, mainly in terms of limit of detection (0.01 ng/mL), limit of quantitation (0.05 ng/mL), linearity range (0.05-50 ng/mL), matrix effect, accuracy (intraday and interday accuracy errors were 9.2 and 10.3% respectively) and precision (intraday and interday precision errors were 5.3 and 12.8% respectively), is shown and discussed.
Methods: Milk samples containing enterotoxin A were treated with excess amounts of trypsin. Specifically, 900 mL of 50 mM ammonium acetate buffer (pH 8.0) containing 2 mg/mL modified trypsin was added to 100 mL milk and incubated at 57°C for 4 h. The undigested proteins were precipitated by adding 100 mL formic acid. The analyte solution was centrifuged, and 20 mL of the supernatant was analyzed.
Standard enterotoxin A standard solutions (1000 ng/mL) were prepared in milk matrix to develop a calibration curve. Enterotoxin A was digested with modified trypsin and protein were precipitated as previously described. This stock solution was diluted with milk matrix to obtain the following standard solutions: 0.005, 0.01, 0.05, 0.5, 2, 5, 10, 20 and 50 ng/mL. Each standard solution was analyzed in triplicate.
Results: 50 milk samples were spiked with different amounts of enterotoxin A and quantitated to verify the accuracy and precision of the developed approach. The intraday accuracy error was 9.2%, whereas the precision error was 5.3%. The data were then analyzed over the span of 3 days to verify the interday accuracy and precision errors. The same 50 samples were stored at 4°C and then analyzed each day to obtain the interday accuracy and precision errors. The interday precision error was 12.8%, whereas the accuracy error was 10.3%.
Finally, two milk samples containing unknown amounts of S. aureus were analyzed to verify the efficiency of LC-CEC-SACI-MS/MS (Table 1). Enterotoxin at a concentration of 0.08 ng/mL was detected in sample 2 by LC-CEC-SACI-MS/MS. In sample 1, the analyte concentration was lower than the LOQ (<0.05 ng/mL). The presence of enterotoxin A in the milk samples was also confirmed using an immunoenzymatic approach, which verified the MS/MS approach. The toxin level in sample 1 was not detected because of low amounts of enterotoxin A, whereas sample 2 was positive or staphylococcal enterotoxins. The two samples were identified by multiplex PCR as containing S. aureus 23S rRNA, and they were also positive for coa and nuc (Figure 2). The two samples also contained sea, although based on the immunoenzymatic approach, only sample 2 was positive for the presence of enterotoxin A. Sample 1 showed a lack of correlation between the presence of sea and its expression. The presence of a gene does not necessarily indicate detectable levels of its expression, and differences between the presence of a gene and its expression can be resolved by further molecular tests.



Sample number	Enterotoxin conc (ng/mL)	Immunoenzymatic results
1	0.01	-
2	0.08	+

Figure 1 Figure 2 Table 1

Conclusions: A reliable and sensitive approach for screening milk samples for S. aureus contamination based on LC-CEC-SACI-MS/MS was developed. The results clearly show that this LC-CEC-SACI-MS/MS approach is more sensitive than immunoenzymatic testing and can also generate quantitative information that is not provided by the commercially available immunoenzymatic kits. Future developments will be focused on using the same approach for the determination of other toxins in food samples if there is an available commercial standard.

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EFFECTS OF PHENOL ON *ACINETOBACTER RADIORESISTENS* S13 MEMBRANES: A PROTEOMIC APPROACH.

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Acinetobacter radioresistens S13 is a Gram–negative bacterium selected for bioremediation purposes due to its ability in aromatic degradation. It is able to grow on phenol (more than 400mg/L) as the sole carbon source, displaying a rapid growth rate . It is also able to metabolize benzoate with a lesser efficiency, and , when both aromatics are supplied together, benzoate is the preferred substrate, in spite of the lower conversion rate to central metabolism compounds. On the hypothesis of a toxic effect exerted by phenol on *Acinetobacter radioresistens* S13 membranes, comparative proteome at both acidic and alkaline pIs, on phenol-grown cells versus acetate-grown, has been set up. The experimental results reveals differential expression of proteins involved in maintaining the integrity of the outer membrane (TolA, LolA) and in peptidoglycan and phosphatidylethanolamine synthesis (PSD). This demonstrates that phenol can disaggregate lipopolysacharides (LPS), lipoproteins and phospholipids, damaging the cell wall up to the murein component. As well as inducing membranes modification, phenol activates overexpression of other proteins functyionally belonging to 3 classes: 1) Proteins involved in emulsification (e.g. OmpA-like protein) 2) Proteins involved in pH homeostasis (e.g. Na^+/H^+ Antiporter) 3) Proteins involved in facilitating the transport of anions (especially phosphate) These results allow us to better understand the effects of phenol on the overall bacterial physiology.

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PROTEOMIC INVESTIGATION ON A POTENTIAL ZINC CONCENTRATING PROBIOTIC *STREPTOCOCCUS THERMOPHILUS*

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Background. Zinc is an essential trace element for humans. It is involved in several metabolic processes, immune functions, and mucosal tropism and it is often present in insufficient dietary quantities.
The use of a probiotic Streptococcus thermophilus strain, as vector to supply the metal to the human organism, could solve this problem.
The aim of this work is to investigate, with a proteomic approach, the protein synthesis pattern of the microorganism in order to understand whether zinc is released in the human intestine by means of specific transporter rather than by cell lysis.
Methods. In the present work the total cell proteome of Streptococcus thermophilus was analyzed by 2DE and mass spectrometry. Two conditions were compared: growth in M17 medium and in the same medium fortified with zinc sulphate. Biomass for proteome analyses was harvested at the end of the exponential phase. 4-7 pI maps and 6-11 pI maps have been performed. The differentially expressed spots have been submitted to a MALDI TOF-TOF analysis.
Results. For the acid maps the gel image analysis highlighted 12 soluble proteins differentially expressed in the two considered conditions: 11 were upregulated and 1 was downregulated in presence of zinc.
However 4-7 pI maps showed a great quantity of spots not clearly resolved in the acidic region; so a new protocol to obtain narrow range pI (3-5.6) maps is going to be set up. For 6-11 pI maps gel image analysis showed that 2 proteins were downregulated in presence of zinc.
Five of twelve proteins in the acidic range, and one of two in the alkaline range, have been correctly identified.
Conclusions. Preliminary results reveal a differential expression of proteins involved in purine metabolism, in replication events and in peptides catabolism.
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OLIGOMERIC CHARACTERIZATION OF THE PHOTOSYNTHETIC APPARATUS OF RHODOBACTER SPHAEROIDES R 26.1 BY NON DENATURATING ELECTROPHORESIS METHODS

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Background: Blue and Clear Native gels are powerful instruments for understanding protein networks in biological membranes. This is particularly challenging since membrane proteins tend to self-associate into non-covalent multimers due to their hydrophobic nature. In this work R. sphaeroides R26.1 has been chosen as a model to investigate the supramolecular organization of its photosynthetic apparatus by these two techniques and compare the results with what reported in the literature performed by atomic force microscopy.
Methods: Rhodobacter Sphaeroides R 26.1 was grown in Sistrom's minimal medium A containing succinate. Photosynthetic cells were harvested and ICM vesicles were extracted and subjected to 2DE. The first native dimension (1D BN-PAGE or 1D CN-PAGE) was performed at 4°C using a Protean II xi Bio-Rad electrophoresis system (180 × 160 mm, 0.75 mm thick). For both BN and CN PAGE the same amount of protein (100 µg) was loaded onto a 0.75 mm thick 4% to 13% w/v acrylamide gradient gel. Second-dimensional SDS-PAGE was done in a large format (180 x 160 mm, 1 mm thick) gel camera (Protean II xi, BioRad). The lanes were then layered onto 14% w/v acrylamide and 6 M urea SDS gel. 2DE spots were excised and digested with trypsin, peptide mixtures were separated using a nanoflow-HPLC system. Peptides were eluted directly into a High Capacity ion Trap (model HCTplus, Bruker-Daltonik, Germany). The scan range used was from 300 to 1800 m/z. Protein identification was performed by searching in the National Center for Biotechnology Information nonredundant database (NCBInr) using the Mascot program.
Results: our findings, besides confirming most of previous data on the organization of photosynthetic apparatus obtained by microscopic investigations, revealed the presence of newly discovered gene products localized to the intracytoplasmatic membrane (ICM) vesicles. This study, besides providing for the first time an accurate biochemical characterization of Rb. sphaeroides ATP-synthase in terms of subunit stoichiometry, supports at the same time the hypothesis that proteomic approaches may bridge gaps existing in fully understanding the functional and structural assembly of biological systems, such as in this case the photosynthetic apparatus of Rhodobacter.
Conclusion: the usage of simple and reproducible techniques, such as BN and CN gels, allowed us to build the photosynthetic membrane assembly of R. Sphaeroides R26.1, chosen as a model. Through this innovative proteomic approach, our investigation also revealed the existence in these complexes of new gene products allowing to know their location and hypothesize their functional relationship to the process of solar energy utilization. It is worth while to point out that these results have been reached by using low amount of sample, with respect to other alternative techniques and by simple instrumentation.

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A STRUCTURAL PROTEOMICS APPROACH TO STUDY THE EFFECT OF NITRIC OXIDE ON BIOFILM FORMATION IN THE HUMAN PATHOGEN *PSEUDOMONAS AERUGINOSA.*

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Background: Among human pathogens, the Gram-negative *Pseudomonas aeruginosa* is popular for its metabolic versatility and its ability to cause nosocomial and communities infections. In particular, the *P. aeruginosa* chronic lung infection is the major cause of death in patients of cystic fibrosis (CF), a genetic disease affecting 1/2500 newborns in Europe. In CF patients, *P. aeruginosa* form a drug-resistant biofilm that leads to lung destruction. Nitrate and nitrite in CF mucus support anaerobic growth and metabolism of *P. aeruginosa.*
Exposure to non-toxic concentrations of NO causes biofilm dispersal and restores *P.aeruginosa* vulnerability to conventional antimicrobials. NO induces global changes in gene expression, enhancing metabolic activity and motility and decreasing adhesion and virulence. The regulatory pathway implicates c-di-GMP, a bacterial second messenger involved in the formation of biofilm, whose intracellular levels are controlled by the combined effect of specific diguanilate cyclases (DGC) and phosphodiesterases (PDE) enzymes. The molecular details on the role of c-di-GMP in the modulation of biofilm are still lacking, but are essential to design effective antimicrobial drugs. A major problem is that the number of genes putatively involved in the control of c-diGMP levels is highly variable in different bacterial species and may be as high as 61 (such as in Vibrio cholerae). This large number of genes suggest that the c-diGMP levels might be temporally and spatially regulated in the bacterial cell to achieve the desired effect, ie biofilm formation, in response to different environmental stimuli. As pointed out by several groups in the field, a complete understanding of the role of c-diGMP will therefore require a system biology approach.
The aim of this project is to identify and study the molecular targets of nitric oxide (NO) involved in the regulation of biofilm formation in *Pseudomonas aeruginosa,* by using a structural proteomics approach. Particular attention will be paid to the proteins involved in c-diGMP turnover (DGCs and PDEs).
Methods: Medium-throughput methods for cloning, expression and crystallization of target proteins have been employed. A facility for independent growth of 24 liters of bacterial cultures at temperatures from 10 to 37°C is available to produce the target proteins. Protein crystallization screenings have been set up using an automated nanodrop protein crystallization robot.
Results: 39 genes with putative DGC and/or PDE domains have been identified in the *P.aeruginosa* genome that are possibly involved in the modulation of c-di-GMP levels. They have been selected on the basis of the presence of conserved residues in their active site, ie GGDEF or EAL for DGC and PDE, respectively. Many genes contain both domains (DGC and PDE) fused in a single sequence. Using a bioinformatic approach we have started the identification of possible targets of NO action among the *P. aeruginosa* genes involved in c-di-GMP metabolism. Four possible targets have been identified as genes which respond transcriptionally to NO: those are PA0575, PA2072, bldA and morA. These proteins contain PAS domains, a widespread signal sensor domain, which often function using an associated cofactor, such as heme or flavin. In *P. aeruginosa* there are 8 genes (beside those listed above) which contains PAS domains and precisely PA0290, PA0338, PA0847, PA0285, PA0861, PA1181, PA5017, PA5442. Genetic studies are available which suggest a link between NO and some of these c-di-GMP-related proteins, such as PA0290, morA, bldA and PA5442 genes. Overexpression in the *P. aeruginosa* PA14 strain of genes PA0338 and PA0847 have been shown to increase biofilm formation; genetic disruption of other genes (PA0861, PA1181, PA5017) impair biofilm formation to various degrees. Interestingly, 3 proteins of the GGDEF/EAL family also contain a GAF domain (PA2771, PA2567, PA5017), which may bind a cofactor (iron or heme) able to sense NO, as found in other known bacterial gas sensors. After this preliminary analysis, we have started the structural studies on two genes, ie PA0575 and PA5017. Both genes have a complex organization, which includes sensor domains (PAS or GAF) and both catalytic domains (DGC and PDE) together with one or more transmembrane helices. All the soluble fragments have been cloned; individual clones for the single domains have also been obtained. Expression studies and crystallization screenings are currently undergoing for these genes and domains.
Conclusions: We have undertaken a structural proteomics study on the molecular targets of NO involved in the regulation of biofilm formation in *Pseudomonas aeruginosa.* At least 15 proteins have been identified for structural characterization; 2 genes have been studied in more detail. Future goals include: (i) cloning, protein purification and crystallization of the other candidates, (ii) set-up of a reliable method for the rapid

identification of the enzymatic activity displayed by these proteins, in order to determine whether they function as DGC or PDE or both and (iii) in solution and structural studies of compounds previously identified as possible inhibitors by other groups.

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PROTEOMIC INVESTIGATION ON THE ENERGY PRODUCING PATHWAYS OF *LACTOBACILLUS* VS. *LACTOCOCCUS*.

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Introduction: Lactic acid bacteria (LABs) are microaerophylic Gram-positive microorganisms involved in several industrial processes like fermented food production. They lack the ability to biosynthesize heme, so they can't obtain metabolic energy by respiration without external heme supplementation. For these reasons they have developed alternative strategies to complementate glycolysis in energy production. Among these systems the best known are the PLP- or pyruvoyl- dependent decarboxylation of amino acids and the ADI (arginine deiminase) pathway. In this work we tried, by means of a proteomic approach, to understand whether these two metabolic pathways can go on in parallel, as a global response to high energy requirement, or if competitive interactions among them occur. To gain this goal we worked on two different LABs: *Lactobacillus hilgardii* ISE 5211 able to decarboxylate histidine into histamine and *Lactococcus lactis* NCDO 2118 able to produce γ-aminobutyric acid (GABA) from glutammic acid. These strains are reported in literature as able to perform ADI pathway. We analyzed both cytosolic and membrane districts for *L. lactis* and only cytosolic proteins for *L. hilgardii*. We set up comparative proteomic experiments based on two-dimensional electrophoresis followed by mass spectrometry identifications (MALDI and ESI).
Materials and Methods: We considered, as stimulated conditions, cultures performed in a medium fortified respectively with 4 g/L of histidine and 5 g/L of glutamate. Equivalent amounts of cells were treated in each protein preparation. Cells were harvested by centrifugation and were resuspended in 3 ml 50 mM Tris–HCl pH 7.3 plus EDTA 1 mM containing 10 µL/mL of Nuclease mix, sonicated and clarified. In order to obtain soluble proteins the samples were centrifuged (100000xg for 1h at 4°C) and the supernatant was dialized and precipitated with methanol/chloroform. To obtain a fraction enriched in membrane proteins the samples were centrifuged (100000xg, 8h, 4°C), were solubilized in 20mM Tris, pH 6.8, 1 mM $MgCl_2$, centrifuged again (45000xg, 5h, 4°C) and resuspended in 200 µL of 50 mM ammonium bicarbonate. The sample was supplemented with 1mL of 2:1 v/v trifluoroethanol/chloroform and incubated at 0°C for 1 h vortexing it for 10 s every 5 min. Samples were centrifugated (10000xg, 5min, 4°C) and the upper phase was recovered and dried using a vacuum centrifuge. Protein concentration was evaluated by 2D-Quant kit. Soluble protein were solubilized in 7.0 M urea, 2 M thiourea, 4% CHAPS, 1% triton X-100, 0.02 M Tris, 0.5% IPG buffer pH 4–7, 1% DTT, while membrane enriched fraction was solubilized in 6.5 M urea, 2.2 M thiourea, 1% p/v aminosulfobetaine-14, 5 mM Tris-HCl pH 8.8, 0.5 IPG buffer, 100 mM DTT. IEF was performed using a 13 cm IPG strip, the strips were sealed at the top of the 1.0 mm vertical second dimensional gels with 0.5% agarose in 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3. Gels were automatically stained and were digitized with Personal Densitometer SI. Image analysis and detection were performed with Progenesis PG-200 v. 2006 software.
Results: In the present results we observed an up-regulation of histidine decarboxylase, in *L. hilgardii*,; on the contrary glutamate decarboxylase was not expressed in a significantly different ratio among the two conditions in *L. lactis* either in cytosolic or in membrane extracts. We also observed a down-regulation of ADI pathway enzymes and glycolytic enzymes in the stimulated condition in both *L. hilgardii* and *L. lactis* strains.
Conclusion: We propose a biosynthetic control exerted by the precursor amino acids on histidine decarboxylase in *L. hilgardii*, while, for *L. lactis* glutamate decarboxylase, a catalytic control was suggested. The regulation of ADI pathway enzymes and glycolytic enzymes suggest a competition among these energy metabolic pathways in both strains.

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IMPROVEMENT OF MEMBRANE ALKALINE PROTEOME ANALYSIS IN BACTERIA BY TWO-DIMENSIONAL ELECTROPHORESIS

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Bacterial membranes have a very important role because they allow the discrimination among compounds present in the extracellular environment. The study of proteins present in this cell district is quite complex because of their hydrophobicity leading to difficulties in their extraction and maintainance in solution. As regards the proteomic field, for these reasons, the trend is to approach the problem of membrane proteins by means of gel free techniques.
Nevertheless, in this study, we tried to set up a procedure useful to obtain two-dimensional maps representing a protein fraction enriched in membrane proteins by *Acinetobacter radioresistens* S13, a Gram-negative bacterium. To gain this goal we tested two different methods for membrane protein extraction based on sodium carbonate and on long ultracentrifuge cycles followed by an organic solvent extraction, respectively. These extraction methods have been applied in combination with isoelectrofocusing based on anodic cup loading useful to overcome some problems connected with alkaline proteins isolectrofocusing.
Both the tested extraction protocols allowed us to obtain two-dimensional acid maps of a protein fraction enriched in membrane proteins. Nevertheless, the sodium carbonate method revealed to be not compatible with alkaline protein isoelectrofocusing, probably due to the presence of salts impossible to fully remove even with several dialysis cycles or with protein precipitation procedures. On the contrary by combining the second extraction method with anodic cup loading isoelectrofocusing we have been able to obtain two-dimensional alkaline maps of a membrane-enriched proteins fraction. The membrane nature of proteins present in the maps was confirmed by both comparison with cytosolic protein maps and mass spectrometry identification of randomly chosen spots. The efficiency of this procedure was tested also in *Enterococcus faecalis* DISAV1022, a Gram-positive rod leading to satisfactory results.
In conclusion the protocol we set up allowed us to obtain a membrane protein enriched fraction useful to perform two-dimensional electrophoresis maps with a good resolution by using both acid and alkaline pI gradient strips.

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PROTEOMIC ANALYSIS OF *B. CLAUSII* STRAINS

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Introduction: The spore-bearing alkaliphilic *Bacillus* species constitute a large, heterogeneous group of microorganisms which have relevant applications and commercial interest.
Bacillus probiotic species have been proved to contribute in preventing and treating various gastrointestinal disorders by improving the host's intestinal microbial balance They are resistant to clinically important antibiotics, including macrolides and aminoglycosides The clinical benefits are mainly attributed to the substances produced by probiotic strains and to their immunomodulatory effects. Currently, the best-documented probiotic bacteria used in human therapy are lactic acid bacteria. In contrast, studies aiming to characterize proteomic profile and identify the bioactive molecules responsible for the probiotic beneficial effects of bacillus, are rare. Proteomic analysis using two gel electrophoresis and mass spectrometry has been evolved as a powerful method that provides valuable information about the cellular protein expression. The focus of this paper are four probiotics *B. clausii* strains which display a low level of intraspecific genome diversity and exhibit a high degree of genomic conservation through time.
Aim of this study is: first, the characterization of the protein expression profile and metabolic responses to the environment of the four B. clausii strains L1, L2, L3 and L4. Second, the identification of proteins differentially expressed among the four strains or protein that can identify each strain, finally, the identification of secreted enzyme that can be useful in industry and domestic life and metabolic bottlenecks that can contribute to improve the process optimization of their production.
Methods: The shaking flasks containing 50 mL of the LB medium were inoculated with a glycerol stock of the four *B. clausii* strains and cultivated 150 rpm a 37°C. Cell and secretome proteins were extracted to the stationary phase as described previously by Wang., (2006). The protein concentration was determined using the Bio-Rad Protein Assay kit. For isoelectric focusing, 250 mg of proteins were loaded on 24 cm IPG strips with a linear gradient from pH 3-10. Focusing was carried out at 20°C by IPG-Phor Isoelectric Focusing System (GE Healthcare) to 70 kVh. The second dimension was an 12.5% SDS-polyacrylamide gel electrophoresis using the buffer system of Laemmli (1970) at a constant current of 15mA gel^{-1} and at 10°C. The gels were stained with Coomassie brilliant blue R-250 and analysed with IMAGEMASTER 2D PLATINUM V.5 software (GE Healthcare). The protein spots showing significant differences were excised from 2-D gels and were subjected to tryptic digestion using Trypsin Profile IGD Kit (Sigma) according to the manufacturer's instructions and analyzed with ESI-Q-Tof MS/MS mass spectrometry.
Results A proteome analytic method based on two-dimensional polyacrylamide gel electrophoresis in combination with mass spectrometric protein identification has been established in our laboratory and applied for the investigation of protein expression of four *B. clausii* strains.
We present a preliminary comparative proteomic analysis of the protein expression and the extracellular complement secretome of the four B. *clausii* strains. As the result, the four strains which belong to a unique genospecies, exhibited surprising considerable variations in both proteins expression pattern and the secretome during the stationary phases of the cultivation process. Spectrometric identification of protein differentially expressed is in progress.
Comparative proteomic analysis of four B. *clausii* strains is aimed at establishing the protein expression profile of each strains as well as the identification of metabolic pathways and cellular processes closely related to the production and secretion of specific bioactive molecules.

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REVIVAL OF *M. TUBERCULOSIS* FROM LATENCY: STRUCTURAL AND FUNCTIONAL STUDY OF THE KEY GROWTH FACTORS RipA AND RpfB

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Background: The interaction between M. tuberculosis and the human host after infection may manifest itself as a chronic disease or as a latent (or dormant) infection, a state capable to evade host responses. The probability of reactivation from dormancy is strongly affected by the type of host immune response and it is significantly enhanced in immuno-compromised patients, e.g. suffering from AIDS. Understanding and controlling the entry and exit from dormancy is important in the development of new anti-tubercular therapies. Surprisingly, it has been demonstrated that mechanisms which regulate bacterial exit from dormancy are the same which are responsible for cellular division. Indeed, proteins which have been identified as responsible for resuscitation, like Resuscitation Promoting Factors (RpfA-E) and the Resuscitation Promoting Factor Interacting Protein (RipA) play a key role in bacterial division, as they localise at bacterial septa and are likely responsible for septal peptidoglycan degradation. Among these proteins, RipA has remarkable effect on bacterial phenotype. Indeed, the deletion of the gene encoding for RipA induces decreasing growth and an abnormal M. tuberculosis phenotype, consisting in branching and chaining bacteria. This makes RipA an excellent candidate as a drug target against Tuberculosis.
Methods: Both RipA and RpfB were cloned into the pET vectors. Proteins were expressed in BL21(DE3) *E.coli* cells and purified under native condition by affinity cromatography. Molecular masses were checked by LC-MS analysis performed on an LCQ DECA XP Ion Trap mass spectrometer equipped with an OPTON ESI source and with a complete Surveyor HPLC system. Crystallization trials were performed at 293 K using the hanging-drop vapour-diffusion method. Structures have been solved by Multiwavelength Anomalous Dispersion using the anomalous signal from the Se atoms of selenomethionine-labeled enzymes. For functional characterization of the enzymes, lyophilized cells of *M. lysodeikticus* labeled with FITC, by covalently linking FITC to amine groups, were used as substrate. The FITC-labeling reaction was carried out at 20°C for 16 hours, protected from light. The reaction mixture was stopped and centrifuged to remove the surnatant. The labeled material was adequately separated from the excess of unreacted fluorochrome and resuspended in a suitable buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 2mM $MnCl_2$, 25mM KH_2PO_4, 0.01%(v/v) CHAPS). Recombinant proteins were incubated with the FITC-labeled cells at 30°C. The buffer alone was used to determine background release of FITC. After 2 days incubation, the insoluble substrate was centrifuged (16,000 x g) and soluble FITC was measured with filters for excitation 492 nm and emission 518 nm.
Results: RpfB and RipA were cloned, expressed and purified in native conditions. Crystals of both enzymes were obtained using vapour diffusion techniques. Best RpfB crystals were obtained using iso-propanol as a precipitant agent whereas RipA crystals grew from poly-ethylene-glycol solutions. Crystal structures of RipA and RpfB were solved using the Multiple Anomalous Dispersion (MAD) method. The crystal structure of RpfB allowed us to establish a model for cell wall interaction and cleavage and to identify the structural determinants important for cell wall anchoring. The structure of RipA is assembled in an $\alpha+\beta$ fold. Structural comparisons with DALI indicated that this enzyme belongs to the NlpC/P60 cysteine protease family. The enzymes' functionality and specificity was studied using both fluorescence and mass spectrometry studies.
Conclusions: A structural and functional characterization of two key enzymes for growth and resuscitation from latency of M. tuberculosis has been performed. Crystal structures allowed us to definitely identify protein functions by structural comparisons with known enzymes. Cell wall degradation experiments revealed that both enzymes are active as cell wall hydrolases. The highest activity was measured for RipA, which is predicted to act as a cysteine protease and, as such, to cleave cell-wall peptide crosslinks. In parallel, mass spectrometry studies of the processed cell wall material provided clues of the peptidase specificity. Altogether, these results provide the basis for the design of low molecular weight enzyme inhibitors, to be used in Tuberculosis treatment.

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PROTEIN EXPRESSION CHANGES INDUCED IN MURINE PERITONEAL MACROPHAGES BY STREPTOCOCCAL INFECTION.

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Background. Recognition of pathogens by macrophages, chased by interaction of proteins expressed on cell surface with pathogen-associated molecular patterns, activates multiple responses by switching on a complex interplay between host defence mechanisms and strategies of the microbial pathogens to evade them. After recognition, macrophage entraps pathogens in phagosomes that afterward fuse with lysosomes. In phagolysosomes, microorganisms are attacked by degradative enzymes and reactive oxygen and nitrogen radicals. Overproduction and release of reactive oxygen species is associated with a raise of glucose metabolism, providing macrophage with the energy required for activating ATP-dependent defences and with the NADPH needed to synthesize superoxide radicals in phagolysosomes. Although changes induced in macrophages by some bacterial and fungal pathogens have been investigated at proteomic level, protein changes occurring after infection with group B streptococcus (GBS) was never approached.
Methods. Protein expression changes induced in mouse peritoneal macrophages by infection with GBS are described. Thioglycolate-elicited macrophages were infected with either viable or heat-inactivated streptococci. After two hour incubation, proteins were solubilized and separated by two-dimensional electrophoresis. Changes in protein expression were evaluated by comparing stained gels with gels obtained by submitting non-infected macrophages to the same incubation and separation procedures. Digital gel images obtained from control macrophages and from macrophages infected with viable or inactivated streptococci were analyzed by using the PDQuest software (version 7.2, Bio-Rad, Hercules, CA, U.S.A.). After filtering gel images, spot detection and quantification were carried out in both automatic and manual modes. Well-resolved spots appearing in all gels were fixed as landmarks before comparing images in a match set. After matching and normalization, 2DE maps of individual groups were grouped together by using the “Replicate Group” function. “Replicate group consensus” tool was used to discard spots not appearing in at least six out of the nine gels from the same group. Non expression-related differences in spot intensity were corrected by using the PDQuest “total quantity in valid spots” normalization mode. Only spots whose staining intensity was significantly different in the three experimental groups and exhibiting a fold change minimum of 2 were considered for mass spectrometric identification. Statistical evaluation of PDQuest-normalized spot intensity values was performed by using the SPSS v 16.0 software. Normality of spots distribution was evaluated using Shapiro-Wilk test ($p<0,05$). As the results highlighted a non parametric distribution of spot intensities, the Kruskal-Wallis ($p<0.01$) test was used to assess overall statistical significance of differential expression among the three experimental groups. Mann-Whitney test with Bonferroni correction was applied for inter-groups comparisons ($p<0.05$). Differentially expressed proteins were identified by tandem mass spectrometric analysis on a ProteomeX apparatus (Thermo Electron Corporation, San Jose, CA, USA) equipped with two Hypersil-Keystone BioBasic C18 capillary columns (0.18 x 100 mm) and configured in the Protein ID mode. Experimental MS/MS data were matched with polypeptide sequences deposited in protein databases of both mouse and firmicutes.
Results. Following statistical and minimum expression threshold criteria described above, 75 polypeptides spots were found to be differentially expressed in GBS-infected macrophages, as compared with control cells. Mass spectrometric analysis of differentially expressed polypeptides allowed to identify 57 distinct murine proteins. Changes in the expression of proteins involved in both positive and negative modulation of phagocytic functions, stress response and cell death were induced in macrophages by pathogen infection. Relevant changes in the expression of metabolic enzymes were also observed. In particular, most of the glycolytic enzymes were down-regulated in macrophages infected with viable streptococci. In addition, streptococcal infection appears to disregulate adenine nucleotides interconversion in macrophages. Indeed, downregulation of synthase F1 complex subunit α was paralleled by overexpression of adenylate kinase 2 in GBS-infected cells, as compared to control macrophages. The increased expression of mitochondrial glutamate dehydrogenase, of citrate synthase and of dihydrolipoamide dehydrogenase suggests that entry of glutamate carbon chain in citric cycle is enhanced in macrophages infected with viable streptococci. Moreover, evidence was obtained that GBS infection induces a many-sided impairment of macrophage defences based on free radicals production. In fact, NADPH-producing dehydrogenases of pentose phosphate pathway and the subunit p40-phox of the NADPH dehydrogenase complex were underexpressed in GBS-infected cells. Finally, overexpression of arginase and underexpression of argininosuccinate synthase entails an impairment of NO synthesis in infected cells.

Conclusions. Once phagocytosed by macrophages, GBS activates a multifaceted strategy to get a complete control of host cell, by manipulating multiple metabolic pathways and more than one step in the same pathway. The inventory of proteins whose expression is altered in infected cells endows with a valuable basis for investigating on the mechanisms underlying the same changes.

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DIFFERENTIALLY EXPRESSED PROTEINS IMPROVED THE TOLERANCE TO ARSENIC IN *PTERIS VITTATA* COLONIZED BY ARBUSCULAR MYCORRHIZAL FUNGI.

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Background. *Pteris vittata* can tolerate soil As concentration up to 1500 ppm and rapidly accumulates the metalloid in its fronds. However, its tolerance to As has not been completely explored. Arbuscular mycorrhizal (AM) fungi colonize the root of most terrestrial plants, including ferns. Mycorrhizae are known to affect plant responses in many ways; indeed, it has been shown that AM fungi improve plant nutrition, modify root architecture, promote plant tolerance or resistance to pathogens, drought, salinity and heavy metal stresses. It has been observed that plants growing on arsenic polluted soils are usually mycorrhizal and that AM fungi enhance arsenic tolerance in a number of plant species, including *Holcus lanatus*, *Medicago truncatula*, *Helianthus annuus*, *Lens culinaris*, *Lolium perenne* and *Trifolium repens*, mostly increasing plant phosphorus status and restricting arsenic uptake. It has recently been suggested that, as AM fungi can inactivate the direct phosphorus uptake pathway, via root hairs and epidermis, they could decrease arsenate uptake into roots suppressing the plant high-affinity phosphate/arsenate transporters.
The aim of the present work was to study the effects of the AM fungi *Glomus mosseae* and *Gigaspora margarita* on *P. vittata* plants treated with As. The interest was focused on proteomic changes in the fronds.
Methods. For proteomic analysis, leaf proteins were extracted by the TCA-acetone method and separated by 2DE. After the classical image analysis (PDQuest) a further validation step was the statistical analysis with Staview and manual control of the differentially expressed spots ODs. Spots of interest were identified by MS/MS analysis on ESI-Q-TOF (Q-STAR). Cross-species identification was performed, since *P. vittata* genome is still unsequenced.
Results. Image analysis showed that As treatment, in absence of AM symbiosis, affected the expression of 88 spots. In particular arsenic induced the down-regulation of the enzymes involved in sugar metabolism, bioenergetics, photosynthesis and carbon fixation. The presence of AM fungi restored the control values or reverted the effect of arsenic stress. In details, AM colonization, in absence of As, induced the up-regulation of 25 spots and the down-regulation of 71 (26 spots disappeared in plants colonized by both fungi respect to the control). Instead, in presence of As, AM fungi induced the differential expression of 174 leaf protein spots, belonging to glycolysis, photorespiration, tricarboxylic acid cycle, electron transport and sulfur metabolism. It is interesting to note that both AM fungi improved tolerance to arsenic, confirmed by morphological parameters. Besides only in mycorrhizal ferns, arsenic induced the up-regulation of glyceraldehyde-3P-dehydrogenase and triose-P-isomerase, supporting their possible involvement in arsenate reduction.
Conclusions. The proteomic approach has been extremely useful in underlining the specificity of each AM fungi symbiosis in the arsenic metabolism of *P. vittata*. The fern fronds displayed different protein profiles, reflecting different molecular strategies adopted to tolerate/detoxify arsenic.

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A COMBINED GENOMIC-PROTEOMIC APPROACH FOR THE ISOLATION OF TRANSCRIPTION FACTORS FOR Lhcb1 GENES IN SPINACH

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Background: Multigene families represent an excellent system for the analysis of molecular mechanisms governing promoter responses to different developmental and environmental signals. Identification of similar, but functionally different, regulatory regions of multigene families is the first step for deciphering specific regulatory networks in which their single genes are involved. Under this view, the availability of both DNA regulatory motifs and cognate transcription factors can be useful to analyze, also on a quantitative basis, their specific interactions.
In order to study the regulatory system of a plant multigene family we started the identification of the spinach *Lhcb1* gene regulatory motifs and the isolation of the relative transcription factors. Lhcb1 (light harvesting complex 1) proteins in higher plants are usually encoded by multigene families, whose members are differentially regulated by typical light responsive elements. We already reported about the identification of three spinach full-length cDNA sequences, whose expression levels is variously regulated by light changes.
Here we report about the identification of regulatory regions for five different *Lhcb1* genes and their use as bait for the isolation of relative transcription factors.
Methods: A genome walking approach was applied to identify regulatory regions upstream of three spinach *Lhcb1* cDNA sequences. Main steps were: primer extension, dC-tailing of single-strand DNA and PCR. Gel-shift analysis with spinach nuclear proteins was carried out in order to validate putative regulatory regions. DNA-affinity chromatography allowed the isolation of interacting proteins to be characterized by mass-spectrometry.
Results: We have developed a genome walking strategy that does not require either preliminary digestion of genomic DNA with restriction enzymes, ligation of DNA linkers or use of degenerate PCR. The method allows consecutive rounds of walking on the same DNA preparation and, thanks to the adopted strategy, appears particularly suitable for the study of multigene families. The Genome Walking method allowed to identify the regulatory regions of three genes for the spinach *Lhcb1* genes, plus two additional members of the Lhcb1 multigene family.
Functional analysis of identified regulatory elements was preliminarily obtained by gel-shift analysis carried out with spinach nuclear proteins. The isolation of potential transcription factors was performed by affinity-chromatography using biotinylated regulatory DNA regions as ligand. Purified proteins were visualized by SDS-PAGE and analyzed by mass spectrometry.
Conclusions: Analysis of spinach genomic sequences allowed us to identify both additional members of the *Lhcb1* multigene family and variously composed regulatory regions.
The identification of cognate transcription factor(s), which is currently in progress, will allow to put the basis for future studies on the differential regulation within the *Lhcb1* multigene family.
The same strategy, which takes advantage from the combined application of genomic and proteomics approaches, appears of general interest to study the differential expression of multigene families.

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PROTEOMICS COMPARATIVE ANALYSIS OF THE RIPE BERRY SKIN OF FOUR DIFFERENT CULTIVARS REVEALS FURTHER PECULIAR TRAITS IN GRAPE RIPENING PROCESS

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Background: Grapevines produce a non-climacteric fruit that develops following a pattern of two successive sigmoidal cycles, each with distinctive characteristics. The ripening of this fruit is characterized by deep changes in primary and secondary pathways that are linked to the quality of wine, being strictly dependent on metabolic composition of the fruits. A central role in the development of main secondary compounds (*e.g.* anthocyanins) is played by skin. Their accumulation, which occurs predominantly in this tissue, begins at "véraison" and is responsible of the colour change in black cultivars. Moreover, skin constitutes a barrier to prevent pathogen infections. The relevance of the exocarp is well-witnessed by some works that using Affymetrix GeneChip® technology emphasized the specificity of the skin profile, in which the gene categories related to housekeeping processes such as protein fate, cell cycle and DNA processing are under-represented while those related to secondary, amino acid and lipid metabolisms are highly expressed. According to these studies, the comparison of the proteomes of five different ripening stages of *cv* "Barbera" showed that numerous skin soluble proteins involved in responses to stress, glycolysis and gluconeogenesis, C-compounds, carbohydrate and amino acid metabolisms changed their expression. Among the obtained results, an activation of the glycolytic pathway was found, which had been described as down-regulated in previous analyses performed on the whole fruit. To obtain further knowledge about the physiological role of this tissue we performed a comparison among the proteomes of the ripe berry skin of four genotypes that are known to differently accumulate anthocyanins. For this purpose the study was conducted on a white cultivar "Riesling Italico" and on three red cultivars with an increasing anthocyanins content "Pinot gris", "Pinot noir" and "Croatina".
Methods: The study was conducted considering samples collected in two following vintages (2005 and 2006). After extraction, proteins were separated by 2-DE, using a pH 4-7 linear electrofocusing gradient in the first dimension and 12.5% polyacrylamide homogeneous gels in the second dimension and stained with cCBB. The most significant differences among genotypes were isolated through multivariate statistic techniques after gels comparison performed using the ImageMaster® Platinum 6.0 software. By means of LC-ESI-MS/MS the spots of interest were finally characterized.
Results: The comparison of the proteomes of different genotypes showed significant differences. Through Forward Stepwise – Linear Discriminant Analysis (FS-FDA) performed on PCA scores, it was possible to distinguish the samples and to isolate a panel of spots characterized by a high discriminating power. Spot characterization allowed the identification of proteins involved in physiological processes such as stress, defence, carbon metabolism and energy conversion, such as HSP70, glutamate decarboxylase, NADH-ubiquinone oxidoreductase, SOD, PPO, ASR protein and methionine synthase. Moreover, differences in the expression of proteins involved in secondary metabolism were found. A good correlation was observed between anthocyanin accumulation and the expression of some enzymes involved in the flavonoid pathway (*i.e.* flavone-3-hydroxylase and leucoanthocyanidin dioxygenase). Moreover, different isoforms of some enzymes, such as isoflavone reductase, were specific for some cultivars. Similarly, the levels of many glycolytic and Krebs cycle enzymes showed a good association with the anthocyanin contents of the cultivars. Among the differences observed, proteins involved in anaerobic metabolisms (e.g. alcoholic fermentation and GABA shunt) were found.
Conclusions: The data show how comparative proteomic analyses among genotypes with different chemical profiles are a good tool in order to characterize the secondary metabolism, detecting peculiar activities and relating protein expression patterns to the abundance of interesting compounds.
Through multivariate statistical analysis it has been possible to distinguish the four analyzed cultivars as well as the order in which they were grouped may reflect both their relative anthocyanin content and their genetic relationship. The choice of using berries berries of two following vintages permits to reduce the effects of the environment and to correlate better the biochemical and physiological events occurring during the ripening process. This work confirms the strict link between primary and secondary metabolisms, thus suggesting that a high expression of many glycolytic and Krebs cycle enzymes is required to sustain biosynthetic activities such as anthocyanin production. Moreover, this work provides new evidences that some traits of anaerobic metabolisms, such as GABA shunt and alcoholic fermentation, may take part in ripening processes.

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PROTEOMIC ANALYSIS OF *Cucumis sativus* (L.) ROOTS UNDER Fe DEFICIENCY CONDITION

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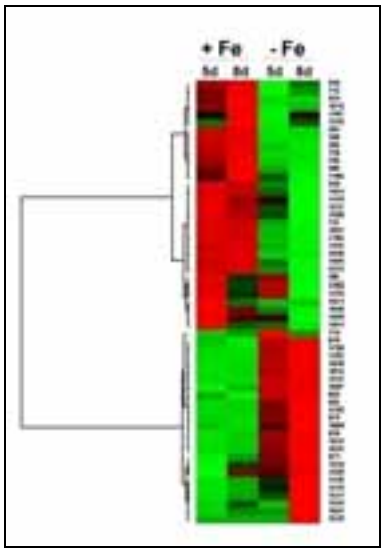
Background: Iron deficiency induces in Strategy I plants the activation of a Fe^{3+}-chelate reductase and a H^{+}-ATPase that increase the request of the energetic substrates, such as NAD(P)H and ATP. Recharging of such substrates requires the acceleration of energetic metabolic pathways. Many processes are increased to efficiently sustain the response to iron depletion. In particular, the activity of phosphoenolpyruvate carboxylase has been shown to increase several fold. The rate of carbohydrate catabolism is also increased under these conditions and the activity of several glycolytic enzymes has been shown to be enhanced, as well. Furthermore, other metabolic pathways and free cytosolic enzymatic activities are increased during Fe deficiency, such as some cytosolic $NAD(P)^{+}$-dependent dehydrogenases belonging to the oxidative pentose phosphate pathway.
Methods: The proteomic pattern of soluble proteins from roots grown in the presence or absence of Fe for 5 and 8 days has been investigated. 400 µg of proteins were loaded on pH 4-7, 24 cm IPG strips. After IEF, strips were loaded on 10% polyacrilamide homogeneous gels for the second dimension. Gels were stained and a comparative analysis was then carried out, using the ImageMaster Platinum software. Only spots showing at least a two-fold change in expression and with an average of their relative spot volumes greater than 0.08 were considered for successive steps. All values were log(z+1) transformed and a two-ways ANOVA ($p<0.001$) was performed. Significant differences linked to the factor iron deficiency were analyzed through the two-ways hierarchical clustering methodology. Spots corresponding to differentially expressed proteins were analysed by LC-ESI-MS/MS.
Results: Twenty-six protein spots out of 57 were overexpressed in the absence of Fe with an increase between the pairwise after 8 days. Most of these proteins belong to the glycolytic pathway. Thirty-one out of 57 protein spots resulted downexpressed under Fe deficiency with some proteins belonging to the biosynthesis of



Hierarchical clustering analysis (HCA) of the 57 spots differentially expressed in the four experimental conditions.

sucrose and complex structural carbohydrates, to structural and stress-response proteins and to proteins containing Fe (such as Aconitase and peroxidase).
Conclusions: Implication of metabolism has been inferred from the microarray analysis performed on Fe-starved Arabidopsis in which it has been shown that several genes encoding enzymes of these metabolic pathways were increased. Notwithstanding, there is not a direct correlation between the increase in the mRNA transcripts and the correspondent protein levels, since other regulatory mechanisms might be present, such as post-translational modifications. To study a global change in the expression of proteins the new proteomic technologies are undoubtedly of great help. Cucumber plants had shown very rapid responses to Fe deficiency and this work describe very important changes not only in the classical responses of Strategy I plants, but also in the metabolic rearrangement induced by this starvation. In fact,

some of the identified proteins are related to structural proteins and other functions while others are involved in carbohydrate metabolism (i.e. glycolysis) and organic acid metabolism. Some of the results obtained are in good agreement with the increased activities seen in previous works performed in our laboratory, in addiction new proteins differentially expressed have been identified.

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CHARACTERIZATION OF *DUNALIELLA TERTIOLECTA* AND *ISOCHRYSIS GALBANA VAR TAHITIANA* PROTEOMES BY TWO-DIMENSIONAL GEL ELECTROPHORESIS (2-DE) AND MASS SPECTROMETRY.

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Background: *Dunaliella tertiolecta* (Dt), class of Chlorophyceae, and *Isochrysis galbana* var *tahitiana* (Istai), class of Chrysophyceae, are unicellular algae commonly used for aquaculture purposes. The exploration of these organisms for pharmaceutical target has revealed important chemical prototypes for the discovery of new agents, stimulating the use of sophisticated physical techniques and new syntheses of compounds with biomedical application. The high cell content of β-carotene, glycerol and protein have made Dt an attractive candidate for commercial production of Fine Chemicals by the nutraceutical and pharmaceutical industry, to obtain novel bio-active compounds and natural integrators. *Dunaliella* can produce metabolites which have several properties: anti-hypertensive, bronchodilatator, analgesic and relaxing for the muscles, anti-oedema.
Among marine microalgae, the golden-brown flagellate *Istai* can be considered as a good source of n-3 PUFA (Polyunsaturated Fatty Acids). In particular, the docosahexaenoic acid (DHA), one of n-3 PUFA, plays an important role in human health, prevention of heart and circulatory disease and brain development in infancy. Microalgae biomass from these two strains have been cultivated in standard condition under artificial light, following the current procedure used in aquaculture. There is very limited information on studies on algae's proteome, and none on Dt and Istai. We performed a preliminary proteomic study of the two algae strains to assess the differences of proteomic 2-DE maps.
Methods: We used a simple and fast method for the extraction of proteins from the two different microalgae strains, by the use of *Trizol* (phenol/ guanidine isothiocyanate). Frozen dryed biomass of Dt and Istai were acheived and dry powder stored at -20°C until laboratory analysis. The same amount of Dt and Istai powder was treated with *Trizol* to extract proteins according to the described procedure, and protein quantification was obtained by Bradford's method. Isoelectrofocusing was carried out using 18 cm IPG strip of pH 3-10 non linear range at 42kVolt hour total. The second dimension SDS-PAGE was carried out on home-made polyacrylamide (12%) slab gels in SDS-PAGE running buffer at a constant current of 35mA/gel for 7hours. Analytical 2-DE gels were Sypro Ruby stained and analysed using PROXPRESS 2D Proteomic imaging system.
Results: The use of *Trizol* reagent allowed to obtain high-quality protein sample for 2-DE from microalgae, which is very difficult to achieve for their high endogenous level of salts, nucleic acids, polysaccharides, pigments and other interfering compounds. The microalgae *Dt* is characterized by a higher amount of proteins (15% yield) beside considerable content of essential Fatty Acids (ESA) compared to *Istai*, which shows lower amount of proteins (8.5% yield) beside a high content of PUFAs. 2-DE protein patterns of Dunaliella and ISTAI were quite different presenting a distinct number and spatial distribution of protein spots.
Conclusions: This is the first 2-DE proteome characterization of *Dunaliella tertiolecta* (Dt) and *Isochrysis galbana* var *tahitiana* (Istai). This preliminary study, which will follow protein identification by mass spectrometry (MALDI-TOF/MS and nanoLC-MS/MS) allows to investigate the protein expression changes in the different microalgae strains useful to explorate the pharmaceutical and nutritional value of these organisms and specially to highlight their successful use for some artificial food chains in aquaculture.

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UNRAVELLING THE TRANSITION FROM THE PRE-CLIMACTERIC TO THE CLIMACTERIC STAGE IN PEACH FRUIT: A COMPARISON BETWEEN CULTIVARS

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Background: Fruit ripening is a very complex phenomenon, since it is regulated by a huge number of genes and is also subjected to environmental influence. Many important metabolic events take place during this process, the most remarkable of which are chlorophyll degradation and pigments biosynthesis, simple sugars and organic acids accumulation, volatiles production and flesh softening. According to the patterns of ethylene evolution and respiration, fruits are classified as climacteric and non climacteric. The latter show a gradual decline in respiration all throughout the ripening process, while the former, which comprise economically relevant species like tomato and peach, are characterized by a peculiar surge in ethylene evolution and respiration rate at the onset of ripening. The role of ethylene has been largely studied and it has been demonstrated to be involved in many of the physiological processes mentioned above. Among the enzymes of the biosynthetic pathway, the levels of ACC oxidase (ACO), which catalyzes the conversion of 1-aminocyclopropane-1-carboxylic acid into ethylene, have been proved to mirror ethylene evolution. According to their flesh phenotypes at ripening, peach (*Prunus persica* [L.] Batsch) fruits are grouped into melting flesh (MF) and non melting flesh (NMF): they both soften but this event is more evident in mature MF than in NMF fruits. This behaviour also has economical consequences since it makes MF fruits soft and juicy and consequently attractive to the consumers but also extremely susceptible to handling and physical injuries; NMF peaches, on the contrary, are less appreciated by the consumers but have good keeping qualities. In this work we applied the proteomic approach to study some biochemical and physiological features of peach fruit ripening by comparing the protein profiles of two peach cultivars with different flesh firmness characteristics, sampled immediately before and during the climacterium.
Methods: At least 15 fruits of the *cv* Bolero (MF) and Oro A (NMF) at the pre-climacteric and climacteric stages were sampled. The different samples were established by a Western blot analysis using anti-ACO antibodies. The mesocarp fraction of every ripening stage was isolated and total proteins from freeze-dried powder were directly extracted with phenol after setting up an adequate extraction protocol. Proteins were resolved by 2D-PAGE on a 24 cm linear pH 3-10 gradient and 12.5% polyacrylamide gels; gels were stained with colloidal Coomassie Brilliant Blue G-250. Gel images and statistical analyses were conducted with ImageMaster Platinum 5.0 and the ANOVA test ($P<0.01$) respectively. By means of LC-ESI-MS/MS the spots of interest were finally characterized.
Results: Preliminary experiments were focalized to optimize the extraction procedure for peach mesocarp tissue.
Gel analyses revealed 53 spots having a statistically significant and 2-fold expression change; 43 among them were analyzed. The spots were grouped according to their expression trends during the transition from the pre-climacteric to the climacteric phase: among the proteins up-regulated in both cultivars at the climacterium we identified two isoforms of ACO. Among the down-regulated spots we isolated Rubisco, two isoforms of S-adenosyl methionine synthase (MAT1 and MAT2) and beta-cyanoalanine synthase. Moreover we found some enzymes involved in the detoxification of Reactive Oxygen Species (ROS): a catalase whose expression levels were decreasing during ripening and a superoxide dismutase which, on the other hand, increased with the climacterium. Finally we identified a malate dehydrogenase cultivar specific, since it was only present in *cv* Bolero and absent in *cv* Oro A.
Conclusions: The extraction procedure set up in this work allowed to obtain high quality, reproducible protein patterns, as confirmed by the gel comparison and the hierarchical clustering analyses.
The expression trends of the spots referring to proteins directly involved in the ethylene biosynthetic pathway were in agreement with the literature: we found two forms of ACO, one of which was particularly abundant (it represented 2% of the total relative spot volume in the climacteric pattern of *cv* Bolero) displaying a dramatic increase in ethylene evolution at the climacterium. Moreover, we found a beta-cyanoalanine synthase, which is responsible for the detoxification of cyanide, a by-product of ethylene evolution. Surprisingly, between the two isoforms of S-adenosyl methionine synthase, MAT2 decreased in both cultivars, while a decrease of MAT1 was found in *cv* Bolero. Moreover, both MAT1 and MAT2 were less expressed in *cv* Oro A than in *cv* Bolero. This feature could be due to the different genetic traits of the two cultivars but also reflect a different ripening trend. Nevertheless, this apparent discrepancy may be linked to different roles of S-adenosyl methionine. This metabolite, in fact, is involved as methyl donor in a *plethora* of other biosynthetic processes than ethylene production (*e.g.* DNA methylation).
During ripening fruit turns from green, where photosynthesis is still active, into a sink organ in which simple

sugars are accumulated. According to this event we observed a decrease in a spot corresponding to Rubisco and an increase in a sucrose synthase levels.
The ripening process has also been defined as a protracted form of senescence: we found three spots referring to proteins involved in the detoxification of ROS with different expression trends during the pre-climacterium-climacterium transition. Two spots corresponding to a catalase and a peroxidase decreased while a superoxide dismutase increased during the transition, suggesting that the ripening process is characterized by a modulation of the different antioxidant enzymes, some of which are also involved in the synthesis of secondary compounds.

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MONO- AND TWO-DIMENSIONAL ELECTROPHORESIS AND LIQUID CHROMATOGRAPHY MASS SPECTROMETRY FOR THE IDENTIFICATION OF RICE (*Oryza sativa*) BRAN PROTEOME

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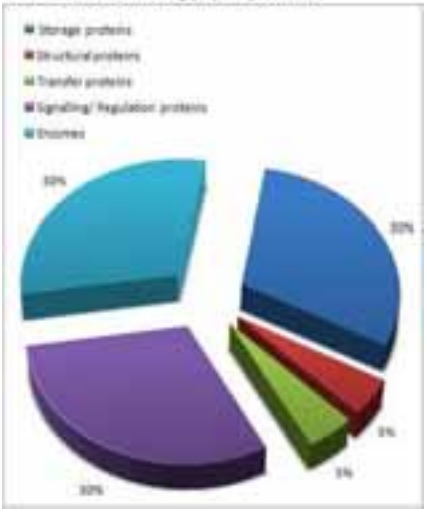
Background: Rice is a very important agricultural resource whose main utilization is for food. While in converting raw rice into table/white rice a large number of interesting by-products is obtained, a relative little amount of this material is still utilized. In particular it appears wasteful the present use of rice bran, a by-product obtained from rough rice milling process. For a better understanding of the functional properties of this resource, knowledge of its qualitative and quantitative protein components is an essential aspect. Thus, aim of the present investigation was to achieve the qualitative characterization of protein pattern in rice bran.
Methods: To extract the largest number of proteins, three different extraction protocols [a) acetone-trichloroacetic acid, b) sodium bisulfite and c) SDS-urea solution] have been applied either on full-fat or on defatted rice bran.
The proteins have been identified by applying mono- and two-dimensional electrophoresis (1-DE and 2-DE), combined with *in situ* tryptic digestion and Mass Spectrometry (MS). To validate these data the mixture of proteins extracted from rice bran was also submitted to in-solution digestion with trypsin; α-chymotrypsin and endopeptidase V_8 from *Staphylococcus aureus*. Liquid Chromatography Mass Spectrometry (LC-MS/MS) was used for the separation and identification of protein digests.
Results: These approaches allowed to unambiguously identify the 43 proteins listed in Table 1. As shown in Figure 1, they were classified as signalling/regulation proteins (30 %), proteins with enzymatic activity (30%), storage proteins (30 %), transfer (5 %) and structural (5 %) proteins. As expected, while a few of these are common to other rice tissues, others have never been observed before and their identification may be useful for completing the knowledge of proteins expressed in this model plant.

Table 1. List of proteins identified in rice bran

Protein	Accession n°	Protein	Accession n°
Glutelin type-A 1	P07728	Glutenedomin-C8	P35142
Glutelin type-A 2	P07730	Glutelin B1	P14323
Glutelin type-A 3	Q08151	Glutelin type-B 5	Q8RE23
Glutelin type-B 2	Q02897	Late embryogenesis abundant protein, group 3	P9C3A4
Glutelin type-B 4	P14314	Embryonic abundant protein 1	P49320
19 kDa globulin	P29835	Seed allergenic protein RA5	Q01881
Prolamin PPROL 14P	Q42485	Salt stress-induced protein	Q6Z5J9
Prolamin PPROL 14E	Q6ZD45	Seed allergenic protein RAG1	Q01883
Putative globulin (cupin family protein)	Q8ZB69	Enolase	Q42971
Late embryogenesis abundant protein 1	A3A2S2	Peroxiredoxin-2E-1, chloroplastic	Q8PTT4
Elongation factor 1-alpha	O64937	Lactoylglutathione lyase	Q94ET6
16.9 kDa class I heat shock protein 1	P27777	Non-specific lipid-transfer protein 1	A2ZHF1
Alpha-amylase subtilisin inhibitor	P29421	Non-specific lipid-transfer protein 2	A2XBX5
17.4 kDa class I heat shock protein 1	Q84Q77	Peptidyl-prolyl cis-trans isomerase	A2WZU9
Oleosin 15 kDa	A2XL05	Phosphoglycerate kinase, cytosolic	Q8H8C7
Oleosin 16 kDa	Q42980	Seed allergenic protein RAG2	Q01882
Glucose and ribitol dehydrogenase	Q7XKJ0	Bowman-Birk type bran trypsin inhibitor	A2WE30
Triosephosphate isomerase cytosolic	P48494	Lectin	Q9SDB8
1-Cys peroxiredoxin A	P9C3C9	10 kDa Prolamin	A2XSB2
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	A2XQT7	13 kDa Prolamin C	P17048
Fructose-bisphosphate aldolase cytoplasmic isozyme	P17784	Malate dehydrogenase, cytoplasmic	Q7XDC8
Aspartate aminotransferase, cytoplasmic	P37833		

Figure 1. Categorization of proteins identified in rice bran according to their Gene Ontology (GO) terms



Conclusions: Aim of the present investigation was to obtain the qualitative characterization of proteins in rice bran. To identify an unprecedented number of proteins, which has greatly enhanced our current knowledge, data collected from 1-DE, 2-DE and gel-free procedures have been combined. The fact that all extraction and identification procedures have been performed in triplicate and quadruplicate with an excellent reproducibility of data, provide a rationale for considering the platform of proteins shown in this study as the potential rice bran proteome. It also represents a source of information by which to evaluate better the qualities of rice bran as food resource.

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2-DE POLYPEPTIDE MAPS FROM OLIVE (OLEA EUROPAEA L) FRUIT TISSUES AND COMPARATIVE ANALYSES OF PROTEINS EXPRESSION DURING RIPENING

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Background: 2-DE is one of the most efficient and powerful methods to study complex patterns of gene expression at the level of proteins. However, the electrophoretic separation of proteins from plant tissue extracts is often complicated by other nonprotein contaminants indigenous to the plant, such as organic acids, lipids, polyphenols, pigments, terpenes, etc. Olive (*Olea europaea* L.) tissue is notoriously recalcitrant to common protein extraction methods due to high levels of interfering compounds. When proteins are extracted by directly homogenizing olive leaf in aqueous buffers and then precipitated by organic solvents, polyphenolic and other contaminants will copurify with the proteins, consequently the resultant brownish pellet, due to polyphenolic oxidation, is hard to be dissolved and is not suitable for 2-DE (our observations). Previously, high quality protein preparation from olive leaf is obtained for proteomics (Wang et al, 2003). The extraction protocol optimized for leaf it was not suitable for proteins extraction from fruit tissues. So that we developed an optimized protocol that attempts to minimize the presence of such compounds as lipids, pigments, polyphenols, polysaccharides, nucleic acids, etc. The protocol produces high quality proteins suitable for 1-DE and 2-DE from olive fruit. In this study we demonstrate well-separated patterns of olive fruit proteins by 2-DE, performing a comparative analysis of protein pattern during fruit ripening. In addition, as an example we demonstrate immunoblots of olive fruit extracts probed with antibody against the b-glucosidase, an enzyme that modulates its activity during ripening and defence response against herbivores (Spadafora et al, 2008).
Methods: Fruits were harvested from a well established grove at 80, 120 and 160 days after flowering (DAF). These three ripening stages correspond to the green and green-brown fruits. Mesocarp tissue was pulverized under liquid nitrogen and proteins were precipitated by adding TCA/H_2O. Then pellet was rinsed in TCA/acetone and finally in 80% acetone. Proteins were extracted and purified by adding a phenol Tris-buffer pH 8.8. Proteins were recovered by adding ammonium acetate to the phenol phase and further washings in acetone. Purified proteins were then dried and redissolved in a rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate), 50 mg/ml DTT, 0.5% carrier ampholyte (3-10 NL IPG buffer; Amersham Biosciences). Proteins were first separated by iso-electrofocusing (IEF), using an immobilized nonlinear pH gradient of 3-10 (Immobiline DryStrip, 18 cm; Amersham Biosciences). The equilibrated strips were placed on vertical 12.5% polyacrylamide gels. Molecular weight standardization markers, covering a 10 to 100 kDa range.Gels were placed at 4° C in a running buffer (Laemmli, 1970) and protein spots on each gel were visualized using colloidal Coumassie Brilliant Blue (CBB). The gels were analyzed using QuatityOne and PDQuest softwares (Biorad). Spots differentially expressed were excised from the gels and identified. To obtain the peptide profile of each spot, the spots were excised from the gels for tryptic digestion (Shevchenko et al., 1996) and the peptides micro-sequenced on a LC-MS. Sequence tags were interpreted from the MS/MS spectra and peptides identified using mass spectrometry-driven BLAST (MS BLAST) to match extracted proteins with those of protein and genome databases. Each peptide was identified with the accession number and MASCOT (Mass Coverage Tool) coverage (%). Peptide sequences which were not identified through these databases were further analysed using *de novo* peptide sequencing and analysis of hypothetical proteins was conducted using software at servers accessible on the Internet: free MS-BLAST Mass Spectrometry software and driven BLAST (http://dove.embl-heidelberg.de/Blast2/msblast.html).
Results: A crossing comparative study of the protein profiles generated from analytical 2-DE gels between three ripening stages was conducted and spots differentially expressed during ripening were revealed. Large part of identified proteins belong to the primary metabolisms such as photosynthesis and sugar metabolism. As relevant results PPO was found in one major protein of 55 kDa. During the last stages of fruit maturation, a second 36 kDa protein was observed, indicating that this protein could serve as a marker of the final phase of ripening. The amount of PPO protein increased significantly during fruit ripening. Besides, the expression of RuBisCo drastically decreases, while chlorophyllase was up-regulated starting from 120 DAF. Finally we found that levels of b-glucosidase, an enzyme which specifically idrolyses oleuropein leading the fruit debittering (Mazzuca and Uccella, 2002) were constant, in disagreement with the drastic decreasing of its activity in ripe fruits (Mazzuca et al., 2006)
Conclusion: Comparative proteomics by means the 2-DE mapping of expressed proteins is a powerful tool to investigate the physiology of olive fruit ripening. Unfortunately genomic resourses from olive is lacking. Few sequences are available in the common databases. For this reason a lot of differentially

expressed proteins did not found their identification. Improuvement of qualitative and quantitative proteomics could be given by new insigths on the olive trascriptome sequencing.

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COMPARATIVE LEAF PROTEOMICS IN POSIDONIA OCEANICA GROWING AT DIFFERENT SEA DEPHTS BY MEANS OF 1D OFF LINE GEL ELECTROPHORESIS AND LC-USIS-MS/MS

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Background: *Posidonia oceanica* is a marine plant (seagrass) which forms widespread meadows along the coastline of the Mediterranean basin; it is one of the few seagrass species which lives up to a depth of 40 m in clear waters. This feature pose the question about how the species adapts to different quality and quantity of light. With the aim of improving knowledge on low light acclimation in this species, the proteomic approach has been performed in plants growing in shallow (high-light) as in deep (low-light) sea water along a well preserved meadow; about twenty-six proteins were found differentially expressed in leaves of plants grown under low-light in respect to those expressed in high-light condition, but only twelve of them were identified by conventional tandem mass spectrometry (MS/MS) and time of flight mass spectrometry, (TOF)[1]. One of the main limits of mass spectrometry is the lack of ionization efficiency and the specificity of the usually employed ionization sources. In order to increase the sensitivity and consequently the ratio of detected protein in leaf proteome analysis, we applied the highly innovative Universal Soft Ionization Source[2] coupled with off line 1D-SDS electrophoresis[3] and Liquid Chromatography – Ion Trap - Tandem Mass Spectrometry (LC-USIS-MS/MS)[2].
Methods: We applied these methods on two different plant populations growing at -5 m and -27 m depths. Purified leaf proteins were separated on Laemli 1 D SDS-PAGE, thus obtaining more than 80 polypeptide bands ranging from 200 to 6 kDa. In-gel protein digestion was made on the whole lane of the preparative gel containing leaf proteins and was sequentially cut into 40 portions, independently of staining intensity. The 40 gel portions were washed with 100 □l of 25 mM NH4HCO3, followed by dehydration with 100 □l of 50% acetonitrile in 25 mM NH4HCO3. Proteins were reduced by incubation with 10 mM DTT (1 h at 57°C), and alkylated with 100 □l of 55 mM iodoacetamide (45 min at room temperature). Gel bands were further washed with NH4HCO3, dehydrated as described above and dried in a vacuum centrifuge.The proteins were digested overnight at 37°C by addition of 15 □l trypsin. Tryptic digest of high-ligth and low-ligth protein bands has been carried out in H_2O- and D_2O-based buffer solutions respectively. The resulting peptide mixture was acidified by the addition of 1 □l of an aqueous solution of formic acid (1%, v/v), and stored at 20°C. Nano-LC-MS/MS analyses of the digested proteins were performed
Results:
USIS is a strong improvement in the ionization source field and joins the benefits obtained by two well known and consolidated ionization sources: 1) Surface Activated Chemical Ionization[4] and 2) Electrospray (ESI)[5]. The joining of the two ionization effects leads to strongly increase the peptides ionization efficiency and consequently the sensitivity and the protein identification coverage. The leaf proteome of *Posidonia* at two light conditions are analyzed both by means of the classical electrospray ionization source (ESI) and USIS technologies. Protein identification is made through comparison with available Expressed Sequence Tags (ESTs), obtained from a single *P. oceanica* cDNA library. The benefits on protein identification coverage achieved using USIS are shown and discussed.

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COMPARATIVE PROTEOMICS ANALYSIS OF DIURNAL RESPONSES IN CRY2-OX AND WILD-TYPE TOMATO PLANTS

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Background: light is an essential environmental factor in the progression of plant growth and development, affecting almost all physiological processes, from seed germination to floral induction. As sessile organisms, plants have developed complex yet efficient systems to integrate the variables of light signals such as quality, quantity, direction and periodicity. Information on environmental light is perceived first by photoreceptors. In higher plants, three photoreceptor systems have been reported: the red and far-red light-absorbing phytochromes, the UV-A/blue light-absorbing cryptochromes/phototrophins, and the as-yet unidentified UV-B light-absorbing receptors. The day/night cycling of gene expression is controlled, mainly, by light and temperature and, secondarily, by a free-running internal molecular timekeeper known as the circadian clock.
Microarray analyses in our lab have established that in wild-type (wt) tomato diurnal rhythms in gene expression affect a large portion of the transcriptome. Transcription analyses, between wt and transgenic tomato overexpressing cryptochrome 2 gene (*CRY2-OX),* have shown that the CRY2 controls the diurnal transcription fluctuations of several phytochrome and cryptochrome genes.
Methods: total soluble proteins were extracted from tomato plants (wt and *CRY2-OX*) grown in LD conditions (daily light cycle of 16h light/8h darkness) and collected at ZT0 (presumptive dawn), ZT8 (eight hours after dawn), ZT16 (presumptive dusk) and ZT20 (four hours after dusk). We have performed a direct screening of the protein profiles using two-dimensional difference gel electrophoresis (2D-DIGE) and mass spectrometry analysis, comparing wt and *CRY2-OX* plants. Three gels representing independent biological samples were analyzed for each genotype and time point. Protein patterns were determined by gel image analysis, and protein spots were quantified by DeCyder version 6.5 software (GE Healthcare). For intra-genotype comparisons, analysis of variance (ANOVA) was used to identify spots exhibiting significant diurnal changes ($p \leq 0.05$) of protein expression during the time course. For inter-genotype comparisons and to verify the consequences of the over-expression of CRY2 under diurnal cycles, differentially accumulated protein spots at each time point were selected based on the Student's t-test analysis ($p \leq 0.05$).
Results: a comparison at each time point was carried out to reveal changes in the accumulation of specific proteins during the diurnal cycle in wt and *CRY2OX* genotypes. A total of 69 and 13 protein spots were detected as differentially expressed in wt and *CRY2OX* genotypes, respectively. Among the 69 spots, 67 were excised from gel for MS analysis and a total of 58 diurnal-regulated protein spots were successfully identified. All the 13 spots differentially accumulated in *CRY2OX* genotypes were excised from gel for MS analysis and 11 of them were successful identified.
Inter-genotype analyses, *CRY2OX vs* wt, revealed 98 CRY2-regulated protein spots, 78 of them successfully identified. Generally a significant decrease of the protein levels was observed in *CRY2OX*, especially at presumptive dusk (ZT16), suggesting that over-expression of CRY2 had a negative impact on the overall protein expression during the presumptive night.
Conclusions: we report that during a diurnal cycle, several classes of proteins are differentially accumulated in both and between genotypes. This study provides a useful overview of how cryptochromes can affect day/night protein expression in tomato.

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COMPARATIVE PROTEOMICS ANALYSES AMONG FLOWERS OF THREE *Petunia hybrida* LINES IN WHICH ANTHOCYANIN BIOSYNTHESIS OCCURS OR IS BLOCKED BY TWO DIFFERENT MUTATIONS

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Background: Plants produce a large number of secondary metabolites divided into three chemically classes (*i.e* terpenes, phenolics and nitrogen-containing compounds) that have important ecological functions, such as plant protection against herbivores and microbial pathogens, pollinators and seed–dispersing animals attraction, plant-plant competition and plant-microbe symbiosis.
Anthocyanins and anthocyanidins, the major subgroup of flavonoids, play a central role in determining the colour of flowers, fruits and seeds. Moreover, because of their antioxidant and metal chelator activities, increasing interest is addressed to the nutraceutical characteristics of flavonoids, which appear to be cancer preventive agents.
In flower epidermal cells, anthocyanin synthesis, localized in the cytosol, branches from the phenylpropanoid pathway and is subject to a multifaceted regulation that includes developmental, transcriptional and enzymatic levels. Moreover, the synthesis of these compounds involves different cellular compartments and is linked to their final accumulation in the vacuoles, where pH and metal ions take part in colour determination. The biochemical pathways, therefore, involve intracellular transport processes and the sub-cellular organization, in which the set up of compartments pHs appears a crucial factor.
In particular, many studies were conducted using flowers of *Petunia hybrida*. In fact, for this species a very large collection of mutants is available allowing to investigate many traits of anthocyanin biosynthetic pathway. At the moment, no proteomic studies have been conducted on Petunia flowers, thereby the information on the translational and post-translational events that occur during flowering are still lacking. In this view, a possible strategy is the comparison of the proteomes of pigmented and white flowers, in which the proteins involved in the anthocyanin synthesis should be differently expressed. In detail, we used as wild type the R27 line with red flowers and two white lines: the W186 line, a mutant for *AN3* gene, encoding flavonoid-3-hydroxylase enzyme and the W225 line, a mutant for *AN1* gene, encoding a transcription factor involved in the regulation of both anthocyanin synthesis and vacuolar pH. Therefore, in this last line the typical acidification of vacuoles that occurs during flowering is also lacking.
Methods: Total protein fraction of limbs was extracted by using two different methods (*i.e*. SDS and phenol methods). Afterwards proteins were precipitated in the pre-cooled 0.1 M ammonium acetate in methanol, washed one time in the same solution and three times in 80% acetone. Proteins were then separated by 2-DE, using a pH 4-7 linear electrofocusing gradient in the first dimension and 10.0% polyacrylamide homogeneous gels in the second dimension. Gels were stained with colloidal Coomassie Brilliant Blue G-250 and analyzed by ImageMaster® Platinum 6.0 software. The most significant differences among genotypes were isolated through the ANOVA test. Moreover, data were analyzed by multivariate statistics. By means of LC-ESI-MS/MS some spots of interest were characterized.
Results: Between the two tested extraction procedures, the phenol method permitted to obtain higher quality gels as well as a larger number of spots detected. Gel analyses revealed about 1300 spots. The Principal Component Analysis, performed on the spot volume dataset, indicated that there was a clear separation between the R27 line and the other two lines. Seventy-one spots showed statistical differences at ANOVA test (P<0.001) with at least a two-fold expression change. The two-way hierarchical cluster analysis showed a good reproducibility of the 2-DE gels as well as it enabled to distinguish many spots with opposite trends in the white flowers respect to the wild type (*i.e.* red flowers). Moreover, the comparison between W186 and W225 lines revealed some spots differently expressed between the two genotypes. The spot characterization allowed the identification of proteins involved in both primary and secondary metabolisms.
Conclusions: This is a first proteomic study performed on Petunia flowers. The preliminary experiments brought to define an extraction protocol characterized by a high quantity of recovered proteins and a good pattern resolution. The differences detected among the three lines appear mainly related to anthocyanin biosynthesis. Spot characterization, though still in progress, revealed that some proteins involved in the carbon and energetic metabolisms appeared more expressed in red flowers, according to the larger request of C-skeletons and energy. In conclusion, this proteomic approach appears suitable to obtain information at translational and post-translational levels and to deepen the picture of metabolic and physiological bases of the flowering process. In this view, the choice of studying flower tissue of wild type *versus* mutants for genes involved in specific traits, like the pH control and/or anthocyanin biosynthesis, appears particularly useful.

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DIFFERENT EXPERIMENTAL APPROACHES TO HIGHLIGHT THE RELATIONSHIP BETWEEN INDUSTRIAL PROCESSING AND PROTEIN/PEPTIDE INTEGRITY IN BIOACTIVE PEA PROTEINS

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Background: During the last decade food industry has increased its attention on functional foods, defined as any food or food ingredient able to provide an health benefit beyond the nutritional properties. The nutraceutical properties depend on the presence of bioactive components such as proteins or peptides with an *in-vivo* biological effect. Several literature data reported many examples of food bioactive proteins characterized by different biological activities such as hypotensive, hypocholesterolemic and hypoglycemic activity. Recent studies carried out in the European Craft Project "Bioprofibre" have highlighted specific biological properties of proteins from *Pisum sativum*, in agreement with previous literature results.
Since data regarding the consequences of industrial processing on the quality of pea protein are scarce, the present investigation was designed to evaluate the effects of mild and harsh thermal and mechanical treatments on a industrial pea protein isolate. In fact, industrial processing is known to cause protein denaturation with either deleterious or beneficial effects on the nutritive value and nutraceutical properties of proteins.

Methods: The total protein extract (TPE) from pea seeds (*cv Attika)* and an industrial pea protein isolate, (PPI, Pisane®) were examined. PPI was treated thermally in an oven from 65°C to 200°C for different exposure time, and mechanically (ultraturrax, high pressure homogenizer). After each treatment, the soluble proteins were extracted and the degradation behavior of the major storage proteins was studied.
Differential Scanning Calorimetry (DSC) and proteomic techniques, such as 2D-electrophoresis and mass spectrometry, were used to investigate the effects of different processing conditions on the protein profile and to assess the availability of intact/resistant peptides after treatments.
Results: After a prolonged heating treatment no native proteins were present and the proteins solubility was greatly decreased, even if some spots with high intensity were still present on 2D-gels. The identity of these gel spots was confirmed via HPLC-Chip-MS/MS showed that the vicilin 30 kDa fragment and legumin acidic subunit were the most resistant. The tryptic peptides derived from them were still available after 30 min dry heating at 120°C. The results of DSC analysis, used to determine the content of native/degraded proteins, showed a decreasing denaturation enthalpy with increasing of thermal and mechanical treatments.
On the other hand, the mechanical stress applied did not show any relevant difference in the DSC thermograms and in 2D-gels, meaning that these kind of mechanical treatments did not affect the native structures of the protein fractions.
Conclusions: This study could help in understanding the relationship between the thermal and mechanical effects on protein integrity, by using 2D-gels, Differential scanning calorimetry (DSC) and gel filtration chromatography. The results indicate that after heating treatments pea protein isolate partially or completely lost their protein integrity, however the HPLC-Chip-MS/MS shotgun proteomics analysis was enabled, especially after harsh treatments, to determine the presence of specific and intact available peptides with putative biological activity.

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ALMOND'S SEEDS (*PRUNUS DULCIS*) PROTEOME: EFFECTS OF REHYDRATION.

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Background: We evaluated the total proteins expression in the seeds of almond (*Prunus dulcis*), by two-dimensional electrophoresis combined with mass spectrometry. The subject of our research is a variety of autochthon almond originate from an area in the south of Italy, near to Bari. This work is a part of an industrial research project which aim to individuate high quality almonds and to develop new methods for the extraction and processing of the raw material to obtain healthy biological foods. We start to investigate the rehydration effect on bottle dried preserved almonds by comparing their proteomes before and after rehydration procedure (reconstitution of dried products in water).
Methods: Preserved and rehydrated almond seeds were mechanically homogenized until to obtain a fine powder and proteins were extracted by a denaturating buffer suitable for electrophoresis analysis. Preserved almond's seed were rehydrated in water at room temperature for 16 hours. Proteins isoelectrofocusing was carried out using 13 cm IPG strip of pH 3-10 non linear range at 40kVolt hour total producted by overnight run. The second dimension SDS-PAGE was carried out on home-made polyacrylamide (12%) slab gels in SDS-PAGE running buffer at a costant current of 30mA/gel. Analytical 2-DE gels were silver stained and analysed using Image Master 2D Platinum.
Results: We obtained high-quality 2-DE map of almond's seeds. Proteome of almond's seeds after rehydration process, although presenting a minor number of proteins spots (323±60 (media±DS) for rehydrated almonds, 477±72 for preserved almonds) is almost similar as spatial distribution of protein spots, to that before rehydration. Comparative proteomic approach identify two protein spots up-regulated and two protein spots down-regulated in rehydrated almonds compared to the preserved ones. Three of these protein spots have been identify by both MALDI-TOF/MS and nanoLC-ESI-MS/MS.

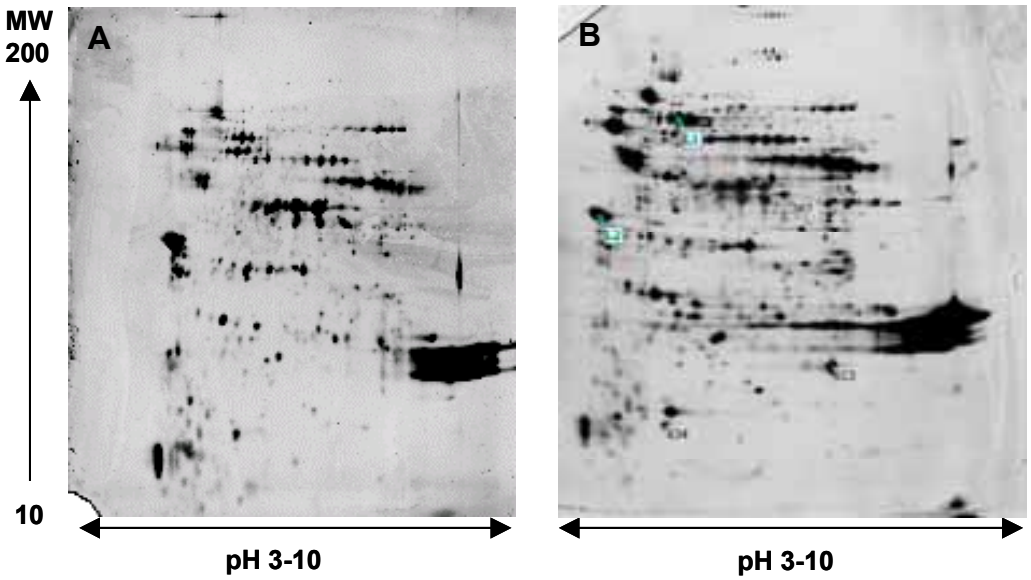

Figure 1. 2-DE protein maps of rehydrated almonds (gel A) and preserved dried almonds (gel B).

Conclusions: 2-DE combined to mass spectrometry constitute a powerful tool to explore differences in the proteome changes of almond's seeds. We hypothesized two molecular events to govern the response to rehydration process, one implicating a major solubilization in the acqueous resuspending buffer of the hydrofilic proteins, the other involving the activation of enzymatic process which could be responsible of proteomic changes, however, these will have to be investigated further. Our finding has laid a solid foundation for further identification, characterization, and standardization of proteins in the seed of almond

(*Prunus dulcis*). Considering that many of them are allergenic proteins this study would also have an important role in the food allergies studies.

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A PROTEOMIC APPROACH TO INVESTIGATE THE SPATIAL AND TEMPORAL RESPONSE OF *POPULUS NIGRA* ROOTS TO MECHANICAL STRESS

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Background: In response to mechanical stress and to improve their anchorage, plants have developed complex mechanisms to detect mechanical perturbations and to induce a suite of modifications at anatomical, physiological and molecular level. To investigate the mechanisms involved in root response to mechanical stress, we have analyzed the alterations occurring in a poplar (*Populus nigra*) taproot bent to an angle of 90°.
Methods: Morphological, genomic and proteomic analysis of poplar taproot was performed according to methods already published (1, 2).
Results: We compared the proteomes and lignin content of the control (non bent) and three different regions (above the bending, bending, and below the bending) of the bent taproot, in three different times during the active-dormancy cycle. Compared with the control, the bent poplar root displays asymmetrical alterations in lignin content and proteome alterations. Almost one hundred and sixty protein spots were found to be differentially expressed spatially (three regions of the bent root) and temporally (three times during active-dormancy cicle) in the stressed root. MALDI-TOF-MS analysis indicates that among the differentially expressed proteins, several are involved in the signal transduction pathway, detoxification, metabolism and stress response. Multivariate statistical analyses (ordering and clustering), were carried out to discriminate proteins regulated by the stress factor from the ones regulated by the active-dormancy cycle, and to identify possibile stress markers. Lignin content was very different between control and all three regions of stressed root during the active-dormancy cycle. Morever, the highest lignin quantity was measured in the middle portion of the stressed root during the full vegetative phase.
Conclusions: The findings reported in our work may provide important cues in understanding the biology of root development under environmental stress conditions.

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THE PROTEOME OF LENTIL (*LENS CULINARIS* MEDIK.) SEEDS: IDENTIFICATION OF MARKERS DISCRIMINATING BETWEEN LANDRACES

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Background: Lentil (*Lens culinaris* Medik.) is one of the most ancient crops of the Mediterranean region. Extensive differentiation of *L. culinaris* over millennia has resulted in a myriad of different landraces. As consequence of environmental and socioeconomic issues, the disappearance of many landraces occurred in more recent times. To promote the survival of endangered lentil landraces, we have investigated the genetic relationship between two ancient landrace cultivated in Capracotta and Conca Casale (Molise, South-Central Italy) and widely spread commercial varieties by using an integrated approach consisting of studies at morphological, DNA and protein level.
Methods: Morphological, genomic and proteomic analysis of lentil seeds was performed according to methods already published (1, 2).
Results: A reference proteomic map of lentil seed was obtained by resolving total protein extracts on 2-D gel electrophoresis. The proteome map showed 134 well resolved protein spots that were further identified by mass spectrometry (MALDI-TOF-MS and/or nLC-ESI-LIT-MS/MS) and classified into nine different functional groups (storage, energy and metabolism, protein synthesis and transcription, stress defence, signal transduction and transported, secondary metabolism, cell cycle and structure and miscellaneous). The comparison of the 2-D lentil seed maps of seven lentil populations belonging to the local ecotype (Capracotta) and five commercial varieties, revealed that 103 protein spots were differentially expressed within and between populations. The multivariate statistical analyses carried out on the 103 differentially expressed proteins, showed that 24 proteins were essential for population discrimination to be further proposed as landrace markers.
Conclusions: The correlation found between the genomic and proteomic data supported the potential of proteomics as a tool in phylogenetic studies and highlighted the possibility to identify proteins that may be specific markers of different landraces.

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PEANUT ALLERGIC PATIENTS CROSS-REACT WITH LUPIN PROTEINS: 34 CLINICAL CASES

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Background: Peanut allergy is a significant health problem because of its high and rising prevalence and the life threatening nature of reaction. In developed countries it affects about 0.4–0.6% of children and 0.3–0.7% of adults. It has been estimated that 30% to 68% of peanut-allergic individuals also react to lupin flour. Lupin seeds are a useful source of human food products because of their composition, being high in protein and dietary fiber but low in fat and starch, and for its role in cardiovascular disease prevention. The most common specie of lupin consumed by humans is *Lupinus albus* L.; however the use of *Lupinus angustifolius* L. is increasing.
Some individuals experience allergic reactions upon ingestion or inhalation of lupin products with symptoms ranging from mild local reactions to anaphylaxis. In 2007 lupin was declared as a new food allergen and was officially added to the EU list of known allergens (Commission Directive 2006/142/EC).
Despite several study in literature, there is no consensus about the presence of one or more major allergen/s in lupin flour. In this context, the aim of the present investigation was to compare the two lupin species, and the proteins contained therein, with respect to the allergenic properties, based on their IgE-reactivity using sera from 34 peanut-allergic patients. In order to get of more general overview, different cultivars of each specie were taken in consideration.
Methods: A comparative protein profile study was carried out in order to determine if the allergenicity of the two species differs: the main seed storage proteins (α-, β-, and γ-conglutin) were selectively purified and tested separately for IgE binding. ELISA assays (indirect and inhibition), western blotting using individual and pooled serum, and mass spectrometry analysis were performed in order to identify the IgE-binding proteins. Sera from 34 peanut-allergic patients were provided by several hospitals in Netherlands (Utrecht, Amsterdam, Arnhem, Ede, Velp).
Results: The screening revealed significant differences in the chromatographic behaviour of all the globulins between the two species. Peanut sera reacted both with *L. albus* and *L. angustifolius* proteins with differences in magnitude of response: a stronger reactivity for α- and β-conglutin and no or less binding with γ-conglutin. The ELISA assays and the western blotting analysis highlighted several reactive spots, indicating the presence of different important allergenic proteins, rather than one major allergen. The different cultivars tested for each species had similar responses, indicating the presence of a related protein pattern with comparable IgE binding affinity.
Conclusions:
The IgE response of purified lupin protein fractions has varied among the peanut allergic patients, with differences of intensity. The results indicate that there is a direct relationship between the peanut-specific IgE level and the cross-reactivity to lupin proteins: the lupin allergenic response of each patient is unique and individual and strongly depends on the IgE level towards peanut proteins.
In conclusion, in order to promote the use of lupin proteins in lupin-based novel food, it is essential that more should be understood about their allergenicity. More studies are needed to establish the prevalence of allergic reactions to lupin in peanut allergic individuals as well as in the population of allergic individuals.

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PROTEOMIC ANALYSIS OF CHROMATE-INDUCED MODIFICATIONS IN *PSEUDOKIRCHNERIELLA SUBCAPITATA*

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Background: Chromium (Cr) contamination of groundwater has become a worldwide problem, due to the important use of Cr in various manufacturing and military industries. In the present study, we investigated the Cr responsive proteins in *P. subcapitata* cells. We have focused our attention on concentrations of potassium dichromate of 0.2 and 1 ppm because they correspond to environmentally-relevant Cr concentrations close to the WHO limits for water. Moreover, the exposure to these concentrations allowed us to investigate the more sensitive protein targets related to Cr stress, avoiding a strong oxidative burst. Toxic effects of heavy metals on microalgae have often been studied as these organisms occupy the lowermost position in the food chain in aquatic ecosystems. In particular, due to its sensitivity, *P. subcapitata* (Chlorophyceae), an unicellular green alga commonly encountered in most freshwaters, is widely used for biomonitoring water quality.
Methods: the algae were inoculated in 150 mL of culture medium, and after 17 h to allow for cell adaptation, they were treated for 72 hours with potassium dichromate at the final concentrations of 0.2 and 1 ppm corresponding to 0.07 and 0.35 $\mu g g^{-1}$ of Cr, respectively, as revealed by Flame Atomic Absorption Spectroscopy. At the end of treatment, cells were pooled for pigment, polyamines (Pas) and protein analysis by two dimensional electrophoresis (2DE).
Results: *P. subcapitata* cells when treated with Cr did not show any appreciable differences in growth kinetics relative to the control samples until 72 hours of treatment. However, in cells Cr-treated we observed a lowering in Chl*b* content causing an increase in the Chl *a/b* ratio. This increase can be interpreted as being due to a decrease in the LHC content of the PSII external antenna. 2-DE analysis and the MS/MS analysis have allowed the identification of 16 statistically confirmed protein variations between control and potassium dichromate-treated samples. Proteins differentially expressed are principally involved in photosynthesis, amino acid metabolism, and detoxification. Finally, the analysis of PAs revealed that the content of free PAs does not change significantly in cells exposed to 1 ppm of potassium dichromate compared to untreated ones, while a conspicuous accumulation in putrescine in the free form (by more than 8 times compared to controls) was found after treatment with 10 ppm metal.
Conclusions:
Our analysis showed that very low Cr doses, which do not affect the growth of the alga, induce changes in the proteome composition. The main targets of Cr-induced damage in *P. subcapitata* are several photosynthetic proteins such as RuBisCO, RuBisCO activase, light harvesting Chla/b protein complex, and LHCSR protein. These data, combined with changes in the photosynthetic pigment composition, indicate an adaptation of the chloroplast antenna.
Cr treatment also altered the expression of some proteins involved in the metabolism of the amino acids glutamine, arginine and methionine. The increase in arginine succinate synthase could be indirectly responsible for the accumulation of Put (that started at 1 ppm but became conspicuous at 10 ppm chromium dichromate) by increasing the availability of arginine. Both ornithine and arginine decarboxylase (ODC and ADC) have been detected in *Chlorella vulgaris*.
Little is known about the role of Pas in algae. In the unicellular green alga *Dunaliella salina*, a transient increase of free PAs was registered a few minutes after subculture into a hyper-saline medium. In a comparative study between the wild-type and a PA-deficient strain of this alga, the latter was reported to be more severely affected by both subculture and salt stresses, with longer recovery times.
An interesting future task would be to complete the proteomic analysis with the study at metabolic level, especially for the amino acid metabolism in order to better understand how the functional status of *P. subcapitata* is affected by chromium stress.

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COMPARATIVE ANALYSIS OF MITOCHONDRIAL PROTEOME BY BN-PAGE AND 2D-DIGE IN GASTROCNEMIUS AND TRICEPS RAT MUSCLES .

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Background: Study of muscle mitochondrial metabolism plays a key role in physiological (ageing), physiopathological (hypoxia) and pathologic conditions (mitochondrial miopathies and neuromuscular disorders). Define its changes could be a good starting point to identify possible biomarkers related to mitochondrial metabolism and to understand the modulation of the mitochondrial function. Skeletal muscles are characterized by a different fiber type compostion directly related to their metabolic properties: muscle with type I fibers (slow) show an oxidative metabolism while muscles characterized by fiber type II (fast) utilise a prevalent glycolytic metabolism. Muscles with different metabolism and different mitochondrial localization are differentially influenced in a number of paraphysiological and pathological conditions, for such a reason it is essential to define mitochondrial proteome of muscles characterized by different function and different fiber type distribution. To approach this difficult task the mitochondrial proteomes of gastrocnemius and triceps muscles, which are structurally and functionally different, were analyzed, in order to highlight possible differences in the mitochondrial proteome under physiological conditions such as ageing. Blue Native electrophoresis followed by SDS-PAGE and fluorescent stain was adopted in order to separate protein complexes, keeping their native condition and enzymatic function. Beside this study a 2D-DIGE analysis was conducted in order to correlate differential expression of soluble proteins to mitochondrial protein complexes.
Methods: Mitochondria enriched fractions from rats gastrocnemius and triceps muscles were obtained using differential centrifugations in density gradients utilising Schagger protocol.
BN-PAGE: In order to maintain complexes integrity, mitochondria were treated with N-dodecil-ß-D-malthoside and stained by Serva Blue-G 250, a non-denaturant dye which binds to complexes. The first dimension was performed in a 5-15% T, 2.5% C polyacrylamide gel gradient. The native electrophoresis was followed by a SDS-PAGE in order to separate the whole complexes into single subunits according to their molecular weight. Each sample was run in triplicate in the same gel to minimize gel variability. Gels obtained were stained by Sypro Ruby and the images were acquired by a laser scanner. Quantitative data were achieved using Progenesis SameSpots software (NonLinear Dynamics), adopting t-test ($p<0.01$) for statistical analysis.
2D-DIGE: proteomic analysis was conducted on protein extracts from each muscle. Each extract was labelled with Cy5 dye (GE Healthcare), while the internal standard, generated by pooling together an aliquot of each muscle extract, was labelled with Cy3 dye (GE Healthcare). The minimal labelling was performed according to manufacturer's recommendations. The first dimension was performed on 24 cm, pH 3-10, NL gradient IPG strips using an IPGphor electrophoresis unit (GE Healthcare), the second dimension was carried out in 20x25 cm 12%T-2.5%C PAGE gels using Ettan Dalt II system (GE Healthcare). Images were acquired using a Typhoon 9200 scanner (GE Healthcare) and analyzed by ProGenesis SameSpots.
Spots of interest from 2D-DIGE and BN-PAGE were excided from gel and digested by trypsin, peptides obtained were analyzed using a MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) and the spectra were analyzed using Mascot software.
Results: 40 spots were visualised by laser scanner on BN-PAGE gels. Mass spectrometry identified 31 subunits from OXPHOS complexes. Of them, 16 subunits were identified in Complex I, 5 subunits in complex V, 5 belonging to complex III, 5 subunits in complex IV and 2 subunits belonging from complex II. Comparative analysis didn't highlight any qualitative change in OXPHOS complex subunits, while a number of spots appeared to be peculiar of each muscle type. Differential protein expression was also evaluated using 2D-DIGE analysis. The latter, revealed 112 spots differentially expressed ($p<0.01$) (41 up-regulated and 71 down-regulated in triceps vs. gastrocnemius) by using the Student's *t*-test provided by Progenesis SameSpots.
This study shows a general deregulation in proteins involved in respiratory chain, Krebs cycle, transport, stress control and protein synthesis.
Conclusions: Proteomic approach by BN-PAGE and 2D-DIGE revealed changes in mitochondrial proteome of two functionally different muscles. The combination of these two separative methods allowed to combine

intact complex qualitative analysis and quantitative assessment of mitochondrial proteome providing better insights into the relationships between mitochondrial complexes and cellular metabolism.
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SAMPLE VARIABILITY OF SERUM "ALBUMINOME" MALDI SIGNATURES FOR EARLY DIAGNOSIS OF HEPATOCELLULAR CARCINOMA (HCC): COMPARISON OF DIFFERENT METHODS OF PREPARATION

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Background. The serum proteome contains information that directly reflects pathophysiological states. In particular, tumor cells and the surrounding environment might generate peptides of different type and/or at different concentrations than normal cells in a "normal" environment. This "fingerprint" can be detected and compared with controls to identify cancer-specific changes. Hepatocellular carcinoma (HCC) is the fifth leading cause of cancer-related death worldwide, and an earlier HCC detection improves the percentage of treatable patients. In the present work, we consider if HCC could be diagnosed and staged by analyzing a specific serum "subproteome", the subset of low molecular weight (LMW) proteins or peptides bound to Human Serum Albumin (HSA), hereafter referred to as "albuminome". The majority of LMW peptides form complexes with high abundant serum proteins, like HSA, which preserve the bound molecules from clearance and allow them to remain in circulation. Published MALDI-TOF (and SELDI-TOF) procedures are often criticized for the lack of data about analytical reproducibility and the use of inadequate data processing procedures. This study aims to compare the reproducibility of MALDI-TOF data obtained from two different "albuminome" preparation approaches.

Methods. In the first method, segregation of albumin was conducted with DakoCytomation® anti-HSA polyclonal antibody coated SiMAG-Cyanuric® paramagnetic silica-based particles. After purification samples were mixed with CHCA matrix and spotted on MALDI target. Acquisition of spectra was performed with an Applied Biosystems® Voyager-DE PRO® instrument. All experiments were carried out in positive, linear ion mode, in evaluating two different mass range, namely from 2500 to 5000 and from 5000 to 15000 Daltons. Preprocessing of raw spectra was performed using in-house developed MATLAB® procedures.

In the second method samples underwent affinity column enrichment method, with Calbiochem® ProteoExtract® Albumin Removal Kit. After denaturation and desalting samples were mixed with CHCA matrix and eluted on the MALDI target. MALDI-TOF MS analysis was performed with the same Applied Biosystems® Voyager-DE PRO® instrument in the mass range of 2000 to 20000 Da. Preprocessing of raw data was performed using both the SpecAlign© (Cartwright Group®, University of Oxford) program and our MATLAB procedures.

As no generally accepted measure of reproducibility of MALDI-TOF spectra is available, we report here data describing the "mean" coefficient of variation (CV) of the ion intensities. Mean CV was computed as the slope of the regression line correlating mean peak intensity and the corresponding standard deviation, for each peak, under predetermined experimental procedures. The following intra-subject coefficients of variation were computed: *intra-sample CV*, computed as the mean CV of ion intensities in a single MALDI-TOF run were a standard serum sample was spotted in different positions; *intra-day CV*, computed as the mean CV observed in independent MALDI-TOF runs carried out on the same sample in the same day; *inter-day CV*: computed as the mean CV observed in independent MALDI-TOF runs carried out on the same sample in different days; *inter-extraction CV*, computed as the mean CV observed in independent MALDI-TOF runs carried out on the same sample after separate extraction procedures.

Results. The in-house developed MATLAB procedure and the SpecAlign program gave similar results when applied to the same data sets. Following the magnetic beads method mean *intra-sample CV* was 30.0% (95%CI: 20.5%-37.8%); mean *intra-day CV,* 38.0% (95%CI: 25.8%-45.7%); mean *inter-day CV*, 43.9% (95%CI: 33.6%-47.1%); mean *inter-extraction CV,* 47.6% (95%CI: 46.7%-56.4%). For affinity column method the corresponding values were: mean *intra-sample CV*, 21.6% (95%CI: 6.5%-54.6%); mean *intra-day CV,* 26.3% (95%CI: 17.7%-39.9%); mean *inter-day CV*, 39.5% (95%CI: 15.0%-67.7%); mean *inter-extraction CV,* 94.9% (95%CI: 81.9%-144%).

Conclusions. The MALDI-TOF magnetic beads method was efficiently applied to classify 522 spectra obtained from serum of 45 patients into three groups: HCV-related cirrhosis, small-unifocal HCCs and advanced HCCs. Notwithstanding the large inter-sample variability, 433/522 samples from patients and

295/299 quality control samples were correctly classified using the RandomForest algorithm, a machine-learning procedure resistant to the high-dimensionality–small sample (HDSS) problem that afflicts the classical statistical methods when the number of variables is of the same order of the available cases and strong correlation exists between variables. The approach which exploits the ProteoExtract® Albumin Removal Kit as an enrichment tool is faster and less expensive than magnetic beads method, and a better spectral reproducibility can be achieved. Inter extraction CV is however still unsatisfactory. We are now to optimizing this extraction step to obtain a better reproducibility, in order to apply the method in of a large prospective study designed to use proteomic fingerprinting in the diagnosis and staging of hepatocellular carcinoma.

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METABOLIC LABELING AND PROTEIN LINEARIZATION TECHNOLOGY ALLOW THE STUDY OF PROTEINS SECRETED BY CULTURED CELLS IN SERUM-CONTAINING MEDIA

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Background. Proteins actively secreted by cells participate in interactions with the environment and are key players in a number of biological processes. Their study can provide a better understanding of the molecular mechanisms underlying these processes in health and in disease conditions. Because proteins can be secreted at very low abundances, the *in vivo* characterization of the cellular secretome is very challenging. Thus, the most common approach is the analysis of media conditioned by cultivated cells. To avoid the contamination with serum proteins, cells are usually cultivated in serum-free medium (SFM). But serum deprivation can influence cell proliferation and increase cell death, thus causing the release of intracellular proteins into the conditioned medium (CM). Moreover, the presence/absence of serum during cell growth can significantly affect the pattern of secreted proteins. Here we present a LC-MS based approach to study CMs containing serum, based on metabolic labeling and protein concentration linearization. The strategy was applied to the differential analysis of two media conditioned by breast cancer cells lines derived from different stages of tumor progression.
Methods. MDA-231 and MCF-7 breast cancer cells were cultivated in presence of 10% fetal bovine serum (i.e. in “usual” conditions). Cellular proteins were metabolically labeled by adding an excess of deuterated valine (D8-Val) to the medium. After 3 days of incubation, the CMs were collected and treated with the Proteominer hexapeptide resin to reduce the dynamic range of protein concentrations. The proteins in the two CMs were analyzed by geLC-MS on an Orbitrap instrument, using stringent statistical validation criteria. After protein identification, the presence of the D8-Val “tag” was used to confirm the cellular origin of the identified peptides. Relative quantification of validated secreted proteins in the two samples was performed using both spectral counts as well as Selected Reaction Monitoring (SRM) on a QTRAP instrument.
Results. By simply adding an excess of isotope-labeled amino acid in a regular culture medium, we obtained 75% labeling for human valine-containing peptides. Treatment of the CM with the hexapeptide resin led to a significant reduction of the dynamic range of protein concentrations as shown by SDS-PAGE. GeLC-MS returned a total number of identified proteins 3-fold higher following the linearization, compared to untreated samples. Accordingly, the numbers of spectra matching to serum albumin and serotransferrin, very abundant proteins in bovine serum, showed a significant decrease after linearization (5-fold and 20-fold, respectively, in the MDA-231 CM).
To focus on the secreted proteins, the spectra obtained from the shotgun analysis of both the MDA-231 and MCF-7 equalized CMs were searched against the human database. The presence of at least 2 D8-Val containing peptides was used to confirm the cellular origin of the identified proteins. By applying this constraint, we validated as truly cellular 39% (114) and 54% (303) of the proteins identified in the CM of MDA-231 and MCF-7 cells, respectively. The relative amount of validated proteins present in the two datasets was estimated using spectral counts. To focus on reliable and significant hits, we retained only proteins matched by at least 10 spectra and considered as significant only relative differences in spectral counts higher than 3-fold. Using these criteria, we found 10 proteins significantly more abundant in the MDA-231 medium and 99 in the MCF-7 one. The SRM-based quantification confirmed the differential expression in the two CMs for some selected proteins.
Conclusions. We presented a LC-MS based approach to study CMs containing serum, combining the advantages of metabolic labeling and protein concentration linearization. Metabolic labeling was essential to distinguish the cellular proteins from the unlabeled serum ones to avoid misleading identifications due to the high homology between bovine and human sequences. In fact, the relevant amount of untagged proteins matched to the human database can be in good part explained by such sequence homology (false positive hits). On the other hand, it is possible that the limited incubation period (3-days) and the lack of valine residues in the sequence of some peptides produced some false negative hits, too. However, we found that the 75% labeling performs reasonably well for the purpose of discriminating between true cellular proteins and bovine serum ones.
The equalization process efficiently reduced the amount of the more abundant proteins (i.e. serum proteins), reducing the dynamic range of protein concentrations and allowing the detection of a significantly higher number of proteins.

Interestingly, 9 over the 10 proteins found significantly more abundant in the MDA-231 CM are known to be secreted and are involved in cell migration/growth. Instead, 90 of the 99 proteins more abundant in the MCF-7 CM have a known intracellular localization. This fact can be explained by passive release of proteins in the medium upon cell death/lysis. The variable degree of contamination of the CMs by intracellular proteins represented indeed a confounding factor in our analyses and it should be carefully evaluated during experimental setup.

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IMPROVED SUBPROTEOMIC ANALYSIS OF THE *CRYPTOSPORIDIUM PARVUM* RELIC MITOCHONDRION BY USING ITRAQ LABELING TECHNIQUE.

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Background. *Cryptosporidium parvum* is a protozoan parasite that causes widespread diarrhoeal disease in humans and animals and is responsible for large waterborne outbreaks of cryptosporidiosis. Many aspects of the cell biology and biochemistry of *C. parvum* are still poorly understood, and little is known about its mitochondrion. In our study an analysis of the mitochondrial subproteome of *C. parvum* has allowed us to infer preliminary metabolic pathways.
Methods. Organellar proteomics was performed on mitochondrial (A) and mitochondrial/ endoplasmic reticulum (B) fractions, obtained by differential centrifugation and lysis from sporozoites, purified from oocysts/sporozoites mixture by using a DEAE cellulose/ pH gradient chromatography. The iTRAQ semiquantitative labelling (iTRAQ 116 and 115 for A and B fractions, respectively) was exploited to produce peptides further analysed by RP-HPLC/MS-MS. Each individual peptide, selected with confidence ≥ 97, was matched against CryptoDB database (http://cryptodb.org/cryptodb/) to identify closest protein hits. The protein list was analysed for EC and GO classification, and then submitted to the motif prediction SignalP (www.cbs.dtu.dk/services/SignalP/), TMHMM (www.cbs.dtu.dk/services/TMHMM/) and mitochondrial localization Mitoprot (ihg2.helmholtz-muenchen.de/ihg/mitoprot.html) algorithms. Finally proteins were classified according to literature, homology data and biochemical criteria.
Results. A total of 138 proteins were identify of which 31 never characterized before, 29 ribosomal, 38 hypothetical, 9 glicolysis-related, 10 associated to the oocyst wall, 7 involved in electron transport, 15 of nuclear origin, 7 related to the cytoskeleton, 7 implicated in importing processes and chaperons activities and 16 associated to other functions. Particularly, 7 ribosomal proteins were annotated as associated with the mitochondrion, 4 proteins involved in mitochondrial electron transport, 5 implicated in the mitochondrial importing, exporting and trafficking processes and 1 associated to ATP generation. All this evidence seem to support the current idea of a functional aerobic-modified mitochondrial metabolism, probably developed to adapt the *C. parvum* parasite to the gastrointestinal low oxygen conditions of its hosts.
Conclusions. Our data aim to provide new insights into the metabolism of *C. parvum*, describing peculiar biochemical pathway of the mitochondrial subproteome network. Our approach therefore seems to be capable to overcome limitations encountered in previous global proteomic studies exploited to characterize mitochondrial proteins of this parasite.

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DETECTION OF PROTEIN-ACRYLAMIDE-ADDUCTS IN HEATED FOODS FOR QUALITY ASSURANCE AND CONTROL

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Background: Acrylamide (AA) is produced naturally in heated foods from the Maillard reaction between amino acids (Asn, Gln and Met) and reducing sugars (D-fructose, D-galactose, lactose, glucose) up to levels of 10^3 ppb. AA can act as mutagen (towards either mammalian somatic and germ cells), neurotoxic (with ataxia, mental status changes, skeletal muscle weakness) and carcinogen (classified by the International Agency for research on Cancer as "probable human carcinogen" 2A group). The AA toxicity is related to its chemical reactivity with a large range of compounds; towards proteins AA can act as alkilating agent particularly on cysteine residues (on sulphydril groups). The European Food and Drink Federation (CIAA) coordinated the efforts and pooled results to investigate possible process methods to reduce acrylamide levels in heated foods, and suitable analytical tools are demanded for detection of acrylamide in free form and in protein-acrylamide-adduct forms to better monitor its formation in food making process. In this study the development of a method by mass spectrometry techniques is traced for the detection of protein-acrylamide-adducts in heated foods.
Methods: The investigation was started using model and semplified systems (protein aqueous solutions) and was then extended to raw and roasted almond and halzenut samples and spiked samples (100 ppb). For the model solutions the reaction with acrylamide was carried out for 16 h at 45 °C. After acid hydrolysis with HCl 6 M, the obtained amino acids were detected by GC/MS as TMS-derived. The cysteine-acrylamide-adducts were identified by comparison with the expected fragmentation ion masses. The reaction with acrylamide in the model solutions was also monitored by MALDI/ToF-MS. The extraction of protein-acrylamide-adducts from roasted samples was effected through several step of clarification and purification (extraction in phosphate buffer, fractionated precipitation with ammonium sulphate, etc.).
Results: The results indicated that in the model solutions the reaction with acrylamide on cysteine residues presents high yields at moderate temperature (45 °C). The elaborated analytical strategy appeared suitable for proposed purpose. The recovery analysis indicated that the traced method could be suitable for the monitoring of protein-acrylamide-adduct production up to low levels (10 ppb) in roasted almonds and hazelnuts.
Conclusions: The elaborated analytical strategy indicated that advanced mass spectrometry techniques combined with traditional analytical techniques of extraction and purification could be efficient analytical tools for the detection of protein-acrylamide-adducts in heated foods for quality assurance and control.

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OPTIMIZATION OF PEAK CAPACITY IN ONE- AND TWO-DIMENSIONAL NANOLC

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In order to obtain the best compromise between peak capacity and analysis time for highly complex proteomic samples it is necessary to optimize column technology and LC conditions in one- and two-dimensional HPLC. The effects of gradient time, column temperature, flow rate, and column length were studied to maximize the peak-capacity-to-analysis-time ratio for peptide samples in one-dimensional RP gradient nanoLC. An offline 2D-LC approach was developed with a microfractionation option of the autosampler allowing automated fractionation of peptides separated in the first-dimension ion-exchange separation and reinjection of the fractions onto a 15-cm long second-dimension RP nanoLC column. To get the highest peak-capacity-to-analysis-time ratio, effects of ^{1}D gradient time, sampling time, and ^{2}D gradient time on performance were studied. Optimal 2D-LC conditions at undersampling conditions were obtained applying a short (10 min) ^{1}D gradient and 20 min ^{2}D RP gradients. For separations requiring a maximum peak capacity of 360 1D-LC was superior to the off-line 2D-LC approach in terms of analysis time. Although a 1D peak capacity of 450 can be easily obtained applying 120 min gradients, for separations requiring a peak capacity higher than 360 it is more profitable to perform a 2D experiment due to its higher peak-capacity-to-analysis-time ratio. Finally, the potential of offline 2D-LC coupled to tandem mass-spectrometric detection is demonstrated with the analysis of a tryptic digest of a 20 protein mixture and an E. coli digest.

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TECHNICAL ADVANCES IN PROTEOMICS MASS SPECTROMETRY: IDENTIFICATION OF POST TRANSLATIONAL MODIFICATIONS

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Background: The importance of post-translational modifications (PTMs) of proteins is notably increased in the proteomic era as they play a critical role in modulating cellular functioning and can vary in response to different stimuli, tuning cellular mechanisms. The assessment of PTMs on a proteomic scale is a challenging task since they are substoichiometric, transient and reversible. Moreover, the amount of post-translationally modified proteins is generally very low as compared to their unmodified counterparts.
Existing methodologies for PTMs identification essentially rely on specific enrichment procedures able to selectively increase the amount of modified peptides. These procedures have to be integrated with sophisticated mass spectrometric experiments to address the identifications of PTMs.
Although the strategies developed so far are not endowed with general applicability and they are far from being satisfactory and productive, a number of examples will be given where the combination of innovative separation methods with advanced mass spectrometric analyses provided positive results.
These experiences are opening up the way for a next generation of proteomic approaches for the identification of a wide range of post translational modifications.
Methods: Recently our group introduced an easy to hand strategy to give preliminary insights for the comparison of glycoproteomes in healthy and pathological human sera, by using a single affinity chromatography step coupled with mass spectrometry techniques. The N-linked glycopeptides resulting from trypsin digestion of sera samples were selectively enriched in order to enhance identification of N-glycosylation sites using LC-MS/MS and to obtain glycoforms profiles by MALDIMS analysis. Moreover, a novel strategy for PTMs identification specifically developed to selectively label phospho-Ser/Thr and nitrated residues is proposed. We suggested a derivatizing step by using dansyl chloride including a chemical moiety capable to fragment in a peculiar and specific way, thus allowing selective mass spectral analysis of modified peptides through the exploitation of the enormous potential of MSn techniques.
Results: Human sera from two different groups of pathologies (myocardial lesions and hepatic carcinoma) were used for the glycosylation analysis as they represent different examples of inflammatory and tumor diseases. Glycans from pathological samples were characterized by abnormal glycosylation patterns, taking into account branching, sialylation and fucosylation. Both of them showed an excessive expression of peculiar structures, the persistence of incomplete and truncated structures, the accumulation of precursors and the appearance of novel structures, thus leading to a higher degree of heterogeneity with respect to the healthy one. As an example, the presence of more than sixty different glycoforms was observed in the hepatic carcinoma serum.
To address a larger number of PTMs should be regarded as particularly attractive and advantageous, providing increased confidence in the PTMs identification, we proposed a general strategy (RIGhT, Reporter Ion Generating Tag), to indicate a common derivatization method based on the labelling of target residues with reagents capable of generating reporter ions in MS2/MS3 experiments. The RIGhT method is based on a) the selective modification of target residues with dansyl chloride or other available dansyl reagents and b) the selective detection and identification of labelled peptides by their characteristic fragmentation pathway in MS/MS experiments as well as the diagnostic 234-170 m/z transition in MS3 mode. Precursor ions scan of the 170 m/z fragment can then be combined with MS2 and MS3 experiments to specifically detect only the parent ions producing the 234-170 m/z fragmentation. We employed the RIGhT strategy to the selective identification of phosphoSer/Thr peptides and to the identification of nitration sites in complex mixtures.
Conclusions: The assessment of substoichiometric and transient post-translational modifications on a proteomic scale is still challenging. Existing procedures are not endowed with general applicability and the successful identification of PTMs still depends on a number of factors including the nature and the extent of the modifications, the amount of sample available etc. Despite advances in technologies and methodologies, the proteomic approach to PTMs seems still far from being satisfactory and productive.
The recent trend shown by research in this field suggests that scientists are moving along two main paths that might also be combined together. Areas that are receiving prompt attention are related to sample preparation and separation technologies with particular emphasis on specific enrichment procedures. The goal is to develop selective methodologies able to largely increase the amount of modified samples as compared to the unmodified one.

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NEW AFFINITY CHROMATOGRAPHY FOR THE HIGHLY SELECTIVE ENRICHMENT OF MONO- AND MULTI-PHOSPHORYLATED PEPTIDES IN PHOSPHOPROTEOME ANALYSIS

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The most challenging analytical task facing phosphoproteome determination requires the isolation of phosphorylated peptides from the myriad of unphosphorylated species. In the past, several strategies for phosphopeptide isolation have been proposed in combination with subsequent mass spectrometric investigations. Among these techniques, immobilized metal affinity chromatography and titanium dioxide have been recognized as the most effective. Here, we present an alternative method for the enrichment of phosphopeptides using an insoluble form of calcium phosphate. In this way we developed an efficient method for the selective separation and fractionation of phosphorylated peptides. The effectiveness and efficiency of recovery for this procedure was assayed using tryptic digests of standard phosphorylated protein mixtures. Based on the higher affinity of multi-phosphorylated peptides, the introduction of a phosphate buffer gradient for stepwise peptide elution resulted in the separation of mono-, di-, tri-, and multi-phosphorylated peptides. Thus, e demonstrated that this technique is highly selective and independent of the the degree of peptide phosphorylation.

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CROSS-LINKING BETWEEN SUMO-1 AND PROTEASOME INHIBITION: A PROTEOMIC APPROACH

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Background: Small ubiquitin-related modifier (SUMO) is a member of the superfamily of ubiquitin-like proteins that are covalently linked to various intracellular target proteins regulating their function, location, and half-life. Despite the similarity between SUMO and ubiquitin, the molecular consequences of these two modifications are distinct. In some cases, like IkBalpha modification, SUMO plays an antagonistic role to ubiquitin, competing for the same lysine. In other cases, as for NEMO/IKKgamma, SUMO and ubiquitin are conjugated in a sequential manner in response to a toxic stress; in further cases SUMO may regulate protein localization, stabilizing substrate, independently from ubiquitination, as for Smad4 modification. The mechanism of exchange between SUMO and ubiquitin remains unclear. Several recent studies indicate that there is a crosstalk between ubiquitinated and SUMO modified proteins in concomitance with proteasome activity. In order to study the cellular role of SUMO in the ubiquitin-proteasome system, we have investigated the effect of proteasome inhibition on conjugated proteins.
Methods: We have analyzed the subcellular distribution of sumoylated proteins in HeLa cells upon MG132 treatment and we have identified nucleolar SUMO-1 targets by mass spectrometric techniques. Moreover we have measured the effect of MG132 stimulus on modified targets by SILAC (Stable Isotope Labeling by Amino acids in Cell culture). To validate the *in vivo* identified SUMO-1 targets, we have performed an *in vitro* sumoylation reaction on HeLa nuclear extract as source of SUMO-1 targets. Then novel interactors were confirmed using coprecipitation and pull-down tests.
Results and conclusions: In this study we have demonstrated that a cross-talk exists between SUMO-1 and the ubiquitin-proteasome system. In particular, we have observed that, upon MG132 treatment, there is a complete redistribution of SUMO-1 targets from nuclear dots into nucleolar structures. Interestingly, using proteomic approaches, we have identified 114 SUMO-1 nucleolar targets involved in ribosome biogenesis, protein complex assembly, RNA splicing and metabolism, chromatin packaging and remodelling and DNA replication. In addition, thanks to quantitative analysis, we found that 58 of these substrates change their level of sumoylation in response to proteasome inhibition. These results confirm that there is a relationship between SUMO-1 and the ubiquitin-proteasome system suggesting that SUMO-1, together with ubiquitin, may ensure the integrity of nucleolar organization. Further investigations on the biochemistry and cell biology of SUMO-1 target proteins identified in this work should help to understand the mechanism by which SUMO-1 acts together with the proteasome machinery.

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PROTEOMIC ANALYSIS OF HUMAN TEAR FLUIDS USING DIFFERENTIALLY LABELED TAGS iTRAQ WITH LC- MALDI MS AND MS/MS

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Proteome profile of tear fluids can be used for disease diagnosis.
The ocular tear film is a complex mixture of ions, small molecules, glycoproteins and proteins, forming a thin film over the surface of the cornea and conjunctiva, protecting the eye against microbial challenge and preserving visual acuity; changes in tear film conditions are usually related to several eye diseases.
The aim of this work was to study the quantification of proteins in the human tear proteomic profiles of healthy and patients in order to find biomarkers for disease diagnosis.
We thoroughly studied the tear proteomic profile of healthy subjects and patients with VKC (vernal keratoconjunctivitis) by LC - MALDI MS and MS/MS, focusing in particular the low mass range region, in which bioactive peptides are present.
The samples were previously tagged using the 4-plex iTRAQ™ technique to determinate relative protein levels in four samples simultaneously; then the samples were pooled and fractionated by liquid chromatography; the collected fractions were mixed with a matrix solution and analyzed by MALDI MS to determine the mass of the precursor peaks of interest and by MALDI MS/MS to fragment the labeled peptides for identification and quantitation.
iTRAQ™ Technology (Multiplexed Isobaric Tagging Technology) uses a chemical tagging reagent which allows multiplexing of two to eight protein samples and produces identical MS/MS sequencing ions for all eight versions of the same derivatized tryptic peptide. Quantitation is achieved by comparison of the peak areas and resultant peak ratios for either four MS/MS reporter ions, each of which has a different mass (114.1, 115.1, 116.1 or 117.1 Da). The analysis of the intensity of reporter ions allows the simultaneous sequencing and quantification of labeled peptides
To verify the proteins identified we used the Mascot MSMS search engine and SwissProt database.

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EXPANDING THE ROLE OF ITRAQ® CHEMISTRY: QUANTITATIVE ANALYSIS OF POST-TRANSLATIONAL MODIFICATIONS

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Background: Several labelling strategies have been developed to target specific amino acid residues and/or post translational modifications (PTMs) enabling the enrichment of subfractions via affinity clean-up. The presence of phosphorylation, glycosylation, nitration, and specific types of oxidation are examples of PTMs that can be targeted.

Stable isotope methods have been introduced into MS-based proteomics to allow relative changes in protein expression to be determined. The principle of these methods is the incorporation of a stable isotope derivative in one of the states to be compared. Stable isotope incorporation shifts the mass of the peptides by a predictable amount. The ratio of analyte between the two or more states can then be determined accurately by the measured peak ratio between the heavy and light derivatized samples.

A novel methodology for quantitative analysis by mass spectrometry makes use of iTRAQ (acronym for Isobaric Tag for Relative and Absolute Quantification) technology, a newly developed method by Applied Biosystems for relative and absolute quantification of proteins.

Methods: The iTRAQ reagents are specifically reactive towards primary amino groups (namely N-terminal of proteins and peptides and amino groups of lysine) and marketed in four different forms called iTRAQ 114, 115, 116 and 117, depending of m/z value of reporter group. The iTRAQ reagents allow the simultaneously multiplexed analysis of four or eight samples. In our strategy, the tryptic digests from the entire protein mixtures are labelled with iTRAQ and directly analyzed by MS on a hybrid triple quadrupole/linear ion trap mass spectrometer. Discrimination between nitro- and unmodified peptide is based on two selectivity criteria obtained by combining a precursor ion scan and MSMS analyses.

Results: So far the iTRAQ chemistry has been limited to primary amines. We are planning to wide the chemistry of the reagent to address quantification of function of other that primary amines by taking advantage of the experience made with dansyl chemistry. In fact, our group has already reported a new approach involving dansyl chloride labelling of the nitration sites that rely on the enormous potential of MSn analysis. Here we report a new strategy based on the use of iTRAQ reagents coupled to mass spectrometry analysis for the selective labelling of several post translational modifications suche as nitration, phosphorylation, carbonylation. This derivatization introduces an iTRAQ moiety that fragments in a peculiar way, according to previous data. Using the great capabilities of a new hybrid mass spectrometer equipped with a linear ion trap analyzer, one can take advantage of the distinctive m/z 114-117 and m/z 145 fragments in MS^2. We found that to enhance the selectivity of LC/MSMS analysis good results were obtained increasing the signal threshold of PIS using the great difference in signal intensity between the iTRAQ reporter ions and other ions occurring in the same mass range. We inferred that our LC-MS/MS method resulted to be a good compromise between selectivity and sensibility that are fundamental parameters in nitro-proteome analysis because of the sub-stoichiometric characteristic of protein nitration. In fact we showed that, without any chromatographic nitro-peptide enrichment, just using the mass spectrometer gas phase fractionation features, we had eliminated about 96% un-desired peptides detected with a classical LC-MS/MS approach, thus leading to the selective identification of post-translational modification sites.

Conclusions: This method was proved to lead to the simultaneous localisation and quantification of modified sites both in model proteins and in biological systems.This work represents the first innovative application of iTRAQ labeling strategy to the analysis of post translational modification, thus providing a powerful analytical method to identify nitrated protein, localise tyrosine nitration sites and to quantitate the extent of protein nitration in a single experiment.

Here the strategy proposed, taking in account the availability of linear trap to select specific labeled peptides giving rise to diagnostic MS/MS product ions, resulted to be of interest in the selective detection of protein nitration, phosphorylation and carbonylation. Moreover, because of its operational simplicity, avoiding long-lasting and time-consuming fractionation procedures, this new strategy seems to be well suited for large-scale quantitative profiling of PTMs.

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QUALITATIVE PROTEOMIC STUDY OF TEAR FLUIDS BY LC-MALDI TECHNIQUES.

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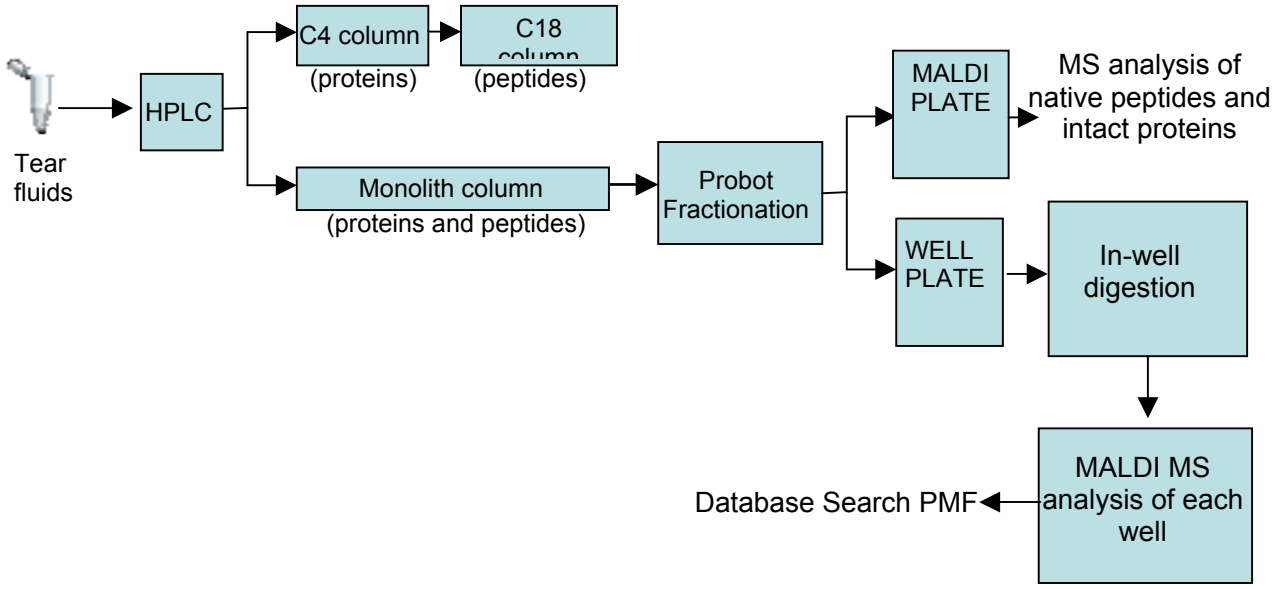
The tear film is a thin layer of fluid that covers the ocular surface and is involved in lubrication and protection of the eye. The composition is complex, both in the variety of protein present as well as the dynamic concentration range which can span up to 10 orders of magnitude. Little is known about the protein composition of tear fluid but its deregulation is associated with disease states, such as diabetic dry eyes, Sjögren's syndrome, conjunctivitis, blepharitis, inflammatory process.
Protome profiles of tear fluids may play an important role in ocular diseases diagnosis and prognosis. This makes body fluid an interesting candidate for in-depth proteomic analysis.
Unfortunately, only a small volume of tear fluid can be collected from pathological and healthy subjects, which makes proteome profiling a challenge.
We have developed an analytical method to study proteomic profile of human tear fluids based on RPLC and MALDI MS. In order to analyzing simultaneously protein and peptides in an only chromatographic run, we use PS-PDB monolithic column instead of coupling C4 and C18 columns.



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AN EASY ALTERNATIVE PROCEDURE FOR MICROSCALE SOLID-PHASE PERMETHYLATION OF OLIGOSACCHARIDES FROM GLYCOPROTEINS

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Background: Glycosylation is among of the most frequently occurring post-translational modifications of proteins and is related to physicochemical (folding, conformational stability, solubility, resistance to proteases) as well as functional properties (cellular recognition, cell-cell interaction, immune response, protein transport). Prior to structural analysis by mass spectrometry (MS), glycans are usually released from glycoprotein(s) by exoglycosidases (N-glycans) or β-elimination (O-glycans). Although most sugars can be analyzed in the native form by either MALDI or electrospray MS, their derivatization by permethylation enormously enhances the analytical sensitivity, stabilizes the sialic acids residues and permits the comprehensive mapping of both neutral and acidic oligosaccharides in a single spectrum acquired in the positive ion mode. Permethylated sugars are detected in the MALDI/MS positive ion mode as Na^+-adducts while they can occur as multi-charged protonated ions in the electrospray MS. MALDI/MS of permethylated sugars is a powerful analytical approach to the structural characterization of glycans which also provides quantitative information. Even though the complete and detailed structure of biologically originated glycans relies on a variety of integrated analytical methodologies, permethylated oligosaccharide structures are relatively simple to assign by measuring molecular mass and considering the natural micro-heterogeneity associated to glycoproteins. Sometimes, for structural assignment it can be necessary to perform MS/MS analysis. The "in solution" permethylation pioneered by Ciucanu and Karak has recently been improved by Mechref and Novotny with a solid-phase strategy which makes use of spin columns or fused silica capillary as reactors, and allows the characterization of sugars deriving from sub-microgram amounts of glycoproteins. Here, we describe an easy alternative procedure of solid-phase permethylation performed in disposable GeLoader Tips (Eppendorf) on glycans in low-picomol amounts, prior to MALDI/MS analysis.
Methods: N-linked glycans were released by peptide N-glycosydase F hydrolysis (PNGase F of *Flavobacterium meningosepticum*) of tryptic peptides of standard chicken ovalbumin (1 µg) and bovine fetuin (1 µg). Samples were dissolved in 20 µL of 50 mM ammonium bicarbonate pH 8.0 followed by addition of 0.5 mU PNGase F and incubated at 37 °C for 18h. Glycans were purified by loading the reaction mixtures onto manually packed C18 (with R2 resin, Applied BioSystems) microcolumns and collecting the flow-through and 40 µL of a 0.1% TFA eluate. Glycans were lyophilized and then permethylated adapting the procedure by Mechref and Novotny utilizing the GeLoader Tips as disposable capillary reactors. Sugars were dissolved in 93 µL DMSO containing 3% (v/v) of H_2O followed by addition of 33.6 µL of CH_3I. The reaction mixture was passed eight times through a GeLoader Tip packed with roughly ground NaOH (filled up to ~1-1.5 cm of height), applying a gentle pression with a plastic 1mL syringe. Chloroform (400 µL) was added to the eluate and three-fold extracted with 400 µL water. The $CHCl_3$ layer, containing the permethylated glycans was evaporated to dryness and reconstituted in 20 µL of 50% EtOH (v/v) / 0.2% formic acid and 1 mM sodium acetate. The same procedure was also carried out on the glycans obtained from a bleu-silver stained SDS-PAGE band of *Phaseolus vulgaris* phytohemagglutinin (200 ng) in gel trypsinized. MALDI/MS experiments were carried out on a Voyager DE-Pro (Applied BioSystems) instrument operating in the reflector positive ion-mode, using DHB as the matrix.
Results and conclusions: The performances of all the solid-phase permethylation strategies are clearly superior to the in-solution method, since they avoid excessive sample handling, prevent degradation and formation of partially methylated sugars or undesired by-products resulting from the use of extremely basic aqueous solution. The drastic conditions of the in solution method, in fact, are known to induce peeling reactions or oxidative degradation of glycans.
The method in "GeLoader Tips" is a fast, easy and inexpensive alternative procedure of solid-phase permethylation of glycans. It was tested on neutral and acidic mixtures of N-linked glycans, including high-mannose, asyalilated and syalilated complex structures, with the possibility to be extended to the analysis of O-linked oligosaccharides. Loss in the permethylation efficiency was observed for complex branched glycans, which apparently require a slightly higher concentration of methyl iodide for complete derivatization. The described strategy combine the easiness of the in "spin column" solid-phase procedure with the increased sensitivity of the "in fused silica capillary" approach. With respect to the latter procedure the in GeLoader Tips procedure does not require electronic pumps to deliver solvent to the micro-reactor or any elaborate apparatus of tube connections. The overall time required for permethylation of a sample ranges within 5-10 min and no further sample clean-up steps are necessary before MS analysis. The method was

successfully applied to profile glycans released from fetuin and ovalbumin as low as 1 microgram level. Furthermore, the two N-linked glycan structures obtained from 200 ng of in gel electrophoresis digested *P. vulgaris* phytohemagglutinin were distinctly detected and assigned by MALDI/MS analysis in the reflector mode. This demonstrates that the procedure is capable to be further scaled-down to the analysis of sugars from glycoproteins at the proteomic level. The relative mass signal intensity reflected the abundance of each glycoform, thus suggesting that efficient permethylation occurred for all glycans. In addition, by analogy with the previous reported strategies, it can be inferred that the recovery of the derivatized glycans was complete. Therefore, we suggest the comparison of glycomic maps in studies of functional glycomics.

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IMPROVEMENT OF MEMBRANE PROTEIN RESOLUTION BY DIFFERENT 2DE APPROACHES

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Background. Membrane proteins comprise approximately 30% of human proteins, and may account for substantially more cellular functions. Despite the importance of membranes in any living system, the compositional analysis of membrane subproteomes is still an obstacle. Classical proteomic approaches allow only a limited protein analysis, in fact there is an almost complete lack of hydrophobic proteins on 2-DE gels. Due to this limitation in separating membrane proteins, various alternative techniques in gel-based, e.g., blue native-PAGE (BN-PAGE), clear-native-PAGE (CN-PAGE), benzyldimethyl-n-hexadecylammonium chloride (16-BAC), and SDS/SDS (dSDS-PAGE) have been employed in membrane proteomics. In particular our research focalizes on protein composition of plasma membrane microdomains, small, heterogeneous, highly dynamic, sphingolipid- and cholesterol-dependent lateral assemblies, involved in fundamental cellular functions, like signalling, and in neoplastic transformation. Regarding plasma membrane microdomain proteome, it is an open challenge in spite of the significant progresses in the last years. In order to get more insignt into this issue, we tackled the study of microdomain proteins by different and combinatorial types of in gel electrophoresis separations: classical 1D and 2D PAGE, 16-BAC/SDS-PAGE and 2D BN/SDS-PAGE.
Methods. Subcellular fractions, microdomain-enriched, were prepared by differential centrifugation from rat kidney and kidney cell lines, as models to set up novel analytical tools, and from surgical samples of RCC and adjacent normal kidney (ANK), following nephrectomy. After treatment with Triton X-100 (1%), in which microdomains are insoluble (Detergent Resistant Membranes, DRM), the fractions floating after sucrose density gradient ultracentrifugation, were isolated, and analyzed by different PAGE techniques, followed by Coomassie Blue staining and immunoblotting for known microdomain proteins (caveolin-1 and aquaporin.1). In our experiment we compared classical 2DE with 16-BAC/SDS-PAGE using the cationic detergent benzyldimethyl-n-hexadecylammonium chloride in the first and the anionic detergent SDS in the second dimension. Moreover we performed on the same samples the separation of native membrane protein complexes through the application of blue native gel electrophoresis (BN-PAGE); after the set up of solubilisation conditions of each type of sample (different dilution series of different non-ionic detergents), second dimensions were performed.
Results. We tested alternative bidimensional electrophoretic techniques to separate membrane proteins: 16-BAC/SDS-PAGE (2D 16-BAC) and 2D BN/SDS-PAGE (2D BN), against the traditional IEF/SDS-PAGE (2DE). Results show that overall quantitative recovery of membrane proteins is increased using 2D 16-BAC separation, in comparison with the conventional 2DE. Recovery of known membrane proteins was checked, by comparison with monodimensional separation, by electrophoresis and immunblotting, followed by MALDI-TOF MS. 2D BN gives us different and additional information. The samples behave differently after the same detergent treatment, owing to their distinct molecular composition: especially, it is evident in RCC microdomains. Moreover we can observe the localization of typical microdomain proteins in different protein complexes: in fact, immunoblotting performed on rat kidney and RCC samples show that aquaporin-1 belongs to a complex with a molecular mass around 500 kDa, while caveolin-1 to a larger one (800 kDa). This last finding is confirmed by the identification on caveolin-1 in excised bands at high molecular weight derived from RCC microdomain samples separated by 1DE (altro poster).
Conclusions. The application of these different electrophoresis approaches shows potential in overcoming the usual hydrophobic protein under-representation in proteome analysis. Conventional 2DE is a very high resolving power, but it is applicable to the analysis of membrane proteins with minor hydrophobicity. In contrast, 16-BAC/SDS-PAGE is very efficient at separating integral membrane proteins. Moreover, 2D BN/SDS-PAGE allow to determine the compositions of membrane protein complexes. Therefore, a combinatorial approach is suitable for maximum coverage of a membrane proteome, identifying also physiological protein–protein interactions, and therefore eases the search for candidate tumour biomarkers among membrane proteins. The present work has been supported by FIRB: Rete Nazionale per lo studio del proteoma umano (n. RBRN07BMCT).

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APPLICATION OF MULTIVARIATE METHODS FOR THE ANALYSIS OF PROTEOMIC SPOT VOLUME DATASETS FOR THE IDENTIFICATION OF PANELS OF POSSIBLE BIOMARKERS

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Background: Two-dimensional gel-electrophoresis (2D-PAGE), is certainly one of the most powerful and widespread techniques for protein separation. It is widely exploited for the identification of possible biomarkers in several research fields as clinical proteomics, botany, toxicology, microbiology, food safety and analysis. However, the high complexity and often low reproducibility affecting 2D gel-electrophoresis partially hamper its application in both biomarker discovery and development of diagnostic tools. The double problem of high complexity of the maps obtained and their low reproducibility has been tackled by our research group by the development of different methods for the comparison of sets of 2D-PAGE maps; these methods are based on the use of multivariate pattern recognition (Principal Component Analysis and Cluster Analysis) and classification methods (Linear Discriminant Analysis, SIMCA, PLS-DA). Multivariate tools are in facts much more effective than classical univariate statistical tests, as Student's t-test, in the identification of panels of possible biomarkers since they allow to properly consider the relationships, i.e. the correlations, existing between variables.
Methods: The problem of low reproducibility and high complexity characterising 2D-PAGE maps has been dealt with by our research group *via* two different approaches: 1) the analysis of spot volume datasets coming from the analysis of 2D-PAGE maps by dedicated software packages (i.e. PDQuest, Melanie III etc.); 2) the direct analysis of 2D-maps images by multivariate tools. Here, different strategies for spot volume data analysis will be described in more detail reporting the most recent results obtained from different cases study. Pattern recognition and classification tools have in facts been applied in the last few years by our research group to spot volume data; in particular, principal component analysis (PCA) coupled to classification tools as SIMCA, linear discriminant analysis (LDA) and partial least squares discriminant analysis (PLS-DA) were applied to different datasets of different complexity.
Results: Different datasets are considered: 1) *Neuroblastoma,* consisting in 8 maps from adrenal mouse glands extracts, divided in two classes of 4 samples each (532 spots revealed): control and diseased; 2) *Cell lines*, consisting in 10 maps from human lymphoma cells, divided in two classes of 5 samples each (264 spots revealed): GRANTA (commercial cell line) and MAVER (new established cell line); 3) *Nuclei,* consisting in 11 samples of colon cancer cells described by 779 spot each: control (6 samples) and treated by a HDAC inhibitor (5 samples); 4) *Pancreas*, consisting in 18 samples from human tumoral pancreatic cell extracts, divided in 4 classes (435 spots revealed): control samples of PACA44 and T3M4 cell lines and samples from the same two cell lines treated with Trichostatin-A. The four datasets represent some typical problems the researcher has to face in proteomics: in diagnosis/prognosis, the identification of the differences in the proteomic profile of control and diseased samples (*Neuroblastoma* data set); in the field of product development, the identification of the differences occurring between two different cell lines in order to evaluate the possibility to commercialize a new established cell line (*Cell lines* data set); in drug design, the identification of the role played by drugs on the proteomic profile of the target cell (*Nuclei* data set) or the effect of a particular active principle on different cell lines (*Pancreas* data set). Different multivariate tools were applied to the selected datasets: PCA, SIMCA, LDA, PLS-DA and allowed the identification of pools of possible biomarkers. The selected cases represent some of the most recent applications published by our research group.
Conclusions: The results obtained pointed out the effectiveness of multivariate tools in the identification of panels of possible biomarkers. The results from multivariate analysis were compared to those obtained by standard univariate analysis and showed that multivariate methods allow the identification of a larger number of significant spots thus improving the possibility of identifying actual effective biomarkers. Some of the selected significant spots were also characterised by mass spectrometry.

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DIFFERENTIAL PROTEIN EXPRESSION BY LC-MALDI ANALYSIS OF MICROGRAVITY EFFECT ON CARDIAC TISSUE

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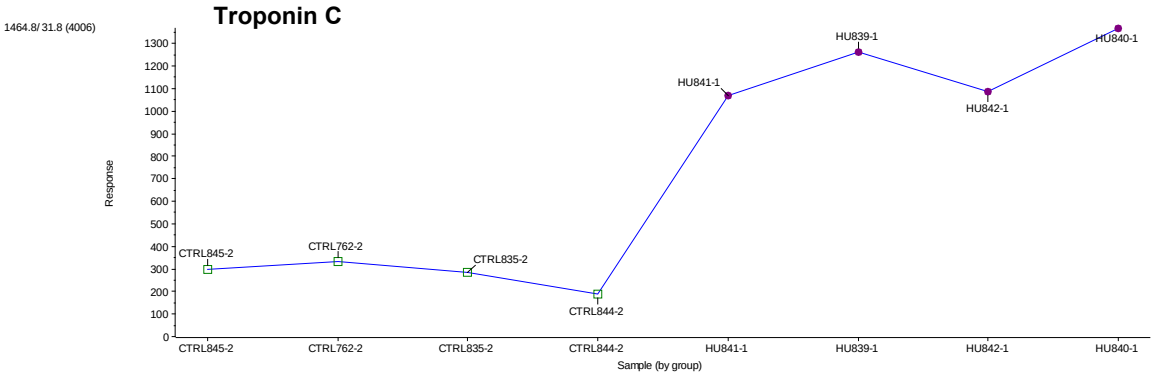
Background: The influence of microgravity on corporal fluids is known as “fluid shift”. The cardiovascular system will adopt a respond to the shift which can produce reduced contraction, cardiac stress, hypoxia, ischemia. Usually, it is associated with oxidative stress after return to Earth's gravity and with a differential expression of proteins involved in cardiac stress and metabolism which could be analyzed by proteomics investigation. We use a label-free LC-MALDI-based approach to analyze the differential protein expression between case/control mice heart samples.
Methods: This kind of stress (microgravity) has been applied by suspending four healthy mice in special cages for 2 weeks, with the body inclined at 30-40° from the horizontal plane. At the end of suspension period, the stressed group and the control group of mice were sacrificed and the hearts removed and frozen promptly in liquid nitrogen. Whole mouse heart tissue was subjected to a differential solubility extraction procedure, obtaining a neutral protein extract and an acidic one. After enzymatic digestion of the two protein classes, the peptide mixture was fractionated by nano-HPLC, using a C18 column. The fractions were collected by a robotic system and spotted on the target for MALDI TOF/TOF analysis. A mass range from 900 to 4000 Da was considered for peak selection. The top 15 masses in each spot (12-s chromatography time) were then selected for MS/MS analysis. Searches were performed against the IPI protein database for Mus Musculus. Variable modifications such as methionine, proline and cysteine oxidation were applied to Mascot search to evaluate the increase in oxidative stress. A positive identification was accepted at the 97% confidence level. Peak lists obtained were processed using MarkerView 1.2 (AB/SCIEX) and t-test between case/control groups was perfomed together with principal component analysis to identify most discriminative ions.
Results: Thanks to a very high resolution of the LC-separation and a very high performance of the MS-MS analysis, we obtained the identification of 500 acidic proteins and 420 neutral proteins. The overlap between the two extracts was under the 20%, that indicates the selective extraction validity. The analysis of the differential modifications between case/control samples show an increase in oxidative modification in cardiac proteins as: aconitase, creatine kinase, cytochrome c oxidase, desmin, malate dehydrogenase, myosin, troponin, tropomyosin etc as reported for cardiovascular diseases oxidative stress. A multivariate analysis was applied to the case/control samples after perform a global normalization of the peak lists. The statistical methods evidenced a differential expression in contractile proteins (as troponin C and tropomyosin) as well as in proteins involved in cell metabolism (as malate dehydrogenase, NADH dehydrogenase and creatine kinase).



Conclusions: In conclusion a differential extraction together with the LC-MALDI analysis allowed us to identify a large number of cardiac tissue proteins (920 total proteins). Additionally, the high performance MSMS analysis offered the possibility to evaluate the redox alterations in cardiac tissue under stress conditions. Finally, multivariate analysis and a bioinformatics approach were applied to the protein profiles for the comparative study. The results indicate variation in contractile and stress proteins and in marker involved in cell metabolism and energy supply.

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INTEGRATING CHIP BASED NANOELECTROSPRAY INTO MODERN LC/MS APPLICATIONS

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Background: Nanoelectrospray and LCMS are the preferred techniques for analysis where sample size is limited and continues to be the golden standard for acquiring qualitative and quantitative information from complex samples. Applications such as Metabolomics, biomarker discovery, lipid screening and proteomics involve the analysis of known and unknown species with a desire to quantify and characterize each component, but sample complexity limits the amount of time for MS/MS and MS^n during an LC run.
Methods: The Advion TriVersa Nanomate system integrates a chip based Nanoelectrospray emitter source with automated Infusion at very low flow rates, the coupling to nanoLC and HPLC systems, as well as the collection of LC-MS and LC fractions simultaneously.
Results Lipids: The fully automated infusion capability is used for Quantitative Lipid profiling by Multiple Precursor Ion scanning coupled on a Q-TOF type instrument. Tissue, Cell or organelle sample are directly infused after Lipid extraction and quantified and analysed by lipid profiler software. Up to 47 different lipid species are screened in a single experiment.
Results UPLC-FTMS: The combination of UPLC separation with high resolution MS is accomplished by using the simultaneous LCMS analyses and fraction collection capability with use of the Nanomate post-column splitter. The Infusion of LC fractions enables the averaging and optimization of MS/MS parameters such as collision energy or the use of different fragmentation techniques. Plant samples are grown under normal and $^{13}CO2$ conditions and extracted before injected to a UPLC system coupled to the TriVersa Nanomate and LTQFT. After the online run, interesting fractions are reanalysed by direct infusion using IRMPD fragmentation enabling the identification of low level compounds.

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A NOVEL CHROMATOGRAPHIC METHOD ALLOWS ONLINE REANALYSIS IN PROTEOMIC INVESTIGATIONS AND ACQUIRING SIGNIFICANT MORE INFORMATION FROM BIOLOGICAL SAMPLES.

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Background: Liquid chromatography combined with electrospray ionization is the standard for the analysis of polar molecules by mass spectrometry (LCMS). The online coupling in LCMS is a major strength, but has the limitation that the mass spectrometer is not able to analyze all co eluting compounds immediately. Multiple injections or fraction collection can overcome this problem but time, sample limitation and the difficulty to fraction collect low volumes from a nanoLC system makes this unpractical.

Method: A new chromatographic strategy which enables to analyze a LCMS run twice with a single injection is described. After column separation the flow from a 75 µm column at a typical flow rate of 250 nL/min is split, so that part is directed to the mass spectrometer for analysis whilst the remainder flows to a capillary tube where it is stored. After the direct LCMS run, the flow is switched and the portion stored in the capillary is analyzed (‘replay run’). Since electrospray is a concentration dependent process the splitting system maintains full signal at decreased flow rates. An additional short column between the storage capillary and the mass spectrometer refocused the stored peaks in the second analyses so that width and intensity is identical to the initial run.

Results: To qualify the set up, the chromatographically performance and MS intensity was compared with a standard nanoLC set up using BSA tryptic digest for typically 60 min separations and mouse liver homogenate for 120-180 min LCMS runs. The chromatography performance and peak intensity for the normal setup are identical with the first and second run of the RePlay setup. Furthermore to explore the ability to analyze a LCMS run twice, examples of combining exploratory and targeted analysis are shown including the quantitation, using different fragmentation techniques, combining MSMS and MSn analyses or different mass spectrometers are shown.

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PRIMARY STRUCTURE AND POST TRANSLATIONAL MODIFICATIONS OF SILICATEIN BETA FROM *PETROSIA FICIFORMIS (POIRET, 1789).*

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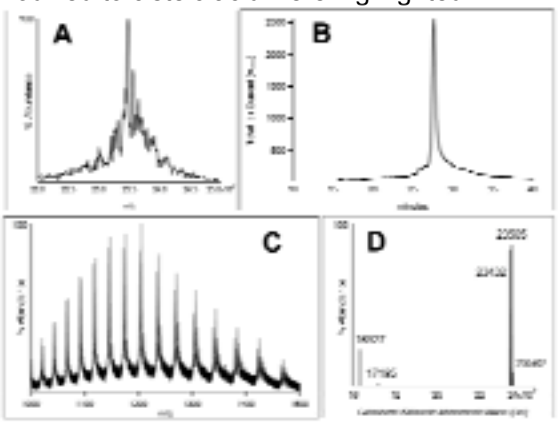
INTRODUCTION Biosilica is an amazing example of natural order and complexity. Siliceous sponge spicules, in particular, are characterized by a large variety of dimensions and shapes, with an ultrastructure based on silica nanoparticles strictly packaged around an axial filament constituted by a family of proteins called silicateins. These peculiar proteins have a high sequence homology with cathepsins and they play a double role of enzyme and template in the control of biosilica precipitation. However, their natural structural organization inside the spicules is far from being understood in details. Silicateins are involved in the biosynthesis and condensation of biosilica in spicules from Demospongiae (phylum Porifera). In this work, axial filaments extracted from spicules of *P. ficiformis* have been extensively analyzed by mass spectrometry.

INSTRUMENTAL AND METHODS Samples coming from dissolution of spicules were reduced with dithiothreitol and alkylated. Protease digestion was performed with trypsin or chimotrypsin. Peptides were injected on NanoLC system coupled with an qTOF mass spectrometer. Both web-based MASCOT and in-house sequence search algorithms were used for sequence coverage analysis and post translational modifications search. Manual de novo sequencing was extensively exploited to analyze ambiguous results, to confirm borderline peptide identifications and unusual post translational modifications.

RESULTS Intact Silicatein appears as a multiply charged pattern of three molecules, very close in mass, at 46865, 47010 and 47125Da respectively (Fig. 1 panels B and C). These masses correspond to dimers of three isoforms of 23432, 23505 and 23562Da respectively (Fig. 1 Panel D).These data match with results from MALDI-MS, where monomers are detected (Fig.1 panel A). The “bottom-up” analysis of the total axial filament protein content allowed the identification of the N-terminus of mature silicatein. Several post translational modifications were also detected in the sequence and three phosphorylation sites Tyr 97, Ser 213 and Ser 66 were found. Other interesting PTMs, such as an extensively oxidized His15 and Cys157 modified to cisteic acid were highlighted.



LPETVDWRTGGAVT$_{oxH}^{15}$VKDQLRCGCSYAFA AVGALEGAAALARGRTASLSEQNVVDCSVPY GNHGC$_{pS}^{66}$CEDVNNAFMYVIDNGGLDTTSSYP YVSRQY$_{pY}^{97}$CKFKSSGVGATATGIVTISSGDES SLESALATAGPVAVYIDASHSSFQFYKYGVLNV PN$_{caC}^{157}$SRSKLSHAMILIGYGTTSSKKYWLLK NSWGPNWGISGYIKMSRGMSNQCGIATYA$_{pS}^{21}$ ^{3}FPTL

Table 1: Primary sequence of Silicatein and post translational modifications

CONCLUSIONS The experimental results here described were obtained exploiting different techniques with the aim to verify the protein composition of axial filament and to characterize the mature isoform of silicatein beta, whose cDNA sequence was described in a previous work of this research group. For the first time in literature, both MALDI and nanospray mass spectra of the intact protein extracted from spicules were acquired, in order to estimate the molecular mass of the proteins contained inside the axial filament. A better understanding of the mechanism of biosilica precipitation and its nanoscale organization, must pass by a complete elucidation of details on native silicatein structures. This work was partially supported by EC fund (NMP4-CT-2006-031541) to MG, by Liguria Region, area marine biotechnology funds to MG, by Italian research ministry fund (PRIN 2007) to MG.

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HIGH THROUGHPUT FULLY AUTOMATED PLASMA/SERUM FRACTIONING METHOD FOR PROTEOMIC ANALYSIS

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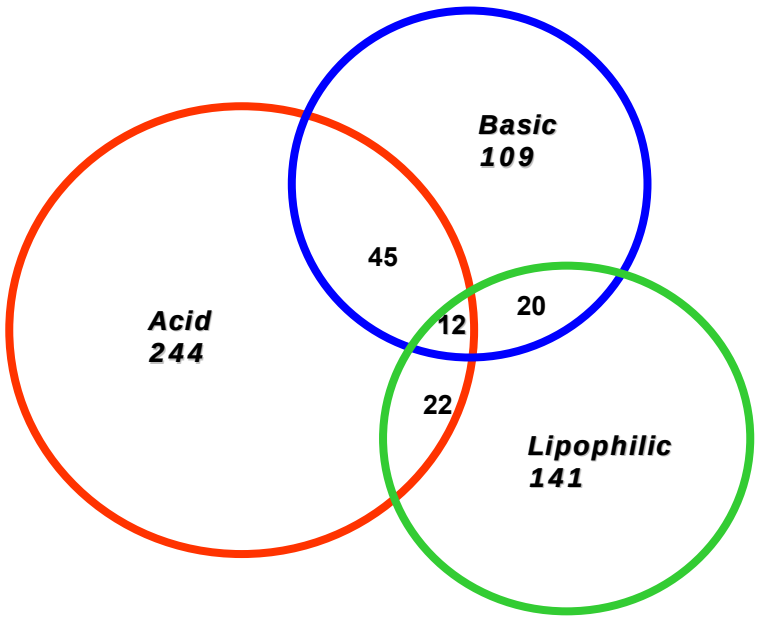
Background. The human blood plasma, including whole body tissues proteome subsets, is the most complex known proteome. The major obstacle in a proteomic study is the wide dynamic range of protein abundance present in plasma, the albumin alone accounting for the 55% of total protein content. To overcome the masking effects of the most abundant proteins and thus identify the less expressed ones, we developed and validated a method able to reduce the sample complexity utilizing a reproducible, semi-automatic robotic system.
Methods. Identical aliquots of mixed plasma samples, obtained by pooled specimens from healthy donors, were independently processed in order to optimise the automation and to test the reproducibility of the method. In a first step, the most abundant proteins were physically removed by a 30kDa cut-off ultra-filtration. In the second step, recovered flow-through was processed by original parallel multi-dimensional liquid chromatography technology (pMD-LC; patent n° PI/2008/A/000102). The different sub-families obtained (i.e. acid, basic, lipophilic proteins) were then subjected to enzymatic digestion and the peptide fragment mixtures automatically loaded (third step) on a reversed phase C18 capillary column (nano-HPLC) and fractionated (final dimension). The column effluent, mixed to MALDI matrix, was spotted by a robotic device directly on the spectrometer analytical plate. The last step of this process was the mass analysis performed on 4800 MALDI TOF/TOF (Applied Biosystems/Sciex).
Results. we used n=8 technical replicates in independent experiments. Preliminary results obtained with three subsets of proteins by the pMD-LC method indicated a very high reproducibility as shown by the comparison of UV nano-HPLC profiles of single subset.



Mass spectral data enabled us to identify 323 acid, 186 basic and 195 lipophilic proteins. The net identified proteins were 593 and among them a very low overlapping percentage was observed (2% between all three class, 14% between acid and basic proteins, 12% between basic and lipophilic and the 8 % between acid and lipophilic). Pearson correlation coefficient performed over all technical replicates showed the high reproducibility of the method and validate all three separation techniques being over 0,73.
Conclusions: The automated method we developed for processing and analysing complex plasma protein mixtures resulted highly innovative and advantageous. It reduced dramatically the analysis time over the conventional gel-electrophoresis based methods (2 week instead 3 months) improving significantly the number of simultaneously processed samples (8 instead 3). Moreover, the robotic semi-automatic sample

processing, avoiding human errors, made highly reproducible specimens analyses. Finally, the reduction of sample complexity enabled us to identify a large amount of proteins with a total ion score superior than 97%.

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MOUSE BRAIN PROTEOMICS TO STUDY THE RELATIONSHIPS BETWEEN ALZHEIMER'S DISEASE AND B-VITAMIN DEFICIENCY

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Background. Hyperhomocysteinemia is associated with Alzheimer's disease, and has been hypothesized to promote neurodegeneration. Homocysteine (HCY) level is regulated by the presence of folate, vitamin B12 and B6. Folate and vitamin B12 mediate the remethylation of homocysteine (HCY), which affects the production of the methyl donor S-adenosylmethionine (SAM). B6 is the cofactor in trans-sulfuration reactions, responsible for HCY utilization in glutathione synthesis.
In a previous study (Mol. Cell. Neurosci., 37: 731-46, 2008), B-vitamin deprivation has been shown to induce hyperhomocysteinemia and imbalance of SAM and S-adenosylhomocysteine (SAH) in transgenic TgCRND8 mice (overexpressing an Amyloid Precursor Protein gene), and that this effect was associated with amyloid- production. To further study the mechanisms linking B-vitamin deficiency, hyperhomocysteinemia and amyloidogenesis, a proteomic approach was applied to detect differential protein expression in brain tissue derived from TgCRND8 mice and wild type mice fed with a control diet (diet A), a B-vitamin deficient diet (diet B) or a diet B + SAM (diet C).
Methods. TgCRND8 and wild type (129Sv) mice were fed either on a diet A, B or C for three months after weaning and then sacrificed. Proteins from brain samples were obtained as described in Wang et al. (Proteomics 2007, 7: 4008–15) and analyzed by two-dimensional gel electrophoresis. Three replicate gels were acquired from brain protein samples derived from three different mice for each condition (transgenic or wild type mice, diet A, B or C). Gel image analysis were performed using the Bio-Rad PDQuest 7.1 software and differentially expressed spots were identified by mass spectrometry. Protein expression profiles has been also evaluated by unsupervised cluster analysis using the Gene Spring software (Agilent Technologies).
Results. Differential analysis detected a group of proteins which, both in transgenic and wild type mice, presents modified expression under condition of vitamin B deficiency (diet B). Diet C (diet B + SAM supplementation) partially restores the protein expression pattern observed in control mice. Significant changes in proteome profile between transgenic and wild type mice have not been detected. Cluster analysis shows that brain protein profile in mice under diet C is more similar to that observed in control mice compared to mice fed with diet B.
Conclusions. Identification of a group of proteins whose brain expression is affected by vitamin B deprivation may help to understand the molecular pathways that mediate the effects played by HCY/SAM metabolism alterations in Alzheimer's disease development.

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NEUROTOXIC EFFETCS OF THE FOOD-CONTAMINANT POLYCHLORINATED BIPHENYL 52 ON PRIMARY NEURONS PROTEOME

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Background. Polychlorinated biphenyls (PCBs) are persistent ubiquitary microcontaminants that bioaccumulate in humans mostly through contaminated foods of animal origins, such as meat and fish. As a consequence, diet represents the main source of low-dose chronic human PCBs exposure. PCBs can be divided into two different groups based on their biochemical and toxicological properties: the dioxin-like PCBs (DL-PCBs) and the non-dioxin-like PCBs (NDL-PCBs). NDL-PCB comprise the vast majority of congeners of PCB residues in food and data on their occurrence refers usually to the sum of six congeners including PCB52. It has been shown that their chronic exposure can affect nervous system development and function. However, the cellular and molecular mechanism underlying neuronal damage are not clear.
The *aim* of the present study was to investigate the possible neurotoxic effects evoked by the key congener PCB52, using a proteomic approach on primary cultures of rat cerebellar neurons. The proteomic approach is hypothesis-independent and might therefore provide new insight into unexplored mechanisms of NDL-PCBs toxicity. To date no proteomic data addressing the neurotoxicity of PCBs exist.
Methods. High purity NDL-PCB key congener PCB 52 has been used to avoid any interference by DL-PCBs, whose biological mechanisms are likely to be fundamentally different from those of NDL-PCBs. Primary neuronal cultures were prepared from cerebellum of 8-day-old Wistar rats. Long-term *in vitro* treatment was performed adding PCB52 (1 microM and 10 microM) to the culture medium 24h after seeding the neurons. Exposure to PCB52 was for 12 days in culture.
Analysis of differential protein expression in the total neurons lysate of untreated and PCB52-treated cells has been carried out using two-dimensional gel electrophoresis (2-DE), computerized gel image, univariate and multivariate statistical analysis for comparative proteomics, in-gel digestion and tandem mass spectrometry (LC/MS-MS).
Results. Seventeen individual proteins, out of approximately 800 spots/2-DE gel visualised by fluorescent staining, showed a statistically significant change in abundance of 1.5 times or more, as a result of long-term exposure to the highest dose of PCB52 (10 microM). No significant modulation of the protein profile was observed at the lowest dose (1 microM). Differential proteome analysis of cell lysates showed an overall perturbation in cell metabolism and energy pathways (*e.g.* alpha enolase, aconitase 2, fumarate hydrase, ATP synthase) in PCB52 exposed neurons. Moreover, exposed neurons showed an altered expression of proteins involved in folding processes (*e.g.* ER chaperones), and in neuron-specific proteins (*e.g.* GFAP; SNAP25)
Conclusions. This is the first report investigating at the protein expression level, the effect evoked by the food-contaminat PCB52 on neurons. Our findings suggests the dysfunction of mitochondrial energy metabolism due to compromised TCA cycle and oxidative phosphorylation. A compensatory response to increase ATP production might explain the up-regulation of glycolitic enzymes we observed. Reduced intracellular ATP levels would be particularly detrimental to the survival of neurons as the reestablishment of Ca^{2+} gradient and of general homeostasis following neuronal signalling, which is highly ATP-dependent. The down-regulation of the specific neuron protein SNAP25, a regulator of Ca^{2+} signalling, might aggravate this scenario. Indeed, PCB52-exposed neurons showed a decreased level of intracellular Ca^{2+} and a basal increased concentration of nitrates/nitrites and cyclic guanosine monophosphate (cGMP), suggesting that PCB52 might influences the signal transduction glutamate-nitric oxide-cGMP pathway, which play a crucial role in the modulation of intracellular events and intercellular communication, including long-term potentiation, the basis of some type of learning.

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DEVELOPMENTAL EXPOSURE TO THE FOOD CONTAMINANT POLYCHLORINATED BIPHENYLS 138 PERTURBS BRAIN PROTEOME PROFILE

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Background. The increasing prevalence of neurodevelopmental disorders cannot be explained entirely by genetic mechanisms. This has led to an active search of environmental exposures that modulate normal neurodevelopment. From such efforts, polychlorinated biphenyls (PCBs) have emerged as a credible risk factor for neurodevelopmental disorders. PCBs are persistent ubiquitary microcontaminants that bioaccumulate in humans mostly through contaminated foods of animal origins, such as meat and fish. As a consequence, diet represents the main source of low-dose chronic human PCBs exposure. The present PCBs exposure of the general population in many countries (Europe included) is in the same order of magnitude as the exposure level where subtle neurotoxic effects have been observed in infants following perinatal exposure. PCBs can be divided into two different groups based on their biochemical and toxicological properties: the dioxin-like PCBs (DL-PCBs) and the non-dioxin-like PCBs (NDL-PCBs). However, the relative contribution of DL- versus NDL-PCBs congeners to PCBs toxicity is still unclear for a large number of different end-points established in several toxicological studies, including the central nervous system. Both individual NDL-PCBs congeners and food-relevant NDL-PCBs (PCB28, 52, 101, 138, 153, 180) mixtures are poorly characterised from a toxicological point of view, and to date, there is no health risk assessment for NDL-PCBs. It is evident that efforts are still highly needed to identify toxicological mechanisms and outcomes, which are specific for NDL-PCBs.
The *aim* of this study was to assess whether prenatal exposure to the key congener PCB138 may alter the proteome profile of brain regions associated with cognitive functions when the pups are young (PND110), in order to gain insight into the mechanisms involved in neuroresponse to NDL-PCB138 exposure. To date no proteomic data addressing the neurotoxicity of NDL-PCBs exist. What makes our toxicoproteomic approach of relevance is that it is not hypothesis-driven, thus allowing the possible discovery of perturbations of pathways not previously known to be affected by NDL-PCBs.
Methods. High purity NDL-PCB key congener PCB138 has been used to avoid any interference by DL-PCBs, whose biological mechanisms are likely to be fundamentally different from those of NDL-PCBs.
Pregnant female Wistar rats were treated orally with PCB138, 1 mg/Kg/day or vehicle, during pregnancy and lactation (gestational day 7 to post-natal day 21, G7-PND21). Cerebellum from young male rats (PND110) was then analysed for its protein profile.
Analysis of differential protein expression in the total protein extract of cerebellum from prenatally unexposed and exposed rats has been carried out using two-dimensional gel electrophoresis (2-DE), computerized gel image, statistical analysis for comparative proteomics, in-gel digestion and tandem mass spectrometry (LC/MS-MS).
Results. We found that PCB138 affects the proteome profile of male cerebellum changing significantly the abundance of 27 protein species corresponding to 15 individual proteins. These proteins were mostly involved in energy metabolism (*e.g* malate dehydrogenase, glyceraldehyde 3-phosphate, aldolase C, ATP synthase), cytoskeleton reorganization (*e.g*. actin, tubulins), channel transport (*e.g*. voltage-dependent anion channel) and neuronal defence signalling (*e.g.* 14-3-3). Pathway analysis showed an overall increase in glycolisis in the cerebellum of prenatally PCB138 exposed rats. This may be a long-lasting compensatory effect to oxidative stress and mitochondrial energy dysfunction caused by PCB138. The subsequent impaired control on Ca^{2+} homeostasis and ATP levels together with the observed increased expression of actins and tubulins, cytoskeleton elements important for the clustering of proteins involved in the neuronal glutamate-nitric oxide-cGMP pathway, might contribute to the alteration of signal transduction pathways associated with NMDA receptors, that play a crucial role in some types of learning.
Conclusions. This is the first report showing significant changes, at the protein expression level, on the cerebellum of neonatally PCB138 -exposed rats. Our findings suggest that PCB138 exposure during development causes long-lasting protein changes in cerebellum, with possible consequences in the cognitive functions associated with this brain area (*e.g.* learning).

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SEQUENCE DETERMINATION OF α_s-CASEINS FROM DONKEY'S MILK BY MASS SPECTROMETRY

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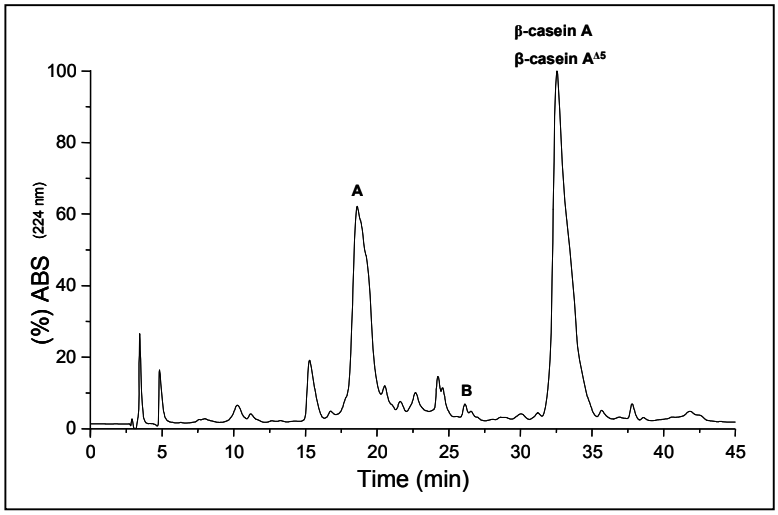
Background: Several clinical studies suggest that donkey's milk could represent a safe and alternative feeding to cow's milk for infants affected by cow's milk proteins allergy. Although the molecular basis of the reduced hypoallergenicity of donkey's milk with respect to cow's one is still unknown, it is reasonable to hypothesize that the hypoallergenic properties of donkey's milk can be related to structural differences of their protein components with respect to the counterparts of bovine milk. Unfortunately, at present time equine milk (*Equus caballus* and *Equus asinus*, Perissodactyla) has been less studied than bovine milk and limited data are available for its genetic polymorphism. Major information are available for whey proteins, whereas data for casein fraction are still scant.
We report here the sequence determination of four α_{s1}-casein variants from donkey's milk and the preliminary data concerning the verification of the mRNA derived sequence of α_{s2}-casein (GenBank Acc. N. CAV00691) by mass spectrometric methods.
Methods: The equine milk was collected at middle lactation stage from an individual donkey belonging to the Ragusano breed in the East of Sicily. After milking, the sample was defatted. The casein fraction was precipitated from skimmed milk at pH 4.3 with sodium acetate/acid acetic buffer and dephosphorylated using alkaline phosphatase from bovine intestinal mucosa. Enriched fractions of α_{s1}-CNs and α_{s2}-CN were obtained by separation of the dephosphorylated casein mixture by analytical RP-HPLC. Then, the α_{s1}-CN fraction was subjected to a 2D-PAGE analysis and the visualized protein spots were in-gel digested with modified porcine trypsin. The α_{s2}-CN fraction was directly subjected to trypsin digestion. Finally, the mixtures of enzymatic peptides were characterized by MALDI-TOF MS and capillary RP-HPLC/nESI-MSMS.
Results and Conclusion: The MALDI-TOF MS spectra of the chromatographic peaks A and B showed the presence of four components having M_r 23658, 23786, 24278 and 24406 ± 10 Da, which coelute in peak A and the presence of a protein with M_r 26030 ± 10 Da eluting in peak B.



In order to separate the four unknown components coeluting in peak A, an enriched fraction of this peak was subjected to a 2D-PAGE run. The 2DE map of this fraction revealed the presence of 2 main spots noted as A1 and A2, with apparent molecular mass of 25 kDa and p*I* 6÷6.3.
Coupling in-gel trypsin digestion, MALDI-TOF MS and capillary RP-HPLC/nESI-MSMS analysis, the investigation of these two spots was performed. The four components were identified as four donkey's α_{s1}-CN variants and their sequences characterized completely, using the known mare's α_{s1}-CN as reference (GenBank accession number AAK83668).
In comparison with the mare's α_{s1}-CN, the component with M_r 24406, migrating in spot A1, present two amino acid substitutions (Q→R[8] and H→Y[115]) and shows the insertion of the domain H[34]TPRE[38]. All together, these differences account for the increase of 656 Da in the M_r of the donkey's α_{s1}-CN, here named variant A, with respect to the mare's counterpart. The component with M_r 24278, co-migrating in spot A1 together with the variant A, lacks of the glutamine residue at position 88 and was named α_{s1}-CN sub-variant A. The components having M_r 23658 and 23786, co-migrating in the spot A2, differ from the two above reported donkey's α_{s1}-CN for the absence of the domain H[34]TPRE[38], respectively. These two donkey's α_{s1}-CN were named variant B (M_r 23786) and sub-variant B (M_r 23658).

Investigation of the tryptic digest of the component with M_r 26030, eluting in the chromatographic peak B, allowed to identify the donkey's α_{s2}-CN. Preliminary results show that the primary structure of the donkey's α_{s2}-CN is in agreement with its recently published mRNA derived sequence (GenBank Acc. N. CAV00691).

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DIFFERENTIAL EXPRESSION OF ODORANT BINDING PROTEINS BETWEEN NEWLY EMERGED AND FORAGER WORKER HONEYBEES (*Apis mellifera* L.)

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Background: In the honeybee society females are divided into two castes, queens and workers. Within the worker caste the same bee could perform different roles and behaviours such as foraging, larval feeding, grooming, patrolling and others. These behaviours are generally related to age and to gland development and can be regulated by different sets of pheromones. The recent description of the complete honeybee genome provides a potent new tool to investigate, by a proteomic approach, the protein phenotype of specific organs during aging. The honeybee genome shows 21 genes encoding Odorant Binding Proteins (OBP). In a previous investigation by the same Authors a very complex and differentiated pattern of OBP expression has been described indicating the need to outline the expression of specific genes encoding for OBP proteins only in a specific time range of the bee life span or in response to a specific physiological state or behaviour. The aim of this study was to detect and identify those OBP that are differentially expressed in newly emerged and forager bees.
Methods: To study OBP expression in the worker honeybee antennae, samples of 280 antennae were dissected from newly emerged workers sampled from a single wax frame and from foragers sampled at their return to the hive. Antennae were frozen in liquid nitrogen, powdered and extracted by vortexing for 50 min in one ml of 50 mM Tris-HCl buffer, pH 8.0, in presence of protease inhibitors. Samples were centrifuged at 14000 rpm for 20 min. The supernatant were precipitated in 10% TCA 20mM DTT in acetone. The pellet were re-suspended in 400 microliter of buffer containing 7M urea, 2M thiourea, 2% (w/v) CHAPS, 0,5%IPG buffer and 20mM DTT. Two different extracts per age were prepared and for each extract two 2-D gels were performed. The Protein content was quantified by Bradford. Isoelectrofocusing was performed on IPG strips (3-10, 11 cm). 300 microgram of antennal proteins were loaded on each strip. The separation of proteins was carried out using self-cast 15% acrylamide gels. Protein spots in the gels were visualised by MALDI compatible silver staining.
40 antennae were dissected from newly emerged and forager workers then were frozen in liquid nitrogen, powdered and the soluble low molecular weight extracted with water and 1% TFA.. Both extracts were analysed by MALDI TOF mass spectrometry.
Results: We measured an average of 3,05 microgram and 3,34 microgram of protein per antenna in newly emerged (N=140) and forager workers (N=140). To detect differentially expressed proteins the images of 2-D gels were compared for different newly emerged and forager bees extracts. 2-D gels revealed some differences in spot number and shape. Newly emerged bees antennae shows at least 14 spots differentially expressed in the molecular range between 45 and 66 KDa while forager bees shows 15 differentially expressed spots in the molecular range 14 – 20 KDa. MALDI TOF analysis of both newly emerged and forager bees antennal extracts revealed m/z signals corresponding to the value expected for three different OBPs were frozen in liquid nitrogen, powdered and extracted (OBP 1, OBP 2 and OBP 16). The identification of these proteins has been confirmed by the analysis through MS and MS/MS of the peptides obtained by enzymatic digestion of spots obtained in the 2D separation.
The analysis of the whole protein extract showed that OBP 1 was expressed in both the newly emerged and forager bees while OBP 2 were expressed specifically in the forager bee antennae. OBP 16 was highly expressed in the newly emerged bees and poorly expressed in the forager ones..
Conclusions: Proteomics appear to be a suitable approach to detect and identify OBPs avoiding the use of specific antibody. This method allowed us to determine that among the 21 OBPs predicted from the honeybee genome only three are expressed in the antennae. In particular OBP 16 is expressed only in young imago and is absent in old imago where OBP 2 arises. Expression of OBP 1 do not differ between the two investigated ages. Further investigation is needed to individuate the time range of the worker life span where OBP 16 expression end and where OBP 2 expression really starts.

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PROTEOMIC CHARACTERIZATION OF SACCHAROMYCES CEREVISIAE DURING ADAPTATION TO CHANGES IN ITS METABOLISM: FROM FERMENTATION TO RESPIRATION.

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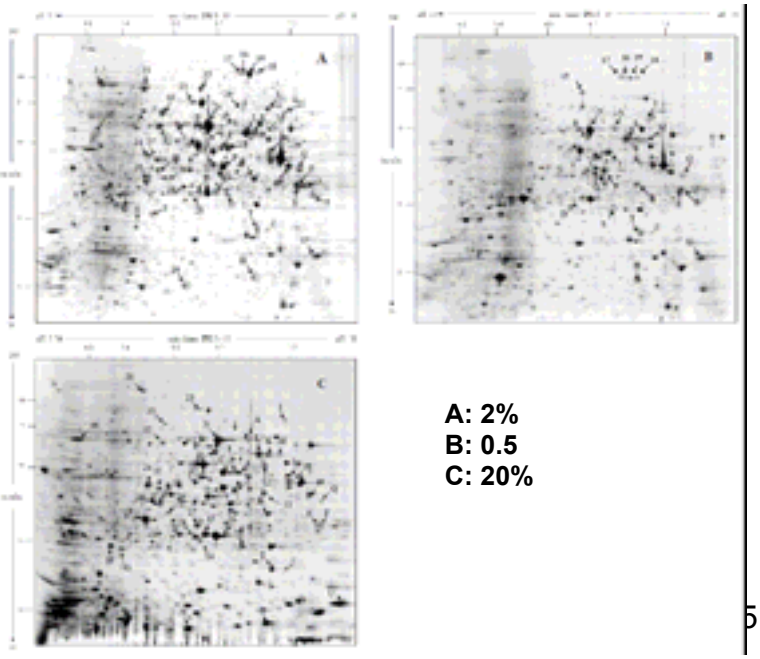
Background: Yeast is a facultative anaerobe and is able to live on various carbon sources. When it is grown on fermentable substrates like glucose, the metabolic energy essentially originates from glycolysis. If the concentration of glucose is high, the Crabtree effect is observed: cells continue to ferment irrespective of oxygen availability, due to glucose repressing / inactivating respiratory enzymes or due to the inherent limited respiratory capacity of cells. To survive to changes in the nutritional environment, yeast is able to detect the availability of nutrients and to adapt its metabolism rapidly. Studying how yeast adapts to changes in its environment, is important because it might be relevant for the optimization in industrial applications and also because it might be relevant to understand some important biological processes characteristic of eukaryotic cells. Ethanol fermentation is clearly one example of a process in which yeast cells have to adapt to significant variation throughout the whole process. We performed a proteomic study to understand how *S.cerevisiae* adapts its metabolism during the exponential growth in synthetic complete (SC) medium supplemented with three different concentrations of glucose, 0.5%, 2% and 20% respectively. Growth in 2% and 20% glucose were selected because both induce a fermentative metabolism and a concentration of 20% glucose makes the medium more similar to natural must. Yeast model of glucose restriction (0.5% glucose) has been chosen in order to study the effect of glucose limitation on the proteome of a single organism.
Methods: The cells were grown up to 0.8 O.D./ ml. At this point, cells grown in 0.5% glucose reached the natural diauxic shift, stop fermenting and start to respire, thus reprogramming part of their metabolism. On the contrary the cells grown on high glucose concentration are submitted to a high osmotic stress. We analyzed the growth rate, the ethanol production, the glucose consumption and the ROS production of yeast cells in these different conditions. We performed a proteomic analysis by 2D-GE to understand how yeast cells adapt to these different glucose conditions.
Results: Cells grown in 0.5% and 2% glucose show a higher growth rate than cells grown on 20% glucose; this may be due to a high osmotic stress under high glucose, which induce a decrease in cell growth. We saw that the ethanol production in 20% glucose was no so high as expected, in comparison to the ethanol produced in 0.5% and in 2% glucose. Furthermore the glucose consumption in 20% glucose was lower than in 0.5% and 2% glucose. We also observed a higher ROS production in 20% glucose in comparison to the others two conditions. Our proteomic analysis pointed out a lot of proteins that change their expression level in 20% and 0.5% glucose in comparison to the 2% glucose, chosen as control. These spots are indicated in figure. We saw that the expression of many proteins involved in glycerol biosynthesis and in stress response (Ssa2p and Ssb1p) was induced in 20% glucose in order to defend the cells from osmotic and oxidative stress. In high glucose concentration we found an up-regulation of several proteins that play major roles in glycolisis and gluconeogenesis and a down-regulation of Adh1p (fermentative pathway). Among the proteins detected in glucose 0.5%, during the transition from fermentation to respiration, we identify proteins involved in aminoacid synthesis and mRNA biogenesis. It is interesting to note that in both conditions (high glucose or glucose restriction) we found over-expressed the Peroxiredoxin, involved in protection against oxidative stress insult.



A: 2%
B: 0.5
C: 20%

Conclusions: The information obtained in our work validates the application of proteomic approach for the identification of proteins which expression change as consequence of environmental variation

such as fermentation in high glucose and growth in condition of glucose restriction.

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EVALUATION OF POST-MORTEM CHANGE IN BOVINE FEEDED WHIT DIFFERENT DIET SUPPLEMENTATION

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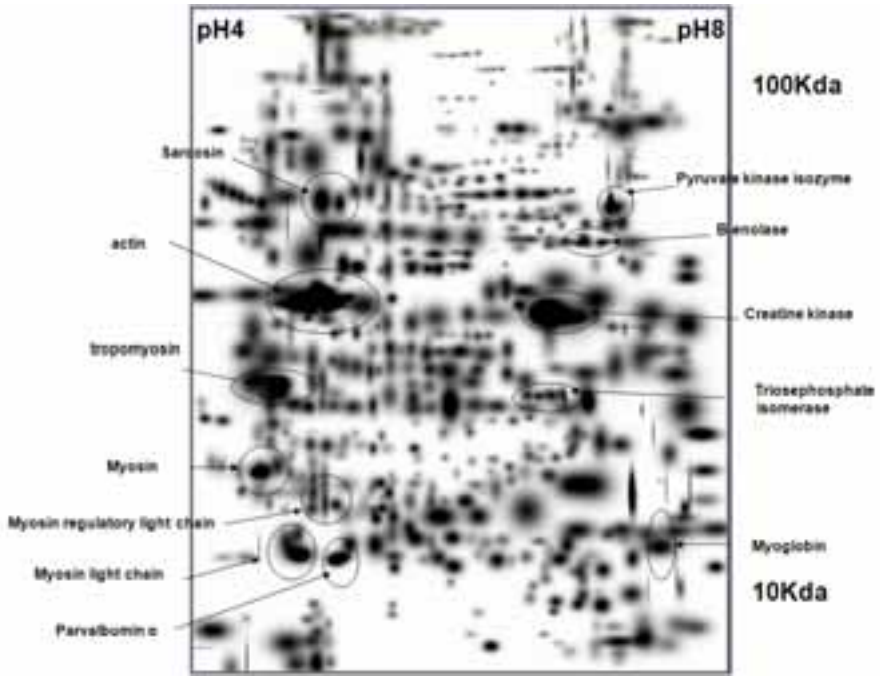
Background: The consumer's growing sensitivity about food characteristics was at first focused for bovine meat on the need to know products origin and then on specific information about meat quality, breeding methods, breed characteristics, animal welfare etc. Sensory properties of meat have an important influence on the purchasing behavior of consumers. Color, juiciness, flavor and tenderness has been shown to be most variable and important sensory component affecting satisfaction with beef. Many factors can affect final meat quality, such as animal welfare, breeding, feeding and transport conditions, slaughtering conditions, electrical stimulation, and chilling conditions. Supplementation of the diet with vitamin E, vitamin C and selenium during the growing and finishing periods may improve meat quality because are a potent antioxidant and has been demonstrated to decrease lipid oxidation, decrease drip loss, increase meat stability and improve the color of meat cuts.
Methods: 90 *limousine* bulls were divided at random into three dietary groups: control, supplementation with vitamin complex and selenium for 30 days before slaughtering and supplementation with vitamin complex and selenium for 60 days before slaughtering. *Longissimus Thoracis* muscle from the 9th thoracic ribs was collected 2 hours after slaughtering and stored at -80°C. Amounts of 100 mg for each muscle sample have been suspended in 1ml of OMNIzol with addition of protease inhibitor and 20µl of 0.25M EDTA. After homogenization on ice, protein samples have been extracted by OMNIzol Extraction protocol (EuroClone). Protein pellets were suspended in 8M Urea, 4% CHAPS, 1% DTT, 15mM Tris, 2% Ampholine 3.5-10. First dimension was performed using IPG strips pH pH 3-10 NL 18cm and second dimension in 10% polyacrilamide gels. Gels were stained by standard silver nitrate staining protocol. Image analysis was performed with Image Master 2D Platinum (GE) and Progenesis SameSpots software (Nonlinear Dynamics).
Results: Identification of the muscle proteome changes relationship to different diet supplement was based on a comparison of muscle samples taken immediately from six animals after slaughter. A synthetic map of bovine muscle proteins was building up as show in Figure 1. A synthetic gel consists of a representative set of spots generated from several registered gel images. The expression of desmine are decreased respectively, in animal fed with vitamin complex and selenium for 60 days before slaughtering, for 30 days before slaughtering and control animal (Fig. 2).



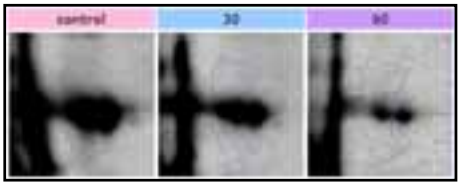


Fig.2: decrease in portein expression of desmin in three different diet

Fig.1: synthetic map of bovine muscle

Conclusions: 2-DE-based proteome analysis is a useful tool for characterizing expression and metabolism of muscle protein in relationship to different diet or supplementation. Besides, analysis of expression profiles could be a key to find protein markers for meat quality, and give deep understanding of characteristics of different welfare, breeding, feeding, transport conditions and slaughtering conditions. In particular, the decrease of desmin may correlate a better color and tenderness of meat.

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COMPARATIVE PROTEOMIC ANALYSIS OF HUMAN FIBROBLASTS EXPRESSING WILD TYPE AND MUTATED DNA LIGASE I.

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Background: DNA ligase I (LigI) is the replicative ligase in human cells. It plays an essential role in DNA metabolism by catalyzing the joining of both single and double stranded breaks in an ATP-dependent reaction. LigI deficient cells (46BR1.G1) were isolated from skin fibroblasts of a patient with immunodeficiency, sun sensitivity and growth retardation, who died at the age of 19 of lymphoma. We have recently shown that 46BR.1G1 accumulate replication-dependent DNA damages that are accompanied by phosphorylation of the protein kinase ATM (Soza et al., Mol Cell Biol 29:2032-41). The phenotype of 46BR.1G1 cells is efficiently corrected by the stable expression of the wild type LigI (7A3 clone). Complementation of the LigI defect results also in a shorter proliferation time. In order to identify proteins whose level or post-translational modification pattern is altered we have started a comparative proteomic analysis of cell lines expressing wild type and mutated LigI.
Methods: Cells were maintained in monolayer culture in DMEM with 10% fetal bovine serum. To prepare total cell extracts, cells were harvested by centrifugation and resuspended in Urea, Chaps and DTE.
Sample was analyzed by 2-DE and mass spectrometry. Protein exstract was precipitated with TCA and resuspended in solubilisation buffer; 1.3 mg of proteins were loaded on gel strips (length 18 cm) with a nonlinear pH 3-10 gradient range. After isoelectrofocusing, the strips were loaded onto 20 x 18 cm, 9-16% SDS-polyacrylamide gels. The two dimensional gels were stained with Blue silver colloidal. Gels were scanned using the Versadoc Imaging Model 3000 System (Bio-Rad) and it were analised using the PD-Quest 7.1 software (Bio-Rad). Statistically significant differences in spot intensity were obtained with the t-Student test. Proteins were identified by matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry (MS).
Results: 2-DE analysis was performed on 7 gels on each cell line. Using PD-Quest software 652 spots were mapped in 46BR1.G1 and 641 spots in the 7A3 clone.
The comparative analysis showed 557 common spots, 22 of which differentially expressed in the two cell lines. In addition we identified 95 spots only detectable in 46BR1.G1 and 84 spots only detectable in 7A3 cells. Proteins identified by mass spectrometry can be ranked in three major groups: stress response, cytoskeleton organization and RNA metabolism. Validation of the results obtained is in progress.

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PROTEOME PROFILING OF RAT MITOCHONDRIA AFTER PARTIAL HEPATECTOMY

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Introduction: The liver is usually a quiescent organ, but it is able to regenerate itself and replace lost tissue, after transplantation and upon the loss of cells, following chemical and viral injury, and partial hepatectomy (PH). Liver regeneration is mainly divided in two phases: the prereplicative phase, during which the liver's energy demand increases, and the replicative phase, during which increased DNA synthesis and mitosis occurs. In the prereplicative phase (0-24 h after PH), mitochondria show a decrease in oxidative phosphorylation capability and production of oxygen free radicals .We applied a proteomic approach to mitochondria isolated 6 h after PH, to characterize mitochondrial proteins that are involved in the prereplicative phase of liver regeneration.

Methods: Rats were subjected to PH at 6h and mitochondria were isolated. Mitochondria from sham-operated rats were used as controls. Mitochondrial protein expression pattern were studied by 2-DE electrophoresis. 2DE And Image Analysis: Isoelectric focusing (IEF) was performed on immobilized pH gradients (IPG; pH 3–10, 13 cm) with IPGphor (Amersham Biosciences). A total of 60 mg of protein was used for analytical runs, and 800 mg of proteins was used for preparative runs to a total volume of 250 ml of rehydrating buffer . Strips were rehydrated for 12 h. Separation of the second dimension was performed in 12.5% SDS/polyacrylamide gels using the Hofer SE 600 Ruby System (Amersham Biosciences). Coomassie R-250 staining protocol was used to visualize protein spots in preparative gels. The stained two-dimensional gels were scanned and image analysis was performed using the Imagemaster 2D Elite software version 3.1. The stained spots were excised and digested in gel with trypsin using the Trypsin Profile IGD Kit (Sigma) according to the manufacturer's instructions. The resulting peptides were extracted and separated by a CapLC system (Waters, Milford, USA) at a flow rate of ca 300 l/min. The mass spectrometer operates in positive ion mode with a source temperature of 100 °C and a voltage of 3.5 kV was applied to the probe tip.Mass spectra were acquired with the Q-Tof analyser in the V-mode of operation and spectra were integrated over one second intervals. Data-directed analysis was employed to perform MS/MS analysis on up to fourthly charged precursor ions. MS/MS Ion Search was employed for protein identification by using Mascot (http://www.matrixscience.com).

Results: Compared to the sham-operated control group, 1 protein was up-regulated and 13 proteins were down-regulated at 6 h. Until now, we identified by mass spectrometry 8 differentially expressed proteins that were associated with lipid metabolism, the OXPHOS system, biotransformation and other metabolic pathways.

Conclusion: The role of mitochondria in the early phase of liver regeneration, is still under challenging. Among the identifed proteins, of particular interest is SOD2 (MnSOD), a matrix mitochondrial enzyme. Superoxide dismutase (SOD) is a detoxification enzyme that converts superoxide to hydrogen peroxide, which can subsequently be converted to water. This finding supports the involvemnt of ROS species in the early phase of liver regeneration.

The systematic characterization of the mitochondrial proteins correlated with the prereplicative phase of liver regeneration, will give a better understanding of the metabolic processes involved in the liver regeneration.

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PROTEOMICS FOR THE STUDY OF CASEIN DEGRADATION IN THE WALL PAINTINGS OF MONUMENTAL CEMETERY IN PISA

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Background: The 14th century frescoes from the Monumental Cemetery in Pisa had been detached in 1945 from the wall and relocated on an asbestos cement support, using a glue based on a mixture of casein and calcium hydroxide.
The degraded glues and support (still present in some important frescoes) now need to be removed.
Due to the various restorations made in the course of history, several materials, some of which are unknown, have been employed. Moreover, the frescoes were detached from the wall and restored at a time when synthetic materials began to be used for restoration. New materials were thus used with little or no knowledge of their ageing behaviour and reactivity towards proteinaceous adhesives (animal glue and casein).
We have recently proposed strategies for the identification of proteins in binders of paintings that rely on proteomic approaches (Leo et al., manuscript in preparation), that have paved the way to the characterization of the effect of aging on artworks.
Methods: To provide an insight of the degradation products in the glues, a proteomic procedure, based on the enzymatic hydrolysis of the proteinaceous matter in the artistic samples without any pre-treatment of the sample, followed by nanoLC/nano ESI-MSMS, has been applied using a CHIP MS Ion Trap XCT Ultra equipped with a capillary 1100 HPLC system and a chip cube (Agilent Technologies, Palo Alto, Ca). The results are integrated by the determination of amino acid composition obtained by a GC/MS procedure.
Results: Preliminary experiments on samples from frescoes detached from the walls easily identified the presence of several proteins from bovine milk (alpha casein S1 and S2, beta casein and kappa casein) and provided a first insight in the degradation products in the naturally aged casein.
Conclusion: In the context of artistic and historic objects, the identification of organic paint constituents, and of proteinaceous components in particular, is still a challenging task, above all because of the very low amount of sample available, but also because of the complex and quite variable chemical composition of the paints itself. Herein we present proteomic approaches as a valuable tool not only for the identification of the constituents, but also for the characterization of the natural and unnatural aging products in the masterpieces.

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NATIVE GEL-BASED ELECTROPHORETIC ANALYSIS OF F_1F_0-ATP SYNTHASE ASSEMBLY IN MITOCHONDRIAL BIOGENESIS: A MULTITASKING ROLE OF IF1

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Background: Differentiation is often accompanied by mitochondrial biogenesis, mainly when differentiation lineage leads to cell phenotypes characterized by high energy demand such as cardiomyocytes. Nevertheless, the connections with the assembly of OXPHOS complexes have not been established yet. F0F1-ATP synthase, the OXPHOS complex V, consists of the catalytic soluble F1 sector, the first subcomplex to be assembled during biogenesis, and the membrane-bound Fo sector functioning as a proton channel. It is now accepted that stable interactions exist between individual F0F1 complexes, which in the inner mitochondrial membrane are arranged as functionally-optimised oligomeric structures. Gel-based electrophoretic approaches, e.g. blue-native PAGE (BNE), have been important for achieving high-resolution separations and visualising some F0F1 oligomeric forms (dimers as the more active forms) from most mitochondrial sources. An accurate selection is required of the best detergent conditions that upon solubilization of membranes preserve both enzymatic activity and supramolecular organization. This study focused on *in vitro* cardiomyogenic differentiation, with the aim to verify the hypothesis that it affects mitochondrial F0F1-ATP synthase biogenesis/assembly concomitant with the observed increase in mitochondria proliferation and differentiation. Functional and electrophoretic analyses are reported, focusing on F0F1 supramolecular assembly state and on natural regulatory peptide IF1 functional / structural roles. IF1 involvement in F0F1 oligomerization remains controversial, although it has been reinforced by recent evidence for *in situ* regulation of both ATP synthesis and hydrolysis activity.
Methods: Cardiomyogenic differentiation was induced in the rat cardiomyoblast line H9c2 by culturing cells in the presence of retinoic acid and serum deprivation. One-step detergent extraction of F0F1-ATP synthase from mitochondria of both parental H9c2 (C) and 1%FCS RA-treated cells (D) was performed, followed by BNE for separation of native multiprotein complexes. Digitonin, a mild nonionic detergent was used to solubilize mitochondria under conditions preserving both enzymatic activity and oligomeric structure. Because the proportion of oligomeric enzyme visualised by BNE is strongly dependent on the amount of detergent used for extraction, we carefully titrated detergent-to-protein ratios in C and D mitochondria from 2 to 7 g/g, to reveal the efficiency of solubilization together with the detergent-sensitivity of the complexes. The condition of better dimer recovery appeared 3g/g. 1D-BNE/ immunoblotting analysis with antibodies anti-β subunit of F1 or anti-IF1 was performed to detect the sub- and super-complexes and bound IF1 in both cell types. Alternatively, after migration the slices of BN gel were cut and submitted to 2D-SDS PAGE and to immunoblotting. The ATP synthase activity was measured spectrophotometrically using a hexokinase:glucose-6-phosphate dehydrogenase coupled assay.
Results: After digitonin solubilization, F0F1-ATP synthase was separated in BNE as dimeric (Vd) and monomeric (Vm) forms in both cell types and the two forms were identified also by in-gel activity staining. Colorimetric quantification of protein amounts by densitometric analyses of BNE stained with Coomassie blue showed a noticeable increase in the amount of fully assembled F0F1-ATP synthase dimer in detergent-solubilised mitochondria isolated from D cells (D: Vd/Vm ratio 0.41±0.06 *vs.* C: Vd/Vm ratio 0.19± 0.02 n=4), suggesting that cardiomyogenesis favours the supramolecular F0F1 assembly in our model. In accordance, functional analysis documented an increase of ATP synthesis activity in D cells (0.23±0.02 U/mg n=6) with respect to C cells (0.12±0.02 U/mg n=6), which was higher (92% increase) if compared to that of the enzyme expression levels in D cells (about 50%) measured by quantitative western blots. 2D-SDS/immunoblotting analysis revealed a greater IF1/β ratio in Vd of D cells (D: 0.97±0.09 *vs.* C: 0.35±0.02 n=3). In addition, BNE (coomassie and activity staining) and immunoblotting analyses revealed in C a marked amount of bound IF1 associated to unassembled F1 subcomplex. This is in accordance with the faint band revealed by activity staining thus supporting the canonical functional role of the inhibitor peptide.
Conclusions: Overall these data suggest that i) an increased stabilization of the dimeric form of F0F1-ATP synthase occurs upon cardiomyogenic differentiation in H9c2 murine cardiomyoblasts, ii) the concomitant increase observed of ATP synthase specific activity as exceeding the protein expression up-regulation is in accordance to the better catalytic efficiency ascribed to the F0F1 oligomeric structures, iii) these effects could be mediated by the natural regulatory peptide IF1 in line with recent reports proposing an additional structural role for IF1. In fact, the increased IF1/β ratio in the stable dimer in D cells points to this peptide as a candidate to contribute to stabilization of the oligomeric structures. The canonical IF1 role in activity

regulation when the enzyme works in reverse, (e.g. in cardiac ischemia) is well known and may be elicited in undifferetianted parental cells by inhibiting the unassembled F1. On the other hands, IF1 may stabilize dimer interface thereby improving efficiency of ATP synthesis as resulted in this study in cells induced to cardiomyogenic differentiation. The binding properties of IF1 eliciting such a role will be further investigated.

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CHARACTERIZATION OF PROTEIN PROFILE AND TARGET PROTEINS OF OXIDATION IN SACCHAROMYCES CEREVISIAE CELLS LACKING SCO1 BY A PROTEOMIC APPROACH

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Background. Cytochrome c Oxidase (CcO) is the terminal enzyme in the aerobic respiratory chain of eukaryotes and some bacteria. *Yeast* CcO comprises three core catalytic subunits, COX1, COX2, and COX3, which are encoded by the mitochondrial genome, and nine additional nuclear encoded subunits. CcO also requires multiple cofactors, including two heme molecules, three copper ions, and magnesium, sodium, and zinc ions. So assembly of CcO, is a sophisticated, multistep process that involves a number of auxiliary proteins. Some of these proteins have a specific role, such as chaperones or facilitators of the assembly process. Among them there are a series of copper-binding proteins implicated in the assembly of the *CuA* and *CuB* copper centers of mature CcO complex. Two of these proteins, Sco1 and Sco2, are important for assembly of the *CuA* site and have been proposed to function specifically in copper transfer and loading.
Sco is a family of proteins ubiquitous to all kingdoms of life. Eukaryotic genomes contain two paralogs, Sco1 and Sco2, that code for mitochondrial metallochaperone proteins. Mutations in human Sco1 and Sco2 produce tissue-specific CcO deficiencies associated with distinct clinical phenotypes, although are ubiquitously expressed.Until this time the function of both Sco1 and Sco2 was predicted to be solely attributable to formation of CcO copper centers, but lately, the structural resemblance of Sco1 to peroxiredoxins and thioredoxins, leaded to belive the possibility that Sco proteins may function as a thiol:disulfide oxidoreductase to maintain the *CuA* site cysteines in the reduced state ready for metallation. Alternatively, Sco1 was suggested to function as a redox switch, in which oxidation of *Cu(I)* to *Cu(II)* induces release of the *Cu(II)* ion, in this way, the oxidation state of copper in Sco could act as a trigger for redox signaling, In support of a redox role for Sco1, Δ Sco1 *yeast* cells were observed to be sensitive to *hydrogen peroxide*. Moreover it was hypotesize that the lack of Sco1 may destabilize COX2 subunit sufficiently that it fails to stably interact with COX1 leading the accumulation of a *heme A*-COX1 *pro-oxidant intermediate* that may result in generalized reactive oxygen damage. The final aim of this work is to elucidate the potential implication of Sco proteins in cellular redox state and also to compare the protein profile of cells lacking Sco1 with the Wild-type one, growth in medium containing different carbon sources (fermentable and non-fermentable).
Methods. In order to identify the proteome and target proteins of oxidation in cells lacking Sco1, we performed a two dimensional gel electrophoresis analysis combined with immunoblotting with specific antibodies for carbonyl groups. Gels from every condition were compared, to evaluate and analyse the presence of qualitative and quantitative differences.
Results and Conclusions. Our first results demonstrate that carbonylated state is increased in ΔSco1 mutant growing on glucose (fermentation) compared to ΔSco1 mutant growing on glycerol (respiration) media. All the carbonylated proteins have been identified by mass spectrometry technologies and will be presented.

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COMPARATIVE ANALYSIS OF LIVER MITOCHONDRIAL PROTEOME OF ADULT AND OLD RAT

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Background: Mitochondria play a key role in aging since they are the major source of intracellular reactive oxygen species (ROS) and, at the same time, the primary target of their action. Mitochondria, which utilize 90% of cellular oxygen, produce ROS as by-products of the respiration: in physiological conditions 0.2% of the utilized oxygen is converted into ROS. ROS are highly reactive molecules harmful to the cell macromolecules: mitochondrial macromolecules, which are nearby the major source of ROS, are primarily damaged. ROS production increases during aging and seems to be responsible for the oxidative damage to mitochondrial macromolecules verified in different aging tissues. The aim of this work was the identification, by differential proteomics, of the quantitative changes and post-translational modifications of mitochondrial proteins in aged liver. The liver mitochondrial proteome from adult and old rats was analysed by using two-dimensional electrophoresis.
Methods: Experiments were performed on two groups of 12-month-old and 28-month-old male Fisher-344 Charles-River rats. Mitochondria were prepared by differential centrifugation from freshly excised livers and mitoplasts by digitonin treatment of mitochondria. Mitoplast proteins were solubilised in rehydration buffer and separated in first dimension on 18 cm 4-7 and 6-11 pH gradient IPG strips and in second dimension on 10% SDS polyacrylamide gels. The samples were run in triplicate and, after silver staining, the gels were compared using Image Master 2D Platinum 6.0 software. Differentially expressed spots were subjected to statistical analysis and excised from the Coomassie stained gels. Spots were in-gel digested with trypsin and proteins were identified by MALDI and nano ESI MS/MS mass spectrometry. Prx III identification were confirmed by western blot on mono- and two-dimensional gels.
Results: Statistically significant variations of 66 spots were found. Forty-five different proteins were identified by mass spectrometry. The 62% of identified proteins belong to different metabolic mitochondrial metabolisms: oxidative phosphorylation system proteins, OXPHOS-related proteins, transport proteins, fatty acids catabolism, ketone bodies metabolism, Krebs cycle proteins, urea cycle proteins. Moreover, differential expression in mitochondrial antioxidant enzymes were found. In fact, in old rats, the accumulation of an acidic spot of Peroxiredoxin III (Prx III) was identified. Prx III is a mitochondrial antioxidant protein belonging to a family of antioxidant and ubiquitous peroxidases. By MALDI analysis the overoxidation of the catalytic Cys to Cys sulphonic acid was identified, this post-translational modification inactivate the enzyme. Furthermore the increase, in old rat, of two spots identified as aldehyde dehydrogenase 2 was found, this enzyme is involved in ethanol catabolism and participate to the detoxification of toxic acetaldehydes and lipid peroxides. Mitochondrial inner membrane proteins of unknown function were also identified.
Non-mitochondrial proteins such as cytoplasmic proteolytic enzymes and chaperonins were also identified.
Conclusions: The qualitative and quantitative alterations of mitochondrial proteins here found will raise new questions for the research about age-related processes. In particular is reported the increase of some proteins of Krebs cycle and subunits of the respiratory complexes content in aging rat liver that is in contrast with the age-related decrease activity of these enzyme already reported. This phenomenon could be explained with an increased synthesis to counteract the decrease activity or due to a decrease in proteolysis of damaged proteins.
The overoxidized form of Prx III has been found here, for the first time, in vivo, in rat liver. However the native Prx III form is still present in old rat and in the same amount as in the adult counterpart. The accumulation of the inactive form of Prx III is probably due to the age-related oxidative stress condition already reported in this tissue and to the decrease of protease activities involved in the degradation of oxidized proteins such as the mitochondrial ATP-stimulated Lon protease.

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ISOFORMS AND PHOSPHORYLATION PATTERN OF THE NDUFS4 SUBUNIT OF COMPLEX I OF THE RESPIRATORY CHAIN OF HIGH EUKARYOTES

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Background: In murine and human cell cultures, activation of the cAMP cascade promote, under condition in which they result in activation of complex I and counteract ROS accumulation, serine phosphorylation in the subunit of the complex encoded by the nuclear NDUFS4 gene (18 kDa subunit). The human, mammals and bird NDUFS4 gene encode for homologous proteins. These proteins, but also those of Drosophila and Anopheles, which present a low overall homology with the human species, have a highly conserved C-terminus in which a canonical RVSTK phosphorylation site in the last residues, with the highest phosphorylation score for PKA, is present. Mutations in the NDUFS4 gene in patients with neurological diseases, impair the assembly and the activity of complex I. Edman sequencing provided results indicating phosphorylation of Ser173 in the C-terminus of the protein encoded by the NDUFS4 gene. Mass spectrometry carried out by us and other groups failed however to show phosphorylation of this serine. The present work deals with the phophorylation of this subunit of complex I.

Methods: Immunodetection of the NDUFS4 subunit of complex I in bovine heart mitochondria and human fibroblasts was performed using anti-N-terminus, anti-C-terminus and anti-phosphorylated C-terminus antibodies after SDS-PAGE separation. Complex I was purified from bovine heart mitochondria and radiolabbeled with [γ^{32}-P]ATP and PKA. After phosphorylation and SDS gel separation, the radioactive band corresponding to 18 kDa was cutted and subjected to IEF. The expression and purification of recombinat NDUFS4 proteins was performed used the QIAexpress Type IV kit (Qiagen). Purification of the heterologous 6xHis tagged NDUFS4 protein was performed. The heterologous proteins were phosphorylated in the presence of [γ^{32}-P]ATP and PKA. Acid-hydrolyzed amino acid mixtures for the analysis of phosphoamino acids were obtained by the procedure of (Kamps and Sefton Annal.Biochem. 176, 22-27).

Results: Analysis of high eukaryote isoforms of the NDUFS4 subunit of complex I and its phophorylation pattern was examined. The conserved RVS site in the C-terminus was found to be costitutively phosphorylated in the isoforms examined. 2D- SDS/IEF PAGE of the [^{32}P]-phosphorylated 18 kDa band of the bovine complex I shows the presence of a PKA-dependent [^{32}P]-labelled spot, recognized by a specific antibody directed against the P-Ser173 of the NDUFS4 C-terminus of the protein, with an alkaline pI and a MW proper of the NDUFS4 protein.Heterologous precursor and mature forms of the human NDUFS4 gene, expressed in E. Coli, incorporated [^{32}P] in a PKA dependent fashion. TLC aminoacid mapping showed phosphorylation of threonine and serine in both the precursor and mature protein. The expression of heterologous mature forms, carring the serine/alanine sobstitution in the C-terminus phosphorylation site, resulted in lower [^{32}P] phosphorylation and TLC mapping showed phosphorylation of threonine.

Conclusions: The present immunochemical analysis shows that the conserved RVS site in the higly homologous carboxy-terminus is phosphorylated in vivo in the species examined. The results show that PKA catalyzes phosphorylation of Ser173 in the RVS C-terminal site as well as of threonine, and possibly other serine residues. The Nano-LC-MS/MS analyses failed, however, to reveal phosphopeptide masses in the heterologous human NDUFS4 protein samples in which TLC identified phosphothreonine and phosphoserine. There are various reasons why mass spectrometry can miss protein phosphorylation. The relative ratio of phosphorylated/non phosphorylated protein can vary substantially depending on the prevaling, in vivo and experimental, conditions. Detection of the phosphorylated form in the presence of the non phosphorylated one can be hampered by competition for ionization of the two forms, especially when the phosphorylated one is present in a low pecentage. The specific location of Ser173 at the very end of the carboxy-terminus of NDUFS4 protein could hamper detection of the small phosphorylated peptide resulting from proteolysis (if this is not abrogated by phosphorylation). The question arises here to what extent negative mass spectrometry results on protein phosphorylation can stand against positive results provided by 32P-radiolabelling, immunochemical detection, 2D electrophoretic analyses and identification of phosphoaminoacids by TLC aminoacid mapping.

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MODIFICATION OF THE BRAIN CORTEX PROTEOME AFTER TRANSIENT ISCHEMIA IN A MOUSE MODEL

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Background. A brief ischemic insult, below the threshold of damage, evokes endogenous defense mechanisms that confer protection against a subsequent severe ischemic attack. This phenomenon is termed ischemic preconditioning and the resulting protection is known as ischemic tolerance. Ischemic preconditioning and ischemic tolerance can occur in different tissues and organs and have been shown in experimental models and in humans too, but the mechanisms underlining cell survival have not been clarified yet. In the brain, ischemic preconditioning triggers several adaptive molecular responses of cells to injury, characterized by different time frames during which proteins seem to play a relevant role. For instance, a rapid and short-lasting protection can be established within minutes of exposure to ischemic attack, as a result of post-translational modification of proteins. A second phase develops more slowly and requires gene activation and *de novo* synthesis of proteins. Since proteins are thought to be very much involved in the development of ischemic tolerance, we used a proteomic approach to study the modulation of the protein profile induced after transient ischemic attack (TIA) in a mouse model with the aim of identifying possible ischemic tolerance mediators and hence new drug targets for stroke therapy.
Methods. A TIA model consisting of a short period (7 min) of transient Middle Cerebral Artery Occlusion (tMCAo) was developed. Since when TIA is performed 4 days before a typical ischemic insult a significant reduction of the ischemic lesion occurs, experimental groups of mice (n=10) were sacrificed 4 days after TIA, severe ischemic injury (30min tMCAo) or sham surgery (used as controls) and brain cortex proteome analysis was performed. The brain was rapidly removed, the right cortex was excised, immediately frozen and stored at -70 °C until protein extraction. Our aim was to identify biological changes above the technical variations, thus we decided to work on pooled samples. Three pools, one per experimental group, were created by mixing equal amounts of proteins from individual protein extract. Proteins (100 micrograms) were separated by two dimensional gel electrophoresis and 5 replicates per group were run. Protein spots were revealed by colloidal blue Coomassie staining, gel images were digitalized and analyzed using the Progenesis SameSpot software (Nonlinear Dynamics, UK) to compare protein expression in the experimental groups (Multivariate analysis). Statistical analyses (Anova and multiple comparison Tukey test) were performed by JMP software (v 6, SAS, Cary, NC, USA). In all instances significance was set at $p<0.05$. Proteins significantly differentially expressed and with expression changes >1.3 were manually excised from the gel, digested with trypsin and the tryptic peptide mixtures were analyzed by nanofluidic liquid chromatography coupled to tandem mass spectrometry (Chip-LC-MS/MS). Tandem mass spectra were analyzed using the search engines Mascot (Matrix Science, www.matrixscience.com) and Phenyx (GeneBio, Switzerland, www.phenyx-ms.com, phenyx@genebio.com) against the Uniprot-SwissProt database (v.54.0). The list of the identified differentially expressed proteins was uploaded as their Swiss-Prot IDs to MetaCore (GeneGo, St. Joseph, MI, USA) to map the proteins into biological networks. The biological process enrichment was analyzed based on GO Ontology processes. A network of these proteins was generated using the shortest paths algorithm.
Results: A 7 min tMCAo represents the longest period of ischemia that does not cause tissue damage, whereas a 30 min tMCAo was able to induce brain injuries in the cortex and striatum, as determined by ischemic volume and neurodegeneration measurments (Neutral Red and Fluoro-Jade staining, respectively). Overall image analysis indicated that 31 protein species were differentially expressed, 27 out of these, were successfully identified by Chip-LC-MS/MS, but only 11 proteins met the requirements of expression fold change >1.3 and $p<0.05$ (Tukey test). Most expression differences were observed in 30 min tMCAo vs. Sham, whereas the expression of only three proteins, namely glial fibrillary acidic protein, glycerldehyde-3-phosphate dehydrogenase, and ATP synthase subunit beta, was different in both 7 min and 30 min tMCAo vs. Sham animals, suggesting their possible involvement in preconditioning and ischemic tolerance induction. Nine out of 11 proteins were overexpressed in the 30 min tMCAo group compared to Sham or 7 min tMCAo groups, down regulation being observed only for glycerldehyde-3-phosphate dehydrogenase and septin-11. Pathway analysis indicated that several proteins were important nodes in the network generated by MetaCore, with prohibitin being the node with the highest number of interactions.
Conclusions: The results show the validity of the proteomic approach to characterize the effects of mild and severe ischemia on the brain cortex protein profile. The proteins differentially expressed were involved in energetic, apoptotic, and regenerative processes, confirming that these processes are often activated in

response to ischemic insults. Studies are ongoing to establish the role of these proteins in preconditioning and ischemic tolerance induction.

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OXIDATION OF PLASMA PROTEINS DURING AEROBIC EXERCISE IN TRAINED AND NOT TRAINED SUBJECTS.

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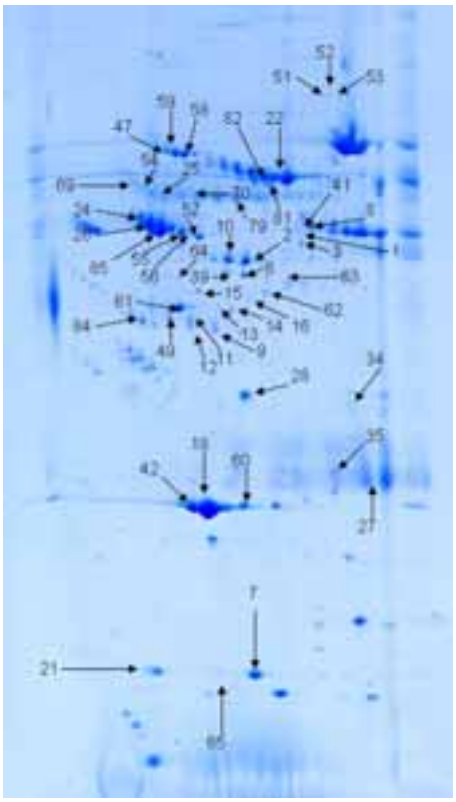
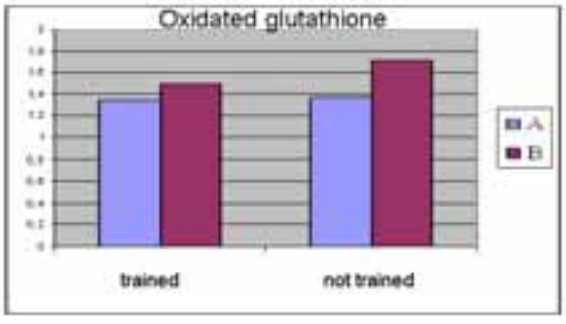
Background: The physical inactivity is a recognized risk factor for the development of obesity, diabetes and cardiovascular disease and the health benefits due to a regular exercise and moderate are incontrovertible. However, physical activity leads to a temporary imbalance between the production of free radicals and their disposal, and this phenomenon is called oxidative stress. An excessive accumulation of free radicals such as reactive oxygen species (ROS) and nitric oxide (RNOS), leads over time to damage proteins, lipids and nucleic acids. The sport continues in our body leads to an increase in endogenous defenses against this type of stress, therefore reducing the damage. Several manufacturers of supplements focuse on sales of antioxidants to reduce the production of radicals (ie oxidative stress), thereby facilitating the recovery between workouts or competitions. However, there are currently significant limitations of the knowledge of the relationship between performance and oxidative stress Free radicals can be produced even in the absence of oxygen (anaerobic conditions) in other cellular compartments as a result of enzyme (xanthine oxidase, NADPH oxidase, etc..) or other substances present in these compartments (calcium, iron, etc.). The correlation between production and physical activity is not known but is thought likely to be due to an anaerobic efforts, or particularly intense.
The aim of this project involves the characterization of plasma protein carbonylation (a type of oxidation induced by an increase in free radicals) in response to a prolonged aerobic exercise. Indeed, the data currently in the literature have clearly demonstrated the increase of oxidized proteins in the plasma of athletes after exercise, but did not identify which are the targets of this oxidation.

Methods: To our research 12 volunteers subjects cooperated, 7 of whom are accustomed to intense daily physical activity (defined as "trained" in this work) and 5 to an occasional physical activity (defined as "not trained" in this work). Plasma samples of all subjects were taken at rest condition and after an hour of run.
To study the protein target of carbonyl we analyzed the plasma of volunteers before and immediately after exercise, using two-dimensional electrophoresis followed by western blot with specific antibodies against oxidized proteins. We then analyzed the two-dimensional images by identifying several proteins that are targets of carbonylation after physical activity.
Results: We first assessed the level of total carbonylated protein in the plasma, but due to the low sensitivity of the method, it was not possible to find large variations between subjects trained and not trained in terms of total carbonylated protein before and after the activity physics. Through the determination of glutathione (GSH + GSSG and GSSG) we could show a decrease in the levels of total glutathione and increase levels of oxidized glutathione in subjects not trained after physical activity. The proteins identified by overlapping with the 2D map of plasma in database are indicated by numbers in the figure and these identifications were subsequently confirmed by mass spectrometry.





A : before run
B : after run

Conclusions:
These methodologies allowed us to have an overview of how the plasma proteome changes in response to an aerobic exercise and to identify new markers of physiological stress.

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DIGE ANALYSIS IN FOOD QUALITY CONTROL: THE STUDY OF MILK FAT GLOBULES FROM *OVIS ARIES*

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Background: the fat fraction of milk consists of micro-lipid droplets composed mainly of triacylglycerols and proteins, surrounded by a membrane bilayer. These vesicles, called Milk Fat Globules (MFGs), are formed in endoplasmic reticulum, unidirectionally transported to apical region of epithelial cells and secreted by an exocytotic mechanism into the lumen of mammary gland during lactation. MFGs contain proteins derived from apical region of secreting epithelial cells and are of extreme importance to correlate the different protein expression of mammary epithelium with the general physiopathological state of the animal. Usually, for commercial use, milk is classified into 3 different quality categories, based on Somatic Cell Count (SCC).
Methods: we collected 48 milk samples from *Ovis aries* derived from two different lactation periods. The MFG fraction was obtained by centrifugations of milk samples at 5000 xg for 15 min at 25°C. The obtained pellets were washed three times with PBS (pH 7.4) and treated with a specific delipidation/precipitation protocol, based on chloroform and methanol for the quantitative precipitation. Then the pellets were resuspended in Urea/CHAPS/TRIS buffer, labeled with Cy2, Cy3 or Cy5 fluorescent stains (GE Healthcare), separated by 2D-DIGE technology (Two Dimensional-Differential Gel Electrophoresis) and analyzed by DeCyder Differential Analysis Software (GE Healthcare). Proteins identification was performed by mass spectrometry.
Results and using our proteomic approach we have highlighted 51 spots from the first lactation animal group and 66 spots from the second with significant quantitative variations. Among these spots we identified Adipophilin, Casein 1, Casein 2 and Annexin 2, which vary through the different quality classes.
Conclusions: the identified protein species could be important as multivariate protein markers together with the Somatic Cell Count (SCC) to assess the quality of milk and its derivates.

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BIOMECHANICAL PROPERTIES AND LIPOPOLYSACCHARIDE -INDUCED PROTEOME MODIFICATIONS IN INF-B TREATED ASTROCYTES

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Background: Interferon-beta (IFN-β) is a pleiotropic cytokine with a disease modifying effect in patients with multiple sclerosis. The exact mechanism by which IFN-β mediates its activity has been object of study in several works, but, its action on central nervous system (CNS) is still debated and not completely elucidated. In this work, we evaluated the effects of IFN-β on primary cultures of control and LPS-treated rat astrocytes by using a proteomic and nanotechnological approach.
Methods: Primary cultures of astrocytes were prepared from 27 newborn Wistar rats. The cells were plated at a density of 1.5 x 10^5 cells/cm^2 onto flakes or Petri dishes and characterized in culture by immunofluorescence using a monoclonal antibody against glial fibrillary acidic protein (GFAP) coupled with a red quantum dot. Cells were treated with 100U/ml IFN- β for 24h or with 10ug/ml LPS for 48h, some others with 10ug/ml LPS for 24h, followed by 100U/ml IFN-β for 24h. A scanning force microscopy (SFM), together with confocal microscopy, was used to investigate morphological and mechanical properties of astrocytes treated with IFN-β. Young's moduli of control and treated cells were determined by measuring a force curve at each position of interest on the cell surface (Fig. 1). For confocal microscopy analysis, cells were fixed in 3.7% formaldehyde in PBS for 5 min, permeabilized with Triton X-100 (0.1% in PBS) followed by a 30 min incubation at room temperature with phalloidin-TRITC. For 2-DE studies, cells were dissolved in sample buffer and isoelectric focusing of protein samples was carried out by using commercial 13 cm IPG polyacrylamide strips (pH 3 to 10). Separation in the second dimension was carried out in 12% SDS-PAGE gels. Silver stained gels were analyzed by the Image-Master 2-D Platinum software. The identity of proteins was determined by a MALDI-TOF analysis and a database search.
Results: The effects of IFN-β on cell growth were determined by MTT assay, which revealed that the proliferation of astrocytes was influenced by IFN-β. Immunoblotting with monoclonal antibody against GFAP and with monoclonal antibody against Vimentin, following by a secondary antibodies peroxidase conjugate, evidenced an astrocytes reaction after IFN-β and LPS treatment, respect to controls with a more prominent gliosis after IFN-β treatment and no attenuation of the response after LPS/IFN-β treatment. Furthermore, IFN-β modified the cell modulus probably by altering the structure of the cytoskeleton. In fact, Phalloidin-staining of actin filaments, showed an alteration in the organization of stress fibres after 24 h of treatment with IFN-β. In addition, several proteins involved in the cytoskeletal structure and organization were found to be differentially expressed after 2-DE analysis. Western blotting confirmed the increase in cofilin-1 and profilin at the protein level.

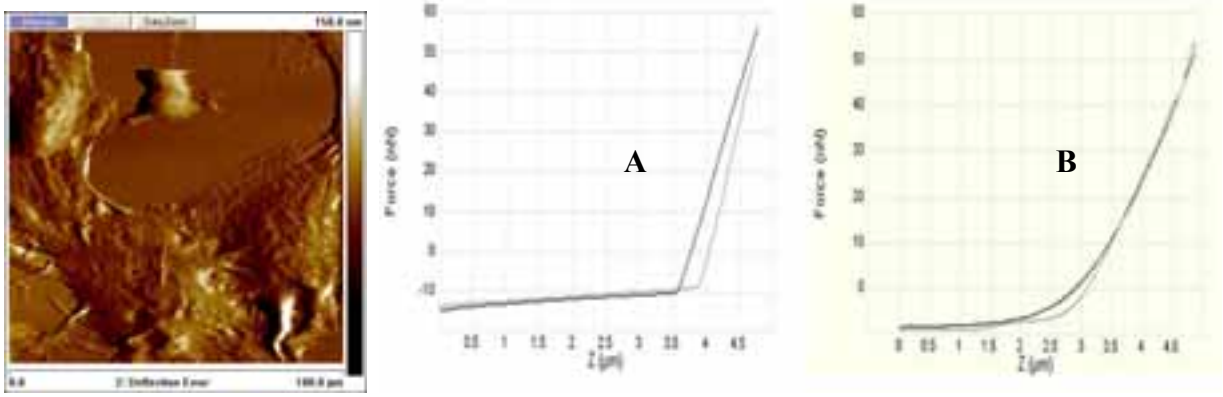

Figure 1. Deflection image of living astrocytes (left). Representative force versus Z-position measured in liquid for the substrate (A) and cell (B) (right).

Conclusions: Following treatment with IFN-β astrocytes increase their elastic properties probably through the regulation of several proteins including cofilin-1 and profilin. Both proteins have been implicated in actin reorganization during cell invasion and migration. Further studies may lead to understand how these

modifications can affect the physiology of astrocytes in vivo considering that these cells provide supportive and mechanical activities essential for CNS functions, including the formation of the blood-brain barrier.

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YOMICS – AN INFORMATION SYSTEM FOR THE IDENTIFICATION OF NEW DIAGNOSTIC AND THERAPEUTIC MARKERS

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Background: YOMICS aims at the development of a library of antibodies for the identification of new diagnostic and therapeutic biomarkers; membrane proteins, acting as signal receptors and transducing signals as growth factors and hormones, are important targets of pharmacological agents. Membrane proteins are hard to solubilize and purify because they have both hydrophobic and hydrophilic regions; their structures are therefore difficult to determine experimentally. Computational methods have come to be the first choice for the identification of membrane proteins and for the prediction of their topology. The main aim of this research is to design and implement a computational framework for transmembrane protein detection.
Earliest computational methods have been Artificial Neural Networks (ANN), due to their capability to capture non linear signals: despite their prediction accuracy, ANN are black boxes with no indication of the biological features that the computational model is expected to capture. More recently Hidden Markov Models (HMM) have become the method of choice: the elements of a HMM correspond closely to the biological entities being simulated.
Methods: We apply a classification model composed by a set of HMM, whose states have an associated probability distribution over the 20 amino acids characterizing the compositional bias in the corresponding regions. In addition, the model architecture specifies the interconnection of states within each set or sub model and also specifies how these sub models are connected to one another. Transitions among states within a given sub model determine the length distribution of the corresponding regions whereas transitions from one sub model to another reflect how the different regions are arranged to form the entire protein. The transition probabilities, along with emission frequencies, enable the model to capture correlations among signals. The training of this model has been performed on a data set of known transmembrane proteins of different biological organisms. We apply it to identify which, among the human proteins, are potentially located in the cell membrane.
In order to maximize the effectiveness of information delivery we present the sequences of amino acids in an aggregated and structured way, which masks to the user the access to multiple information sources, and implements an automatic approach for the filtering and selection of the candidate proteins. The result is a computational framework whose architecture is composed by the *data integration layer* and the *data analysis layer*.
The *data integration layer* provides homogeneous access to different data sources, both publicly available (EntrezGene, Uniprot) and private repositories, ensuring synchronization and updating of the information stored, preserving data quality through filtering and managing the integration of heterogeneous data formats.
The *data analysis layer* provides the functionalities of filtering data through HMM, classifying proteins on the basis of the presence of transmembrane domains and the signal peptide, identifying also their topology.
Results: The main outcome of the research work has been the production of an integration platform, which supports studies on proteins databases through data integration and allowing to researchers to concentrate on the interpretation of the data rather than in their searching and understanding. Furthermore, the data analysis tools that we offer supports, in the preliminary phase of the analysis, the discovery of proteins of potential interest; these, after having being selected though YOMICS, can be further investigated through laboratory analysis for their characterization.
Conclusions: The system is being applied for the analysis of the whole human genome, with the objective of identifying 2.000 proteins of interest. The performance of the above system both in terms of flexibility and prediction accuracy makes it a suitable candidate for estimating the number of membrane proteins as well as the frequency of proteins of different topologies to be expected in a given genome.

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