CNR DSV CONSIGLIO NAZIONALE DELLE RICERCHE
DIPARTIMENTO SCIENZE DELLA VITA

ItPA

Dipartimenti di:
Biochimica Medica, Biologia Medica e Fisica Medica
Biochimica e Biologia Molecolare "*Ernesto Quagliarello*"

Italian Proteomic Association

3rd Annual National Conference

Congress Center "Hotel Sierra Silvana"

11-14 June 2008

Selva di Fasano (Brindisi), Italy

Organized by

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11th June

15.00-17.30 **Registration**

17.45 **Conference opening**

Chairman: Prof Sergio Papa

18.00 **Plenary lecture**

Lilia Alberghina-*Università di Milano Bicocca*
Proteomics and Systems Biology for a better understanding of cellular functions

19.30 **Welcome Cocktail**

12th June

Session I: Medical Proteomics (Clinical Proteomics/Biomarkers)

Chairman: Prof Lilia Alberghina, Dr Lorenzo Citti

9.00 **James H. Scrivens** *University of Warwick-Coventry(UK)*
An evaluation of profiling and differential proteomics approaches to the characterisation of biochemical pathways in newly discovered prokaryotes

9.40 **Cecilia Gelfi** *Università degli Studi di Milano*
Muscle: a target tissue for biomarkers discovery

10.00 **Andrea Urbani** *Università di Chieti-Pescara*
Investigating systematic factors affecting MS molecular biomarker discovery

10.20 **Michela Di Michele** *Università Cattolica Campobasso*
Glycoproteomics of paclitaxel chemoresistance in ovarian cancer cell lines

10.40 **Poster Session (Med 1-15) - Coffee break**

Chairman: Prof Maurizio Simmaco, Dr Domenico Garozzo

11.10 **Rosa Terracciano** *Università Magna Graecia Catanzaro*
Mesoporous beads: new tools and strategies in body fluids proteomics

11.30 **Roberto Raggiaschi** *Siena Biotech SpA Siena*
Protein expression analysis by 2D-DIGE of different areas of GBM from patients biopsies

11.50 **Ida Pucci-Minafra** *DOSAC Università di Palermo*
Breast cancer proteomics: from research to clinical applications

12.10 **Patrizia Cancemi** *DOSAC Università di Palermo*
Fibroblast's role in breast cancer: a proteomic approach

12.30 **Gianluca Di Cara** *DOSAC Università di Palermo*
Multiple effects induced by Herceptin® on 8701-BC breast cancer cells

13.00 **Lunch - Poster Session (Med 16-43)**

Session II: Cellular Organelle Proteomics

Chairman: Prof Cecilia Gelfi, Prof Fulvio Magni

16.00 **Albert Sickmann** *DFG-Forschungszentrum für Experimentelle Biomedizin Würzburg (Deutschland)*
Organelle Proteomics - Application to yeast mitochondria and platelet plasma membranes

16.40 **Salvatore Scacco** *Università degli Studi di Bari*
Isoforms and phosphorylation pattern of the subunit of complex I encoded by the NDUFS4 gene in high eukaryotes

17.00 **Clara Musicco** *IBBE CNR Bari*
The mitochondrial proteome of adult and old rat liver: quantitative and qualitative changes

17.20 **Poster Session (Cell 1-2; Sys 1-4) - Coffee break**

17.50 **Claudia Cirulli** *Università di Milano Bicocca Milano*
Studying the yeast cell cycle regulator SIC1 by functional proteomics

18.10 **Elena Silvestri** *Università degli Studi del Sannio Benevento*
3,5-diiodo-L-thyronine-induced proteomic changes in mitochondria from fatty liver: evidences from two-dimensional and blue native electrophoresis.

19.00 **ItPA Member meeting**

13th June

Session III: Proteomics/Systems Biology

Chairman: Prof Paolo Mocarelli, Prof Martin Larsen

09.00 **Holger Prokisch-** *Institute of Human Genetics University Munich-(Germany)*
Mitochondriomics

09.40 **Rita Casadio-** *Dipartimento di Biologia Evoluzionistica Sperimentale Università di Bologna (Italy)*
Filtering proteomes for sequence annotation: Subcellular Localization and GPI anchor prediction in Eukaryots

10.20 **Tiziana Bonaldi** *IEO Milano*
Combined use of RNAi and quantitative proteomics to study gene function in *Drosophila*

10.40 **Poster Session (PTM 1-8) - Coffee break**

Session IV: Post-Translational Modifications

Chairman: Prof Piero Pucci, Prof Andrea Urbani

11.10 **Martin Larsen-***Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense M, (Denmark)*
Phosphoproteomics – technologies and application to the study of depolarization-dependent protein phosphorylation in nerve terminals.

11.40 **Mark McDowall-***Waters UK Limited(UK)*
Accurate intact mass analysis and structural characterisation of a monoclonal antibody by ion mobility coupled with mass spectrometry

12.20 **Giuseppe Palmisano** *Università degli Studi di Bari*
Analyses and Implications of Complex I Phosphoproteome

12.40 **Angela Amoresano** *Università di Napoli Federico II*
Combined use of MS/MS/MS and Dansyl chemistry in proteomics.

13.00 **Lunch - Poster Session (Mic 1-5; Pl 1-9)**

Session V: Microbial Proteomics

Chairman: Prof Enrica Pessione, Dr Andrea Scaloni

15.30 **Samuel Kaplan** *Department of Microbiology and Molecular Genetics, University of Texas, Texas (USA)*
Post-genomic tools and the study of photosynthesis in Rhodobacter Sphaeroides

16.10 **Dirk Benndorf** *Max Planck Institute for Dynamics of Complex Technical Systems Bioprocess Engineering (Germany)*
Metaproteom analysis of microbial communities.

16.40 **Francesca Italiano** *IPCF CNR Bari*
Response of *Rhodobacter sphaeroides* R26.1 to heavy metals exposure

17.00 **Poster Session (Others 1-10) - Coffee break**

17.30 **Marco Gobbetti-***Università degli Studi di Bari (Italy)*
Proteomics of cell-cell communication in food related lactic acid bacteria

18.00 **Antonio Gnoni** *Università degli Studi di Bari*
Proteomic approach to the characterisation of the antibiotic producer *Nonomuraea sp ATCC 39727*

18.20 **Ramona Kuhn** *IRSA CNR Bari*
Metaproteomic approach to MBR biomass characterization

18.40 **Alessandro Pessione** *Università degli studi di Torino*
A proteomic study reveals a membrane-bound localization of tyrosine decarboxylase in *Enterococcus faecalis*

21.00 **SOCIAL DINNER**
Hotel Sierra Silvana

14th June

9.00 **Poster Award**

9.15 **Egisto Boschetti** *Biorad Laboratories C/o CEA-Saclay France*
The ProteoMiner and the FortyNiners: searching for gold nuggets in the proteomic arena

Session VI: Plant Proteomics

Chairman: Prof Lello Zolla, Prof Raffaele Gallerani

9.40 **Jean-Benoit Peltier** *INRA-Montpellier (France)*
Introduction to proteomics of post-translational modifications in plants

10.20 **Maria Giulia Egidi** *Università della Tuscia Viterbo*
Proteomics as a complementary tool for identifying unintented side effects occurring in transgenic maize seeds as a result of genetic modifications

10.40 **Coffee break**

11.10 **Paolo Laino** *Università della Tuscia Viterbo*
Comparative proteomic analysis of heat stress on the metabolic seed protein fraction in the widly grown italian durum wheat cultivar Svevo

11.30 **Mariasole Di Carli** *ENEA Casaccia-Roma*
A combined approach to analyze grape berry (*Corvina*) ripening and withering

11.50 **Linda Bianco** *ENEA Centro Ricerche Trisaia Rotondella (MT)*
Description of major proteins expressed in strawberry fruit during ripening

12.10 **Angiola Desiderio** *ENEA Casaccia-Roma*
Proteome analysis of resistant plant expressing antiviral antibodies

ORAL PRESENTATIONS

PROTEOMICS AND SYSTEMS BIOLOGY FOR A BETTER UNDERSTANDING OF CELLULAR FUNCTIONS

Lilia ALBERGHINA and Marco VANONI

Dipartimento di Biotecnologie e Bioscienze, Università di Milano-Bicocca,
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The highthroughput technologies are allowing genome-wide descriptions of relevant cellular components (mRNA, proteins, metabolites) in different physiological and pathological conditions. The understanding of complex cellular functions (such as cell cycle, differentiation, transformation, etc.) requires that this information be structured in a way to extract the hidden pattern behind and to acquire ability to predict the effects that specific perturbations will have on the function under consideration.

Systems Biology, with its integration of molecular analysis, mathematical modeling and computer simulations, is the new approach that aims to give structure and predictive ability to biological information.

In this talk it will be presented an analysis of how proteomics could be integrated into systems biology, showing, among other findings, our recent data on the interactions of cell metabolism and cell cycle, monitored by transcriptome and proteome analysis. The results are taken together and discussed in the frame of the cell cycle model described by Barberis et al, in PLoS Comput. Biol 2007.

AN EVALUATION OF PROFILING AND DIFFERENTIAL PROTEOMICS APPROACHES TO THE CHARACTERISATION OF BIOCHEMICAL PATHWAYS IN NEWLY DISCOVERED PROKARYOTES

James H Scrivens[1], Vibhuti Patel[1], Konstantinos Thalassinos[1], Susan E. Slade[1], Andrew Crombie[1], J. Colin Murrell[1], Joanne B. Connolly[2]

[1] Department of Biological Sciences, University of Warwick, Coventry, UK;

[2] Waters, Manchester, UK

Methanotrophic bacteria are environmentally important as they utilise atmospheric methane, a greenhouse gas as sole carbon and energy source. Until recently, all methanotrophs were unable to utilise substrates containing carbon-carbon bonds. A newly discovered genus Methylocella has been shown to grow on acetate and ethanol whilst retaining the ability to grow on single carbon sources. Identification of the enzymes involved in metabolism of multi-carbon substrates is essential. Methane grown Methylocella uses the soluble form of methane monooxygenase. When acetate was added to the culture, methane oxidation appeared to be abolished. In order to assess the impact of the Methylocella genus on methane flux in the environment, it is necessary to quantitatively determine the complex relationship between substrate availability and growth.

To characterise the proteins expressed using the different substrates it is essential to obtain confident identifications of as large a number of proteins as possible over a wide dynamic range with the ability to measure differential expression. Mass spectrometry based proteomics based on a bottom up approach has been well established as a technique capable of providing this information. Differential expression can be measured using a variety of labelled and non-labelled experimental techniques. In order to establish a consistent, validated method that could be applied to a number of expression experiments two approaches have been evaluated.

1. Enzymatic digestion of the protein mixture, labelling with iTRAQ reagent, followed by the use of 2D liquid chromatography (strong cation exchange (SCX) and reversed phase (RP)). Each fraction is then analysed using nanoLC-ESI-MS/MS.
2. Enzymatic digestion of the protein mixture followed by the use of a non-labelled alternative scan nanoLC-MS/MS approach Identity^{E} recently introduced by Waters.

The results obtained have been evaluated with respect to quantity of protein required, experimental and instrument time needed, number and confidence of proteins identified and differential expression measurements for the two substrates.

MUSCLE: A TARGET TISSUE FOR BIOMARKERS DISCOVERY

Cecilia Gelfi [1,2]

[1]*Dept. of Sciences and Biomedical Technologies, University of Milan, Segrate (MI), Italy*
[2] *Institute of Molecular Bioimaging and Physiology, CNR, Segrate (MI), Italy*
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The peculiarity of muscle tissue is the ability to produce an adaptive remodelling in response to different stimuli. This adaptive remodelling due to muscle disuse was observed in humans under paraphysiological conditions such as space flight, bed rest and immobilization. The muscle atrophy found after disuse appears to be similar; suggesting that a common mechanism is operating. In animal models, unloading remodels postural muscle phenotype, inducing catabolic events that result in a shift of muscle profile from slow-oxidative to fast-glycolytic. In humans, bed rest produces similar adaptive changes which occur at both structural and functional levels.

Atrophy is also a result of denervation, aging, starvation and a number of diseases defined as distinct clinical syndromes. The knowledge of the molecular mechanism at the basis of an imbalance in protein anabolism and catabolism (occurring in skeletal muscle) and the signalling events regulating these changes have only been partially investigated. Regardless of the enciting event, skeletal muscle atrophy is characterized by a decrease in protein content, fiber diameter, force production, and fatigue resistance. As a result, the activation of these signals can influence protein turnover by differentially modulating the protein synthesis and degradation. Results from proteomic investigations support the importance of skeletal muscle in the detection of signalling molecules related to muscle and non muscle diseases, such as neuromuscular disorders (ALS) and in cancer cachexia. The muscle tissue well summarizes the pathological processes that lead to the degeneration of motor neurons in ALS and the massive catabolic activation observed in cachexia. The use of comparative proteomics may represent a significant approach in the identification of clinically relevant biomarkers even in disorders not directly related to the muscle.

INVESTIGATING SYSTEMATIC FACTORS AFFECTING MS MOLECULAR BIOMARKER DISCOVERY

Damiana Pieragostino[1,2,3], Piero Del Boccio[1,2,3], Francesca Petrucci[1,2], Dante Mantini[1,2], Barbara Pavone[1,2], Santina Lupisella[3,4], Maurizio Ronci[1,2,3], Federica Forli[1,2], Paolo Sacchetta[1,2], Carmine Di Ilio[1,2], Giorgio Federici[3,4] and Andrea Urbani[1,2,3]

[1]Centro Studi sull'Invecchiamento (Ce.S.I.), Fondazione "G.D'Annunzio",[2]Dipartimento di Scienze Biomediche, Università "G.D'Annunzio", Chieti-Pescara, Italy; [3]Centro Europeo Ricerca sul Cervello (CERC), IRCCS-Fondazione S. Lucia, Roma, Italy [4]Dipartimento di Medicina Interna, Università di Roma "Tor Vergata", Roma, Italy

The development of reliable approaches from sample collection up to the separation of real MS signals from noise are still a critical factors for clinical proteomics investigations. We have pursued a systematic evaluation of pre-analytical factors affecting the biomarker discovery from sera and CSF. Moreover we have evaluated the sensitivity and specificity of peak detection algorithms most frequently employed for MS signals annotation. We present the development of novel computational methods to address these issues.

Serum and CSF MALDI-TOF mass spectrometric analysis was performed using linear MALDI-TOF experiments. Semi-quantitative performances were developed by an internal standard approach. Independent component analysis (ICA) has been used to separate distinct underlying signals from mixed recorded signals, on the basis of their statistical properties.

The validation of the proposed method for the separation of MALDI-TOF-MS signals from noise address both the effectiveness of signal processing and the detection of reliable protein peaks. For this purpose, we used mass spectra from in-vitro purified protein mixtures and simulated data for testing the developed algorithms and commercial solutions under controlled conditions. Human sera samples were then applied for validating the performances. APEX and centroid algorithms allow a peak detection hit rate of 30% and 25% while the application of ICA in combination with novel techniques for background noise reduction and baseline removal (LIMPIC) allows a hit rate of 100%.

We applied this novel strategy to the identification of pre-analytical bias in sera MALDI-TOF-MS profiling both from healthy donors and from Multiple Sclerosis affected subjects. Our data outline different molecular aging effects according to the pathological conditions. The differential signals have been identified by nLC-MS/MS peptide sequencing. A multivariate PCA model for sample classification have been constructed with a cumulative proportion of the explained variance of 0.89 on the first two components.

We have develop a novel noise reduction strategy for clinical proteomics investigations in particular by application of Independent component analysis. We have pursued an identification of key molecular features of sample aging affecting molecular biomarkers investigations.

GLYCOPROTEOMICS OF PACLITAXEL CHEMORESISTANCE IN OVARIAN CANCER CELL LINES

Di Michele Michela[1], Della Corte Anna[1], Cicchillitti Lucia[2], Del Boccio Piero[3], Urbani Andrea[3], Ferlini Cristiano[2], Scambia Giovanni[2], Donati Maria Benedetta[1], Rotilio Domenico[1]

[1] Research Laboratories, [2] Department of Oncology, "John Paul II " Centre for High Technology Research and Education in Biomedical Sciences, Catholic University, Campobasso, Italy; [3] Centro Studi sull'Invecchiamento (Ce.S.I.), Chieti, Italy

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Glycosylation is an important post translational modification and plays a fundamental role in carcinogenesis and drug resistance processes. Ovarian cancer is the leading cause of gynaecological cancer mortality. Paclitaxel is used in the first line treatment, but acquired resistance represents a major obstacle to a successful therapy (Ferrandina et al., 2006). A comparative proteomic approach was applied to the human epithelial ovarian cancer cell lines A2780 (paclitaxel sensitive), A2780TC1 and OVCAR3 (acquired and inherently resistant) to screen resistance biomarkers for the development of tailored therapies (Di Michele et al.).
Proteins differentially expressed in paclitaxel sensitive and resistant ovarian cancer cell lines were identified by DIGE coupled with mass spectrometry (MALDI-TOF and LC-MS/MS). Moreover, the glycoproteome of sensitive and resistant cell lines was analysed by: i) glycoprotein enrichment with lectin affinity chromatography and ii) total proteins separation by 2D SDS-PAGE and glycoprotein specific fluorescence staining, both followed by MS analysis.
Statistical analysis based on the protein expression of the paclitaxel sensitive and resistant ovarian cancer cells clearly discriminated the three cell lines (Fig 1). 151 proteins resolved by DIGE resulted to be differentially expressed in pairwise comparisons among the three cell lines and were further identified. Most of the proteins were related to the category of stress response (24%), metabolism (22%), protein biosynthesis (15%) and cell cycle and apoptosis (11%). Several glycoproteins were found to be up- or down-regulated in resistant compared to sensitive cell lines, such as calumenin, 150kDa oxygen regulated protein and glucosidase 2.
This work represents the first direct proteomic comparison of paclitaxel sensitive and resistant human ovarian cancer cells with the characterization of chemoresistance modulated glycoproteins.

References:
Di Michele, M., Della Corte, A., Cicchillitti L., et al. 2008. Submitted
Ferrandina, G., Zannoni, G.F., Martinelli, et al. 2006. Clin Cancer Res, 12 (9): 2774-9.

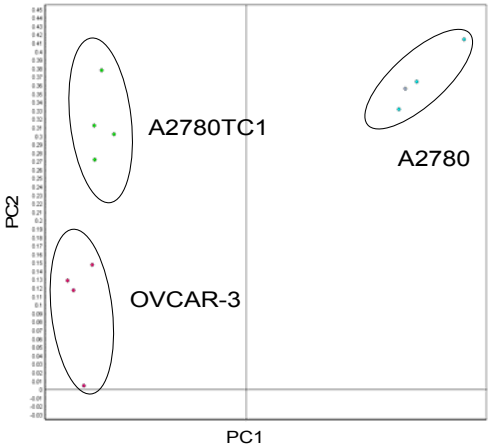

Figure 1. Multivariate analysis of DIGE results. Principle Component Analysis (PCA) discretely clustered the 12 (four replicates for each of the three cell lines) individual protein expression maps into the A2780 (paclitaxel sensitive), A2780TC1 and OVCAR3 (paclitaxel acquired or inherent resistant, respectively) cell lines.

MESOPOROUS BEADS: NEW TOOLS AND STRATEGIES IN BODY FLUIDS PROTEOMICS

Rosa Terracciano[1], Francesca Casadonte[1], Stella Frascà[1], Maria Immacolata Preianò[1], Luigi Pasqua[2] and Rocco Savino[1]

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[2]*Dep. of Chemical Engineering and Materials, University of Calabria, Cosenza, (Italy).*
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The delineation of a rapid and sensitive protein profiling method for biomarker discovery is an essential prerequisite for high-throughput and large scale clinical studies. The design and generation of material-based platforms for capturing "molecular signatures" from body fluids has gained increasing interest in recent years (Villanueva J. et al., 2004). We have therefore developed a strategy based on mesoporous silica beads for plasma low molecular weight peptides profiling (Terracciano R. et al., 2006). According to IUPAC nomenclature porous material are divided into 3 classes: microporous material with pore diameter below 2 nm, mesoporous material with pore diameter between 2-50 nm and macroporous material with the pore diameter above 50 nm. We speculated that mesopores might have the right dimension to be tunable for peptides entrapment, while micropores might be small and macropores might be too large. Given the high surface area mesoporous silicates offer the desired adsorptive capacity for binding and enrichment of low molecular weight peptides present in body fluids. Moreover, the absorption properties can be modified, since the pore walls, exhibiting high concentration of silanol groups at the surface, can be functionalized with different chemical species. Taking into account these characteristics, the application of mesoporous silicates for plasma and urine profiling as has been developed by our research group. We have thus designed new "hybrid" mesoporous silicates in order to differently modulate selective peptides enrichment. Plasma, serum and urine peptides were extracted from new derivatized mesoporous silica beads and then profiled by MALDI-TOF Mass Spectrometry. Different panels of peptides repertoires have been thus collected and could be used in biomarker discovery for disease diagnosis.

References:

Villanueva J. et al., 2004, Analytical Chemistry, 76:1560–1570.

Terracciano R. et al., 2006, Proteomics, 6: 3243-3250.

PROTEIN EXPRESSION ANALYSIS BY 2D-DIGE OF DIFFERENT AREAS OF GBM FROM PATIENT BIOPSIES

Raggiaschi Roberto [1], Matteoni Silvia[1], Mangiola Annunziato[2], Magnoni Letizia[1], Gotta Stefano[1], Rossi Marco[1], Valensin Silvia[1], Kremer Andreas[1], and Bakker Annette[1]

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Glioblastoma multiforme (GBM) is by far the most common and most malignant of the glial tumours. The median duration of patient survival is 1 year. The adverse prognosis of GBM is mainly due to its high propensity for tumor recurrence that is an inevitable event. In this study proteomics and bioinformatics investigations were undertaken in order to compare brain biopsies of tumor cores and periphery tissues from 6 GBM patients. This analysis aims to the identification of proteins that could potentially be involved in migration and invasive processes demonstrated to be important in tumor recurrence.

Liquid nitrogen frozen brain biopsies were pulverized and solubilized using DIGE-labeling compatible solution in order to label proteins with Cy-dyes for subsequent 2D-DIGE analysis. Proteins were separated on a 24 cm format by performing the 1st dimension on 3-10NL IPG strips and the 2nd dimension on a 9-16% SDS PAGE. Image analysis was performed with DeCyder-2D v6.5 while statistical analysis was carried out using Matlab R2007b (The MathWorks). Statistically significant spots were identified employing LC-orbitrap MS/MS. Networks and pathways analysis was performed using GeneGo's Metacore and Ingenuity IPA. The proteomics analysis of the 18 brain biopsies of 6 GBM patients derived from the enhanced lesion (the tumor in sensu strictu) the two adjacent sections, one from 1 cm outside the border of tumor (P1) and the other from more than 1 cm outside the border of tumor (P2) resulted in the selection of 54 protein spots differentially expressed. An unsupervised clustering of all 18 samples using normalized volumes of the 54 spots allowed clustering together 5 out of 6 tumor cores while the P1 and P2 were completely mixed. Database search of MS spectra was performed using Mascot software and ENSEMBL human database and it allowed the identification of 284 proteins that were imported into our in-house proteomics database. The identified proteins/genes were uploaded into GeneGo's Metacore and Ingenuity IPA for further analysis. The initial network analysis revealed major processes within the dataset including: metabolic processes, cytoskeleton and organization, anti-apoptosis and development-related. In an ad hoc experiment only few statistically significant differences were detected between close and far tumor peripheries.

BREAST CANCER PROTEOMICS: FROM RESEARCH TO CLINICAL APPLICATIONS

Pucci-Minafra, Ida[1,2], Cancemi, Patrizia[1], Albanese, Nadia Ninfa[1], Di Cara, Gianluca[1], Marabeti, Maria Rita[1], Marrazzo, Antonio[1,3], Minafra, Salvatore[1,2]

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Current clinical parameters for breast cancer diagnosis and cure 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 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 higher than previously thought. Therefore, proteomic screening for differential protein expression in subsets of tumour samples is an essential tool to generate data bases, to contribute to the knowledge of cancer-related biological pathways and to allow a molecular classification of cancer subtypes. In our laboratories we have developed adequate extraction procedures for proteomic profiling of breast cancer cells (Pucci-Minafra et al., 2006) and biopsy tissues (Pucci-Minafra et al., 2007). To minimize the limitation induced by heterogeneity of breast cancer tissues, we have recently introduced, as objective criterion to compare proteomes of different tissues, the normalization of the expression levels of individual proteins, for actin content in each tissue extract (Pucci-Minafra et al., 2008). With the aim to identify new sets of candidate markers, useful for patient's stratification and clinical correlations, in the present study we have increased the casistic of the surgical tissues of patients diagnosed for ductal infiltrating breast cancer. Differentially expressed proteins were identified by 2D-IPG coupled with Peptide Mass Fingerprint (PMF) and N-terminal microsequencing. Among the tumour-derived protein spots, a large proportion was found present in all patients, while other proteins, showed sporadic presence and high expression level, which suggests their possible role for patient stratification.

Pucci-Minafra I., Cancemi P., Fontana S., Minafra L., Feo S., Becchi M., Freyria A. M., Minafra S. Proteomics 2006, 6(8), 2609-2625

Pucci-Minafra I., Cancemi P., Marabeti M. R., Albanese N. N., Di Cara G., Taormina P., Marrazzo A. Proteomics Clinical Applications 2007, 1, 118-129.

Pucci-Minafra I, Cancemi P, Albanese NN, Di Cara G, Marabeti MR, Marrazzo A, Minafra S. J Proteome Res. 2008 Feb 20; [Epub ahead of print]

FIBROBLAST'S ROLE IN BREAST CANCER: A PROTEOMIC APPROACH

Cancemi, Patrizia[1], Albanese, Nadia Ninfa[1], Di Cara, Gianluca[1], Minafra, Luigi[1], Marabeti, Maria Rita[1], Pucci-Minafra, Ida[1,2]

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Fibroblasts are the major mesenchymal cell types committed to the matrix formation and renewal. Moreover they are the main source of paracrine factors that influence the growth of epithelial cells of neighbouring tissues. For these properties they may be involved in tumourigenesis, either by remodelling the tumor-associated extracellular matrix (ECM), and by the production of paracrine factors that influence the growth of carcinoma cells. Studies of fibroblasts associated to carcinomas have documented their phenotypic modifications, including abnormal migratory behaviour in vitro and growth factors altered expression. (Schor & Schor, 2001). In addition, fibroblasts often recruit inflammatory cells involved in the stimulation of angiogenesis, probably by the proteolytic release of sequestered angiogenic activators (Tlsty & Hein, 2001). Reciprocally, cancer cells may regulate the biosynthetic activities of fibroblasts, thus altering the ECM of the tumor, which in turn exerts some influence on neoplastic cell behaviour. The aim of the present work was to extend our previous observations on the effects of microenvironment factors on neoplastic cell behaviour (Pucci-Minafra et al., 2008, in press), utilizing the well characterized breast cancer-derived cells, 8701-BC (Minafra et al. 1989). The neoplastic cells were exposed to influences of fibroblasts either in a co-culture system or through incubation with the theirs conditioned media. In this report we show that the fibroblasts affect neoplastic cell behaviour by: increasing cell proliferation rate, rising the migration and invasion properties of cells in boyden chamber assays, and inducing a transition of cytoskeletal filament expression.
Further, these data were confirmed by proteomic approach that allows to evaluate multiple response of cells subjected to external influences.

Schor, S.L. & Schor, A.M. 2001. Breast Cancer Research, 3: 373-379.
Tlsty, T.D. & Hein, P.W. 2001. Current Opinion in Genetics & Development, 11: 54-59.
Pucci-Minafra, I., Albanese, N.N., Di Cara, G., Minafra, L., Marabeti, M.M., Cancemi, P. 2008. Connective Tissue Research, in press.
Minafra, S., Morello, V., Glorioso, F., La Fiura, A.M., Tomasino, R.M., Feo, S., McIntosh, D. & Woolley, D.E. 1989. British Journal of Cancer, 60: 185-192.

MULTIPLE EFFECTS INDUCED BY HERCEPTIN® ON 8701-BC BREAST CANCER CELLS

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Herceptin, an anti-neoplastic humanized monoclonal antibody (Herceptin®, Roche, CH), has been shown to be active against breast cancer cells over-expressing HER-2 receptor. HER-2 is a cell membrane protein that belongs to the epidermal growth factor receptors family and that results over-expressed in the 25-30% of breast cancer patients. The over-expression of HER-2 is considered a predictive and prognostic marker for breast cancer malignancy and invasiveness. On these bases, we aimed to analyze the effects caused by Herceptin treatment on 8701-BC breast cancer cells (Minafra et al., 1989). Firstly we evaluated the effects of Herceptin on the growth rate of 8701-BC cells. To this purpose, parallel cell cultures were daily treated with 0.5, 1, 1.5 and 3 μgr/ml of drug respectively, for a period of 7 days. Cell number was determined by a MTS assay. The resulting growth curves have shown a dose-dependent pharmacologic response, where cells treated with 3μgr/ml of Herceptin underwent 50% inhibition after 7 days. Therefore this concentration was selected to evaluate phenotypic effects exerted by the drug on the surviving cells. In a branch of experiments, the expression level of a group of pivotal protein classes was assayed, both at proteomic and immunological level. Concurrently, MMP-2 and MMP-9 activity in conditioned media of treated and untreated cells was assayed by zymographic assays. This investigation is justified by previous observation of our group, showing a certain degree of correlation between gelatinase expression and HER-2 overexpression in vivo (La Rocca et al., 2004). The results so far obtained, have shown that Herceptin treatment induces a strong inhibition of gelatinolytic activity and modulates the expression levels of some proteins involved in cancer progression, such as cytoskeletal proteins, cell cycle regulators, membrane receptors and associated proteins and some metabolic enzymes. We suggest that present data may contribute greatly to the knowledge of the multiple effects exerted by Herceptin on breast cancer cells.

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ORGANELLE PROTEOMICS - APPLICATION TO YEAST MITOCHONDRIA AND PLATELET PLASMA MEMBRANES

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Cellular organelles are of utmost importance for cellular metabolism, protein maturation, signal transduction and many further physiological processes. In this talk strategies for the isolation and analysis of two important organelles (mitochondria and plasma membranes) will be presented and discussed in detail.

Because of its sensitivity and high-throughput capabilities proteomics has become an important method in protein research regarding the analysis of complex protein samples. This in turn represents the basis for functional characterization and exploration of the biological relevance of proteins and complete protein networks. Platelets represent an optimal field for proteome research because of their anucleate nature, which renders genomic techniques inappropriate. Additionally, platelets are of major relevance to a broad range of cardiovascular diseases including coronary heart disease, myocardial infarction, and stroke. Membrane proteins and receptors act as signal acceptors, mediators, enhancers, and multipliers and therefore work generally as key molecules in many cellular functions in many cases. This is expressed in the fact that about 50% of all current small molecule drug target plasma membrane receptors as well as other membrane proteins. Therefore, proteomics represents a very promising technology for the comprehensive analysis of platelets and platelet membrane proteins to discover new potential membrane receptors or other relevant proteins that could be potential new drug targets as well as missing links for a basic understanding of platelet function.

Mitochondria play a central role in many cellular functions, including bioenergetics, apoptosis, and the metabolism of amino acids, lipids, and iron. Many diseases have been attributed to mitochondrial defects. According to the currently available information, however, only ≈ 70–80% of all presumed mitochondrial proteins have been identified so far. Thus, our knowledge about the physiological functions of mitochondria is still limited, and many mitochondrial diseases cannot be analyzed on a molecular level.

ISOFORMS AND PHOSPHORYLATION PATTERN OF THE SUBUNIT OF COMPLEX I ENCODED BY THE NDUFS4 GENE IN HIGH EUKARYOTES

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In murine and human cell cultures, activation of the cAMP cascade or direct addition of cAMP 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. Mutations in the NDUFS4 gene in patients with neurological diseases, impair the assembly and the activity of complex I. In isolated mammalian mitochondria cAMP promotes phosphorylation of 18 kDa subunit(s) of complex I. Edman sequencing provided results indicating phosphorylation of Ser145 in the C-terminus of the protein encoded by the NDUFS4 gene (Papa et al., Biochim. Biophys. Acta, 2008, in press). Mass spectrometry carried out by other groups failed however to confirm phosphorylation of this serine (Chen et al. J. Biol. Chem., 279:26036-42, 2004). 2D-IEF/SDS PAGE from our laboratory shows the presence in complex I of a PKA-dependent [^{32}P]-labelled band, recognized by a specific antibody directed against the P-Ser145 of the NDUFS4 C-terminus of the protein, with an alkaline pI of 9.52 and a MW proper of the NDUFS4 protein. In other experiments NDUFS4 protein was produced by heterologous expression of the entire cDNA (*presequence* plus mature protein: *M A A V S M S V V L R Q T L W R R R A V A V A A L S V S R V P* ***pT*** *R* ***pS*** *L R T* ***pS*** *T W R L* A Q D Q T Q D T Q L I T V D E K L D I T T L T G V P E E H I K T R K V R I F V P A R N N M Q S G V N N T K K W K M E F D T R E R W E N P L M G W A S T A D P L S N M V L T F S T K E D A V S F A E K N G W S Y D I E E R K V P K P K S K S Y G A N F S W N K R T R V **pS** T K; bold: candidate phosphoaminoacids; boxed: putative protein kinase consensus sites). The heterologous protein was [^{32}P]-labelled in a PKA dependent fashion. TLC of the protein digest showed phosphorylation of Thr and Ser residues. Work is in progress to identify the sequence position of the residues phosphorylated by PKA.

THE MITOCHONDRIAL PROTEOME OF ADULT AND OLD RAT LIVER:QUANTITATIVE AND QUALITATIVE CHANGES

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Mitochondria are a major source of reactive oxygen species (ROS) in tissues during aging. ROS initiates oxidative damage to phospholipids, proteins and nucleic acids; this damage may be a major cause of degenerative diseases and aging. The development of strategies to prevent the impact of aging requires to understand the molecular mechanisms that underlye this process. The aim of this work was the identification by proteomic tools of the quantitative and qualitative changes of mitochondrial proteins in old liver, highlighting the post-translational modifications.

We analysed the mitochondrial proteome of liver from adult (12 months) and old rats (28 months) by using two-dimensional electrophoresis. Proteins were separated on 18 cm 4-7 and 6-11 pH gradient IPG strips and 10% SDS polyacrylamide gels. Differentially expressed proteins were identified by MALDI and nano ESI MS/MS mass spectrometry. Results led to highlight 66 spots with altered expression in old rats. Mass spectrometry led to the identification of 45 proteins. These proteins belong to different mitochondrial pathways: beta oxidation of fatty acids; Krebs cycle enzymes; subunits of the respiratory chain complexes; urea cycle. In particular, we found differential expression in mitochondrial antioxidant enzymes: an increase of glutathione peroxidase 1; an increase of the mitochondrial aldeyde dehydrogenase, a detoxicant enzyme probably involved in the metabolism of 4-Hydroxy-2-nonenal; a decrease of catalase. Furthermore we identified, in old rats, for the first time in vivo, a post-translational modified form of Peroxiredoxin III. The total content of PrxIII in old rats was double than in young ones for the accumulation of the modified form. MALDI analysis revealed that this form was due to the overoxidation of the catalytic cysteine to cysteine sulphonic acid, this modification inactivates the protein irreversibly. The proteomic study here reported permitted to appreciate quantitative and qualitative changes in the protein expression of liver mitochondria of old rats. These results suggest new informations and open new lines of investigation on the involvement of mitochondria in aging.

STUDYING THE YEAST CELL CYCLE REGULATOR SIC1 BY FUNCTIONAL PROTEOMICS

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In the cell, many processes are governed not only by the relative abundance of proteins but also by their post translational modifications. Complex biological processes such as cell cycle, cell growth, cell differentiation and metabolism are orchestrated and tightly controlled by reversible modification events that modulate protein activity, stability, interaction and localization (1). In particular, the cell cycle is strictly regulated by a molecular interaction network, which involves the periodic synthesis and destruction of cyclins that bind and activate cyclin-dependent kinases that are present in nonlimiting amounts. Phosphorylation of specific serine, threonine and tyrosine residues led protein kinases to alter the function of their target proteins and cyclin-dependent kinase inhibitors contribute to cell-cycle control (2).

In the yeast, Sic1 is a cyclin-dependent kinase inhibitor (Cki) that inhibits the Clb5,6/Cdk1 complex whose activity is required to start S phase (3). Mutation of a phosphorylatable serine within the Sic1 casein kinase 2 (CK2) phosphorylation consensus sequence (mutations S201A or S201E) alter cell growth/cell-cycle coordination at the G1 to S transition (4).

Our approach to study the function of Sic1 in cell cycle regulation entails the identification of its post-translational modifications (phosphorylation) and of its molecular partners. To this end yeast cells are grown under different environmental conditions (i.e. using either glucose or ethanol as carbon source) and analyzed in different cell-cycle phases.

We present a first set of data regarding nutritional and cell cycle modulation of Sic1 phosphorylated by CK2 and outline a strategy to obtain the complete phosphosignature of Sic1 under the same conditions described above performing mass spectrometry analysis.

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3,5-DIIODO-L-THYRONINE-INDUCED PROTEOMIC CHANGES IN MITOCHONDRIA FROM FATTY LIVER: EVIDENCES FROM TWO-DIMENSIONAL AND BLUE NATIVE ELECTROPHORESIS

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The liver participates in energy and substrate metabolism, controlling blood glucose levels and modulating the balance between glycogenolysis and gluconeogenesis. It is involved in fat oxidation and storage being the major site of fatty acid synthesis in presence of excess dietary cardohydrates, amino acids or ketone bodies. Diet-induced obesity is commonly associated with fatty liver and steatosis. It is now more and more evident that these conditions are strictly correlated to alterations of mitochondrial functions. 3,5-diiodothyronine (T2), a naturally occurring iodothyronine, is able in affecting energy metabolism and mitochondrial efficiency. It has been reported that long term treatment with T2 powerfully reduces adiposity in rats fed high fat diet stimulating hepatic fatty acids oxidation and increasing mitochondrial uncoupling, leading to a less efficient utilization of lipid substrate reducing body weight-gain and liver adiposity.

To study how the mitochondrial phenotype responds in terms of protein expression to T2-treatment, we combined two-dimensional gel electrophoresis (2D-E), mass spectrometry and blue native PAGE. Liver mitochondrial proteome from standard diet -fed control rats (N), high-fat diet-(HFD) fed rats and T2-long term (30 days) treated HDF rats (HFD+T2) was analyzed. The identification of differentially expressed (2-fold changes and $P< 0.05$) proteins (from 2D-E maps) among the experimental groups, allowed us to obtain an integrated view of the phenotypic/ metabolic adaptation (oxidative stress, lipid metabolism, urea cycling and respiratory chain activity) occurring in liver mitochondrial proteome during HFD and after T2- treatment. Interestingly, T2-treatment counteracted several changes induced by HFD. Blue native page and subsequent in gel-activity measurements of OXPHOS complexes, revealed, quantitatively, a significantly modified profile of individual complexes in HFD mitochondria vs N ones. This pattern was re-normalized in mitochondria from T2-treated animals with a suggested higher oxidative capacity as indicated by the colorimetrically stained enzymatic activities of the essayed complexes. The present study lead to a metabolic interpretation consistent with the notion that, in a situation of cellular and mitochondrial overload of fatty acids, T2 is able to affect mitoproteome in a way putatively associated with no-steatosic liver following high fat diet. The identified metabolic pathways may be responsible for the beneficial effect of T2 on liver adiposity and energy metabolism. Overall, the data supply novel informations for elucidating the mechanisms of T2 action on liver mitochondrial phenotype and metabolism in fatty liver.

MITOCHONDRIOMICS

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The impact of mitochondria on several fundamental cellular processes is reflected in their involvement in the pathophysiology of common diseases such as Parkinson's disease, diabetes, and obesity and a wide range of monogenic disorders primarily associated with energy impairment or metabolic diseases. The importance of mitochondria is also reflected by the steep increase of proteins, which has been localized to this organelle. In yeast, more than 500 of the expected 700-800 mitochondrial proteins are already annotated. In the mammalian species, the expected numbers are estimated to be in the range of 1500-2000 proteins, and the currently annotated entries reach almost 700. In addition to the studies dealing with single proteins, there are many high-throughput approaches that improve the description of the mitochondrial proteome. They include computational predictions of signaling sequences, proteome mapping, mutant screening, expression profiling, protein-protein interaction, and cellular sublocalization studies. The MitoP2 database (http://www.mitop2.de/) was established to structure, explore, and customize the available data on mitochondrial proteins, functions, and diseases. MitoP2 provides a comprehensive picture of the mitochondrial proteome by focusing on (1) the orthology between species, including *Saccharomyces cerevisiae*, mouse, and humans; (2) the definition of mitochondrial reference sets in these species; (3) the integration of data predictive for mitochondrial localization or function stemming from genomewide approaches; (4) the allocation of a gateway for functional data from model systems and genetics of mitochondriopathies; and (5) the calculation of a combined score for each protein summarizing the indirect evidence for a mitochondrial localization.

Although many mitochondrial proteins have been identified, understanding their functional interrelationships has been a challenge. Using the integrated data, we construct a comprehensive network of the mitochondrial system in their cellular context, including extra-mitochondrial proteins. The network brings together annotated and predicted functions into a single framework. This enabled, for the entire system, a survey of mutant phenotypes, evolution, and disease susceptibility. Furthermore, we derived novel functional contexts for hundreds of uncharacterized proteins. Our network thus advances the understanding of the mitochondrial system in yeast and identifies properties of genes underlying human mitochondrial disorders.

FILTERING PROTEOMES FOR SEQUENCE ANNOTATION: SUBCELLULAR LOCALIZATION AND GPI ANCHOR PREDICTION IN EUKARYOTS

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Bioinformatics tools in the postgenomic era may help in transferring information from the biological data bases of structures and sequences to non-annotated sequence targets. To this aim the annotation of the subcellular localization is a major step in protein functional annotation. This is particularly important in eukaryotic cells, which contain several subcellular compartments enclosed by membranes hosting different relevant functions (Casadio et al., 2008). Methods are available for the 'ab initio' prediction of the subcellular localization of globular proteins, relying both on the explicit search of sorting signals in the residue sequence, or on the capture of intrinsic features coded in the protein residue composition. We developed BaCelLo, a SVM-based predictor that outperforms other available methods when the major localizations are discriminated: extracytoplasmic space, cytoplasm, nucleus, mitochondrion and chloroplast (Pierleoni et al., 2006).

With BaCelLo it is possible to predict subcellular localisation when the data base search for homology is not sufficient to locate the sequence. Alternatively when the protein is predicted to be a membrane protein, its topology is assigned with ENSEMBLE (Martelli et al., 2003), specifically implemented for the prediction of topological models of all-alpha membrane proteins. By this it is possible to filter eukaryotic proteomes and annotate the correspondent protein sequences (Pierleoni et al., 2007).

As a second relevant feature the detection of lipid anchors may also help in predicting the protein function. A common way for targeting globular proteins to the membrane surface is the attachment with a lipid anchor. The most common and studied lipid anchor modification is the glycosylphosphatidylinositol (GPI) linkage to the C-terminal residue, upon the cleavage of a 20-30 residue long peptide. We developed GPIPE, a method based on HMMs and SVMs that is able to accurately discriminate GPI-anchored proteins in a proteome and to determine the position of the cleavage site. The integration of these predictors with others from the our group in a platform, already implemented in house, allows the collection of different structural and functional features for each filtered protein sequence in a fast and automatic way.

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COMBINED USE OF RNAi AND QUANTITATIVE PROTEOMICS TO STUDY GENE FUCNTION IN Drosophila

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Introduction

RNA interference is a powerful way to study the function of genes of interest. Upon depletion of a gene product, the most common global and unbiased assay is microarray analysis, which allows the measurement of thousands messenger RNAs. Clearly, it would be desirable to have a similarly global 'ready out' at the protein level for the characterization of loss-of-function phenotypes. We introduce such a technology by simultaneously applying Stable Isotope Labeling by Amino acids in Cell culture (SILAC) (1) and RNA interference (RNAi) to Drosophila SL2 cells.

Methods

SL2 cells were grown in custom Schneider Medium. 'Heavy' and 'light' media were prepared by adding 13C615N4 L-arginine and 13C615N2 L-lysine or the corresponding non-labeled amino acids, respectively (1). For RNAi, SILAC-labeled SL2 cells were seeded in custom serum-free medium containing amino acid isotopes. dsRNA was added and cells were incubated for 1 hour at 26°C. Serum-containing heavy or light media was added and cells were grown for 7 days until harvest. Production of GST or ISWI dsRNA was performed as described in (Worby et al., 2001). Subsequently, whole cell extracts was separated by SDS/PAGE, lanes were cut and processed for mass spectrometry. Peptides were analyzed by nanoflow liquid chromatography an Agilent 1100 LC system coupled to LTQ-Orbitrap (Thermo Electron). The raw data files were analyzed with an in-house developed quantitative proteomics software MaxQuant, (2), in combination with a Mascot search engine.

Results

By Liquid Chromatography- Tandem Mass Spectrometry (LC-MS/MS), we obtained a quantitative proteome to a depth of more than 4000 proteins in this single cell type, providing an excellent platform for global proteomic profiling of different cellular states. After knockdown of ISWI, an ATP- hydrolyzing motor of different chromatin remodeling complexes (3), we quantitatively compared protein levels by automated analysis of mass spectrometric results. ISWI itself was reduced ten-fold as quantified by SILAC. In addition, several hundred proteins were significantly regulated and clustered into distinct functional categories, as expected from the diverse ISWI-dependent biological processes. Interestingly, Acf-1, a direct interaction partner of ISWI, is severely depleted at the protein but not the transcript level, most likely resulting from reduced protein stability.

Proteome-wide phenotypization upon RNAi should become a powerful new tool in the post-genomic arsenal.

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PHOSPHOPROTEOMICS – TECHNOLOGIES AND APPLICATION TO THE STUDY OF DEPOLARIZATION-DEPENDENT PROTEIN PHOSPHORYLATION IN NERVE TERMINALS

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Depolarization of neurons rapidly collapses the membrane potential leading to an influx of calcium into nerve terminals. This initiates the release of neurotransmitters from small synaptic vesicles in nerve terminals and also initiates the recycling of the empty synaptic vesicle (endocytosis) for reuse. Nerve terminals, synaptosomes, can be rapidly isolated from neurons, however due to contamination with e.g., mitochondria, further purificaiton using percoll gradients are necessary to achieve pure synaptosomes. Depolarization-dependent calcium influx activates a phosphatase calcineurin which triggers dephosphorylation of key endocytic proteins and activates endocytosis. It also stimulates calmodulin-dependent protein kinases to phosphorylate proteins involved in synaptic vesicle pools for exocytosis. Only a small number of phosphosites in these proteins have been identified and quantified after depolarization to date.

Phosphoproteomics relies on methods for efficient purification and sequencing of phosphopeptides from highly complex biological systems using low amounts of starting material. We have previously developed highly selective and sensitive methods for enrichment of phosphorylated peptides from complex mixtures including TiO_2 chromatography (Larsen MR *et al*., MCP, 2006) and the SIMAC strategy (Thingholm TE *et al.*, MCP 2007). In addition, we have shown that a significant amount of hydrophilic phosphopeptides are lost because they are not retained by normal reversed phase material. However we introduced graphite columns to capture these phosphopeptides for mass spectrometry. In this study we combined peptide quantification using iTRAQ, peptide pre-separation strategies, our selective phosphopeptide enrichment procedures and reversed phase and graphite chromatography's to achieve comprehensive phosphoproteome analysis of living nerve terminals. These results we have compared with resutls obtained by traditional pull down strategies combined with phosphoproteomics strategies.

ACCURATE INTACT MASS ANALYSIS AND STRUCTURAL CHARACTERISATION OF A MONOCLONAL ANTIBODY BY ION MOBILITY COUPLED WITH MASS SPECTROMETRY

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Over the past 15 years electrospray ionisation combined with mass spectrometry has proven to be a very powerful combination for determination of intact protein mass. The differences observed between expected and actual masses can provide insight into the presence of post translational modifications. Using a Bayesian based algorithm, we show that sub 1ppm mass measurement accuracies can be obtained on an intact glycoprotein in excess of 30kDa.

Structural characterisations of intact mouse antibody molecules are challenging due to their high molecular mass, hydrophobic nature and presence of sugar moieties. Conversely the analysis of the intact proteins also advantageous because it reduces dramatically the time for sample preparation and data interpretation, compared with peptide mapping and sequencing. Furthermore, it also minimizes the chance of introducing putative modification, which are often observed during peptide mapping. The aim of this study is to perform structural characterisation on a monoclonal antibody by ion mobility mass spectrometry. We show how it is possible utilising ion mobility to perform Time Aligned Parallel (TAP) fragmentation and gain full glycan and peptide sequence coverage of a glycopeptide of interest.

ANALYSES AND IMPLICATIONS OF COMPLEX I PHOSPHOPROTEOME

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Phosphorylation is one of the most abundant post-translational protein modifications. In higher eukaryotes more than 30% of proteins are phosphorylated [1, 3]. Different biological processes such as cell cycle, growth, differentiation and metabolism are regulated by reversible phosphorylation events that modulate protein activity, stability, interaction and localization [1-3]. Furthermore the regulation of protein phosphorylation has a major role in human disease, most notably in cancer [4].

A large number of mitochondrial proteins have been found to be phosphorylated, among which subunits of complex I, complex IV and complex V of the oxidative phosphorylation system [5-7]. Complex I (NADH-ubiquinone oxidoreductase, E.C. 1.6.5.3) is the largest enzyme of the respiratory chain. In bovine heart complex I consists of 45 subunits [8] and its deficiency represents one of the most severe and frequent cause of the disorders in the mitochondrial energy metabolism associated with human diseases [9, 10]. Complex I dysfunctions are also associated with various neurodegenerative diseases [11].

In the last years phosphorylation of complex I subunits has been investigated by various groups with different methodological approaches; in the present study we have extensively analyzed the phosphoproteome of bovine heart complex I using a newly developed procedure based on non-denaturing gel electrophoretic separation of the complex from small quantities mitochondria samples, in-gel digestion, phosphopeptide enrichment by titanium dioxide (TiO_2) [12] and phosphopeptide-directed MS^3 mass spectrometry analysis. The results, besides confirming serine phosphorylation of the 42kDa, ESSS subunits and B14.5a subunit revealed threonine phosphorylation of the B14.5b (human gene NDUFC2) and B16.6 (Grim-19) subunits of bovine complex I. The analysis also revealed presence of programmed cell death protein 8 (AIF) [13] in both the native and the purified complex I samples. The possible physiological relevance of phosphorylation of these proteins is interesting and it will deserve further investigations.

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COMBINED USE OF MS/MS/MS AND DANSYL CHEMISTRY IN PROTEOMICS

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The challenge of proteomics, the next frontier of biological and biomedical research, is essentially based on archetypal "Analytical Chemistry" strategies, namely: i) separation and ii) qualitative (identification) and quantitative analysis of proteinaceous analytes. Furthermore mass spectrometry, one of the most powerful tool in modern Analytical Chemistry, is the core methodology in proteomics. However it is well known that either separation methodologies (2D gel electrophoresis) and mass spectrometric techniques (mass fingerprinting) were actually introduced much time before than proteomics advent. In this field, MS has been recognized as a 'Gold Standard' tool for the identification and analysis of proteins. Despite the advances in technology and methodology, proteomics is still far from having reached the stage of productivity and utility that it is necessary for it to be critical to biological and biomedical research in the post-genome era. Areas requiring a prompt attention are related to sample preparation, separation technologies, quantitative methodologies, full exploitation of modern mass spectrometers.

Our research is aimed at facing some of the above challenges by focusing on the analytical chemistry core of proteomics through the integration of innovative separation methods and mass spectrometry techniques for a next generation proteomics.

A new hybrid instrument has recently been introduced, that combines the capabilities of a triple quadrupole and an ion trap in a single platform through the use of novel triple quadrupole linear ion trap mass spectrometry technology. The system couples high selectivity triple quadrupole functionality such as precursor ion scanning, with high performance linear ion trap performance for very sensitive full scan MS and MS/MS data. More importantly, combining source collision-induced dissociation (CID) and tandem mass spectral acquisition in a MS3 experiment using a linear ion trap results in a highly selective and sensitive approach to identifying proteins in mixture.

Since 1968 [1] we demonstrated, for the first time, that the very low IP of the DANS moiety led to relatively intense molecular ions in MS. Since DANS derivatives have an intense fragment at m/z: 170, we applied "metastable refocusing" for the analysis of amine mixtures [2,3]. Revisiting our previous findings a method for proteomic analysis based on the

use of dansyl chloride and linear ion trap is thus suggested. In a recent paper [4], we set up a new approach to label selectively phospho-Ser/Thr residues by exploiting the features of novel linear ion trap mass spectrometer in a next generation proteomic approach. For the first time we introduced the novel acronym RIGhT (Reporter Ion Generating Tag), to indicate a general derivatization method based on the labelling of target residues with reagents capable of generating reporter ions in MS2/MS3 experiments. The method is based on a) selective modification of target residues with dansyl chloride or other available dansyl reagents and b) on the selective detection and identification of labelled peptides by exploiting the characteristic fragmentation pathway of dansyl derivatives. The dansyl derivatization in fact, introduces a basic secondary nitrogen into the molecule that enhances the efficiency of signal ionization; and using a linear ion trap mass spectrometer, one can take advantage of the typical 170 m/z and 234 m/z fragments in MS2 and the diagnostic 234-170 m/z fragmentation in MS3 mode.

Experiments combining a precursor ion scan with a MS3 linear ion trap scan modes can be made at femtomolar sensitivity. The selected precursor ions are submitted to a combined MS2 and MS3 experiments to specifically detect only the ions that produce the 234-170 m/z fragmentation.

Now using chemical procedures coupled with dansyl chloride labeling we showed a methodology capable of large-scale proteomic detection of post translational modifications in complex protein mixture. So far, dansyl-cysteamine has been successfully exploited to detect phosphorylated peptides [4] and dansyl chloride labelling has been useful for the identification of 3-nitrotyrosine residues [5] in complex protein mixtures.

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POST-GENOMIC TOOLS AND THE STUDY OF PHOTOSYNTHESIS IN RHODOBACTER SPHAEROIDES

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Rhodobacter sphaeroides is a non-sulfur, purple, facultative photosynthetic bacterium belonging to the alpha-3 subgroup of the proteobacteria. It is best characterized by its remarkable ecological versatility, able to grow either aerobically or anaerobically as a heterotroph or lithotroph, utilizing either respiratory or photosynthetic electron transport chains. Evidence suggests that it is representative of a group of organisms leading to the development of mitochondrion on the one hand and of the Cyanobacteria on the other, which were the precursors of the chloroplast. In our laboratory we have been studying how light and oxygen control the development and abundance, as well as structure-function of the inducible photosynthetic membranes or ICM. We have employed several major approaches to address these problems, namely: genetics, biochemistry, transcriptomics and proteomics. Although the major ICM components comprising the pathways which are involved in harvesting light energy and its conversion to chemical energy are known, we have suspected for sometime that other components of the ICM constitute important structural and functional activities. In a combination of genetic and proteomic studies we have begun to identify and to characterize the protein and cognate gene pairs which comprise these structures (Zeng, et al., 2007). To do this we have had to characterize the proteomic composition of the various sub-cellular fractions of R. sphaeroides in order to precisely identify new proteins which are part of the ICM structure. Because R. sphaeroides undergoes distinct morphological changes when it transitions between different growth modes we have employed time-dependent transcriptome and proteome anyalyses to better understand the regulatory cascades that accompany these transisitions
(Arai et al., 2008). The technologies developed at PNNL have enabled us to carry out kinetic analyses as cells are shifted between alternative growth states. Assisting in all of these analyses has been the use of both structural and regulatory mutations. In general terms, utilizing the proteome analyses developed by the PNNL laboratory, we have found that gene expression profiles as determined from the proteomic data are detectable at approximately half the efficiency as determined from transcriptome analysis. For example, under almost all growth conditions, transcriptome profiles reveal gene expression levels of 63-73%, whereas proteome analysis shows expression levels of approximately 32-36%. Finally, bringing together the power of genetics and the post-genomic tools enabling the analysis of both the transcriptome and proteome have opened a new chapter in our ability understand this very versatile organism.

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METAPROTEOME ANALYSIS OF MICROBIAL COMMUNITIES

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Micro-organisms represent a significant proportion of biomass on earth and drive many natural cycles. They are also involved in the development and prevention of diseases in humans, animals and crops. Although they live naturally in communities, mostly pure cultures have been analyzed in the lab. Current approaches allow the description of the structural and functional microbial diversity in ecosystems by proteins (metaproteome analysis). While nucleic acids and phospholipid fatty acids are often used as biomarkers for microbial identity and metabolic potential in environmental samples, extracellular and intracellular proteins are also promising markers of actual biological activities. The advantage of proteins is that they confer insight into actual functionality, including catalysis of metabolic reactions and regulatory cascades, and hence are better markers for microbiological activity than DNA or even RNA.

The presentation gives an overview on several examples for successful metaproteome analysis in literature. Furthermore results from own experiments are presented including samples from soil and ground water (1) as well as from defined mixed cultures of pathogens involved in lung infections of patients suffering from cystic fibrosis. After protein extraction with liquid phenol, the proteins were separated by 2D-electrophoresis. It became obvious that sample preparation is the most critical step by analyzing samples with high concentrations of contaminants, e.g. humic compounds. Several proteins were identified by mass spectrometry. Chances and limitations of the metaproteomic approach will be discussed with special focus on protein identification.

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RESPONSE OF RHODOBACTER SPHAEROIDES R26.1 TO HEAVY METALS EXPOSURE

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Chromate is a highly soluble and toxic non-essential oxyanion for most organisms. A number of chromate resistant bacteria, including *P. fluorescens*, *E. coli*, *Bacillus* sp, *Arthrobacter* sp. have been investigated each showing different resistance mechanisms [1]. The chromate influence on the photosynthetic growth of the facultative anaerobic bacterium *Rhodobacter sphaeroides* strain R26.1 is presented. An efficient resistance mechanism to chromate is suggested both by the high EC50 value and the lag-phase lengthening induced by concentrations above 0.05 mM. *R. sphaeroides* cells completely reduce chromate 0.2 mM within 20 h from light exposure and exhibit residual chromate-reducing activity up to 0.5 mM; cell growth starts upon complete chromat reduction. Increasing the metal concentration produces a progressive increase in the reduction time, mirrored by an increase in the lag-phase duration. Chromate reductase activity results preferentially associated with the protein soluble fraction.

Chromate effect on soluble enzymes is investigated by a proteomic approach: reproducible bidimensional electrophoretic maps of *R. sphaeroides* R26.1 soluble proteins were obtained from cells grown in plain and chromate-exposed mediums. More than 600 independent spots were found along with numerous differences between the two growing conditions. Protein identification via Peptide Mass Fingerprint is currently in progress.

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PROTEOMICS OF CELL-CELL COMMUNICATION IN FOOD RELATED LACTIC ACID BACTERIA

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Bacteria synthesize, release, detect and respond to small signalling molecules, termed "autoinducers". These signalling molecules accumulates and trigger cascade genetic and proteomic events when a "quorum" is reached. Only recently, a few studies have considered the mechanisms of cell-cell communication in food related lactic acid bacteria (1). Such mechanisms have been studied through a proteomic approach in sourdough lactic acid bacteria (2). As determined by two-dimensional electrophoresis analysis, associations between the most common sourdough lactic acid bacteria revealed variations in the levels of expression of cytoplasm proteins. After mass-spectrometry analysis, most of these proteins were shown to be involved in the energy metabolism, environmental stress adaptation and also in "quorum sensing" mechanisms. Gene expression studies showed variations in the levels of the *luxS* and *metF* genes driving the intra-species cell-cell communication. Presumptive furanone like molecules were found as the signalling molecules to determine cell-cell communication in sourdough lactic acid bacteria. Under the same food ecosystem, cell-cell communication was studied based on the synthesis of the autoinducer-I (AHL-type) involved in the inter-species mechanisms of "quorum sensing" (3). Other genes, HPK and RR, specifically involved in "quorum sensing" were identified in sourdough lactic acid bacteria and their levels of expression was determined under conditions simulating sourdough fermentation. The same mechanisms seemed to drive the microbiota of the natural whey starter used for Parmigiano Reggiano cheese making during propagation (4). The resulting population is a mixture of different phenotypes mainly determined by the gradient of temperature that traditionally affected the preparation of the above natural starter.

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PROTEOMIC APPROACH TO THE CHARACTERISATION OF THE ANTIBIOTIC PRODUCER *Nonomuraea sp ATCC 39727*

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Introduction: Actinomycetes are well known producers of secondary metabolites. *Nonomuraea sp ATCC 39727* is a strictly aerobic actinomycete, industrially important as producer of the glycopeptide A40926, used as a precursor of the semi-synthetic antibiotic dalbavancin. In previous studies it was shown that the production of A40926 is depressed by calcium, but promoted when L-glutamine or L-asparagine are used as nitrogen sources instead of ammonium salts (1). In this study the proteome of *Nonomuraea sp ATCC 39727* has been analysed in two different antibiotic production conditions achieved by simple chemically defined growth media. Changes in the 2DE pattern in the different growth conditions tested have been detected, and the spots showing significant differences have been analysed by mass spectrometry.

Materials and methods*:* The strain was maintained and cultivated as described (1). The chemically defined media used in this study are the MM-103/Ca (strong depression of A40926 production) and MM-103/Gln (overproduction of A40926). *Two-dimensional gel electrophoresis (2DE)* Proteins were separated by 2DE on 24-cm IPG strips that provided a linear gradient from pH 3 to 10. The second dimension was a Laemmli SDS-PAGE (12.5%) and the gels were analysed by ImageMaster 2D V.5. *Protein identification by mass spectrometry* The stained spots were digested with trypsin. The resulting peptides were extracted and separated by RP-HPLC and then analysed by a Q-Tof mass spectrometer. *Enzymatic assay* The enzymatic activity of MDH was performed as in (2).

Results and discussion The pattern of protein expression has been analysed in two different growth conditions and analysed by 2DE. Of the variable spots, one showing a decrease in the overproduction condition, was identified as a malate dehydrogenase (MDH). The different expression level in the two growth conditions of MDH is of particular interest since no significant changes in the expression levels of enzymes involved in the central metabolism could so far be associated with A40926 overproduction. Direct measurements showed a significant decrease of the MDH enzymatic activity in the extract from the bacteria grown in the A40926 overproduction conditions. Modifications in the TCA cycle have been proposed to be involved in the overproduction phenomenon in different model systems (1). It is known that production of secondary metabolites like A40926 depends on a high supply of acetyl-CoA. It is conceivable that the observed decreased expression of MDH, impairing the TCA cycle activity, will make more acetyl-CoA available for A40926 production.

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METAPROTEOMIC APPROACH TO MBR BIOMASS CHARACTERIZATION

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Membrane bioreactors (MBR) are a relatively new and promising technology in the sector of advanced wastewater treatment (Yang et al., 2006). The combination of a bioreactor with a set of membrane modules enables the straightforward separation of treated sewage from activated sludge. MBRs compete by many advantages to conventional activated sludge processes (CAS), but its greatest feature leads to the support of both non-flocculating and flocculating bacteria. Therefore MBRs can be operated on higher loading rates and provide very high effluent quality.

Nevertheless, MBRs still tend to membrane biofouling that impedes further process optimization. Biofouling is mainly caused by extracellular polymeric substances (EPS) and soluble microbial products (SMP) that accumulates onto and into the membrane. Consequently the permeate flux declines while simultaneously the pressure on the membrane (transmembrane pressure - TMP) inclines. Until recently, much research has been carried out to minimize biofouling effects. Engineering tools were mainly applied with regard to process optimization and monitoring. In contrast, biological tools to were rarely applied due to the fact that biofouling have been strongly related to the occurrence of EPS and SMP that consist mainly of polysaccharides and proteins. Thus, studies on DNA and/or RNA have become impractical in studying biofouling phenomena. Both engineering and biological tools did not reveal sufficient inside to the linkage between biofouling and biomass dynamics. There still exists a lack of knowledge about enzymatic activities and microbial dynamics of the MBR bioconsortium.

However, a novel approach called “metaproteomics” has been recently proposed to explore environmental samples and activated sludge (Wilmes and Bond, 2004; Kan et al., 2005). This approach can deliver important information about the microbial enzymatic activity and can reflect reaction/adaptation of a biocommunity to its environment. It is for this the reason that metaproteomics will gain the understanding about biofouling in MBR.

Here we present the first application of metaproteomic approach to MBR sludge, an extremely heterogeneous sample often found in environmental systems. We have developed a novel extraction and purification method based on phenol, especially for environmental samples (Benndorf et al., 2007). First studies were carried out on steady stage development of

the MBR biomass and its specific reactions to a punctual salt shock load. It could be demonstrated that conventional engineering tools to monitor the reactor performance were not as sensitive as proteomic tools to reveal reactions and adaptations of the MBR biomass. Important protein-protein interactions could be detected by 2D-PAGE application only and provided a new inside into the complex nature of the MBR bioconsortium.

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A PROTEOMIC STUDY REVEALS A MEMBRANE-BOUND LOCALIZATION OF TYROSINE DECARBOXYLASE IN *ENTEROCOCCUS FAECALIS*

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Enterococcus faecalis APT TYRA 10-2 2 is a food-contaminant bacterial strain, isolated from a sample of Robiola of Roccaverano, a Piedmont cheese. Its ability to produce biogenic amines was determined by HPLC [1]: these analyses showed a strain ability to biosynthesize high amounts of tyramine and traces of 2-phenylethilamine. The amine production kinetic suggests that the same enzyme was able to catalyse the decarboxylation of both tyrosine and phenylalanine, as already described in Enterococcus faecium [2].

In the present work Enterococcus faecalis proteome was analyzed in correlation with the highest amount of tyramine produced both in stimulated (CDM fortified with tyrosine and phenylalanine) and control conditions (CDM). Both cytosolic and membrane districts were analyzed by bidimensional electrophoresis. Cytosolic proteins were resolved by 4-7 pI and 4-5.5 pI maps; membrane proteins were analyzed by 4-7 pI maps. On the complex our investigations highlighted 48 proteins differentially expressed: 35 overexpressed and 13 downregulated in stimulated condition. Most of these proteins belong to 9 main metabolic pathways. We highlighted the different expression of tyrosine decarboxylase: it was expressed in the membrane district and its expression was higher in stimulated condition. Membrane-bound localization of TDC was hypothized by long time [3] but no certain evidences were available.

We also tried to evaluate the strain ability to produce biogenic amine precursors by mean of proteolytic enzymes. To this aim we investigated extracellular protein expression by zymograms and 4-7 pI 2DE maps. Zymograms seemed to indicate the presence of proteases, but we were not able to find them in bidimensional maps. Actually analyses of extracellular fraction by LC-MS MS are in progress.

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THE PROTEOMINER AND THE FORTYNINERS: SEARCHING FOR GOLD NUGGETS IN THE PROTEOMIC ARENA

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The present lecture will cover modern aspects of combinatorial ligand libraries (CLL), as used for analyzing the "low-abundance proteome" in association with mass spectrometry. First, the capturing properties of baits of different lengths (from single amino acid to hexa-peptides) are described, to show that a plateau is rapidly reached above a tetra-peptide in length, thus confirming the validity of having adopted hexapeptides for the considered application. The mechanism of interaction with proteins from very complex proteomes and the ability to decrease the dynamic concentration range is demonstrated with the help of mass spectrometry analysis. Examples are given on how treatment with CLLs dramatically improves the detectability of peptides in mass spectrometry analysis and permits one to detect a very large number of proteins as compared with control, untreated samples. The use of complementary libraries is discussed with the aim to discover additional low-abundance species that escaped the first library. The lecture will end by discussing the possibility to discover extremely rare gene products, and the quantitative aspect of the technology when associated with mass spectrometry. Some insights on the applications for hidden, low-abundance biomarkers are also presented. The samples to be dealt with: the cytoplasmic proteome of the red blood cell, egg white proteomics, cerebrospinal fluid, human sera and urines. Last, but not least, the use of CLLs for the discovery of a large number of previously undetected host proteins in recombinant DNA products.

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INTRODUCTION TO PROTEOMICS OF POST-TRANSLATIONAL MODIFICATIONS IN PLANTS

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The initial goal of proteomics was the large-scale identification of all protein species in a tissue. From this tantalizing task, research in proteomics moved at first to subcellular level discovering the paralog issues and slid later to protein-protein interactions named "interactome". The picture became even more complex by the need to analyse protein status that frequently undergo post-translational modifications (PTMs). Post-translational modifications of proteins are reversible covalent processes modifying the primary structure of proteins. PTMs affect enzymatic activity, intracellular localization, protein-protein interactions, stability and greatly increase protein complexity and dynamics. More than 300 different types of PTMs have been identified. In plants, phosphorylation, thiol/disulfide redox modulation, acylation, formylation, glycosylation, nitrosylation, ubiquitination, SUMOylation, palmitoylation are important modifications and specific methodologies have been developed to study them. The quantitative analysis over the time of different PTMs represents currently a challenge that will help to decipher regulation of biological events.

PROTEOMICS AS A COMPLEMENTARY TOOL FOR IDENTIFYING UNINTENDED SIDE EFFECTS OCCURRING IN TRANSGENIC MAIZE SEEDS AS A RESULT OF GENETIC MODIFICATIONS

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In order to improve the probability of detecting unintended side effects during maize gene manipulations by bombardment, proteomics was used as an analytical tool complementary to the existing safety assessment techniques.

Since seed proteome is highly dynamic, depending on the species variability and environmental influence we analyzed the proteomic profiles of one transgenic maize variety (event MON 810) in two subsequent generations (T05 and T06) with their respective isogenic controls (WT05 and WT06). Thus, by comparing the proteomic profiles of WT05 with WT06 we could determine the environmental effects, while the comparison between WT06 and T06 seeds from plants grown under controlled conditions allowed us to investigate the effects of DNA manipulation. Finally, by comparing T05 with T06 seed proteomes it was possible to get some indications about similarities and differences between the adaptations of transgenic and isogenic plants to the same strictly controlled growth environment. Approximately 100 total proteins resulted differentially modulated in the expression level as a consequence of the environmental influence (WT06 vs. WT05), whereas 43 proteins resulted up- or down-regulated in transgenic seeds with respect to their controls (T06 vs. WT06), which could be specifically related to the insertion of a single gene into a maize genome by particle bombardment. Transgenic seeds responded differentially to the same environment as compared to their respective isogenic controls, as a result of the genome rearrangement derived from gene insertion.

To conclude, an exhaustive differential proteomic analysis allows to determine similarities and differences between traditional food and new products (substantial equivalence), and a case-by-case assessment of the new food should be carried out in order to have a wide knowledge of its features.

COMPARATIVE PROTEOMIC ANALYSIS OF HEAT STRESS ON THE METABOLIC SEED PROTEIN FRACTION IN THE WIDELY GROWN ITALIAN DURUM WHEAT CULTIVAR SVEVO

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In Central and Southern Italy, where durum wheat is mostly grown and represents one of the most important crops, grain filling occurs between April and May, when sudden increases in temperature may take place. High temperature during grain filling has already been recognized to cause a deviation from expected properties and quality characteristics of bread wheat doughs. This was a consequence of differential accumulation of gluten proteins that resulted in an alteration of their ratios that, in turn, modify technological properties of doughs. Wheat grain proteins are typically classified according to their solubility properties into albumins (water soluble), globulins (salt soluble) and prolamins (gliadins and glutenins). These latter make up the gluten, and are mostly responsible for rheological properties of wheat doughs. Non-prolamin fractions include proteins with metabolic activity or structural function. Many of these proteins may generate allergies or intolerance in sensitive individuals. In order to verify the consequences of heat stress on endosperm protein accumulation in durum wheat, we submitted the widely grown cultivar Svevo to two thermal regimes (heat stress vs. control), by producing four biological replicas for each treatment. Two-dimensional electrophoresis (IEF/SDS-PAGE) was carried out on the metabolic (non-prolamin) fraction. IPG strips (18 cm long) in the pH range 3-10 were used to perform three different technical replicas for each biological replica. Protein spots in 2-dimensional gel electrophoresis were revealed with Coomassie Brillant Blue (CBB) and analyzed with the software Progenesis SameSpots (Nonlinear Dynamics, UK), in order to identify differentially expressed polypeptides between heat stressed and control plants. This analysis revealed 132 differentially expressed polypeptides (both up- and down regulated). These spots were cut out and the polypeptides were identified after in-gel digestion by MALDI TOF and MALDI-TOF-TOF MS. Approximately 50% of the picked spots revealed by 2-DE analysis as being differentially regulated were identified by NCBInr and TIGR Wheat protein databases search. The identified proteins were functionally diverse and included: Heat Shock Proteins, proteins related to ATP synthesis, proteins involved in glycolysis, carbohydrate metabolism, and signal transduction.

A COMBINED APPROACH TO ANALYZE GRAPE BERRY (*CORVINA*) RIPENING AND WITHERING

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The considerable economic impact makes wine grape one of the most important fruit species in many countries wordwide. Moreover, consumption of grapes and wine has numerous nutritional benefits for humans. For these reasons there is a great interest in studying and improving grape berry development. Molecules related to aroma and taste are produced during fruit maturation, being their synthesis profile specific for each variety. During the ripening process, changes in berry softness, pigmentation, sugar and water content contribute to confer peculiar quality characteristics. In this work we present expression analysis of grape berry (*Corvina* variety) during ripening and withering processes using a combination of transcriptomic and proteomic approaches.

Total protein extracts of berries from seven ripening stages were analyzed by DIGE (Differential in Gel Electrophoresis, GE Healthcare) technology. The comparative analysis revealed that numerous soluble proteins evolve during maturation with specific distribution at different stages. A total of 800 spots were detected in the 2-DE maps, 80 protein spots resulted differentially expressed during berry withering and were identified by MS analysis. The majority of these proteins were related to metabolism, energy and defence activity.

As for the transcriptomic approach, total mRNA extracts from the same berry samples of seven ripening stages were analyzed by Combimatrix GeneChip oligonucleotide microarray technology.

Expression profiles at proteomic and trascriptomic level were compared, identifying correlations and divergences.

Our work represents the first study where dynamic changes of gene expression during withering process have been correlated to the levels of the corresponding protein products. These results may significantly contribute to the knowledge of the role played by defined proteins and genes involved in the physiological ripening/withering process, in the perspective of both quality improvement and *Vitis vinifera* variety characterization.

DESCRIPTION OF MAJOR PROTEINS EXPRESSED IN STRAWBERRY FRUIT DURING RIPENING

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Strawberry is worldwide appreciated for its unique flavour and as a source of vitamins and dietary beneficial compounds (i.e. antioxidants). Both nutritional and taste features are closely related to fruit ripening process. Here we report the first attempt to elucidate the complex physiological process of strawberry fruit ripening at proteomic level. As a prelude to isolation of differentially expressed proteins during fruit ripening, we planned to describe the proteome picture of the strawberry fruit using a combination of different approaches.

A proteome reference map of strawberry fruit from Queen Elisa genotype was achieved by 2-DE analyses of proteins extracted from berries at three different ripening stages: white, turning and red. In order to isolate a set of proteins commonly present in fruit during ripening, spots having overlapping coordinates among the compared stages were identified.

To implement proteomic data on strawberry fruit deriving from 2-DE approach, the same protein extracts were also analysed by two complementary approaches. In the first, protein samples were analysed by SDS-PAGE coupled to nLC-ESI-IT-MS/MS for identification of protein components. Subsequently, the same protein extracts were analysed by MudPIT, where a complex peptide mixture was directly analyzed by using biphasic capillary liquid chromatography-tandem mass spectrometry. A number of different proteins, simultaneously identified in both methods for each ripening stage was detected.

To correlate fruit protein expression with ripening-related quality traits of Queen Elisa elite genotype, differential in-gel electrophoresis by 2-DE DIGE system (GE Healthcare) was performed, comparing the total protein content of the different ripening stages. Moreover, we extended 2-DE DIGE analysis also to Queen Elisa parental genotypes, Miss and USB 35, comparing protein extracts derived from fruit at red stage.

It is interesting to note that, despite of the lack of strawberry species-specific sequences in protein and nucleotide databases, a significant number of proteins were identified. This was accomplished by the combination of gel-based and gel-free approaches for comparative proteomic analysis, providing the first characterization of the strawberry fruit proteome and the description of its variation during ripening.

PROTEOME ANALYSIS OF RESISTANT PLANTS EXPRESSING ANTIVIRAL ANTIBODIES

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Since viral diseases are not sensitive to common chemical treatments of crops, the possibility to obtain self-protecting plants against viral attacks is of critical importance for agronomical applications. Transgenic plants may represent an effective solution but their acceptance is strictly related to the safety of genetically modified products. The insertion into the plant genome of exogenous genes coding for proteins able to confer pathogen resistance might lead to the production of undesired compounds or suppression of endogenous genes.

We investigated whether the expression in plant of protective recombinant antibodies may alter the physiological protein profile. Hereby, we analyzed two different transgenic plant lines (*Lycopersicon esculentum* cv. MicroTom and *Nicotiana benthamiana*) expressing recombinant antibodies against different plant viruses (cucumber mosaic virus and tomato spotted wilt virus) in different cell compartments.

Proteomes of plants expressing antibodies were compared with wild-type counterpart by 2D-DIGE (Differential in Gel Electrophoresis, GE Healthcare) technology. Approximately 2000 spots were detected in the 2-DE maps and only 17 proteins resulted differentially expressed in each transgenic plant model analyzed. Most of the proteins identified by combined MALDI-TOF PMF and LC-ESI-IT-MS/MS analysis were related to photosynthesis and defence activities. The limited variations in both transgenic models, either as number of proteins or changes in expression levels (average ratio ≤ 2), suggest that the antibody expression does not substantially alter the natural expression profile of these plants. Our results confirm that the expression of antiviral antibody in plants may represent a valid strategy to create plants protected from viral attacks in the respect of human and environment safety.

POSTER

MEDICAL PROTEOMICS
(CLINICAL PROTEOMICS/BIOMARKERS)

PEPTIDOME PROFILING OF OVARIAN CANCER BY MALDI-TOF/MS

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Ovarian cancer is a morphologically and biologically heterogeneous disease, its prognosis being strongly dependent on tumor classification and early detection. Tumors are classified based on a subjective histopathological evaluation. CA125 is the most thoroughly assessed biomarker for ovarian cancer, but it lacks of sensitivity for early stage diagnosis. Proteins are the logical targets, both for use as biomarkers for screening, as well as drug target. The challenge of this work is to identify statistically valid markers using plasma peptide profiling obtained by MALDI-TOF mass spectrometry.

5 mL of fresh blood of 20 healthy volunteers and 20 patients (first diagnosis: *pelvic mass*) were centrifugated at 3000 rpm for 20 minutes (4°C); plasma samples were diluted 1:4 with 10%ACN and centrifuged for 30min at 14000g in Centricon tubes at 30000 Da cut-off, the filtrates were purified by ZipTip C18. Spectra were acquired by Voyager-DE PRO, (matrix: HCCA; calibration: internal); 3 spectra were acquired for each sample and aligned by SpecAlign. ANOVA, PCA and LDA analysis were performed for maximizing the separation between the objects (samples) and the selected groups. Evaluation of tissue biopsies from malignant lesions collected at surgery allowed to define 1-3 degrees of tumor differentiation.

Data exploration was carried out on the complete MALDI-TOF spectra with PCA/LDA: the analysis shows a good separation between the group of patients and the control group.

Lesions were classified based on histological evaluation and included 5 benign, 2 borderline, 7 malignant samples and 6 samples with different gynaecological disorders.

This analysis provides the possibility for early detection and better monitoring of tumor progression; indeed figure 1 shows the separation among 7 patients with different degree of cancer differentiation. The three groups of patients, with different degree of tumor differentiation are well-separated.

Mass spectrometry can generate a proteome/peptidome fingerprint of plasma. Advances in proteomics lead to new and improved approaches in molecular diagnostics, capable of classifying, by statistical models, patients into subgroups with different prognosis and response to individual treatments.

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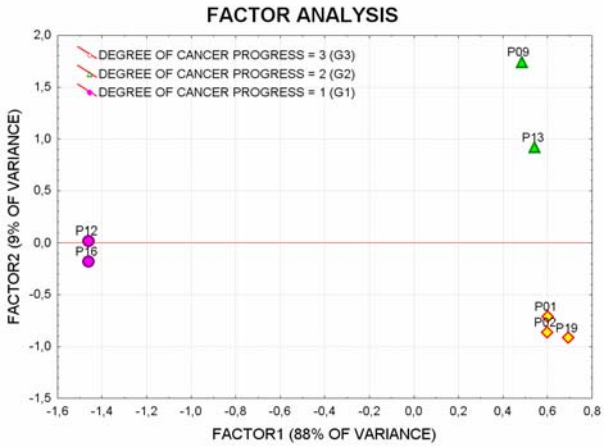

Figure 1. Principal component analysis, on the basis of plasma profile allows to distinguish patients with different 1-3 degrees of tumor differentiation (1=well differentiated; 3=poorly differentiated).

PROTEIN CARBONYLATION IN SERA FROM ALS PATIENTS

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Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disesase with no effective biochemical markers for clinical diagnosis. ALS exist in both familial and sporadic form. The first suggestion of a possible role of the involvement of reactive oxygen species production mechanism in this desease derives from the observation that mutations in the Copper-Zinc Superoxide dismutase-1 (SOD1) gene are responsible for approximately 20% of familial ALS [1]. A bulk of evidence support the hypothesis that oxidative stress, mithocondrial dysfunction, SOD1 mutation, protein aggregation [2] and other process like protein nitration [3] and glutathionylation can be impicated in sporadic ALS (80%).

In this study we tested the hypothesis that proteomic analysis will be able to identify protein biomarkers in the human sera that provide insight into desease pathogenesis and are diagnostically useful.

We applied a proteomic approach to determine specific targets of protein oxidation in sporadic ALS and control sera, by coupling immunochemical detection of protein carbonyl groups with two-dimensional polyacrylamide gel electrophoresis. The amount of carbonyls is considered a biomarker of oxidative stress and it is used to quantify the level of oxidative damage in polypeptide chains. 2D-immunoblotting images were analyzed by Immage Master Platinum and the relative presence/absence and frequency of each carbonylated protein have been reported. In particular, 81 spots were more frequently carbonylated in the ALS sera, whereas. 31 in the control ones.

The interesting protein spots were excised from preparative 2D gel and identified by MALDI-TOF and ESI-IonTrap.

Between them we can recognise albumin and IgG heavy chain which are known to play a role of oxidation-sensitive proteins, acting as “antioxidant buffers” to protect critical protein again oxidation. Some others, such as ceruloplasmin have antioxidant activity, demostrating the occurance of an oxidant/antioxidant imbalance.

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PROTEOME ANALYSIS OF BRONCHOALVEOLAR LAVAGE IN INDIVIDUALS FROM METSOVO, NON-OCCUPATIONALLY EXPOSED TO ASBESTOS

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Inhabitants of Metsovo, NW Greece, have been exposed to an asbestos whitewash, resulting in malignant pleural mesothelioma (MPM) and pleural calcifications (PCs). Interestingly, those with PCs (PC+) are less prone to MPM. They also have lymphocytic alveolitis, and differences in BAL proteins, compared with those without (PC-). This may mean a different response to the fiber leading to different susceptibility to neoplasia. To further evaluate this, a proteomics analysis of BAL proteins was performed.

BAL fluids from four Metsovites of each group were analysed and compared to three controls, using 2D-electrophoresis (2DE) and subsequent image analysis (MELANIE-4). Selected spots were identified by mass spectrometry.

Some of the most interesting proteins identified are rappresented by fragments of Albumin, S100-A9 protein and HSP-27 which are increased in Metsovites compared to controls, and Acid ceramidase and glutathione S-transferase, which have an increased expression only in the PC- group.

Intense expression of albumin fragments, S100-A9 protein and HSP-27 suggests ongoing inflammation. The strong presence of acid ceramidase and glutathione S-transferase (involved in carcinogenesis) only in Metsovites without PCs is further evidence of different reaction of this group to the asbestos fibre and increased susceptibility to neoplasia.

COLLAGEN VI AND SPECTRIN a-CHAIN EXPRESSION IN RENAL CELL CARCINOMA

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Renal cell carcinoma (RCC) account for about 85% of renal cancer for which more than 54000 new case and about 13000 deaths are expected to occur in 2008 in United States. The variety of histological subtypes, the lack of early clinical symptoms, the resistance toward conventional cancer therapies, and the absence of molecular markers preclude the availability of prognostic information and treatment guides. A combination of different proteomic approaches seem to be a powerful strategy to identify biomarkers for early diagnosis, prognosis, and clinical management. We have studied 9 RCC and autologous normal kidney by 2-DE combined with mass spectrometry. Comparative analysis allowed to highlight 14 spots differentially expressed: 43% were up-regulated and 57% down-regulated in RCC, with statistical significance by Student's t test ($p<0.05$). The abundances of Collagen VI and Spectrin a-chain in RCC were confirmed by SDS-PAGE Western Blot analysis on 16 RCC and autologous normal cortex tissues, and by 2-DE Western Blot of 6 sample pairs. The results showed Collagen VI abundance increased in 12 RCC (75%), whilst in 4 RCC (25%) it was decreased. The immunoblotting results of Spectrin displayed two different bands at about 40 and 45 kDa. The 40 kDa weight band was found overexpressed in 75% of RCC compared with normal cortex; the 45 kDa band was only present in 8 normal cortex and in 2 RCC tissue. The evaluation of TNM stage of patients shows no correlation with expression of the two proteins that are also independently modulated in tumor samples. Collagen VI is known to be correlated to tumorigenesis since it stabilizes cyclin D activity, and its overexpression in renal cancer is in agreement to reports on human colon cancer and mouse mammary tumor [1, 2]. Spectrin a-chain functions in non-erythroid cells are not well understood; but several studies suggest its possible role in the maintenance of specialized membrane subdomains, stabilization of cell–cell contacts, vesicle trafficking and interaction with specific apoptotic proteins (calpain and caspase).

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EVALUATION AND QUANTIFICATION OF TWO DIMENSIONAL GEL ELECTROPHORESIS ERROR VARIANCE

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Protein profiles obtained by proteomic approaches investigate changes in one or more protein spots, and the resulting data constitute complex multidimensional outcomes. Statistical models have been proposed for the analysis of a single outcome, but often, the analysis of multiple outcomes is restricted to study each response separately [1]. The most common method, powerful in protein separation, utilized in proteomics is 2-DE. Since this method shows some limit of reproducibility, great attention is directed to quantify variability associated with protein expression. Therefore, the aim of the present study was to quantify the error associated with 2-DE including proteins as numerous variables, and experimental gel replicates as a small number of observations. The data set of 15 2-DE gels derived from two samples of cortex renal cells and three samples of renal cell carcinoma were utilized and processed in triplicate. Finally, 118 spots common to all gels, with Mw and pI ranges of 102-12 kDa and 4.4-7.7 respectively, were analysed after log transformation of the % spot volume. We defined the variability within replicates associated with each spot, and by an empiric investigation we observed that it did not follow a chi-square distribution with 10 degrees of freedom (DF), as expected, but with lower DF, or a mixture of distributions. These results were due to the correlation of spots values also present in the same gel within replicates. Therefore, when multiple outcomes need to be analysed simultaneously, a joint model is required for a robust standard error estimation of the true sample variability [1-3]. Random-effect approaches applied to joint models of multivariate profiles, recently at limelight, might be able to identify molecules associated to renal cancer and to point out new diagnostic markers.

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PROTEOMIC ANALYSIS OF KIDNEY RAT WITH TYPE I DIABETES

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Diabetic nephropathy, worldwide in adults of western Countries, is the primary etiology of chronic kidney disease and kidney failure and its progression is associated with structural and functional renal changes. The precise mechanisms starting and promoting diabetic nephropathy complications have still to be elucidated. Therefore, proteome approaches can be useful to better understand the pathogenesis inducing renal damage in diabetes. To define changes in protein expression associated to diabetic nephropathy, we compared kidney 2-DE gel images of Sprague Dawley rats: six streptozotocin-induced type 1 diabetes, five treated with insulin and six control. The analysis was carried out by Image Master 2-D Platinum 6.01 software. Differentially expressed spots were excised and proteins were identified by MALDI-TOF mass spectrometry. Among these, Ezrin and Tubulin beta-2A chain showed some isoforms in the diabetic group but not in the other two. The different pattern of both proteins was confirmed by SDS-PAGE and 2-DE Western Blot immunodetection.

Ezrin acts as a linker between the plasma membrane and cytoskeleton actin and its up-regulation is associated with epithelial tumour metastasis and signal transduction molecules, whilst its over-expression seems to play a role in major kidney disease [1]. Tubulin, a member of a small family of globular proteins, makes up microtubules. The changes in its pattern expression can be related to alteration of the cytoskeleton microtubule in proximal renal tubules of diabetic rats, as already suggested for a-Tubulin [2]. Our results pointed out different modulation of post-traslational modifications revealing isoforms associated to disease status.

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COMPARATIVE PROTEOMIC ANALYSIS OF NORMAL AND DIABETIC RAT KIDNEY WITH/WITHOUT INSULIN TREATMENT

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Type 1 diabetes (Insulin-Dependent Diabetes Mellitus, IDDM) may cause end stage renal disease (ESRD). In fact, despite a good glicemic control, 30-40% of Type 1 diabetic patients develop nephropathy after 10-20 years of disease (Andersen et al, 1983). Molecular mechanisms responsible for diabetic nephropathy are complex and not completely known.
In order to evaluate this issue we have performed a multiple comparative proteomic analysis in kidney from: i) streptozotocin-induced diabetic rats, ii) streptozotocin-induced diabetic rats treated with insulin, iii) control rats. After comparison of 2DE-PAGE maps from three different groups of animals, spots containing over and underexpressed proteins were analysed by MALDI-TOF/MS for identification. Mass spectrometry analysis identified 104 protein spots representing 83 unique proteins. Using some bioinformatic tools, such as Ingenuity Pathway Analysis (IPA), it has been possible to draw pathways of functional associations among proteins found to be modulated in three different rats groups. Moreover, in order to identify major biological processes and molecular functions involved, proteins dataset were subjected to Genome Ontology (GO) annotation using database DAVID, Panther and Babelomics. Further sorting for relation to metabolism was also performed on the basis of KEGG pathway terms and bioinformatic means. In particular, we found several proteins involved in glucolysis/gluconeogenesis (such as alpha-enolase, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase) and also in leukocyte transendothelial migration (such as ezrin and myosin light polypeptide 6).

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PROTEIN PROFILING OF SERUM IN RENAL CELL CARCINOMA (RCC) PATIENTS FOR DISCOVERY OF POTENTIAL BIOMARKERS

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Renal Cell Carcinoma (RCC) is the most common cancer of the kidney with the main histological subtype being clear cell (ccRCC) which accounts for around 75%. The incidence of RCC is increasing worldwide each year. The prognosis of patients with metastatic RCC is poor due to a lack of sensitivity to radio- and chemio-therapies (Banks et al., 2007). Therefore the need of new diagnostic and predictive tools for efficient detection of early RCC is urgent. In this study we have investigated serum protein profiles of control subjects and RCC patients by using ClinProt/MALDI-TOF-MS aimed to search candidate biomarkers for RCC. We have purified with C8 coated magnetic beads serum of 29 healthy subjects and 33 ccRCC patients by ClinProt technique. Statistical analysis with ClinProTools 2.1 software showed a cluster of three signals (A = m/z 1083 ± 8 Da, B = 1445 ± 8 Da and C = 6879 ± 8 Da) able to discriminate control subjects from patients. Evaluation of the diagnostic efficacy by Receiver Operating Characteristic (ROC) curve provided an AUC value of 0.917 (sensitivity of 85% and specificity of 90%) for marker A, and lower AUC values in range of 0.7-0.9 for markers B and C. These signals together improved the diagnostic capability in the training test with specificity of 100% and sensitivity of 90% and also the external validation confirmed both specificity and sensitivity. The recognition capability of this model to distinguish patients based to TNM tumour stage showed 100% of sensitivity for patients in pT2 (n=5) and pT3 (n=8) and 85% for pT1 patients (n=20). With the progress of disease the area of markers A and C constantly decreased, while marker B increased in stages 1 and 2 reaching a plateau for tumor at pT3. These preliminary data, after their validation with more patients, may represent an useful tool for RCC diagnosis.

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PROTEIN PROFILING OF NON-INVASIVELY COLLECTED AMNIOTIC FLUIDS IN PREMATURE RUPTURE OF MEMBRANES

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Premature rupture of the membranes (PROM) complicates 10% of all gestations and 2-4% of preterm pregnancies. It is associated with significant maternal, fetal, and neonatal risks and it affects infant neuro-development (Thadikkaran et al.,2005). The characterization of the proteome of amniotic fluid could be useful to identify proteins involved in pathogenesis and complications of PROM.

The ClinProt methodology allows to reduce the complexity of biological samples through the use of magnetic beads (MB) with activated surface. This technique can be carried out by robot in an automated way on a large number of samples. Moreover it allows to obtain the protein profile of biological fluids by MALDI-ToF MS and to retain part of the eluted sample for characterization of peaks of interest by LC-ESI-MS/MS.

Leakage of amniotic fluid into vagina is a frequent event in PROM, so our study was focused on the feasibility of ClinProt prepurification before MALDI-ToF analysis on amniotic fluids non-invasively collected in PROM and preterm PROM (pPROM).

Amniotic fluid was collected from vaginal posterior fornix of women with PROM (group 1, n=10) and pPROM (group 2, n=10). Samples were prepared with hydrophobic interaction MB and analyzed with MALDI-ToF MS in order to generate protein profiles.

It was possible to generate and compare the protein profiles of the studied groups. Furthermore 3 signals were observed to be overexpressed in pPROM patients.

This cluster of signals was able to discriminate the two studied populations. These results, after being validated on a wider number of patients, may be useful to enlighten the molecular mechanisms of PROM and to define diagnostic tools.

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EVALUATION OF URINE PROTEOME PROFILES IN RCC PATIENTS BY CLINPROT/MALDI-TOF-MS

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Renal Cell Carcinoma (RCC) is a major histological subtype of malignancies of the kidney. No currently satisfactory biomarkers are available that can be used for the RCC diagnosis at an early stage, for its prognosis and follow up. In this study we have evaluated urine profiles of control subjects and RCC patients by using ClinProt technique, which couples a cromatografic step to MS analysis allowing direct investigation of biological fluid (Villanueva et al., 2004). With the aim to identify possible biomarkers for RCC, we have purified with C8 coated magnetic beads urine of 29 healthy subjects and 39 clear cell RCC patients by ClinProt method. A cluster of three signals (A = at m/z 1827 + 8 Da, B = 1914 + 8 Da and C = 1968 + 8 Da) was found to be able to discriminate controls from patients through the statistical analysis with ClinProTools 2.1. Diagnostic efficacy, evaluated through Receiver Operating Characteristic (ROC) curve analysis, showed a sensitivity of 85-90% and a specificity of 90% for markers A and B, while a sensitivity of 70% and a specificity of 100% for marker C. Combination of three markers allows an improvement of recognition capability with specificity and sensitivity of 100% and 95%, respectively, in the training test and of 100% and of 85% in the external validation. Ability of this pattern of signals to discriminate RCC patients grouped on TNM tumour stage provided a sensitivity of 100% for patients at pT1. In addition a signal used in the cluster, down expressed in RCC patients, was identified as a fragment of Tamm-Horsfall Protein (THP) that is not already described in literature (Mischak et al., 2004). In conclusion these preliminary results are promising in the possibility to be used as multiple biomarkers for ccRCC diagnosis.

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PRIMARY CELL CULTURES FROM NORMAL KIDNEY AND RENAL CELL CARCINOMA: MOLECULAR AND PROTEOMIC STUDIES

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Renal cell carcinomas (RCCs) represent 3% of all human malignant diseases. Their diagnosis is difficult and often late without validated RCC markers. Primary cell cultures from human RCC and normal renal cortex were established to get round the problem of cell heterogeneity of tissue that makes difficult to find RCC markers by genomic and proteomic studies.

Primary cultures from 39 matched samples of normal cortex and RCC have been studied. Their cytological characteristics have been evaluated analyzing cellular morphology and composition by immunofluorescence and FACS analysis. Normal cortex cultures were composed of epithelial cells (>90% were positive for cytokeratin, E-cadherin and also vimentin), of proximal (CD13 positive) and distal (PNA positive) tubule, with a very low contamination (0.5%) of endothelial (CD31 positive) cells. Tumor cultures were positive (>90%) for the epithelial markers with high expression of proximal tubular markers. 2-DE analysis of normal cortex and RCC primary cultures evidenced 63 differently expressed protein spots; among the 44 proteins identified by MS, 11 were more abundant in RCC. Some of these proteins are related to VHL/HIF-1 angiogenesis pathway and others are involved in cytoskeleton remodelling pathway. For the molecular validation of 2DE/MS results the expression of two of these proteins, Annexin A3 and Lasp-1, has been analyzed by Real-Time PCR and 1D western blot, evidencing a different isoform expression pattern between normal and RCC cultures. Transcript and protein expression of Arg, a non-receptor tyrosine kinase involved in cytoskeleton regulation, has been also studied. RCC cultures show a higher Arg transcript level and a different isoform pattern than normal renal cultures. These well-characterized in vitro model may be useful to evaluate as eventual biomarkers the differentially expressed proteins, that will be also investigated by activation or silencing of specific genes and/or pathways in which are involved.

PROTEOMIC STUDY OF DIABETIC TUBULAR EPITHELIAL-MESENCHYMAL TRANSITION USING AN IN VITRO MODEL OF TUBULAR PRIMARY CELL CULTURES

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Epithelial-mesenchymal transition (EMT) plays an important role in embryogenesis and organ formation, and is involved in tumor progression, metastasis formation and progressive tissue fibrosis. In diabetic nephropathy the progressive renal fibrosis arises from 'activation' of renal fibroblasts but also from tubular epithelia cells that, through EMT, become collagene-producing. The process of tubular EMT involves (1) loss of epithelial cell adhesion; (2) de-novo α-SMA expression, loss of E-cadherin expression and actin reorganization; (3) disruption of tubular basement membrane; (4) enhanced cell migration and invasion. To understand the molecular mechanisms of EMT in diabetic nephropathy and to find molecular markers for early detection of EMT, we used an in vitro model of diabetic tubular EMT establishing primary cultures of human tubular cells treated with high glucose concentration. Tubular cells from normal cortex grown in DMEM and 10% foetal bovine serum were cultured, after a 48 hour treatment in serum-free medium, with different glucose concentrations: 100mg/dl (control) and 450 mg/dl (high glucose) for 6-96 hours. The cellular in vitro model has been well characterized. The metabolic activity of cell cultures was measured by MTT assay and apoptosis by annexin V/propidium iodide double staining FACS analysis. The development of EMT has been proved by cellular morphological changes evidenced with contrast phase microscopy and by immunophenotypic analysis with FACS. The α-SMA expression, which increases at 72 hours of high glucose treatment, and E-cadherin expression, which decreases at 96 hours of high glucose treatment, have been analyzed by Real-Time PCR and Western blot. A MUDPIT (Multidimensional Protein Identification Technology) approach is ongoing on control and treated cell lysates to profile changes in protein expression patterns. The proteins putatively found differentially expressed may help the comprehension of molecular mechanisms underlying the pathophysiology of diabetic nephropathy and might be used as EMT markers.

LONG-LASTING CONSEQUENCES ON RAT PREFRONTAL CORTEX SYNAPTOSOMES PROTEOME DUE TO THC EXPOSURE DURING ADOLESCENCE

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This work is part of a study about long term neurobiological effects of marijuana consumption during adolescence.

On this purpose female adolescent rats have been chronically treated with Δ9-tetrahydrocannabinol (THC) during adolescence (35-45 post natal days, PND) and mood and cognitive parameters have been evaluated at 75 PND (adulthood). Adolescent THC exposure produced a depressive-like behaviour with anhedonia at adult age and cognitive impairments. On this basis, we decided to evaluate protein expression profiles in synaptosomes from prefrontal cortex, one of the most relevant brain areas involved in cognitive performance, by two-dimensional electrophoresis (2-DE) in order to get a deeper insight into the biochemical mechanisms at the ground of the observed effects. We decided to focus our analysis on synaptosomes, artificial structures produced by fractionation after selective centrifugation of nervous tissue homogenates, because they are rich in proteins involved in the synaptic transmission and eventually in neuronal functionality.

2-DE proteomics conducted on these artificially obtained membranous sacs revealed long-lasting alterations in response to THC administration.

In particular we observed a down-regulation of fructose-bisphosphate aldolase A, cytochrome b-c1 complex subunits 1 and 2, ATP-synthase alpha and beta subunits, pyruvate kinase isozymes M1/M2, heat shock cognate 71 kDa protein, glyceraldehyde-3-phosphate dehydrogenase, ubiquitin-carboxyl-terminal hydrolase isozyme L1 (UCHL1), phosphatidylethanolamine binding protein1 (PEBP1). We additionally observed an up-regulation of an isoform of β-actin.

As a whole, these findings support the view of a persistent deficit in synaptic activity in prefrontal cortex as a consequence of THC assumption during adolescence.

PROTEOMICS INVESTIGATIONS ON RENAL CELL CARCINOMA SECRETOME: A MUDPIT APPROACH

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Aberrant secretion or shedding of proteins is commonly associated with disease, including cancer. It is conceivable that either the tumor itself or its microenvironment could be sources for biomarkers and potential therapeutic targets. However secreted proteins are not easily detected in the culture medium due to the low concentration and moreover they are often masked by high amounts of protein supplements or cell debris. We evaluated the feasibility of MUDPIT (MUltidimensional Protein Identification Technology) approach to investigate the secretome of normal and Renal Cell Carcinoma (RCC) primary cell cultures. Short-term cultures from the tumor and the normal adjacent tissues, previously shown to retain a great phenotypic similarity with original tissue can be used as a simplified model system for studying biochemical and molecular changes associated to this specific neoplastic status (Perego et al., 2005). The method is based on a starvation in serum free medium for 48 h, time required for the cells to secret detectable amounts of protein without increasing cell death rate, on several washing steps with phosphate buffered saline, on dialysis to eliminate salts and on lyophilisation to concentrate proteins. The two-dimensional (strong cation exchange followed by reversed-phase) nano LC-MS/MS strategy on ESI ion trap has provided the identification of 57 proteins in the normal primary cell culture medium and a total of 107 in the tumoral counter party, with 17 proteins sharing; The data analysis were performed with an in-house MASCOT search (vers. 2.2) using the resulting pooled raw mass spectra from fractions of each sample. Due to the complexity LC-MS/MS searches moreover with overwhelming volume of data produced by MUDPIT we show identifications subdivided in groups of confidence. Many of those proteins were known to be secreted or have a signal peptide or a transmembrane domain. Preliminary evaluation of our findings revealed several proteins that mediate important cell-cell and cell-matrix interactions, and many supplementary candidates that need to be further characterized.

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CHARACTERISATION OF CLASS III BETA TUBULIN COMPLEXES

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Tumor microenvironment is characterised by glucose deprivation, acidosis and severe hypoxia. In particular, the decrease in oxygen levels is shown to impede the cell cycle and thereby result in resistance to most chemotherapeutic agents which normally target rapidly proliferating cells. Among the factors related to drug resistance, class III beta tubulin (TUBB3) plays a prominent role in several tissues. TUBB3 has assumed an important role as a clinical marker of drug resistance in ovarian, lung, gastric, and breast cancer patients and has been discovered as a marker of poor prognosis (Ferrandina et al., 2006). We have recently found that TUBB3 expression level is activated by hypoxia and identified two different isoforms of TUBB3 characterized by specific posttranslational modifications, such as phosphorylation and glycosylation, that determine its sub-cellular localization and result to be related to drug resistance (Cicchillitti et al., 2008). To better characterize the pathway involved in TUBB3 posttranslational modifications and to identify its interaction partners, we performed immunoisolation with anti-TUBB3 antibody of clarified total lysates from the human epithelial ovarian cancer cell lines A2780 (paclitaxel sensitive), A2780TC1 and OVCAR3 (acquired and inherently resistant). Proteins eluted from the beads were separated by 1D or 2D SDS –PAGE and silver stained. Resolved bands detected in all cell types studied were excised and proteins identified by MALDI-TOF mass spectrometry. We identified several proteins involved in the response to oxidative stress and glucose deprivation, in particular chaperones and protein that catalyze protein folding, as GRP78, ERp57 and HSP70. This data indicate that TUBB3 is inserted in a functional network of factors involved in the oxidative stress response and in the endoplasmic reticulum stress pathway and can provide a novel target for developing therapeutics.

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T-CELLS PROTEOME ANALYSIS IN PATIENTS AFFECTED BY LYMPHOPROLIFERATIVE PATHOLOGIES

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B-lymphoproliferative diseases represent a wide range of neoplastic syndromes: Hodgkin's lymphoma, aggressive and indolent lymphoma, chronic lymphocytic leukemia, plasma cell dyscrasias.

T-lymphocytes play a key role in regulation of immunity and act in both stimulation and suppression of B-lymphocytes. Recent studies show that, in patients affected by solid tumours or lymphomas, the tumour-infiltrated lymphocytes may have an important part in regulation of tumour progression (1). Furthermore, T-lymphocytes from peripheral blood has been shown to be feasible molecular sensors of gene expression modification in physiological and pathological conditions (2). Thus, proteomic mapping of circulating T-cells derived from patients affected by different B-lymphoma might contribute to understand their functional role in these pathologies.

The protein pattern of Pan T-cells derived from patients affected by diffuse large b-cell lymphoma (DLBCL) was acquired by two-dimensional gel electrophoresis (isoelectrofocusing: pH 3-10 NL, 18 cm; SDS-PAGE: 9-16% gradient gel, 18x20 cm) and compared with a reference expression profile obtained by pooling protein extracts of T-lymphocytes from 10 healthy donors. Differentially expressed proteins were identified by peptide mass fingerprinting using a Voyager DE MALDI-ToF mass spectrometer (Applied Biosystems).

Unsupervised analysis of proteomic data using the GeneSpring GX 7.3 expression analysis software (Agilent Technologies) highlights the alteration of T cells proteome profile in diseased patients.

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DEVELOPMENT OF NEW SERUM MOLECULAR MARKERS FOR DIAGNOSIS CUTANEOUS MELANOMA

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A proteomic study on serum proteins from patients undergoing surgery for suspected melanoma was performed in order to achieve a proteomic characterization of serum in presence of cancer and its progression and define new molecular markers of disease. After albumin and IgG depletion, serum proteins from 48 individuals were separated by 2DE and image analysis of proteomic maps revealed the modification of the expression pattern in relation with the presence and progression of the tumor in 5 regions of the map.

Differently expressed spots were identified by MALDI-TOF mass spectrometry after in gel trypsin digestion. Significant increase of expression were found for an high molecular weight transthyretin (TTR) and angiotensinogen (AGT) while vitamin D binding protein (DBP) expression was decreased in presence of melanoma. Other proteins, identified as RBP (retinol binding protein), ZAG 2-glycoprotein), low molecular weight TTR, ficolin and haptoglobins,α(zinc- didn't show significant variations after statistical analysis in the group of all melanoma patients. Interestingly, when patients were divided according to the timing of surgery (before and after one month from surgical removal of the tumor), results showed better correlation with the presence of malignancy. In general, protein expression came back to control values in stage I and II of the disease after one month from surgical removal of suspected melanoma (7 controls and 18 melanoma patients), as well as after one year as investigated in stage I patients (5 controls and 5 stage I melanoma patient). In particular, a significant increase of high molecular weight TTR appears as a marker of melanoma. After surgical removal it is still over-expressed only in stage III, but not in early stages. TTR involvement in melanoma is of big interest since it is the major carrier of thyroid hormones and retinol binding protein which can act as modulators of the expression of several genes. Moreover retinoic acid is an inhibitor factor of cell proliferation, invasion and metastasis and is responsible of melanogenesis in melanoma cell lines. So over-expression of RBP observed before surgery (normal after surgical removal) appears of big interest in the characterization of the tumor progression.

Other statistical relevance was found in the expression of DBP and AGT in the group of patients analyzed before surgery. In particular AGT, a potent antiangiogenic factor, is over-expressed in stage I melanoma while it's low in advanced stages. Since the AGT is known to block in vivo tumorigenesis by suppression of intratumoral vascularization, it could be the physiological response to early tumor growth. After surgical removal AGT is significantly decreased in the same group of patients (stage I).

DBP spot is significantly reduced in melanoma patients compared to control individuals. After surgical removal it is still down-expressed only in stage III, but not in early stages.

We related the decrease of this spot (hypothesized to be a glycosylated isoform of DBP, from preliminary analysis based on molecular weight and isoelectric point) to the impaired immune response of cancer patients. In fact cancer cells release an endoglycosidase (alpha-N-acetylgalactosaminidase) that can deglycosylate DBP, whose glycosylated form is known as precursor of DBP-MAF (Macrophage Activating Factor) mediated macrophage activation cascade, finally leading to immunosuppression in advanced cancer patients. In order to confirm this hypothesis we performed an enzymatic assay to evaluate specific enzymatic activity of serum alpha-N-acetylgalactosaminidase. It was significantly higher in stage III of melanoma patient, but not in early stages.

For the fist time a pathophysiological profile of the modifications associated to cutaneous melanoma insurgence and its progression has been drawn by using a proteomic approach. An extended analysis of a larger number of patients it's necessary to confirm our results which open the way to the development of new strategies of early diagnosis and drug targeting of this tumor, whose clinical outcome is currently not predictable by known serum biomarkers being histopathological assay after surgical removal the only reliable tool for melanoma diagnosis.

LEVELS OF CIRCULATING GELATINASES AND PROTEOMIC CORRELATIONS IN BREAST CANCER PATIENTS.

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Breast cancer represents the most frequent and potentially aggressive type of cancer, as well as the leading cause of cancer deaths in women in the western countries. Moreover, the biological and clinical evolution of breast cancer is one of the most enigmatic and poorly predictable, probably because it includes several different forms that behave differently among patients. Matrix metalloproteinases (MMPs) play a critical role in the conversion of in situ breast cancers to invasive lesions. Recent evidence has shown multiple functions of MMPs, which include the mobilization of growth factors and processing of surface molecules, besides the extracellular matrix degradation. For these reasons MMPs have received great attention in recent years, as putative tumor markers. Previous studies by our group have shown that levels of MMP- 2 and MMP-9 are significantly enhanced in breast and colon cancer patients (Pucci-Minafra et al., 2001, La Rocca et al., 2004). In the present study we analyzed the proteomic profiles of surgical tissues of patients diagnosed for ductal infiltrating breast cancer and the corresponding activity levels of MMP-2 and MMP-9, detectable in the tissue extracts by zymographic assays. A prototype of this combined assay is presented in figure 1. Quantification of gelatinolitic activities was performed by the ImageQuant TL software, while the proteins expression levels were calculated as the volume of the proteomic spots (V%) by ImageMaster 2D Platinum. Interestingly, a positive correlation between the gelatinase activity levels and some proteins was observed. These results suggest that the combination of these two assay procedures may be of great utility to identify new patterns of protein/enzymes involved in cancer progression.

NEW POSSIBILITIES IN THE ELUCIDATION OF JURKAT T CELLS PROTEOME BY HEXAPEPTIDE LIGAND LIBRARY: 2-DE AND MALDI-TOF ANALYSIS

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The large number of gene and gene splice-variants that encode proteins, as well as the extensive post-translational modifications of eukaryotic proteins renders proteomic studies extremely difficult. The detection of specific, disease-related protein markers, notoriously difficult to identify, can be extremely challenging on classical two-dimensional electrophoresis (2-DE) where highly abundant proteins could obscure the rare ones. Consequently, there have been an extensive investment into developing techniques and methods capable of revealing the so called "hidden proteome". A new approach based on a combinatorial library of hexapeptides has been recently developed and applied to the study of different biological samples including serum, urine and bile. However, the use of this method in combination with 2-DE analysis has not been completely elucidated in the case of cellular proteome and currently applied only to investigate the platelet proteome. In this study by using 2-DE and MALDI-TOF we evaluated for the first time the potential of commercial hexapeptide library for the proteome analysis of human leukaemia Jurkat T cells.
Jurkat cell line was maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and antibiotics. Cells were harvested by centrifugation followed by lysis via sonification in Tris buffer containing protease inhibitors. Varying amounts of cell lysate (2.5 mg/ml, 5 mg/ml, 14 mg/ml, 50 mg/ml) were subjected to column chromatography over a solid-phase combinatorial ligand library (ProteoMiner, Biorad). Following washing, each individual column was subjected to two distinct elutions using a RS solution (2 M thiourea, 7 M urea, 4% CHAPS) and a RSA solution (2 M thiurea, 7 M urea, 4% CHAPS, acetic acid to pH 3.3) respectively. RSA eluted sample was precipitated with 2D Clean-Up (GE Healthcare) and resuspended in RS.
For 2-DE studies, 80 μg of proteins 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 NL). 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.
These studies have demonstrated that initial protein sample concentration strongly influences the apparent enrichment of low abundance proteins from Jurkat cells by Proteominer. Additionally, different proteome profiles were observed in the RS versus RSA elutes. In the RS elutes, there is an enrichment of the basic proteome, meanwhile the RSA elutes appear to contain a general enhancement of total protein elution in comparison to the RS buffer. Interestingly, this is contrary to previously published data for the platelet proteome which showed an enrichment in the acidic proteins following chromatography. The low abundance proteins, for which there was an observed enhancement following elution from the Proteominer column in respect to the control sample, were identified by MALDI-TOF.
We find that extensive information on the protein composition of complex samples such as Jurkat proteins can be obtained using ligand library with an enrichment proteomic tool box. In this work, we provide evidence that this technique can be used to detect particular proteins useful to the field of lymphocyte biology and contribute significantly towards the discovery of new biomarkers of diagnostic relevance.

METASTATIC PROGRESSION IN BREAST CANCER INVESTIGATED BY DIFFERENTIAL IN-GEL ELECTROPHORESIS

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In spite of very important improvements in cancer therapy, breast cancer is still the most frequent cause of death for cancer among women in the Western world. One of the major reason for this poor prognosis is the tumor relapse often leading to the metastatic disease, a complex phenomenon that leads cancerous cells to invade the surrounding stroma, enter the vessels and spread to distant organs like lung, pleura, liver, brain and most commonly to the bones. It has become clear that understanding the molecular background of carcinogenesis and to predict the metastatic behaviour of the tumor can lead to more approriate treatments and improve patients'survival. In this study we used 2D-Difference in Gel Electrophoresis (2D-DIGE) and tandem mass spectrometry to compare metastatic and nonmetastatic breast cancer samples. Our aim was to identify proteins that can be used as biomarkers of metastatic progression and as prognostic indicators of the metastatic risk. We compared fifty heterogeneous breast cancer samples whose classification was based only on their level of invasion. Differential protein expression was evaluated using 2D-DIGE on pH 4-7 and pH 6-11 IPG strips. Differential analysis, using the Student's t-test provided by DeCyder software, revealed five spots differentially expressed ($p<0.05$). Among these proteins, three were more abundant in metastatic samples and two were less abundant. These spots were identified by MALDI-ToF and some of them were tested on the same samples used for the proteomic study by immunohistochemical assay and Western blotting. Up-regulated proteins included Glucose regulated protein 78 kDa, Selenium binding protein 1 and Annexin A5. Down-regulated proteins included Tetrahydrofolate synthase and Guanine Nucleotide–binding protein G(I)/G(S)/G(T) beta subunit 3. Proteomic results were validated by immunohistochemistry in a group of 181 breast carcinomas from patients with a 9 years follow up. Protein expression levels were correlated to clinical and prognostic features of the tumour and patients follow up.

A DIGE APPROACH FOR THE ASSESSMENT OF RAT SOLEUS MUSCLE CHANGES DURING UNLOADING: EFFECT OF ACETYL-L-CARNITINE SUPPLEMENTATION

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After hind limb suspension, a remodeling of postural muscle phenotype is observed. This remodeling results in a shift of muscle profile from slow-oxidative to fast-glycolytic. Such transition produces an adaptive remodeling which occurs at both structural (shift from type I to type II fibers, selective loss of contractile proteins, reduction in fiber diameter) and functional levels (reduced force generation capacity and resistance to fatigue). These metabolic changes, accompanied by a fiber type shift, increase muscle fatigability. Acetyl-L-carnitine (ALCAR), the acetylic ester of carnitine, has been found to control the skeletal muscle phenotype, particularly in muscles where the oxidative type I fibers prevail, such as the soleus. Several studies have pointed out a positive role of ALCAR dietary supplementation in the improvement of the skeletal muscle oxidative metabolism during unloading. In the present study we applied a 2D-DIGE, mass spectrometry and biochemical assays, to assess qualitative and quantitative differences in the proteome of rat slow-twitch soleus muscle subjected to disuse. Meanwhile, the effect of acetyl-Lcarnitine administration on muscle proteomic profile and in both unloading and normal-loading conditions were evaluated. The results indicate a modulation of troponin I and tropomyosin complex to regulate fiber type transition. Associated, or induced, metabolic changes with an increment of glycolytic enzymes and a decreased capacity of fat oxidation were observed. These metabolic changes appear to be counteracted by acetyl-L-carnitine treatment which restores the mithochondrial mass and decreases the glycolytic enzyme expression, suggesting a normalization of the metabolic shift observed in unloaded animals. This normalization is accompanied by a maintenance of body weight and seems to prevent a switch of fiber type.

SKELETAL MUSCLE WASTING IN CANCER CACHEXIA INVESTIGATED BY A 2D-DIGE APPROACH

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Skeletal muscle wasting occurs in human muscles as a result of disuse (e.g., immobilization, denervation, muscle unloading), aging (i.e., sarcopenia), starvation, and a number of disease states (i.e., cachexia). Although all these conditions can be defined as distinct clinical syndromes, our understanding of the molecular mechanism at the basis of the imbalance in protein anabolism and catabolism occurring in skeletal muscle is complicated by a certain degree of overlap and poorly defined at the molecular level. Regardless of the enciting event, skeletal muscle atrophy is characterized by a decrease in protein content, fiber diameter, force production, and fatigue resistance. Nevertheless, the different types of conditions producing atrophy imply different types of molecular triggers and signaling pathways for muscle wasting. This can influence the protein turnover by differentially modulating the protein synthesis and degradation processes. In our previous studies we used a 2D-DIGE proteomic approach to investigate muscle atrophy associated to physiological (aging) and para-physiological (unloading) states. The same approach was adopted in the present study in order to highlight the variation in protein expression in rectus abdominis muscles from healty subjects and patients affected by gastroenterological cancers and chronic pancreatitis. The aim of the study was to define the contractile and metabolic proteins changes in order to understand the mechanisms involved in a pathological process such as cancer cachexia and compare the results with aging and unloading.

Protein extracts were labeled and separated in the first-dimension using IPGstrips (non linear pH 3-10 gradient). The second dimension was carried out in 12 % w/v polyacrylamide SDS-PAGE. Samples were run in triplicate and analysed using DeCyder software. ANOVA was used to detect quantitative protein changes between healthy and diseased subjects. After statistical analysis, we found significant changes (significative level $p < 0,01$) in 136 spots; the differentially expressed proteins were excised, digested in gel with trypsin and characterized by MALDI and Tandem Electrospray Mass Spectrometry (ESI MS/MS).

PROTEOMIC CHANGES IN MURINE REVERSINE-TREATED FIBROBLAST CELLS

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Reversine (2-(4-morpholinoanilino)-6-cyclohexylaminopurine) is a synthesized purine recently discovered involved in the control of molecular pathways of cell specialization. This small molecule may induce the reversion of adult cells to various differentiated cell types under appropriate conditions (1).

Despite its great potential to regenerate damaged tissues (2), the mechanisms triggered by reversine and the genes and proteins involved are not well-known. Proteomic techniques represent a powerful tool to identify the change in protein abundace modulated by the reversine treatment in order to investigate the effects of the latter on cellular pathways. In our study the proteomic pattern of cellular extract from murine reversine-treated fibroblast and control cellular extracts were compared. The differences were investigated using 2D-DIGE and mass spectrometry: spots that showed a statistically significant difference in abundance were utilized to generate a list of candidate spots for protein identification. These spots were matched on the preparative gel, excised, subjected to in-gel enzymatic digestion and identified by MALDI and ESI-TRAP mass spectrometry. Identifications were carried out comparing experimental MS and MS/MS data with a set of theoretically predicted fragments in MASCOT database.

The differences between reversine-treated and control cells indicated that proteins belonging to protein folding, response to chemical stimuli, regulation processes and purine nucleotide binding are involved.

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PLATELET TRANSCRIPTOME AND PROTEOME ALTERATION IN PATIENTS AFFECTED BY FIRST ACUTE MYOCARDIAL INFARCTION

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Platelets play a fundamental role in hemostasis and in wound repair contributing to the formation of vascular plugs. Under pathologic circumstances, unwanted platelets activation is implicated in the onset of myocardial infarction (MI) which is the single most critical event in coronary disease. The dynamics of platelet activation precipitating myocardial infarction are unknown but of great importance in terms of risk assessment and antithrombotic therapy. Platelets lack nuclear DNA but retain megakaryocyte mRNAs, some of which are translated upon activation. Thus, their transcriptome and proteome analysis could provide information about the molecular changes preceding the coronary event. The aim of the study was to compare mRNAs and proteins levels in platelets extracts from patients affected by a first acute MI (FAMI) compared to control subjects in order to identify potential precipitating factors.

Whole blood was collected within 6 hours from the acute event and purified platelets were used to profile their mRNAs population by DNA microarrays and their protein content applying 2D-DIGE and MALDI-ToF mass spectrometry. Both platelet transcriptome and proteome revealed quantitative differences between FAMI patients and controls. In particular, we identified 713 transcripts specifically modulated in FAMI platelets, the majority (666) were down-regulated, and only 46 genes were up-regulated. Proteomic analysis revealed 66 differentially expressed spots matching 43 proteins. The majority were involved in the cytoskeleton organization and biogenesis, in cellular signaling and processing. The comparison of transcriptome and proteome data indicates that a down-regulation of some transcripts is accompanied by a corresponding protein increment. The present study highlights that platelets from FAMI patients are in an activated state suggesting that an activation could take place before the acute event: these small anucleated cells are emerging as a promising target for the prevention of MI in terms of diagnosis and drug development.

HUMAN PLASMA PROTEOME CHANGES ASSOCIATED WITH SNAKE VENOM OF ECHIS CARINATUS

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Metalloproteases and Serine protease are major components of snake venom and has been studied over the years (Guerranti et al., 1999). The proteases of Echis carinatus venom (EV) are prothrombin activator like Ecarin that exhibits very strict substrate specificity and Carinactivase that is strongly dependent on Ca2+ ions for activation of prothrombin (Yamada et al., 1996). Also SVTLE comprise a number of serine proteases functionally and structurally related to thrombin (Castro et al., 2004). It is well known for their effect on coagulation. In this preliminary studies of Echis carinatus proteases effects on plasma proteome were analysed in vitro incubating crude EV with human plasma pooled from healthy volunteers. This mixture was analysed at different incubation times and varying the ratio EV proteases/plasma proteins using SDS-PAGE to find out the best conditions. By Total lab software we obtened the V% of the singular band and many modifications were identified. Then 2D-E was employed to see the effects of EV proteases on plasma proteins after 60 mins incubation, and another incubation with only plasma in assay buffer served as control. By 2D Image Master Platinum software the images were spotted and mached. Lots of variation were found and spots showing a significant change, then this we analysed by MALDI-TOF. The identification of these proteins will help us in discovering other pathophysiological process activated by EV protease apart from that involved in the coagulation disorders. In conclusion a clear effect of EV proteases on plasma proteins is related to EV toxicity through mechanism alternative to coagulation.

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A PROTEOMIC APPROACH TO IDENTIFY AUTOANTIGENS IN CELIAC DISEASE

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Celiac disease (CD) or gluten sensitive enteropathy is a permanent inflammatory disease of the small intestine. It is activated in genetically susceptible individuals by the dietary ingestion of proline- and glutamine- rich proteins that are found in wheat, rye, and barley. CD is the result of both environmental (gluten) and genetic factors (HLA and non-HLA genes), and the distribution of these two components can probably be used to identify the areas of the world at risk for gluten intolerance. Active celiac disease is also accompanied by the presence of autoantibodies in sera of patients (1). Sera of patients suffering from celiac disease contain a high amount of antibodies to gliadin and Immunoglobulin A (IgA) antiendomysial. Moreover, CD is often associated with other autoimmune diseases, in particular type 1 diabetes, but also thyroiditis, hepatitis and autoimmune-related osteoporosis and low bone mass .

The aim of the work is the identification of potential biomarkers of CD through the comprehensive analysis of the reactivity of the sera from CD patients. In order to set up 2-DE conditions and to perform preliminary experiments with CD sera, we used protein extracts from Human Myeloid Leukemia cells K562 and Human Embryonic Kidney 293T cells. Cellular extracts were resolved by 2-DE, transferred onto nitrocellulose or PVDF membranes and probed with CD patient's serum. Several protein spots (n=30), residing in the gel region characterized by pI range 4.5–8.5 and mass values of 30–80 kDa, were recognized by the CD sera. Thirteen proteins have been identified by MALDI and ESI mass spectrometry. Among them, only enolase has been previously reported to induce antibody response in CD patients and thereby considered an autoantigen (2). The annotated spots include putative novel CD autoantigens, which will be further confirmed on samples from intestinal biopsy.

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PROTEOMIC ANALYSIS OF SCIATIC NERVE IN A TRANSGENIC RAT MODEL OF CMT1A PERIPHERAL NEUROPATHY: PRELIMINARY DATA

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Charcot-Marie-Tooth (CMT) disease comprises a group of clinically and genetically heterogeneous disorders, affecting the human peripheral nervous system, characterized by distal weakness and atrophy, pes cavus deformity, and severely slowed motor and sensory nerve conduction velocities. The most common form (CMT 1A) is caused by a 1.5 Mb tandem duplication in chromosome 17p11.2 containing the PMP22 gene. In this work we used a proteomic approach, including a basic two-dimensional gel electrophoresis separation system, to individuate differentially expressed proteins in sciatic nerve from CMT 1A transgenic rats. We used sciatic nerves of 10-15 mg in weight derived from 3 control (wild type), 2 hemizygous and one homozygous rats. We carried out the 2DE separation loading the proteic extracts on 7cm or 13cm pH 3-10 IPG strips and using a linear 9-16% SDS-PAGE in the second dimension. The 2DE maps obtained in duplicate have been visualized by Sypro Ruby and/or silver staining and a comparative analysis has been performed by Image Master 2D Platinum (version 6.0) software. Some protein spots have been excised and digested with trypsin. Peptide Mass Fingerprinting analysis has been performed on a MALDI-TOF MS Voyager DE-PRO (PerSeptive Biosystems) equipped with a delayed extraction device.

By comparison, we observed a drastically reduction of expression in the myelinic proteic components such as the protein P0 and P2, in the homozygous and hemizygous rats, according to the dysmyelinization and/or demyelinization that occurs in this form of CMT.

Moreover some other proteins, not yet characterized, showed significant qualitative and/or quantitative differences.

The complete characterization of the differential proteic pattern, will let us to highlight on the molecular mechanism responsible of this inherited peripheral neuropathy.

DIFFERENTIAL PROTEIN PROFILING OF MULTIPLE SCLEROSIS CSF SAMPLES USING A DIRECT MALDI-TOF STRATEGY

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Multiple Sclerosis (MS) is a complex disease of the central nervous system and several pathophysiological processes like inflammation, demyelination, axonal damage and repair mechanism contribute to the heterogeneity in its phenotypic expression. Despite MS is generally believed to be a disease of autoimmune origin, the real pathogenic mechanism, at the molecular level, remains poorly understood.

Proteomic analysis of cerebrospinal fluid (CSF) in MS patients has been reported to be a powerful methodology to provide new insight into the disease and to identify potential specific biomarkers. In the details we used a direct molecular profiling strategy, using matrix-laser desorption ionization mass spectrometry, for identifying phenotypic markers. The experiments were conducted to directly analyze the proteome of the CSF. An aliquot of each sample, control (n=10) and patients (n=8), underwent a desalting/concentration step over ZipTip C18 (Millipore Corporation, Bedford, MA, USA). After protein purification samples were typically mixed at a 1:1 vol/vol ratio with CHCA matrix solution (10 mg/ml in 50% ACN and 0.1% TFA), 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, Foster City, CA, USA) equipped with a 337-nm nitrogen laser and delayed-extraction (DE) technology. Separate spectra were obtained for a restricted mass-to charge (m/z) range (1000-25000 Da) in linear mode geometry, by applying an acceleration voltage of 25 kV . The acquired spectra, assayed in duplicate, were then processed for automated advanced baseline correction and noise. For the statistical analysis the peak lists were imported for data transformations and quantitative data were obtained presenting the signal intensity of each peptide/protein as a percentage of peak area.

The organized data, then, were subjected to Mann-Whitney U-test and cluster analysis. By this way, we have obtained differential protein profiles and in particular we have been able to identify 53 ion signals differentially expressed, 19 over expressed and 34 down-expressed Moreover the cluster analysis applied on this signals separated correctly in two distinct group 8 out of 10 control samples and five out of 8 MS samples while an other group comprising the remaining samples represented an overlapping group.

This preliminary data demonstrated the validity of the used MALDI-TOF protein profiling analysis of CSF in searching for peptide/small protein markers of neurological diseases.

COMPARATIVE PROTEOME PROFILING AND FUNCTIONAL ANALYSIS OF CHRONIC MYELOGENOUS LEUKEMIA CELL LINES

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Chronic myelogenous leukemia (CML) is a myeloproliferative disorder characterized by the (9;22) reciprocal chromosomal translocation producing the so-called Philadelphia (Ph) chromosome. This rearrangement results in the production of the chimeric Bcr/Abl oncoprotein with a constitutive tyrosine kinase activity. CML is usually a triphasic disease, having a chronic, an accelerated, and a blast phase, during which progressive resistance to imatinib (IM, the major drug for CML treatment) is acquired. New evidence suggests that CML blasts arise from leukemic progenitors (rather than leukemic stem cells) that have restored self-renewal capacities. However, a more clear characterization of this cell population, responsible for CML progression, is necessary to better understand this disease and to develop new treatment strategies. The aim of the present study was the molecular profiling of different Ph+ CML cell lines (LAMA84, K562 and KCL22) by a proteomic approach (based on two-dimensional gel electrophoresis combined with mass spectrometry analysis). Multivariate analysis highlighted significant differences in the global proteomic profile of the three CML cells lines and the detailed analysis of differentially expressed proteins revealed that LAMA84 cells preferentially expressed proteins associated with an invasive behaviour, while K562 and KCL22 cells preferentially expressed proteins involved in drug resistance. These results demonstrate that these cell lines, representing the same pathological phenotype, show characteristics in their protein expression profile that suggest different phenotypic leukemia subclasses. These data contribute to a new potential characterization of the CML phenotype and may help to understand interpatient variability in the progression of disease and in the efficacy of treatment. Moreover we have started the molecular characterization of IM-resistant phenotype by using LAMA84R cells as "in vitro" model. Interestingly these cells, compared with the sensitive counterpart, show a significant increasing of proteins involved in drug resistance (the same observed in K562 and KCL22 cells). Some of these proteins(such as HSP70s and Annexin 1) are inhibited by Carboxyamidotriazole (CAI), a drug studied in our laboratory to treat IM-resistant CML. These data indicate that CAI may determinate the reversion of resistant phenotype, as supported by functional assays.

INNATE IMMUNITY PROBED BY LIPOPOLYSACCHARIDE ARRAYS AND PROTEOMICS

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Lipopolysaccharides (LPSs) are ubiquitous and vital components of the cell surface of Gram-negative bacteria. They are amphiphilic macromolecules composed of a hydrophilic hetero-polysaccharide (comprising the core oligosaccharide and O-specific polysaccharide) covalently linked to a lipophilic moiety termed lipid A, which anchors these macromolecules to the outer membrane. In animal and plant cells innate immune defenses toward microrganisms are triggered by the perception of pathogen associated molecular patterns (PAMPs). These are conserved and generally indispensable microbial structures such as LPSs that are fundamental in the Gram negative immunity recognition.

Aim of this project is the development of lipopolysaccharide microarrays methodology for the delucidation of mechanisms involved in the LPSs-mediated molecular recognition and anti-infection responses in vegetal and animal organisms. The investigation will be pursued through the identification of the protein partners specifically interacting with these effectors by functional proteomics strategies. The complex protein/lipopolysaccharide tagged with biotin will be isolated on streptavidin column and the proteins will be fractionated by gel electrophoresis and identified by mass spectrometry. The experiments will be carried out in duplicate, using cellular extracts treated and not treated with lipopolysaccharides. Lipopolysaccharide intended to be used as baits will be purified from the authentic vegetal and animal pathogen bacteria involved in the infection of the cell that yield the protein extract.

DIFFERENTIAL EXPRESSION OF BRAIN PROTEINS IN THE NUCLEUS ACCUMBENS OF ISOLATION-REARED RATS

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Most current animal models of schizophrenia are based on invasive paradigms or pharmacologically-induced states. These non-naturalistic approaches, albeit useful to represent specific aspects of the disorder, are poorly suitable for the study of neurochemical and functional imbalances in psychosis. An experimental model that more closely captures the neurobiological relationship between environmental stress and schizophrenia is the isolation-rearing (IR) paradigm. This manipulation consists in subjecting rats to the deprivation of social interactions for 6-9 weeks after weaning. In this research it has been examinated protein expression profiles in selected brain areas from healthy and isolation-reared rats using 2-DE based proteomics. Image analysis revealed that 3 protein spots were differentially regulated in the nucleus accumbens of isolation- and socially-reared rats, when compared to control rats. These spots were identified by mass spectrometry (MALDI-TOF and MS/MS). Altered proteins (heat shock proteins 60, alpha synuclein, 14-3-3 protein zeta/delta) have previously been connected to schizophrenia. These results show the high potential of using proteomic methods for the validation of animal models of schizophrenia and it result a valid method to identify new proteins involved in the pathophysiological mechanisms of schizophrenia.

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PROTEOME ANALYSIS OF MULTIDRUG RESISTANCE IN METHOTREXATE-RESISTANT HUMAN OSTEOSARCOMA CELL LINE U-2OS/MTX

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Resistance to anticancer drugs is a major obstacle in the effective treatment of tumors. Many molecular mechanisms that are responsible for drug resistance are known whereas others have yet to be discovered. Methotrexate (MTX) is widely included in the multidrug chemotherapeutic regimens for high-grade osteosarcoma (OS), with doxorubicin, cisplatin and ifosfamide. To understand the mechanisms responsible for methotrexate-resistance, a proteomic approach was used to identify proteins that were expressed at different levels by the methotrexate-resistance human osteosarcoma cell line, U2OS/MTX, and its parental cell line, U2OS. Two-dimensional gel electrophoresis (2-DE) and image analysis was used to determine which proteins spots were expressed in different levels by the two cell lines. Protein identification was performed by PeptideMassFingerPrintig (PMF) and by tandem mass spectrometry using both MALDI-MS/MS and nLC-MS/MS approaches. All the 24 differential proteins between the two cell lines were identified, and the differential expression levels of the partial proteins were confirmed by Western blot analysis. Based on the proteome map, we found that MTX causes a markedly increased in the levels of HSP70 and HSP90, whereas the level of the HSP27 was not significantly altered in MTX-treated U2OS cells.

We, thus, conclude that these proteins likely contribute to the resistance selected in the U2OS/MTX cells, and their altered expression in tumors may cause clinical resistance to chemotherapy.

NUCLEAR PLC BETA 1 SIGNALING: eEF1A2 IS A NOVEL PHOSPHO-SUBSTRATE OF PKC BETA 1 IN MYOBLAST

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Introduction - Phospholipase C beta 1 (PLC-β1) is a key player in the regulation of nuclear inositol lipid signaling and of a wide range of cellular functions, such as proliferation and differentiation (1,2,3). PLCbeta1 signaling depends on the cleavage of phosphatidylinositol 4,5-bisphosphate and the formation of the second messengers diacylglycerol and Inositol tris-phosphate which activate canonical protein kinase C (cPKC) isoforms. Here we describe a proteomic approach to find out a potential effector of nuclear PLCbeta1 dependent signaling during insulin stimulated myogenic differentiation.

Results - We have previously shown that PLC-β1 is greatly increased at the nuclear level during insulin-induced myoblasts differentiation and that this nuclear localization is essential for induction of differentiation. Thus, nuclear proteins of insulin stimulated C2C12 myoblasts, were immunoprecipitated with an anti-phospho-substrate cPKC antibody. After Electrophoretic gel separation of proteins immunoprecipitated, several molecules were identified by LC-MS/MS. Among these most relevant and unexpected was eukaryotic elongation factor 1 alpha 2 (eEF1A2). We found that eEF1A2 is phosphorylated by PKCbeta1 and that these two molecules co-immunolocalized at the nucleolar level. eEF1A2 could be phosphorylated in many sites among which both threonine and serine residues. By site direct mutagenesis we demonstrated that it is the serine residue of the motif recognized by the antibody that is specifically phosphorylated by PKCβ1. The silencing of PLCβ1 gives rise to a reduction of expression and phosphorylation levels of eEF1A2 indicating this molecule as a target of nuclear PLCβ1 regulatory network during myoblasts differentiation.

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PROTEOMIC ANALYSIS OF CISPLATIN RESISTANCE IN OVARIAN CANCER

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Cisplatin [cis-diamminedichloroplatinum (II)] is one of the most potent and highly effective chemotherapeutic agents for treatment of several types of cancer including ovarian, cervical, and testicular cancers. However, the development of acquired resistance has limited its effectiveness, especially in ovarian cancer chemotherapy. Resistance to cisplatin is not completely understood and it is thought to occur through many different mechanisms. Therefore a great deal of investigation has concerned the ways to overcome drug resistance, including the development of new chemotherapeutic compounds, addition of translational agents which modify signal transduction pathways or investigation of new methods for drug administration.

In the present study we search for protein networks associated with drug resistance by comparing protein expression in two human ovarian carcinoma cell lines one sensitive and the other resistant to cisplatin, respectively 2008 and C13* cells. The resistant subline, isolated after prolonged exposure to low drug doses, is approximately 20-fold more resistant to cisplatin than the sensitive one. Comparative analysis was performed by two-dimensional liquid chromatography system (PF-2D, Beckman Coulter), i.e. chromatofocusing (1st dimension) and reverse phase chromatography (2nd dimension). About eighty peaks showing a differential expression of at least 100% between the two cell lines where further analysed and identified by MALDI-TOF mass spectrometry. To obtain insights into potential cellular pathways that may be modified in two cell lines, an explorative in silico analysis was undertaken using IPA (Ingenuity Pathway Analysis) software. This application was used to identify direct and indirect molecular interactions, pathway associations, and functional assignments involving the proteins of interest. IPA resulted in the generation of two main protein networks of protein and/or gene associations. The most interesting network emerging from this analysis featured 17 of the 44 identified proteins and main functions associated with the components of this network were gene expression and lipid metabolism: protein COX-2, progesterone and estradiol are central nodes of this network. Moreover, protein classification by Biological Process shows several proteins involved in Immunity and Stress response: Peroxiredoxin-2, heat shock protein 90kDa alpha and beta, Glutathione S-transferase and Thioredoxin domain containing 2 (Sptrx-1), most of them are upregulated in cisplatin resistant cell line. Particularly, Sptrx-1, known as testis specific, was found in an ovarian cell line and associated to cisplatin resistance for the first time.

AN APPROACH TO WHOLE BLOOD SERUM PROTEOMICS USING FLOW FIELD-FLOW FRACTIONATION WITH MULTIANGLE LASER SCATTERING DETECTION AND NANOCHIP-ION TRAP MASS SPECTROMETRY

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Current approaches for serum proteomics can show limitations related to composition of the proteome and to the different protein expression levels, then giving method-dependent results. Separation methods to reduce sample complexity are often used as methods for MS-based approaches to serum proteomics. Flow field-flow fractionation (FlFFF) is a separation technique in which proteins are separated according to their difference in diffusion coefficient. FlFFF with multi-angle laser scattering (MALS) detection allows for the absolute determination of the molar mass and size parameters of the fractionated analytes (1). The gentle separation mechanism of FlFFF maintains the intact structure of protein and protein complexes. FlFFF-MALS has been recently proposed for intact lipoprotein characterization in whole human serum samples (2). In this work, FlFFF-MALS was used to fractionate whole blood serum before MS-based proteomics. Fractionation was performed by injecting undiluted serum samples and collecting fractions corresponding to the elution times of high abundance proteins and lipoproteins. The molar mass range values of the serum fractions was on-line determined by MALS. Each fraction was then digested with trypsin, and the peptide mixtures were further analyzed by nanochip/liquid chromatography coupled with electrospray HCT ion-trap MS (ESI/MSMS). The protein present in the different fractions were identified by using MASCOT software. The protein complexes were identified by grouping interacting proteins in network and using bioinformatics tools commonly available on the WEB. The preliminary results show promising for the development of a method based on integrated techniques for the identification of proteins and protein complexes natively present in complex biological samples such as blood serum.

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LYMPHOCYTES PROTEOMIC ANALYSIS IN PARKINSON'S DISEASE PATIENTS REVEALS CYTOSKELETAL PROTEIN DEREGULATION.

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Parkinson's disease (PD) is a progressive neurodegenerative disorder of unknown etiology. It affects mainly the elderly, although a small portion of PD patients develops the illness at a much younger age. It has been found that in both patients and experimental animal models of PD neuroinflammation appears to be an ubiquitous findings. These cases present phagocyte activation, increased synthesis and release of proinflammatory cytokines and complement activation (Whitton, 2007).

Using two-dimensional gel electrophoresis (2DE) and mass spectrometry protein identification we analyzed changes in lymphocyte protein expression of patients affected by idiopathic PD in order to identify peripheral biomarkers. 2DE maps from PD patients under pharmacological treatment were matched with those from controls and from patients under subthalamic nucleus deep brain stimulation (DBS) to evaluate the effect of therapy withdrawal.

This analysis permitted to correlate alterations of actin cytoskeletal proteins of peripheral lymphocytes of PD patients to the illness supporting the hypothesis of cytoskeletal involvement in the neurodegenerative disorders (Gourlay & Ayscough, 2005).

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UTILIZING DISCOVERY DATA TO EXPEDITE TARGETED PROTEIN QUANTITATION METHODS

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Considerable number of proteomics laboratories utilize LTQ-based platform for initial screening of proteins, peptides and expression levels, rendering a list of protein biomarker candidates that must be further validated. From these discovery experiments, a wealth of knowledge and thousands of MS and MS/MS spectra have been accumulated which can be used to direct more selective SRM-based analyses. We present here the targeted quantitation workflow incorporating software and libraries that identify optimal peptide and product ions to be used in a subsequent SRM assay. The addition of top two or three product ions not only increases the selectivity for identifying the targeted peptide, but can dramatically increase the detection and quantification capabilities if the resulting method. In our observations, utilizing product ion abundancies from LTQ-based experiments provides a higher probability of matching the most abundant SRM transition than relying on a rule-based approach.

ELASTIC FIBER CALCIFICATION: IDENTIFICATION OF PATHOGENETIC PATHWAYS BY PROTEOME ANALYSIS

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Pathological mineralization of soft connective tissues may frequently occur in several cardiovascular complications, in diabetes, in renal disorders and with aging. Within soft connective tissues, elastic fibers are the components most susceptible to calcification. In order to investigate on the mechanisms responsible for the occurrence of ectopic mineralization in different organs, we have used fibroblasts isolated from patients affected by Pseudoxanthoma elasticum (PXE), a genetic disease due to progressive calcification of elastin fibers and characterized by wrinkles, laxity and redundancy of the skin, by premature cardiovascular complications and by a severe visual impairment similar to macular degeneration. We have recently shown, by 2D gel electrophoresis and Western blot on whole cellular protein extracts, that dermal fibroblasts from PXE patients exhibit reduced expression of carboxylated Matrix Gla Protein (Gla-MGP) (Gheduzzi, 2007). In the present study we demonstrate that in PXE fibroblasts there is a significant up-regulation of calumenin, an endoplasmic reticulum chaperone that plays a central role in the regulation of gamma-carboxylation of vitamin-K dependent proteins, such as MGP. Calumenin acts by inhibiting the gamma-carboxylase as well as the vitamin K 2,3-epoxide reductase (VKOR). This last enzyme produces reduced vitamin K, a required cofactor for gamma-carboxylation of vitamin K-dependent proteins. Furthermore, VKOR has been shown to harbor a thioredoxin-like CXXC center involved in reduction of vitamin K1 2,3-epoxide (Vit.K>O). This reduction is linked to the dithiol-dependent oxidative folding of proteins in the endoplasmic reticulum by protein disulfide isomerase (PDI). In PXE fibroblasts there is also a significant downregulation of PDI, consistently with the impairment of the complex system responsible for the correct carboxylation of MPG. These data get new light on the importance of MGP carboxylation in the pathogenesis of ectopic calcifications. Work supported by grants from EU (Geneskin-CA-512117 and Elastage- CT-2005-018960)

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SERUM PROTEIN PROFILING IN PATIENTS WITH CROHN'S DISEASE

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Crohn's disease (CD) is a chronic autoimmune pathology that involves the gastro-enteric tract. It is a multi-factorial and polygenic disease of unknown aetiology. Currently, the diagnosis is difficult and it is based only on clinical, endoscopic, radiological and histological methods.

Aim of this work is to evaluate the serum protein profile of healthy and Crohn's disease patients using 2D electrophoresis and mass spectrometry.

Image analysis of 2D maps showed that 1-antitrypsin,αseveral proteins involved in inflammatory response (1-antichimotrypsin, haptoglobin and complement factor 3) are increased inα Crohn's disease. Overexpression of complement 3 was statistically significant and in particular C3c showed a relevant increase (Fig1). The most significant change was detected for transthyretin. In CD patients there was a shift in isoelectric point from pH 5.52 to pH 4.7 (identification confirmed by mass spectrometric analysis) (Fig 2); that suggests a possible transthyretin PTM role linked to Crohn's disease. This findings may be useful to better understand the pathological mechanisms of Crohn's disease and to find putative markers for a more specific diagnosis.

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HUMAN URINE PEPTIDOMICS FOR BIOMARKER DISCOVERY: A MALDI-TOF-MS INVESTIGATION

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Prostate cancer (PCa) is the most common cancer in males with a high mortality in the western society. Actually, the existing screen for prostate cancer based on serum prostate specific antigen (PSA) can not distinguish between prostate cancer and benign prostate hyperplasia resulting often in unnecessary prostate biopsies (Stamey et al.,2002; Thompson et al. 2004). New diagnostic biomarkers to univocally identify PCa at the early stage are then necessary. Large-scale protein analysis by means of mass spectrometry (MS) for the identification of biomarkers in the urinary proteome may represent an important step to reach a non-invasive diagnosis of this disease (McKee et al., 2000; Hochstrasser et al. 2002). The profiling of urine peptidome (without prior trypsinization) could be followed by MALDI-TOF-MS, that could represent an excellent tool for distinguishing patients with PCa. However, to avoid false positive in biomarker discovery, there is a critical need for development of standard protocols to control the three major sources of variability: pre-analytical, analytical and biological factors (West-Nørager et al., 2007). In many cases, some peptides initially claimed as biomarkers were simply found to arise from sample manipulation, bad storage or sample degradation on time. Then, in the first part of this work the influence of these variables in the human urine peptidomic profiling of healthy donors was studied by means of MALDI-TOF-MS and statistical analysis. Once an ideal protocol able to minimize these issues was optimized, the peptide profiles (1000-12000 Da) arising from healthy donors and subjects with prostate cancer were compared. Our study shows the appearance of different polypeptides in urine samples of prostate cancer patients that could be potentially used as markers for early detection of cancer. Further work in this direction is currently in progress to identify the peptides and to validate these results.

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A OPTIMIZED 2-D PAGE PROTOCOL FOR HUMAN URINE ANALYSIS

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Human urine is a readily obtainable, non invasive biological sample that can be used to investigation biological markers. Sample preparation is fundamental in urine proteomic studies in order to isolate and to concentrate urinary proteins and to eliminate the interfering salts and low molecular weight contaminants. We report a optimized protocol for isolate and concentrate urinary proteins. Immediately after collection, urine samples were centrifuge and the sediment of broken cells in the pellet were discarded. The ultrafiltration of urine samples (supernatants) were achieved by means of Amicon Ultra-15 Centrifugal Device (Millipore) and thereafter the samples were subjected to protein precipitation in a mixture of acetone and methanol . The pellets were solubilized in 2D sample buffer containing 7M urea, 2M thiourea, 4% CHAPS, 1% DTT, 15mM Tris, 2% ampholine 3.5-10 (1:10, W/v). Protein concentrations were determined using the 2D Quant Kit (GE). The protocol gave a good reproducibility for protein concentrations (CV 3.31%), valuated after repeated processing of the same sample (Fig. 1). Isoelectric focusing was performed on 13 cm immobilized pH 4-7 L IPG strips on a Multiphor II system (GE). The second dimension was performed using a 8-15% (w/v) polyacrylamide gradient vertical SDS-PAGE slab gels. The spots were identified by MALDI-TOF mass spectrometry. This method enabling a good recovery of total proteins, as well as better evaluation of the distribution and expression and it can be represent a good tool for an analysis of urinary proteins from different biological samples.

PROTEOMIC AND METABOLOMIC PROFILES OF MURINE MICROGLIA

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Neuronal loss and an aberrant deposition of fibrillar proteinaceous material (amyloid) are the hallmarks of a group of neurodegenerative disorders, such as Alzheimer's disease (AD), the Huntington's chorea (HD) and Transmissible Spongiform Encephalopathies (TSE or prion diseases). In all these pathological states, amyloids are generated in a disease-specific manner from structurally unrelated proteins. Biochemical features of the neuronal compartment made it vulnerable to free radical injures, an unbalanced redox state has been proposed as an earlier event common in all amyloidogeneses. In fact, in neurons and astrocytes a continuous production of oxidant agents and depletion of antioxidant agents has been detected during neurodegeneration.

Microglia, a primary immune effector cell of the central nervous system affects homeostatic, neuroprotective, regenerative and degenerative outcomes in health and disease. Despite these broad neuroimmune activities linked to specific environmental cues, a precise profile for microglia in the context of neurodegenerative disease has not been elucidated so far. Our investigation is pointed on depicting protein expression and metabolic changes in microglial cells upon activation from a large panel of amyloidogenic peptides, structurally unrelated.

With this aim, BV2 cells, a murine cellular line preserving most of functional activity of the microglia, has been selected as cellular model. Cultured cells, rested or stimulated with peptides or chemical oxidant agents, have been characterized on the basis of the proteome (mainly through 2DE-MALDI/TOF approach), the GSH/GSSG ratio and by profiling activities of a large panel of antioxidant enzymes.

Preliminary results show that also in microglia compartment, an inpairment in the redox homeostasis occurs upon stimolation with amyloidogenic peptides. Changes in the proteome will be discussed in terms of a correlation between the observed unbalances in the antioxidative defences and amyloidosis.

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SPECIFIC PROTEINS IDENTIFIED IN HUMAN WHOLE SALIVA FROM RHEUMATOID ARTHRITIS PATIENTS

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Rheumatoid arthritis (RA) is a chronic autoimmune disease of unknown etiology, characterized by the development of synovitis, which is directly responsible for cartilage and bone degradation in multiple joints. This inflammation disorder is initiated by self-attack using own immune system and several candidate antigens such as type II collagen, glucose-6-phosphate isomerase, chondrocyte glycoprotein 39 and protein chaperon BiP, have been proposed. Recently, to identify RA-specific biomarker, different proteomic studies have been performed in serum, synovial fluid and cells (synoviocytes, lymphocytis) from RA-patients.
In the present work, for the first time, we used two-dimensional gel electrophoresis (2-DE) in combination with mass spectrometry (MS) to obtain the whole saliva (WS) protein map of RA patients and to compare it with WS from healthy subjects in order to evaluate the global changes of the protein profiles which occur in this disease. WS was collected from 18 patients with diagnosis of RA and 15 healthy volunteers with similar mean age. Salivary protein patterns were obtained as previously described (Giusti et al., 2007). Protein expression profiles for each sample were generated by 2-DE and protein spots of interest were identified using MALDI-TOF/TOF analysis. Quantitative and qualitative differences in the WS protein pattern comparing the synthetic gels of the disease with respect to the controls were observed. A significant up-regulation of apolipoprotein 1, haptoglobin, leucocyte-elastase inhibitor alfa-1 antitrypsin and 14-3-3 protein isoforms was found and also specific proteins of disease were identified (i.e protein chaperon BiP). The present study is the first reporting the WS protein pattern of RA patients and comparing the differences between the WS of RA patients and the WS of healthy subjects. In this study, newly identified proteins were detected in WS that might have a pathological significance for RA. It is possible that some of them can be defined as new therapeutic targets or diagnostic markers for RA disease.

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DIFFERENTIAL EXPRESSION PROTEOMICS IN FIBROBLASTS FROM PATIENT WITH EARLY-ONSET PARKINSON'S DISEASE

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Parkinson's disease (PD) is the most common neurodegenerative disorder affecting human and is characterized clinically by classical parkinsonism and pathologically by progressive preferential loss of dopaminergic neurons in the substantia nigra. The aetiology of PD is complex and poorly understood but it likely involves both genetic and environmental factors. Reduced complex I activity has been reported in PD. This and other observations led to the hypothesis that mitochondrial dysfunction contributes to the aetiology of disease. A direct link between mitochondria and Parkinsonism-associated gene comes from the discovery of mutations in PTEN-Induced Kinase 1 gene (*PINK1*) in several pedigrees of autosomal recessive familial PD [1].The *PINK1* gene encodes a 581 amino-acid protein with a predicted N-terminal mitochondrial targeting sequence and a highly conserved serine/threonine kinase domain. PINK1 protein has been shown to localize in the mitochondria and exhibit activity to phosphorylate downstream effector molecular chaperone HSP75 (TRAP1) [2] and serine protease HtrA2 [3]. In the attempt to identify proteins that may be associated with the process of dopaminergic neuronal cell death we have analyzed the protein expression changes of primary fibroblast cultures from *PINK1*^{W437X} familial PD patient by using proteomics technologies including protein separation by 2-DE gel electrophoresis and identification by LC-nano-ESI-Q-TOF masspectrometry. The results show differences in the expression pattern of some proteins in fibroblasts of a patient whit the homozygous mutation whit respect to control sample. Among these proteins the S100A4 calcium binding protein was identified. (Swiss_Prot P26447). This protein was significantly down expressed in the PINK1 cells. An appealing hypothesis is that down-regulation of the S100A4 protein, could result in increased cytosolic calcium concentration due to the reduced buffering capacity of this protein, whit increase of the mitochondrial calcium uptake, in turn involved in cytochrome c release from mitochondria.

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PEPTIDOMICS ANALYSIS OF HUMAN URINE FOR PREDICTION OF DIABETIC KIDNEY DISORDER

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Diabetic nephropathy (DN) can develop as a complication of both type 1 and type 2 diabetes, and, if not well treated, could lead to end-stage renal disease. Microalbuminuria (MA) is presently the only earliest putative diagnostic sign of diabetic nephropathy. However, MA is not specific for diabetic renal damage, because it may also reflects a general vascular injury or renal damage from other causes, such as hypertension (Wolf & Ritz, 2003; Ziyadeh & Sharma, 2003). Thus a great enhancement in the management of patients with diabetes mellitus would be the discovery of additional protein markers for early non-invasive detection of diabetic renal damage. Large-scale protein analysis by means of mass spectrometry (MS) for identification of biomarkers in the urinary proteome may represent an important step in the non-invasive diagnosis of kidney diseases (Jungblut et al.,1995; McKee et al., 2000; Hochstrasser et al., 2002). Among the recently developed strategies, the so-called top down approach (O'Riordan et al., 2004; Nguyen et al.,2005), where the native proteins and peptides are analyzed without prior trypsinization, looks to be promising. This approach allowed to examine peptides and small proteins up to 10-20 kDa that may represent excellent markers of a given pathophysiological entity. In our study, we have applied proteomics technologies such as MALDI-TOF-MS and statistical analysis for the examination of urine to detect novel biomarkers that could play a critical role in diabetic nephropathy. Once optimized a protocol that could minimize the major sources of variability, the human urine peptidomic profiling arising from 40 healthy donors and 60 diabetic subjects were analyzed by means of MALDI-TOF-MS in the range of 1000-12000 Da. Our first results shown the presence of some distinguishing polypeptides in urine samples of MA positive diabetes patients that could be used as markers for specific clinical analysis of diabetic nephropathy. Further work in this direction is necessary to validate these results.

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PROTEOMIC ANALYSIS OF SALIVARY PROTEINS PATTERNS AS POTENTIAL TOOL TO DISCRIMINATE TYROID DISEASE: A PRELIMINARY STUDY

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Thyroid nodules are the most common thyroid disease, with an incidence of 4-7% in iodine sufficient areas that markedly increases in iodine deficient countries. Thyroid nodules are defined as adenomas, carcinomas or hyperplastic lesions. Classification of thyroid nodules into distinct groups is based on their hystopathological appearance and it is based on specimen obtained after fine-needle aspiration on cellular characteristics and cell-specific antigens only. Specific molecular or proteic markers are still lacking. Recently, we proposed the proteomic analysis of FNA as a rapid, non-invasive and preoperative method for the discovery of specific tumoral markers (Giusti et al., 2007). Now, we carried out a preliminary study to evaluate if proteome of whole saliva may be source of potential markers for diagnosis of thyroid diseases. Ten patients with multinodular goiter, nine patients with microfollicular nodules, seven patients with papillary carcinoma and ten healthy subjects were enrolled in the study. Salivary protein patterns were obtained, as previously described (Giusti et al., 2007), using 2-DE technique. The pathological classes showed different protein patterns in comparison to control group and especially the samples from patients with multinodular goiter had a typical protein profile. A comparison of WS maps of different classes of thyroid diseases with respect to the controls was performed to evaluate the potential usefulness of salivary proteomic analysis as a complementary tool of FNA in the diagnosis of tyroid disorders.

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CELLULAR ORGANELLE PROTEOMICS

CELL TRAFFICKING OF SUMF1 DESCRIBED BY FUNCTIONAL PROTEOMICS EXPERIMENTS

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Sulphatase Modifying Factor 1 (SUMF1) activates all sulfatases in the ER operating a post-translational modification of a specific cysteine of their catalytic domain into formylglicine. This unique modification is essential for sulfatase activity, In fact, mutations in the SUMF1 gene result in the inactivity of all the sulfatases and in the onset of the Multiple Sulfatase Deficiency (MSD) disease (Cosma et al., 2003). Previous studies demonstrated that SUMF1 is secreted, and when taken up by other cells it is re-localized to the ER, where it activates endogenous sulfatases (Zito et al., 2007). However, the molecular pathways by which SUMF1 is secreted by the cells or is taken up from the medium are still largely unknown. To dissect out the mechanism controlling its folding, trafficking and function, we searched for proteins interacting with SUMF1 and focused on molecules potentially involved in its retention/escape from the ER. In order to identify SUMF1 partners, extract from stable HeLa cells expressing SUMF1-3XFLAG, were immunoprecipitated with immobilized anti-FLAG antibodies. The eluted protein complexes were separated by SDS-PAGE and the protein bands were analysed by nanoLCMSMS and identified by database search using the MASCOT software. Among all the SUMF1 putative partners we focused our attention on PDI, ERp44 and ERGIC-53. We demonstrated that these interactions are crucial for controlling SUMF1 trafficking and function.

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MITOCONDRIAL F1F0ATPSYNTHASE ASSEMBLY AND OLIGOMERIZATION UPON CADIOMYOGENIC DIFFERENTIATION OF MURINE CARDIOMYOBLASTS

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The mitochondrial F1F0ATPsynthase harbors two major structural domains, a transmembrane component (F0) containing a proton-permeable channel and a peripheral, matrix-localized, catalytic component (F1) where the ATP is synthesized. F1F0ATPsynthase forms homo-dimers and larger oligomers that contribute to mitochondrial cristae morphology. Cell differentiation is often accompanied by mitochondrial biogenesis, but the connections with bioenergetics and assembly of OX-PHOS complexes have not been established yet. This study focused on cardiomyogenic differentiation (markers troponin I and myosin heavy chain) induced in the rat cardiomyoblast cell line H9c2 by culturing in the presence of retinoic acid and serum deprivation. Mitochondrial biogenesis was documented by confocal microscopy with development of a typical elongated network organization. In order to check for F1F0ATPsynthase assembly modifications, we used blue native-PAGE to compare the amounts of native monomeric and oligomeric F1F0, as well as of incomplete F1F0 and unassembled F1, extracted from control and differentiated mitochondria. The bands containing monomeric F1F0 (complex V) and unassembled F1 was resolved from dodecyl maltoside mitochondrial extracts and assigned by in-gel development of ATPase activity and by the subunit pattern found in second dimension SDS-PAGE. To detect small amounts of incomplete F1F0, individual BN-PAGE lanes were submitted to 2-D SDS-PAGE and anti-alpha/beta Western blotting. To explore F1F0 oligomerization in the parental and differentiating cardiomyoblasts, mitochondrial digitonin extracts were subjected to BN-PAGE followed by 2-D SDS-PAGE and alpha/beta immunodetection. Active bands and alpha/beta subunits were found at the positions corresponding to the migration in BN-PAGE of monomeric F1F0 (V) and oligomeric forms, from which one had an apparent mass of 1200 kDa thus corresponding to dimeric F1F0 (Vd), whereas larger F1F0 oligomers showed lower mobility. To compare by densitometry the proportion of oligomeric and monomeric F1F0 between differentiating cadiomyoblasts and their controls, we carefully titrated detergent-to-protein ratios in view of possible changes in protein expression and/or lipid content between control and differentiated mitochondrial preparations. The results showed that cardiomyogenic differentiation favours in H9c2 both monomeric F1F0 assembly and dimerization/homo-oligomerization, thereby overcoming functional and assembly/stability defects in the whole F1F0 observed in the parental line. To define F0 subunit/s lacking in incomplete F1F0, we are planning 2-D immunoblotting and MS analyses.

PROTEOMICS/SYSTEMS BIOLOGY

HSP90 IS A COMPONENT OF THE CAP-BINDING COMPLEX DURING DROSOPHILA OOGENESIS

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In eukaryotes, translation initiation is facilitated by the cap structure, m7GpppN, which is present at the 5' of all mRNAs. This structure is recognized by the eukaryotic translation initiation factor 4E (eIF4E), the rate-limiting component for cap-dependent translation, which associates with either stimulatory or inhibitory factors to modulate protein synthesis. In order to identify novel factors that act at the translational level during Drosophila oogenesis, we performed an affinity chromatography purification of eIF4E complexes on m7GTP-Sepharose beads followed by LCMSMS identification of individual proteins. Among many others, we isolated the product of the hsp83 gene, the evolutionary conserved chaperone Hsp90, as a specific component of the cap-binding complex. Here we report that Hsp90 interacts in vitro with the translational repressor Cup. In addition, we show that hsp83 and cup interact genetically, since lowering Hsp90 activity further impairs the oogenesis alterations linked to diverse cup mutant alleles. Hsp90 and Cup co-localize in the cytoplasm of the developing germ-line cells within the germarium, thus suggesting a common function from the earliest stages of oogenesis. Taken together, the present work starts elucidating the role of Hsp90 during Drosophila female germ-line development and strengthens the idea that Cup has multiple essential functions during egg chamber development.

OPTIMISATION OF TAP-TAG PURIFICATION IN PED/PEA-15 INTERACTOMA STUDIES

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Phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocytes (PED/PEA-15) was originally identified as a major astrocyte phosphoprotein and as type II diabetes overexpressed protein. PED was found to be widely expressed in different tissues and highly conserved among mammals. Several studies revealed that PED/PEA-15 regulates multiple cellular functions by binding to distinct components of major intracellular transduction pathways. PED/PEA-15 has been shown to exert antiapoptotic action through distinct mechanisms by phosphorylation carried by PKC, CaMKII and AKT.

In order to elucidate new molecular pathways involving and regulating the PED/PEA-15 activity, a functional proteomic strategy has been carried out. PED/PEA-15 was expressed in the lung adenocarcinoma A459 cell line with a double tag consisting of Protein A and Calmodulin binding peptide (CBP) separated by a TEV cleavage site. This constructed was employed for the Tandem Affinity Purification of PED/PEA-15 protein partners using the procedure first developed by Rigaut et al in 1999 for yeast complexes purification and successively adapted for mammalian systems. Although theoretical advantages are expected in protein complexes isolation by Tap Tag, this system did not find a large application in functional proteomic studies as the one step (immuno)affinity purification. The standard protocol, in fact, provides several steps characterised by a very low yield, as in the case of the TEV proteolytic cleavage. In order to improve the purification yield during the purification of PED/PEA-15 TAP-Tag complexes, several modifications of the original protocol have been developed and the results are be reported in this presentation.

NEW METHODS TO IMPROVE PROTEIN-PROTEIN INTERACTIONS STUDIES

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Functional proteomics approaches are addressing two major topics, the elucidation of biological function of unknown proteins and the definition of cellular mechanisms at the molecular level. In the cells, many proteins display their biological functions through the rapid and transient association within large protein complexes. Understanding protein functions as well as unravelling molecular mechanisms within the cell is then depending on the identification of the interacting protein partners. The association of an unknown protein with partners belonging to a specific protein complex involved in a particular process and/or cellular signalling pathways would in fact be strongly suggestive of its biological function. The strategies employed in the protein complexes characterisation are mainly based on (immuno)affinity procedures, followed by protein fractionation by SDS-PAGE and identification by LCMSMS. All these procedure require preventive pre-cleaning steps in order to reduce the number of false positive interactors that bind not specifically the matrix used for the protein complex purification. In many cases pre-cleaned samples still contain proteins unrelated to the molecule of interest which could impair the right protein-protein interaction association. In order to overcome this drawback, other methods have been investigate as integration to the (immuno)affinity purification procedures. These methods are essentially addressed to a rough fractionation of native complexes, as achieved by ultracentrifugation in glycerol/sucrose gradients and/or gel filtration chromatography. The fractions containing the protein of interest are selected by western blot and then submitted to a following (immuno)affinity purification procedure. These procedures have been set up for the identification of Chromobox 7 (CBX7) protein partners, in order to elucidate CBX7 biological function. On the basis of protein homology with other CBX proteins, it is supposable for CBX7 a role as transcription repressor, but to date there are no direct evidences of this function. Moreover, the cbx7 gene has been found differently expressed in several tumours, suggesting specific and still uncharacterised roles for the protein CBX7.

A NOVEL APPROACH TO EXTRACT BIOLOGICAL KNOWLEDGE FROM PROTEOMICS DATA

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Proteomics today generates vast amounts of peptide and protein identification data with high accuracy. However, a growing issue is how to interpret the results, particularly when analyzing protein and peptide identities made by automated database search engines, and when trying to extract biologically meaningful information from mass spectrometry experiments.

This poster shows the application of a new bioinformatics tool, ProteinCenter, that manages these protein and peptide lists and puts them in a biological context. The tool was developed specifically to help researchers rapidly obtain a biologically-relevant overview in large-scale proteomics studies by using biological annotations from multiple resources. Within minutes, output generated by protein database search engines can be translated into biological information.

In the current study we used ProteinCenter to analyze a quantitative proteomics study of the yeast pheromone signaling pathway (1). Using only the protein list, it was possible to deduce which cellular processes were perturbed. Among the top over-represented Gene Ontology (GO) cellular components were: site of polarized growth, mating projection , cell projection and cellular bud neck . This correlated perfectly with the design of the experiment: the mating projection as a part of cell projection is formed by unicellular fungi in response to mating pheromone. More detailed analysis showed that mating projection is caused mainly by the mating projection tip rather than the mating projection base.

Similar analyses were performed at the level of GO Biological Process and GO Molecular Function and a comparison with the whole yeast proteome from reference SwissProt database was made.

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POST-TRANSLATIONAL MODIFICATIONS

A PROTEOMIC CHARACTERIZATION OF WATER BUFFALO MILK FRACTIONS

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Water buffalo has been studied in relation to the exclusive use of its milk for the manufacture of high-quality dairy products. Buffalo milk presents physicochemical features different from that of other ruminant species, such as a higher content of fatty acids and proteins. We report here a detailed proteomic analysis of buffalo skim milk, whey and milk fat globule membrane fractions. Notwithstanding the poor information available on buffalo genome, identification of protein isoforms corresponding to 72 genes was achieved by a combined approach based on 2-DE/MALDI-TOF PMF and 1-DE/microLC-ESI-IT-MS-MS. Major protein components, i.e. alphaSl-, alphaS2-, beta-, and alpha-lactoglobulin, were characterized for beta-lactalbumin kappa-caseins, post-translational modifications, providing a scientific basis to coagulation/cheese making processes used in dairy productions. Minor proteins detected emphasized the multiple functions of milk, which besides affording nutrition to the newborn through its major components, also promotes development and digestive tract protection in the neonate, and ensures optimal mammary gland function in the mother. Defence against pathogens is guaranteed by an arsenal of antimicrobial/immunomodulatory proteins, which are directly released in milk or occur on the surface of secreted milk lipid droplets. Proteins associated with cell signalling or membrane/protein trafficking functions were also identified, providing putative insights into major secretory pathways in mammary epithelial cells.

SIGNAL TRANSDUCTION PATHWAYS OF MANTLE CELL LYMPHOMA: A PHOSPHOPROTEOME-BASED STUDY

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Protein phosphorylation is a post-translational modification that affects a significant subset of the proteome and plays a key role in signal transduction, regulation of gene expression, and cell cycle control in eukaryotic organisms. Abnormal regulation of protein phosphorylation due to mutation or overexpression of signalling proteins often results in various disease states. The aim of this study was to identify a "core phosphoproteome" that might be cell line-independent and representative of real mantle cell lymphoma (MCL) samples. We analysed the global phosphoproteome profiles of 7 different MCL cell lines (Granta-519, Jeko-1, MAVER-1, NCEB, Rec-1, UPN1, and UPN2). We provide here a list of phosphorylated protein identified by immobilized metal affinity chromatography (IMAC) combined with two-dimensional gel electrophoresis and nano-reverse phase-high performance liquid chromatography (RP-HPLC) coupled with tandem mass spectrometry (MS/MS). We attempted to predict the protein phosphorylation sites and the pathway membership of identified phosphoproteins by using bioinformatics tools (such as NetPhos 2.0, PhosphoBase 6.0, and PANTHER). The phosphoproteins were further classified with respect to their cellular component, biological process, and molecular function using GO annotation. Interestingly, out of the all the identified proteins, 23% were involved in RNA splicing and translation, 18% in cell proliferation, cell cycle and apoptosis, 13% in chromatin and cytoskeleton organization, and 9% in signal trasduction. We believe that the information about proteins that are subject to phosphorylation in mantle cell lymphoma can be extremely useful in the study of their specific roles within certain pathways. Moreover, these findings may provide the basis for more rational, targeted therapy.

NEW APPLICATIONS OF iTRAQ IN PROTEIN CHEMISTRY AND PROTEOMIC

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A number of tagging (or 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. The iTRAQ reagents are specifically reactive towards primary amino -amino groups of lysine)εgroups (namely N-terminal of proteins and peptides and 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. 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 phosphorylation and nitration sites. This method was proved to lead to the simultaneous localisation and quantification of modified sites both in model proteins and in biological systems. This derivatization introduces an iTRAQ moiety that fragments 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 MS2.

THE REDUCTIVE DESULFURIZATION OF MET AND CYS RESIDUES IN BOVINE RNASE A IS ASSOCIATED WITH TRANS LIPIDS FORMATION IN A MIMETIC MODEL OF BIOLOGICAL MEMBRANES

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Damage to bovine pancreatic RNase A, due to the H• atom and/or solvated electron attack at protein sulfur-containing residues, was investigated by Raman spectroscopy and mass spectrometry techniques. To the already known desulfurization process affecting Met residues, novel reactivity was observed involving disulfide moieties, leading to the chemical transformation of Cys into Ala residues. Mapping experiments demonstrated that desulfurization selectively affected Met79, Cys110, Cys58 and Cys72 during first stages of reaction. While this reaction was performed on protein species added to large unilamellar vescicles, desulfurization yielded sulfur radicals able to induce a cis-trans isomerization of lipids at the onset of irradiation. These findings reveal new scenarios on reactions generated by radical stressing conditions, thus suggesting the need for specific assays and for future investigations to detect these modifications in proteins and lipids within challenged cells.

Ferreri, C., Chatgilialoglu, C., Torreggiani, A., Salzano, A.M., Renzone, G. & Scaloni, A. 2008. J. Proteome Res., in press

IMPORTIN BETA PARTNERS IN CELL CYCLE PROGRESSION

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Importin beta, one of the major nuclear transport receptor, is emerging as a multifunctional protein involved in various cellular processes (Harel. & Forbes, 2004). Although the mechanism by which importin beta regulates nuclear protein import in interphase is now well established, the way by which this protein acts as negative regulator in mitotic spindle assembly and centrosome dynamics is still poorly understood. It is known that the importin beta nuclear import machinery is regulated in a cell cycle-dependent manner through the modulation of interaction modes. Furthermore, immunoblotting analyses have shown that importin beta is phosphorylated exclusively in mitosis (Yasuhara et al. 2004), suggesting that phosphorylation at mitotic onset could play a pivotal role in spindle assembly, whereas dephosphorylation at mitotic exit could be important for reformation of the nuclear envelope. To get more insight into the mitotic roles of importin beta in mammalian cells, we have immunoprecipitated endogenous importin beta both from mitotic and interphasic HeLa cells. Pull-down experiments with recombinant versions lacking specific domains (e.g. mutants lacking either the Ran-interacting domain, or the importin alpha-binding domain, or a double deletion mutant) were also performed. Cell cycle phase-specific profiles were determined for both the interaction partners and the post-translational modification pattern of importin beta by a comprehensive approach, encompassing immunoblotting detection, 2DE maps, phosphorylated peptides enrichment.
Results will be mainly discussed in terms of how diverse phosphorylation states of importin beta and of its interactors may modulate cell cycle progression.

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PHOSPHOPEPTIDE ENRICHMENT FROM COMPLEX BIOLOGICAL SAMPLES BY THIN FILM TITANIA-COATED MAGNETIC BEADS (PHOS-TRAP™)

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Reversible phosphorylation of proteins is an important regulatory mechanism that occurs in both prokaryotic and eukaryotic organisms; phosphorylated proteins may play crucial roles in many cellular mechanisms, therefore the characterization of this post translational modification, is of the utmost importance in proteomics investigations. The analysis of the entire complement of phosphorylated proteins in a cell is certainly a feasible option. However this is strictly dependent upon the optimization of enrichment protocols for phosphoproteins and/or phosphopeptides a better fractionation techniques using advanced chromatography and improvement of methods to selectively visualize phosphorylated residues in mass spectrometry analyses. Although the current procedures for phosphoproteomic analysis are satisfactory there is still sample loss and inconsistencies with regards to sample preparation, enrichment and instrumentation. Another limitation is that some relevant proteins will likely be missed since no extraction condition is all encompassing. It is possible that proteins with low stoichiometry of phosphorylation, in very low abundance, or phosphorylated as a target for rapid degradation will be lost. Previous procedures to isolate phosphorylated proteins included radioactive labeling with 32P-labeled ATP followed by SDS polyacrylamide gel electrophoresis or thin layer chromatography. These traditional methods are inefficient because it is impossible to obtain large amounts of proteins required for phosphorylation analysis. Therefore, the current and simplest methods to enrich phosphoproteins are affinity purification using phospho-specific antibodies, immobilized metal affinity chromatography (IMAC), strong cation exchange (SCX) chromatography, or titanium dioxide chromatography (Phos-trap™). However, enrichment and recovery of phosphopeptides using an IMAC system strongly depends on the type of metal ion and column material, and is often hampered by the non-selective enrichment of acidic peptides(1). The Phos-trap™ Phosphopeptide Enrichment Kit is based on robust enrichment of phosphopeptides using titanium dioxide coated magnetic beads. Fully optimized buffers and assay protocols allow efficient fractionation of complex biological samples, such as milk, serum or protein digests containing complex mixtures of peptides, in minutes. The resulting samples are ready to be analyzed by mass spectrometry or other detection tool of choice. The application of this phosphopeptide enrichment will be shown both on a single protein and on a peptide/phosphopeptide mixture from an in situ idrolysis of a 2DE fractionated protein .

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POST TRANSLATIONAL MODIFICATION IN MILK FROM CLONED CATTLE[

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The association between food and health is well established and recent studies have shown that modifiable risk factors seem to be of greater significance for health than previously anticipated. Prevention of disease may in the future be just as important as treatment of diseases. Indeed, many consumers are highly aware of health-properties of food, and the market for healthy food and food with special health benefits is increasing. Cloning could provide animals with a better quality of meat and other products, such as dairy, offering the possibility of creating strains of animals with increased disease resistance or other better quality. The US Food and Drug Administration has just approved the use of meat and milk from cloned cattle, pigs, and goats and from the offspring of clones of any species traditionally used as food.

Aim of this study is to evaluate possible differences between proteomic profiles, in particular post translational modifications (PTM), in milk coming from cloned and non cloned cattle in order to verify safety of this food.

Our results have not been shown critical differences between proteins of milk coming from cloned and not cloned cattle, either expression or in the post translational modifications. Lack of differences between these type of milks, if will be confirmed on other animals, should permit to use 'cloned' milk in human feeding.

MACROSCOPIC POST-TRANSLATIONAL MODIFICATIONS OF THE ARCHITECTURAL TRANSCRIPTION FACTORS HMGA

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Nuclear processes relies on the coordinated action of several factors which modulate chromatin dynamics. Among these factors HMGA (HMGA1a, HMGA1b and HMGA2) proteins constitute a family that has been implicated in development and differentiation and whose altered expression has been involved in different pathologies including neoplastic transformation, obesity and type II diabetes.

The causal involvement of HMGA proteins in cancer development has been well established and linked to their ability of affecting multiple and different proliferative and regulative pathways. In addition, proteomic data evidenced that HMGA are highly connected proteins in the chromatin network and suggested an involvement of these proteins also in RNA processing, DNA repair and chromatin structure dynamics. Their multifunctionality is probably due to their small size (about 100 aa residues), natively disordered status, and modular organization in four domains (3 highly conserved DNA binding domains and an acidic C-terminal tail) that confer them the ability to interact with several different DNA sequences and protein partners. HMGA proteins are heavily post-translational modified and these PTMs affect both DNA and protein binding thus modulating their functions. We systematically screened by LC/MS the post-translational state of HMGA proteins in several cell lines of different origin and phenotype. The comparison of high quality mass spectra of the three entire HMGA proteins allow to evidence both intra-family and inter-sample differences regarding both the levels and type of HMGA post-translational modifications. Since no data are currently available about HMGA2 post-translational modifications we further characterized the PTMs of this protein demonstrating that CK2 can be responsible for the phosphorylation at the C-terminal tail.

MICROBIAL PROTEOMICS

IDENTIFICATION OF MEMBRANE COMPLEXES OF THE PHOTOTROPHIC EUBACTERIUM *RHODOBACTER SPHAEROIDES* STRAIN R26.1 BY BLUE NATIVE ELECTROPHORESIS AND MASS-SPECTROMETRY. ITS APPLICATION FOR STUDYING ADAPTATION TO HIGH CONCENTRATIONS OF HEAVY METALS

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The phototrophic bacterium *Rhodobacter sphaeroides R26.1* is known for its capacity to survive to high concentrations of heavy metal ions [1, 2]. Indeed, the bacterium population size at stationary phase is halved at concentration (EC50) ranging from 0.02 mM to 39 Mm (depending on the metal), that is about one order of magnitude higher than for *Escherichia coli* [1]. In particular Co2+ is among the most highly tolerated ions (EC50 = 12 mM), which was also found to negatively influence the synthesis of light harvesting complexes [3]. *R. sphaeroides R26.1* response to high Co2+ concentration was investigated by analyzing its soluble proteome. Among the observed alterations of protein profiles, the down regulation of porphobilinogen deaminase, a key enzyme in the porphyrin and bacteriochlorophylls biosynthetic pathway, is of particular interest allowing to justify the light harvesting complex diminished synthesis. Noteworthy the down-expression of periplasmic proteins MdoG (putative transmembrane protein) and ModA (ABC molybdate tranporter, periplasmic binding protein) might indicate a possible active resistance mechanism [4]. To gain more insight into the response of the bacterium membrane to high Co2+ concentrations, the characterization of its proteome was started by establishing a reliable extraction protocol and by seeking the proper conditions for the Blue-Native electrophoresis (BN-PAGE). Preliminary results obtained by mass-spectrometry analysis of membrane complexes resolved by BN-PAGE will be presented for cultures obtained with either control and Co2+-enriched media.

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ANTIBIOTIC RESISTANCE AND PATOGENICITY IN ESCHERICHIA COLI

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Escherichia Coli is a Gram negative bacterium, widely studied because it represents an integrating part of the human enteric flora, even if various strains are pathogen. Moreover such strains are zoonotic agents and they can be isolate also in ruminants in which cause diarrhoea and edema. Human infection occurs via fecal-oral pathway and animals are reservoirs for this human pathogen. Lesions are characterized by intimate bacterial attachment to the host cell membrane and the destruction of microvilli at the site of bacterial adherence, caused by the accumulation of signal proteins leading to the rearrangement of cytoskeletal proteins, in particular, filamentous actin, resulting in pedestal formation at the apical cell membrane.

In recent data, the evaluation of membrane proteins, phosphoproteome and the study of oxidative stress, can contribute to understanding the phenomenon of antibiotic resistance to molecular level and to define new strategies for the design of highly selective therapeutic agents.

Evaluation of protein profiles respect to various mechanisms of stress, i.e. the resistance to antibiotics or the modification related to the antibiotic resistance, represents a valid and integrating approach for the study of new therapeutic strategies.

In the current study, comparative proteomics was applied to identify changes in proteins responsible for antibiotic resistance in different in vivo isolates Escherichia coli. In particular it has been studied strains with same virulence factors, but an antibiotic profile completely different, isolates from different organs of the same animal.

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DE NOVO SEQUENCE ANALYSIS AND INTACT MASS MEASUREMENTS FOR CHARACTERIZATION OF PHYCOCYANIN PRIMARY STRUCTURE FROM APHANIZOMENON FLOS-AQUAE, A BLUE-GREEN ALGA USED AS A NUTRIENT SUPPLEMENT.

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Aphanizomenon flos-aquae (AFA) is a fresh water unicellular blue-green alga that spontaneously grows in copious amount in Upper Klamath Lake (Oregon, USA). It is a nutrient-dense food source and is consumed for its health-enhancing properties. This microalga contains large amounts of a blue protein called phycocyanin (PC) belonging to the photosynthetic apparatus and with demonstrated antioxidant and radical scavenging properties. An efficient method for its separation has been set up: PC can be purified by a simple single step chromatographic run using a hydroxyapatite column (ratio A620/A280 of 4.78), allowing its usage for health-enhancing properties while eliminating other aspecific algal components. Proteomic investigation and HPLC analysis of purified AFA phycobilisomes revealed that, contrary to the well-characterized Synechocystis and Spirulina spp., only one type of biliprotein is present in phycobilisomes: phycocyanins with no allo-phycocyanins. Two subunit polypeptides of PC were also separated: the β-subunit containing two bilins as chromophore and the α-subunit containing only one. Moreover, the characterization of phycocyanin primary structure from Aphanizomenon flos-aquae was determined by mass spectrometry de novo sequencing with the aid of chemical derivatization. The SPITC-derivatized peptides underwent facile fragmentation, predominantly resulting in y-series ions in the MS/MS spectra. Combining N-terminal sulfonation by SPITC and MALDI TOF/TOF analyses facilitated the acquisition of sequence information for both lysine and arginine-terminated tryptic peptides from AFA phycocyanin without the need for additional pretreatment, such as guanidination of lysine amino group. This strategy allowed us a peptide fragment fingerprinting and de novo sequencing of several peptides belonging to both α- and β-phycocyanin subunits, obtaining a sequence coverage of 67 % and 75 %, respectively. The presence of different protein isoforms was also revealed and confirmed by Intact Mass Measurements (IMMs).

PROTEOMIC CHARACTERISATION OF BIOTOXINS PRODUCED BY CYANOBACTERIA BLOOM IN ITALIAN LAKES

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Cyanobacteria, known also as blue-green algae (BGA), are prokaryotic organisms widely distributed in surface waters, producing blooms and scums. These phenomena in lakes and freshwaters are seasonal, caused by several species belonging to the genera Microcystis, Planktotrix, Anabaena, Nostoc, Oscillatoria, the most frequently observed. Some BGA genera produce peptide biotoxins, that can be toxic to wildlife, domestic livestock and even to humans; not all cyanobacteria strains are toxic within one species. Microcystins (MCs), the most abundant and dangerous toxins produced by cyanobacteria, are cyclic heptapeptides of about 1000 g mol-1. A wide variety of other peptide biotoxins produced in freshwaters are known, such as cylindrospermopsin, anatoxin-a and analogues, anabaenopeptins, nodularins, cyanopeptolins, microviridins, oscillapeptins, saxitoxin-analogues. As the presence of cyanobacteria in Italian freshwaters is common in the last years, it is urgent to monitor the risks for consumer's health deriving from possible intake of biotoxins from contaminated foods. Herein, we present a peptidomic method for profiling peptide biotoxins based on the combined use of MALDI-TOF-MS for rapid peptide detection and nanoLC/ESI-MS/MS for structural characterization and quantitative analysis. The different analytical systems were compared to evaluate their performances and reliability in monitoring environmental outbreaks. The method was applied to the analysis of water and algae samples from Averno Lake, near Naples and from other freshwater sources, as a consequence of a cyanobacteria blooms, as well as to fish and livestock tissues from the same areas. In this way, we characterised the complete peptide pattern of biotoxins produced, which allowed an evaluation of the possible health risk related to their presence in the lake, and to plan strategies of monitoring and intervention.

COUPLING OF NATIVE LIQUID PHASE ISOELECTROFOCUSING AND BLUE NATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS: A POTENT TOOL FOR NATIVE MEMBRANE MULTIPROTEIN COMPLEX SEPARATION.

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In this study, a new 3D native electrophoretic protocol is proposed for an exhaustive separation and identification of membrane protein. It is based on native liquid phase isoelectrofocusing (N-LP-IEF) of multiprotein complexes in the first dimension, followed by blue native polyacrylamide gel electrophoresis (BN-PAGE) in the second dimension, where both the pI and the molecular masses of multiprotein complexes (2D N-LP-IEF-BN) were used to separate them in their native form. Finally, each single component can be resolved using denaturing electrophoresis (3D N-LP-IEF-BN-SDS-PAGE). The pI-based MicroRotofor has a number of advantages over BN-PAGE; it does not require the addition of any chemicals and separation of complexes is based on the protein's real physico-chemical properties which inevitably change when dye is added. Results were more easily reproduced than with BN and the pI of each native complex was also determined. Although some fractions still contained comigrating complexes after MicroRotofor, these were subsequently separated by BN for further analysis. The photosynthetic membranes of Spinacia oleracea and Rhodobacter sphaeroides were chosen as models for setting up analytical methods suitable for any membrane proteins. Thus, highly hydrophobic complexes, such as ATP-synthetas and Cyt b6/f, were separated in native form. SDS-PAGE revealed almost all the subunits from the multiprotein complexes, indicating that by using 3D N-LP-IEF-BN-SDS-PAGE it is possible to achieve a greater degree of component identification than with 2D BN-SDS-PAGE.

Poster

PLANT PROTEOMICS

PROTEOMIC, PIGMENT COMPOSITION, AND ORGANIZATION OF THYLAKOID MEMBRANES IN CADMIUM TREATED SPINACH LEAVES.

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The changes induced in the photosynthetic apparatus of basal and apical spinach (Spinacia oleracea L.) leaves upon addition of cadmium to hydroponics solution were characterized separately. Two proteomic approaches coupled with chlorophyll, xanthophylls and phytocheletins analysis and in vivo measurements of photosynthesis were used. In the basal leaves, during the first ten days the concentration of chl a were greatly reduced more than chl b. Lutein, neoxanthin and violaxanthin slightly increased and no zeoxanthin and anteroxantin were produced, indicating that the violaxanthin-zeaxanthin de-epoxidation cycle was not involved. Cadmium reduced significantly the amount of antenna proteins of PSI, while PSII antennae were effected in minor extend, with exception of isomeric Lhcb1.1 which decreased significantly just at onset of treatment. Cytochrome b6/f and the ATP-synthase complex remained unchanged. However, no new protein was formed and no specific protein disappeared in the photosynthetic apparatus, while an increasing amount of phytochelatins was recorded over time. Fluorescence analysis revealed that damage to PSI only influences photosynthesis when light intensity drives high rates of electron transport, which in turn require large rates of RuBP regeneration.

In the apical leaves the total chloroplasts remained unchanged, a slightly increase of chlorophyll is recorded over time and no phytochelatins were observed.

Upon removal of cadmium a rapid re-synthesis of both chlorophylls was detected, lutein decrease again and a significant re-synthesis of Lhcb1.1 mRNA antenna was observed, especially in the presence of zinc.

All data supported the hypothesis that cadmium affects aspecifically the photosynthetic apparatus by its replacement to other metal ions inside proteins. Plants do not react by specific mechanisms but localize it into the basal leaves by an over over-production of phytochelatins which avoid its diffusion to the new leaves.

THE EXPRESSION OF TOMATO PROSYSTEMIN GENE IN TOBACCO PLANTS HIGHLY AFFECTS HOST PROTEOMIC REPERTOIRE

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Systemin, an octadecapeptide isolated from tomato, is the primary signal molecule involved in the local and systemic responses to pest attack, elicited by activation of a set of defence genes. It derives from processing of prosystemin, a prohormone of almost 200 amino acids. Prosystemin orthologues have been found in other Solanaceae species but not in tobacco, where are present hydroxyproline-rich peptides functionally but not structurally related to tomato systemin. Molecular events leading to the release of signalling peptides from protein precursors are unknown in plants; the occurrence of a family of signal molecules suggests that initiation of wound response may involve different processing mechanisms. It has been previously shown that the protein product from an engineered tomato prosystemin gene is processed in tobacco, thus suggesting that the components responsible for its post-translational modifications are present in this species. By analyzing the proteome repertoire of transformed tobacco plant leaves with 2-DE, here we demonstrate that the constitutive expression of the tomato prosystemin gene highly affected host protein synthesis. In particular, engineered plants showed a number of differentially synthesized proteins that were identified by PMF MALDI-TOF and microLC-ESI-IT-MS/MS experiments as polypeptide species involved in protection from pathogens and oxidative stress, or in carbon/energy metabolism. Significant differences in over-produced proteins were observed with respect to previous data reported on systemin-engineered tomato plants. Our results strongly support the need of using proteomic approaches during systematic analysis of plant tissues to investigate the principle of substantial equivalence in transgenic plants expressing a transgene for a signalling molecule.

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED PROTEINS IN THE FLESH OF BLOOD AND COMMON ORANGES

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In Italy the sweet orange production is characterized by red pigmentation, due to the anthocyanin content. The anthocyanins are natural red, purple and blue pigments, mostly present in plant epidermal cells, into the vacuoles, in which give colour to different tissues. They can act as antioxidants, phytoalexins or as antibacterial agents. The antioxidant activity of anthocyanins gives cause for a variety of medicinal usage: prevention of cancer, anti-inflammatory activity and anti-arteriosclerosis activity. On citrus mature fruits anthocyanins are exclusively expressed in blood oranges and its hybrids. Anthocyanin greatly varying content is strictly related to genotype and environmental conditions, moreover expression levels vary considerably among flesh and rind, these facts causing trouble to marketing of the product. In the present work we analyzed differentially the flesh proteome of Moro nucellare 58-8D-I, one of the cultivar with the highest anthocyanins content, and of Cadenera (a common orange) using 2D-gel electrophoresis separation. The tryptic digest of each spot was characterized by LC-MSMS and the proteins were identified by searching both protein and EST databases.

The results of the proteome analysis were compared with the differentially expressed genes previously identified in blood and blond oranges.

Licciardello, C., Russo M. P., Vale' G. & Recupero R. G. 2008. Tree Genetics & Genomes, 4: 315-331

PEPTIDE FINGERPRINT OF HIGH QUALITY *Campania* WHITE WINES BY MALDI-TOF MASS SPECTROMETRY

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Food traceability is strictly connected with "food safety" and "food quality", as it can help to preserve the identity of unique quality traits, against frauds or commercial disputes. The European regulation 178/2002, from January 1st 2005, imposes rigorous requirements for food traceability. In this framework, there is a growing demand of new traceability systems for the collection and authentication of any information of units/batches of food ingredients and products.

In the past years, the development of biological identification technologies *(i.e.* DNA fingerprint, chemical and biochemical characterization, etc.) has greatly contributed to support and check traceability systems. In parallel, computer technology provided many new and innovative tools for products tracing. Simple hand-written or printed labels are being rapidly replaced or supplemented by computer-readable identification codes *(e.g.* bar codes, radio frequency tags). These electronic identifiers allow the simple and fast tracking of items by automatically gathering and storing information in computer databases.

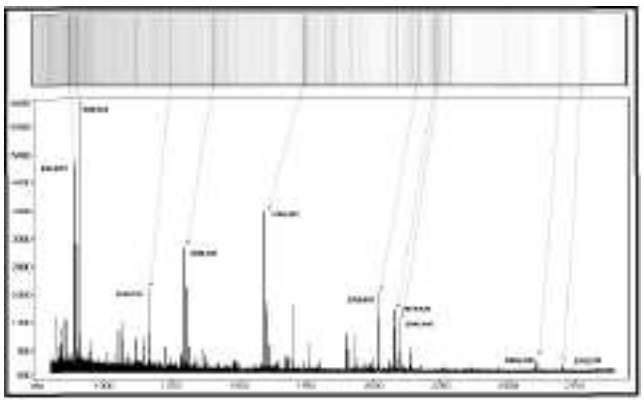

Figure 1. Example of the graphical view of a typical "mass code"

In the present work we propose the application of peptide profiles derived from whole wine protein tryptic digests to obtain a signature of high quality *Campania* white wines by MALDI-TOF mass spectrometry. To this purpose, the MALDI-TOF spectral traces have been converted into simulated images to obtain a graphical representation of the peak lists. The obtained "mass codes" constitute a simple but effective tool to display differences between samples, suggesting their potential use as "biological bar codes" for food authenticity and traceability.

PEG FRACTIONATION IMPROVED DETECTION OF LOW ABUNDANT PROTEINS BY 2D ELECTROPHORESIS ANALYSIS IN GLOBE ARTICHOKE

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Globe Artichoke, Proteomics, Low-abundant proteins, RuBisCO, Polyethylene glicol (PEG)

The globe artichoke (Cynara cardunculus var. scolymus L., Compositeae, 2n = 2x = 34) has long been used for culinary purposes, but it also has a number of known therapeutic effects, mainly due to the abundance of phenylpropanoids (PPs) and sesquiterpene lactones (SLs). Despite its economical, pharmacological and nutritional importance, little research efforts have been addressed to study both its genome and functional genome. For this reason, we have established an efficient proteomics method for detecting globe artichoke proteins and evaluating their variation under stress conditions.

The first leaf proteome map of globe artichoke has been obtained using a polyethylene glycol (PEG) fractionation approach (modified from Kim et al., 2001) for overcoming the presence of the highly abundant ribulose-1,5-biphosphate carboxylase-oxygenase complex (RuBisCO), which can be tought of as the plant analogue of albumin in mammalian serum and plasma preparations. RuBisCO overwhelms many low-abundant proteins and affects gel resolution in vegetable samples. To validate our approach, 2-DE gels obtained from total extract and PEG fractions were compared with Image Master 2D Platinum software (GE Healthcare).

The resulting 2-DE maps fractions showed: i) more clear patterns, ii) a diminished representation of RuBisCO and iii) new protein spots, otherwise undetectable using the total extract. Following the PEG fractioning, 73 additional proteins were observed in the same area of RuBisCO which were analysed with a nano LC-MS/MS mass spectrometry and identified by sequence homology through Mascot MS/MS Ion Search.

Future studies will be addressed to study the variation of the globe artichoke proteome under stress conditions (biotic and abiotic), for identifying key enzymes/regulatory factors differentially expressed in the phenylpropanoids and sesquiterpene lactones metabolisms.

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PRELIMINARY APPROACHES TO THE DEVELOPMENT OF A "LABEL-FREE" ABSOLUTE QUANTIFICATION OF GAMMA CONGLUTIN IN COMPLEX PROTEIN MIXTURES

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Quantitative proteomics based on mass spectrometry has been used in food quality control for detecting and quantifying proteins in tiny amounts which could have either negative or beneficial effects on human health: allergens and bioactive proteins respectively.

In literature two main approaches are reported in order to profile the relative abundance of target proteins in different samples: stable isotope labeling (SIL) techniques and stable isotope label-free (SIF) techniques. SIL tecniques, in spite of their potency, have some limitations such as the use of expensive labeled reagents; therefore they are certainly not applicable in the area of food analysis.

The actual increasing interest for lupin proteins is based both on their nutritional and technological characteristics, that permit to use them as ingredients in the formulation of a large range of different food products, and on their potential nutraceutical properties (Arnoldi et al., 2007). In particular gamma-conglutin, a mature seed storage protein composed by a heavy and a light chain linked by disulfide bonds, seems to be the hypoglycemic component and recent studies have demonstrated that it may be the major allergen in lupin seed (Magni et al., 2005).

The aim of this work was the development of a HPLC-Chip mass spectrometric Multiple Reaction Monitoring (MRM) assay for the quantification of Lupinus albus gamma-conglutin in complex protein mixtures. Specific tryptic peptides are selected as stoichiometric representative of the target protein and quantified against a spiked internal standard (bovine serum albumin, BSA) to provide absolute quantitation of protein concentration.

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TOWARD THE FUNCTIONAL CHARACTERIZATION OF THE SUMO PATHWAY DURING THE PLANT RESPONSE TO ABIOTIC STRESSES

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The transient conjugation of the Small Ubiquitin-like MOdifier (SUMO) protein to target proteins is a post-translational modification playing an influential role in a wide variety of cellular processes by regulating protein-protein interactions and subcellular location or by antagonizing ubiquitination. In yeast and human, SUMOylation target proteins are factors involved in replication, transcription and translation, RNA-binding proteins, cytoskeleton components and transport factors as well as metabolic enzymes. SUMOylation is conserved in plants, but a few number of plant SUMO-conjugates have been isolated so far. Western analyses revealed accumulation of SUMO targets in wheat seedlings as an early and transient response to different kind of abiotic stresses, like drought, heat and cold. These targets as well as their modification are therefore potential regulators of the plant molecular response to abiotic stresses and their identification could integrate the current knowledge on plant stress tolerance. To functionally characterize the SUMOylation pathway in plants and to identify its protein targets, three proteomic approaches have been initiated in Arabidopsis and Triticum durum. An immunoaffinity chromatography with the anti-SUMO1 immunoglobulin followed by LC-MS/MS analysis have been used to identify wheat proteins conjugated to SUMO after heat stress. Transgenic Arabidopsis plants overexpressing the SUMO conjugating enzyme AtSCE1a fused to the TAP tag have been produced to isolate protein complexes of the SUMOylation pathway. A wheat cDNA library from cold treated leaves has been screened by yeast two-hybrid analysis to isolate potential E2 interactors (SUMO-conjugates and E3 enzymes). The preliminary results of these strategies are shown.

SON OF PROTEIN PILOT AND MASCOT SOFTWARES FOR THE ANALYSIS OF MS/MS DATA

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Phytoremediation studies very often deal with unsequenced or poorly characterized plant genomes, thus protein identification from 2D map spots is performed using cross-species identification tools. Spot digestion is followed by LC/MS/MS analysis and peptide fragmentation data are evaluated by different algorithms.

We have employed leaf protein extracts from Pteris vittata, a fern that hyperaccumulates arsenic in its fronds in concentrations higher than 1500 ppm (Ma et al 2001). Up to now the NCBI database contains only 23 protein sequences of the P. vittata specie and 51 protein sequences of the Pteris genus.

Spots of interest were trypsin digested, the peptide mixtures were separated and analysed on nanoHPLC ESI-Q-TOF mass spectrometer (QSTAR- Applied BioSystem). Cross-species identification was performed using two different MS/MS data algorithms: the Mascot MS/MS Ion search and the Paragon algorithm of Protein Pilot software.

By using Mascot scoring algorithm (Perkins et al., 1999), in the case of unsequenced protein the aim is to pull out those entries which exhibit the closest homology, often equivalent proteins from related species with the same peptides. In Protein Pilot, the algorithm (Shilov et al. 2007) makes possible to include a larger set of aminoacid modifications and substitutions using the moderate PC computing resource. We have chosen to analyse with the two different softwares a set of proteins, including some of them identified with only one peptide and some others with a large coverage.

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Shilov et al. 2007. Mol Cell Proteomics, 6, 1638.

PROTEOMIC APPROACH TO STUDY THE CROSSROAD BETWEEN OXIDATIVE STRESS AND PCD IN PLANT CELLS

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To date, there have been few reports of proteome analysis that focused on the PCD signalling pathway in plants. The aim of this work was to investigate the events involved in the early phase of programmed cell death (PCD) and the crossroad between oxidative stress and PCD in plant cells.

We used as plant model system the tobacco TBY-2 cells: it is known that they normally grow at 27°C and, when heat shocked for 10 minutes at 35°C and 55°C, undergo respectively oxidative stress and PCD in absence of added PCD inducers.

TBY-2 samples treated for 10 minutes at 35°C and 55°C and then recovered at 27°C for 3 or 6 hours have been analyzed by 2D electrophoresis. The protein patterns have been acquired and the electrophoretic images compared by using Image Master 2D Platinum by superimposing of pictures. Spots having interesting expression behaviour have been excised by gel, subjected to enzymatic digestion "in situ" and analyzed by LC-ESI-MS/MS.

The identified proteins were categorized in different classes. As expected, the global protein-profiling revealed significant differences in many enzymes involved in oxidative stress response, as a function of time and severity of heat stress: their putative role in PCD is discussed. Many metabolic enzymes (such as pyruvate decarboxylase, pyruvate kinase) were affected by heat stress treatment, indicating that extensive metabolic changes are associated with programmed cell death. Moreover, we identified several molecular chaperone proteins that were differentially-regulated by both heat treatments. These proteins play a role in the stabilization and facilitation of refolding of proteins that have been denatured during exposure to various stresses. Strikingly, we found the down-regulation of the Heat shock protein Hsp70 and Hsp90 whose elevated expression in human promotes cancer by inhibiting PCD. Several lines of evidence argue that plant PCD shares some characteristic features with animal PCD. However, the molecular components of the plant PCD machinery remain obscure and comparing the functions of key components involved in this process between kingdoms may serve fruitful for understanding the regulation of PCD in both.

OTHERS

PROTEOMICS IN CULTURAL HERITAGE.

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Science can interact with art in many ways. One way, and possibly the most important one nowadays is providing tools for safeguarding our cultural heritage to ensure that future generations have the opportunity to appreciate artistic masterpieces. Determination of chemical composition of colour layers is an essential step required by restorers to choose a proper restoration technique as well as for dating of art works. The characterization of the colour layer composition includes determination of its inorganic pigments, organic pigments (e.g., madder lake) and binding media (oils, resins, wax or proteinaceous material). Over the centuries, different organic binders have been used by artists, ranging form eggs, milk or glue, used alone or mixed together or with oils and other organic materials. Different methods have been proposed in the literature to characterize the proteinaceous binders, and, so far, the identification has been prevalently performed using high-performance liquid chromatography, gas chromatography (GC)-mass spectrometry (MS), pyrolysis-GC-MS and Fourier transform IR spectroscopy. More recently, mass spectrometry was successfully used for protein painting analysis, generating promising results. As in a classical proteomic procedure, the proteinaceous binders are digested with specific proteases. A fingerprint mixture of peptides is thus generated from a protein that is much more individual than amino acid ratios. This resulting mixture can then be analysed by mass spectrometry. In this work our attention was focused on binders, and in particular we have optimized procedures for suppressing the interference arising from protein contaminations and for the reliable and efficient identification of proteinaceous material in painting models by nanoLC/nano ESI-MSMS, allowing the identification of the proteins in artworks without ambiguity. The method tested on model samples was then successfully applied to real samples, coming from masterpieces of the vaults of the Assisi Basilica.

INVESTIGATION OF THE αS1-CASEIN SEQUENCE FROM DONKEY'S MILK BY MASS SPECTROMETRY

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The wide use of cow's milk in infant diet, as substitution of breast feeding, has shown that a considerable percentage of newborns are allergic to cow's milk proteins. When mother's milk is not available or may not be advisable, alternative HA formulas and soy bean-derived formulas have been used. They provide adequate nutrition of infants, even if it should be considered that their allergenicity is reduced, but never completely suppressed (Walker-Smith J., 2003; Klemola et al., 2002). Recent clinical studies seem to suggest that donkey's milk could represent an alternative feeding to cow's milk for infants affected by cow's milk proteins allergy (CMPA) (Businco et al., 2000). However, these studies are not supported by adequate investigations at molecular level and the molecular basis of the presumed hypoallergenicity of donkey's milk with respect to cow's one is still unknown. It is reasonable to hypothesize such hypoallergenic properties of donkey's milk can be due to structural difference of the various protein components of the two types of milk considered, but we cannot to exclude the possibility that a genetic polymorphism of Equidae milk can play an important role. On this respect, limited data are available for the genetic polymorphism of donkey's milk and the knowledge of its protein profile has not been fully investigated. Major information are available for whey proteins (Cunsolo et al., 2007), whereas data for casein fraction are still totally absent. We report here the first results aimed to the sequence determination of the αs1-casein from donkey's milk sample by coupling direct RP-HPLC/ESI-MS, enzymatic digestion, MALDI-TOF MS, RP-HPLC/nESI-MS/MS and bioinformatic tools. Direct RP-HPLC/nESI-MS allowed the detection of two donkey's αs1-casein forms coeluting in the same chromatographic fraction, which show a molecular weight of 23798 Da and 24416 Da, respectively. Preliminary characterization of these components, carried out taking as reference the two horse's αs1-caseins sequence, allowed the coverage of about 75% of the sequences.

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LC-MS METHOD FOR THE QUANTIFICATION OF WHEY PROTEIN TRACES IN MIXED-FRUIT JUICES

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Whey proteins due to their wide ranging nutritional, biological and functional properties are often utilized by the food industry in the preparation of foods. They are valuable ingredients of protein fortified beverages such as fruit juices and sport drinks. Despite their high nutritional and functional properties, whey proteins, are also recognized as powerful allergens. Therefore, the widespread use of milk and whey proteins by the food industry poses a serious threat to the health of milk allergic consumers.

Within the European Union, Directive 2003/89/EC states that the 12 most common food allergens, including milk, have to be declared on the label when they have been intentionally introduced in a foodstuff. Therefore, the availability of accurate and sensitive detection methods for food allergens is crucial for the food industry to ensure a correct labelling of their products in order to protect allergic consumers. A method using solid-phase-extraction and liquid chromatography coupled to mass spectrometry was developed in order to detect traces of the three allergenic whey proteins lactalbumin, lactoglobulin A and B in mixed fruit juices. Different sample pre-treatments were compared and the best recoveries were obtained with a method employing a solid-phase extraction cartridge. Recoveries ranging from 68% to 79% were achieved and the limit of detection was set at 1 ug/ml. Both full scan mode and multiple ion monitoring acquisition modes were investigated and compared. The method was utilized to analyse 15 mixed-fruit juices collected from the market and was found to be capable of positively identifying all three milk proteins. The developed method enables the unambiguous determination of allergenic whey proteins in mixed-fruit juices and can assist in the protection of milk allergic individuals.

AUTOMATED 2D LC-MS USING A SPLIT-FREE, NANOSCALE LC SYSTEM

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In proteomic studies, two dimensional liquid chromatography (2D-LC), using a strong cation exchange (SCX) separation followed by gradient elution on a reverse phase (RP) medium, is frequently applied prior to MS analysis to facilitate analysis of complex samples. Most LC systems require sophisticated ternary or quaternary gradient generation set ups to be able to deliver the salt elution steps from the ion exchanger onto the RP column and the accurate gradient elution to elute the peptides into the MS. Here we describe a simple, automated SCX/ RP 2D separation strategy achieved using a split-free, nanoscale LC system already optimized for use within an LC-MS set up.

To evaluate system performance we analyzed the proteome of mouse placental cells. The cytosolic fraction was subjected to cysteine reduction and alkylation with iodoacetamide followed by trypsin digestion. The lysate was purified and analyzed by 1D and 2D methods on a split-free, nanoscale LC system (EASY n-LC, Proxeon, Odense, Denmark) coupled to LTQ-Orbitrap (Thermo-Fisher, Bremen, Germany). The 2D separation was performed using a 10 step salt elution varying from 0.05 to 0.5 M ammonium acetate.

The auto-sampler component of the EASY-nLC and standard injection programs were used to deliver the salt elution steps, thereby transforming a 1D LC system into a 2D system. The viability of this method was demonstrated by comparing LC-MS data obtained following 1D and 2D separations. The 1D analysis yielded about 600 protein identifications whereas 2D analysis identified approximately 2000 proteins. These results clearly demonstrate the significant improvement which can be achieved by slight modifications of the 1D nanoscale LC system. Far more information was obtained with only minimal modification of the LC component in the LC-MS analysis.

HOST-PARASITOID INTERACTION PROBED BY A FUNCTIONAL PROTEOMICS APPROACH

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Host-parasitoid interaction provides the opportunity to isolate genes and molecules with potential insecticidal activity.

During ovideposition, parasitic wasps (parasitoids) inject factors that inhibit the host immune system in order to create a suitable environment for their progeny. Such factors include venom and ovarian secretions, sometimes containing viruses of the Polydnaviridae family. These viruses are associated with ichneumonid and braconid wasps so that they are classified as Ichnovirus and Bracovirus, respectively. They express proteins acting as regulation factors involved in the host immune response suppression. Gene expression takes place without viral replication and induce numerous endocrine alterations, which interfere with the normal development and metabolism of the larvae.

In Cortesia congregata (CcBV) and Toxoneuron nigriceps (TnBV) genomes, a number of IkB-like genes are present. These proteins consist mostly of ankyrin repeats, display significant sequence similarity with proteins of the NF-kB pathway, but lack regulatory domains.

Three and six open reading frames have been found by genome sequencing in TnBV and CcBV, respectively, and have been denoted TnBVank1-3 and CcBVank1-6.

In this work TnBVank1 gene has been transfected in Drosophila melanogaster S2 cells, together with V5 peptide sequence as a tag. In order to identify the network of proteins involved in ANK1 mediated immune response to viral infection, immunoprecipitation experiments have been performed, using agarose beads coated with anti-V5 antibody. As a control, protein extracts have been preincubated with agarose beads coated with an aspecific antibody.

Immunoprecipitated complexes were fractionated by SDS-PAGE and protein bands were excised and in situ hydrolysed. Peptides mixtures were analysed by LC-MS/MS and proteins identified by bioinformatic softwares.

UV-LASER INDUCED PROTEIN CROSSLINKING AS A NOVEL TOOL FOR STUDYING PROTEIN-PROTEIN INTERACTIONS.

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The detection and analysis of protein-protein interactions is one of the central tasks of proteomics in the postgenomic era. Most biological processes are controlled by dynamic molecular network of enormous complexity rather than by individual proteins.

The comprehensive analysis of any interactome will require the continued development of the technologies used to examine it. Therefore, novel strategies and methodologies that could help in depicting the complex traffic map of the rapidly changing sets of protein interactions are of extraordinary importance for understanding cellular processes.

Chemical crosslinking combined with mass spectrometric analysis has become a viable approach for studying protein-protein interactions and protein complexes at low-resolution. It is, in principle, a formidable tool to freeze transient or labile protein interactions. Moreover, in addition to identifying which proteins interact within a complex, crosslinking approaches can also pinpoint where proteins interact. However, the use of crosslinking to study multi-protein complexes or to search for binding partners has been limited by the relative slow and often inefficient chemistry employed by most classical crosslinkers. UV-laser crosslinking will offer an alternative, potentially powerful tool to investigate such protein-protein interactions. It could obviate many of the problems associated with standard chemical crosslinking reagents, and put crosslinking in a proteome-wide position for the characterizations of protein-protein interactions, especially transient interactions, because the number of photons required for covalent complex formation can be delivered very rapidly, and the high energy of the pulses should result in efficient crosslinking.

The purpose of this study was to investigate the feasibility of exploiting UV-laser induced crosslinking as a tool for the analysis of protein-protein interactions. The experiments presented in this study explored UV-induced photochemical crosslinking yield as a function of dosage and intensity for a model oligomeric protein samples. Moreover, in the current study, we combined crosslinking and high resolution mass spectrometry to experimentally identify the actual crosslinks introduced in the molecule and to gain some insights in the molecular mechanism of the reaction.

INNATE IMMUNITY PROBED BY LIPOPOLYSACCHARIDE ARRAYS AND PROTEOMICS

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Lipopolysaccharides (LPSs) are ubiquitous and vital components of the cell surface of Gram-negative bacteria. They are amphiphilic macromolecules composed of a hydrophilic hetero-polysaccharide (comprising the core oligosaccharide and O-specific polysaccharide) covalently linked to a lipophilic moiety termed lipid A, which anchors these macromolecules to the outer membrane. In animal and plant cells innate immune defenses toward microrganisms are triggered by the perception of pathogen associated molecular patterns (PAMPs). These are conserved and generally indispensable microbial structures such as LPSs that are fundamental in the Gram negative immunity recognition.

Aim of this project is the development of lipopolysaccharide microarrays methodology for the delucidation of mechanisms involved in the LPSs-mediated molecular recognition and anti-infection responses in vegetal and animal organisms. The investigation will be pursued through the identification of the protein partners specifically interacting with these effectors by functional proteomics strategies. The complex protein/lipopolysaccharide tagged with biotin will be isolated on streptavidin column and the proteins will be fractionated by gel electrophoresis and identified by mass spectrometry. The experiments will be carried out in duplicate, using cellular extracts treated and not treated with lipopolysaccharides. Lipopolysaccharide intended to be used as baits will be purified from the authentic vegetal and animal pathogen bacteria involved in the infection of the cell that yield the protein extract.

INTERDISCIPLINARY STUDY FOR THE EVALUATION OF BIOCHEMICAL ALTERATIONS ON MUSSEL MYTILUS GALLOPROVINCIALIS EXPOSED TO A TRIBUTYLTIN-POLLUTED AREA

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An interdisciplinary approach was employed to monitor the concentration and the effects of butyltin compounds in mussels (Mytilus galloprovincialis). Tissues from animals exposed to a marine area (Vado Ligure harbour) with a high concentration of tributyltin (TBT) were analysed and compared with control samples. TBT concentrations were measured by gas chromatography-mass spectrometry and the protein pattern in gill tissues was studied by proteomic analysis. Several proteomic signatures associated with contaminant exposure were observed; spots that were significantly increased in all contaminated samples were identified by mass spectrometry as fragments of beta-tubulin. The degradation of beta-tubulin was then confirmed by western blot analysis with specific anti-beta-tubulin antibody. The effects observed on mussel gills after exposure in the TBT-polluted area are discussed.

EXPLORING THE CHICKEN EGG WHITE PROTEOME WITH COMBINATORIAL PEPTIDE LIGAND LIBRARIES

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The use of two types of peptide ligand libraries (PLL), containing hexapeptides terminating either with a primary amine or modified with a terminal carboxyl group, allowed discovering and identifying a large number of previously unreported egg white proteins. Whereas the most comprehensive list up to date (Mann, Proteomics, 2007, 7, 3558-3568) tabulated 78 unique gene products, our findings have almost doubled that value to 148 unique protein species. From the initial non-treated egg it was possible to find 41 protein species; the difference (107 proteins) was generated thanks to the use of PLLs from which a similar number of species (112 and 109, respectively) was evidenced. Of those, 35 proteins were the specific catch of the amino-terminus PLL, while 33 were uniquely captured by the carboxy-terminus PLL. While a number of these low-abundance proteins might have a biological role in maintaining the integrity of the egg white and protecting the yolk, others might be derived from decaying epithelial cells lining the oviduct and/or represent remnants of products from the magnum and eggshell membrane components secreted by the isthmus, which might ultimately be incorporated, even if in trace amounts, into the egg white. The list of egg white components here reported is by far the most comprehensive at present and could serve as a starting point for isolation and functional characterization of proteins possibly having novel pharmaceutical and biomedical applications.

PROTEOMIC CHARACTERIZATION AND CYTOMODULATORY PROPERTIES OF PEPTIDES FROM MOZZARELLA DI BUFALA CAMPANA (PDO) CHEESE WHEY

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Milk proteins have attracted attention in the biological and medical research community not only because of their nutrient value but as substances which constantly supply the organism with bioactive compounds. Bioactive peptides are hidden in a latent state within the primary sequences of milk proteins, requiring enzymatic proteolysis for their release. Therefore, peptides with physiological effects can be found in fermented milks and cheeses or in by-products of dairy industry such as waste whey. Antimicrobial, immunomodulatory, antithrombotic, opioid and antioxidant activities, enhancement of mineral absorption and/or bioavailability, blood pressure-lowering (ACE inhibitory) effect are some of the biological activities attributed to milk-derived peptides. However, the great complexity and the wide dynamic range of relative peptide abundance in these products severely challenge the capabilities of existing analytical methodologies. A major step forward in this field has been achieved by combining the biochemical and biological approach with the tools of proteomics technology. Herein, we have applied proteomic methodologies to characterize the protein and peptide fraction of Mozzarella di Bufala Campana PDO cheese waste whey and assayed the antioxidant and cytomodulatory effects of isolated peptides on hydrogen peroxide-induced oxidative damage in CaCo-2 cell lines. After 12 h treatment, we found that a specific peptide fraction decreased the production of reactive oxygen species (ROS) (-42%) and enhanced cell differentiation (27% alkaline phosphatase activity/min). These data demonstrated that a specific peptide fraction from Mozzarella di Bufala whey can be regarded as a potential health-enhancing nutriacetical. The information acquired can lead to a deeper definition of the structure-activity relationship in milk-derived peptides, driving research in the development of dietary supplemenents for functional foods and of novel drugs for pharmaceutical industry.

AUTHOR INDEX

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