Italian Proteomic Association
2nd Annual National Conference

Marina Palace
Aci Trezza (Catania),Italy

26th-29th June 2007

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POSTERS

O R A L S

Molecular imaging and profiling of tissue section using mass spectrometry: applications in biogical and clinical research. [O1]

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Profiling and Imaging MALDI MS can be used to assess the spatial distribution of peptides and proteins in biological samples, and is especially effective in its application to tissue sections. Applications range from low-resolution images of peptides and proteins in selected areas of tissue to high resolution images of tissue cross sections. Using a raster of the tissue surface by a laser beam, images of samples are produced in specific m/z values, or ranges of values. Each spot on the sample irradiated by the laser is approximately 30-50 microns in diameter and typically covers the m/z range 1000-100,000. Individual m/z values can then be assembled from the mass spectra to produce selected m/z images. Sections obtained from any tissue type can be imaged to locate tissue specific peptides and proteins in X, Y coordinates of the tissue. We have employed the technology in studies of a variety of diseases, including several types of cancers, neurodegenerative diseases and kidney diseases, comparing proteins differentially expressed in diseased tissue with those in the corresponding normal tissue. This will be illustrated with studies of breast tumor biopsies and also those for human glioblastomas. In the latter, MS patterns have also been correlated with patient outcomes. This has been applied to a protocol termed histology-directed molecular analysis of tissue and biopsy specimens. Imaging MS has also been applied to drug targeting and metabolic studies with analysis of specific tissues after systemic drug administration. Whole animal sagittal sections have been imaged to measure molecular changes in proteins in multiple organs and correlating this with drug concentrations in these same organs.

Proteomics goes intelligent: Pathway analysis of proteomics expression data. [O2]

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In the absence of reliable and inexpensive protein arrays, shotgun proteomics is the fastest way of obtaining information on the identity and expression level on hundreds of proteins in a single experiment. The data interpretation is a difficult task, as even a simple stimulus can result in abundance change of hundreds of proteins. Most of these proteins are distantly related to the originally stimulated pathway. Thus direct mapping of up- and/or down-regulated proteins onto the known pathways seldom produce correct identification, and the data interpretation is often limited to grouping the proteins according to their origin and/or function using one of the available gene ontology databases. At the same time, interpretation of the integral proteomic experiment is very important, as it can give insight into the late stages of the pathway. The global proteomic expression data are also more relevant for clinical samples and the search in them of disease biomarkers.

Here we show that meaningful interpretation of integral proteomics data is possible using a reliable database of known pathways and a specially developed interpretation procedure. Briefly, the procedure combines the results of direct mapping and transcription factor analysis. In both cases, the analysis is performed via regulatory molecules (key nodes). Key node analysis is shown to be an essential step ensuring optimal combination of sensitivity and specificity.

In the current work we propose and validate the procedure using original as well as publish proteomics data. We also demonstrate that useful biological information that can be obtained with the proposed approach.

Proteomic Studies on Low- and High-Grade Human Brain Astrocytomas. [O3]

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Human brain astrocytomas range from the indolent low-grade to the highly infiltrating and aggressive high-grade form, also known as glioblastoma multiforme. The extensive heterogeneity of astrocytic tumors complicates their pathological classification. We compared the protein pattern of low-grade fibrillary astrocytomas to that of glioblastoma multiforme by 2D electrophoresis. The level of most proteins remains unchanged between the different grade tumors and only few differences are reproducibly observable. Fifteen differentially expressed proteins, as well as seventy conserved spots, were identified by mass spectrometry. Western and immnunohistochemical analysis confirmed the differential expression of the identified proteins. These data provide an initial reference map for brain gliomas. Stable cell lines from low- and high-grade tumoral tissues have been obtained to test if additional differences can be detected at the cellular level. The protein expression profile of these cell lines is currently under investigation. Our findings contribute to deepening our knowledge of the factors that characterize this class of tumors and, at the same time, can be applied toward the development of novel molecular biomakers potentially useful for an accurate classification of the grade of astrocytomas.

Combined Proteomic and Metabonomic analyses of urine in three genetic Forms of the renal Fanconi Syndrome(FS). [O4]

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Introduction: Fluids secreted or excreted from a living organism provide a unique window into its biochemical status. Characterization of a biofluid composition can carry detailed biochemical information on organ dysfunction, as a consequence of a genetic mutation, a drug toxicity or a pathology. This study aimed at the exploration of the molecular composition of the urine of patients affected by Fanconi syndrome (FS). FS is a proximal tubular defect that can cause glucosuria, amino aciduria, phosphaturia and most consistently, low molecular weight proteinuria (LMWP). The origin of FS can be genetic or acquired. Among the genetic forms there are Dent's disease and Lowe syndrome that are X-linked forms and an autosomal dominant (ADIF) form, phenotypically similar to Dent's, but it's gene defect is unknown. The acquired forms appears as a consequence of environmental factors as drug treatment or heavy metal exposure.

Aims: To assess if their respective gene products are ultimately involved in a common reabsorptive pathway for proteins and low molecular mass endogenous metabolites, we compared renal Fanconi urinary proteomes and metabonomes with normal (control) urine using mass spectrometry and 1H-NMR spectroscopy, respectively. In a first experiment we compared Dent's disease, Lowe syndrome and normal urinary proteomes by 2DE and MALDI-TOF analysis. In a second set of experiment urinary proteomes and metabonomes of Lowe syndrome, Dent's disease and ADIF were compared by a combined analysis, using tandem mass spectrometry and 1H-NMR spectroscopy. We also included urine from patients with LMWP secondary to ifosfamide treatment (TP).

Results: Comparative analyses of the three genetic forms of FS and TP (a drug-induced form), with normal urinary proteomes showed that the FS proteome is quantitatively and qualitatively different from normal urine (e.g., uromodulin most abundant protein in normal urine, albumin in FS urine). Consistent with the proteomic findings, the 1HNMR data also revealed clear differences in the metabolic profiles of FS urine versus normal urine, due mainly to amino aciduria. However, we also found well defined differences among the different forms of FS in their urinary metabolite and protein compositions. An unsupervised analysis of the data (using cluster analysis as for microarray data) grouped the Lowe and Dent's urinary proteomes and metabonomes together, whereas ADIF and TP clustered together separately.

Conclusion: Our findings demonstrate a distinctive 'polypeptide and metabolite fingerprint' that can characterize the renal Fanconi syndrome; they also suggest that more subtle and cause-specific differences may exist between the different forms of Fanconi syndrome that might provide novel insights into the underlying mechanisms and cellular pathways affected.

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A new general approach for the purification of proteins from biological extracts for identification by mass spectrometry [O5]

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The definition of an isolation process in view of formal identification for a protein of interest selected during mass spectrometry investigations of proteomes, represents a major difficulty when starting from very crude protein extracts.

The approach reported in this presentation is a fast and easy process involving two main steps and followed by protein digestion and the classical processes of peptide finger printing and/or peptide sequencing.

Basically the protocol consists of an initial rational selection of few sorbents out of several dozen and then the definition of the sequence of selected media.

From the first step, one sorbent is selected for its properties to capture the protein to purify, regardless whether other protein impurities are also co-adsorbed; then 3-7 other complementary sorbents are identified to remove impurities but without interacting with the target protein under the same buffering conditions.

The second step consists in superimposing sorbents under a cascade manner with the sorbent in charge to capture the target protein located in the last position. Impurities are thus progressively removed by the sorbent sequence while the target protein is captured by the last sorbent from where it is collected using an optimized gradient.

All operations are performed with a single adsorption buffer for all columns and all monitoring performed by means of mass spectrometry.

Examples of protein isolation/ identification from biological fluids will be shown.

Proteomics investigations of proteins degradation arrest by selective proteasome inhibitors [O6]

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Late-onset neurodegenerative diseases are often associated with the formation of toxic intracellular aggregates which should be substrates of cell degradation pathway such as the ubiquitin-proteasome system. In fact, aggregates of ubiquitinated proteins have been observed in the CNS of patients affected by Alzheimer's, Parkinson's and Huntington's disease.These aggregates might be suggestive that dysfunctions in the ubiquitin-proteasome system might contribute to the pathology of various neurodegenerative disorders.

In order to investigate the early molecular events in the accumulation of ubiquitinated proteins we have employed selective proteasome inhibitors such as epoxomicin and PSI on an in vitro human cell line model of neuroectodermic origin (Neuroblastoma, SH-5YSY). A combination of flow citometry and proteomics experiments by 2D-PAGE protein separations coupled to MALDI-TOF-MS and nLC-Q-TOF-MS/MS were applied to characterise the differential protein profiles.

Increased levels of poli-ubiquitinated proteins were found associated to the activation of the drugs induced apopotosis as shown by western blot analysis of caspase-8, p21 and p53. Nevertheless a sub-population of the cell lines is capable to overcome cell death by the proteasome inhibitor toxicity. We have performed a proteomics investigation on the different cell populations before and after exposure to epoxomycin by employing cell citometry analysis combined with cell sorting harvesting. Our results identified a direct involvement of various intracellular pathways such as the heat shock protein machinery (HSP60, 70), of Trans Golgi Network (TGN) and of nucleotide metabolism. Interestingly the resistant cell population shows higher level of VGF inducible factor.

Identification of a New Truncated form and Deamidation Products of Fibrinopeptide B Released from Human Fibrinogen by Thrombin [O7]

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Quantification of fibrinopeptides release is widely used to investigate the early steps of fibrinogen activation. In the reverse-phase (RP) HPLC or capillary electrophoresis analyses of fibrinopeptides mixtures derived from the thrombin-promoted activation of human fibrinogen, a few unidentified peaks are consistently present. The composition of these peaks was studied by RP-HPLC/MS, revealing a single major anomalous peptide having a molecular mass of 1384.4. A further MS/MS analysis allowed the identification of this form, corresponding to a N-terminally truncated fibrinopeptide B (FPB) lacking the first two residues (pyroglutamic acid and glycine). This previously unidentified, relatively low-abundance form (~7%) has been found in all the fibrinopeptides preparations tested by us, and it is likely present in circulating fibrinogen. Moreover, deamidated forms of all FPB species (including desArgB), resulting from the conversion of an asparagine in an aspartic acid, were also identified. Overall, these previously not described forms constitute a substantial amount of FPB (up to ~17% of the total), and should be taken into account for a reliable quantitative analysis of FPB release. An assessment of the incidence of these new forms on circulating human fibrinogen and their possible biological relevance is awaiting further studies.

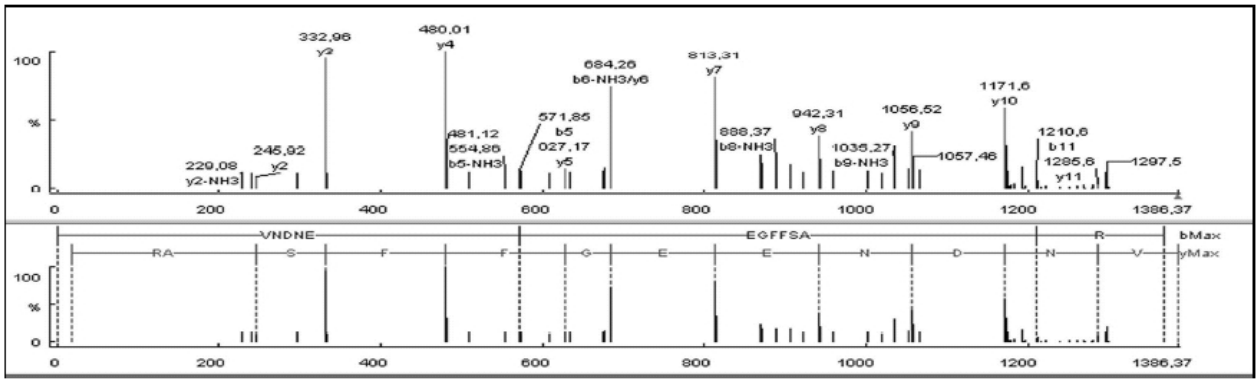

Figure 1

Figure 1:***"De novo"* analysis of one of the unknown peaks.** Semi-automatic analysis yielded the VNDNEEGFFSAR sequence corresponding to a truncated form of fpB (fpBY2).

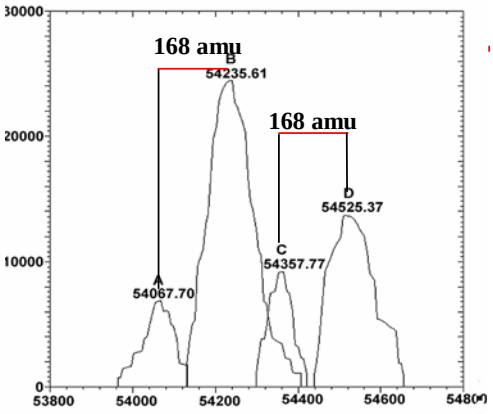

Figure 2

Figure 2: **Deconvolution of the ESI/MS raw mass spectrum of purified Bβ chain from human fibrinogen.** The spectrum puts in evidence, apart from the main mono- e di-sialylated forms of the Bβ chain, also the signals of two shorter forms differing by 168 Da, consistent with the removal of the dipeptide pE-G.

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Native two dimensional electrophoresis for resolving plasma proteins [O8]

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A two dimensional (2D) gel electrophoresis system in which a native first dimension is followed by a second denaturing SDS-PAGE separation has been developed. The first separation is characterised by a native gel with a molecular sieve of T=3.5-16% w/v, and a monomer concentration of cross-linker C=6% w/v. Di-Hydroxy-Bis-Acrylamide (DHBA) has been used as monomer because of its greater hydrophilic characteristic respect to usual monomer. We have combined this novel separation approach to an orthogonal apposition of the second dimensional SDS gel.

This method allows us to resolved a larger number of plasma proteins, fragments and crosslink-products of proteins and to have a major evidence of the high molecular weight protein repertoire (>500 KDa). Indeed, over an hundred forty plasma protein spots have been identified by Mass Spectrometry and western blot analysis, many of them has been described for the first time with a gel 2D-PAGE. Moreover, this technology can be used to identify protein-protein interaction partners even on extremely complex biological samples such as the human plasma. Our data, shows that this native two dimensional electrophoresis is an additional proteomic tool for separation of proteins to coupled with standard 2-D electrophoresis gel.

Coupling Microfluidic Networks and Surface Plasmon Resonance Imaging for Proteomic Biosensing [O9]

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Surface Plasmon Resonance Imaging (SPRI) [1,2] is a surface-sensitive technique for detecting biomolecolar interactions from arrays of molecules attached to chemically modified gold surfaces. It expands the label-free capability of the standard SPR technique [3-4] by allowing a multiplexed approach to the evaluation of biomolecular interactions and to the sensing of chemical and biological analytes. [5-7]

In order to take fully advantage of the SPRI approach, a precise control of both the patterning of biomolecules onto the sensor surface as well as the fluidic of the analyte solution are imposed. In this perspective, the use of microfluidic devices provides SPRI compatible convenient means for manipulating very small amounts of sample and for controlling the patterning of a variety of different biomolecules (i.e. DNA, peptides, proteins, carbohydrates).[8-9]

In this context, we have directed our efforts towards the development of new approaches which allow rapid and cheap prototyping of microfluidic devices and their successive implementation to the SPRI apparatus. We have developed different microfluidic platforms integrated with SPRI for the monitoring of binding events on micro-patterned protein arrays. The microfluidic devices have been fabricated in poly (dimethylsiloxane) by replication from vinyl masters.

In order to demonstrate the sensing properties of the SPRI platform, the Biotin/Streptavidin interaction was investigated by using two sets of parallel microchannels. Intersections of the array with the microchannels define the regions of the sensor surface where the interactions take place while the surrounding areas are useful for non specific interactions and refractive index changes corrections (Figure 1). A limitation of a similar double step approach is represented by the need to further manipulate the gold substrate after the patterning of the biomolecular array, as it is necessary to remove the first PDMS device used to array the surface and to place in contact to the functionalized surface the second PDMS device used to allow the flowing of the analytes. Degradation of the surface integrity can result from such manipulations. The use of PDMS microfluidic networks such as the one shown in Figure 2 is presented as a way to carry out SPRI experiments without the need for the above mentioned double step approach. As an alternative way to carry out SPRI experiments, the new approach employing Y shaped microfluidic networks was used for the study of the interaction between the Datura Stramonium Agglutinin (DSA) and the Asialofetuin. In this case, both the spatially controlled pattering of selected biosystems onto solid surfaces and the simultaneous and independent monitoring of biospecific binding events is demonstrated to be feasible without any degradation of surface integrity.

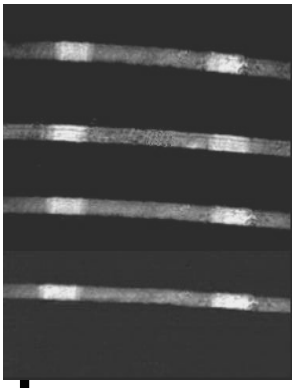

Figure 1: SPRI difference image of a surface patterned by using a microfluidic network. The bright area is caused by the Biotin/ Streptavidin interaction.

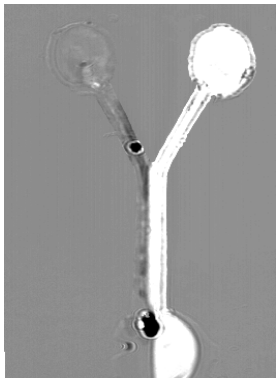

Figure 2: SPRI difference image of a surface patterned by using a Y shaped microfluidic network. The bright area is caused by the anchoring of DSA onto the sensor surface.

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Identification of molecular markers in early events involved in vascular smooth muscle cells activation [O10]

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Endothelial injuries in arterial walls initiate a cascade of events concluding with to the activation of Smooth Muscular Cells (VSMC). Hormones, cytokines and growth factors have pivotal roles in this process that conveys to the acquisition of a migratory-proliferative phenotype, eventually causing the occlusion of the vase.

This study investigates with a proteomic approach the molecular changes that promote VSMC switching from quiescent to activated-proliferating phenotype. In particular, it focuses on the modulation in tyrosine-phosphorylation due to cells activation by serum or single growth factors such as Insulin-like Growth Factor 1 (IGF-1) and Platelet Derived Growth Factor (PDGF-BB).

Comparison of the 2D-PAGE profiles resulting from quiescent or activated-proliferating VSMC let us recognize a number of differences in protein expression. Several differentially expressed proteins were identified by mass spectrometry and their time course changes in tyrosine-phosphorylation recorded from time zero till 48 hours after stimulus.

We documented a general decrease of the tyrosine-phosphorylation level within the first minutes after stimulation followed by a recovery that is quick and dramatic for some chaperones and redox enzymes, otherwise it is not so significant for glucose metabolism enzymes. As far as the cytoskeleton components are concerned, no remarkable fluctuations were detected at the earliest time points except for those relative to á-actin which displays an impressive decrease.

A comparison of the early stages of cell stimulation after serum or single growth factors administration enlightened important differences in the phosphorylation of chaperones suggesting their crucial role in VSMC activation.

In order to establish the role of each signalling pathway on the molecular changes, a gene "knock-down" investigation approach was planned. We focused on growth factor signalling, and very preliminary results have been obtained on VSMC cells depleted by PDGF-BB receptor and stimulated with PDGF. In this way proteins that could be significant in committing cells to migration should be identified.

Structural Proteomics: then and now

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One of the key areas of study in systems biology is how proteins function within cells and how that function translates to health. The integration of advanced imaging technologies and computational science with basic molecular and cellular research offers insight into how living systems function. In this respect, x-ray crystallography is the workhorse imaging technique, complementary to NMR, microscopy and mass spectroscopy, contributing an essential role in the technology platform for an integrated systems biology approach. A detailed analysis of protein-protein, and protein-ligand interactions is a crucial goal for systems biology and determination of three-dimensional protein structure is particularly important to proteomic research, since subtle changes in structure can affect the proper function of a protein, changing its activity, and possibly leading to its instability and ultimate destruction by the cell.

Enormous advances in molecular and structural biology have enabled large scale, high throughput structural proteomics research. As a result, such initiatives are solving protein structures and generating data at an unprecedented rate. Now one of the major challenges for the post-genomics era is to functionally assign and validate the large number of novel target genes and their corresponding proteins. Consequently, functional genomics approaches have gained considerable attention in the quest to convert this massive data set into meaningful information.

To exploit the vast data emerging from structural proteomics research and to contribute to the generation of functional hypotheses, we are developing an automated procedure for probing proteins of unknown function, employing distributed computing methods and the programme GRID (Goodford, *J Med Chem*, 1985, **28**, 849-57). The programme GRID is a computational method for determining energetically favourable binding sites on molecules of known structure, based on discrete chemical functional groups (ex. metal ions, phosphate), small molecules (ex. carbohydrates, lipids), and amino acids. The use of GRID to probe structures of unknown function has important applications for understanding many biological processes and forms the basis of most drug design strategies. Our efforts in this area form part of a larger scale collaborative project with ACET (www.acet.rdg.ac.uk) to provide workflow environments for biological computing using distributed networks and immersive technologies.

An overview of the current state-of-the-art for structural proteomics, and our recent efforts to provide an automated server for generation of functional hypotheses will be presented.

Sequence similarity is more relevant than species specificity in probabilistic backtranslation [O12]

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Background. Backtranslation is the process of decoding a sequence of amino acids into the corresponding codons. All synthetic gene design systems include a backtranslation module [1]. The degeneracy of the genetic code makes backtranslation potentially ambiguous since most amino acids are encoded by multiple codons. The common approach to overcome this difficulty is based on imitation of codon usage within the target species [2].

Results. EasyBack [7], a new parameter-free, fully-automated software for backtranslation using Hidden Markov Models [8] is described. EasyBack is not based on imitation of codon usage within the target species, but instead uses a sequence-similarity criterion. The model is trained with a set of proteins with known cDNA coding sequences, constructed from the input protein by querying the NCBI databases with BLAST. Unlike existing software [3, 4, 5, 6], the proposed method allows the quality of prediction to be estimated [8]. When tested on a group of proteins that show different degrees of sequence conservation, EasyBack outperforms other published methods in terms of precision (Figure 1).

Conclusions. The prediction quality of a protein backtranslation method is markedly increased by replacing the criterion of most used codon in the same species with a Hidden Markov Model trained with a set of most similar sequences from all species. Moreover, the proposed method allows the quality of prediction to be estimated probabilistically.

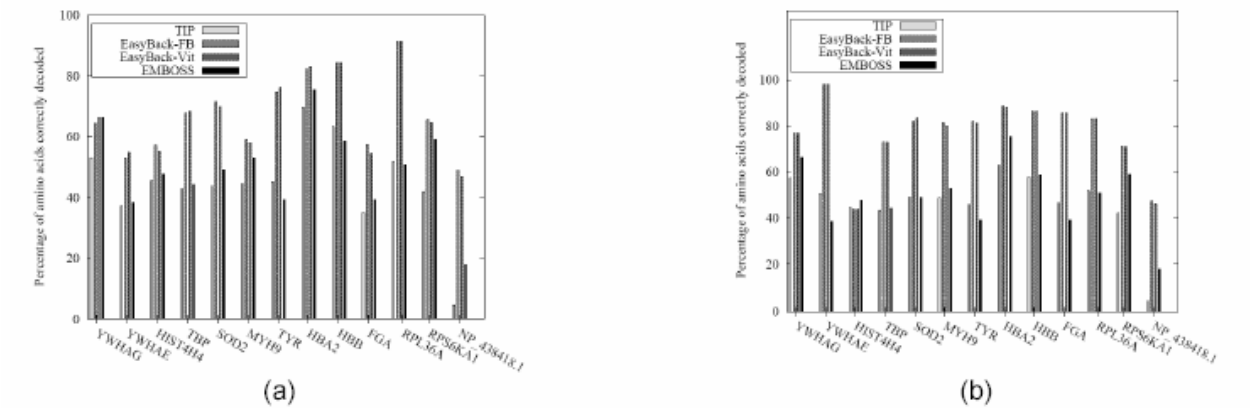

Figure 1: EasyBack vs TIP and BACKTRANSEQ. Performance of EasyBack compared with TIP and BACKTRANSEQ based on percentages of amino acids correctly decoded. Easyback and TIP were tested using BLAST All-Species training sets. BACKTRANSEQ used Species-Specific training sets. (a) For all systems each training set comprised 100 sequences. (b) The training set is the minimal subset of the query output sufficient to make a prediction obtained by a binary search strategy. Classical Viterbi algorithm (EasyBack-Vit) and a posterior decoding technique (EasyBack-FB) were used to make prediction.

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Algorithms and techniques for large biological networks analysis [O13]

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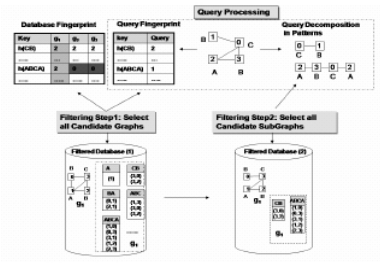
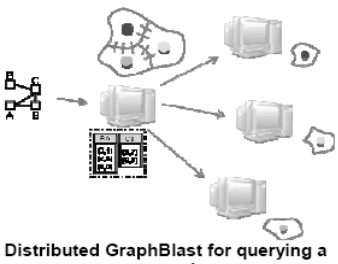
Background. Application domains such as bioinformatics, cheminformatics represent data as graphs where nodes are basic elements (i.e. proteins, atoms, etc..) and edges model relations among them. In these domains, a key role is played by systems that (1) search for all exact or approximate occurrences of a given query graph in a collection of graphs (query to database [7]) or (2) find common subgraphs in k graphs (alignment of networks [6]). For example, locating subgraphs matching a specific topology is useful to find higher-order connectivity motifs of networks that may have functional relevance, predict protein function, predict protein interaction. However, finding subgraphs is an hard problem since it entails detecting isomorphisms. Therefore indexing, filtering and mining techniques are used to make this problem feasible.

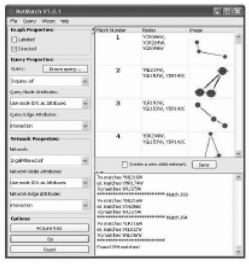
Results. GraphBlast [3], Netmatch [2] and NetAl are presented. GraphBlast performs a graph query search in a database of graphs. It implements efficient graph searching algorithms together with advanced filtering techniques. It allows to select candidate subgraphs rather than entire graphs. It implements an efficient data storage based also on low-support data mining. Since biological data are increasing exponentially and searching for subgraphs is very hard, a distributed version of GraphBlast for searching in a large network is provided. NetMatch is a Cytoscape plug-in which allows searching biological networks for subcomponents matching a given query. It extends graph theoretical algorithms to efficiently deal with biological network properties. Cytoscape is a bioinformatics software platform for the visualization and analysis of biological networks. To make the query creation process easy, a drawing tool is provided. In the above systems queries may be approximate in the sense that certain parts of the graph query may be left unspecified. NetAl extends an efficient graph searching algorithm [1] to find all common subnetwoks of two networks. The search is optimized by finding all non-redundant common subgraphs in the following way. It starts with the entire graphs, finds local maximal subgraphs, marks nodes of such subgraphs, and continues the search on the unmarked nodes.

Conclusions. GraphBlast is compared with Frowns [4], GraphGrep [7] and gIndex [5]. Experiments show that GraphBlast outperforms all the compared systems both on real and synthetic databases. The proposed low-support mining technique which applies to any searching system also allows a significantly indexing space reduction. NetAl has been compared with several network alignment tools [6] on real biological networks. Future work includes an extension of NetAl for the alignment of k networks.



GraphBlast: querying a database of graphs


Distributed GraphBlast for querying a network


NetMatch: Cytoscape plug-in for querying a network

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Homology driven proteomics expands the organismal diversity of biochemical research [O14]

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Homology driven proteomics enables biochemical characterization of unconventional, yet biologically interesting model organisms from a variety of phylogenetic classes, including plants, fungi, insects, reptiles, among many others. Confident identification of isolated proteins is achieved by taking advantage of even marginal similarity between sequences of fragmented peptides and homologous sequences from phylogenetically distant species already available in a database. Within the presented proteomics pipeline, a single dataset of MS/MS spectra is acquired at the uncompromised sensitivity and database searches are performed in a layered manner. Stringent searches (Mascot) rapidly identify most conserved proteins and, via scripted automation, help to rectify the dataset for automated de novo sequencing followed by sequence similarity searches by Mass Spectrometry driven BLAST (MS BLAST). Typically, MS/MS spectra from a few hundreds of precursors are interpreted de novo and several candidate sequences per each precursor are allowed in the same search, which, altogether, encompasses more than 2000 peptide sequence candidates and enables confident protein identifications in mixtures. In organisms with known genomes, the approach provides new means of validating the confidence of database searching hits and unbiased identification of polymorphic sequences, sites of alternative splicing and post-translational modifications.

Flow field-flow fractionation with multi-angle laser scattering detection for blood lipoprotein profiling [O15]]

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Over the last fifty years, recognition of pathological association of lipoproteins, and of their constituent molecules, with cardiovascular diseases, the major contributors to morbidity and mortality throughout the world, has focused increasing attention. Determination of lipoprotein parameters including triglycerides (TG), total cholesterol, and cholesterol associated to high-density lipoprotein (HDL) and low-density lipoprotein (LDL) has become the most popular method to assess lipoprotein abnormalities and, consequently, coronary artery disease (CAD) risk in clinical diagnosis. Recently, intensive research has identified new risk factors able to provide new insights into the atherosclerotic process, and to enhance diagnostic power in risk assessment. LDL size is one of these emerging risk factors. [1] The LDL size is mainly measured by non-denaturing polyacrylamide gel electrophoresis (PAGE) [2] or high-performance gel-filtration chromatography (HPGC) [3]. Both PAGE and HPGC can however give accurate size estimation only if standard of known size are available. Moreover, recent studies have shown that lipoproteins may be discoid rather than spherical. LDLs were also reported to be discoidal particles. PAGE and HPGC cannot however give information on particle shape.

Flow field-flow fractionation (FlFFF) is a separation technique in which macromolecules and particles are separated in an empty, capillary channel according to their difference in diffusion [4]. No stationary phase is present, and the separation mechanism is sufficiently gentle not to alter the native structure of proteins and protein complexes. By FlFFF it is possible to obtain the diffusion coefficient of proteins, and then their hydrodynamic radius (Rh), from their retention time values.

Hollow-fiber (HF) FlFFF is a prototype, microchannel variant of FlFFF. It employs as channel a piece of porous HF [5].

Recently, HF FlFFF reached a performance that is comparable to that of commercial FlFFF. The interest in HF FlFFF is increasing not only because the channel is of reduced volume, but also because it is simple to construct and inexpensive to make it potentially disposable. Disposable FlFFF channels should represent key advantage in clinical analysis, where run-to-run sample carryover must be strictly avoided.

Multi-angle light scattering (MALS) is one of the few methods available for the absolute determination of molar mass (Mw) and particle size over a broad range. On-line FlFFF-MALS makes it possible to size-sort macromolecules and particles, and to standardless characterize in size and shape narrowly dispersed fractions of them. This is because it can be obtained, for even broadly dispersed samples, the distribution of Rh, of the root mean square gyration radius (Rg), and of the absolute Mw values. [6] In this study, we propose feasibility of FlFFF-MALS for size and shape characterization of lipoproteins from whole human serum samples. Sera were analyzed with no previous treatment or, in some experiments, after serum staining with Sudan Black B, a dye for lipid components which generates a specific absorption maximum at 600 nm. The method performance is compared using either commercial (Eclipse 2, from Wyatt Technology Europe) or prototype HF FlFFF systems. HDL, LDL and VLDL are separated by FlFFF, detected and quantified by UV absorbance using lipid-specific dyes and/or by online MALS. According to the FlFFF theory, lipoproteins are eluted at retention times proportional to their diffusion coefficient. Assumed a spherical shape for lipoprotein particles, the Rh values of the different lipoprotein classes were then obtained from the retention time values of the relevant bands in the fractogram. From the MALS signal it was also possible to evaluate the corresponding Rg values. By determining the Rg/Rh values, information about the lipoprotein particle shape could be therefore obtained. The experimental Rg/Rh values obtained by FlFFF-MALS for a set of serum samples were found in the range 0.88 ± 0.5. These values are higher than those expected for either a homogeneous sphere (Rg/Rh=0.774) or for a disk-like model (Rg/Rh = 0.787-0.830). However, they are compatible with a disk-like model if the density distribution of LDL, which are core-shell particles of non-homogeneous density, is considered. Finally, from band intensities the relative abundance of the different lipoprotein classes can be determined: high intensity of band 4, for instance, reflects the correlation existing between high TG level and high VLDL content. FlFFF-MALS shows to be a promising method for size and shape characterization of lipoproteins. The use of miniaturized, potentially disposable HF FlFFF channels exhibits comparable performance. Being FlFFF a flow-assisted separation technique, it is suited to the implementation of post-column FIA methods to specifically determine, by enzymatic or immunometric methods, analytes in the lipoprotein fractions which are of clinical interest, such as cholesterol, TG and apolipoproteins.

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Identification and Quantification of Proteins Expressed in Embryonic Mouse Stem Cells by 2D LC-MS/MS and LC-MS^E Analysis [O16]

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Mass spectrometry is recognized as an essential tool for proteomics studies in biological sciences and biomedicine. In fact, identification and quantification of all expressed proteins in cells, tissues and organisms is one of the greatest challenges in the post-genomic era. While 2DE is a powerful technique for protein separation, it has a number of limitations which needs new technological alternatives. Furthermore, the small sample amounts often available for analyses require efficient sample preparation and separation procedures together with very sensitive spectrometric methods. In this scenario, liquid chromatography (LC) is often used as a methodology complementary to 2DE for protein or peptide separation in complex mixtures. During the last years, remarkable efforts have been focused on the molecular profiling of stem cells, with the aim to define a protein "stemness" signature and to identify proteins involved into molecular pathways responsible for the two main characteristic of stem cells: self-renewal and pluripotency.

Therefore, stem cells represent an ideal model system for early development studies or, in clinical research, for understanding mechanisms involved in organs development.

Here we present the results of a relatively high throughput 2D LC-MS/MS methodology, used to qualitatively characterize the proteome of a mouse stem cell line under different biological conditions. The adopted 2D LC approach is based on the separation of a complex tryptic digest mixture using a nanoscale LC system connected on-line to an instrument capable of data directed switching between the MS and the product ion MS/MS mode. An efficient SCX step-gradient elution method has been optimised for on-line peptide separation. The research has enabled the recording of large data collection loading with low sample amounts. Protein identification has been achieved via databank searching of the ESI-MS/MS spectra, providing qualitative information on the proteins included in the complex mixture. A large number of MS/MS spectra have been acquired in a fully automated fashion, resulting in the identification of a multitude of proteins from a single LC-MS/MS experiment. The identified proteins have then been classified in terms of subcellular localization, molecular function and biological process as defined by their associated Gene Ontology annotation. Selected subsets from the differentiated and undifferentiated samples have been qualitatively and quantitatively compared – utilizing the results of the LC-MS^E experiments – to obtain a comparative view of their respective proteomes.

Functional Proteomics of Barley Seeds. Spatio-temporal Analysis and Thioredoxin Target Proteins [O17]

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Seed proteomes were monitored for malting barley during grain filling and maturity including protein identification by in-gel digestion, mass spectrometry and database searches.[1-5] Comparison of 2DE patterns of a doubled haploid population with malt analysis data and genetic markers allowed direct correlation between 2DE spots, quality parameters and the chromosome map which can be used in breeding. This coupling also applied to a series of cultivars of varying malting quality. Early germination was monitored on a few seeds (to emulate the steeping process) and showed rapid disappearance of proteins associated with desiccation and appearance of proteins related to oxidative stress notably for the dissected embryo.[6] In a different study changes in response to gibberellic acid were monitored in proteomes of aleurone layers from the experimental cultivar Himalaya by 1DE and 2DE, by western blotting for specific proteins and mass spectrometry. In order to correlate mRNA and protein levels, gene expression was analysed in parallel. The experimental system also enabled analysis of release of hydrolytic enzymes as accumulating in the culture medium. The release of enzymes is a key function of the aleurone layer that is not amenable to analysis in intact seeds. The aleurone plasma membrane proteome was isolated by two-phase partitioning, reversed phase C4 with stepwise isopropanol elution, and 1DE leading to identification by LC-MS of integral membrane proteins with up to 10 membrane spanning domains.[7] Despite the fact that much is known about proteins synthesized by aleurone layers, this approach has led to identification of new proteins with unknown functions. These experiments form the basis for studying changes in aleurone layer transcriptomes and proteomes in response to GA and ABA. Finally, thioredoxin isoforms identified in the seed proteome were cloned and expressed and used for identification of thioredoxin target proteins,[8,9] and specifically of the reduced disulfide bond.[10] The thioredoxins were crystallized to reveal structural determinants needed for recognition of target disulfides as highlighted in a complex made by formation of mixed-disulfide bonded thioredoxin and the target protein barley a-amylase/subtilisin inhibitor.[11]

Supported by the Danish Research Agency and Research Council for Technology and Production.

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Congenital disorders of glycosylation: a never ending story [O18]

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Congenital disorders of glycosylation (CDG) are a large family of genetic diseases due to deficient or increased glycosylation of glycoconjugates. Some 28 defects have been identified since Jaeken et al reported the first CDG patients in 1980. Clinical features of the different CDG types are heterogeneous and range from severe to mild and from multisystem to mono-organ involvement, but neurological manifestations are mostly predominant. There are three main groups of CDG: diseases of protein N-glycosylation, of protein O-glycosylation and of lipid glycosylation. N-glycosylation defects can be due to defective assembly (cytosolic and endoplasmic reticulum (ER) defects) or to defective processing (ER and Golgi defects) (Table).

Isoelectrofocusing (IEF) of serum transferrin (Tf) is the method of choice to screen for N-glycosylation defects accompanied by deficiency of sialic acid, a negatively changed sugar. The abnormal IEF patterns show a cathodale shift and can be grouped into 2 types: type 1 with a decrease of tetrasialoTf and an increase of disialo- and asialoTf pointing to an assembly defect, and type 2 with an increase also of the uneven bands trisialo- and/or monosialoTf, pointing to a processing defect. In the latter case, intermediate and/or abnormal glycans are accumulating, and MALDI-TOF is an important tool in the elucidation of these glycan structures and in the elaboration of a hypothesis about the basic defect.

In case of a type 1 lipid-linked oligosaccharide analysis is indicated in the further investigation of the patient. As to O-glycosylation defects, isoelectrofocusing of apolipoprotein CIII is a screening method for mucin-type O-glycosylation defects.

Table: Human genetic disorders of glycosylation

A. Defects in protein N-glycosylation

Disorders	Defective protein	Defective gene
CDG-Ia	Phosphomannomutase II	*PMM2*
CDG-Ib	Phosphomannose isomerase	*MPI*
CDG-Ic	Dol-P-Glc: Man_9-$GlcNAc_2$-P-P-Dol glucosyltransferase (glucosyltransferase I)	*ALG6*
CDG-Id	Dol-P—Man: Man_5-$GlcNAc_2$-P-P-Dol mannosyltransferase (mannosyltransferase VI)	*ALG3*
CDG-Ie	GDP-Man: Dol-P-mannosyltransferase (Dol-P-Man synthase I)	*DPM1*
CDG-If	Lec35 (Man-P-Dol utilization 1)	*MPDU1*
CDG-Ig	Dol-P-Man: Man_7-$GlcNAc_2$-P-P-Dol mannosyltransferase (mannosyltransferase VIII)	*ALG12*
CDG-Ih	Dol-P-Glc: Glc_1-Man_9-$GlcNAc_2$-P-P-Dol glucosyltransferase (glucosyltransferase II)	*ALG8*
CDG-Ii	GDP-Man:Man_1-$GlcNAc_2$-P-P-Dol mannosyltransferase (mannosyltransferase II)	*ALG2*
CDG-Ij	UDP-GlcNAc: Dol-P-GlcNAc-P transferase	*DPAGT1*
CDG-Ik	GDP-Man:$GlcNAc_2$-P-P-Dol mannosyltransferase (mannosyltransferase I)	*ALG1*
CDG-Il	Dol-P-Man:Man_6-and Man_8-$GlcNAc_2$-P-P-Dol mannosyltransferase (mannosyltransferase VII-IX)	*ALG9*
CDG-Im	Dolichol kinase	*DK1*
CDG-IIa	N-acetylglucosaminyltransferase II	*MGAT2*
CDG-IIb	Glucosidase I	*GLS1*
CDG-IIc	GDP-fucose transporter	*SLC35C1/FUCT1*
CDG-IId	β-1,4 galactosyltransferase	*B4GALT1*

B. Defects in protein O-glycosylation

Disorders	Defective protein	Defective gene
O-xylosylglycan defects multiple cartilaginous exostoses Ehlers-Danlos syndrome	Glucuronyltransferase/N-acetylglucosaminyltransferase β-1,4-galactosyltransferase 7	*EXT1/EXT2* *B4GALT7*
O-N-acetylgalactosaminylglycan defect familial tumoral calcinosis	Polypeptide N-acetylgalactosaminyltransferase 3	*GALNT3*
O-mannosylglycan defects Walker-Warburg syndrome muscle-eye-brain disease	O-mannosyltransferase 1 O-mannose β-1,2-N-acetylglucosaminyltransferase	*POMT1/POMT2* *POMGNT1*
O-fucosylglycan defect spondylocostal dysostosis type 3	O-fucose-specific β-1,3-N-acetylglucosaminyltransferase	*SCD03*

C. Combined protein N- and O-glycosylation defects

Disorders	Defective protein	Defective gene
Hereditary inclusion body myopathy	UDP-GlcNAc epimerase/kinase	*GNE*
CDG-II/COG7	Conserved oligomeric Golgi complex subunit 7	*COG7*
CMP-sialic acid transporter deficiency	CMP-sialic acid transporter	*SLC35A1*
CDG-II/COG1	Conserved oligomeric Golgi complex subunit 1	*COG1*

D. Defects in lipid glycosylation

Disorders	Defective protein	Defective gene
Amish infantile epilepsy	Lactosylceramide α-2,3 sialyltransferase (GM3 synthase)	*SIAT9*
Glycosylphosphatidylinositol deficiency	Phosphatidylinositolglycan, class M	*PIGM*

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Glycoproteomics of congenital disorders of glycosylation and secondary glycosylation disorders. [O19]

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Congenital disorders of glycosylation (CDG) are genetic diseases of glycan synthesis including defects of protein N- and O-glycosylation [1]. CDG group I or CDG-I refers to glycan assembly disorders which result in protein site glycosylation underoccupancy. CDG-II comprise defects in the glycan processing leading to protein glycosylated with incomplete glycans [2].

Secondary glycosylation disorders have been reported in genetic diseases not primarily due to defects in the glycosylation pathways, such as galactosemia (GALT deficiency) and hereditary fructose intolerance (aldolase B deficiency).

The physiological roles of glycan structures in the fine-tuning of multiple biological processes is depicted in CDG clinical spectrum which is broad and virtually involves all organ systems. Glycoproteomics of CDG and secondary glycosylation disorders [3] is aimed to investigate glycosylation changes of proteins with the following purposes: 1) biomarker detection for diagnosis and treatment monitoring (whenever possible); 2) characterization of defective glycan structures to pinpoint possible enzyme/gene defects in patients with unknown basic defect; 3) insight into the pathogenesis by elucidating structure-function relationships.

A major challenge in CDG research concerns the possibility to screen different native intact glycoproteins for their possible macroheterogeneity due to site glycosylation underoccupancy which is mandatory for characterization of CDG-I defects. In addition, the investigation of glycoprotein microheterogeneity (structural abnormalities of N-glycan structures at glycosylation sites) is required for detecting CDG-II disorders.

We set up a new method based on IgY immunoaffinity separation coupled with MALDI MS, as schematised in figure, for clinical proteomics of post-translational modification (glycosylation).

This approach enabled to obtain high quality MALDI mass spectra so as to differentiate single glycoforms due to lack of occupancy of the glycosylation sites (macroheterogeneity) and, following PNGase F digestion, to perform the glycan profiling (microheterogeneity). The method was validated by the analyses of human underglycosylated serum glycoproteins in CDG-I, CDG-II and secondary glycosylation disorders and applied for biomarker detection in the diagnostic procedure (CDG-I and II) and treatment monitoring (galactosemia and HFI).

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N- and O-glycosylation of proteins: a paradigma for mass spectrometry? [O20]

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For implementation of rapid and accurate glycoproteome analysis high demands on techniques related to mass determination accuracy, sensitivity of detection, directed fragmentation and speed of data acquisition are required. Identification of glycoconjugate structure calls for efficient strategies similar to those for identification of proteins, but additional structural parameters relevant for their biological interaction specificity are the site of the glycosidic bond attachment, patterns of branching, and stereochemistry at anomeric centers. According to the natural microheterogeneity on the same glycosylation site it is important to set the analysis parameters for high and low abundant glycoforms. High speed mapping and sequencing of complex glycomixtures by MS became suitable for glycoproteomics introducing the on-line capillary electrophoresis and automated chip-based sample admission [1, 2]. In-capillary digestion method for glycoproteins with subsequent MS and MS/MS analysis represent a novel interesting option in terms of efficiency and speed [3,4].

High mass resolution and accuracy can be achieved on Fourier Transform Ion Cyclotron Resonance (FT-ICR) MS at 9.4 T along with the possibility of electron capture dissociation and infra red multiphoton dissociation for determination of glycosylation patterns [5-7]. Sample admission by chips coupled to FT-ICR gave a significant rise in sensitivity and speed of analysis [7-10].

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Phosphoproteomic Analysis of Protein Kinases using Immobilised Kinase Inhibitors and Mass Spectrometry [O21]

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Protein kinases are major players in the phosphorylation cascades central to intracellular signalling and, therefore, represent attractive targets for therapeutic intervention in a wide range of human diseases. However, proteomic techniques do not always permit the comprehensive characterization of protein kinases as its performance depends on the efficiency of the affinity purification method and the sensitivity of the subsequent MS analysis.

In this investigation, we combined immobilised low-molecular-weight inhibitors [1-3] for the enrichment and an LTQ-Orbitrap mass analyser for the identification of a variety of protein kinases from complex mixtures. The small molecule inhibitors bisindolylmaleimide X and purvalanol B were coupled to Sepharose resins and the affinity matrices were initially connected in series in order to immobilise different protein kinases. Upon sample loading, the affinity columns were disconnected from each other and retained proteins were released specific elution procedures. After gel electrophoresis and tryptic digestion of the bands in different eluates, peptides mixtures were analysed by online LC-MS/MS with an LTQ-Orbitrap using multi-stage activation. This approach permitted the purification and the identification of up to 50 protein kinases after their separation from the majority of the other cellular proteins.

Moreover, phosphopeptides were screened initially by performing multiple reaction monitoring (MRM) on a 4000 Q-Trap mass spectrometer and subsequently by generating an accurate mass inclusion list on the orbitrap, to allow the sensitive detection of phosphopeptides and identification of phosphorylation sites. This technology was used to design a phosphorylation site readout assay for protein kinases isolated from either cell or tissue lysates, which it would be very useful for studying the phosphorylation of protein kinases in experiments where a phosphospecific antibody is not readily available. To investigate the efficiencies of the two methods, identical sample amount was injected and compared by monitoring the extract ion chromatogram (XIC) of the 2+ and 3+ charge states of the phospho-peptide.

Current results indicate that the order of magnitude of the intensity of the XICs are not equivalent, confirming the higher sensitivity of the Orbitrap instrument in comparison with the 4000 Q-Trap. Future work will involve the use of higly selective reaction monitoring (H-SRM), which allow a quick and efficient analysis of complex mixture without the need of MRM methods and the use of EGF stimulated HeLa cells.

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Ion Mobility Spectrometry Coupled With Time-of-Flight Mass Spectrometry for High Definition Analysis of Peptides, Proteins and Protein Complexes [O22]

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Electrospray mass spectrometry (MS) is an established method for the characterisation of peptides, intact proteins and protein complexes. However, the complexity of MS and MS/MS spectra obtained from large polypeptides can limit the information recovered. Very high resolution mass analysers (e.g. FT-ICR MS) are traditionally advocated to resolve such spectral complexity. Ion mobility spectrometry (IMS) in dynamic combination with MS has emerged as an alternative strategy - enabling complex populations of ionised polypeptides, and their collision induced dissociation (CID) fragments, to be deconvoluted with high definition.

We have recently described a hybrid quadrupole/IMS/TOF (Q/IMS/TOF) mass spectrometer that enables high definition analysis of polypeptide species based upon a combination of their ion mobility and m/z ratio [1,2]. Q/IMS/TOF analysis enables the cross-section of intact proteins to be calculated and the stoichiometry of protein complexes to be determined.

Furthermore IMS enables CID fragment ions generated from intact proteins (or tryptic peptides) to be fractionated by charge state - greatly simplifying the resulting MS/MS spectra.

We have studied the GroEL complex, intact lysozyme and beta-lactoglobulin to evidence the analytical characteristics of this Q/IMS/TOF system. In addition, we have evaluated the potential of Q/IMS/TOF MS for rapid screening of tryptic peptide digests.

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The Protein Profile of Confluent Human Dermal Fibroblast is Modulated by Serum Withdrawal [O23]

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The importance of serum factors for cell growth and cell maintenance in vitro is well known as well as the influence of serum withdrawal on cell cycle. By contrast, few data are available on the effect of serum deprivation on quiescent cells in primary cell culture. Since fibroblasts may frequently experience changes in growth factors availability, as during wounding, aging or as a result of fibrotic proceses, aim of the present study was to investigate the response of human dermal fibroblasts in primary cell culture to serum withdrawal applied for 48 hours after cell confluence. We have selected quiescent fibroblasts, in order to avoid the effect of serum deprivation on the cell cycle and because quiescent cells are in a condition more similar to the in vivo situation.

In order to investigate the role of serum deprivation of the protein profile and behaviour of quiescent cells, normal human dermal fibroblasts were grown for 48h after confluence, in 5% CO2 and 21% O2 in the presence or absence of 10% FBS. Cell viability, cell morphology and reactive oxygen species (ROS) production were evaluated by light microscopy and FACS analysis. Moreover, proteome was investigated by two-dimensional gel electrophoresis. Differentially expressed proteins were analyzed by mass spectrometry.

Serum withdrawal, although causing cell shrinkage, did not significantly modify the total cell number. ROS production was markedly increased after serum deprivation. By proteome analysis, 41 proteins appeared to change their expression and, of these proteins, 31 were identified by mass spectrometry. One of the most significant changes occurring upon serum withdrawal is the reduced expression, at the protein level, of numerous enzymes involved in the glycolytic pathway (figure 1). These data have been validated by measuring the amount of lactate, the ultimate product of glycolysis (figure 1).

Interestingly, in our experimental conditions, annexin 2 exhibited the most dramatic changes, being down- regulated more than ten folds in the absence of serum. Reduced expression of annexin 2 may cause altered distribution of lipid microdomains on plasma membrane, thus causing a weakness of membrane resistance and/or altered secretion of molecules from fibroblasts into the extracellular space. Moreover, it has to be mentioned that serum starvation caused an upregulation of calreticulin, a molecular chaperone being part of the primary quality control of protein folding within the ER. However, beside the role as chaperone, calreticulin may be also considered a stress response protein. Present data seem to indicate that it may be a specific target of serum withdrawal. Moreover, it has been recently hypothesized that calreticulin can modulate cell apoptosis and its increased expression can be regarded as a first sign of the apoptotic pathway activation that may occur for longer serum starvation.

In conclusion, it could be suggested that dermal fibroblasts can adapt themselves to environmental changes and that these cells represent a good experimental model allowing to investigate the response of quiescent cells to serum deprivation, highlighting the pathways that may prelude to irreversible changes and to cell death after a prolonged serum withdrawal.

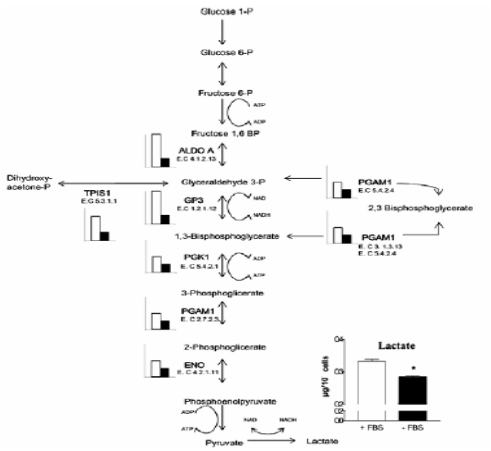

Figure 1: Drawing that indicates the reduced expression of proteins involved in the glycolytic pathway upon serum withdrawal, as evaluated by proteome analysis and by lactate measurement.

Work supported by grant from Elastage LSHM-CT-2005-018960

Mesoporous Silica Beads Surface Tailoring, a Wide-Ranging Strategy for Plasma Biomarker Discovery in Ischemic Heart Injury [O24]

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Recognition of myocardial ischemia is critical both for the diagnosis of coronary artery disease and the selection and evaluation of therapy. Recent advances in proteomic and metabolic profiling technologies may offer the possibility of identifying novel biomarkers and pathways activated in myocardial ischemia. Recently we described an approach based on porous silica beads for selective binding and enrichment for plasma low molecular weight proteins (LMWP) (1,2). The use of porous particles for sample pretreatment is more sensitive than surface capture on chips because particles have larger combined surface areas than small-diameter spots.

As a part of an ongoing project aimed to develop high capacity mesoporous silica beads (MSB) for plasma/serum proteomic analysis, we have chemically modified silica surface in order to increase the selectivity of the method towards positive or negative charged peptides. Varying pore size distributions and surface chemistry MSB allowed selection of molecular species not only by molecular cut off but also by electrostatic interactions. We are now evaluating the ability of our MSB-MALDI-based approach to identify a diagnostic molecular pattern for human myocardial ischemia.

This study was planned to assess the effects of myocardial ischemia induced by transient balloon occlusion during Percutaneous Coronary Interventions.

Five patients men (from 63 to 75 years) scheduled for elective single lesion angioplasty of the left anterior descending artery (LAD) were prospectively included in the study. No patients had acute coronary syndromes, previous myocardial infarction (MI), diabetes mellitus, congestive heart failure (CHF), systemic hypertension, or high plasma cholesterol levels. Blood samples were obtained at baseline, just before maximal tolerated myocardial ischemia (from 30 to 70 seconds) and 5 min, 30 min, 6, 12 and 24 hours after ischemia. The samples were then submitted to MSB-MALDI/TOF analysis.

All detected peaks were normalized relative to internal standards. The changes in plasma profiles caused by experimentally induced myocardial ischemia were quantified for each plasma LMWP as peak-height ratios between signals at each time point post-ischemia and the signal at baseline.

Four potential biomarkers (2210 Da, 2256 Da, 2348 Da, 4130 Da,) were found to be significantly elevated 24 h post-ischemia.

From these preliminary results MSB-MALDI emerges as a reliable platform for the discovery of proteomic pattern-based biomarkers.

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Differential Proteomics Analysis for Target Discovery in Huntington's Disease [O25]

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Huntington's disease (HD) is a neurodegenerative disorder caused by an abnormally elongated polyglutamine (PolyQ) tract in the large protein huntingtin (htt).A proteomics approach has been carried out to discover potential drug targets in this monogenetic autosomal dominant disease. Three repetitions of differential proteomics analysis have been carried out using the 2D-DIGE technology that allows the comparison of proteomes from different samples on the same electrophoretic gel. Three different fluoresce dyes were used in the analysis performed on stable transfected PC12 cell clones bearing an inducible form of the wild type (17Q) or of the mutated (136Q) htt gene. Variation in protein expression was evaluated as a consequence of the induction of the transcription of htt at different time points i.e. after 12 and 48 hours.

Based on the results of these three independent differential proteomics experiments, the differentially modulated proteins, identified in at least two experiments, were analyzed using bioinformatics tools, and a subset of 48 proteins modulated by the expression of PolyQ htt was considered for further bioinformatics analysis refinement by inclusion into protein networks.

The combination of proteomics and pathway analysis increased the number of potential targets since effectors of the differentially modulated proteins have also been considered potential targets for the cure of HD.

Proteomic Detection of Breast-Cancer Subpopulations [O26]

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Among breast cancer histotypes, the infiltrating ductal carcinoma represents the most common and potentially aggressive form. Despite the current progress achieved in early cancer detection and treatment, including the new generation of molecular therapies, there is still need for identification of multiparametric biomarkers capable of discriminating between cancer subtypes and predicting cancer progression for personalized therapies. We have already reported the identification of a set of differentially expressed proteins between paired ductal infiltrating breast carcinomas and non-tumoral adjacent counterparts (Pucci-Minafra et al. Proteomics-Clinical Applications, 2007). In the present study we report a further advance on the protein annotation and classification, performed on 30 new breast cancer samples with the aim to identify candidate markers for clinical correlations and patient's stratification, as well as to contribute to the knowledge of breast carcinogenesis. The surgical tissues utilized in this study are part of a tissue bank collection present at the La Maddalena Hospital. Expression levels of identified protein spots were statistically compared by the ImageMaster 2D Platinum 6.0 software.

Work supported by Por Sicilia misura 3.4 (project DIAMOL).

Tumor Microinvironment Analysis of Renal Cancer by SERPA [O27]

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Renal cell carcinoma (RCC), represents the most common neoplasm affecting the adult kidney and is characterised by a variety and hetereogeneity of histological subtypes, drug resistance and absence of molecular markers that make patient management difficult.

Several studies have shown that the immune system of cancer patients is activated against tumor specific antigens, mutated gene products, differentiated and overexpressed proteins. Therefore, the serological identification of tumor antigens by patient autoantibodies may provide a set of potential biomarkers and diagnostic and prognostic information [1]. More recently, new proteomic approaches provide the opportunity to identify autoantigens to better understand the pathophysiology of renal cell carcinoma onset and growth. Moreover, new evidence in cancer research has suggested that tumor progression is dependent not only on cancer cell themselves, but also on numerous factors present in the tumor microenvironment. In fact, not only cancer cells, but also stromal and inflammatory cells play a crucial role in tumor angiogenesis, and the mechanism of carcinogenesis seems to arise from a chaotic process of inflammation and repair [2,3].

Hence, the aim of the present study was to identify microenvironment proteins from tumor interstitial fluids that elicit an immunoresponse in renal cancer patients applying Serological Proteome Analysis (SERPA). After protein separation by 2-DE, immunoblotting was performed employing autologous sera of 15 RCC patients and the ones of 7 healthy donors as primary antibodies. The analysis of immunoreactive proteins from normal and neoplastic interstitial fluids pointed out 4 different tumor specific autoantigens with autologous sera common to all RCC patients. These proteins were not immunoreactive with the healthy donor sera. One of these proteins, calmodulin, a calcium trasducer involved in tumor cell-endothelial cell adhesion and in angiogenic cellular responses, appears as a potential marker in RCC [4].

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The ErbB2 epitope identified by the novel human anticancer immunoagents ERB-hcAb and ERB-hRNase [O28]

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Recently, we have prepared and characterized human immunoagents directed to the ErbB2 receptor, and effective as anticancer agents in vivo and in vitro on ErbB2-positive carcinoma cells. Mass spectrometry and biochemical investigations have revealed that these immunoagents recognize on the ErbB2 receptor an epitope distinct from those recognized by all other previously described anti-ErbB2 antibodies, including Trastuzumab and Pertuzumab.

Comparative proteomic and immunohistochemistry analyses to study alphaenolase/ MBP-1 isoforms expression in breast cancer [O29]

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Ductal infiltrating carcinoma (DIC) is one of the most common breast cancers. Recently, gene expression profiling techniques have provided many insights into the complexity of gene regulation in breast cancer, indicating that DIC could be classified into several subtypes. Therefore, novel specific markers that may predict or indicate response to therapies or other physiological perturbations are needed. A growing number of evidences suggest that alpha-enolase is involved in cancer. A variant form, termed MBP1, binds and negatively regulates c-Myc gene transcription suggesting that Myc-induced glycolysis is subjected to a negative-feedback control by alphaenolase gene products. In addition, MBP1 exerts tumor suppressor activity in human breast and prostate cancers. We have used comparative proteomic and western blot analysis, in breast cancer cell lines, normal breast tissues and primary breast cancers (DIC) to study the differential expression of alpha-enolase isoforms and MBP1. Furthermore, cytoplasmic and nuclear alphaenolase localization in normal and tumor tissues was evaluated by immunohistochemistry using anti-enolase mAbs. The data obtained indicates that alpha-enolase is overexpressed in breast tumors and that the presence of MBP1 is a significant predictor of good outcome, suggesting that analysis of alpha-enolase isoforms expression could be used to predict the biological behaviour of the tumor.

A computational Tool Kit for the Classification of 2D-Electrophoresis Maps by Combination of Unsupervised Machine Learning Techniques. [O30]

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Abstract

Classification of non-optimized 2D-map samples is a demanding job because of the high inter- and intra-map variability, as background distribution inhomogeneities and geometric/intensity gel distortion, attributed to the experimental uncertainty [1].

Data set of 2D-maps generated from CA3-CA1 hippocampal neuron synaptosomes versus cytosol was used. After image cropping we propose a single image correction based on novel pre-processing techniques of noise removal filtering and background subtraction, by an integrated strategy using image filtering, contrast enhancement and 3-D mathematical morphology [1].

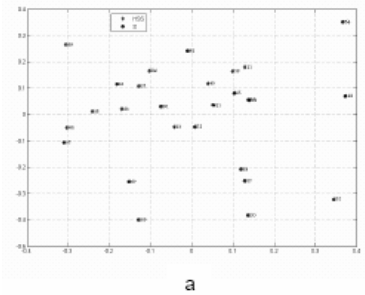
The image registration is founded on control points as pI and Mr markers, and proteins that are not affected by experimental changes. We perform the registration transformation of every gel of the experiment versus one of them chosen as reference [1].

Thus we exploit a recently proposed approach where quantitative image descriptors, extracted on the basis of pixel intensities quantified in the different zones in which the image is subdivided, are analysed by multivariate statistic techniques of dimensionality reduction [2].

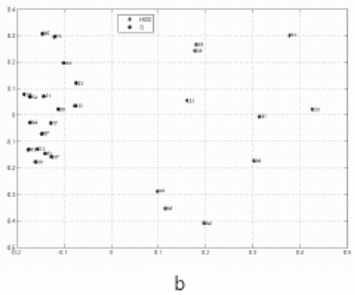
Dimensional reduction of the data set comes from original images (Fig. 1a) compared with the one that comes from the pre-processed images (Fig 1b), shows the effects of the corrections.

The ultimate step is a clustering analysis of the pre-processed data set in the new projection space obtained by dimensionality reduction: this phase is based on a late generation of pattern recognition algorithms stemming from message passing and belief propagation theory [3].

Results in Fig. 2 show an excellent classification due to the high performance obtained by combination of image preprocessing and dimensionality reduction by unsupervised machine learning techniques. This suggests that unsupervised classification of high-throughput proteomic data deriving from non-optimized 2D-maps is even possible in spite of the high variability and experimental uncertainty. Moreover, the use of the direct image method [1], considering the total amount of pixel information without spot detection, demonstrates - in support of the recent trend in computational tool kit - high capability to save the pattern information stored in the original images.



a



b

Figure 1. Representation of the compared data sets after dimensional reduction: (a) original images; (b) pre-processed images.

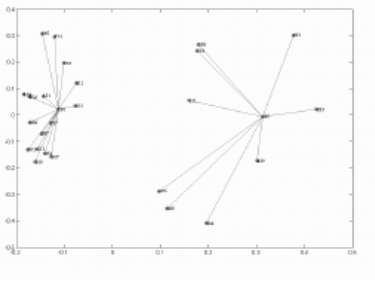


Figure 2. The unsupervised classification comes out excellent.

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A Web Application for Storage, Management and Visualization of Comparative Proteomics Data [O31]

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We present a web application created for efficient storage and comparison of proteomics data sets composed of mass spectrometric fragmentation data (MS/MS) as well as related identification data. The architecture is composed of a web interface, a database back-end containing the proteomic data sets and a series of scripts dedicated to data management (See Figure 1).

The data storage component is implemented under the MySQL database system (v. 5.0.27), containing the following information: sample data, MS analysis parameters, MS results, protein identification search parameters, protein identification results. Data are retrieved from the database in the form of pairwise comparison sets (e.g. a proteomic analisys of a protein subset from a recombinant organism along with the analogous control sample from the wild-type organism) in order to show both shared and exclusive identifications. For each identification, the following data are served: sequence of identified peptides; significativity score; sequence coverage and alignment on the amino acid sequence of each identified protein for the MS experiments being compared; support for evaluation of covalent modifications; comparison of the MS/MS spectra of the identified peptides. The user interface of the program is a web page; a group of pages is dedicated to data and user management (user subscription, user login, file upload), while another group of pages is dedicated to visualization of results in text or graphics form.

Most software components of the application dealing directly with composition of the web pages are written in the PHP programming language (v. 5.1.4); some lower level components are written in PERL (v. 5.8.6); the "fastacmd" tool from the BLAST package version 2.2.15 is used for protein sequence retrieval. Finally, data are served through an Apache web server (v. 2.2.2).

This architecture allows an efficient and flexible data access: I) The interpretation of the MS/MS analysis (the "biochemical" data layer) is directly accessible and less dependent on understanding of MS data. MS and MS/MS data are nonetheless accessible; II) The data can be served through a network, with two main consequences. One is the possibility of remote, concurrent and multiple data access; this is a progress when compared to the case of data analysis based on dedicated workstations with proprietary mass spectrometry software; III) The different components of the web application (web server, database server, ancillary software) can be hosted on different computer clusters, allowing a great scalability when required.

An example of comparative evaluation of data from proteomics analysis of mutant yeast DNA binding proteins will be described.

LC-Q-TOF method for the detection of milk biomarkers in cookies. [O32]

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Introduction

Cow milk allergy is a widespread food allergy most prevalent in early childhood [1]. Studies carried out on a large population have identified the most abundant milk proteins such as lactoglobulins, caseins and lactalbumin to be the major allergens recognised by sensitive patients [2]. A multitude of food products can trigger this type of allergy since cow milk proteins are used as processing aids in a number of food products and beverages. In order to protect sensitive consumers Directives 2000/13/EC and 2003/89/EC have been issued, [3, 4] stating 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. The availability of accurate and sensitive detection methods for food allergens is crucial for the food industry to inspect and control their production processes, and to ensure the correct labelling of their products in order to protect allergic consumers.

Heating and other technological food processing can lead to changes in the target protein structure and these modified proteins are often hard to detect using an immunochemical approach. For these reasons there is a need for confirmatory tests to assess the presence of allergens in foods and in this perspective Mass Spectrometry has become an important tool.

The combination of a quadrupole mass selector and quadrupole collision cell with orthogonal acceleration TOF-MS was used in this study for the identification of milk biomarkers in milk spiked cookies and the results were compared with ELISA analyses. To investigate the influence of the baking process on the detection of milk allergens in food products, blank cookies were prepared and spiked either before or after baking. Different extraction methods were investigated in order to obtain the highest extraction yields. Results show that LC-ESI-TOF is a useful tool to identify milk traces in food products such as cookies although the sensitivity of the method was found to depend on the processing the sample had undergone.

Materials and methods

Sample preparation consisted of five main steps: extraction, protein precipitation, reduction, alkylation and tryptic digestion. Chromatographic separation was carried out by using a gradient of water and acetonitrile containing 0.2% of formic acid. The flow rate was 1 µl/min after splitting at a 1/10 ratio. A volume of 1 µl tryptic digest was pre-concentrated onto a µ-precolumn PepMap C18 before injecting into a C18 column, 150 µm i.d., 150 mm, 3 µm particle size. Q-TOF experiments were carried out in full scan mode and MS survey mode allowing the fragmentation of the most intense multicharged peptides. All eluted peptides and fragments were identified by searching the Swiss-Prot/TremBL database and the reduced databank of milk proteins. ELISA analyses were carried out according to the manufacturers' instructions.

Results

We developed a method based on capillary LC-Q-TOF for identification of potential biomarkers in milk spiked cookies. ELISA tests were carried out on the same samples submitted to LC-MS analysis and results were compared. ELISA analyses have shown that the detection of milk allergens strictly depends on the duration of baking, with samples exposed to longer baking times yielding lower milk protein concentrations. Cookies spiked before baking were compared to ones spiked after baking and results showed that incorporation of the spike before heating has an impact on the extraction and/or detection of milk allergens. Cookies fortified at different concentrations were submitted to the LC-Q-TOF-MS analysis and Table 1 reports an example of results obtained from cookies spiked with 100 and 1000ppm where the majority of peptides detected were found to be modified as a consequence of the heating applied. As shown some milk proteins were identified with a good coverage by the software. This approach provides a valuable tool for the final confirmation of milk allergens in complex matrices.

Table 1. Data searched against the milk databank

Cookies spiked at 100 ppm		
Protein identified	**Peptides matched**	**% of coverage**
α S1- CN	8	49.5
BSA	15	36.2
α S2- CN	10	31.5
Cookies spiked at 1000 ppm		
α S2- CN	14	72.1
α S1- CN	7	63.1
LF	6	51
BSA	31	50.4
β CN	4	10.7

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Proteomic Analysis of Milk Proteins from Cloned Cattles [O33]

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Animal cloning for food production purposes is going to become an established practice as US Food and Drug Administration has just concluded that food from cattle, swine, and goat clones and their progenies is as safe to eat as food from animals of those species derived by conventional means. EFSA reported that the European Commission had recently asked it for advice on the implications of animal cloning on food safety, animal health, animal welfare and environment implication of live cloned animals, obtained through somatic cell nucleus transfer (SCNT) technique, their offspring and of the products obtained from those animals. 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 and other desirable qualities.

In the present study, a proteomic approach based on two dimensional electrophoresis and staining of 2-DE gels with dyes specific for post-translational modifications (PTMs) such as glycosylation and phosphorylation has been used to evaluate the differential expression and post-translational processing capabilities of milk proteins, in particular caseins, from cloned and non-cloned animals in different lactation states. Post translational modifications has been confirmed by MS analysis.

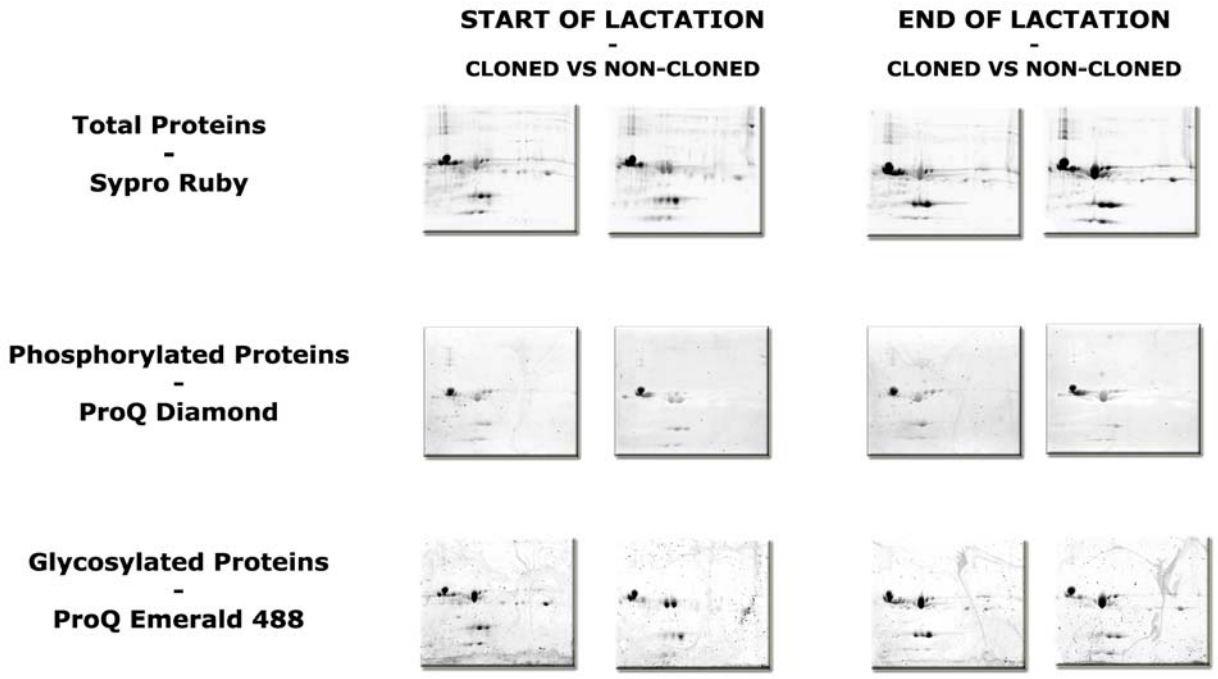

Figure 1: two dimensional electrophoresis of milk samples at different lactation states coming from cloned and non-cloned cows; in addition to total protein profiles (sypro ruby stain), both phospho- and glyco-protein profiles have been investigated.

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Molecular Characterization of a Glycoallergen from Olive tree *Olea Europaea* pollen [O34]

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In Mediterranean area sensitization to olive tree pollen is a wide-spread phenomenon. More than 30% of the population in this area is affected by type I-allergy during the pollination season. The allergens are substances that have antigenic property, in fact when they are in contact with sensitive patients induce the production of specific antibodies, IgE. The majority of olive pollen allergic subjects are sensitive to the major olive pollen allergen, Ole e 1 [1]. This last one is the major allergen of *Olea Europaea*, and one of the best-characterized allergens of Oleaceae family; it has been isolated, purified and biochemically characterized, and its specific cDNA was cloned and sequenced (GenBank™/ EMBL Data Bank accession numbers S75766 and X76395, respectively) [2].

Ole e 1 shows 85-95% identity with Lol p 11, Lig v 1, Syr v 1, Fra e 1 and Pla l 1 allergens and 36-38% identity with the deduced amino acids sequences from LAT52, Zmc13 and OSPSG genes from tomato anthers and maize and rice pollens, respectively. Ole e1 is a glycoallergen, with an N-linked glycoside on Asparagine 111. The glycoside portion of an allergen is really important, because it is involved into cross-reactivity events. For this kind of vegetable protein, the glycosidic core comprises at least three mannose and two N-acetylglucosamine, but the entire glycosil structure is more complex [3].

The antigenic profile of *Olea europaea* pollen from different Mediterranean cultivars was obtained by MALDI mass spectrometry using a simple procedure of chemical fractionation [4,5].

The experimental approach requires an extraction with saline solution of ammonium bicarbonate (50mM), than the extracted proteins are denaturated by means of organic solvents and the obtained pellet is fractionated into two hydrosoluble fractions (**a,b,**), while fraction **s** is the lyphophilic supernatant (**Chart 1**).

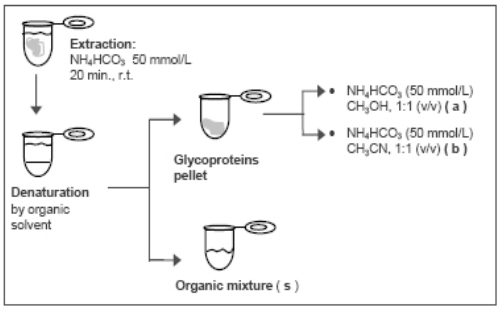

Chart 1

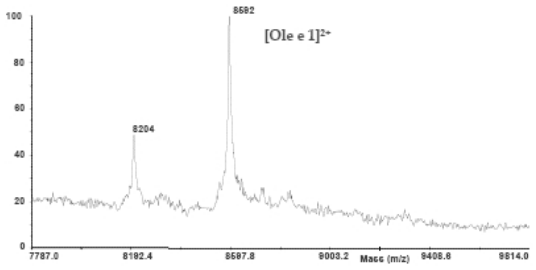

Figure 1

The olive (*Olea Europaea*) pollens of the Mediterranean cultivar Villacidro was chosen to characterize isoforms pool of the Ole e1, occurring in natural material, in particular fraction **b** of pollen Villacidro (**Figure 1**) was preferred for a complete characterization of Ole e 1. The fraction was treated with DTT (50mM) under magnet stirring, then incubated with trypsine, with and without previous deglycosylation with PNGase F. Tryptic digest and peptide mass fingerprinting were performed by MALDI-TOF-MS in order to obtain additional structural details of Ole e 1.

Database search (by mean of Mascot program) using peptide masses from MALDI reflectron spectrum of Villacidro fraction **b** without deglycosylation, confirms that the most important component of the fraction is the main pollen allergen [Olea europaea] gi[13195753, the principal isoform of Ole e I, with 83% sequence coverage and 10 peptides matched. MSMS experiments confirm peptides identification and also reveal the presence of a number of glycopeptides, that induce to believe the glycosyl portion as a very high mannose structure. ExPASy-GlycoMod tool (www.expasy.ch/tools/glycomod) and the program CarboCalc were used to complete the glycopeptides characterization.

Finally, database search using peptides masses from MALDI reflectron spectrum after deglycosilation gives nine additional proteins, that are know isoforms of Ole e 1. Some of these are confirmed by MS/MS experiments.

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Plant Proteomics for investigatine the Molecular Mechanisms of Metal Tolerance: the exemple of *Cannabis sativa* and *Pteris vittata*. [O35]

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Trace elements enter agroecosystems through both natural and anthropogenic processes. Copper is essential to plant growth, but it also an heavy metal, so becomes toxic at high concentrations. Other trace elements, like the metalloid arsenic, are non-essential elements, and have toxic effects on living organisms. Phytoremediation is the plant-based technology for the removal of contaminants from soil and water; the success of a phytoremediation activity relies on the plant tolerance to toxic elements.

The proteomic approach, 2-DE followed by MS/MS analysis, has been applied to investigate the response to copper in Cannabis sativa and the effect of arsenic in the hyperaccumulator Pteris vittata; both the hemp and the fern are nonmodel plants, whose genome is still almost totally unsequenced.

After growing C. sativa in presence of copper (150 ppm), the root system accumulated the metal and underwent growth inhibition (1); the differential expression analysis revealed that five proteins increased, two proteins disappeared, and seven proteins decreased. A possible defence network could be presented, including the up-regulation of the copper scavenger protein aldo/keto reductase, the involvement of other stress proteins (such as formate dehydrogenase, enolase and elicitor-inducible protein); other implicated proteins were those which confer greater copper resistance and provide an efficient reducing system (thioredoxin peroxidase, peroxidase and cyclophilin) and those which regulate root growth (actin, ribosomal proteins and glycine-rich protein).

Pteris vittata hyperaccumulated As in the fronds. Since arbuscular mycorrhizae (AM) increase the As translocation factor (the ratio of metalloid concentration in shoots to that in roots) in P. vittata (2), the proteomic changes in the leaves of the fern infected with AM fungi Glomus mosseae or Gigaspora margarita have been investigated. Each AM experimental system has been evaluated in absence and in presence of As (after 60 days of a weekly-treatment with 25 ppm As), in order to evaluate the fungi role in the plant molecular response to the metalloid. The proteomic analysis of the fern leaf 2-DE maps, revealed that thirty spots were affected by As and/or fungi treatments, many of them belonging to the RuBisCO system, indicating a damaging effect of As on the photosynthetic apparatus.

In addition, semi-quantitative RT-PCR showed that the presence of both, AM fungi and As, modified the level of expression of large and small subunits of RuBisCO.

In both models, C. sativa (root) and P. vittata (leaf), the identification of metal/metalloid toxicity molecular targets will be helpful in improving the features of plants used as phytostabilizer and/or hyperaccumulator in the phytoremediation of contaminated soils.

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How to Discriminate Between Leucine and Isoleucine in Tryptic Peptides by Low Energy ESI-TRAP MS^n [O36]

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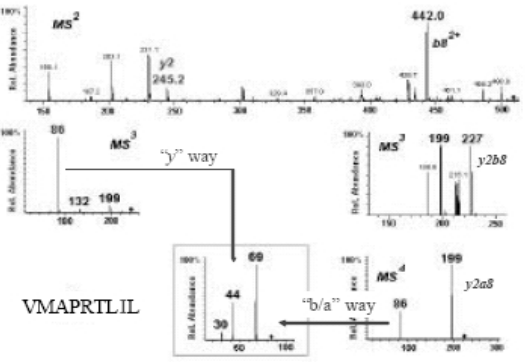
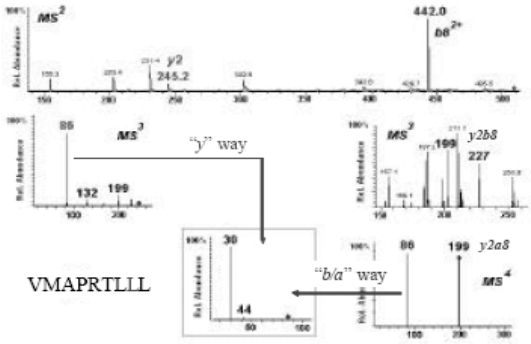
In peptide sequencing experiments involving a single step tandem mass acquisition, leucine and isoleucine are indistinguishable because both are characterized by a 113Da mass difference from the other peptide fragments in the MS^2 spectrum. In this work [1], we propose a new method to distinguish between these two amino acids in consecutive MS^n experiments, exploiting a gas-phase fragmentation of isoleucine that leads to a diagnostic 69Da ion, previously reported in literature [2]. We used this method to assess the Leu/Ile residues of several synthetic peptides. The procedure was then tested on a tryptic digest, assigning the correct amino acid in the majority of the peptides. This work was performed with an old and low-resolution instrument, thus demonstrating that our method is suitable for a wide number of ion trap mass spectrometers, not necessarily expensive or up-to-date. With more performant instruments, our method will hopefully be included in routine LC-MS analysis. We are currently working on the characterization of the breakdown products of Leu and Ile in order to assign a convincing structure for the 69 Da ion. The process starts from an appropriate y or b fragment that, through a MS^3 step, produces a $\mathbf{y_n b_m}$ ion carrying the Xle residue. This species is then fragmented and produces, with one or two consecutive breakdown reactions, the immonium ion. Only the MS^5 (or MS^6) of Ile immonium ion generates a 69Da fragment that allows the correct assignment. Since our method gave good results on myoglobin tryptic digest, despite several severe instrumental limitations, such as slow electronics, extended "low mass cutoff" phenomena and low resolution, we believe that new generation ion traps should be able to routinely address the Leu/Ile issue.





CA) ion trap mass in a formic acid 0.1% rect infusion analysis ıe MS^n experiments, ع to the precursor ion

Legends to figures.
The process for Xle assessment of two isomeric peptides: the immonium ion is the key of the entire method, because Isoleucine, unlike Leucine, produces the diagnostic 69Da fragment. Starting from the MS^2 spectrum of a peptide, both y and b ions can produce the desired information through MSn fragmentation of an opportune $\boldsymbol{y_n b_m}$ ion.

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Optimization of Protein Extration for Two-Dimensional Electrophoresis and Study of the Protome Changes in the Grape Berry Skin During Ripening. [O37]

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Grape is a non-climateric fruit whose development is characterized by a double sigmoidal curve, in which two periods of active growth are separed by a phase during which no dimensional changes occur. Ripening is the second growth period and starts at the moment of véraison, the onset of color change in black cultivars. During this stage, also called maturation, many events occur: the fruit softens and increases its sugar content; the titratable acidity drops and many molecules related to the secondary metabolism, such as taste and aroma compounds, make their appearance. All these phenomena are strictly linked to the quality and technical features of both table and wine grapes. In order to get more information on them it is essential to characterize the skin tissue because of its central role in maturation. In addition of being a very metabolically active tissue and the physical barrier between the environment and the fruit, the skin is also the place of the synthesis of key compounds such as anthocyanins. Despite its relevance, studies aimed at unravel the behaviour of this tissue during ripening, especially at the proteomic level, are few, probably because of the difficulties that have to be faced in approaching such an interfering-compounds rich sample. In fact, skin protein extraction is affected by the high contents of molecules such as tannins, anthocyanins and terpenes that are typical of many grape cultivars.

The aim of this work was to study the ripening-associated proteome changes of the berry skin of Barbera, a red cultivar widely cultivated in Northern Italy. The berries were sampled from plants grown at the experimental station of Riccagioia (PV) in five different dates from véraison till full ripeness. A protein extraction protocol, suitable for both véraison and ripe berries, which are defined by a different chemical composition, was set up. It has been necessary to adapt a method used to extract protoplast proteins, which involves a phenol-based phase separation, introducing a preliminary step during which the homogenized sample is washed with cold acetone in order to remove phenolic interfering compounds. Proteins were separated using two-dimensional electrophoresis techniques (2-DE), using a pH 3-10 linear electrofocusing gradient in the first dimension and 12.5% polyacrylamide homogeneous gels in the second dimension. Five replicates for each condition were obtained. The gels were stained with CCBB and their analysis was carried out using the ImageMaster Platinum software. About 80 spots significantly changed their expression during ripening. In order to characterize them, the LC-ESI-MS/MS spectrometric approach was used.

The identified proteins were involved in sugar metabolism, in response to biotic or abiotic stresses, in cellular and in secondary metabolism. In particular, as far as it concerns the relative abundance, the dominant proteins were involved in defence mechanism suggesting that proteins such as chitinase and β-1,3-glucanase accumulate as the berry ripens.

The bidimensional hierarchical cluster analysis suggested that ripening is a continuous process in the sense that the behaviour of most proteins during the whole maturation period reflected the expression change occurred in the first weeks after véraison while proteic pattern of the last month of maturation is quite conserved.

A new two dimensional blue native method coupled with mass spectrometry for proteomic study of thylakoid membrane proteins under stress conditions. [O38]

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Functional proteomics of membrane proteins is an important tool for the understanding of protein networks in biological membranes. Photosynthetic apparatus of higher plants presents in the thylakoid membrane represent a good model for setting up analytical methods suitable for membrane proteins. Isoelectric focusing in a first dimension followed by denaturating sodium dodecylsulfate polyacrilamide gel electrophoresis (SDS-PAGE) in a second dimension is an effective way to resolve large numbers of soluble and peripheral membrane proteins. However, it is not applicable for isolation of native proteins complexes or for the separation of highly hydrophobic membrane proteins. In alternative, the proteome of the photosynthetic apparatus of high plants, obtained by analysis of thylakoids without any previous fractionation, was mapped by native electrophoresis (2-D/BN) followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as the second dimension two-dimensional-blue native (2-D/BN)/SDS-PAGE) [Zolla (2006)]. This protocol provided an excellent alternative to the 2-D-isoelectric focusing/sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 2-D separation of the most hydrophobic thylakoid proteins. Monocots and dicots showed significant differences in the first dimension while in the second dimension patterns appeared similar. Identification of each spot was performed by internal peptide primary sequence determination using both nano-electrospray ionization tandem mass spectrometry and, to a lesser extent, peptide mass fingerprinting matrix-assisted laser desorption/ionization-time of flight using MALDI-TOF. Thus, this method was used for the comparative analysis of thylakoid membranes among plants under stress conditions. To this regard the time course of changes induced in the photosynthetic apparatus of spinach (*Spinacia oleracea* L.) seedlings exposed to iron deficiency shortly after germination were characterized with two proteomic approaches. Concerning the two photosystems, within each photosystem all core components were present, with no particular protein missing, which indicates that iron deficiency does not inhibit altogether the synthesis of a specific core protein. Regarding antenna protein as revealed by 2 BN-SDS, it is interesting that the decrease in Lhcb trimers and increase in the monomeric form, as well as the decrease in Lhca dimerization, was substantial after only 10 days of iron deficiency and tended to be reversed as iron deficiency continued, suggesting that a plant adaptation mechanism may be activated during exposure to iron-deficient conditions, and that such mechanisms may be at the basis of the observed de-novo formation of Lhcb trimers and Lhca dimmers [Zolla *et al.,* 2003, Timperio and Zolla 2005]. In contrast, although the total amount of antenna proteins was significantly reduced in both photosystems, the stoichiometry of antenna composition of PSI was not compromised, whereas specific antenna of PSII were strongly reduced, such as Lhcb4 and Lhcb6, Lhcb2, and the isoform of Lhcb1 (Lhcb1.1), while Lhcb3 increased. In Chlamydomonas, specific components of Lhca were strongly reduced at the onset of iron deficiency, leading to an overall down-regulation of Lhca, whereas Lhcb abundance remained fairly constant. Thus, it may be speculated that iron deficiency-induced damage to the photochemical apparatus of higher plants is different from that observed for algae. Regarding the individual components of the photochemical apparatus, specific antennae of PSII; Lhcb4 and Lhcb6, Lhcb2 and its isoform Lhcb1.1, were all reduced, while Lhcb3 concentrations increased. 2 BN-SDS revealed that the trimeric organization of Lhcb and the dimerization of Lhca were greatly affected in the first ten days of iron deprivation, but this was partially reversed when de-epoxidation status of the xanthophylls changed. Since both chlorophylls and xantophylls changed during plants grown Thus, it is speculated that the observed changes in the monomer-trimer equilibrium of major PSII antenna is possibly the result of xanthophyll and chlorophyll fluctuations as an adaptation strategy to iron deficiency in higher plants, playing a role in the energy dissipation mechanisms, as here assessed by changes in the non-photochemical quenching of fluorescence.

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Characterisation of Low Molecular Weight Glutenin Subunits in a Translocated Durum Wheat [O39]

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Glutenin polymers are formed by high (HMW-GS) and low molecular weight glutenin subunits (LMW-GS). The latter group of subunits have been less characterised compared to the former due to their great number and heterogeneity. LMW-GS have been subdivided into B, C and D groups on the basis of their biochemical differences, though only subunits included in the B group are considered typical LMW-GS. In fact, it has been demonstrated that C and D groups correspond to mutated gliadins, in which a different organisation of their cysteine residues has enable them to form intermolecular disulphide bonds. Typical LMW-GS have been classified into three major groups, namely LMW-s, LMW-m and LMW-i types, according to their first amino acid residue. The latter group of subunits has been found to have a few peculiarities compared to the first two, such as a different distribution of cysteine residues (which could affect glutenin polymer formation capability of these subunits and dough quality characteristic) and encoding genes associated only to the chromosome 1A. In order to gain more information on the LMW-GS we have used a durum wheat line carrying a 1BL.1RS translocation, in which the short arm of the chromosome 1B is replaced by the short arm of the chromosome 1R of rye. This line was obtained using the durum wheat cultivar Cando, in which the translocation is present, crossed and back-crossed three times with the Italian durum wheat cultivar Svevo.

LMW-GS extracted from the durum wheat cultivar Svevo and line carrying the 1BL.1RS translocation were characterized by 2D gel electrophoretic separation and mass spectrometry.

The spots of two gels were excised, digested by trypsin and analysed by MALDI-TOF MS and RP-HPLC/nESI-MSMS.

The data obtained allowed to compare the protein differently expressed. We revealed the lack of LMW-m and LMW-s gluten subunits in the translocated line while the LMW-i subunits, coded by chromosome 1A, remain expressed.

The genetically modified Svevo cultivar is currently under evaluation in order to determine the effect of the absence of the B-type LMW-GS on the viscoelastic properties of the dough.

Effect of Cobalt ions on the soluble proteome of Phototrophic Eubacterium *Rhodobacter sphaeroides* Strain R26.1 [O40]

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Rhodobacter sphaeroides is a well characterised photosynthetic facultative bacterium whose genome has been completely sequenced and is available to the scientific community (1). Interest in the characterization of its proteome is due to the observed capacity to grow in heavy metals polluted environments (2) and hence in its potential use in bio-remediation strategies. In particular, the strain R26.1 shows tolerance to Co^{2+} ions, up to 10 mM concentration. Interestingly tolerance to Co^{2+} is accompanied with a noteworthy detrimental effect on the photosynthetic apparatus of the microrganisms, and in details on the biosynthesis of photosynthetic pigments.

In order to have a wide overview of the effect of cobalt ions on the soluble proteome of *R. sphaeroides* grown in heavy metal contaminated environments, we started a comparative analysis of its water-soluble proteins from cells cultured in both normal and Co^{2+} enriched media. Comparative analysis was achieved by 2DE (two-dimensional gel electrophoresis) and peptide mass fingerprinting protein identification. Isoelectrofucusing (IEF) was performed with 4-7 pH IPG strips, improving a previous published protocol for *R. sphaeroides* (3), see figure 1.

By comparison of optimized 2DE maps it was possible to identify about 100 statistically different spots involved in cobalt response. Functional annotation could be made for 21 of them. Generally, a strong reduction of same anabolic pathways'enzymes (nitrogen organication, protein biosynthesis, DNA replication and chlorophyll biosynthesis) is evident in bacteria grown in Co^{2+} rich media, while catabolyc enzymes are expressed at higher concentrations, as also a hypothetical signal peptide protein, the RNA polymerase beta subunit and the aspartyl/glutamyl-tRNA amidotransferase subunit B.

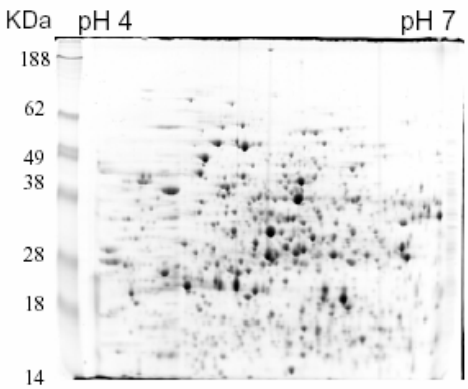

Figure 1: Control 2DE Map of *Rhodobacter sphaeroides* total soluble protein fraction.

In addition, the cobalt responce produces the down-regulation of two different periplasmic proteins: ModA (periplasmic component of an ABC transporter) and the MdoG (probably involved in a polyanionic periplasmic glucan biosynthesis). We propose, therefore a cobalt active resistance mechanism based on their downregulation in Co^{+2} grown cells leading to a reduced transport of cobalt ions into the cell.

Other studies are necessary, however, to better characterize both the overall response and specific pathways' regulation involved in the bacterium response. Under this view proteomic analysis of other specific *R. sphaeroides* sub-proteomes (membrane and secreted proteins) can contribute to the general description of the adaptation.

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P O S T E R S

Analysis of Emmer (*Triticum dicoccon* Schrank) Mature Seed Soluble Proteome [P01]

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The Italian word "farro" designates a group of cereals whose seeds are characterized by a hulled habit; in fact, after threshing, their grains still appear covered by the spikelet glumes. Differences in their ploidy levels are quite distinctive in that these species are, respectively, diploid (2n = 14; *T. monococcum* L.), tetraploid (4n = 28; *T. dicoccon* Schrank) and hexaploid (6n = 42; *T. spelta* L. syn. *T. aestivum* subsp. *spelta*), and correspond to the hulled wheats commonly known as einkorn, emmer and spelt. Emmer is the farro species commonly cultivated in Italy.

The cereal seed, besides the embryo, comprises the endosperm tissue which in turn is constituted by the bulky starchy endosperm (flour) and the surrounding aleurone (bran). The aleurone tissue contains exclusively soluble storage proteins that, on the other hand, represents only a minor fraction of the starchy endosperm total proteins. In mature seeds the starchy endosperm is made of dead cells while the aleurone cells mantains full vitality and plays many regulatory functions in the seed physiology; these functions are mediated by several soluble proteins. Among these proteins are also included factors possibly responsible for the emmer adaptability to adverse ecological conditions that, on the other hand, may result in compounds negatively affecting human nutrition (antinutrients and allergens).

We analyzed and compared aleurone and starchy endosperm 2D-maps (IEF, pH range 3-10 X 12% SDS-PAGE) of the soluble proteins, to reveal possible pattern of qualitative differential expression.

Protein spots particular to one of the tissues, and therefore deemed relevant to its characterization, were excised from the gels and analyzed by mass spectrometry and bioinformatics screening.

Comparative proteomics of an old sicilian durum wheat [P02]

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Timilia is an old Sicilian durum wheat cultivar largely used in the first part of the last century, characterized by high resistance to dry conditions and other abiotic and biotic stresses. *Timilia*'s bread is a long shelf-life product and shows flavour quality, probably due to its protein composition. For this reason, it holds remarkable interest as a "biodiversity source" of *Triticum* durum genus.

In this work, the proteomic profile of *Timilia* was compared with that of *Simeto*, an Italian durum wheat variety representative of the most currently used commercial cultivars. Initially, the water soluble (metabolic proteins) fractions of *Timilia* and *Simeto* were separated by 2D-PAGE. The spots of two gels were excised, digested by trypsin and analysed by MALDI-TOF MS. The mass spectral data were used for database-searching. The investigation of 50 spots in *Timilia* and 70 spots in *Simeto* allowed to identify 22 proteins in 43 spots, and 29 proteins in 59 spots, respectively. Some proteins (Peroxidase 1, Endogenous Alpha-Amylase/Subtilisin Inhibitor, Glyceraldehyde-3-phosphate dehydrogenase, 27K Protein, Triosephosphate isomerase and Dehydroascorbate reductase) are present both in *Timilia* and *Simeto.* Both cultivars also share homologous proteins belonging to the Globulins and Alpha-Amylase Trypsin Inhibitors family. In contrast, two Globulins, one Secretory protein, one Heat shock Protein class I, two Tritins, one Actin and a group of Serpins are expressed only in *Simeto* but not in *Timilia.* All these proteins differentially expressed in *Simeto* have a protective function and their absence in *Timilia* can be explained supposing that the defense mechanism of the ancestral *Timilia* is different from that of other current varieties. The absence of these proteins in the *cv. Timilia* could provide a differentiation method of its flour in comparison with the flours derived from the common cultivars.

The research was also extended to the storage proteins. In particular, in the *Timilia* a Bx- and a By-type High Molecular Weight Glutenin Subunit (HMW GS) were identified by *Peptide Mass Fingerprint* (PMF). The sequences of the two proteins were compared with the corresponding HMW GS of *Simeto* and the differences were characterized. Diagnostic fragments, produced by tryptic digestion of these Bx and By Glutenin Subunits, permit to identify unambiguously the *Timilia* flour when it is present as a mixture with other cultivars.

Identification of wheat glutenin proteins by proteomic analysis [P03]

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A proteomic-based approach was used for characterizing glutenins from an Italian wheat cultivar (Svevo). A two-dimensional gel electrophoresis (2-DE) map of roughly thirty individual spots was obtained by submitting the 50% isopropanol protein extract to isoelectric focusing on immobilized pH gradient across two pH gradient ranges, i.e., 3-10 or pH 6-11 in the first dimension, and to sodium dodecyl sulfate-polyacrylamide electrophoresis in the second dimension. The tryptic digest of each spot was characterized by MALDI TOF MS. Due to the low presence of lysine and arginine within the glutenin amino acid sequence, for accurate identification of the parent protein, it was necessary to analyze the tryptic peptides at either low or high molecular mass range. The spot digests were analyzed after both oligo Poros R2 and R1 resin purification. This step was particularly important to improve high molecular mass detection. The measured mass values were subsequently scored in Mascot databases for sequence determination. By mass fingerprinting, we identified High and Low Molecular Weight glutenins such as those of the A-, B-, and D-type. These results give insight into the complex nature of gluten heterogeneity. This approach also provided us with sound reference data for differentiating gluten composition amongst wheat varieties.

Immunogenic proteins and peptides in beer: a proteomic approch [P04]

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Malt derived from germinated barley or other celiacogenic cereals is the basic ingredient used in brewing. Because of the uncertainties in assessing the presence of toxic gliadin-like epitopes, usually beer is not allowed in the coeliac diet on a "*a priori*" basis.

The average protein content of beer is 0.2-0.6 g/100 ml, originating mainly from barley and in an almost negligible amount from yeast. Beer contains both intact proteins and products of proteolytic and chemical modifications formed during brewing. Major amounts of two polypeptides, Z_4-barley protein and non-specific Lipid Transfer Protein, deriving from the water soluble fraction of barley endosperm and, recently, sub-nanomolar amounts of a non-degraded hordein (the ethanol soluble fraction of barley) have been detected in beer. Z_4 and ns-LTP are particularly resistant to thermal treatments and are implicated in induction of asthma, urticaria and even IgE-mediated anaphylaxis in predisposed patients, in a way non related to coeliac disease.

We report on a proteomic approach based on electrophoretic/mass spectrometry and on "bottom up" LC-MS/MS analysis for polypeptide identification in two Italian national beer samples, made with different processes by the same producer. We aTimed at characterizing the main low molecular mass polypeptide fragments generated in the brewing process and to evaluate the presence of possible immunogenic epitopes in beer. By combining a competitive ELISA assay and MS analysis, we are restricting to a very limited number of candidates the epitopes potentially responsible for triggering intestinal inflammation in coeliac patients.

Proteomic Pattern and Phenols content: a Promising Tool to Monitor *Posidonia* Meadows Health State [P05]

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The endemic seagrass Posidonia oceanica (L.) Delile colonizes mobile substrates producing highly productive meadows that play a crucial role in coastal ecosystems dynamics. Human activities and natural events are responsible for a widespread meadows regression; to date the identification of "diagnostic" tools to monitor conservation status is a critical issue. In this study the feasibility of a novel tool to evaluate ecological impacts on Posidonia meadows has been tested: a stress indicator, i.e. phenols quantification, coupled to 2-D electrophoretic protein analysis of rhizome samples. The overall expression pattern from Posidonia rhizome was determined using a preliminary proteomic approach, 437 protein spots were characterized by pI and molecular weight. We found that protein expression differs in samples belonging to sites with low or high phenols: 22 protein spots are peculiar of "low phenols" and 27 unique spots characterize "high phenols" samples. Posidonia showed phenols variations within the meadow, likely reflecting heterogeneity of environmental pressures. Comparison of the 2-D electrophoresis patterns allowed to highlight qualitative protein expression differences in response to these pressures; differences may account for changes in metabolic/physiological pathways as adaptation to stress. The combined approach phenols content/proteomic pattern seems a promising tool to monitor Posidonia medaws health state.

Application of shotgun proteomics with HPLC-Chip system to the differential analysis of the major storage proteins in legumes [P06]

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In the last few years there is a growing demand of innovative methods able to assure the traceability of specific food ingredients along the production chain. When the components to trace are proteins, in particular in the case of allergens or bioactive proteins, proteomic methods have a major role. Liquid chromatography coupled with mass spectrometry (LC-MS) allows to profile large numbers of proteins in complex mixtures (1).

In shotgun proteomics, a complex protein sample is digested with trypsin and then the heterogeneous mixture of peptides obtained is analysed with classical reversed–phase LC-MS. The main advantage of these methods is the reduction of the reliance on protein fractionation by chromatographic or 2-DE approaches. When applying this technique, the efficiency of the chromatographic system is a very critical point as the peptide mixtures are very complex. Consequently, the application of the new integrated microfluidic device HPLC-Chip appears to be particularly promising to improve the sensitivity and the reproducibility.

Shotgun proteomics is not a quantitative technique per se. Although the intensity of a peptide peak depends linearly on the concentration of the peptide, different peptides have different propensities for ionization. Therefore, two peptides present in equimolar amounts may show different mass spectra intensities. In the case of label-free shotgun proteomics, this results in a relative protein profiling approach.

Recent literature data (2) shows the possibility to develop semiquantitative label-free methods in shotgun proteomics by spiking the complex mixture with a well-know exogenous protein as an internal standard.

Here, we described an innovative analytical method based on shotgun proteomics for the simultaneously identification and relative quantification of legume proteins. The use of the Spectrum Mill MS Proteomics Workbench software for processing raw MS/MS data permitted to develop a reliable approach for the differential analysis of target protein and provides also a statistical evaluation (3) of the results.

Applying this approach to the comparison of the storage proteins of different cultivars of *Lupinus albus*, we demonstrated that it is possible, without any previous fractionation, to detect all main proteins and to compare their relative abundance in the different cultivars.

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3. Perkins PD et al., *53rd ASMS Conference on Mass Spectrometry*, 2005

Analysis of the Proteome Released from *Lupinus albus* Seeds at Early Germination Stages [P07]

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Upon imbibition, the quiescent dry seed rapidly resumes metabolic activity. Cell structures, mRNAs and enzymes necessary for this initial activation are already present within the mature dry seed. In particular, hydrolytic enzymes are synthesized to mobilize the stored reserves and to favour radicle extension through the structures surrounding the embryo. This latter event marks the end of germination and the onset of seedling growth. Although the general mechanisms of germination have been extensively studied, some aspects are still obscure.

During the first steps of germination (i.e. 3-4 days) Lupinus albus, a legume seed, releases various kinds of proteins outside the fully viable seeds. The type and quantities of the polypeptides accumulated in the medium vary continuously during the germination, indicating a modulated mechanism and a functional role of these proteins, never described in detail before.

Aim of the present work is to study the time course of the protein release and to characterize the polypeptides accumulated into the germinating medium by 2D electrophoresis and mass spectrometry. This would help to unveil the role of these polypeptides and the mechanisms of their release outside the seed.

Lupinus albus (var. Multitalia) seeds have been vernalized overnight in deionized water, at 4° C. Then the seeds hav e been manually dehulled, soaked in deionized water (1 mL/seed) and kept at 21° C under mild shaking. The incubation time has been extended up to 72 hours.

Two experimental settings have been performed. In the first, aliquots of the medium have been withdrawn at given times and the concentrations of either the ionic potassium and the released proteins have been determined (Figure 1). In the second setting, the water medium has been collected and replaced every 24 hours. In this case, the proteins have been analyzed by two-dimensional (2-D) IEF/SDS-PAGE and characterized by mass spectroscopy. Figure 2 shows the protein profile of the medium after 24 hours of incubation.

The full set of results obtained indicate that the release of polypeptides in the germination medium is not caused by a passive leakage from the seed but rather it is driven by a specific mechanism, as shown by the release/reabsorption curve of ionic potassium (1). Moreover, the characteristic potassium curve matched that one of protein secretion into the medium. The electrophoretic pattern appeared relatively simple in term of composition, being formed by only few types of spots. Some of them are released immediately after the onset of germination (before 24 hours) and are accumulated for the whole time considered; other polypeptides appeared and/or disappeared in the subsequent timing (48 and 72 hours). The overall quantity of proteins released in the medium decreased with time.

In conclusion, the fact that some polypeptides are specifically released outside the seed during the early stages of germination indicate a well defined role, possibly in the defence mechanisms against microbial pathogens (2).

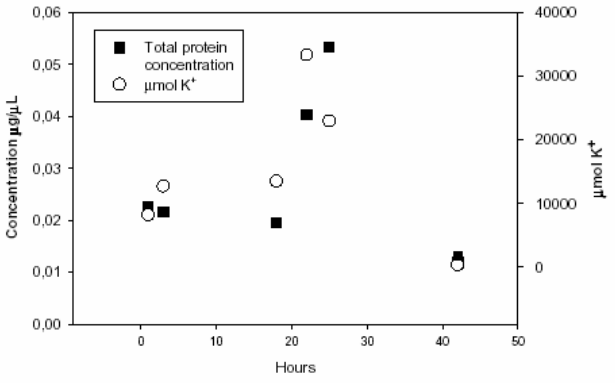

Figure 1: *see text for details*

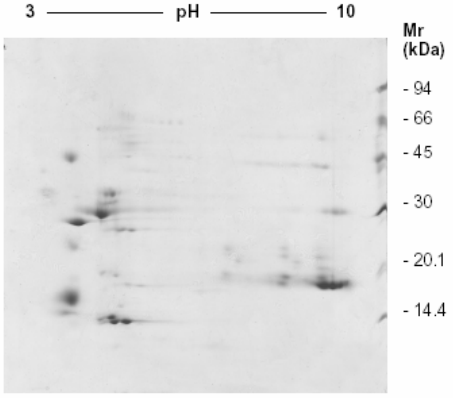

Figure 2: *see text for details*

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Is Proteomics fair to unveil "moonligthing" proteins? The case of CLIC1 [P08]

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Introduction

Multifunctional or "moonlighting" proteins have turned out to be surprisingly common, and examples of proteins with many proposed functions have emerged [1,2]. Cells have been demonstrated to develop sophisticated mechanisms for switching between distinct functions of these proteins [3,4]. CLIC1 is a cellular protein proved to arise membrane chloride channels upon an oxidative stress [5]. According to crystallographic data, it has been suggested that in the structure of the monomeric and soluble form of this protein an oxidative burst does induce, *via* the formation of a specific intramolecular disulfide bond, a dramatic conformational shuffling to a membrane soluble dimer [6]. Very recently CLIC1 overexpression was demonstrated to correlate with human gastric carcinoma and with the insulin signaling in human hematopoietic cells [7,8]. With the aim to investigate on relationships between amyloidogenesis, oxidative stress and perturbation of cellular permeability, recently we started a funded research (PRIN 2005, prot. 2005054591) in which proteomics and metabolomis have been extensively applied. In these studies, CLIC1 has been used as a model of proteins for which changing in expression, structure, localization and biological function induced by perturbing events on cellular environment such as an oxidative burst, could be valuable events.

Results

We demonstrate that in the proteome of microglia cells an oxidative burst induces fast changes in GSH/GSSG ratio and long-term increment in the proteic carbonyl groups, revealing an impairments of the redox defenses. The oxidative stress does not induce changes in CLIC1 expression, as proved by differential 2DE analyses of the two cellular lysates. Conversely, a dramatic change of CLIC1 both in the physico-chemical surface properties (RP-HPLC retention time and GSH affinity) was demonstrated by in vitro assays, and a more complicated proteomic approach based on a previous separation of cellular compartments (allowed us to unveil the different location of CLIC1 from cytosol to cellular membrane) upon an altered redox state. According to this data, CLIC1 is expected to act as an further transductive intracellular element, by which an intracellular oxidative stress can induce significant change in the cellular membrane properties.

Conclusions

Traditional 2DE-dependent proteomics failed to reveal the alternative localization of CLIC1 and thus is not suitable in general to detect all possible changes (*e.g*, intracellular protein trafficking) in the biological properties of a selected proteome. On the contrary, the approach we applied in this study may be valuable to fish "moonlighting protein" for better defining a dynamic proteome.

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Characterization of Olive Seed Storage Proteins from Different Cultivar by SDS-PAGE and MALDI Mass Spectrometry [P09]

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Olive (*Olea europaea* L.) is one of the most important oil-storing crops in many countries, due to its high productivity and the quality of its oil.

Storage proteins are formed during seed development and deposited predominantly in specialized storage tissues, like the cotyledon or endosperm (1). Correctly formed and assembled mature storage proteins are steadily accumulated, and their degradation happens, only after a long period of rest when seeds germinate and seedlings start to grow.

Interest in seed storage proteins is increasing, because of the importance of regulation of gene expression, as a matter of fundamental research and the high biological value of these proteins representing a major parameter of nutritional quality in commonly used crops .

Basic knowledge about the presence of seed storage proteins in olive seeds doesn't exists to date. However, seed storage proteins are the plant proteins most abundantly consumed by humans, but olive seeds are not, because whole fruits are processed for oil production (2).

In this work olive seed proteins profile of six different Mediterranean cultivars (Minuta, Canino, Frantoio, Verdello, Lezze, Tonda di Filogaso) was characterizated by SDS-PAGE and MALDI mass spectrometry.

Portions (50 mg) of seed powder were extracted with aqueous ammonium bicarbonate and also with only water.

The proteins pool is denatured with chloroform/methanol and resuspended in water and/or ammonium bicarbonate.

Electrophoratic pattern of olive seeds extract in non reducing and reducing conditions reveals the same well-resolved bands for each cultivar considered, with molecular weights ranging from 20 to 55kDa (Fig. 1)

Each spot was excised and in-gel digested with trypsin and peptides mixture were analyzed by MALDI/ TOF.

All tryptic digested bands were matched through Mascot programs (www.matrixscience.com) and they are recognised as oleosins.

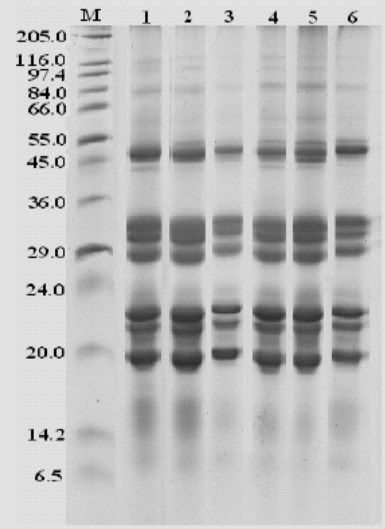

Figure 1.SDS-PAGE separation of Olive (Olea europaea. L) seed proteins 1.Minuta 2.Canino 3.Frantoio 4.Verdello 5.Lezze 6.Tonda di Filogaso. M Molecular weights (in kilodaltons) are shown to the left.

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Proteomic Analysis of Fruits of three Peach (Prunus persica) Cultivars with Different Flesh Firmness Characteristics During Ripering [P10]

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The quality of peach (*Prunus persica*) is mainly linked to the components of fruit taste (*e.g.* sugars, organic acids and volatiles) and flesh texture. The latter is one of the most important factors determining the consumer's perception of fruit quality and commercial characteristics. On the basis of flesh texture, peach fruits are classified as either melting flesh (MF) or nonmelting flesh (NMF).

Decrease in fruit firmness occurs during ripening of both phenotypes, but is more pronounced in MF fruits, where a marked loss of firmness (*i.e.* melting of the flesh) occurs in the final stages of ripening.

Moreover, the ripening of this climacteric fruit is characterized by deep biochemical changes to sustain characteristic changes in the metabolic composition. According to these modifications, using a microarray approach, it has been found that a few hundreds genes are up- or down-regulated during the transition from pre-climacteric to climacteric phase. These genes are involved in specific traits of primary and secondary metabolisms. At present only studies at the transcriptomic level are available, while no proteomic studies investigating the ripening process of the peach fruit have been performed yet. The main problem in resolving protein mixtures is the setting up of a satisfactory protein extraction protocol. This is more evident in fruits where a great number of interfering compounds is present.

The first aim of this work was to improve the protein extraction protocol that was modified starting from a previously proposed one, which consisted of a phenol extraction followed by a methanol/ ammonium acetate precipitation. After setting up a protein extraction method suitable for removing interfering compounds, a 2-DE analysis was performed using a pH 3-10 linear gradient in the first dimension and 12.5% polyacrylamide homogeneous gels in the second dimension. A preliminary two dimensional map of peach fruit proteome was thus obtained. In order to investigate the biochemical and physiological processes that characterize peach fruit ripening, three genotypes with different firmness were compared: i) *Bolero*, a melting flesh cultivar; ii) *Oro A*, a non melting flesh one; iii) *Ghiaccio*, a stony hard cultivar characterized by fruits able to maintain high flesh firmness and that in addition lacks ethylene synthesis.

Using the ImageMaster Platinum software a comparative analysis was then carried out. Sixty-three spots changed significantly their expression among the six conditions. The bidimensional hierarchical cluster analysis showed a similar trend for *Bolero* and *Oro A*, whereas a peculiar and quite different cluster was found for *Ghiaccio*. Moreover, some spots were related to a specific stage of ripening as well as to a specific cultivar only.

The LC-ESI-MS/MS approach was used to analyse the differentially expressed spots. Some of the identified proteins were involved in a few metabolic and physiological processes characterizing fruit ripening, while others were related to the flesh firmness event. These preliminary data underline the power of the proteomic approach to improve the knowledge of ripening processes even to identify molecular markers that could be useful to define, and possibly predict, features of peach fruit.

Differences in the Root and Leave Proteome of Maize (Zea mays L.) Plants Grown in Different Nitrogen Availability [P11]

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Nitrogen is the mineral nutrient most abundantly required by plants. Nevertheless, its availability in the soil is subjected to large fluctuations due to abiotic processes (*e.g.* erosion and leaching) as well as to biotic processes such as microbial activity. Moreover, many crop plants are poorly able to utilize this nutrient; it has been estimated that, under best conditions, the use efficiency of cereals is not higher than 50%. In order to maintain adequate nitrogen availability in the soil, which is an essential condition to obtain high yields, farmers have used larger and larger nitrogen fertilization with the final effect of increasing, besides the production costs, the environmental pollution. Selection of plants with higher efficiency for the use of available nitrogen is therefore an essential prerequisite for sustainable agriculture. The achievement of this objective is strictly linked to an in-depth knowledge of the processes involved in the acquisition of this mineral nutrient by plants, which is mostly present in the form of nitrate in cultivated soils.

Nitrate assimilation, which can occur in both roots and leaves, requires a first step of NO_3^- reduction to ammonium by the enzymes nitrate reductase (NR) and nitrite reductase (NiR) followed by transfer of ammonium onto α-ketoglutaric acid by the enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT). Both systems involved in nitrate assimilation are induced in the presence of the nutrient and are de-induced in its absence or when the nutritional requirement is satisfied. The activation of these processes, together with the possibility of supporting them, is strictly linked to the processes supplying carbon skeletons and metabolic energy, such as photosynthesis, respiration, the pentose-phosphates cycle and other activities such as amino acids and protein synthesis and secondary metabolism; the efficiency in nitrogen acquisition will thus directly depend on the ability of modulating all these cell functions.

To date, many studies have been devoted to the expression at the transcriptional level, whereas only a few are the researches which have analysed expression at the proteomic level. To study this aspect, the effect of nitrogen availability on protein expression in roots and leaves of maize was investigated by comparison between the 2-DE gels of the soluble fractions extracted from plants grown in the absence of nitrogen or incubated in the last 30 hours in the presence of 10 mM nitrate.

The expression of 17 spots in the roots and 16 in the leaves was affected by nitrogen availability. Their characterization by LC-ESI-MS/MS analysis allowed the identification of proteins mainly involved in nitrogen and carbon metabolisms. In particular, in roots, we found an increase of some proteins (*e.g.* cytosolic 6-phosphogluconate dehydrogenase, nitrite reductase, glutamine synthetase) suggesting a reactivation of nitrogen metabolism. Moreover, the addition of nitrate induced changes on secondary metabolism, as inferred by the decrease of phenylalanine ammonia-lyase (PAL). In leaves, the greater changes interested proteins related to the photosynthesis processes, such as a putative RuBisCo subunit binding-protein â-subunit and a putative 33kDa oxygen evolving protein of Photosysthem II. According to an activation of photosynthesis, a decrease of 6-phosphogluconate dehydrogenase was observed. Moreover, two spots were identified as photosynthetic phosphoenolpyruvate carboxylase (PEPC) whose isoelectric point values suggested to be respectively the phosphorylated and un-phosphorylated forms of the same enzyme. A different ratio between the two forms was observed in the plants treated with nitrate in comparison with the starved plants, suggesting an effect of nitrogen starvation on the circadian control of PEPC. Finally, the relative spot volume referable to PAL also decreased in leaves, according to a slight modulation of phenylpropanoid secondary metabolism.

This work provides a first characterization of the proteome changes occurring in roots and leaves of maize plants grown in different conditions of nitrogen availability. These results suggest that some proteins involved in the nitrogen and carbon metabolisms are affected by nitrate availability in their expression and/or in post-translational control. Further studies are necessary now to define in detail these different aspects.

Differences in the Protein Profile of the Ripe Berry Skin of Four Different Grape Cultivars [P12]

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Grape ripening is characterized by deep changes in the metabolic pathway that is necessary to sustain the synthesis of primary and secondary compounds. The quality of wine grapes will be strictly depending to the resulting metabolic composition. A central role in the developing of the main secondary compounds (*e.g.* anthocyanin pigment) is played by skin. Their accumulation that occurs predominantly in this tissue begins at "véraison" and is responsible of the colour-change in black cultivars. Moreover, skin also constitutes a barrier to prevent pathogen infections. Despite of its important role, a few studies have been performed on isolated skin tissue. A first proteomic study comparing three stages of ripening showed that numerous skin soluble proteins involved in photosynthesis, carbohydrate metabolism, stress response and anthocyanin synthesis changed their expression from "véraison" to complete ripeness.

A possible approach to obtain further knowledge about the physiological role of this tissue could be to compare genotypes that are known to be different in the anthocyanin accumulation. In this view, a comparative analysis among the proteomes of the ripe berry skin of four different grape cultivars such as "Riesling Italico", "Croatina", "Pinot gris" and "Pinot noir" was performed. The evaluation of anthocyanin content showed that these compounds were undetectable in the white cultivar "Riesling Italico" while they progressively increased in "Pinot gris", "Pinot noir" and "Croatina" respectively.

After extraction, proteins were separated by two-dimensional electrophoresis (2-DE), using a pH 4-7 linear electrofocusing gradient in the first dimension and 12.5% polyacrylamide homogeneous gels in the second dimension. The analysis of the gels stained with CCBB detected about 1300 spots. The comparison of the proteomes of different genotypes showed significant differences in the expression of about 200 spots. The characterization of some of these spots by LC-ESI-MS/MS analysis allowed the identification of proteins involved in the physiological processes such as stress, defence, carbon metabolism and energy conversion , such as HSP70, glutamate decarboxylase, NADH-ubiquinone oxidoreductase, SOD, PPO, ABA stress ripening protein and methionine synthase.

Moreover, differences in the expression of proteins involved in the secondary metabolism were also found. A good correlation was observed between anthocyanin accumulation and the expression of some enzymes involved in the flavonoid pathway (*i.e.* flavone-3-hydroxylase, F3H and leucoanthocyanidin dioxygenase, LDOX). Different isoforms of some enzymes, such as isoflavone reductase (TPA), were specific for some cultivars. These data suggested that the comparative proteomic analysis among genotypes with different chemical profiles seems to be a good tool in order to characterize the secondary metabolism detecting peculiar activities and relating protein expression patterns to the abundance of interesting compounds.

The hierarchical cluster analysis pointed out that the four analyzed cultivars were clearly distinguishable and the order in which they were grouped may reflect both their relative anthocyanin content and their genetic relationship. In order to reduce the effects of the environment on the observed variations, the analysis is being repeated using berries of the following vintage. These data indicated that such a proteomic approach could contribute to the puzzling grape phylogeny debate, providing a bridge between genome and phenotype.

A proteomic approach to unravel lateral root formation in woody plants under environmental stress condition [P13]

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The formation of lateral roots is an important event in plant morphogenesis, since they form a major component of the root system (Lloret and Casero, 2002). Lateral roots at the correct positions and in appropriate numbers, enable the plant to establish a root system capable to efficiently accomplish water and nutrient uptake and to ensure a good anchorage (McCully and Canny, 1988; Varney and Canny, 1993; Wang et al., 1991, 1994, 1995).

A large number of evidences, show that root morphology is guided by genetic program but the ultimate configuration of a root system is largely determined by environmental factors. The literature on the effects of environmental conditions on root system architecture is very wide (Casimiro et al . 2003; Forde & Lorenzo 2001; Lopez- Bucio, Cruz-Ramirez & Herrera-Estrella 2003). Malamy (2005) proposes that root development depends from two pathways: (1) "intrinsic" pathways that are essential for organogenesis and growth, and that determine the characteristic architecture of the plant; and (2) "response" pathways that determine how plants respond to external signals to modulate the pathways in (1). In the herbaceous model plant, Arabidopsis thaliana, lateral root formation is initiated close to the root tip, with anticlinal divisions in pericycle cells adjacent to the protoxylem poles, giving rise to lateral root primordia (LRP) (Dolan et al., 1993; Dubrovsky et al., 2000; Malamy and Benfey,1997a; Malamy and Benfey, 1997b). The subsequent development of the primordia follows a series of highly ordered cell divisions that ultimately lead to the emergence of lateral roots (Malamy and Benfey, 1997a; Malamy and Benfey, 1997b). However, the positions of lateral root primordia along the main root axis are stochastic and do not follow a pattern, indicating that not all the pericycle cells adjacent to the protoxylem poles will give rise to a lateral root (Dubrovsky et al., 2000). In fact during the establishment of root architecture, signals from inside and outside the plant are transmitted to pericycle cells where the initiation of lateral roots takes place (Bhalero et al., 2002; Malamy and Ryan, 2001). Another intriguing and mostly unexplored aspect, of root development, is that in response to environmental factors plants may modify root architecture by developing lateral roots from a secondary body, where the pericycle is missing. The literature reports several examples of woody plants developing lateral roots from a secondary structure in response to external cues as flooding or artificial root pruning (Wilcox, 1955, 1968; Bogar and Smith, 1965; Kozlowski, 1984).

However, up to now, the physiological and molecular mechanisms involved in lateral roots development from a secondary body of woody plants, have not been investigated yet.

In the present study, the plant model Populus nigra is used to investigate mechanisms involved in lateral roots formation from a secondary tissues. In particular, a proteomic approach is used to analyze the temporal and spatial alteration of the proteome of tap roots subjected to mechanical stress, and to identify gene factors involved in the development of lateral root from secondary tissues.

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Proteomic analysis of grape berry during ripening by DIGE technology [P14]

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INTRODUCTION

Grape ripening is a key growth phase for determining the quality of grapes, as it immediately precedes harvesting. To predict wine quality produced from defined grape varieties, mature berry characteristics, such as skin colour, titratable acidity, soluble solid concentrations, and volatile aroma compounds, are essentially evaluated. Anthocyanin pigment accumulation starts in skin cells at veraison and continues through the ripening phase. Ripening is also characterized by increasing of grape size, softening, and cell expansion resulting in water and sugar accumulation in the mesocarp cell vacuoles.

Therefore, the understanding of molecular mechanisms regulating berry maturation can provide fundamental information useful to optimize vintage quality. Moreover, there is not any published datum concerning protein stability during withering process, which precedes and affects wine preparation. In this work we present a comparative proteomic analysis using DIGE technology on grape berry of *Corvina* variety during ripening/withering process.

EXPERIMENTAL RESULTS

Protein extraction from grape berries is a complex process, because of non-protein contaminants, which interfere with the 2-DE analysis.

We tested different protocols based on TCA/acetone and phenol to extract total soluble proteins from various ripening

stages (from pre-veraison to withering) of *Corvina* berry (skin and pulp). The TCA-based protocol resulted superior to the phenol method, displaying higher proteins yield and resolution. The optimized extraction protocol was applied to seven ripening stages and the corresponding proteomes are being analyzed by 2-DE using DIGE technology.

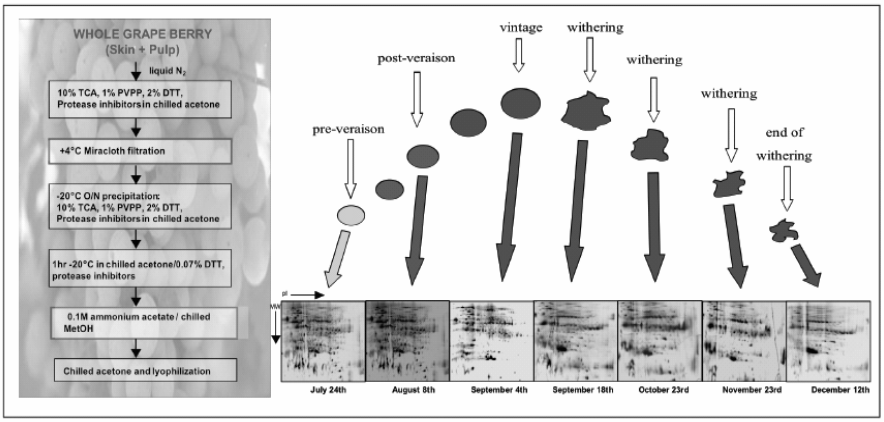

FIGURE - Experimental procedure adopted to obtain an adequate extraction and 2-DE separation of protein samples. Seven berry samples, collected during ripening and withering, were analysed. For 2-DE 18 cm strips pH 3-11NL were used. In this preliminary analysis proteins were revealed using silver staining.

CONCLUSIONS

This comparative analysis will contribute to the knowledge of the physiological role of protein associated to grape berry

development and will provide the first dynamic profile of the grape berry protein expression during maturation and withering. The same grape berry samples used for proteomics analysis are currently under investigation by both transcrittomics and metabolomics methodologies. An integration of the differential results obtained with different technologies will provide an innovative and comprehensive approach able to elucidate the main aspects involved in berry maturation.

AKNOLEDGMENTS

This work is part of the national project "Vigna" ("VItis GeNome Analysis" – "Caratterizzazione Strutturale e funzionale della Vite") funded by Italian Ministry of Agriculture.

Screening of Monoclonal Antibodies to Isolate Grape Maturation-Related Proteins [P15]

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Grape maturation involves biochemical events in both primary and secondary metabolism. In order to isolate grape maturation-specific polypeptides, we searched for monoclonal antibodies aimed at proteins whose expressions undergo variation during the process of grape maturation. Since most of the processes involved in fruit ripening are localized in grape skin, we analysed the proteins from the skin of Barbera, taken at the moment of véraison (immature skin) and also seven weeks later (mature skin).

After immunisation of 6 Balb/c Mice with these two total protein extracts (mature and immature antigen), differential ELISA screening was carried out and we selected a panel of specific monoclonal antibodies for both maturation stages. The specificity of the selected antibodies was verified by two-dimensional electrophoresis: the antigens of greater interest will be identified through mass spectrometry.

The obtained results will allow the definition of a panel of molecular markers to use in a more careful definition of the grape ripening processes.

Integrated Proteomic Approach to Highlight the Differences in Protein Composition between *Lupinus albus* and *Lupinus angustifolius* [P16]

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Sweet lupin proteins are gaining attention from industry and consumers because of their possible role in the prevention of cardiovascular disease. With Australian narrow-leaf lupin (L. angustifolius) now available in the European market it is important to determine whether the potential nutraceutical properties of this lupin species differ from that of white lupin (L. albus) more commonly found in Europe. Three varieties each of L. angustifolius and L. albus were analysed via 2D-electrophoresis, anion-exchange chromatography and chromatofocusing, with CHIP LC-MS/MS also being used as a tool to confirm the identity of some gel spots.

As a first step it was important to guarantee that the protein extraction method was efficient and reproducible. The six varieties were therefore extracted in 100 mM Tris-HCl ± 0.5 M NaCl, pH 8.2.

The protein extracted was iso-electrically precipitated, and the precipitate was resuspended in 100 mM Tris-HCl at pH 8. When separated on a DEAE Fast Flow column with a linear salt gradient it became clear that without salt the protein extraction was not reproducible. Instead, when extracted in the presence of salt, the chromatograms of the three albus varieties overlapped each other, as did those of angustifolius. Another distinguishing aspect was the ratio of vicilin: legumin peak area, with L. angustifolius having an approximate peak area ratio of 1:2, and L. albus 1:1. An exemplary chromatogram is shown below in figure 1.

In order to confirm that these differences in vicilin content were not due to protein loss during precipitation, a total protein extract of the same six varieties were analysed using 2D-electrophoresis. The same trend was found, with fewer spots of the 7S vicilin subunits being present on the gels of L. angustifolius.

Other notable differences on the 2D-maps were found for all the main globulin proteins. For example, in the area of the 2S proteins L. angustifolius had four distinct spots. This protein has previously been shown to be genetically heterogeneous, but this is the first time, to our knowledge that the subunits have been resolved according to their isoelectric point and thus been seen to be so numerous. Another interesting observation was that in the L. angustifolius varieties the small subunit of the 7S conglutin gamma protein has a lower molecular weight than found in L. albus, and the acidic legumin subunits a higher molecular weight. Highlighting these differences in protein composition between L. albus and L. angustifolius could help to understand differences in their bioactivity.

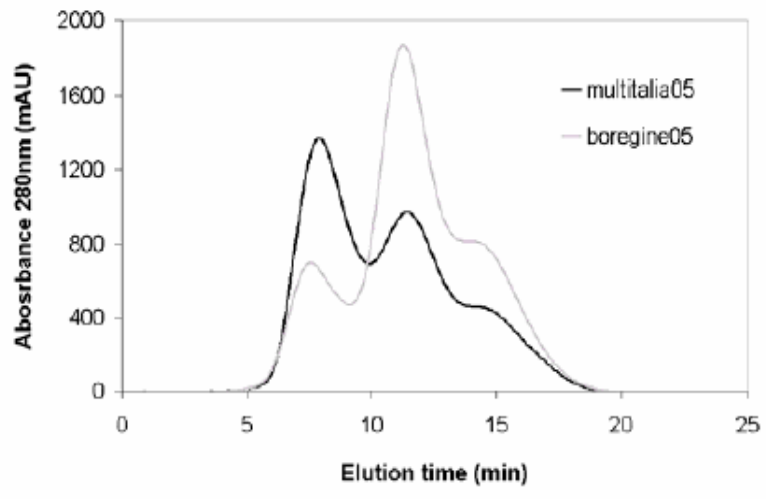

Figure 1: Exemplary chromatogram of a globulin protein isolate extracted from *L.albus* (cv. Multitalia) and *L.angustifolius* (cv. Boregine), eluted from DEAE FF under a linear salt gradient.

Protein Extractibility of the Olive Seed and Proteomic Approach [P17]

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Samples preparation for 2-DE analysis is a critical step and is absolutely essential for good results. In the case of the table olive, the cell wall and the vacuole make up the majority of the cell mass, with the cytosol representing only 1-2% of the total cell volume. The seed represents the 2-4% of the drupe and is the object of this work, which describes the specific problem of the proteins extraction with the aim to identify a molecular marker and define a traceability method applicable to the PDO table olive. The interest was focused on the protein component and because of the recalcitrant kind of tissue, three different methods of extraction, to obtain the best results in terms of amount of proteins extracted and quality of 2-DE maps, were evaluated. The best results were obtained using the phenol extraction and methanol/ammonium acetate precipitation protocol. Furthermore, in this work are shown the proteins 2-DE maps obtained with the different extraction methods. The best maps were used for the identification of proteins by mass spectrometry MALDI-TOF to approach a proteomic study.

Proteome Profiling of Mature Pollen of *Parietaria Judaica* [P18]

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Parietaria judaica pollen is one of the most common causes of airway allergic disease in the Mediterranean area. In order to characterize the protein content of this weed, proteome analysis of mature *Parietaria judaica* pollen was conducted using two-dimensional gel electrophoresis and mass spectrometry. The first reference proteome map was thus established by identifying 71 protein spots (including isoforms) involved in a variety of cellular functions such as carbohydrate metabolism, energy production, cytoskeletal organization, and stress response. The occurrence of allergens causative of widespread pollen allergy was also detected. On the basis of these results, further investigation will be focused on a more detailed characterization of *Parietaria judaica* allergen profile so to improve our comprehension of the pathogenesis of pollen-induced allergic reaction.

* These authors contributed equally to this work

Work supported by ARPA Sicilia

2D-DIGE and BN-PAGE for a new insight into tomato chromoplast proteome [P19]

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The fruit of the tomato plant is composed of elongated tomato cells filled with organelles called chromoplasts (plastids). Plastids are involved in numerous metabolic pathways such as nitrate assimilation, biosynthesis of starch, fatty acids, and assimilation of nitrogent into amino acids. Chromoplasts are found in coloured organs of plants such as fruit and floral petals, to which they give their distinctive colours. The main characteristic of ripening is the dramatic increase in the carotenoids (lipid-soluble pigments) content of the fruit, mainly lycopene in membrane-bound crystals within the plastids, and the disappearance of chlorophyll. Moreover, the chloroplast-chromoplast transition is marked by the degradation of the highly structured thylakoid membrane system, and a reduction in the levels of proteins and mRNAs associated with photosynthesis.To evaluate the metabolic role of chromoplasts during fruit ripening we have undertaken a proteomic study of this organelle. We are now using 2D gels, Blue-native PAGE (BN-PAGE) and Differential Gel Electrophoresis (DIGE). 2D-DIGE is based on direct labeling of lysine groups on proteins with cyanine CyDye DIGE Fluor minimal dyes before isoelectric focusing, enabling the labeling of 2-3 samples with different dyes and electrophoresis of all the samples on the same 2D gel. This capability minimizes spot pattern variability and the number of gels in an experiment while providing simple, accurate and reproducible spot matching. Since membrane proteins are under represented in 2-D gels due to their high hydrophobicity, BN-PAGE was used. BNPAGE is a special case of native electrophoresis for high resolution separation of enzymatically active protein complexes from tissue homogenates and cell fractions. The separation principle relies on binding of Coomassie blue G250 which provides negative charges to the surface of the protein. Chromoplasts from firm-ripe fruits have been purified using sucrose density-gradient centrifugation. The purity and integrity of the isolated chromoplasts have been evaluated by electron microscopy and the use of enzymatic markers (Cytosol, Mitochondria, Peroxisome, ER). After extraction with a phenol based protocol, proteins were labelled with CyDyes DIGE® fluorochromes (GE Healthcare). First dimension (IEF) was carried out on both 18 and 24cm pre-cast IPG strips (GE Healthcare) with a pH gradient of 3-11NL. The second dimension (SDS-PAGE) was performed using the Ettan DALTsix system. Analysis has been done using the DeCyder software® (GE Healthcare). BN-PAGE was performed as described by Reisinger et al. (2006). Spots for identification were excised from 2-D gels and BN-PAGE gels, tryptically digested and analysed by MALDI-TOF/TOF MS or nanoLC/ESI-Q-TOF MS. Acquired spectra were then searched with Mascot (Matrix Science, UK) against the NCBI database and blasted against the Tomato EST database. Chromoplasts from Wt and Transgenic tomato fruits were isolated and resolved using the three methods explained above. Total chromoplast protein fractions have been solubilized and analyzed on 2D-gels. Our preliminary results show clear differences between tomato chromoplasts from Wt and transgenic lines. The most abundant spots have been excised and digested with trypsine and the generated peptides analyzed by both nanoLC/ESI-Q-TOF and MALDI-TOF/TOF. Since tomato genome has not been yet fully sequenced, the obtained sequence fingerprints were matched against all sequence data available on public databases using the Mascot and dBEST engines. The identified proteins can be classified in the following groups: 1) metabolism; 2) ionic channels, ion transporter and related; 3) defence/repair; 4) miscellaneous; 5) Unknown; 6) Not identified. Membrane protein complexes were also studied using BN-PAGE. The BN-SDS PAGE technique has been proven to be a good method to resolve highly hydrophobic integral membrane proteins from chromoplast preparations. These membrane protein complexes are just now being analyzed by nanoLC/ESI-Q-TOF.

Comparative liver mitochondrioma of adult and old rat [P20]

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Oxidative stress has been implicated in aging. 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 aged liver, highlighting the post-translational modifications. No proteomic analysis of liver mitochondria in aged rat has been until now reported. Here we analyse the global changes of mitochondrial proteome by using rat liver mitoplasts from adult (12 months) and old rats (28 months) and two-dimensional electrophoresis.

Proteins were separated on 18 cm 4-7 pH gradient IPG strips and 10% SDS polyacrylamide constant concentration gels. The samples were run in triplicate and, after silver staining, the gels were compared using Image Master 2D Platinum 6.0 software. We choose spots based on statistically significant differences ($P< 0,05$) in the relative volume between adult and aged rats. Differentially expressed proteins were excised from the Coomassie stained gel, in-gel digested with trypsin and characterized by MALDI and ESI MS/MS mass spectrometry and western blot.

Preliminary results led to highlight 43 spots with altered expression in old rats. Mass spectrometry led to the identification of 28 spots that represent 20 different proteins. These proteins belong to different mitochondrial pathways: two proteins are involved in the beta-oxidation of fatty acids; three protein in Kreb cycle enzyme; one flavoprotein and four iron-sulfur proteins are subunits of NADH dehydrogenase complex and one protein of Ubiquinol-cytochrome c reductase. All these proteins have an increased expression in old rats. We found differential expression in antioxidant enzymes, an increase of glutathione peroxidase 1 and a decrease of the mitochondrial aldeyde dehydrogenase a detoxicant enzyme probably involved in the metabolism of 4-Hydroxy-2-nonenal (4-HNE) (Ohsawa et al.,2003). We also identified an increased expression of leucine aminopeptidase 3, a cytoplasmic esopeptidase and a decrease expression of three cytoplasmic chaperones. We identified, in old rats, a post-translational modified form of Peroxiredoxin III, a mitochondrial antioxidant protein. The peroxiredoxins are a family of antioxidant and ubiquitous peroxidases that catalyze the reduction of oxygen peroxide using a thiol as the other substrate. In cell cultures, the administration of H_2O_2 leads to the appearence of acidic spots for peroxiredoxins II and III (Rabilloud et al., 2002). The pI shift of the protein to a more acidic pI in the two-dimensional gel, is caused by overoxidation of the active site cysteine into cysteic acid, thereby adding a negative charge to the protein. This acidic form must be considered as an inactive form of peroxiredoxin. This modification has been found for the first time *in vivo* in rat liver.

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Detection and Sequence Determination of the Lactoferrin from Donkey [P21]

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Cow's milk protein allergy (CMPA) is the most common food allergy in childhood, with a reported prevalence of 2-7.5%. In such instances, and when mother's milk is not available or may not be advisable, hydrolyzed and hypoallergenic cow's milk formulas and soy bean-derived formulas have been used. Although clinical studies have reported that these alternative formulas provide adequate nutrition of infants, it should be considered that allergenicity in these products is reduced, but never completely suppressed. In popular tradition donkey's milk constitutes a good substitute for cow's milk in many children with severe CMPA.

Although milk from donkey has been used in infants with cow's milk allergy, limited data are available for their genetic polymorphism and the knowledge of its protein profile has not been fully investigated.

Compared with bovine milk, donkey's milk shows a very different protein composition. In fact, in donkey's milk whey proteins constitute 40-50 % of the nitrogen fraction, whereas in bovine milk they accounts for only 20% of the nitrogen fraction.

Here, we report the detection and sequence determination of the lactoferrin from donkey, whose amino acid sequence was still unknown, by combined use of RP-HPLC, deglycosylation, enzymatic digestions, mass spectrometry and database search.

Proteomic Tool to Study Fat Globule Membrane Proteins from Horse Milk [P22]

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Milk represents the main nutrition source for the newborns of each mammal during the neonatal period; moreover it is an important mean to transfer the acquired immunity against pathogens from the mother to the newborn. The milk secreted immediately after birth (colostrum) has an unique protein composition, containing high amount of specific immunoglobulins in addition to the major milk proteins (Csapò-Kiss et al, 1995). Mare's milk fat composition, in addition to the protein fraction properties, suggests that this product is more similar to human milk than cow's milk (Malacarne et al., 2002). In spite of several studies dealing with identification of mare's milk proteins, only the major whey proteins and some caseins have been already characterized. Due to the fact that the equine genome in not completely sequenced and the difficulty to recover very hydrophobic proteins, up to now little is known about the proteins associated to the milk fat globules, that have been shown to have an important role in defence mechanisms in newborn.

In the present study we describe a systematic proteomic analysis of mare's milk fat globule membrane proteins (MFGMP), by 2-dimensional electrophoresis (2-DE) including the identifications of the major proteins by mass spectrometry (MS).

Several protocols for sample preparation were tested in order to improve the extraction of the proteins associated to horse milk fat globule membranes. The first problem to solve for the membrane protein extraction, is to find the best solubilization buffer composition that has to prevent protein precipitation and to be able to extract as many proteins as possible. For this reason we evaluated a wide variety of detergents and the best results have been obtained with the buffer containing amidosulfobetaine-14. After extraction, a protein precipitation step was added in order to remove lipids that are the major contaminants in MFGMP preparation. The method suggested by Wessel and Flugge (1984), that is very effective in the removal of lipids, has been chosen. Another problem that had to be solved was the contamination by proteins associated to whey and casein fractions which tend to cluster fat globules, as confirmed by Keenan et al. (1983). For this reason an homogenization process of cream layer and skimmed phase was added. Most of the identifications were obtained by homology searching against Homo sapiens, Canis familiaris, and Bos taurus. Thanks to proteomic tools as De novo MS/MS sequencing and the traditional biochemical technologies as N-terminal amino acid sequencing, we were able to identify the major mare's MFGM proteins, such as butyrophilin, adipophilin, xanthine oxidoreductase and lactadherin. Butyrophilin and xanthine oxidoreductase are suggested to link milk secretory granules to the plasma membrane for secretion by interacting with adipophilin (Heid et al., 1996). Lactadherin is a multidomain protein and its phisiological function is not completely known; however in human milk a protective function at gut level against rotavirus infection has been demonstrated (Newburg et al., 1998).

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Sheep Serum Proteome [P23]

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Veterinary medicine needs sensitive and specific analysis to check physio-pathologic states that can occur during growth, differentiation and reproduction and to study, diagnose and control diseases. However the proteomic analysis of tissues and biological fluids is still poorly utilised in the control of animal health and productivity. This is mainly due to an incomplete characterization of the genome of different animal species. The investigation of serum proteome is still limited to cattle, dogs, pigs and horses1-2. Consequently, we focused on ovine due to their importance in the animal production breeding.

In this study, we present a reference 2-D protein map for sheep blood serum. The 2-D map was obtained by using both total and albumin depleted serum from ewes. The proteins were identified after tryptic digestion by mass spectrometry (MS) analysis using MALDI-TOF/TOF MS and ion trap MS. The latter was connected to a capillary electrophoresis system. MS and MS/MS data were combined and searched using the MASCOT algorithm and standard data bases.

The results obtained provide a reference map for the protein biomarkers which may have a diagnostic and prognostic significance for the sheep pathologies as well as may be used as biological indices of wellbeing.

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Isolation and characterization of high molecular weight proteins from pig epididymosomes by SDS-PAGE and MALDI TOF/TOF [P24]

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The final stages of mammalian sperm maturation occur outside the testis, in the excurrent ducts. The acquisition of fertilizing ability is mainly due to sequential interactions of male gametes with proteins secreted by epididymis in the intraluminal compartment by merocrine and apocrine secretion.The latter provides epididymal fluid with small membranous vesicles (0=25-100nm), named epididymosomes. The transfer of selected proteins from epididymosomes to sperm membrane has been reported in some species, suggesting that these vesicles can mediate modification of macromolecular components of sperm surface during epididymal maturation.

The protein composition of pig epididymis fluid has been investigated to suggest a possible mechanism of protein transfer from epididymosomes to spermatozoa.

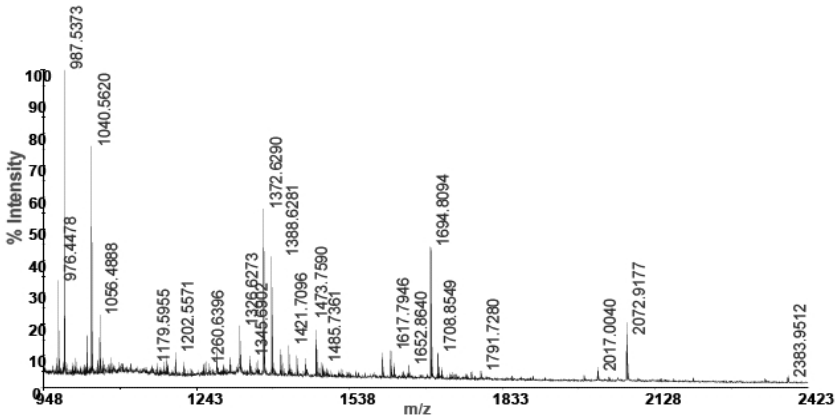

Figure 1: MALDI MS spectra of 65 Beta-hexosaminidase beta chain precursor

Epididymosomes were purified from caput epididymal fluid of *Sus scrofa domestica* by high-speed centrifugation followed by chromatography. The pool of proteins were precipitated with MeOH/ CHC_3 and partitioned with three different acqueous solution at different pH. This chemical fractionation affords to three different protein mixtures (a-c); each mixture was separated by SDS-PAGE. Epididymosomes fractions (a-c) showed a complex protein pattern (MW range of about 14-150kDa) with a major band at -65 kDa.

Protein bands within (60-80) kDa associated with epididymosome fractions (a-c), were chosen for a protein identification by peptide mass fingerprinting after tryptic digest. Ion peaks corresponding to protonated tryptic peptides of protein spots within (60-80) kDa were easily detected, with mass errors of 50 ppm (Figure 1). Data base search allows to identify Alkaline Phosphatase (Q29486) Serum Albumin (gi|2190337), Lactoferrin (gi |17467354) and 65 Beta-hexosaminidase beta chain precursor (gi|6225504). Sequence specific peptides were identified for each tryptic mixture by MS/MS experiments and all tandem mass spectra were evaluated using MASCOT data base searching

The most interesting preliminary results of epididymosome proteomic analysis is the identification of the enzyme 65 Beta-hexosaminidase beta chain precursor (gi|6225504). In fact, this enzyme, has been localized in the principal cells of boar caput epididymis as well as in the ejaculated sperm head of different mammals, where it is involved in the sperm-zona pellucida interaction. Therefore, it is reasonable to hypothesize that epididymosomes could mediate the acquisition of new epididymal proteins by pig spermatozoa during their transit in the epididymis.

Exploitation of Endougenous Protease Activity in raw mastitic milk by MALDI-TOF/ TOF [P25]

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Both quantity and quality of milk is altered in cows affected by mastitis. The intra-mammary infection process causes the elevation of Somatic Cell Count (SCC) and increases proteolytic activity in milk. The correlation of protein and peptide profiling with the inflammatory stage can be a useful tool for a better understanding of the inflammatory processes, for early diagnosis and treatment of mastitis. The proteic profiling of bovine milk produced by cows with subclinical mastitis was obtained by MALDI mass spectrometry.

The milk produced by healthy cows and those showing clinical symptoms of acute mastitis were chosen to study both the proteolytic activity in milk as a function of the resulting peptides. In particular, the milk produced from all quarters (**FL**: front left; **FR**: front right; **RL**: rear left; **RR**: rear right) of both affected ($_{M}$) and uninfected ($_{H}$) animals were selected to determine the protein and peptide profile and to identify and characterize specific proteins.

A simple procedure of chemical fractionation of raw milk was developed, whereby less complex fractions of proteins were obtained prior to Mass Spectrometric [1] and SDS-PAGE analysis.

	SAMPLES	S. C. C. [cell/mL]
1	Front Left quarter Healty cow	$1.1*10^3$
2	Front Right quarter Healty cow	$0.6*10^3$
3	Rear Left quarter Healty cow	$1.2*10^3$
4	Rear Right quarter Healty cow	$1.3*10^3$
5	Front Left quarter Mastitic cow	$0.4*10^6$
6	Front Right quarter Mastitic cow	$1.2*10^6$
7	Rear Left quarter Mastitic cow	$11.6*10^5$
8	Rear Right quarter Mastitic cow	$8.4*10^5$

Table1

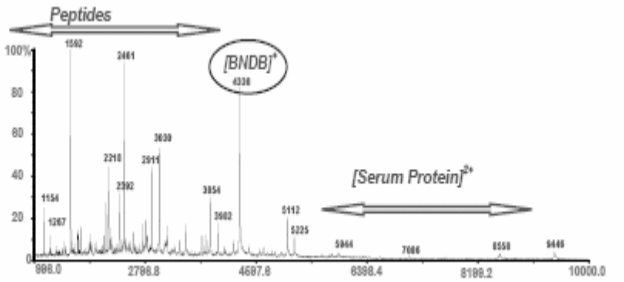

Figure1

50ìL of fresh raw milk taken from each quarter of healthy and infected cows (Table 1) was precipitated with 1mL of CHCl3/CH3OH 1:3 (v/v) yielding the supernatant fraction s and pellet which was partitioned consecutively, under magnetic stirring and at room temperature with (1) 1mL of 50mM NH4HCO3 and (2) 1mL di H2O (TFA 2%). Each step was followed by centrifugation at 11000 rpm for 2 min.

The profiles of milk proteins thus obtained could allow the identification of either early markers of the acute phase of mastitis or endogenous peptide of innate immune response (Figure1) [2].

The most important peptidase families identified in milk are Plasmin and Cathepsin [3,4]. Plasmins are trypsin-like serine proteases with optimal activity at pH 7.5 and 37°C, playing an important role during the ripening of cheese. Cathepsin family includes Cathepsin B, H, L, I (lysosomal cysteine proteases with optimal activity at pH < 7), Cathepsin G that is an alkaline serine protease and Cathepsin D, an aspartyl protease, with maximum activity at pH 3.2.

The activity of the endogenous proteases in raw milk produced from each quarter of healthy and mastic cows was therefore assayed over 24, 48, 96 and 216 hours incubation at 37°C at both physiological and acid pH. Sequence specific peptides were identified for each fraction by MS/MS experiments and all tandem mass spectra were evaluated using MASCOT data base searching.

The results show a specific proteolytic activity of endogenous enzyme towards β-casein precursor (P02666), α-S2-casein (P02663), α-S1-casein (P02662) and κ-casein (P02668).

The mass spectral profiling of the examined samples indicate that different proteic pattern can be found in mastitic milk. Milk from mastitic udders exhibit increased proteolytic activity which is undoubtedly associated to the releasing of PMN from blood. The extent of the breakdown of the caseins in high SCC milk is a function of several enzyme families and on the length of the exposure to enzymes act [5]. These results demonstrate that the proposed experimental procedure can supply valuable information on the presence and activity of numerous cell-derived proteolytic enzymes in bovine milk.

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Characterization of the Protein Fraction of Donkey's Milk, a Promising Substitute in Infant Cow's Milk Allergy [P26]

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Food allergies constitute a problem of social relevance [1]. Particularly milk, which plays a fundamental role in infant diet, constitutes the main source of allergenicity in children. In popular tradition donkey's milk constitutes a good substitute in infant cow's milk protein allergy (CMPA) [2]. However, these properties still need to be demonstrated objectively at scientific level, and therefore a better knowledge of the protein composition of donkey's milk is required. Donkey's milk is considered to have a composition which is closer to that of breast human milk. Because of its high content of polyunsatured fatty acids and vitamins, milk of donkey presents excellent nutritional values and is easily digested. Compared with bovine milk, donkey's milk shows a very different protein composition. The main difference is the proportion of whey proteins in the milk of both species. In fact, in donkey's milk whey proteins constitute 40-50 % of the nitrogen fraction, whereas in bovine milk they accounts for only 20% of the nitrogen fraction. At present time donkey's milk has been less studied than bovine milk and limited data are available for the genetic polymorphism. Here, we report the characterization of the protein profile of donkey's milk samples by combined use of RPHPLC, electrophoresis and mass spectrometry methods.

Direct RP-HPLC/ESI-MS analysis of the whey fraction from an individual donkey's milk sample allowed the detection of four unknown components (Mr 1594, 3043, 14646 and 14230 Da, respectively) and of two minor components, together with the identification of already known whey proteins, such us lysozyme B, α- lactoalbumin, β-lactoglobulin I B and β-lactoglobulin II B. MALDI-TOF/MS and RP-HPLC/ESI-MS/MS analysis of the enzymatic digests of the unknown and minor components resulted in the identification and characterization of: i) two β-casein fragments (Mr 1594, 3043 Da), which reasonably arise by endogenous proteases cleavage of the donkey's β-casein and could be potential biologically active peptides; ii) the oxidized methionine forms of lysozyme B (14646 Da) and α –lactoalbumin (14230 Da); iii) the sequence of donkey's serum albumin which presents the amino acid substitution Val→Ile at position 497 with respect to the cDNA deduced sequence and, iv) the amino acid sequence of donkey's lactoferrin. Comparative isoelectric focusing (IEF) analysis of the protein fraction of forty-six individual donkey's milk samples revealed a remarkable heterogeneity. Most of the milk samples showed a common IEF pattern, whereas some samples were defective of specific IEF protein bands with respect to the reference pattern.

Coupling in-gel trypsin digestion, MALDI-TOF MS analysis and database search resulted in the determination that some milk samples were defective in the casein fraction, lacking the αs1-casein components, whereas others were defective in the whey fraction, lacking the β-lactoglobulin II components.

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A proteomic approach to plasmin and chymosin activity on river buffalo and bovine pure â-casein [P27]

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Plasmin primary proteolysis origins C-terminal and N-terminal â-casein (â-Cn) fragments that are known as ã-Cn and as proteose peptones respectively. Chymosin activity on â-Cn produces C-terminal small peptides. In vitro combined action of these enzymes on the isolated casein has not been well studied and it is not known whether they act in a concerted manner in cheese. To a better understanding of proteolysis phenomenon and identify proteolytic fragments in cheeses we had investigated in vitro proteolysis of pure bovine and river buffalo â-Cn by a proteomic approach. Pure bovine and river buffalo â-Cn were submitted to action of plasmin, of chymosin and their concomitant action for different time. Kinetic reaction was monitored by monodimensional electrophoresis. The electrophoretic profiles that showed a high complexity were further analysed by two dimensional gel electrophoresis. The obtained electrophoretic maps were compared each other and with an electrophoretic map of caciocavallo, a ripened cheese from milk bovine, by image analyses. The common spots were identified by MALDI-TOF mass spectrometry. This analytical strategy allowed us to identify the polypeptide fragments deriving from plasmin and chymosin action on â-Cn in ripened cheese.

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Proteomic analysis of dorsal root ganglia in rats with induced neuropathic pain [P28]

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Introduction

Neuropathic pain is defined as pain initiated or caused by primary lesions or dysfunction in nervous system. Peripheral nerve injuries induce neuropathic pain that is usually persistent, and is related with cellular and molecular changes in peripheral nervous system, spinal cord and brain. There are also evidences that the ongoing pain associated with peripheral nerve injury reflect, at least in part, changes in the excitability of primary afferent neurons. It means that the primary sensory neurons of the dorsal root ganglia (DRG) play a key role in neuropathic hypersensibility. Dorsal root ganglia of four rats with neuropathic pain induced by chronic costriction of sciatic nerve, were compared with those from control rats using 2D-DIGE technology.

Materials and Methods

Painful neuropathy was induced in 4 male Sprague Dawley rats weighing 200-250g. Animals were anaesthetized with sodium pentobarbitaland neuropathic pain induced as described by Bennet & Xie (1988). Briefly, the common sciatic nerve was exposed at the level of the mid thigh and, proximal to the trifurcation of the sciatic nerve, four ligatures were loosely tied around it at about 1 mm spacing so that the epineural circulation was preserved. Sham animals (sciatic exposure without ligation) were used as controls. Two weeks aftre ligation animals were sacrified, L4-L6 root dorsal ganglia (RDG) on the ipsilateral of ligation were dissected and immediatly stored at –80°C. Ganglia samples were solubilized in DIGE lysis buffer and separated in a 24 cm long 3-10 NL pH gradient IPG strips as first dimension; the second was a 12% constant polyacrylamide concentration SDS-PAGE. All samples were run in duplicate, gel images were acquired by Typhoon 9200, which generate overlaid multi-channel images for each gel. Images were processed by DeCyder Differential Analysis Software, which is able to detect, quantitate, match and analyse multiple gel.

Results

Protein changes induced by neuropathic pain after sciatic nerve ligation were observed. After statistical analysis ($p<0.01$), 74 spots were differentiallly expressed compared to control rats, 37 of them were identified by MALDI ToF. Up-regulation of several proteins involved in cytoskeleton organization, cellular defense mechanisms and antioxidative processes were identified.

Toxicoproteomics-Discovery of predictive Biomarker in rat plasma by MALDI mass spectrometry* [P29]

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* *We thank the BMBF (grant 0313137) for funding*

In a previous in vivo study with a genotoxic carcinogen (N-Nitrosomorpholine), we have shown that proteomic analyses can help in identifying predictive safety markers. Here, we extended this study to identify predictive protein markers in plasma of rats treated with prototypical toxicants. For that purpose, we employed the subacute toxicity bioassay in rats (28 days/OECD TG 407) for the prediction of genotoxic and hepatotoxic compounds (fig. 1). Wistar rats were exposed to nine different chemical substances of three categories (genotoxic carcinogens, non-genotoxic and hepatotoxic, non-genotoxic carcinogens) and samples were taken at three different time points (day 3, 7 and 28). In this BMBF-funded project, genomic and proteomic analyses were done on liver tissue and plasma samples. In addition, effects on the gene and protein expression level were correlated with histopathological findings. In order to identify predictive biomarkers in plasma, we analysed these samples by two-dimensional-gel-electrophoresis (2-DE) followed by protein identification using mass spectrometry (MS). Candidate markers in plasma are generally difficult to detect since more than 99% of plasma proteins are high abundant ones. Therefore, we applied several depletion methods for high abundant proteins to increase the sensitivity. By using 2-DE, several differentially expressed proteins could be detected after 3 days as well as 7 or 28 days of exposure. By subsequent MS analysis, we identified some candidate biomarkers such as vitamin D-binding protein and betaine-homocysteine methyltransferase in the genotoxic carcinogen group. Furthermore, several high abundant proteins, e.g., apolipoprotein A1 and complement

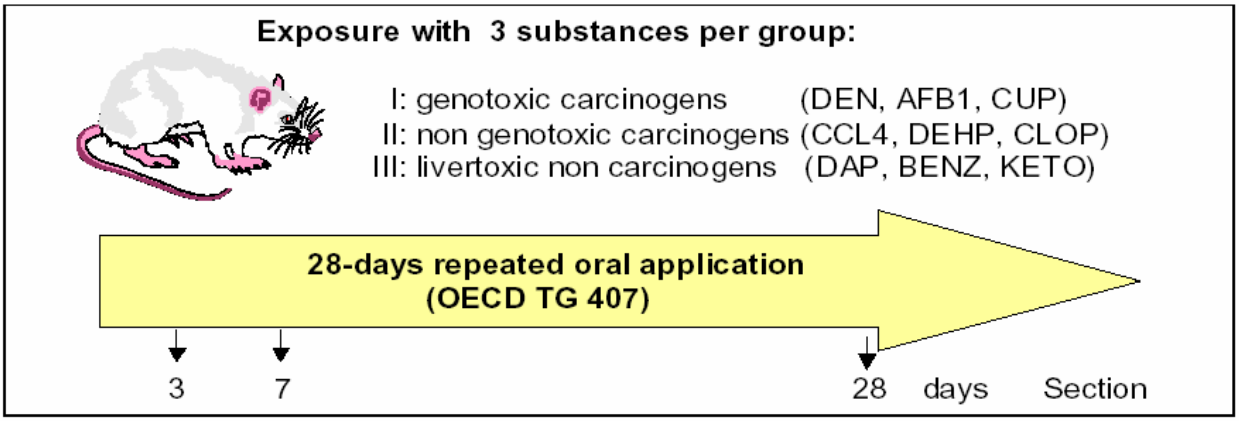

Figure 1. Study design. Wistar rats were exposed to nine different chemical substances of three categories and samples were taken at three different time points. DEN: N-Nitrosodiethylamin; AFB1: Aflatoxin B1; CUP: Cupferron; CCL4: Carbon tetrachloride; DEHP: Diethylhexylphthalate; CLOP: Clofibrate; DAP: Diallylphthalate; BENZ: Benzaldehyde; KETO: Ketoconazol

We are currently
ts with markers

Metabolic Modulation induced by Chronic Hypoxia in Rats Using a Comparativae Proteomic Analysis of Skeletal Muscle Tissue [P30]

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Introduction

Hypoxia occurs when the supply of oxygen to tissues is unable to meet cellulatr demand. Physiological and pathological instances of hypoxia include embryonic development, exposure to high altitude, muscular exercise, inflammation, anemia, infarction of the myocardium or the central nervous system, and in solid tumors[1,2]. Muscular tissue provides a good model of *in vivo* hypoxia adaptation, since its metabolic rate can increase by a 100-fold over basal levels, and it is subjected to fluctuating oxygen levels during exercise. In the present report, we have used 2D-difference in-gel electrophoresis (2D-DIGE) and tandem mass spectrometry to investigate an *in vivo* model in wich Sprague-Dawley rats are exposed to chronic hypoxia.

Materials and Methods

Animals. Ten male 5-week old Sprague-Dawley rats were divided into two groups: control, normoxic rats (N, n=5) breathing room air (F_IO_2=0.2l), and chronic hypoxic rats (CH, n=5) exposed to a normobaric hypoxic atmosphere (F_IO_2=0.10). The treatments were of 2 weeks duration. After the treatments rats were sacrified and the mixed gastrocnemious muscle was isolated and divided: a small part was utilized for total protein extract and the remainder for mitochondrial enrichment.

Muscle Protein Extractions. The frozen muscles were grounded and solubilized in a sample buffer[3] (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, and 1 mM PMSF). The pH of the protein extract was adjusted to 8.5 and protein concentration was determined using 2D-Quant Kit[4].

Preparation and extraction of Enriched Mitochondrial Fractions. A mitochondrial isolation buffer (250 mM sucrose, 0.15 mM KCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.5% BSA fatty acid free) was added to the muscle tissue.

Mitochondria-enriched samples were obtained by differential centrifugation and solubilized by modified DIGE lysis buffer (10 mM Tris-HCl, pH 8.5, 7 M Urea, 2M Tiourea, 5% CHAPS, 1% Triton X-100, 1mM PMSF, and Proteinase Inhibitor cocktail. Citrate synthase activity in enriched mitochondria fractions was assessed by the Srere method[5].

Cy-Dye Labelling. Protewin labeling with cyanine dyes (Cy3 and Cy5) was performed according to the manufacturer's instructions (GE Healthcare) and as previously described[6].

2D-DIGE, Image acquisition and gels Analysis. Samples combined with the standard (50 µg from Cy5 and Cy3) were separated on 18 cm IPG strips (3-10 non linear pH gradient) wirh a voltage gradient from 200 to 8000 V for 70000 Vh total. The second dimension was performed on 20x25 cm SDS gels (12% T) and each sample was run in triplicate. The Cy-Dye-labeled gels were visualized using Typhoon 9200 scanner and images were processed by DeCyder software. The differences between the two groups (normoxic vs hypoxic rats) were assumed to be significant with a p-value<0.05 after t-test.

Protein Immunoblotting. 40 µg of each protein extract (from normoxic and hypoxic rats) were separated by SDS-PAGE and transferred to PVDF membrane. The membranes were incubated with monoclonal anti HIF-1α (1:10000), polyclonal anti PDK-1 (1:30000) and polyclonal anti mTOR (1:5000). MCF cell cultures extract was adopted as positive control of HIF-1α stabilization induced by oxidative stress[7].

Results.

Hypoxia-induced changes of rat skeletal muscle were investigated by two-dimensional difference in-gel electrophoresis (2D-DIGE) and mass spectrometry. We evaluated the differences between normoxic and hypoxic rats in the total Gastrocnemius muscle and in the enriched mitochondria fraction. Our results indicated that prolonged hypoxia down-regulated proteins involved in theTCA cycle, ATP production, and electron transport, whereas glycolitic enzymes and deaminases, involved in ATP and AMP production, were are up-regulated. Up-regulation of the hypoxia markers hypoxia inducible factor 1 (HIF-1α) and pyruvate dehydrogenase kinase 1 (PDK1) was also observed, suggesting that *in vivo* adaptation to hypoxia requires an active metabolic switch. The kinase protein, mammalian target of rapamycin (mTOR), which has been implicated in the regulation of protein synthesis in hypoxia, appears unchanged, suggesting that its activity, in this system, is not controlled by oxygen partial pressure.

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Proteomic profile of gastrocnemious muscle in a transgenic mouse model of familial amyotrophic lateral sclerosis [P31]

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Background

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease characterized by progressive degeneration of upper and lower motor neurons. About 10% of all ALS cases show familial inheritance and roughly one fifth of them are linked to autosomal dominant mutations in the SOD1 gene. Mutant human SOD1 expression in transgenic mice causes progressive loss of spinal motor neurons with consequent muscle paralysis and premature death.

Muscle cells are characterised by a great plasticity that makes muscle a sensitive tissue in respect to pathological events involving other contexts, in particular in motor neurons changes of which muscle is the main effector. Unlike the nervous tissue, skeletal muscle has the advantage of being relatively easily investigated by needle biopsies, allowing the identification of specific diagnostic and prognostic disease biomarkers.

Objectives

Discovering biological markers for early detection of ALS is critical to evaluate prognosis and start neuroprotective therapies as soon as possible. The aim of this study was to detect markers of primary events in animal models.

Methods

In the present study we examined the proteomic profile of the gastrocnemious muscle from hSOD1^{G93A} transgenic (Tg) mutant mice in the pre symptomatic (7-week-old mice) and symptomatic (14-week-old mice) stage of the disease using 2D-DIGE approach coupled to mass spectrometry. The same proteomic approach was performed on gastrocnemious of age-matched control (NTg) mice and on 14-week-old animals that underwent sciatic nerve axotomy (Cr, crush) in order to identify the physiological changes induced in muscle by ageing and by denervation.

Results

Approximately 3500 spots were detected in a 3-10 non linear pH gradient. Differential analysis of muscles at the early pre symptomatic stage compared to NTg, revealed that 82 spots were significantly and differentially expressed in Tg hSOD1^{G93A} versus NTg mice, 24 of them were identified by mass spectrometry. At 14 weeks of age, quantitative differential analysis of Tg hSOD1^{G93A} vs. NTg generated 153 differentially expressed spots, 55 were common to the two different disease stages, and 16 of them were identified. Moreover, we identified 8 proteins that were differentially expressed at 7 weeks but were not changed 7 weeks later. All the proteins were mitochondrial. A total of 7 proteins maintained the same up or down regulation suggesting their role as disease markers.

Ageing induced a significant change in 95 spots, while 153 were changed after denervation, 28 of them were common between ageing and crush. Only spots exclusively changed during disease progression or presenting differential behaviour from ageing and crush were taken into consideration. Overall, 23 proteins were identified, 12 of them were not influenced by the ageing and denervation processes, indicating these changes were exclusively related to disease progression. The remaining 11 proteins showed differential behaviour between pre symptomatic and symptomatic transgenic mice compared to ageing and denervation.

Conclusions

Molecular mechanisms underlying neurodegenerative diseases are still poorly understood. The identification of clinically relevant biomarkers for neurological disorders poses unique challenges that could be overcome by the use of "omics" technologies. In this report a proteomic approach to study, in muscle, the onset and disease progression in a mouse model of ALS was adopted. Disease-specific profiles of protein expression in skeletal muscle were found, indicating that, comparative proteomic studies performed on this tissue could represent a tool for biomarkers identification and for disease progression studies, facilitating the early diagnosis and the follow-up of neurodegenerative disorders.

3-Iodothyronamine (T_1AM) Signalling Pathway Involves Tyrosine Phosphorylation in Rat Heart [P32]

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3-Iodothyronamine (T_1AM) is a novel endogenous thyroid hormone derivative that induces bradycardia and hypothermia in mouse and a dose-dependent negative inotropic effect in rat heart (1). The signal transduction that regulates these effects is still unclear. In cell culture model, nanomolar concentrations of T_1AM activates a G protein-coupled receptor known as trace anime-associated receptor 1 (TAAR1). In rat heart transcripts for 5 TAARs are present, and specific and saturable binding of [^{125}I]T_1AM is observed, with a dissociation constant in the low micromolar range (5 μM); but the cAMP pathway is not activated since myocardium cAMP levels are unchanged upon perfusion with T_1AM (2).

Perfusion with genistein, a tyrosine kinase inhibitor, remarkably increases the T1AM hemodynamic effects, which are attenuated by vanadate, tyrosine phosphatase inhibitor. No effect is produced by inhibitors of others kinase, namely protein kinase A, protein kinase C, calcium-calmodulin kinase II, phosphatidylinositol-3-kinase, or MAP kinases (2).

Using Western blotting analysis with anti-phosphotyrosine antibodies we have previously shown that phosphorylation of tyrosine residues in microsomal and cytosolic proteins at approximately 97, 64, and 28 kDa is changed in the presence of T_1AM (2). These results suggest that the downstream effects of T_1AM may involve the inhibition of tyrosine phosphorylation pathways.

We report here the use of MALDI –TOF MS peptide mapping to identify which proteins are located within the gel bands that show a change in phosphorylation pattern upon treatment with T_1AM. Most of the proteins that we identified belong to the energetic metabolism. In the microsomal fraction, bands at 97kDa were identified as the muscle form of glycogen phosphorylase, hexokinase type I complex with glucose and inhibitor glucose-6-phosphate, and α-ketoglutarate dehydrogenase. At 64kDa, three proteins were found: the mitochondrial calcium binding aspartate/glutamate carriers citrin and aralar, and the long-chain acyl-CoA synthetase. At 28kDa, proteins in the bands were indentified as NADH dehydrogenase (ubiquinone) Fe-S protein 3 and the mitochondrial enoyl-CoA hydratase. In the cytosolic fraction, the modified band (between 97 and 64 kDa) was found to contain the muscle form of the glycogen phosphorylase.

In conclusion, using anti-phosphotyrosine antibodies and MALDI-TOF MS, we have identified cytosolic and microsomal proteins, in rat heart preparations, whose phosphorylation may be inhibited by the treatment with T_1AM. Although these proteins are not currently known to be regulated by tyrosine phosphorylation, our findings suggest that they contain functional phosphotyrosine residues. Additional studies are underway to further characterize the phosphorylation state of the identified proteins.

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Differential Proteome Analysis of the Cerebrospinal Fluid of Normal and Equine Protozoal Myeloencephalitis Affected Horses Using Two-dimensional Fluorescence Difference Gel Electrophoresis and Mass Spectrometry [P33]

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Equine protozoal myeloencephalitis (EPM) is a common and costly neurologic disease of horses caused by *Sarcocystis neurona,* protozoa that parasitize the nervous system. Confirmation of the clinical diagnosis is a challenge for veterinarians because the current immunologic tests lack specificity. Multiplexing strategy approach including two-dimensional fluorescence difference gel electrophoresis (2D DIGE) and mass spectrometry were employed to identify differential protein expression associated with EPM.

Cerebrospinal fluid (CSF) samples were collected from normal and EPM horses. EPM was diagnosed if neurological signs were present, Western blot against *S. neurona* on the CSF was positive and other neurological diseases were excluded by ancillary tests and/or necropsy. CSF was collected under general anesthesia, at the atlanto-occipital site to avoid blood contamination. The CSF proteins were precipitated, labeled with CyDye DIGE fluor dyes and separated on 2D gels. The 2D gels were scanned with Typhoon, and the digitized images were analyzed with DeCyderTM software (GE Healthcare). The differentially expressed proteins were excised with a spot picker (ProPic II, Genomic Solutions) directly from the DIGE gel without post staining, in-gel trypsin digested with ProPrep Investigator (Genomic Solutions), and the tryptic peptides were analyzed with a linear ion-trap mass spectrometer (LTQ, Thermo Electron corporation) equipped with an electrospray ionization interface and a nanoscale liquid chromatography system (Ettan MDLC, GE Healthcare) delivering a flow rate of ca. 200 nL/min online to the LTQ.

Preliminary analysis shows that some of the proteins are altered with disease. A number of differentially expressed proteins were identified in the various horse CSF, including serotransferrin, cyclin T and steroidogenic acute regulatory protein. Various isoforms of serotransferrin were altered with EPM compared to the normal horse CSF. The above proteins, including the isoforms may be associated with EPM, and the characterization of differentially expressed additional proteins could give insight into the pathogenesis and diagnosis of EPM.

Fig. 1: 2D maps of horse CSF proteins labeled with CyDyes showing the overlay view of the three dyes Cy2 (standard), Cy3 (red) and Cy5 (blue). CSF proteins were precipitated, labeled with CyDye DIGE Fluor minimal dyes and were separated on a pH 3-11 IPG strips, followed by 10% gel.

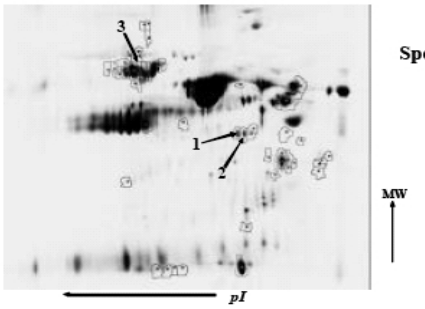

Fig. 2: Differential protein expression. Digitized 2D image from Typhoon were analyzed with DeCyder, and some of the proteins that showed at least 2-fold increase (blue) or decrease (red) in expression compared to normal CSF were excised with a spot picker (ProPic II).

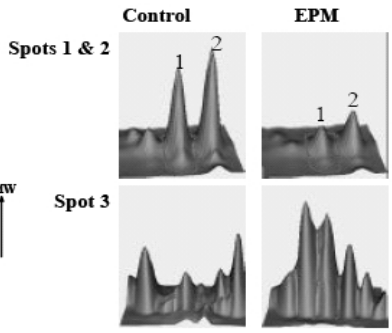

Fig. 3: 3D images of some of the differentially expressed spots in Fig. 2. The digitized images from the Typhoon were analyzed with DeCyder 2-D Differential Analysis Software.

Optimised two dimensional electrophoresis for protein characterization of rat brain tissues

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Introduction

High resolution two dimensional electrophoresis (2DE) remains the core technology for arraying complex protein mixtures, prior to mass spectrometry characterization[1]. Due to the complexity of brain tissue, optimising protein separation techniques is of primary importance to unravel the function of proteins in the brain and their role in disease. So, brain tissue is a very good model to improve resolution due to either hydrophobic then basic characteristic of proteins. It has been described that for wide pH 3–10 gradients, the use of HED could also be advantageous[2]; although not needed for reduction of streaking, it has been result in an improved reproducibility of the basic part of the resulting 2D maps. In this work it has been applied HED to rat brain two dimensional electrophoresis analytical loads in combination with anodic cup application or paper bridge application to compare the better methods to load brain extract.

Methods

Brain samples were collected from healthy rat and proteins prepared for 2-DE using two protocols: (I) direct suspension of tissue in IEF solubilization solution containing 7M urea, 2M thiourea, 2% CHAPS, 2% Triton X-100, 1% DTT or 10 mM TCEP , 1.0% v/v Ampholine (3.0-10) followed by constant stirring and (II) using Omnizol (EUROclone) following the manufacturer instruction. Protein solubilization was subsequently performed using the same solution used for the protocol I. Additional alchilation procedure was performed with 100 mM HED as described[2]. IEF was subsequently performed on IPG strips (GE Healthcare) pH 3-10 NL 18 cm loaded with anodic cup or paper bridge[3] and using a step-voltage program from 100 to 8000 V for a total focusing time of approximately 200 kVh. Proteins were detected by silver stain and gel images acquired using an apparatus Pharos FX (BIORAD). Image analysis was performed using ImageMaster 2D Platinum 6.0.1 (GE Healthcare).

Results and conclusions

Appropriate sample preparation is essential for obtaining reliable results in a proteomic analysis. The recovered protein amount using protocol I was four time more than OMNIzol precipitation. The protocol I is the best choice for extraction of total protein because it allowed better recovered protein amount and a rapid, simple, reproducible using for extraction protein from brain tissue. Image analysis indicate that paper bridge loading combined with HED give very sharp image and a strong reduction of streaking and improved separation and focusing of spots (fig.1A) according to literature; statistical analysis derived from image analysis shows a loss of spots (about 20%) compared with anodic cup loading. Therefore the latter method coupled to HED (fig. 1C) is the best choice for semi quantitative analysis of brain tissue. This method is suitable for high-throughput approaches and can easily be adapted to other biological samples.

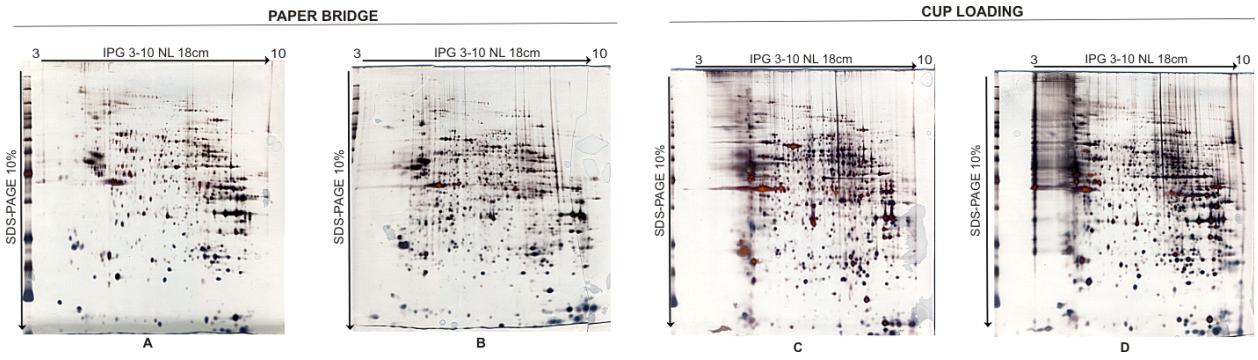

Figure 1: 2-D gel images of rat brain proteins. Separation was performed using different extraction protocols I: (A) HED, (B) no HED, (C) HED, (D) no HED and different loading methods: (A) (B) paper bridge, (C) (D) cup loading. Proteins were visualized by staining with silver nitrate.

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Two dimensional electrophoresis evaluation of milk from bovine with Johne's disease [P35]

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Introduction

Johne's disease is a chronic granulomatous enteropathy affecting all ruminants. The etiologic agent is Mycobacterium avium subsp. paratuberculosis (MAP), a slow-growing, facultative intracellular mycobacteria. There is also a concern that MAP might be a causative agent of some cases of inflammatory bowel disease in humans, especially Crohn's disease (1). However, the 'cause and effect' relationship remains controversial due in part to the difficulties in detecting MAP in human tissues using in vitro culture. Food products including pasteurized bovine milk have been suggested as potential sources of human infection.

In the present study a two dimensional electrophoresis approach has been used to analyse milk from bovines with paratuberculosis in order to characterize two dimensional profile of milk proteins come from infected bovines. This approach could be able to study putative markers for more sensitivity and specific diagnosis of paratuberculosis.

Methods

Milk samples were collected from healthy and infected bovine and prepared for 2-DE by dilution in IEF rehydration solution containing 7M urea, 2M thiourea, 4% CHAPS, 1% DTT, 2.0% v/v Ampholine (2). IEF was subsequently performed on IPG strip with pH range 4-8 and ultra narrow pH gradient 4-5.5 using a step-voltage programme from 100 to 8000 V for a total focusing time of approximately 100 kVh. Proteins were detected by coomassie G-250 and Pro-Q diamond (to detect phosphorylations) and gel images acquired using a dedicated scanner Pharos FX (Biorad).

Fig.1

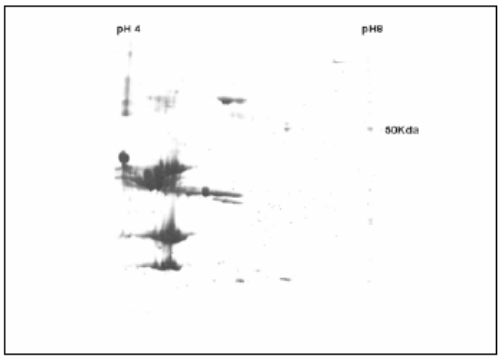

Fig.2

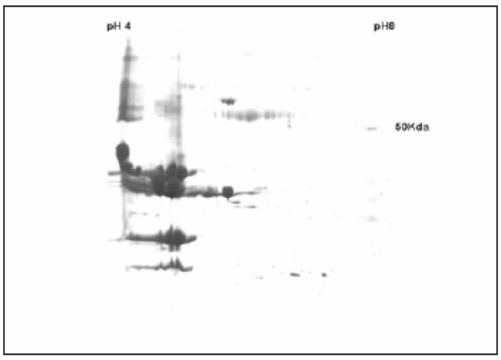

Fig1,2: Coomassie-stained 2D maps of healthy (1) and infected bovine (2).

Fig.1bis

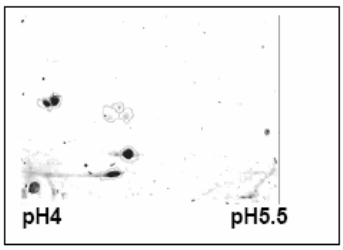

Fig.2bis

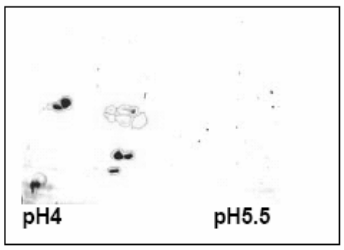

Fig. 1,2 bis: Pro-Q-Diamond-stained 2D maps of healthy(1bis) and infected(2bis) bovine

Conclusions

Quantitative analysis performed on coomassie-stained gels have shown major differences in the area with pH 4.5. Our results have shown that caseins are most abundant in milk from infected bovines that in controls milk. This is unexpected result and further investigations are working on.

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Proteomic characterization of MFGs from *Ovis aries* [P36]

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Milk Fat Globules (MFGs) are droplets of triacylglycerol coated with a cellular membrane. They represent the lipidic fraction of milk samples and are secreted by exocytosis from mammary gland's epithelial cells.

The aim of this project is the proteomic characterization of MFGs from *Ovis aries*, that are very important in the food industry for the production of milk and its derivates.

We have analysed 48 MFG samples from *Ovis aries*, in 2 different lactation periods. The MFG fraction was separated by 2D-DIGE technology (Two Dimensional-DIfferential Gel Electrophoresis) and analysed by DeCyder Differential Analysis Software (GE Healthcare). Protein identification was performed by mass spectrometry.

Using our proteomic approach we have identified 51 spots from the first lactation animal group and 66 spots from the second with a significant and quantitative variation through the 5 classes analysed. These protein species could be important as multivariate protein markers together with the Somatic Cell Count (SCC), to asses the quality of milk and its derivates.

Proteomic Analysis of Maternal Sea Urchin Determinants [P37]

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Spatial segregation of "morphogens" within oocytes and eggs play a functional role in the specification of the cell fate during early development. This asymmetry is obtained by selective localization of specific determinants such as messenger RNA or proteins. To isolate these determinants can help to study cell fate. Centrifugation has been an useful tools in sea urchin developmental research. This technique permits to separate in two halves Paracentrotus lividus eggs, called nucleated and anucleated, and separate different component such as proteins. Moreover, by utilizing specific messenger as marker it has been demonstrated that the cytoplasmic determinants don't change their position during centrifugation. Two-dimentional polyacrylamide gel electrophoresis (2-D PAGE), in which proteins are separated according to charge (pI) by isoelectric focusing in the first dimension and according to size (M_r) by SDS-PAGE in the second one, has the capacity for the resolution of complex mixtures of proteins, permitting the simultaneous analysis of hundred or even thousands of gene products. Proteins extracted by the two halves of P. lividus eggs were first separated in an immobilized pH gradient (IPG) strip 3-11, non linear, and afterwards, to resolve the greater number of spots from crowded areas, in medium range of pH. The second dimension was performed on gradient gel 9-16%, to analyze simultaneously proteins with a wide range of M_r. Silver stained analytical gels showed the presence of several different spots between nucleated and anucleated samples, suggesting that this distribution can be important for their final destination during sea urchin differentiation. It have been excised 91 protein spots and submitted to MALDI-TOF mass spectrometry. 18 proteins (fig. 1; table I) were identified in sea urchin or other genomes. 46 proteins spots, of which the MS spectra has been obtained, didn't find any homology with known sequences. Preliminary analysis indicate that one protein (toposome; fig. 2) is present only in the animal part whereas one protein (beta-tubulin; fig. 3) is present only in the vegetal part of the sea urchin egg. Our date provide a first sea urchin proteomic map and represent a first step to identify specific determinants.

*This work was supported by n° 22 MIUR grant, D .M n° 11005.

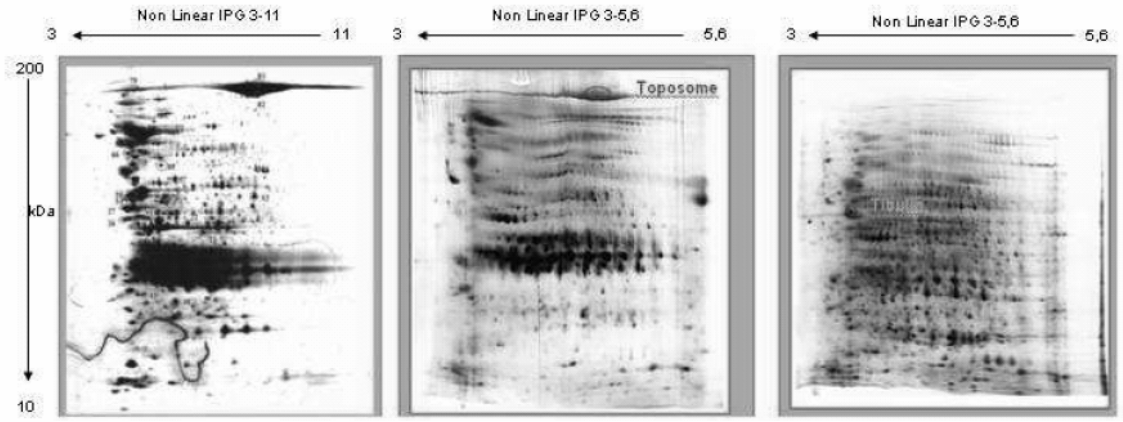

Fig. 1: Unfertilized eggs – Identified spots

Fig. 2: Nucleated halves

Fig. 3: Anucleated halves

Table I: Spot for which it has been found a sequence homology in database.

Spot.	ProteinName	pI	MW	Taxonomy
21	Actin, acrosomal process isoform (Actin-5)	5.29	45057	Strongylocentrotus purpuratus
22	Predicted: similar to gelsolin, partial	5.84	20723	Strongylocentrotus purpuratus
23	Actin, cytoplasmic 1 (beta actin)	5.29	42034	Sigmodon hispidus
26	Predicted:similar to 34/67 kD laminin binding protein	8.93	19020	Strongylocentrotus purpuratus
27	similar to H+-transporting ATPase beta subunit. Confermata in ms/ms	5.14	56111	Strongylocentrotus purpuratus
29	Predicted: similar adenosylhomocystenase	5.89	48061	Strongylocentrotus purpuratus
43	Predicted: similar to LOC496233 protein	4.88	17798	Strongylocentrotus purpuratus
52	mitochondrial ATP synthase alpha subunit precursor. Confermata in ms/ms.	8.16	59798	Strongylocentrotus purpuratus
54	beta tubulin	4.73	50540	Strongylocentrotus purpuratus
55	beta tubulin	4.73	50540	Strongylocentrotus purpuratus
57	Predicted: similar to chaperonin subunit 8 theta	5.14	57696	Strongylocentrotus purpuratus
69	Predicted: ATPase H+ transporting, 70 kD, V1 subunit A, isoform 1	5.40	68770	Pan troglodytes
79	toposome	6.15	155336	Paracentrotus lividus
82	toposome	6.15	155336	Paracentrotus lividus
83	toposome	6.15	155336	Paracentrotus lividus
38	Actin cytoplasmic 1	5,29	42052	Homo sapiens
51	>lcl|hmm208951 Gene (has frameshifts or/and premature stops) predicted by Gnomon on Strongylocentrotus purpuratus Spur_v2.1 genomic contig SpuUn_WGA56874_2 [Un]			
66	>lcl|hmm5592 Gene (has frameshifts or/and premature stops) predicted by Gnomon on Strongylocentrotus purpuratus Spur_v2.1 genomic contig SpuUn_WGA45184_2 [Un]			

Proteins profiling in calves serum: possible applications in food safety issues [P38]

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Despite the ban by European Union, performance enhancing agents (PEAs) are still illegally used in different species to increase the rate of weight gain and/or improve feed efficiency.

Besides the risk for animals, residual amounts of these chemicals in the resulting animal products could be dangerous also for the consumers. Results of the official investigations, in disagreement with it happens in the reality, would suggest the use of a combination of different active principles either at very low dosages and of unknown chemical structure or not included in the national residue monitoring plans. These can lead to the generation of (false) negative data.

There is therefore the urgent need to develop biological assays that could ascertain the exposure of food producing animals to a number of drugs or hormones currently used for the chemical manipulation of animal growth.

Mass spectrometry could represent an innovative and useful approach to provide the extremely accurate data highlighting the eventual modifications of protein expression resulting from the exposure of living animals to such agents. In this study we focused on the development of a robust proteomics platform to study the low molecular range (5-20kDa) of bovine serum with the double purpose on the one hand to characterize the protein profile, on the other hand to find biomarkers of illicit treatments comparing treated and non treated animals.

Through MALDI-TOF and nanoLC-nanoESI-Q-TOF, we analyzed two different groups of animals: the first was treated with a combination of 17-â-estradiol, clenbuterol and dexamethasone; the second was untreated. Additionally, sera from another group of untreated Friesian male calves were successively analyzed.

The employment of ZipTip C_4 approach was chosen as the best extraction method. After fractionating the serum on a C_{18} semipreparative column, we were able to identified nearly 30 proteins; some of them were attributed to fragments of higher molecular weight proteins or not visible in the mass range investigated.

Data from the sera collected from 12 calves (6 control and 6 treated) were independently analyzed by MALDI-TOF-MS after C_4 ZipTip extraction spectrum. They showed nearly 80 peaks and the average MALDI-TOF spectra of the low molecular weight (LMW) fraction of bovine serum for the two groups highlighted two proteins differently expressed in treated and control group. Another group of 20 sera collected freshly from untreated animals have been analyzed with same protocol to verify the effective absence of the discriminant signals.

Data obtained from comparison between treated and non treated animals represent just a first step forward the detection of biomarkers of illicit treatment in veal calves using mass spectrometry approach. Screening a larger number of animals these two peaks seem to be reproducible markers of treatment, nevertheless they represent just preliminary results that need to be confirmed.

Further current investigations are still examining the increasing dynamic range of protein detection and looking for blood biomarkers.

Unconventional prefractionation methods in proteomics research [P39]

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The complexity of proteomes is so large that prefractionation of initial sample is a well-known approach to help finding additional protein species.

Classical chromatography is the most general fractionation method used; however, it suffers from a relatively low throughput and a carryover of proteins that can be found in several fractions. What is proposed here is a rational cascade of chromatography media superimposed likewise several small columns and using a same buffer. As the sample crosses the different adsorbent layers, proteins within are sequentially trapped according to the complementary properties for the solid phase media. Once the loading and capturing is achieved, the sequence of columns is disassembled and each column containing different proteins is eluted separately in a single step and under optimal elution conditions. When compared to classical single-chemistry fractionation the proposed approach shows much lower protein overlap between fractions and therefore greater resolution.

Sequences of media can be randomly selected, or can be of the same family such as lectins or IMAC loaded with different metal ions or even a series of hydrophobic media of different strength. In all cases the sequence is aligned according to the selectivity of the media for the proteins to separate. Generally the narrower specificity is placed first and the sequence ends by the largest specificity. Examples of fractionation will be shown.

Rapid Identification and Characterisation of Tryptic Peptides Using High Linear Velocity Nanobore UPLC MALDI MS/MS and ion mobility separation [P40]

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MS has firmly established itself as the primary technique for identifying proteins due to its unparalleled speed, sensitivity and specificity. Strategies can involve either analysis of the intact protein or smaller peptide sequences resulting from enzymatic cleavage. A common approach to protein prefractionation is 1D- PAGE. The downside of this approach is the number of gel samples to be analysed by LC-MS/MS. With typical HPLC run times of up to 1 hour the time required for the entire analysis can be prohibitive.

Here we describe the use of nanoscale columns packed with sub 2um particles for rapid separations using a nanoUPLC system. Increasing the flow rate of the separation and running a very rapid gradient allows high quality separations to be achieved for tryptic peptides with a sample to sample inject time of 10 minutes. The eluent was combined with matrix solution and spotted onto MALDI target plates. This allows for the rapid characterisation of simple mixtures such as those obtained from 1D gel bands.

We will present exact mass MALDI MS/MS data obtained from standard tryptic digests of simple mixtures and data from in-gel digests. An additional dimension of separation is introduced through the use of a novel ion mobility separation device. This will be compared and contrasted against data acquired using conventional separations and MS analysis.

Proteomic Profiling of Cerebrospinal Fluid and Serum in Schizophrenia by label free exact mass LC MS [P41]

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Schizophrenia is a common, chronic and disabling neuropsychiatric disorder the diagnosis of which relies on a complicated clinical examination and the presence/absence of other disorders. An objective diagnostic test for schizophrenia would improve current diagnosis and aid in monitoring individuals over the course of illness and may also be useful in determining prognosis. In this study we employed a novel label-free exact mass, MS-based approach to analyze the proteomic profiles in conjunction with multivariate data analysis to investigate cerebrospinal fluid (CSF) and sera from first-onset, drug-naive paranoid schizophrenia patients and healthy controls. Control and disease state samples were analyzed by LC-MS.

Protein identifications and quantitative information was extracted using specialised algorithms providing a label-free quantification method, coupled to simultaneous protein identification.

Partial least squares discriminant analysis showed a significant separation of first-onset, drugnaïve schizophrenia patients from healthy controls in these studies. A total of 174 different proteins were identified from the CSF samples. Of these proteins, 98 proteins exhibited a significant expression change, with 52 regulated by more than 30%. In the male samples 34 were up regulated and 18 were down regulated between the schizophrenic and control samples. From this study 23 proteins, some associated with other neurological disorders, are undergoing further validation.

The phosphorylation pattern of structural subunits of complex I of the respiratory chain. What 2-D western blotting, radiolabelling and mass spectrometry can tell us about the phosphorylation sites? [P42]

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Protein phosphorylation, among the several hundred known post-translational modifications, is one of the most abundant and various biological processes such as cell cycle, cell growth, cell differentiation and metabolism are regulated by reversible phosphorylation events that modulate protein activity, stability, interaction and localization.

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.

NADH:Ubiquinone oxidoreductase or complex I is the largest multimeric enzyme of the five mitochondrial electron transport complexes. The mammalian complex I is made up of 45 subunits: seven subunits are encoded by the mitochondrial genome, the remaining by nuclear genes. Fourteen subunits, including the seven encoded by the mitochondrial DNA are conserved from prokaryotes to humans, these contain all the known redox centers of the complex. The functional role of the supernumerary subunits is not yet fully understood. The nuclear NDUFS4 gene encodes for the 18kDa subunit (NADH dehydrogenase-ubiquinone FeS protein 4, AQDQ). The protein products of this gene in mammalian, bird as well as drosophila and anopheles present a highly homologous carboxy-terminal region in which a canonical RVS protein kinase A phosphorylation consensus site is conserved.

2D-electrophoresis, ^{32}P labeling and immunochemical analyses show that this protein is phosphorylated by PKA in mammalian (murine and human) cell cultures, in human skeletal muscle, in isolated bovine heart mitochondria and in purified complex I (1).

Western blotting analyses were performed with specific antisera raised against synthetic peptides with conserved sequences of the human 18kDa amino-terminus (anti-N), carboxy-terminus (anti-C) and carboxy-terminus with the serine phosphorylated of the RVS consensus site (anti-C,P). Control analysis showed that the anti-C and anti-C,P recognized specifically the respective peptide without any detectable cross-reactivity.

The drosophila isoform detected by the anti-C and anti-C,P antibodies was not recognised by the anti-N antibody as expected from the divergence in the N-terminal sequence conserved in the other species. Moreover mouse fibroblast cultures exposed to cholera toxin treatment associated with the production of intracellular cAMP, increased the reaction of the 18kDa protein band with the anti-C,P antibody, whilst the anti-N antibody remained unchanged.

A systematic approach to study the purified bovine complex I incubated with [γ-^{32}P]-ATP and C-PKA was performed. After SDS-PAGE, two radiolabelled bands, in the 18-20 kDa region, were excised, electroeluted and separated by isoelectrofocusing on a 3-10 pH gradient, subjected to a second SDS-PAGE and transferred to a PVDF membrane. On this membrane the radiolabelled protein spot was identified as the 18kDa AQDQ on the basis of its M.W. and isoelectric point (9.6) and reaction by the anti C,P antibody. These results show that the 18kDa subunit is phosphorylated by PKA. The common MS-based methods used to selectively enrich the phosphopeptides are based on chemical and affinity-based approaches. One of the chemical methods use the phosphoramidate chemistry (PAC). The affinity-based methods include immobilized metal affinity chromatography (IMAC) and titanium dioxide resins. So far, however, each method isolates different, partially overlapping phosphopeptides, implying that none of the methods by itself is able to comprehensively analyze a phosphoproteome (2).

Tandem mass spectrometry and Peptide Mass Fingerprint (PMF) analyses identified in the 18kDa SDS gel band of isolated complex I the 18kDa (NDUFS4), ESSS, SGDH, B17 (NDUFB6), B18 (NDUFB7) and B17.2 (DAP13) subunits. The same analysis on the titanium dioxide enriched fraction did not reveal the phosphorylated form of the carboxyterminal peptide of the 18kDa subunit under conditions in which phosphorylation of the ESSS subunit was detected as previously reported (3). In the last years a new phosphorylation pattern of the bovine heart complex I has been depicted according to the newly discovered phosporylated 42kDa, B14.5a, B14.5b and B16.6 subunits which were previously not detected (4).

Mass spectrometry analysis of the purified in vitro PKA phosphorylated NDFS4 protein expressed in E.Coli heterolog system identified the protein with 66% coverage but no C-terminal phosphorylation site was detected after titanium dioxide phoshpopeptide enrichment. Analyses on the synthetic phosphopeptides showed the low ionisation tendency of the phosphorylated carboxy-terminal peptide compared to the ionisation signal of the non phosphorylated one. This difficulty could hamper the identification of this C-terminal phosphorylation site.

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Applications of 2-D Electrophoresis and Western Blot to Analyse and Trace Proteins in Lupin based Pasta Products [P43]

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Two-dimensional (2-D) IEF/SDS-PAGE is a powerful tool to get molecular "pictures" of food proteomes and monitor the processing effect(s) of a given food item on its protein profile. However, the relatively scarce diffusion of proteomic techniques at industrial and analytical level has prevented their application in this promising area so far. In this work, 2-D electrophoresis has been used to monitor the main steps of lupin-based gluten-free pasta production. To the best of our knowledge, this work is one of the rare examples of 2-D electrophoresis application to the analysis of legume seed protein components in the whole production chain of a food product.

The optimised dietary exploitation of protein-rich plant sources, such as legume seeds, is the target of several research and development programs. Among the legume seeds, lupin is an interesting one for various reasons: a very high protein content (1), comparable to that of soybean, a low presence of antinutritional compounds (2), the functional properties of its components in food matrices (3) and the nutraceutical potentialities of some of its proteins (4).

In this work we have studied a lupin-based pasta product, taking advantage of the presence of a single protein source in this product, being lupin used as the unique alternative to semolina, and of a previously published lupin storage protein 2-D maps (5). To this aim, three different production lots of lupin-based pasta were analysed. For each lot, samples at each critical production step, including seeds, raw materials, namely the flour and the protein concentrate, half-processed products and dry pasta, were used to generate the corresponding 2-D electrophoretic maps. The presence, integrity and constancy of proteins throughout the industrial processing have been assessed. Indeed, some differences in the protein profiles between the raw materials, i.e. lupin flour and lupin protein concentrate, were attributed to the different varieties which they arose from. On the other hand, the electrophoretic analyses showed only minor differences among the samples during the industrial processing. In particular no alteration of the covalent continuity of the main polypeptide backbones. The disulphide pattern did not change during the process, as well, and the constancy of the glycosylation pattern, as measured by the lectin Concanavalin A on the blotted maps, indicated that this molecular feature was not affected by the process too.

In conclusion, this work shows that taking 2-D electrophoretic "pictures" of the polypeptides at each relevant step of a food production process can be very useful to both quality control strategies and traceability of specific protein components in force of the high resolution of the technique also in complex food matrices.

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A Functioinal Proteomic Approach for the Identification of Aldolase C Interactors *in vivo* [P44]

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Aldolase C protein (AldC) is the brain form of the glycolytic enzyme aldolase, whose expression increases during mammalian development and is highest in adult cerebellum (1,2). Due to its peculiar distribution in well-delimited cell compartments of human cerebellum and in several other areas of the CNS, we suggested new functional roles for AldC (1). Such an hypothesis is supported by the finding that AldC regulates the stability of the light neurofilament mRNA transcript (3). Moreover, as an interactor of prion protein in mouse brain tissue, AldC could be involved in the modulation of neuronal functions and dysfunctions (4).

To define a comprehensive AldC interactome map, we used a functional proteomic approach. We immunoprecipitated endogenous AldC together with its interactors from adult mouse cerebellum with anti-AldC antibody. Mass spectrometry procedures coupled to bioinformatic tools (5) allowed to identify AldC protein partners and to draw inferences regarding cellular processes that are directly or indirectly concerned with AldC .

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A Blot-Overlay Based Approch for HMGA Molecular Partners Identification [P45]

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The HMGA (High Mobility Group A) chromatin architectural transcription factors are paradigms for the natively disordered proteins. Despite the lack of any defined structure, they are inserted in an articulated interaction network, both with DNA and other nuclear proteins. However, HMGA proteins have not their own activities but rather they modulate the function(s) of other proteins/DNA regions by binding to them. Given the functional data that can be obtained by identifying their molecular partners we exploited the absence of any structural constraint to develop an in vitro strategy for the identification of HMGA molecular partners. We demonstrated that the combination of a RP-HPLC prefractionation procedure, 2D gels, blot-overlay and mass spectrometry can be successfully applied for the identification of HMGA molecular partners and that this approach can also be adopted to evaluate on a large set of protein the involvement of selected regions, aminoacid residues, or PTMs on the binding properties of HMGA proteins.

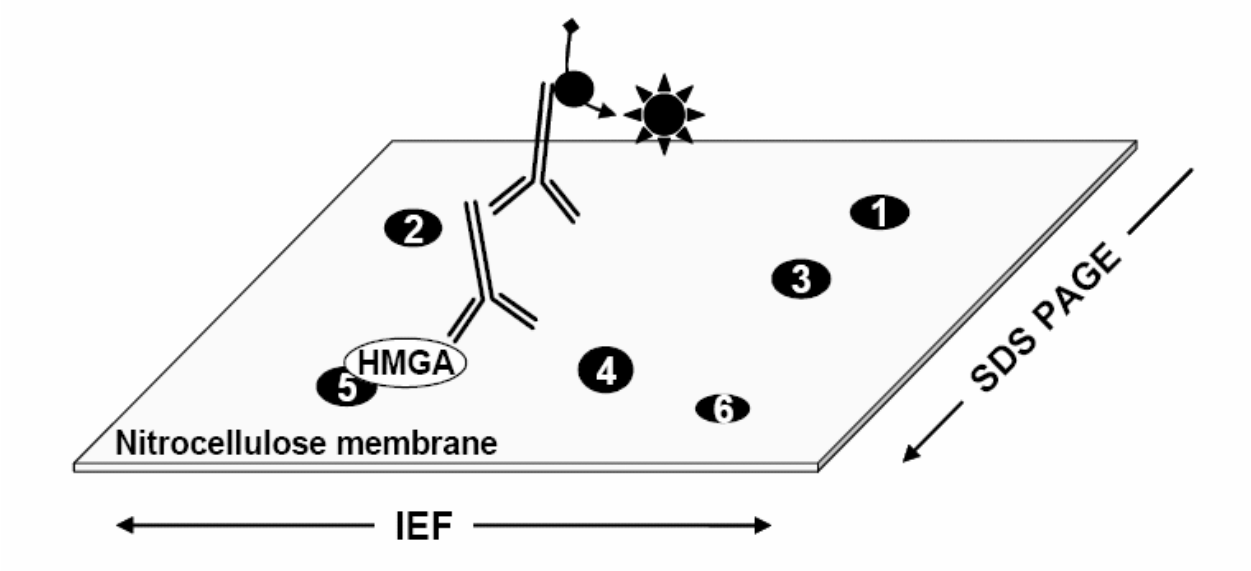

Figure 1: Schematic representation of 2-DE blot-overlay. Nuclear proteins are separated according to their pI (IEF) and molecular weight (SDS PAGE) and transferred onto nitrocellulose membrane. Membrane is then incubated with HMGA1a protein that recognise and bind to its molecular partners. Bound HMGA1a is evidenced by the use of specific a-HMGA antibody and peroxidase-coniugated secondary antibody.

Increased Selectivity for the Ion Mapping of Synthetic and Endogenous Molecules from Tissue Sections using MS/MS on a MALDI Q-TOF Mass Spectrometer [P46]

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Imaging the spatial distribution of molecules in tissue using MALDI MS is a rapidly developing technique. The acquisition of accurate mass data in this type of experiment can be hampered in axial MALDI Tof systems. Even small changes in sample position and laser energy in the source region of this type of mass spectrometer affect mass measurement accuracy and mass spectral resolution. Here, we show how the use of an orthogonal Tof MALDI mass spectrometer circumvents these problems by decoupling the MALDI source from the mass analyser. Imaging data were acquired on a MALDI Q-Tof mass spectrometer. To reduce interference from the biological matrix and enhance specificity the instrument was operated in MS/MS mode, a quadrupole was used for precise precursor ion selection. The sensitivity of specific ions was further enhanced by synchronising the high voltage push of the Tof mass analyser with the arrival of ions of appropriate m/z in the acceleration region.

MALDI imaging information has been obtained from thin sections of rat tissue from animals doped with drugs as well as from untreated animals. Data obtained on the spatial distribution of drugs/ drug metabolites and endogenous species will be presented. Challenges and future directions with MALDI imaging sample preparation are discussed.

A novel approach coupling ion mobility separation with TOF mass spectrometry for real-time, charge state sensitive, data dependant LC-MS/MS analysis [P47]

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Modern mass spectrometers can acquire hundreds of MS/MS spectra in a single run, often a considerable number of these spectra can originate from background related species. We have previously described a hybrid quadrupole oa-TOF mass spectrometer incorporating a travelling wave ion mobility device capable of separating ions by both ion mobility and m/z. This allows singly charged ions to be separated and removed from ions of higher charge states without loss in sensitivity. We have evaluated this technology for data dependent LC-MS/MS of tryptic digest mixtures.

We have previously shown that there is a clear relationship between an ion s charge state, at a given m/z, and its mobility in the ion mobility device. We can therefore generate a rule set that provides the acquisition system with the appropriate drift time vs m/z information, enabling the specific selection of 2+ ions across the m/z scale. This has been applied to LC-IMS TOF MS data for mixtures of differing complexity. Removal of singly charged species from the data reveals information obscured in the TOF MS data. Further, this new approach has been used for realtime, charge state sensitive, data dependant LC-MS/MS analysis. This LC-MS/MS approach provides additional specificity in both the IMS MS survey mode and the MS/MS mode. Examples will be shown where 2+ and 3+ precursor ions of the same m/z can be separated, post MS1 selection, and a specific set of fragments obtained for each precursor ion.

Proteomic Advanced Service for Targeted Analysis (P.A.S.T.A) Database [P48]

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The identification of lot of proteins at one time by shotgun proteomics and the ever increasing volumes of proteomic data have resulted in major issues in data storage and accessibility[1,2].

In this contest we have developed an interactive and free proteomic database called P.A.S.T.A (Proteomic Advanced Service for Targeted Analysis)[3] (**Fig. 1**) with the aim to collect and storage the data related to proteins identified by Italian groups, to make data exchange easier and to represent the state of the art of the Italian proteomic research.

Introduction of data on P.A.S.T.A website, requires a pre-registration (**Fig. 2**) for obtain a login and related password.

Fig.1: P.A.S.T.A Home Page

Each italian group may upload the identified protein list in the database, only after acceptance by a scientific journal with peer review. Everyone, without registration, can query the P.A.S.T.A database (**Fig. 3**) by protein name, publication year, tissue, separation and identification methods and other parameters in order to obtain the infomation about the related publication present in the database.

Also, for each protein present in database, it is possible to obtain automatically the different identification codes (NCBI, IPI, SWISS-PROT), and extra information available in the principal proteomic databases.

PASTA is intended to researchers working on life sciences and it's an on line software service which greatly increases productivity when retrieving information about known proteins. Retrieval and organization of information about a protein can be a laborious and time-consuming task, therefore we have linked P.A.S.T.A with a tool, WebAnts[4], which automatically queries some (about 20) of the most important biological databases distributed across the world and collects several information concerning the selected protein, supplying the user with a web report (including names, symbols, keywords, sequence, cellular localization, function, comments, domains, 3D structure, literature references, cross-references...) which forms a complete and easy-to-consult output card.

We invite submission of additional data from the italian community so as to begin building up the critical mass of public proteomic data.

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MSB-MALDI-MS, Mesoporous Silica Beads-Matrix Assisted Laser Desorption Ionization-Mass Spectrometry: a New Promising Approach for Plasma/Serum Proteomic Analysis [P49]

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Currently efforts in MS-based diagnostic platforms are focused on resolving the "deep proteome" of complex biological fluids. The greatest challenge for biomarker discovery is the isolation of rare candidate proteins within a highly concentrated and complex mixture of high abundant species**.**

The design and generation of new highly selective materials such as new advanced mesoporous silica and silicon are attractive candidates for a wide range of applications in bio fluid proteomics. We have recently developed a platform based on mesoporous silica beads (MSB) for plasma high abundant proteins depletion. Tuning the pore size distribution and surface adsorptive properties MSB selectively capture peptides and small size proteins excluding large size proteins such as Human Serum Albumin. Plasma samples are exposed to MSB, captured molecular species are extracted and then profiled by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDITOF/MS). Given the high surface area MSB offer the desired adsorptive capacity for binding and enrichment of low abundance peptides present in body fluids.

We are now investigating on direct spotting of MSB peptides-loaded suspension on the MALDI target plate as a new fast and improved procedure for plasma/serum peptides detection. Preliminary results emphasize that method significantly improves MALDI spectra quality and also enhances peak signal intensity in comparison to the previous standard protocol.

This new protocol based on MSB-MALDI-MS could therefore provide substantial increase in analysis speed and peptides detection in the low ng/mL range thus allowing a very sensitive detection of plasma/serum peptidome.

Efficient Digestion and Nanoscale LC-MS/MS Analysis of Low-nanogram Amounts of Protein Samples [P50]

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The classical approach to the identification of unknown, partially purified proteins, relies on gel based separation, tryptic digestion and mass spectrometric analysis of the generated peptides. The low yields associated with this method normally allow for the identification of proteins in amounts down to the mid-nanogram range (10-50 ng), whereas MS sensitivity would, in principle, be capable of achieving much lower detection limits.

Off-gel protein digestion and MS analysis, the "shotgun" proteomics approach, has been pioneered by Yates and co-workers[1]. Currently, it is the most straightforward method to characterize protein mixtures, spanning from affinity purified protein complexes to whole cell lysates.

We describe a protocol for the direct off-gel digestion of minute amounts of proteins and for the efficient purification of the resulting tryptic peptides before nanoscale LC-MS/MS analysis. The protocol consist of three main steps: (i) sample digestion in presence of moderate amounts of non-ionic detergent (0.1% - 0.5%); (ii) desalting through standard RP SPE columns; (iii) Ziptip SCX purification for removal of the detergent. Zip tip eluate is directly diluted with chromatographic mobile phase and injected for nanoscale LC-MS/MS analysis.

The method is applicable to partially purified protein mixtures, and it allows the identification of proteins in the low (~1-2) nanogram range from "real-life" conditions, it is compatible with relatively large sample volumes (hundreds of microliters), and it is tolerant to the presence of potential MS contaminants like anionic and non-ionic detergents, urea, non-volatile buffers .

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In-depth analysis of the HeLa phosphoproteome using specific phosphoprotein purification chromatography and MALDI chip based IMAC phosphopeptide enrichment [P51]

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Introduction

The specific phosphorylation of serine, threonine, or tyrosine residues is the most common mechanism for the regulation of cellular protein activity. A wide range of cellular processes and activities, such as transmembrane signaling, intracellular amplification of signals, and cell-cycle control are controlled by reversible protein phosphorylation. Here we present a workflow for efficient purification of phosphoproteins and subsequent MALDI-MS/MS detection of phosphopeptides. This workflow covers the purification of phosphoproteins using affinity column chromatography, protein separation by high resolution 2D-PAGE, and MS-based protein identification. Protein phosphorylation was validated with the help of on-chip IMAC enrichment of phosphopeptides and subsequent analysis by MALDI-MS/MS.

Preliminary results

Processing of 2.5 mg protein by affinity chromatography yielded approx. 300 µg of putative phosphoproteins, which were separated by 2D-PAGE. After staining for total protein with a sensitive fluorescence dye, approx. 400 protein spots were detectable, 200 of which were randomly selected for MS analysis. Direct application of the resulting tryptic digests onto the pre-sublimated CHCA matrix of Mass·Spec·Turbo Chips and subsequent acquisition of MS and MS/MS spectra yielded a high number of unambiguously identified proteins. The subsequent enrichment of phosphopeptides on Mass·Spec·Focus IMAC chips was evaluated by typical spectral features of phosphopeptides such as the occurrence of PSD ions resulting from neutral loss of H3PO4 and HPO3 and matching to the sequence of the respective protein. Further analysis of these phosphopeptide candidates in MS/MS experiments often showed the typical dominant neutral loss fragments (-98 Da) and y- or b-ion series with weaker signal intensities. The precise mapping of phosphorylation sites in a number on-chip purified peptides could be demonstrated.

Novel aspects

Efficient and specific two step pre-fractionation scheme (protein and peptide purification) for in-depth analysis of phosphoproteomes by 2D-PAGE/MALDIMS

New Technological Platform for Undertaking of Preclinical and Clinical Trials of Drugs [P52]

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The aim of the work was a making of the modern technological platform for high-throughput screening of new molecules for development of target drugs. The research idea of the project includes the following stages of the drug development: 1)searching stage of the perspective drugs on base of the genetic and proteomic study; on this stage we are conducted studies in the field of genomics, proteomics, combinatorial chemistry, bioinformatics with using of technologies of polymerase chain reaction, parallel sequencing, two-dimensional electrophoresis in polyacrylamide gel, MALDI-TOF-mass-spectrometry (Autoflex II, Bruker, USA), computer analysis of the three-dimensional structure of the molecular target, computer design of the chemical structure of drug, estimation of biophysical parameters of drug; 2) preclinical stage of the drug development expects the separation of the chemical structure of perspective drug-leader on base of screening and testing with using of cellular technology, laboratory biomodels; in this stage we are conducted pharmacokinetic study (zero-time concentration, maximum and minimum concentration of new drug, drug concentration in the last test, "steady-state" drug concentration in biological liquid and tissue of biomodels by liquid chromatography/mass-spectrometry [Thermo Finnigan, USA], estimation of absorption rate constant, elimination rate constant, half-life period of drug, total clearance, renal and non-renal clearance of drug, time of the achievement of maximum drug concentration in central camera, distribution volume, area under timeconcentration curve, standardized safety margin) and pharmaceutical study of drug (in-vitro dissolution test, estimation of the chemical structure by IF – spectrophotometry [Perkin Elmer, USA]); 3) stage of clinical trial of the drug, expecting using of genotype technology, phenotype technology in pharmacoproteomic and toxicoproteomic studies and the research of single nucleotide polymorphisms of perceiving and metabolizing systems of drug in the human organism; 4) stage of state registration of the drug and introduction new product into the clinical practice with building of population PK / PD drug model.

Consequently, these high-throughput screening technologies are being used to develop improved drugs and discover new biomarkers, as well as for personalized medicine and biodefense.

Application of Two Dimensional Chromatography coupled to Tandem Mass Spectrometry (2DC-MS/MS) in Proteomic Analysis

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One of the aims of proteomics is to obtain large-scale protein identification from highly complex biological specimens, in order to determine the global protein expression patterns and identify proteins whose expressions are altered. Recently, to reduce the complexity of protein mixtures, many new methodologies have been developed, including two-dimensional chromatography coupled to tandem mass spectrometry (2DC-MS/MS)[1]. This approach, called MudPIT (Multidimensional Protein Identification Technology) provides a significant improvement over gel-based analysis, as it represents a fully automated and high throughput technology, that simultaneously allows separation of digested peptides, their sequencing and the identification of the corresponding proteins (Fig. 1). In this way, it is possible to characterize protein mixtures in a wide pI (<4 or >10) and MW (<10 kDa or >250 kDa) range, and membrane proteins are also identified.

In fact, 2DC is configured to separate peptides based on charge and hydrophobicity in the first- and 2nd- dimension respectively and connected to an MS instrument for online analysis of separated peptides. The peptide mixture is loaded onto a strong cation exchange (SCX) column, eluted stepwise with salt injections of increasing molarity and captured onto peptide traps for concentration and desalting prior to final separation by reverse phase C18 column. The two traps are loaded in turn (that is, one is being loaded and equilibrated while the other is being eluted), reducing the cycle time required for each sample. For each step a subset of peptides eluted from C18 column are MS and MS/MS analyzed by a mass spectrometer which operates in a data-dependent mode. The acquired MS/MS spectra are matched to the "in silico" MS/MS spectra of the corresponding protein databases for protein identification via the SEQUEST search engine. In order to compute the large amount of data typically obtained from MudPIT proteomic

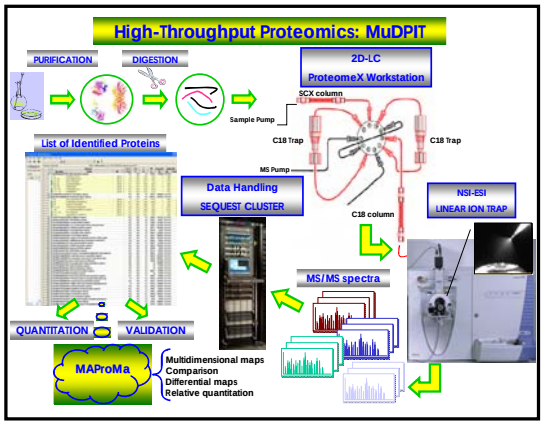

Fig. 1: Scheme of MudPIT.

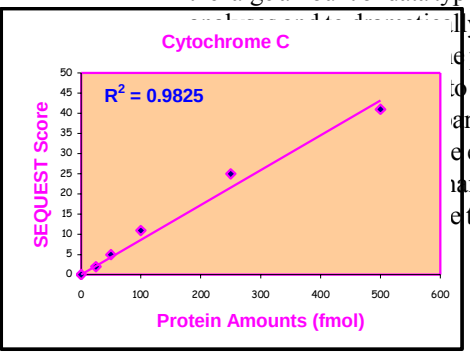

Fig. 2: Protein amounts vs Sequest Score values.

y reduce protein database search times, e power of MS information management o separate tasks with a SEQUEST Cluster arallel on different nodes[2].

employ this technology in order to achieve proteomic profiles of different sample types, such an cells and tissues[4,5]. The structural characterization of proteins is also studied[6].

to obtain a reproducible semi-quantitative evaluation of relative protein abundance in samples utilizing SEQUEST-based proteomic analysis platform, which allows the identification of up- and down-regulated proteins by comparisons performed between normal and modified samples, such as healthy/diseased, treated/not treated or at different stages of growth. Moreover, by analyzing various samples a direct relationship between the SEQUEST score values and relative abundance of identified proteins has been observed (Fig. 2).

Based on these findings, we have also developed a software MAProMa (Multidimensional Algorithm Protein Map) that allows simple, rapid and semi-quantitative comparison of different samples with presentation of results in a user-friendly format for biologists.

The main results will be presented.

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N-terminal isotope tagging stategy for quantitative proteomics [P54]

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Recently, stable isotope-coding strategies have been widely used for the quantitative analysis of proteins in complex biological samples by mass spectrometry. Many reagents are commercially available like ICAT, iTRAQ, followed by LC or MALDI MS and MS/MS analysis. The target of the present work is to apply a new set of reagents used for the identification and quantification of complex protein mixtures. These reagents are trifunctional, stable isotope molecules composed of a "reporter", a "spacer", and a specific reactive group capable of reacting easily with N-terminal and amine side chain groups of proteins.

For this work Dansyl chloride (Dns-Cl ,**1**) is suitable, in fact is a widespread used reagent in biochemistry for its fluorescent properties. It was introduced several years ago in peptide chemistry[1] as a molecular device for driving the gas-phase sequencing of peptides by electron ionization mass spectrometry (EIMS). An increase in BSA protein sequence coverage[2] was quite recently reported when Dns-derivatized tryptic peptides were analyzed by matrix-assisted laser desorption ionization (MALDI), whereas a selective detection and identification of phosphopetides was achieved by electrospray ionization (ESI) of Dns derivatized peptide digests.[3]

For the above application we developed a new method for the synthesis of Dansyl derivatives[4]. According to this, d_6-Dns-Cl (**2**) was synthesized while Dansyl chloride (**1**) is commercial available.

Two sets of standard proteins were chosen to devise the experimental procedure of labelling. The optimal conditions for the labelling achieved in basic pH ($NaHCO_3/Na_2CO_3$ buffer) in rt for 3 hrs. In the above conditions Dns group reacts with the N-terminal group of peptides and the side-chain amine group of arginines. In Figure 1 is reported the MALDI MS spectrum of a tryptic peptide mixture obtained from equimolar dansylated (d_0/d_6) protein. Hopefully we correspond that data results are reproducible when different ratios of protein mixtures tried.

Figure 1: Tryptic Peptides mixture obtained from equimolar dansylated (d_0/d_6) protein.

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Investigation of protein isoforms by proteomics and bioinformatics techniques [P55]

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Mitofilin is an integral membrane protein of the inner mitochondrial membrane. Separation via bidimensional electrophoresis (2DE) of human kidney cortex proteins determines train of spots that are identified by MALDI-TOF spectrometry as Mitofiln. These spots present similar molecular weight (Mw) but consistent differences in Isoelectric point (pI). The coexistence of several isoforms can be explained as due to either alternative splicing, protein degradation or occurrence of post-translational modifications (PTM). The focus of this investigation is the prediction of phopshorylation isoforms using the pI change among all spots identified by western blot.

Theoretical pI values were calculated on the basis of 3D homology modeling structure and pKa values of the involved amino acids, for several combinations of phosphorylation on different sites, in order to attempt an assigment of specific 2DE spots to a specific phosphorylation PTM.

Classical proteomic techniques and bioinformatic tools were combined in order to characterize some of these spots and to propose a prediction of possible PTM. A polyclonal antibody was used to identified all Mitofilin isoforms.

Statistical Approaches in Two Dimensional Gel Electrophoresis [P56]

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The development of analytical tools for rapid analysis and identification of protein profiles in different cells and tissues is currently a significant and suggestive area in biological research. Clinical proteomics is an interdisciplinary field that requires the collaboration and expertise of clinicians, statisticians/bioinformaticians and biologists/biochemists.

Many quantitative proteomics studies based on 2-DE gels, are commonly used to find differences in protein expression between samples under different conditions. A major obstacle to reliably determine quantitative changes is to overcome experiment to experiment variation.

Typically the reproducibility of proteomic technique used, as assayed by regression analysis, coefficient of variation or other variance estimation data, is not reported [1]. A good experimental design should consider the impact of different sources of variation in order to increase the power of conclusions and to minimize the number of false positives [2]. The aim of the present work was to asses the variance components as quantification of errors related to sample preparation, protein concentration assay and 2-DE run, in a basic experiment.

The examined data set consist of spot volume data from 2-DE gels of three samples of cortex renal cells and two samples of renal cell carcinoma analysed in triplicate. Since in differential proteome analysis we use % spot volume, we considered either the total spot volume, as normalizing factor, and % volume of each single spot for each gel. The Bartlett test of total volume did not allow to reject the hypothesis of variance homogeneity ($p = 0{,}853$). The evaluation of random effects contribution to the variance of the total spot volume was calculated applying a nested random effects model. The results highlighted a variability within sample three times higher than that between samples. Therefore, the CV% associated with total spot volume, including the variability in loading sample pipetting, protein quantitation, and other factors, was 12,98%.

Moreover, we have considered four spots in order to evaluate the variability of % spot volume. The CV% obtained was in the range of 18%-29%. Further investigations are necessary to create a model to analyse all spots in the gel [2, 3].

This basic variance model will be helpful in the following phase to identify molecular cluster profiles associated to renal cancer in discovery of new potential diagnostic markers.

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Comparison between fractioning technologies for differential protein analyses [P57]

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A good fractioning method enhances the number of proteins identified by mass spectrometric (MS) analysis and improves their relative quantification. The choice of the fractioning method is therefore critical.

In this study we report the results of two different separation technologies used for differential analysis. Prostate cancer cell lines PC3 and LNCaP were the models for the study. The first technology used was a two-dimensional liquid chromatography system (PF-2D, Beckman Coulter) , i.e. chromatofocusing (1st dimension) and reverse phase chromatography (2nd dimension), followed by SDS-PAGE due to MS method used for identification (3rd dimension). In this way, for each cell line, 1544 chromatographic peaks were observed, that were analyzed on differential map (Delta Vue analysis, fig. 1).

The other technology consisted in electrophoretic-separation: proteins were first separated by liquid isoelectric focusing (1st dimension) and then by gel electrophoresis; however, due to high protein number on gel, running focusing fractions on reverse phase (2nd dimension) column followed by SDSPAGE (3rd dimension) was preferred; 426 peaks were observed and compared for differential analysis.

Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) was used in both approaches for protein identification. Advantages and disadvantages of the two systems are discussed and results are compared.

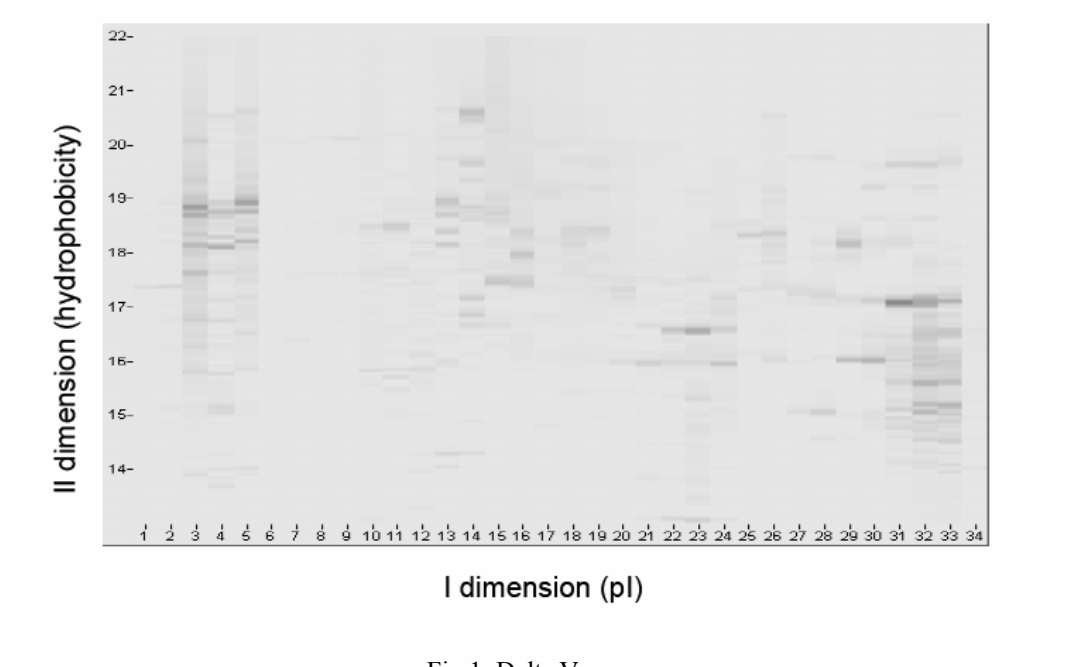

Fig.1: Delta Vue map

A novel method to selectively detect, identify and quantify post translational modifications by MS/MS/MS fragmentation [P58]

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Mass spectrometry, one of the most powerful tool in modern Analytical Chemistry, is the core methodology in proteomics. 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 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.

Treatment with selective derivatizing agent and manipulation of solution chemistry can improve ionization and detectability of analytes by ESMS. Since 1968 [1] we demonstrated, for the first time, that 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 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. 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[4-5] in complex protein mixtures. Moreover, the quantification problem will be addressed by developing novel strategies aimed at the quantification of PTM modifications exploiting new iTRAQ modified reagents coupled with MS3 experiments. We planned to wide the chemistry of the reagent to address quantification of function of other primary amines by taking advantage of the experience made with dansyl chemistry.

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Glycans profiling by a proteomic approach [P59]

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Oligosaccharides characterization is arousing a particular interest, being a challenging analytical problem. These molecules present extremely various structures as concerning the type of monosaccharides they are built of, the branching, the anomericity of the glycosydic linkage and the global structure that the carbohydrate moieties confer to proteins.

Nowadays mass spectrometry is answering to most of the challenges connected to the study of glycans: the problem of glycoforms heterogeneity has been overcome by mass spectrometry methodologies, which allow to achieve qualitative and quantitative information on oligosaccharides structures present in the analyte, with enormous sensitivity.

In this work we provided an overview of the connection between glycan structures and disease progression, especially in cancer and inflammation. This glycobiological approach represents a new opportunity for therapeutics and diagnostics, since oligosaccharides provide signals for cell-cell, cell-matrix, protein-protein interactions and protein targeting. Such signals are involved in several pathological processes concerning cell recognition and adhesion. Therefore comparative study of the N-glycosylation pattern of the entire human serum glycoproteome can be considered an excellent probe for specific pathologies.

In this study we focused our attention on two different pathologies: a serum sample from a patient diagnosed with myocardial lesions and one deriving from a patient affected by hepatic carcinoma.

The glycans profiling of the myocardial lesions disease revealed the presence of hyper-fucosylated signals, whereas the K-hepatic serum was characterized by hyper-fucosylation and hyper-sialylation. Moreover glycopeptides enrichment performed by ConA affinity chromatography on serum tryptic digest coupled LC-MS/MS analysis allowed both the identification of glycoproteins by a proteomic approach and the localisation of several N-glycosylation consensus sequences.

Multidimensional Liquid Cromatography of Proteins Employing Monolithic IEX and RP Columns [P60]

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The development of monolithic columns during the past years has brought a great improvement in the biopolymer analysis field. Monolithic columns offer several advantages over particulate columns due to their macroporous structure leading to fast mass transfer, low backpressure and high resolution. Poly-styrene divinylbenzene (PS-DVB) based monolithic columns are chemically inert, offering high pH stability and excellent chromatographic performance in reversed phase LC. While the application of reversed phase monolithic columns is quite common, the use and availability of functionalized monolithic columns is small. To enable the fast 2D-LC analysis of complex protein samples a monolithic column with anion exchange properties was designed.

By performing fraction collection between the two chromatographic dimensions high method flexibility is achieved. Moreover, protein mixtures are separated into less complex fractions resulting in much higher sequence coverage and hence confidence in the identification.

In the presented work proteins were separated on polymethacrylate based monolithic WAX and PS-DVB based reversed phase columns. The orthogonality, peak capacity and robustness of the 2D-LC method are studied with a set of protein standards and a bacterial protein lysate.

Novel off-line ultidimensional LC Method for Separation and Tandem MS Detection of Tryptic Peptides [P61]

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Introduction

Multidimensional liquid chromatography (MDLC) is a valuable technique for bottom-up and top-down workflows in proteomics. Various approaches of MDLC, e.g. MudPit, have been described for the separation of peptides. Off-line MDLC techniques have several advantages over on-line approaches: i) higher flexibility with respect to column dimensions and mobile phase selection, ii) easier method development and iii) the ability to perform re-analysis of the fractionated effluent. On the other hand off-line LC methods are labour intensive and time consuming.

Here we discuss a novel off-line 2D-LC method for the separation of tryptic peptides. The peptides are separated on a 300 µm i.d. packed SCX column. Micro fraction collection and re-injection of the peptide samples onto a reversed phase monolithic trap column has been automated. The second dimension separation is achieved on a 200 µm i.d. monolithic column which is connected to an ion-trap mass spectrometer.

The method was evaluated with a complex protein tryptic digest sample. Peptide identification by tandem MS allowed the assessment of the quality of the 2D-LC separation. It was found that most of the peptides, around 85%, were present in single or adjacent SCX fractions. The method repeatability was studied by performing consecutive 2D-LC experiments, demonstrating excellent retention time precision data for both the first and second dimension separation.

Quantitative Evaluation of DeCyder 6.5 Software the Performance in Two-Dimensional Difference in gel Electrophoresis Analysis. [P62]

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Efficient analysis of protein expression in the differential proteome using Two-Dimensional Difference In Gel Electrophoresis (2-D DIGE) data also depends on automated image processing. Since DeCyder™ 6.5 is the software platform specifically designed for DIGE analysis, the aim of the present study was to verify and validate the results obtained by this program to improve the accuracy of DIGE outcomes. A set of tests was developed to quantitatively evaluate three essential steps of DeCyder analysis including: spot detection, gel matching, and spot quantitation.

In the first step, the software package by DIA (Differential In-gel Analysis) module attempts to determine whether a spot is, or is not, a protein. To test spot detection the automatically detected protein spots were numbered and compared to the ones manually detected as "real". In the second step, the BVA (Biological Variation Analysis) module, performing gel to gel matching, allows quantitative comparison of protein expression across multiple gels. To study match efficiency, the outcomes of auto-matching algorithm were checked and the percentage of erroneously and correctly matched and unmatched spots were calculated.

Quantitative analysis was performed using different concentrations of two known proteins added to the same protein mixture obtained from normal kidney tissue. Analysis of variance (ANOVA) was applied to matched spots and the data was filtered to retain spots with ANOVA p values of 0.05 or less. The protein levels were quantified comparing the graphs of the predicted standardized abundance with those generated by DeCyder analysis. The percentage of "real" spots obtained by DIA module for each gel, was estimated and showed about 17% of missed, and 27% of extraneous spots. The results obtained by BVA module showed that 80% of the pair proteins was correctly matched and only 1.2 % was unmatched. Moreover, 23% of extraneous spots, such as dust, was erroneously matched. In spot quantitation tests the values of the protein abundance obtained by statistical analysis were similar to predicted ones with the trendline of $R2 = 0.96$. The quantitative analysis is the stronger point of the DeCyder software and it is able to accurately detect differences in protein expression.

Phosphorylation Site Analysis in Complex Samples Without the Use of Antibodies [P63]

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Protein phosphorylation is crucial to regulation of cell signaling pathways. An antibodyfree process workflow for phosphorylation site analysis is described involving orthogonal phosphomonoester-selective binding strategies. First, complex protein samples, such as rat kidney cytosol proteins, are separated by conventional gel electrophoresis.

Phosphoproteins are detected with a selective stain that binds to the phosphomonoester dianion moieties of serine, threonine, and tyrosine residues at neutral pH. Then, phosphoprotein bands are excised and subjected to proteolytic digestion. Constituent phosphopeptides subsequently purified using titanium dioxide thin-film coated magnetic beads at acidic pH. Phosphopeptides are eluted at alkaline pH and directly characterized by MALDI-TOF or tandem mass spectrometry without chemical modification by methyl esterification. Identification is readily performed from as little as 78 fmol of starting material.

Novel 2DE Approach for Plasma-Membrane Proteomics [P64]

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Despite the importance of membranes in any living system, the compositional analysis of membrane subproteomes is still an obstacle. In particular, the widely used 2-DE has major drawbacks regarding the separation of hydrophobic proteins and, as a result of these limitations, they are usually under-represented in common 2-DE maps. Within plasma membrane, our research focalizes on protein composition of caveolae, flask-shaped invaginations of the plasma membrane, characterized by a peculiar lipid and protein composition and involved in fundamental cellular functions, like signalling, and in neoplastic transformation [1].

Subcellular fractions, caveolae-enriched, are prepared and characterized from human renal cell cancer (RCC) and adjacent normal kidney (ANK) to be submitted to comparative subcellular proteomics using novel analytical tools, after set up on rat kidney and kidney cell lines.

We show results of application of an alternative electrophoretic technique for separating membrane proteins: two- dimensional BAC/SDS electrophoresis (2-DB) using the cationic detergent benzyldimethyl-n-hexadecylammonium chloride in the first and the anionic detergent SDS in the second dimension [2].

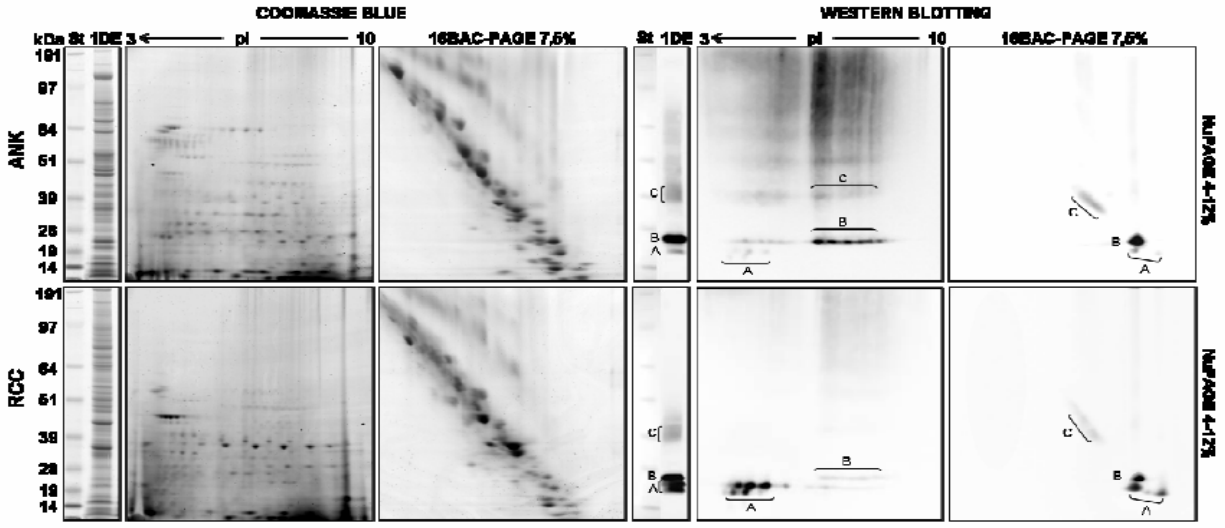

Figure 1: Comparison of monodimensional, conventional 2DE, and 2-DB separation of caveolae-enriched fractions prepared from ANK and RCC. Left panel: CCB staining; right panel: WB with anti-CAV1 (A) and anti-AQP1 (B,non-glycosylated, C, glycosylated isoforms). Equal amounts of total proteins are loaded.

It is then possible to check the quantitative recovery of known membrane proteins and the isoform pattern, by comparison with mono and traditional 2D separation, after CBB staining and WB (Fig.1). CAV1 is known to be up-regulated [3] and AQP1 down-regulated [4] in RCC. Results show that an improvement in hydrophobic proteins representation is achieved in 2-DB, compared to conventional 2DE, more evident for AQP1 than for CAV1, likely due to higher AQP1 hydrophobicity. Also the abundance of the AQP1 glycosylated isoforms is much more reliable in 2-DB, compared to conventional 2DE.

In conclusion, this novel 2DE approach shows potential in overcoming the usual hydrophobic protein under-representation in proteome analysis and therefore eases the search for candidate tumour biomarkers among membrane proteins.

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Proteomic analysis of RBC membrane protein degradation during storage [P65]

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Two-dimensional gel electrophoresis and mass spectrometry were used to identify protein profile changes in red blood cell membranes stored over time under atmospheric oxygen, in the presence or absence of protease inhibitors. New spots with lower molecular masses, ranging between 7 and 15 kDa were observed during the first seven days storage, while over time further fragments and high-molecular-mass aggregates appeared, seen as a smearing in the upper part of the gel. Some of the protein changes turned out to be shifts in isoelectric point, as a consequence of chemical oxidations. All these new spots are the result of protein attack by reactive oxygen species (ROS). Furthermore, protein identification has revealed that most proteins attacked are located in the cytoskeleton. During the first 7 days of storage oxidative degradation is observed prevalently in band 4.2, to a minor extent in bands 4.1 and 3, and in spectrin. After 14 days there were new fragments from beta-actin, glyceraldehyde-3-phosphate dehydrogenase, band 4.9 and ankyrin, among others. Preliminary protein-protein cross-linked products, involving alpha and beta spectrin, were also detected. The cross-linked products continued to increase over time.

However, protein degradation is greatly reduced when oxygen is removed and blood is stored under helium. Interestingly, very few spots are related to enzyme activity and they are more numerous when oxygen is present, indicating that some proteases may be oxygen-dependent.

Proteomics for the Elucidation of Cold Adaption Mechanisms in *Listeria monocytogenes* [P66]

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Listeria monocytogenes is a gram-positive rod-shaped bacterium found in food of different origin (fish meat, milk, soft cheese, etc) and frequently involved in foodborne disease outbreaks. It causes listeriosis, a potentially life-threatening illness. Notably, it is able to survive hostile environments and stress conditions such as those encountered in foodprocessing technologies (high salt concentration, wide range of pH and temperature, low water availability) and also to grow in biofilm mode.

In particular, this psychrotolerant organism has a minimum growth temperature estimated to be just below 2°C. Cold adaptation mechanisms are important in *L. monocytogenes*, enabling this food pathogen to survive and proliferate, thus reaching minimal infectious levels also on refrigerated foods (1). In this light, *L. monocytogenes* represents an interesting and well studied model system; the knowledge of the genome of this pathogen, which has been completely sequenced (2), opened up new prospects to proteomic studies (3).

Proteomics could in fact provide a suitable tool also to gain an improved understanding of survival and cold adaptation mechanisms implemented by *L. monocytogenes.* With this aim we carried out a comparative study on *L. monocytogenes* grown at three different temperatures (4°C, 25°C and 37°C), by integrating two dimensional electrophoretic separation, image analysis of the 2D-maps and identification of proteins differentially expressed by Peptide MALDI Fingerprint strategy.

The 70 differentially expressed proteins were grouped based on their cellular functions and metabolic pathways, using the KEGG (Kyoto Encyclopedia of Genes and Gemomes) resource (http://www.genome.jp/kegg/) (4). This classification helped to highlight a stringent correlation between proteins with similar general function and their regulation in the expression pattern in relationship with the different growth conditions.

Primary results highlighted, as major difference, a lower amount of enzymes involved in carbohydrate metabolism expressed by *L monocytogenes* grown both at 25°C and 4°C. Moreover, other down-regulated proteins included enzymes associated with amino acid metabolism, mainly of small hydrophobic residues, and nucleotide (purine and pyrimidine) biosynthesis, thus indicating that a shift in growth temperature basically induces a reduction in the general growth rate.

Interestingly, in *L. monocytogenes* grown at 4°C, proteins involved in membrane transport resulted to be down regulated, while proteins involved in cell motility, folding, sorting and degradation were up-regulated.

A study of the functional role of the identified proteins clearly suggests that cold stress at 4°C more significantly affects several, seemingly unrelated, cellular processes and a rationalization of results is still in progress to gain a deeper insight in the mechanisms of cold acclimatation. These findings may have an impact in the development of better ways of controlling this pathogen in food and related environments.

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Proteomic analysis of cryptic operon bgl in *Escherichia coli* [P67]

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The *bgl* operon, which encodes the gene products necessary for the uptake and utilization of aryl-β- glucosides, is silent (non-inducible) under all laboratory growth conditions.The cAMP-dependent catabolite regulator protein CRP regulates the *bgl* promoter positively. Various spontaneous mutations relieve silencing of the *bgl* promoter. It is likely that these mutations activate expression of the *bgl* operon by impairing a specific nucleoprotein complex, that represses transcription initiation at the *bgl* promoter under normal conditions. We used a proteomic approach (2Delectrophoresis) to analyze the variations between a wild type strain of E. coli (K12 W3110), a spontaneous mutant (W3110 bgl+), and a E. coli strain harbouring a plasmid (pASK18) that encodes for a sigma factor, psfS, which is known to activate bgl operon.

Redox stress proteins are involved in adaptation response of the hyperthermoacidophilic archaeon Sulfolobus solfataricus to nickel challenge [P68]

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Background

Exposure to nickel (Ni) and its chemical derivatives has been associated with severe health effects in human [1-3]. On the contrary, poor knowledge has been acquired on target physiological processes or molecular mechanisms of this metal in model organisms, including bacteria and archaea. In this study, we describe an analysis focused at identifying proteins involved in the recovery of the archaeon Sulfolobus solfataricus strain MT4 from Ni-induced stress. As result of the elucidation of its genome sequence in 2001 [2], hyperthermoacidophilic crenarchaeon Sulfolobus solfataricus, which grows between 70 and 90°C and in a pH range of 2–4, is an attractive crenarchaeal model organism for functional genomic analysis.

Results

Sulfolobus solfataricus was grown in the presence of the highest nickel sulphate concentration still allowing cells to survive; crude extracts from treated and untreated cells were compared at proteomic level by using a bi-dimensional chromatography approach. We focused on perturbations generated by Ni sulphate on cytosolic proteins. We identified several proteins specifically repressed or induced as result of Ni treatment (Table). Five proteins were detected only in the non-treated sample, whereas eleven proteins were detected only in the nickel-treated sample. Observed up-regulated proteins were largely endowed with the ability to trigger recovery from oxidative and osmotic stress in other biological systems. It is noteworthy that most of the proteins induced following Ni treatment perform similar functions and a few have eukaryal homologue counterparts.

Conclusions

These findings suggest a series of preferential gene expression pathways activated in adaptation response to metal challenge.

Table - Down/Up-regulated proteins detected following challenge of S. solfataricus with 100 mM NiSO4.

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The Transcriptional Machinery Gathered at the *E. Coli Rrnb* P1 Promoter Includes Proteins involved in the Biogenesis of Ribosome and the DNA Repair Mechanisms [P69]

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Functional proteomic studies were carried out to investigate the transcriptional machinery that gathers at the *rrnB* P1 promoter in *E. coli.* A multimer of a 28-mer deoxynucleotide encompassing the upstream and the -35 sequence of the ribosomal promoter was covalently linked to agarose beads and incubated with total protein extracts from K12 *E.coli* cells. A randomised version of the oligonucleotide was also prepared, incubated with an equal amount of the extract. and used as control.

Protein bands only occurring in the sample, i.e. those recruited by the correct oligonucleotide system, were identified by both MALDI-MS and LC-MS/MS analyses of their *in situ* generated tryptic peptides. Some of the identified proteins were cloned and expressed and their ability to bind the oligonucleotide bait was investigated. Verification of the interaction among the different proteins was obtained by comparing the proteomic results with the *E.coli* interactome as recently reported. The results obtained showed that the identified proteins gathered within three biologically distinct functional groups, the transcription machinery, the biogenesis of ribosomes and the AidB related DNA repair mechanism. Functional hypotheses on the possible functional significance of the latter two complexes were suggested.

Competition between GABA decarboxylation and ADI pathway detected by combined proteome and transcriptome in *L. lactis* NCDO 2118 [P70]

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Biogenic amines are compounds commonly present in living organisms in which they are responsible for many essential functions. In cheese, γ-aminobutyric acid (GABA) can be produced by *Lactococcus lactis* by means decarboxylation of glutamate. The GABA is an inhibitory neurotransmitter of the central nervous system and it could have a blood pressurelowering effect. The main enzyme responsible for the formation of GABA is the glutamate decarboxylase, a pyridoxaldependent lyase. This reaction allows to increase the alkalinity of the cytoplasm and to generate ATP. Moreover, in *L. lactis,* also the ADI (arginine deiminase) pathway is involved in both acid resistance and energy production. The aim of this study is to investigate the relationship betweeen these two metabolic routes. A proteomic and transcriptomic approach was used. Both methods reveal that there is competition between the two pathways and in particular the enzymes arginine deiminase, ornithine transcarbamylase and carbamate kinase are down-regulated when glutamate is present. These results are of high value for good industry applications since GABA is a welcome molecule while the ADI pathway generates toxic metabolites like ammonia and ethyl carbamate. So supplementing media with glutamate is a mean to repress the expression of the ADI pathway's enzymes.

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Subcellular proteomes of *Acinetobacter radioresistens* S13 reveal the presence of a membrane-bound biosurfactant [P71]

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Acinetobacter radioresistens S13 is a gram-negative strain selected for its efficiency in phenol biodegradation and lipase production. It is also able to produce a glycoprotein (made-up of three subunits: 16kDa, 31kDa and 39kDa) with surfactant properties named Alasan. *A.radioresistens* S13 has been fully characterized from a proteomic point of view, and both acidic and alkaline cytosolic proteome as well as membrane proteome are available at present. Since the microorganism has not been fully sequenced at present, spots identifications were obtained via *de novo* sequencing. A time-course proteomic investigation demonstrates that the higher molecular mass subunit of Alasan is located mainly in the cytosol during the early exponential phase, in the outer membrane fraction during the late exponential phase and it disappears during stationary phase. Supposing an extracellular location in this growth stage, experiments including ammonium sulphate precipitation, SDS-PAGE and 2DE electrophoresis were performed on culture broths. Three proteins of 16kDa, 31kDa and 45kDa respectively were detected. The difference between this finding and the theoretical expected for the higher molecular mass subunit, can be ascribed to different degrees of glycosylation. The emulsifying activities of the partially purified complex have been determined on different hydrophobic molecules and compared with those of commonly used surfactants.

Mitochondrial proteome of *Saccharomyces cerevisiae* mutants lacking SCO1 or SCO2 (Synthesis of Cytochrome c Oxidase) [P72]

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Sco is a family of proteins ubiquitous to all kingdoms of life. Ortholog and paralog genome browsing has shown that one or more representative of this class are present in most bacterial and eukaryotic genomes (1). Eukaryotic genomes contain two paralogs, Sco1 and Sco2 (2), that code for mitochondrial metallochaperone proteins with essential, but poorly understood, roles in copper delivery to cytochrome c oxidase (COX) (3). Mutations in human SCO1 and SCO2 produce tissue-specific COX deficiencies associated with distinct clinical phenotypes, although are ubiquitously expressed. Mutations in hSCO1 cause fatal infantile hepatoencephalomyopathy (4), whereas mutations in hSCO2 cause fatal infantile cardioencephalomyopathy (5). The reason for the tissue specificity in the two disorders is unknown. To investigate the molecular function of the Sco proteins we characterized the mitochondria of Saccharomyces cerevisiae cells lacking SCO1 or SCO2. Yeast is an irreplaceable model system because it is simple and its gene annotation is excellent. It is currently the only organism in which efficiency of new technologies has been tested at a whole-genome level and the function of a basic set of recently discovered genes conserved in all eukaryotes has been explored in detail by the means of new technologies and also by classical genetics and biochemistry.

First, we assessed the growth phenotypes on a defined growth medium containing different carbon sources (fermentable and non-fermentable). As observed previously, sco1 mutant but not sco2 exhibited growth defect on a respiratory carbon source like glycerol and ethanol. Moreover sco1 mutant but not sco2 grew more slowly and reached a lower cell density on medium containing 0.5% glucose (calorie restriction). The differences in growth rate in sco1 mutant strain resulted from differences in the levels of mitochondrial respiration. In particularly, the rate of mitochondrial respiration decreased dramatically in this strain during the growth on glycerol. On the contrary, in sco2 mutant the respiration rate increased in comparison to the wild type strain. The growth pattern, along with the respiratory deficiency in sco1 mutant, could be related to dysfunctional mitochondria. We explored the possibility of a defective membrane potential by observing the accumulation in cells of the rhodamine B hexyl ester (Rhodamine B), a cell-permeant dye developed for mitochondrion staining. It is often used for detecting the changes in membrane potentials caused by respiratory activity, ion-channel permeability and drug binding (6). The cells from wild type and Ä sco2 strains showed characteristic mitochondrial staining, with rhodamine B accumulating at the inner mitochondrial membrane, while the Ä sco1 strain showed diffused staining pattern with no rhodamine accumulation. This results indicated that mitochondrial membrane potential is lost in Ä sco1 cells.

In order to characterized the mitochondrial proteome of cells lacking SCO1 or SCO2 during the growth on glycerol (respiration), we performed a two dimensional electrophoresis of this organelle. Image analysis of two-dimensional electropherograms revealed the presence of both quantitative and qualitative variations between the two mutants in comparison to the wild type. Protein spots of interest will be identified by Mass Spectrometry.

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A proteomic characterization of Nonomuraea sp ATCC 39727 in two different growth and antibiotic production conditions [P73]

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Introduction:

Actinomycetes are well known producers of secondary metabolites (Demain, 2000). This group of bacteria includes industrially important antibiotic producers, like the glycopeptide producers Amycolatopsis orientalis. The model organisms for this group of bacteria are Streptomyces coelicolor and Streptomyces avermitilis. Nonomuraea sp ATCC 39727 is a strictly aerobic actinomycete, industrially important as producer of the glycopeptide A40926 (Goldstein et al., 1987), used as a precursor of the semi-synthetic antibiotic dalbavancin currently under clinical development. 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 (Technikova-Dobrova et al., 2004). 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 two dimensional electrophoresis protein pattern in the different growth conditions tested have been detected, and the protein spots showing significant differences have been analysed by mass spectrometry.

Materials and methods

Nonomuraea sp ATCC 39727 was obtained from ATCC. The strain was maintained and cultivated as described (Technikova-Dobrova et al., 2004). 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). The cultivation was performed three times in triplicate. The A40926 was analysed by high-performance liquid chromatography (HPLC) as described (Technikova-Dobrova et al., 2004) using pure (>90%) A40926 (kindly supplied by Sanofi-Aventis Bulk) as a standard. The sample preparation was performed starting with stationary phase cultures at the end of the production period (Technikova-Dobrova et al., 2004). Two-dimensional gel electrophoresis (2DE) Proteins were separated by 2DE. For isoelectric focusing 250 ?gr of proteins diluted in the IPG strip rehydration buffer, were loaded on 24-cm IPG strips (GE Healthcare) that provided a linear gradient from pH 3 to 10.The second dimension was a SDS-polyacrylamide (12.5%) electrophoresis using the buffer system of Laemmli. The gels were stained with Coomassie brilliant blue. Analysis of protein patterns Stained gels were scanned with an Image Scanner (GE Healthcare) and analysed with ImageMaster 2D platinum V.5 (GE Healthcare). Spot detection was done automatically using the default settings in the first instance. Spots present in all the gels of both classes and exhibiting an intensity difference between the two growth conditions with a p value < 0.05, using the two-tailored Student's t test. Protein identification by mass spectrometry The stained spots were excised and digested in gel with trypsin. The resulting peptides were extracted and separated by capillary scale reversed-phase liquid chromatography and then separated on a column. The eluate from the column was directed to the ESI source of a Q-Tof Micro hybrid quadrupole/time-of-flight mass spectrometer. Data-directed analysis was employed to perform MS/MS analysis on up to fourthly charged precursor ions. MS/MS Ion Search was employed for protein identification by using Mascot. Enzymatic assay The enzymatic activity of MDH was performed as described in (Laluce et al., 1987).

Results and discussion

The pattern of protein expression has been analysed in two different growth conditions and and analysed by 2D electrophoresis. In order to analyse the proteins differentially expressed in the two growth conditions only the matched spots present in all gels of both classes have been investigated. Using this constrained statistical analysis only seven proteins were considered differentially expressed in the low A40926 production condition respect to the high one. Of the variable spots identified by the analysis of the 2DE images one, showing a decrease in the overproduction condition, was identified with high confidence. This spot has been identified as a malate dehydrogenase (MDH), in all the databases used. The different expression level in the two growth conditions of malate dehydrogenase 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, as compared to that measured in the extract from the bacteria grown in the A40926 low-production conditions. Modifications in the tricarboxylic acid cycle have been proposed to be involved in the overproduction phenomenon in different model systems (Technikova-Dobrova et al., 2004). 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 Krebs cycle activity, will make more acetyl-CoA available for A40926 production. The systematic exploration of the metabolic networks and the identification of the proteins differentially expressed in response to different minimal media will give a better understanding of the bacterial response to different environmental conditions and of the metabolic processes involved in antibiotic production.

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Analysis of Rps19 protein and Rps19 missense mutant proteins interactome [P74]

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Ribosomal protein S19 (Rps19) is a 16 kDa protein found mainly as a component of the ribosomal 40S subunit. Its mutations are responsible for 25% of Diamond Blackfan Anemia (DBA) cases. DBA is a congenital disease characterized by defective erythroid progenitor maturation (1). Dysregulation of Rps19 has therefore been implicated in this defective erythropoiesis, though the link between them is still unclear. A loss of function not directly connected with Rps19 structural role in the ribosome have been proposed. We used proteomic strategies to look for proteins interacting with Rps19 in order to determine its functions (2). Finally we identify proteins interacting with Rps19 missense mutant proteins R62W, R101H and V15F as described by De Costa el al.

Protein complexes are isolated by affinity purification using GST-Rps19, GST-Rps19$_{R62W}$ GST-Rps19$_{R101H}$ and GSTRps19$_{V15F}$ recombinant proteins and identified using LCMS/MS analysis. To define the interactome of Rps19 and Rps19 missense mutant proteins we subtracted species common to the GST. Raw data from μLCMS/MS analyses are converted into a Mascot format text in order to identify proteins by means of the MatrixScience software *(www.matrixscience.com)*.

We identify 159 proteins interacting with wild type Rps19 from the following Gene Ontology categories: NTPases (ATP- and GTPases; 5 proteins), hydrolases/helicases (19 proteins), isomerases (2 proteins), kinases (3 proteins), splicing factors (5 proteins), structural costituents of ribosome (29 proteins), transcription factors (11 proteins), transferases (5 proteins), transporters (9 proteins), DNA/RNA-binding protein species (53 proteins), other (1 dehydrogenase protein, 1 ligase protein, 1 peptidase protein, 1 receptor protein, 1 translation elongation factor) and 13 proteins of still unknown function (2). Proteomic results are validated by western blotting and by co-immunoprecipitation using a monoclonal Rps19 antibody. Many interactors are nucleolar proteins and thus expected to take part in the Rps19 interactome; however, some proteins suggest additional functional roles for Rps19.

We also carry out an in silico proteomic analysis of proteins known to directly or indirectly interact with Rps19 by using databases HPRD Human Protein Reference Database *(www.hprd.org)* and PubMED *www.ncbi.nlm.nih.gov*. A list of primary (direct) and secondary (indirect) protein-protein interactions of RPS19 is created using the web available Human Protein Reference Database. Primary interactions of Rps19 are screened for protein interactors to define an in silico interaction map with the indirect protein partners. This map is then compared with the Rps19 protein partners identified by experimental proteomic strategies. In addition a list of primary interactions is created by HPRD for each identified protein. The list of Rps19 protein partners is compared with the Nucleolar Proteome Database *(http://www.lamondlab.com/NoPDB)* and with the Pre-Ribosomal Network yeast database *(http://www.pre-ribosome.de/Home.html)*. Interactomes of Rps19 mutant proteins are compared to the wild type protein one in order to elucidate the loss of function hypothesis suggested for DBA pathogenesis.

The characterization of Rps19 interacting proteins provides information for studies aimed at more cleary defining the role of the protein in the DBA.

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Design of fermented milks enriched with ACE-inhibitory peptides produced by recombinant DNA technologies [P75]

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Together with their nutritional value, food proteins carry a wide range of functional properties. Several of them are ascribed to the bioactive peptides (BPs) encrypted in their primary structure. BPs need to be activated by enzymatic proteolysis of parent proteins during gastrointestinal digestion or food processing. Milk and dairy products represent an excellent source of BPs and in particular of Angiotensin-I Converting Enzyme (ACE) inhibitory peptides displaying antihypertensive activity. Currently, some potent ACE inhibitory peptides are employed as additives in milks fermented by specific starters to produce new *functional foods*. Since there is a need to develop cost-effective technologies which ensure the production of purified BPs with high yields, here we report the over-expression in *Escherichia coli* of three bovine β-casein derived BPs with known ACE-inhibitory activity as recombinant GST-BP fusion proteins. Following cleavage with a *Lb. helveticus* proteinase, required to release active BPs, the fraction containing polypeptides of molecular weight lower than 10 KDa, purified by RP-FPLC and characterized by LC-MS, was assayed for ACE inhibition and used for the enrichment of fermented milks to design a novel ACEinhibitory milk drink. This new procedure represents a biotech method for the production of *functional foods* containing recombinant BPs suitable for hypertension prevention.

Change in Protein Expression during Progression of Papillary Thyroid Carcinoma [P76]

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Protein expression differences in stage I and stage IV papillary thyroid carcinomas (PTC) bearing the $BRAF^{V600E}$ mutation and in matched normal thyroid tissues were evaluated by 2DE-MS/MS. Tissue specimens from PTC and the corresponding normal thyroid samples from the contra-lateral lobe were snap-frozen at the time of surgery. Tumors were assayed for *BRAF* mutational status as previously described [1]. Histological examination and immunological assays for serum levels of anti-thyroglobulin and anti-thyroperoxidase antibodies were performed to exclude patients affected by lymphocytic thyroiditis from the present study.

Ten samples bearing the $BRAF^{V600E}$ mutation and corresponding control tissues were submitted to proteome analysis.

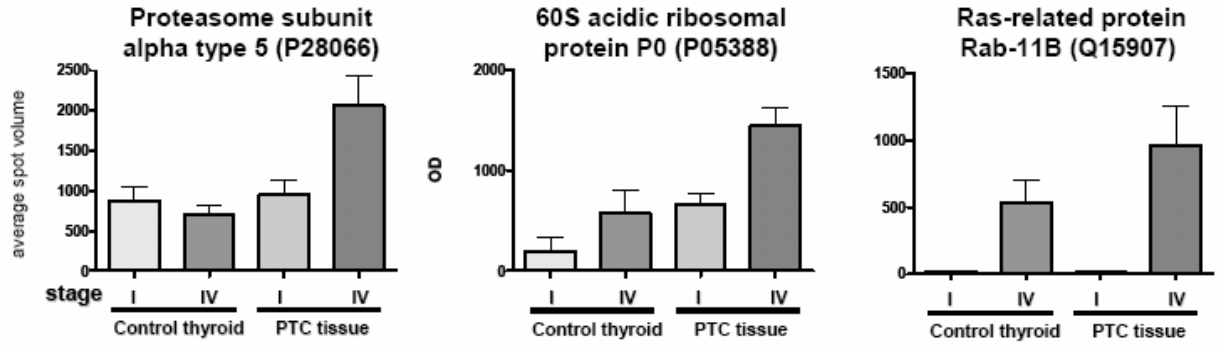
Five PTC samples classified as stage I tumors and five PTC samples classified as stage IV tumors were analysed. Oneway ANOVA test was used to assess statistical significance of expression differences.

Three protein spots, overexpressed in stage IV tumors (five patients) as compared to stage I tumors (five patients) and to the respective control tissues, were identified.

Proteasome subunit á-type 5 (P28066), whose expression was not statistically different in control and tumor tissues in stage I, was overexpressed in stage IV, as compared to matched control thyroid tissues.

Although up-regulated in both stage I and IV tumor tissues, as compared to matched normal thyroid samples, the expression of the 60S acidic ribosomal protein Po subunit (P05388) was more than two times higher in stage IV than in stage I tumor samples and in stage IV control samples.

The third protein whose expression was higher in stage IV than in stage I tumor tissues is the Ras-related protein Rab-11B (Q15907), that was almost undetectable in stage I tumor and control tissue 2DE gels. This protein was overexpressed with high statistical significance in both stage IV tumor and matched control thyroid.



Western blot and immuno-histochemical tests were performed to explore the potential diagnostic use of the present findings.

Although statistically significant in 2DE analysis, the differential expression of proteasome subunit á-type 5 and of ribosomal protein Po tested in stages I and IV tumors by Western blot analysis did not provide any possibility to clear- cut identify tumor stages. Indeed, in some tumors classified in stage I both proteins were more expressed than in tumors classified in stage IV.

On the contrary, the extremely different expression of Rab-11B in stages I and IV, as observed in 2DE analyses, are expected to provide useful information for staging. Unfortunately, suitable antibodies to use for Western blot analysis of tumor samples are not available, at present.

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Proteome and Phosphoproteome Differential Analysis in PC12 Cells Overexpressing Substrate-Trapping Mutants of the LMW-PTP Isoenzymes [P77]

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INTRODUCTION

Low molecular weight protein tyrosine phosphatases (LMW-PTPs) are small enzymes that are ubiquitous in many organisms. They are important in biological processes such as cell proliferation, adhesion, migration and invasiveness (1). LMW-PTP is expressed in mammalian cells as two isoforms (IF1 and IF2) originating through alternative splicing. IF1 and IF2 differ in substrate specificity and in the sensitivity to some modulators (2, 3) and it is reasonable to suppose that they perform distinct physiological functions. The best characterized isoform is IF2; this enzyme plays a key role in cell proliferation control and many proteins involved in signalling pathways are among its substrates: PDGF, insulin, and ephrin receptors, STAT5, p190 RhoGap, caveolin-1, and b-catenin. To investigate the role of low molecular weight protein-tyrosine phosphatases isoenzymes, two mutants (IF1/D129A and IF2/D129S) that do not possess any catalytic activities but appear to bind tightly to their tyrosine phosphorylated substrates have been overexpressed in PC12 cells (rat pheochromocytoma). PC12 cell line is a useful model for study cell signaling since it responds to many growth factors, neutrophins and hormones.

EXPERIMENTAL PROCEDURES

Cell Culture and Differentiation PC12 cells were cultured in RPMI medium supplemented with 10% horse serum and 5% fetal bovine serum. Neuronal phenotype and differentiation was induced by growing PC12 cells in the presence of 50 ng/ml of nerve growth factor for 4-5 days. *Cell stimulation* Cells were treated with 50 ng/ml NGF or 60 ng/ml EGF or 100 nM insulin at 37°C for 5 and 10 min and then lysed in 50 mMTris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EGTA, 1 mM sodium orthovanadate, 60 mM octylglucoside and protease inhibitor mix. The lysates were clarified by centrifugation for 30 min at 20000*g* and analysed by SDS-PAGE/WB or 2D-E. *Pervanadate treatment* Prior to cell lysis, 80-90% confluent cultures of cells were treated for 15 min with 0.1 mM pervanadate. Cells were lysed in 10 mM Tris-HCl buffer, pH 7.4, containing 8M urea, 4% CHAPS, 10% glycerol, protease inhibitor mix and 5 mM iodoacetate. After incubation at 4 °C for 30 min, 10 mM dithiothreitol was added to inactivate any unreacted iodoacetic acid, and the insoluble material was removed by centrifugation. Lysates of pervanadatetreated cells containing tyrosine-phosphorylated proteins were analysed by 2D-E.

RESULTS

The LMW-PTPs are regulators of multiple signal pathways and can both enhance and antagonize several cellular events. The proteome of PC12 cells overexpressing or not the LMW-PTP dominant negative mutants were analysed; the 2D-E maps, obtained from each cell line, don't share substantial differences relative to their protein contents. Moreover we analysed the phosphorylation patterns of cells both grown in normal conditions and after insulin, NGF and EGF stimulation or pervanadate treatment. Preliminary results indicate that the two isoenzymes actually have a distinct role in PC12 cells. In response to insulin stimulation, only the IF1-D129A mutant forms stable complexes with the endogenous tyrosine-phosphorylated IRβ-subunit. The IF2/D129S mutant seems instead to be involved in NGF-induced signalling. As a consequence of EGFR stimulation we noticed a similar behaviour of the LMW-PTPs isoenzymes. Further investigation is needed to settle these issues.

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Proteomic analysis of human tear fluids using LC-MALDI MS and MS/MS [P78]

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Proteome profile of tear fluids can be used for disease diagnosis and prognosis and generate useful information to understand the interaction between the eye and its contacting objects.

The ocular tear film is a complex mixture of ions, small molecules, glycoproteins and proteins, forming a thin film over the surface of the cornea and conjunctiva, protecting the eye against microbial challenge and preserving visual acuity; changes in tear film conditions are usually related to several eye diseases.

We thoroughly studied the tear proteomic profile by LC- MALDI MS and MS/MS focusing in particular the low mass range region, in which bioactive peptides are present. Variations of human tear proteomic profiles are correlated with clinical conditions of the eye, in order to find biomarkers for disease diagnosis.

The aim of this work is to identify most of the proteins is possible and all the peptides present by MS methods, from a small amount of sample.

In fact, only a small volume of tear fluid can be collected in a clinical laboratory under normal operational conditions.

Tears were collected from healthy subjects using a 5µl glass microcapillary tube.

This 5µl portion of tear sample was reduced with 200 mM DTT in 25 mM ammonium bicarbonate and incubated for 1.5 h at 56°C. The sample was alkylated in the dark for 1 h by adding 200 mM IAM in 25 mM ammonium bicarbonate. Trypsin and RapiGest reagent were added into the sample and incubated for 1 h at 37°C. The sample was then desalted using C_{18} ZipTip, dried to evaporate the organic solvent and diluted with 0.1% TFA for the LC- MALDI MS/MS experiment.

Tear proteins were eluted by a reversed-phase high-performance liquid chromatography (RP-HPLC). The LC-PACKINGS system consisted of a Capillary- and Nano- HPLC system ULTIMATE™, an UV- detector ULTIMATE™, a Well Plate Microautosampler FAMOS™, an Advanced Microcolumn Switching Unit SWITCHOS II™. The C_{18} PepMap analytical column was from LC-PACKINGS too. Gradient elution was performed with solvent A (0.05%, v/v, aqueous TFA) and B (0.05%, v/v, TFA in acetonitrile). The fractions were collected onto a 384-well MALDI plate (APPLIED BIOSYSTEMS), by the MicroFraction Collector PROBOT™ (LC-PACKINGS).

The system was under control of CHROMELEON® Software.

The plate was cooled at room temperature and 1µl of matrix solution (3mg/ml á-Cyano-4-hydroxycinnamic acid in TFA 0.1%-acetonitrile 2:1) was directly spotted on top of each spot and allowed to dry at room temperature.

Mass spectra were recorded using a 4800 MALDI TOF/TOF™ Analyzer by APPLIED BIOSYSTEMS. The sequences of the peptide fragments were determined using Mascot software by Matrix Science and the SWISS-PROT protein database.

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Differences in severity of clinical phenotype reflect diversity of glycoproteome in two sibs with CDG-Ia [P79]

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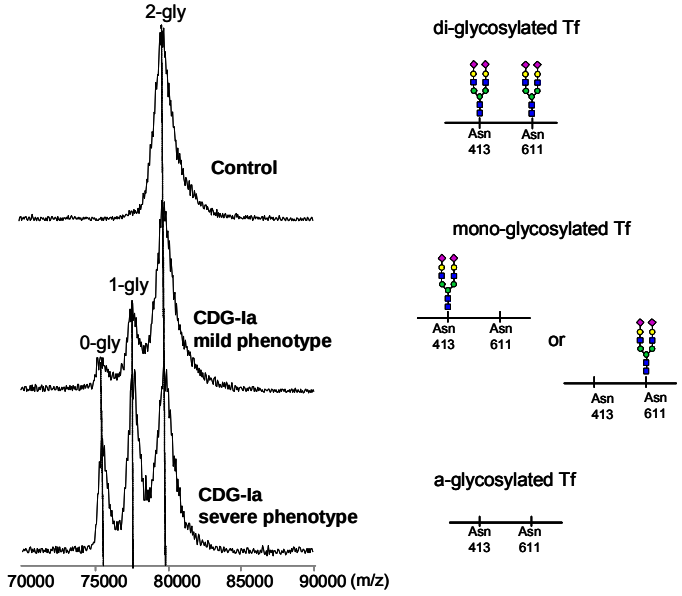
Congenital Disorder of Glycosylation (CDG) type Ia (*PMM* deficiency) is the most common among a family of genetic disorders of protein glycosylation [1]. CDG-Ia has a wide clinical spectrum and genotype-phenotype analyses is limited by extensive allelic heterogeneity [2].

We set-up a new method based on IgY immunoaffinity separation and MALDI TOF MS for the study of protein glycosylation and we applied this strategy to the clinical proteomics of patients with CDG-Ia.

Here we describe the results of the above investigation in two CDG-Ia brothers with proven PMM2 deficiency caused by the V129M/R141H genotype and with different disease severity (Pt. 1, moderate phenotype; Pt. 2, severe phenotype). Glycoproteomic analysis pointed to analyze glycosylation patterns of native serum transferrin and *N*-glycan profiles. As reported in figure, MALDI spectra from the studied CDG-Ia patients show in addition to the main normal peak at *m/z* ~79,5 kDa corresponding to di-glycosylated transferrin, an additional lesser component with *m/z*~77,4 kDa (mono-glycosylated) and a still lesser component with *m/z*~75,2 kDa (a-glycosylated). The increases of mono-glycosylated and a-glycosylated species were significantly more evident in Pt. 2. Differences in serum transferrin N-glycan profiling, presenting with an increased rate of fucosylation and branching, were also observed with the more significant changes being evident in the more severe affected patient.

The application of proteomic analysis of glycosylation and mass spectrometry in CDG may be helpful to elucidate possible relationships between variations of *N*-glycosylation of human serum proteins and pathology.



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PF 2D analysis of plasma from patients with pancreatic and colorectal cancer: profiling the differences [P80]

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Definition of gastrointestinal (GI) cancer encompasses a group of cancers of the digestive system: colorectal, oesophageal, liver, pancreatic and stomach cancer. They can have common symptoms, including general indigestion or abdominal discomfort, loss of appetite or unexplained weight loss, fatigue, but each of them is also characterized by its own symptoms, risk factors and treatments.

In order to achieve better description of peculiar signs of each cancer type, we analysed the plasma protein profiles, acquired by two-dimensional liquid chromatography (PF 2D Beckman Coulter), of pancreatic and intestinal cancers.

For each pathology, ten subjects with homogeneous clinical parameters were selected to create a representative plasma protein pool, whose profiles were compared by the DeltaVue software. The second dimension fractions containing the differentially expressed peaks were further analysed on SDS-PAGE to isolate single protein band, which were identified by peptide mass fingerprinting.

Proteomics as clinical tool: a case report [P81]

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We report the use of two-dimensional (2D) electrophoresis and protein identification by peptide mass fingerprinting to characterize the composition of an anomalous gelatine-like mass (called "yolk", fig. 1) formed in the urine of a subject undergone to kidney transplantation. As the patient developed a severe BK virus nephropathy resistant to treatment, the graft was removed.

A "yolk" slice was solubilized in 8M urea, 2M thiourea, 4% CHAPS and the protein content was quantified by the Bradford assay. Urine surrounding the "yolk" was also collected and quantified as above mentioned. Two-dimensional (2D) electrophoresis was performed on 7cm, pH 3–10 NL IPG strips (Bio-Rad), using 80 mg proteins in 140 ml of re-hydration buffer (8M urea, 2M thiourea, 4% CHAPS, 65mM DTE and 0.5% (v/v) ZOOM Carrier Ampholytes, Invitrogen), on a ZOOM IPGRunner system (Invitrogen). The second-dimension electrophoresis was run on 10x8 cm, 4–12% gradient polyacrylamide gels (Invitrogen), which were then stained with Coomassie Blue R-250. The 2-D gels were analyzed by PDQuest software version 7.1.1 (Bio-Rad) and spots of interest were identified by MALDI-ToF mass spectrometry.

The obtained 2D maps (fig. 2) show that, in the "yolk", specific proteins are present compared with normal urine. Six of them were identified as: Tamm-Horsfall protein (THP, spot 1), alpha- and betafibrinogen (spot 2 and spots 3, 4) and carbonic anhydrase I and II (spot 5 and 6).

THP is the most abundant urinary protein in mammals, known to be involved in the pathogenesis of many renal diseases. Furthermore, abundant THP excretion in urine seems to be related to defence mechanisms against urinary tract infections [1].

The reported case represents a paradigm about the effectiveness of employing proteomic approach to clinical investigation, especially when miniaturization of the system (mini-gel format) allows matching the rapidity, reliability and low-cost requirements of medical research.

Figure 1: "yolk"-like formation in patient urine.

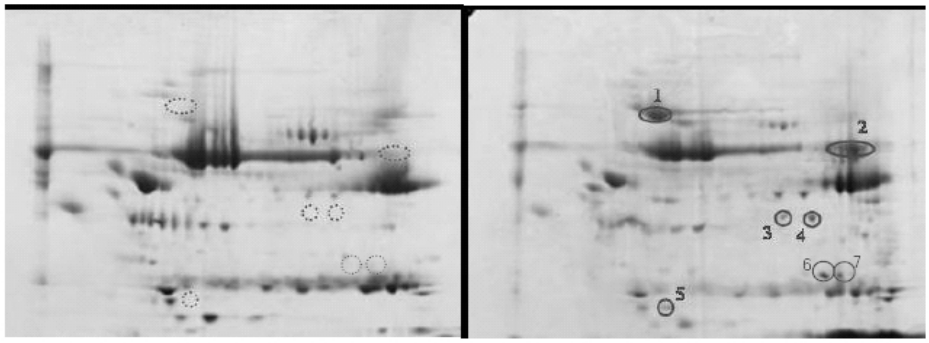

Figure 2: 2D-gel of normal urine (left) and of the "yolk" (right); differentially expressed protein are highlighted in circles.

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Proteome analysis of peripheral T-lymphocytes: a suitable biosensor of strictly related deseases [P82]

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Lymphocytes from peripheral blood may represent molecular sensors and can be used for evaluation of gene expression modification in physiological and pathological conditions. They presents many advantages: i) they are extremely sensitive to the environment modifications, quickly reactive to different agents and able to record different molecular signals at staminal and mature cell levels [1]; ii) the variation of these stimuli determines modifications of the lymphocytes protein pattern which can be estimated by proteomic platforms iii) they are easily available in large number from peripheral blood without any need for invasive and/or risky biopsy. To assess the potentiality and usefulness of differential proteome analysis of peripheral T-cells in clinical investigation we studied two pathologies which, although different, share many clinical and biochemical signs: the polycystic ovary syndrome (PCOs) and the congenital adrenal hyperplasia (CAH). CAH and PCO syndromes are hyperandrogenic diseases often characterized by hirsutism, acne and alopecia, chronic anovulation, infertility, higher frequency of endometrial carcinoma, coagulation defects with hyperfibrinogenemia and higher risk of cardiovascular disorders [2]. Dyslipidaemia, reduced glucose tolerance and diabetes mellitus are also observed. Whereas CAH syndrome, transmitted as a recessive autosomal character, is due to enzyme deficiency (21-hydroxylase in about 90% of the cases) affecting the ability in converting cholesterol into corticosteroid hormones [2], PCO primary causes remain indeterminate. The protein pattern of circulating T-cells derived by 10 PCO- and 5 CAH-affected patients was analysed by twodimensional gel electrophoresis (isoelectrofocusing: pH 3-10 NL, 18 cm; SDS-PAGE: 9-16% gradient gel, 18x20 cm) and its variations were evaluated by comparison with a reference expression profile obtained by pooling protein extracts of Tlymphocytes 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 was also performed, using the GeneSpring GX 7.3 expression analysis software (Agilent Technologies). Comparison with healthy subjects showed that 14 proteins are differentially expressed in both PCOs and CAH, 15 exclusively in PCOs and 35 exclusively in CAH (fig. 1). Seventeen of these proteins have been identified by mass spectrometry analysis. Hierarchical clustering applied to the protein expression data generated a dendrogram (fig. 2) in which PCO and CAH samples segregate in two main groups, highlighting the competence of T-lymphocytes as living biosensor system. The sum of the results supports the employment of T-cells proteome analysis as a useful tool to investigate diseased conditions, even because lymphocytes represent an easily accessible biological sample.

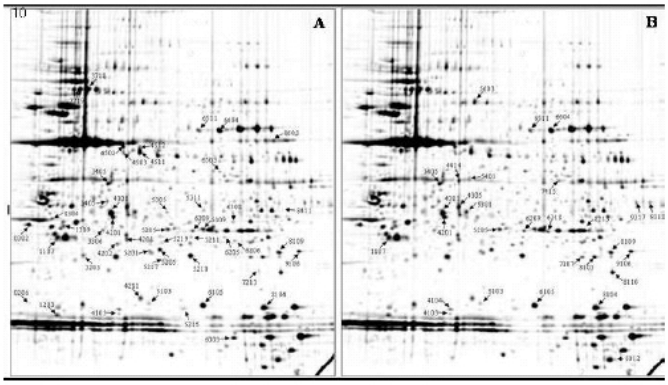

Figure1: Spots marked by arrows and numbers correspond to differentially expressed proteins in CAHs (A) and in PCOs (B) patients

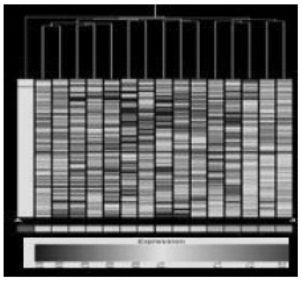

Figure2: Dendrogram derived by hierarchical clustering of protein expression data. Red box: healthy pool; yellow boxes: PCOs samples; azure boxes: CAH samples.

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Proteomic Analysis of Bal from Patients with Langerhans Cell Histiocytosis [P83]

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Following our studies on the identification of potential biomarkers in ILD by a proteomic approach, we analysed the protein composition of BAL from patients with Langerhans cell histiocytosis. There is no data in the literature on BAL proteome of this rare smokingrelated.

ILD with poorly defined pathogenetic mechanisms. BAL samples were obtained from six patients with Langerhans cell histiocytosis and six healthy controls. Twodimensional gel electrophoresis was performed and proteins were identified by different methods (gel matching with 2DE maps of BAL performed in our lab, MALDI-TOF MS and immunoblotting). Electrophoretogram images were obtained by computing densitometer and processed with the ImageMaster 2D Platinum computer system. Quantitative variations in proteins were expressed as relative volumes of spots. Several proteins of interest have been found.

They include apolipoprotein A1 which was significantly increased with respect to controls (0.3083±0.12 vs 0.029±0.019, p=0.002), and alpha1 beta glycoprotein, haptoglobin B snf galectin 1 that show an increasing trend. Our aim is to complete characterization of the LCH BAL protein map in order to identify potential biomarkers of the disease and to obtain insights into its pathogenesis.

Proteomic Map of Peripheral Blood Mononuclear Cells [P84]

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In the last years, the increasing use of proteomics tools in the biomedical field has stimulated the creation of proteomic databases with the aim to identify and catalogue proteins and their isoforms and to collect experimental data. By using databases it is now possible to know phenotype-associated proteins and to have information about the protein expressed in several tissues, cells and organelles in order to facilitate the study of pathologies and to accelerate the process of diagnostic marker discovery and drug development. Specially, enormous progresses have been done in the characterization of blood cellular components. Peripheral blood mononuclear cells (PBMCs) are the sample of choice for many genomic and proteomics studies and they represent a common biological sample for the diagnosis of different diseases. PBMCs play a key role in the innate and adaptive immune response during different pathological conditions like cancer and inflammation. However, the exact role of these cells in many processes like autoimmune diseases is still a matter of discussion.

In this work we want to provide a detailed proteome profile of PBMCs in order to give a better comprehension about their physiology and to highlight strength and limitation in the use of this biological specimen in proteomics applications.

Blood samples were collected from six healthy donors and PBMCs were isolated by a density gradient centrifugation on Ficoll-Plaque Plus. The cells were resuspended in sample buffer and the extracted proteins were separated by 2-DE gel electrophoresis using two different pH range: 4-7 linear and 3-10 nonlinear. Silver stained gels were digitized and analyzed by the Image-Master 2-D Platinum software (Amersham Bioscience). In order to minimize analytical and biological variances, each sample was run in duplicate and a master gel was created by keeping only spots present in all the gels. The identity of proteins was determinated by a MALDI-TOF analysis and a database search. Two hundreds forty-three spots, corresponding to one hundred seventy-three different proteins, have been identified on 2-DE gels of PBMCs. The expression of these proteins reflects many aspects of the immune physiology of PBMCs.

By using information obtained from the harvester database (http://harvester.fzk.de/harvester/) and the literature, the proteins have been clustered in thirteen categories, based on their functional classification and subcellular localization: (1) cytoskeleton and associated proteins, (2) metabolic enzymes, (3) proteins responsible for cellular redox homeostasis, (4) protein and peptides processing, (5) extracellular proteins, (6) mitochondrial proteins, (7) HPS proteins and chaperones, (8) signaling, (9) vesicles, (10) membrane proteins, (11) nuclear proteins, (12) apoptosis and (13) miscellanea. The last categories comprise proteins with not precise function and spots identified with more than one protein as mixture.

Furthermore, a more detailed characterization has been proposed for some classes. The most representative groups of PBMCs proteins are involved in the cytoscheletal organization and in metabolic processes (66 and 37 proteins respectively), while only few proteins represent the class of membrane proteins.

Further studies are in course to study the variation of protein pattern in healthy individuals.

PBMCs protein expression profile in IFN-treated multiple sclerosis patients: relation to clinical and cerebral magnetic resonance imaging findings [P85]

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Multiple sclerosis (MS) is a disease of the central nervous system (CNS) with an autoimmune inflammatory and degenerative component. The relationship between brain atrophy and immune-mediated inflammation is still under investigation. Existing disease-modifying therapies provide a detectable effect on progressive atrophy and disability. In particular, immunomodulatory agents like β-interferon (β-IFN) can positively influence the lesion burden and cerebral atrophy acting on the peripheral blood mononuclear cells (PBMCs) immune response. The aim of this work is to investigate the putative modifications of the protein expression profile of PBMCs in MS patients and their correlations with respect to MRI and clinical parameters. PBMCs were isolated from fourteen Relapsing Remitting (RR) IFN-treated patients, seven Secondary Progressive patients (SP) and ten healthy control subjects. Using a proteomic approach, 2-DE and MALDI-TOF, we identified a set of differentially expressed proteins ($p<0,05$) between RR and control classes. PBMCs from RR treated patients had a higher expression of twenty-one spots in comparison with control individuals, six spots had a decreased expression in patients relative to controls. One additional spot was present in all controls and only four RR patients. In addition, the altered expression of these spots was also observed in SP class. Among the proteins identified there is the well-know marker of IFNaction, MxA and others proteins belong to cell metabolism, actin remodelling or apoptosis. Their expression level was correlated with quantitative clinical impairment (EDSS) and brain atrophy in all MS patients. A correlation, by Spearman's test, was found between brain atrophy, EDSS, disease age and three proteins: Interferon-induced 35 KDa protein (IFP 35), Septin-2 and Serine/Threonine-protein phosphatase. IFP 35 is a leucine zipper protein whose expression is regulated by interferon gamma (IFG). IFG is a proinflammatory cytokine abnormally expressed in serum and cerebrospinal fluid of patients with MS. Serine/Threonine-protein phosphatase is one of the three catalytic subunits of protein phosphatase 1 (PP1), a protein involved in several cellular processes such as protein synthesis, glycogen metabolism and cell division. Septin 2, also known as Nedd5, is a member of the septin family, a group of GTP-binding proteins with a role in cytoskeleton organization and function. Recently the importance of Nedd5 was highlighted by a study that candidate Nedd5 as novel autoantigen in systemic lupus erythematosus. Our results suggest that several functional modifications of PBMCs mirror cerebral neurological processes.

Proteomic on fine-needle aspiration of thyroid nodules [P86]

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Proteomic approach has been recently applied for studying human diseases as breast, brain, prostate and colon cancer. This led to the discover of new putative tumors markers. In this work, with the aim to overcome the complexity of thyroid tissue analysis, we proposed the fine needle aspiration fluid (FNA) as a source of thyroid markers, less complex than tissue or serum. Seventeen patients were enclosed in the study: four with a follicular lesions (2 M/ 2F), 13 (4M/ 9F) with a papillary cancer; in the latter group four patients had a tall cell variant. Immediately after the surgical removal of the thyroid a fine needle biopsy was made on suspicious or already diagnosed malignant nodules and on normal tissue of the controlateral lobe. After passing the needle through the tissue 3 or 4 times, 4 ml of saline solution was aspirated with the same syringe. This fluid was immediately centrifuged at 2300 x g for 20 min at 4°C. Proteins from resulting supernatants were precipitated using TCA. The proteins profile of FNA was obtained by two-dimensional electrophoresis and the difference of proteins expression in follicular adenoma and papillary cancer respect to control was evaluated using Image-Master 2D Platinum (1). The protein spots which determined FNA protein profile and those which determined to shown significant (quantitative or qualitative) difference from comparison of different groups by software, were chosen for excision (Figure 1A and B). Sixty-eight protein spots were picked from the gels digested with tripsin and subjected to MALDI-TOF peptide mass fingerprint analysis Of the 68 spots, 64 (94 %) were identified, yielding 57 different protein identification. In fact, in some cases, the same protein was identified in different spots across the 2D gel, suggesting the occurrence of post-translational modification such as transthyretin (n°56,63), annexin 1(n°7,44), ciclofillin A (n°19,35), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (n°50, 58), phosphoglycerate kinase (PGK-1) (n°10,48), and apolipoprotein 1(n°3,61).

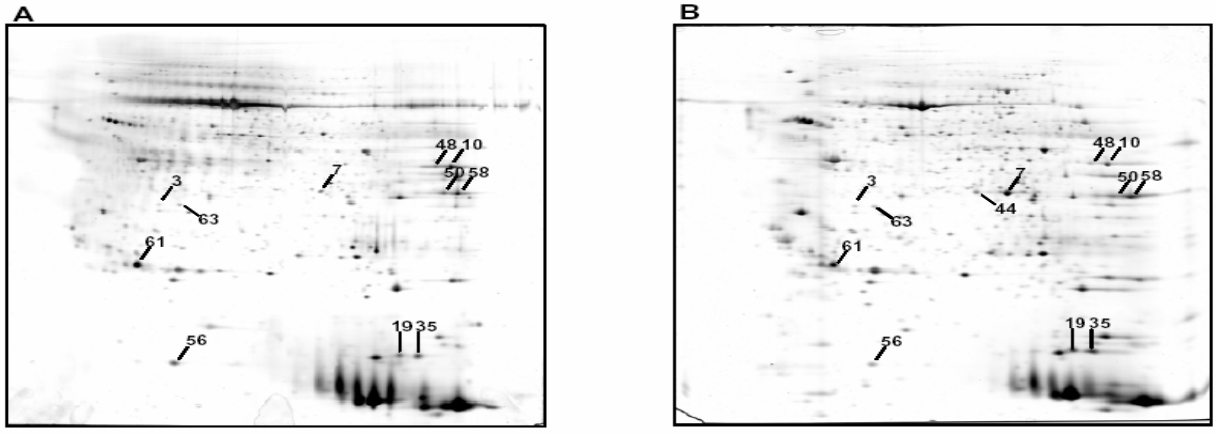

Figure 1A and B 2DE proteins pattern of normal (A) and papillary (B) human FNA.

The proteome analysis revealed a specific fingerprint of FNA with up and down regulation of different functional system: 1. metabolic enzymes, 2. antioxidative defense proteins, 3. motility proteins, 4.serum proteins, 5. pro-apoptotic proteins. Additionally, qualitative differences were found in papillary carcinoma and the expression of proteins such as galectin 3, protein S100 A13, septin and ezrin can be observed. As has been suggested for other fluids or tissues (2,3), the identification of FNA proteins may be the basis for the discovery of potential protein biomarkers in specific thyroid diseases, particularly in the cancer diagnosis. We believe that proteomic analysis of FNA is more advantageous than proteomic analysis of tissue (4) because this new approach is a rapid, non-invasive and preoperative method for the discovery of specific tumoral markers and moreover it might be used during cytological evaluation of FNA avoiding, or at least reducing, the pitfalls of FNA diagnosis. Therefore, our results of could be a starting point to improve the differential diagnosis of thyroid cancer.

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Effect of iron and dopamine on the oxidative Modifications of the DJ-1 Protein [P87]

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Parkinson's disease (PD) is a common neurodegenerative disorder with a mainly sporadic etiology, although a number of monogenic familial forms are known. Most of the motor symptoms are due to selective depletion of dopaminergic, neuromelanin-containing neurons of substantia nigra pars compacta (SNpc) [1].

DJ-1 is a ubiquitous, highly conserved protein. It was first identified as a novel oncogene 2] and has recently been found to be causative gene for familial PD PARK7 [2]. The function of DJ-1 in human brain is not known, although evidences suggest its role in transcriptional regulation [4] and antioxidative stress [5], and loss of its function is thought to results in the onset of PD.

We had determined the oxidative state of DJ-1 by western blot analysis of bi-dimensional protein maps. We analyzed autoptic samples of substantia nigra of patients of PD and we observed that DJ-1 expression becomes altered in a sporadic PD patient under chronic L-DOPA treatment who simultaneously received chronic iron supplement. The change in the isoelectrofocusing pattern is not observed in other patients who received L-DOPA but no iron fumarate. Therefore we reproduced similar conditions in vitro using SH-SY5Y human neuroblastoma cell line as a model. We treated cells for different period of time and we evaluated how the oxidative state of DJ-1 changed.

These findings indicate that DJ-1 was dramatically modified by reactive oxygen species in the midbrain area being examined. Therefore, labile iron complexes like iron fumarate should not be administered in patients treated with L-DOPA.

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Multivariable DIGE/MS-Based Proteomic Profile of Platelets from Patients with Myeloproliferative Disorders [P88]

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Polycythemia vera (PV), chronic myeloid leukaemia (CML), essential thrombocythemia (ET) and myeloid metaplasia and myelofibrosis (MMM) are chronic hematologic diseases which constitute the group of myeloproliferative disorders (MPD). These diseases share common abnormalities of stem cell proliferation and an ill defined hemostatic imbalance resulting in increased risk of both thrombotic and hemorrhagic episodes. The clinical relevance of this hemostatic imbalance is particularly apparent in patients with polycythemia vera and essential thrombocythemia, where thrombohemorrhagic accidents are far more frequent than other complications, such as infections and blastic transformation. The co-existence of enhanced thrombotic and hemorrhagic risk in the same disease as well as its relation to platelet abnormalities have puzzled investigators in this field [1, 2].

In a preliminary study, we have focussed our attention on platelets of patients with MPD, using a proteomic approach. Multivariable DIGE/MS was used to investigate possible changes in expression and/or post-translational modification of proteins from platelets of patients with ET or PV. Platelet-rich plasma (PRP) was obtained by centrifugation of citrated blood collected from MPD patients and healthy volunteers. Proteins were extracted from platelets and labelled with three different CyDye DIGE fluors minimal dyes, separated by 2D-DIGE and visualized using a Typhoon Trio scanner. Triplicate samples were prepared independently and simultaneously analyzed across multiple DIGE gels using a pooled sample internal standard to quantify expression changes with statistical confidence. Principal component analysis and hierarchical clustering of the individual DIGE proteome expression maps provided independent confirmation of distinct expression patterns from the individual experiments and demonstrated high reproducibility among replicate samples (Figure 1, 2).

In MPD samples, ten spots exhibited statistically significant difference ($P<0,01$); six were down-regulated and four were up-regulated as compared to controls.

Among down-regulated proteins, vinculin, fibrinogen á chain with different degrees of phosphorylation and transgelin 2, were identified by MALDI-TOF MS, while actin with two different degrees of phosphorylation was up-regulated.

The advances in proteomic technologies open new perspectives in studying platelets proteomics and in particular myeloproliferative disorders, in fact multivariable DIGE technique and mass spectrometry will be able to increase the speed of acquisition as well as data analysis, providing higher sample throughput in the same period of time.

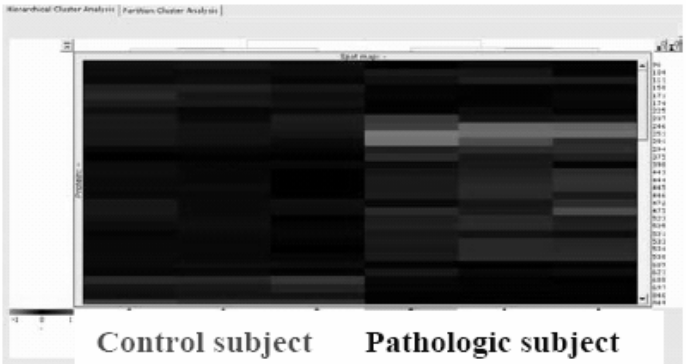

Figure 1: Hierarchical Cluster Analysis

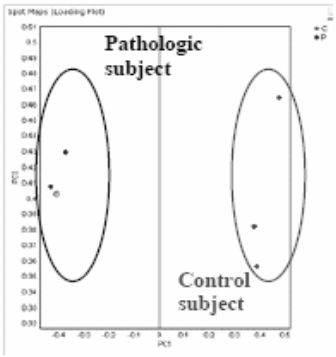

Figure 2: Principal Component Analysis

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Comparative Proteomic Analysis of Paclitaxel Sensitive A270 and Resistant A270TC1 and OVCAR-3 Epithelial Ovarian Carcinoma Cell Lines using DIGE [P89]

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Ovarian cancer is one of the leading causes of gynaecological cancer-related deaths. Largely asymptomatic, most patients are diagnosed at an advanced stage of the disease and, after surgical resection, chemotherapy remains the most effective treatment. Despite the success of paclitaxel in advanced stage ovarian carcinoma, clinical drug resistance poses a major obstacle to the successful use of this agent in the treatment of cancer. Several mechanisms have been implicated in paclitaxel resistance, but they are not sufficient to fully explain the chemoresistance process.

In this work a proteomic approach has been used to analyse proteins differentially expressed in paclitaxel-sensitive and resistant ovarian cancer cells to elucidate mechanisms at the basis of paclitaxel resistance. Paclitaxel sensitive A2780 and two paclitaxelresistant human ovarian cancer cells, OVCAR-3 and A2780TC1, were employed. The study was performed using Difference Gel Electrophoresis (DIGE), an innovative and highly reproducible method. Proteins from paclitaxel-sensitive and resistant ovarian cancer cells were resolved in quadruplicate resulting in about two thousands spots per gel (Fig. 1) and further analysed by the DeCyder software. Statystical analysis clearly discriminated paclitaxel sensitive and resistant cell lines (Fig. 2) and revealed 127 differentially expressed proteins to be further investigated by mass spectrometry. 113 protein spots were identified by MALDI-TOF belonging to different functional classes (Fig 3).The characterization of these variations could contribute to understand the complex mechanisms of paclitaxel resistance in cancer and lead to the development of new biomarkers and molecular targets for novel drugs discovery.

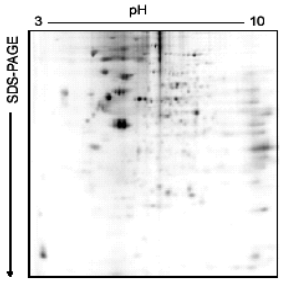

Figure 1: Master gel representative of 2D pattern of A270 and paclitaxel-resistant A270TC1 and OVCAR-3 human epithelial ovarian cancer cells. IEF as the first dimension separation was over a 13cm, pH 3-10 linear strip. The second dimension was carried out by SDS-PAGE in a 12% polyacrilamide gel.

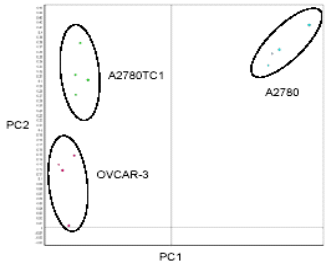

Figure 2: Score plot of the first two components of PCA on the basis of DIGE analysis of the three human cancer cell lines. The gel images are represented as circles. The images of paclitaxel resistant human ovarian cancer cells are clustered

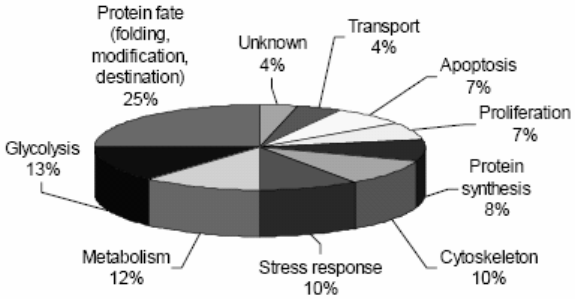

Figure 3: Functional classification of the differentially expressed proteins identified in paclitaxel sensitive (A2780) and resistant (A270TC1 and OVCAR-3) human epithelial ovarian cancer cells.

Proteomic Enalysis of Entracellular Effect of Platelet Lysate on Tissue Regeneration, using a Human Fibroblast model [P90]

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Platelets are a source of growth factors able to stimulate wound-healing and tissue regeneration; the clinical effect of topical administration of platelet factors is well documented (1), but the biochemical basis of this beneficial effect are still unknown. The aim of this study was to evaluate the capacity of a platelet lysate (PL) to reconstitute optimal conditions for somatic cells to be expanded ex-vivo, without xenogenic adjuvants.

The protein expression in human fibroblasts treated with autologous PL was analysed by proteomics. Fibroblasts were obtained from skin biopsies of consenting healthy donors. After 2-DE and image analysis (PDQuest software) of four replicates, differentially expressed proteins were identified by peptide mass fingerprint on Voyager-DE Pro MALDI-TOF. Fibroblast stimulation with PL modified the expression of some cytoskeletal protein, such as different forms of vimentin and actin. Moreover two enzymes (a-glucosidase and aldheyde reductase), the heat shock proteins gp96 and 27kDa, the collagen type VI and the major Vault protein were down-regulated by PL treatment.

The PL-induced mitogenic activity was also investigated through semiquantitative immunoblotting analysis of common signal transduction mediators such as p38 MAPKinase and High Mobility Group Box 1 Protein (HMGB1), as markers of proliferating cells. p38 MAPKinase is involved in transmission amplification and diversification of extracellular responses, while HMGB1, a chromosomal protein involved in architectural functions with functional importance as regulator of transcription, plays a remarkable role as a marker of proliferation.

The proteomic approach is able to clarify the molecular pathways through which somatic cells are stimulated by plateletderived factors as a whole, in contrast with the classical approach in which the effect of a single substance is tested on cellular growth and differentiation. The responses of human fibroblast cell cultures to PL are the first step to extend the PL use from implantology, orthopedics, wound healing to the area of mesenchymal stem cells.

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Differential surface Proteomics of peripheral Blood Lymphocytes as a Tool for Biomarker Discovery in Parkinson's Disease [P91]

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Parkinson's disease (PD) is a progressive neurodegenerative disorder of unknown etiology, characterized by severe motor symptoms such as uncontrollable tremor, slowness of movement, rigidity and postural instability. Pathologically, a marked depletion of dopaminergic melanin-containing neurons of substantia nigra pars compacta is observed. The cause of nigral cell death remains unknown but oxidative stress, mitochondrial dysfunction and environmental toxins are probably implicated. Also immune system is involved in PD. Changes in lymphocyte population in blood and cerebrospinal fluid, cytokines and acute phase proteins production, and synthesis of immunoglobulin have been observed in patients with PD, as well as activated microglia and proinflammatory cytokines in the substantia nigra.

A strong demand that comes from the clinicians is the identification of easily detectable biomarkers in order to delineate the stage and the progression of the pathology, beyond the outcome of the therapy.

Peripheral markers could be searched on the surface of lymphocytes that could change their protein expression and localization on the surface as a consequence of the disease or the treatments.

In order to set up the protocol and to take into account changes in the surface proteome due to the dopaminergic therapy we considered at first a prototypic lymphocyte cell line (Jurkat T-cell leukaemia) eventually challenged with dopamine or L-DOPA. First of all we fixed the conditions of the treatments with L-DOPA and dopamine, choosing concentrations without a cytotoxic effects (tested with MTT assay and Trypan Blue staining of cells) and in the simultaneous presence of catalase to avoid aspecific effects due to oxidation of dopamine. As regards L-DOPA, experiments were performed in the concentration range 1-5 μM corresponding to the plasma level in patients under pharmacological treatment.

Proteins on the surface of the cells are labelled with biotin and affinity captured with immobilized monomeric avidin. Fractions are then separated by standard SDS-PAGE for the subsequent identification. The experimental set up will be later used to analyze surface proteins from lymphocytes of PD patients.

Proteomic analysis of SH-SY5Y human neuroblastoma cells expressing α-synuclein as a model of dopamine susceptibility [P92]

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Recent identification of mutations linked to familial forms of Parkinson's disease has permitted to better define the pathology of this disease at molecular level. In particular, expression of either wild-type or mutant protein in different cell lines has demonstrated that α-synuclein toxicity is dopamine-dependent. The human neuroblastoma SH-SY5Y cell line expresses the dopamine transporter and dopamine receptors, and is able to form storage vesicles, although the low activity of the vesicular monoamine transporter type 2 impairs dopamine storage into vesicles. This cell model has been stably transformed to increase a-synuclein expression levels.

Here, we report the effect of dopamine and copper on the protein expression pattern of this cell line, either wild-type or transformed. Protein extracted from the cells have been separated by two-dimensional electrophoresis, gels were then silver stained and excised proteins identified by internal sequence tagging by using a nanoelectrospray - ion trap mass spectrometer. We have identified about 40 proteins; among them, several are involved in energetic metabolism, oxidant species scavenging pathways, apoptosis, and ubiquitin-proteasome system.

Proteomic Analysys of Cerebrospinal Fluid in Multiple Sclerosis Patients [P93]

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Introduction

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[1]. 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[2]. Thus we have employed a basic proteomic approach, including two-dimensional gel electrophoresis (2DE), to investigate on this pathology.

Methods

To fractionate proteins in samples, 1 mL of CSF from three relapsing-remitting MS patients and three "non MS" control subjects was subjected to a reversed phase solid phase extraction (RP-SPE) using an Oasis HLB cartridge, as described by Yuan and Desiderio[3], obtaining three resulting fractions. Each of them was lyophilized and resuspended with rehydration buffer (Urea 7M,Thiourea 2M, CHAPS2%, DTT 15mM, 3-10 NL IPG buffer 2%). Focusing was carried out on 7cm pH 3-10 NL IPG strips (GEHealthcare). Second dimension separation was performed on a home-made linear 9-16% SDS-PAGE. Each sample was run in duplicate. A comparative analysis between the 2DE maps obtained from the first fraction was performed by Image Master 2D

Platinum 6.0 software (GE Healthcare). Both a Cluster analysis and the Student's T-test were used to evaluate differences in protein expression. P values less than 0.05 were considered significant.

Results and Conclusion

Image analysis performed on the total maps obtained from the first fractions detected approximately 300 spots per gels with a percentage of matched spots comprising between 60% - 80% for the "non MS" control group with a positive coefficient of correlation (0.979±0.01). Same findings were for MS group, that showed matched spots from 54% to 74%, with a coefficient of correlation of 0.986±0.004.

Automatic heuristic clustering carried out by software grouped the analysed samples in the two expected classes (Fig.1). A representative map of control group and MS group is shown in figure 2. By comparing the average value of volume percent of each matched spots, we have identified a set of ten spots whose expression differed significantly between MS and "non MS" subjects, with p values less than 0.05. In particular five proteins were over expresses, two were under expresses and three appeared undetectable in MS group compared to the controls. (Table 1)

Further studies are in progress to confirm these data in a larger group of MS patients and to definitely characterize the differentially expressed proteins by MALDI-TOF analysis.

Fig.1 Euristic Cluster

Match ID	% Volume MS	% Volume Controls	p
574	0.056 ±0.018	0.126 ± 0.022	0.0015
623	0.336± 0.145	0.127± 0.02	0.0119
624	0.33± 0.06	0.151± 0.06	0.0011
706	0.02± 0.008	0.05± 0.02	0.045
740	0.26± 0.13	0.40± 0.06	0.0457
753	0.21± 0.08	1.94± 0.87	0.018
788	Undetectable	0.05± 0.03	0.028
881	0.018± 0.015	0.012± 0.01	0.0105
894	Undetectable	0.07±0.06	0.0049
895	Undetectable	0.06± 0.05	0.0177

Table 1: Protein differentially expressed

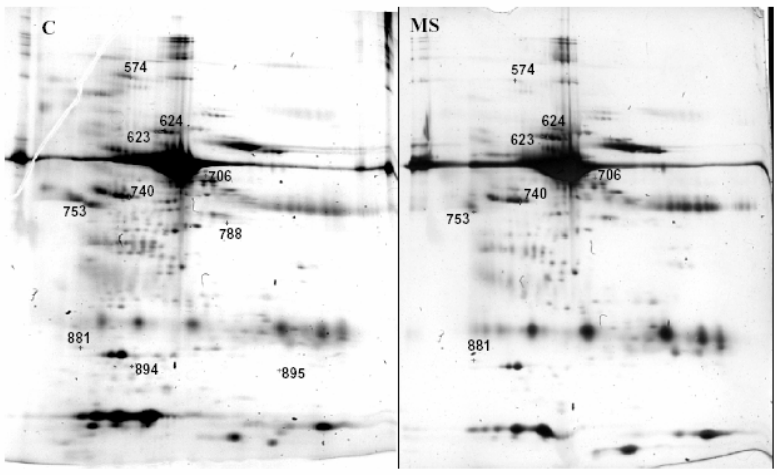

Fig.2 Representative map of Control group and MS group. Red asterisks show the differentially expressed spots.

Identification of a Transthyretin (TTR) Variant by MALDI-TOF Protein Profiling Analysis [P94]

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Introduction

The use of matrix-assisted-laser-desorption ionization mass spectrometry (MALDI-MS) to acquire spectral profiles has become a powerful method for analyzing changes in global protein expression patterns of biological systems. In order to identify specific Cerebrospinal fluid (CSF) and/or Serum protein profiling, from patients with neurological diseases, we have used a controlled micro-chromatography (ZipTip) protein extraction system, combined with MALDI-TOF-MS, to produce highly reproducible protein profiles.

Methods

CSF and serum samples were centrifugated at 13,000 g for 10 min at 4°C to remove insoluble material and an aliquot of 20 µl was processed by micro-chromatography (ZipTip). Peptide/protein profiles were analyzed with a Voyager DE PRO MALDITOF mass spectromer (Applied Biosystem). Separate spectra were obtained for a restricted mass-to charge (m/z) range (1000-25000 kDa). Two dimensional electrophoresis of serum was performed on 5 µl of sample diluted with rehydration buffer (Urea 7M,Thiourea 2M, CHAPS2%, DTT 15mM, IPG buffer 2%) to a final volume of 125 µl . Focusing was carried out on 7cm pH 3-10 NL IPG strips and the second dimension separation was performed on a linear 9-16% SDS-PAGE.

For genetic analysis after amplification, TTR gene sequence was conducted using Big dye terminator v 1.1 and analyzed on an ABI 3130XL DNA sequencer.

Results and Conclusion

By analyzing the MALDI-TOF-MS protein profiles of patients with neurological diseases we have been able to identify in a CSF sample an intense signal at 13709.2 m/z, likely corresponding to a TTR variant (Figure 1). The same signal was found successively also in the patient serum. We have identified the anomalous protein by 2DE of serum, and the abnormal spot/protein was characterized by MALDI-TOF PMF confirming the TTR identity and an Arg124Cys change. Direct sequence analysis of the corresponding exon confirmed a CGC124TGC substitution. This TTR variant appears to be not previously reported in Italian population.

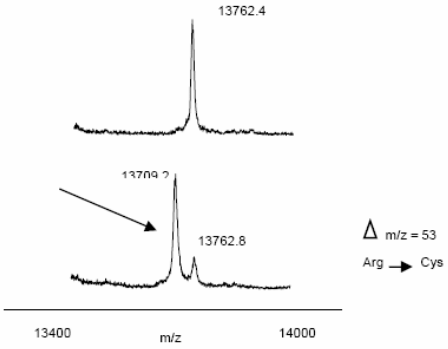

Fig 1 MALDI-TOF profiling of CSF

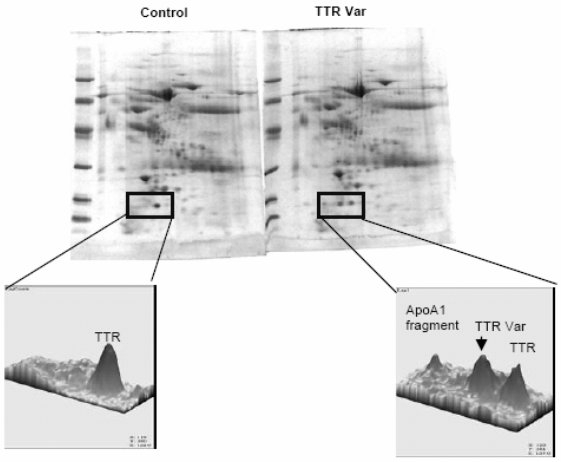

Fig.2 2DE of sera from Control and patient with TTR variant

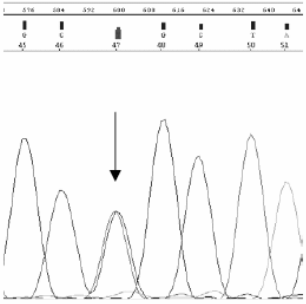

Fig.3 Sequence analysis of TTR gene

Characterization of Primary Cell Cultures Deriving From Normal Kidney and Renal Cell Carcinoma by Molecular and Proteomic Studies [P95]

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Renal cell carcinomas (RCCs) represent 3% of all human malignant diseases and they differ on their genetic basis, natural history and prognosis. The diagnosis is difficult and often late and nowadays validated RCC markers for diagnosis and prognosis do not exist. Primary cell cultures from human renal cell carcinoma (RCC) and normal renal cortex were established to find molecular markers for RCC by genomic and proteomic studies. These primary cell cultures could represent a homogeneous cell population to get round the problem of cell heterogeneity of the tissue, in which the presence of a mixture of tumor and normal cells such as leukocytes and connective tissue cells makes the molecular analysis difficult.

22 primary cultures of normal cortex and RCC have been studied. They have been cytologically characterized evaluating the growth features, and the cellular morphology and composition, by immunofluorescence and FACS analysis at different passages in culture. Antibodies against cytokeratin, vimentin and E-cadherin have been used as markers of epithelial cells in culture; antibody against CD13 and Lotus Tetragonolobus agglutinin (LTA) have been used as markers of proximal tubular cells; Arachis Hypogaea agglutinin (PNA) has been used as marker of distal tubular cells and antibody against WT1 protein has been used as marker of podocytes. The cells in primary cultures were >90% epithelial and mainly proximal and distal tubular cells with a low contamination of podocytes (<10%) in normal kidney cultures. Tumor primary cell cultures were positive (>90%) for the epithelial markers and the expression of tubular markers was different among different cases.

The molecular characterization of these primary cell cultures have been also performed by studying the expression of transcript or protein level of some molecular targets already studied in corresponding tissues. As evidenced by Realtime PCR, MnSOD and Hsp27 mRNA levels were higher in tumor cultures, than in normal renal cultures like in corresponding tissues, while Flotillin-1 trancript expression was heterogeneous among different samples. At protein level, 1-DE Western Blotting confirmed the overexpression of Hsp27 in tumor cultures and the heterogeneity of Flotillin-1. Furthermore Caveolin-1 is overexpressed in tumor cultures like in the corresponding tissue. We also studied the cellular localization of Flotillin-1 and Caveolin-1 by immunofluorescence in both normal and tumor primary cell cultures.

The analysis of 2-DE map of normal cortex and RCC primary cultures evidenced 63 differently expressed protein spots. Among these, 44 were identified by MS: 29 were present only in cortex, 2 only in RCC, 9 were statistically more abundant in RCC and 4 in cortex cultures. For two proteins present only in cortex the transcriptoma analysis by microarray evidenced also a downexpression of corresponding mRNA in RCC cultures. The proteins overexpressed in RCC are directly or undirectly involved in different metabolic pathways. Some proteins are related to the VHL/HIF-1 pathway through the involvement of VEGF and the neoangiogenesis process. Other proteins are involved in cytoskeleton remodelling pathway either by direct interactions with cytoskeleton proteins, or through the activation of tyrosine kinase receptors.

Therefore these primary cultures may be an useful model complementary to the tissue to study the potential use as biomarkers of differentially expressed proteins which may be furtherly investigated by activation or silencing of specific genes and/or pathways.

Supported by PRIN 2004, PRIN 2006 and FIRB 2001

Unravelling the Mechanisms of Resistance to Imatinib Mesylate in Chronic Myeloid Leukemia: a Proteomic Approach [P96]

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Imatinib mesylate is a potent inhibitor of BCR-ABL tyrosine kinase that is implicated in the development of chronic myeloid leukemia (CML).[1] major concern in the treatment of CML is the emergence of resistance to imatinib. We studied an imatinib-resistant cell line (KCL22-r) to characterize proteins that are involved in the mechanisms underlying resistance to imatinib mesylate. By using two-dimensional difference gel electrophoresis (2D-DIGE) we identified about eighty differentially expressed proteins. We focused our attention on three down-regulated proteins: HSP70, HSP27 and Annexin A1. Microarray analysis correlates with proteomics data for the genes that encode HSP70, HSP27 and Annexin A1. We confirmed the down-regulation of HSP70, HSP27 and Annexin A1 in resistant cells by western blotting. We demonstrated that the downregulation of HSP70 could be related to the down-regulation of heat-shock transcription factor-1 (HSF-1). Annexin A1 was shown to regulate the endosomal EGFR trafficking [2] and is involved in mechanisms of resistance to different drugs used in chemotherapy [3]. We are currently investigating the potential involvement of these proteins in the imatinib resistance.

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Identification of the Interactome of Human AF4, a Mixed-Lineage Leukaemia Gene Fusion Partner [P97]

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The *AF4* gene is the most frequent partner of the mixed lineage leukaemia (MLL) fusion gene that is associated with infant pro-B acute lymphoblastic leukaemia, which has a poor prognosis[1,3]. The AF4 protein is a member of the AF4/LAF/FMR2 (ALF) family of proteins, which share the N-terminal homology domain, the ALF domain that contains a proline-serine-rich region, and the C-terminal homology domain. AF4 also contains a transactivation domain and several nuclear localization signals[4,5]. However, besides being a putative transcriptional activator, little is known about AF4 functions and its molecular mechanisms. Using proteomic strategies we looked for proteins that interact with human AF4 in an attempt to shed light on AF4 functions, and on the molecular mechanisms underlying diseases related to AF4-associated fused genes. Proteins were isolated by immunoaffinity purification of Flag-AF4 and identified using liquid chromatography-tandem mass spectrometry coupled to bioinformatics tools[6, 7]. We identified about 60 AF4-interacting proteins that belong to various Gene Ontology Categories. More than 50% of them are known to participate in the transcription regulatory mechanism, including the mediator complex in eukaryotic cells. More than 60% of the identified proteins are localized within the cell nucleus. These findings indicate that AF4 plays a major role in the complex machinery that promotes transcription in human cells. Hence, the interaction between AF4 and RNA polymerase II is a crucial factor also in human cells.

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Integration of Genome Wide Molecular Analysis and Subcellular Proteomics for Renal Cell Carcinoma Biomarker Identification [P98]

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Clear cell renal carcinomas (cRCC) is representing about 3% of all kidney cancers. No biomarkers for early diagnosis of cRCC in asymptomatic patients or for post-surgery monitoring are yet available. In the context of an Italian research project aimed at the discovery of new markers for cRCC, the identification of differentially up-regulated genes associated with regions of increased DNA copy number was carried out, by genome-wide SNP copy number analysis (DNA) and transcriptomic profiling (RNA) using GeneChip Affymetrix technology on 30 cRCC patients tissues. In order to confirm the differential expression at protein level, methods of protein enrichment were developed, preparing subcellular fractions from tumor or adjacent normal kidney tissues (ANK) from the same cRCC patients.

We focused our study on a subset of membrane-associated proteins such as RGS1, CXCR4, CAV1, CAIX, EMMPRIN, that were further investigated using a subcellular comparative proteomic approach. In particular from homogenates (Hom), postnuclear pellet (P_2), and caveolar microdomains (MD) were prepared, enriched in proteins of the caveolin family (CAV1). Caveolae and CAV1 are known to supply compartmentation of various signaling molecules, and, by dysregulation of these signal transduction pathways, they may be involved in oncogenesis and in promoting cancer cell migration and metastasis [1]. EMMPRIN (extracellular matrix metalloproteinase inducer) is a glycoprotein enriched on the plasma membrane of most tumor cells [2]. CAIX is a member of the carbonic anhydrase family, already reported as overexpressed in RCC: it is thought to play a role in the regulation of cell proliferation in response to hypoxic conditions, involved in tumor progression [3]. CXCR4 is a G-protein-coupled chemokine receptor, and it has been recently shown up-regulated by hypoxia in a variety of cell types [4]. RGS-1 (Regulator of G-protein signalling-1) act as deactivator of Gprotein signaling pathways and is supposed to regulate chemokine receptor signaling [5].

Table I: Genome-wide SNP copy number analysis (DNA) and transcriptomic profiling (mRNA) results on a selection of 5 genes.

Entrez Gene ID	Gene Symbol	Cytoband	Fold Change (mRNA)	Copy Number (DNA)
5998	RGS-1	1q31	7,02	Gain
7852	CXCR4	2q21	7,71	Gain
857	CAV-1	7q31.1	8,67	Gain
768	CA IX	9p13-p12	11,67	Normal
682	EMMPRIN	18p13.3	N/A	Normal

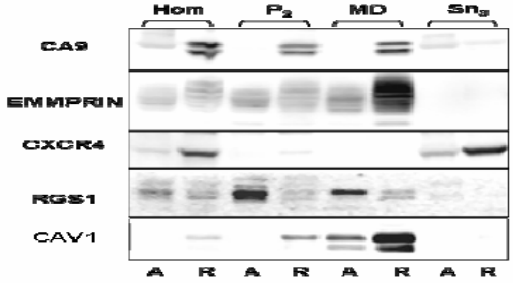

Figure 1: EF/WB analysis of the 5 selected proteins in RCC (R) and ANK (A) subcellular fractions.

Results obtained by SDS-PAGE and western blotting with specific antibodies show a good, but not complete, concordance between genomic profiling, gene expression (Tab.I) and protein levels (Fig1). In fact, CA9, CAV1 and EMMPRIN signals are increased in RCC caveolae (MD), while CXCR4 show higher levels in RCC, but it looks like a cytosolic protein (Sn3); on the contrary, RGS1 is a caveolar protein, but its abundance is lower in RCC. In conclusion, integration of DNA/RNA genome wide analysis with subcellular protein enrichment may provide a promising approach for the discovery of candidate tumour markers of cRCC.

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Study of the interference of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) on estrogen induced proliferation in MCF 7 breast cancer cell line by proteomics [P99]

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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and other compounds, that mimic the activities of estrogen when assumed by diet, are known as endocrine-disrupting compounds (EDC) [1]. Despite many studies, our understanding of the molecular mechanism of Aryl Hydrocarbon Receptor (AhR) and Estrogen Receptor (ER) crosstalk is far from complete.

As it is known, the effects of TCDD are mediated by AhR, a ligand-activated nuclear transcription factor that, at present, has no identified endogenous ligand [2]. When TCDD (or another EDC) binds to the AhR a heterodimer is formed with AhR Nuclear Translocator Protein (ARNT); this transcription complex interacts with Xenobiotic Responsive Element (XRE), located in the 5'regulatory region of Ah-target genes, such as CYP1A1 and CYP1B1 [3]. Many data suggests an inhibitory crosstalk between AhR and ER signalling and several mechanisms have been proposed in order to explain how AhR inhibits ER activity [4].

Because our previous data suggested that MCF 7 cell line is a good estrogen responsive breast cancer model [5], we have studied the effect of co-treatment of TCDD and E2 on MCF 7 proliferation and we found a significant reduction on the proliferation induced by E2. This result has led us to undertake a proteomic study of which we report the preliminary results.

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Oxidized proteins in plasma of patients with heart failure: role in endothelial damage [P100]

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Background

Increasing experimental evidences support the concept that oxidative stress is both increased in the failing heart, and contributes to the pathogenesis of myocardial remodeling and failure (1). Indeed, markers of oxidative stress are elevated in chronic heart failure (CHF) patients and have been correlated with myocardial dysfunction and overall severity of heart failure (1). Furthermore, it has been reported that plasma antioxidative enzyme activities are decreased in patients with CHF (2).

Aim

This study is intended (a) to analyze the presence of carbonylated proteins in plasma of CHF patients; (b) to identify, by a proteomic approach, the specific targets of carbonylation; (c) to analyze the alteration of protein function in in vitro cultured endothelial cells.

Methods

Study population consisted in 20 patients with dilated cardiomyopathy due to ischemic heart disease [n=20, New York Heart Association (NYHA) class III, n=13; NYHA class IV, n=7)], 20 patients with idiopathic dilated cardiomyopathy (normal coronary angiography, n=20, NYHA class III, n=11; class IV, n=9) and 20 healthy volunteers.

Plasma levels of oxidized proteins were measured by immunoassay and analyzed by a proteomic approach.

Results

Plasma levels of oxidized proteins was significantly higher in CHF patients than in control subjects ($p<0.01$). A proteomic approach allowed the identification of two oxidized proteins, α-1-antitrypsin and fibrinogen, which specifically underwent oxidation. Oxidation of α-1-antitrypsin resulted in loss of its protease inhibitor activity, thus leading to endothelial cell death in the presence of elastase. Fibrinogen, when oxidized, became otherwise cytotoxic and induced apoptosis in endothelial cells.

Conclusions

This study shows that plasma levels of oxidized proteins are increased in CHF patients and, by a proteomic approach, permitted the detection of proteins that, otherwise, could not be identified by conventional measurements. Finally, in vitro results highlighted the potential role of oxidized proteins in endothelial cell damage that occurs in CHF.

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New Insights in the Pathogenesis of Pseudoxanthoma Elasticum Revealed by Proteome Analysis

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Pseudoxanthoma elasticum (PXE) is a genetic disease characterized by progressive mineralization of elastic fibers with main clinical alterations affecting eyes, skin and the cardiovascular system. Even though the disease is due to mutations in the ABCC6 gene coding for the membrane transporter MRP6, the pathogenesis of PXE is still unknown. It has been hypothesized that, in PXE, connective tissue alterations could be sustained by abnormal constituents and/or degradation products, as a result of modified cell behaviour. Very recently, we demonstrated that PXE fibroblasts are characterized by imbalanced production and degradation of oxidant species (Pasquali-Ronchetti et. al, J. Pathol. 2006).

The present study was undertaken in order to investigate, by proteome analysis, the protein profile of dermal fibroblasts cultured in vitro from healthy individuals and from PXE patients matched for age and gender, aiming at the identification of pathogenetic pathways responsible for elastic fiber mineralization.

We have demonstrated that 40 proteins significantly changed their expression, among these, 29 proteins were identified by MS. From the analysis of identified differentially expressed proteins it appears that a certain number of proteins are modulated and/or related to oxidative stress, consistently with the recent observation that PXE fibroblasts are characterized by a condition of persistent mild oxidative stress (figure 1).

In particular, we have demonstrated a significant down-regulation of four enolase isoforms. This enzyme catalyzes the formation of phosphoenolpyruvate from 2-phosphoglycerate, but evidence indicates that it may function, other than a glycolytic enzyme, as a modulator of growth control as well as of thermal and hypoxia tolerance. Moreover, enolase may act as a cell surface receptor for plasminogen, suggesting that fibroblasts in PXE may favour proteolytic activities on the cell surface, thus contributing to matrix remodelling and increased degradative potential, consistently with in vitro and in vivo data (Annovazzi L., et al., Eur. J. Clin. Invest, 2004; Quaglino D. et al., BBA 2005).

Moreover, PXE fibroblasts exhibited a significant reduced expression of annexin 2 and glutaredoxin, supporting the hypothesis that the unbalanced regulation of oxidative stress may alter membrane stability, whereas expression of ubiquitin and calumenin appeared significantly increased in pathologic cells. Interestingly, calumenin may cause inhibition of gamma-carboxylase activity and a consequent decrease of the active form of MGP (Matrix-Gla Protein) a potent inhibitor of soft connective tissue calcification, that is significantly less expressed in PXE fibroblasts. These data further sustain the importance of oxidative stress in the pathogenesis of this disorder (figure 1) and may disclose new perspectives for the development of therapeutical approaches in PXE and in PXE-related syndromes (i.e. beta-thalassemia and vitamin-K deficiency), where connective tissue alterations are not associated with ABCC6 mutations (Hamlin N. et al., Br. J. Haematol., 2003; Vannaker O:, et al. J. Invest.Dermatol., 2007).

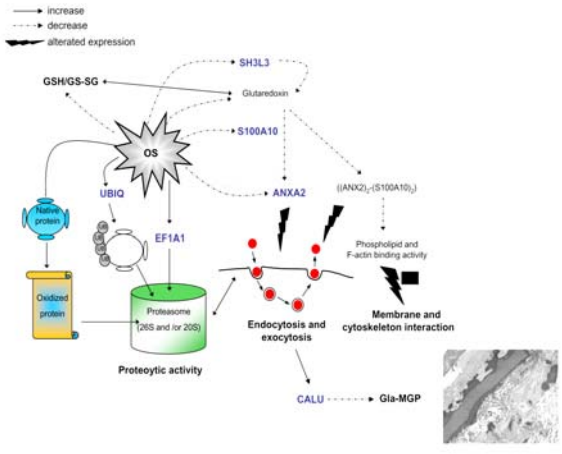

Figure 1: Cartoon showing the pathogenetic pathways that may lead to elastic fiber calcifications, as demonstrated by changes in the protein profile of PXE compared with control fibroblasts.

Work supported by grants from EU (Geneskin-CA-512117 and Elastage- CT-2005-018960.)

AP/MALDI-MS Studies of the Interaction between Insulin Degrading Enzyme and Insulin [P102]

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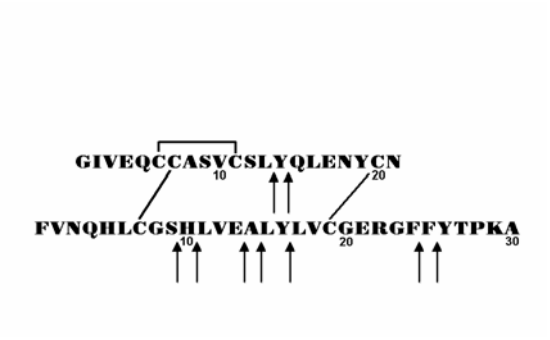
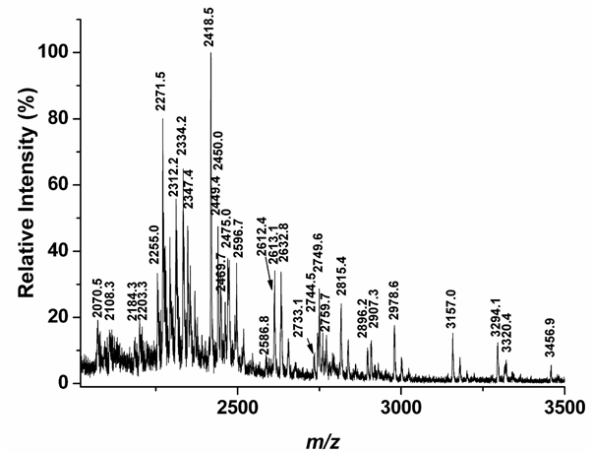
The prominent role that insulin degrading enzyme (IDE) has in the clearance of insulin as well as of other molecules such as amyloid-â has recently drawn a huge interest of the scientific community towards this protease. In order to give an insight into the way by which IDE interacts with its substrates, several papers have been focused on the structure of the IDE/insulin complex. In this scenario, although the cleavage sites involved in the interaction of insulin with IDE are known, a convenient experimental method that is able to identify in a complete and unambiguous way all the peptide fragments generated by such interaction has yet to be found. MS-based experiments have often represented an invaluable tool for the assessment of the cleavage sites, but the reported MS-spectra always show a partial coverage of all the peptide fragments generated by the enzymes interaction, lacking a complete characterization.

In this work we report a new experimental procedure by which an unambiguous as well as complete assignment of all the peptide fragments generated by the interaction of insulin with IDE is described. Atmospheric pressure/matrix assisted laser desorption ionization (AP/MALDI) mass spectra are reported and the data recorded, together with the introduction of a reduction/alkylation step, allow us to fully characterize the cleavage sites of the bovine insulin interacting with IDE. Different experimental conditions are screened and some insights into the IDE/insulin system regarding preferentiality of the cleavage and its dependence on the particular experimental conditions used are also given. Investigation on the tendency that different insulin fragments have towards aggregation is also carried out.

Good reproducibility, global and unambiguous assignment, low time consuming experimental procedure and small amount of enzyme requirements are some of the advantages of the proposed AP/MALDI based approach.



Primary structure of bovine insulin showing the cysteine bridges (lines) and the bonds broken by IDE (arrows).



AP/MALDI mass spectrum insulin in PBS solution after interaction with IDE. The high number of detected peaks is due to the many possible combinations of insulin fragments originating from the two different chains of the molecule and to the presence of sodiated peaks.

Proteomic Profile of Hypoxic Breast Cancer Cells [P103]

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Malignant progression of solid tumors is associated with several microenvironmental changes, including the onset of local hypoxia. This event may stimulate set of genes that allow tumor cells to fruitfully adapt and to survive and spread in a hostile environment.

The aim of present research was to study the effects exerted by hypoxic conditions on the 8701-BC breast cancer cells, in a broad proteomic context. This cell line derives from a ductal infiltrating carcinoma of breast and it has been extensively characterized, also for its proteomic profile (Pucci-Minafra et al Proteomics, 2006). For this purpose, cells were grown at low oxygen content (pO_2 2%) and examined in parallel with normoxic cells (pO_2 20%).

Using the silver stain method, we resolved about 1000 proteins both in normoxic and hypoxic samples. Protein spots of interest were identified by N-terminal or internal sequencing methods. All identified proteins were grouped into functional categories and their quantitative variations were calculated by the ImageMaster algorithms, using the percentage of relative volumes to normalize data. Surprisingly, a large number of proteins belonging to metabolic categories was not altered significantly by growth in hypoxic condition, suggesting that this cell line, isolated from an advanced malignant breast cancer, was already basically adapted to anaerobic metabolism. Major modifications were observed in a small group of proteins which are now considered as cancer-related hypoxic-responsive proteins.

At the same time a set of experiments, performed by semi-quantitative RT-PCR, showed that, while ErbB2 expression levels underwent to low modifications during hypoxia, Myc was increased by several folds.

Work supported by MIUR/PRIN prot. 2004059221

Cytoskeleton and Proteomic Changes Induced by Fibroblasts on Breast Cancer Cells [P104]

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Although it is widely accepted that the cross-talks between cancer cells and stromal cells play a crucial role in tumor progression, the molecular mechanisms involved in this dynamic system remain largely to be defined, due mainly to the multiplicity and complexity of the components implicated.

To investigate the effects of soluble mediators released by normal fibroblasts on breast cancer cells, we investigated the proteomic expression profile of 8701-BC cells co-cultured with fibroblasts in transwell chambers. The identified proteins were grouped in functional categories, as previously reported (Pucci- Minafra et al., 2006). Cytoskeleton and associated proteins resulted the major functional categories undergoing to modulation. Bidimensional western blot revealed a significant down-regulation of cytokeratins (CK8, CK18), and a concurrent up-regulation of vimentin and actin. Moreover, fibroblasts conditioned medium significantly enhanced the proliferation and invasion of 8701-BC. Taken together these results indicate that fibroblasts derived signals can affect significantly the phenotype and activities of the 8701-BC breast cancer cells.

Work supported by MIUR/PRIN prot. 2004059221

Proteo-genomic modulations induced on breast cancer cells by endothelial cells [P105]

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During the invasive process of malignant tumors, carcinoma cells detach from the primary mass, cross the basal lamina and the surrounding extracellular matrix and get into contact with a new microenvironment, usually precluded to cells of epithelial origin. While significant progress has been made in understanding the induction of tumor vasculature by secreted angiogenic factors, little is known regarding the cross-talk between neoplastic cells and endothelial cells. In this work we aimed to study the intercellular cross-talk of breast cancer cells and endothelial cells at proteomic and genomic level. For this purpose breast cancer cells (8701-BC) and microvascular endothelial cells (MVEC) were co-cultured in transwell chambers, and then separated by cell sorting, based on the differential expression of CD31 antigen, highly expressed in MVEC and almost absent in 8701BC cells. Proteomic and genomic analyses, performed on control and cocultured breast cancer cells, were carried out utilizing 2DE and an oligo GEarrays (SuperArray) with 288 breast cancer genes, respectively. Our analyses revealed that endothelial cells exert significant influences on breast cancer cells, both at protein and transcripts levels. For some genes, however, was not possible to correlate the level of transcription with that of related proteins, suggesting that some effects of endothelium on cancer cells may act at post-transcriptional level.

Work supported by MIUR/PRIN prot. 2004059221

Proteogenomic Changes in Differentiating U937 [P106]

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In "vivo" haematopoietic cell differentiation is finely regulated through quantitative and qualitative changes in gene expression leading to the achievement of specific functions of given phenotypes within the proper cell-lineages.

The underlying regulatory mechanisms that are involved in these processes are still poorly understood, while it is known that their disturbance can result in several neoplastic disorders.

The U-937 cell line is an established model to study monocytic differentiation, carcinogenesis and pharmacological responses. The U937 can be induced to differentiate along the monocytic pathway by several inducers, for example, ATRA, vitamin D3 (VitD3) and 12-O-tetradecanoylphorbol-13-acetate (TPA). To investigate the differential proteomic profiles between proliferating and differentiated cells, we submitted the undifferentiated U937 cells were incubated in the presence of TPA for 24h.

Differentiated U937 cells acquire adherence to substrate, reduce proliferation rates and express various cell surface molecules.

In the present report we show for the first time that U937 differentiation is correlated with the expression of a panel of proteins and RNAs, which may represent a new set of differentiation markers.

Work supported by MIUR/PRIN prot. 2004059221

Caveolin-1-dependent invasiveness in human ovarian carcinoma cells probed by proteomics [P107]

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GM3 ganglioside complexed with integrins controls tumor cell motility and invasiveness by regulating the activation state of c-Src. c-Src is activated in cells with low GM3 levels and high invasive potential, whereas artificially induced increase in GM3 levels caused inactivation of c-Src, influencing cell motility. On the other hand, it has been previously reported that caveolin-1 expression inhibits metastasis development and invasiveness in mammary tumor cells and promotes cell-cell adhesion in ovarian carcinoma cells by a mechanism involving inhibition of Src kinases. We found that high levels of gangliosides in ovarian carcinoma cells are associated with phenotypic changes occurring upon acquisition of antitumor drug resistance. Our preliminary results show that resistant cells express higher levels of caveolin-1 than parental sensitive cells. Caveolin-1 is enriched in a detergent-resistant membrane fraction, where it interacts with glycosphingolipids and a multiprotein complex, that includes Src family kinases; experimental manipulations able to increase cellular ganglioside levels in sensitive cells inhibits their invasiveness. These results suggest that glycosphingolipids might be involved in the regulation of caveolin-1-dependent, Src-mediated invasiveness in tumor cells. In this respect, the definition of the molecular mechanisms underlying the possible role of a caveolin-1-based signalling cassette regulated by glycosphingolipids in the modulation of invasiveness in ovarian carcinoma cells represents a crucial step. This goal can be pursued by the identification of the interacting protein partners of caveolin-1 in cells expressing different glycosphingolipid levels and characterized by different invasive properties. The association of caveolin-1 with partners belonging to a specific protein complex involved in a particular mechanism would in fact be strongly suggestive of its biological function. Furthermore, a detailed description of the cellular signalling pathways might greatly benefit from the elucidation of protein-protein interactions in the cell, that could be modulated by glycosphingolipids. On these bases, this work is aimed to study the protein partners interacting with caveolin-1 in functional complexes. Identification of caveolin-1 molecular partners was accomplished by immunoprecipitation-based functional proteomics approaches taking advantage from the use of recently developed specific anti-caveolin antibodies. Caveolin-1 complexes were isolated by immunoprecipitation from total cellular extracts with the specific anticaveolin antibody and the immunoprecipitated material was fractionated by SDS-PAGE, showing the presence of 21 protein bands (14-30 KDa). Protein bands were excised from the gel and digested *in situ* with trypsin. The resulting peptide mixtures were then analysed by MALDI/MS and the caveolin-1 molecular partners were identified by database search using specific softwares available in the lab.

Early adaptation to hypoxia in human skeletal muscle [P108]

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Introduction

Hypoxia, defined as an inadeguate O_2 supply, can trigger redox status disturbances at distinct level of cellular organization leading to an increased oxidative stress with consequent proteins, lipids and DNA damage. Hypoxia adaptation in humans occours in a number of physiopatological events such as exercise, aging, high altitude exposure, cardiovascular deseases, diabetes and cancer.

Human high altitude exposure represents the model for in vivo hypoxia investigations. There are many evidences that this paraphysiological condition induces oxidative damage and a number of cellular deleterious effects. We proposed to investigated this adaptation by analyzing skeletal muscle as target tissue due to is fluctuating O_2 demand and it's sensitivity to a low O_2 supply. Previous studies on the effects of hypobaric hypoxia exposure on muscles showed:

- morphological changes in mithocondria (swelling and cristae degeneration),
- the presence of increased lipofuscin, a product of lipid peroxidation and the activation of the cellular response to hypoxia by the hypoxia-inducible factor 1 (HIF-1) in mountaineers;
- a series of cellular and metabolic adaptations such as a decreased number of mitochondria coupled with a shift of the oxidative metabolism towards a carbohydrates metabolism and a reduced intramyocellular lipid substrates stores in high-altitude natives.

The aim of our study was to investigate the early effects of hypoxia exposure in human skeletal muscle at the molecular level using a proteomic approach (Difference In Gel Electrophoresis and Mass Spectrometry). The study was performed on climbers of Monte Rosa at an altitude of 4,559 m where the P_iO_2 is 87 mmHg and the decrease of O_2 percentage compared to sea level is 41%.

Methods

Vastus lateralis muscle biopsies were obtained from ten subjects before and after a seven-nine days of progressive exposure to hypobaric hypoxia (4,559 m, Monte Rosa, Italy). Protein extracts, labelled with Cy5 dye, together with an internal standard, labelled with Cy3 dye, were separated by two dimensional electrophoresis in both 4-7 and 6-11 pH gradients as first dimension and in 12% constant polyacrylammide gels in second dimension. Images analysis (DIA and BVA) and statistical analysis (paired Student's T-Test) were performed with DeCyder 6.5 software.

Silver stained semi preparative gels and HPLC ESI MS/MS were adopted for protein identification.

Results and conclusions

DIGE analysis revealed a significative difference in 116 spots, of which 83 are underespressed while only 33 are overespressed in hypobaric versus normobaric condition. HPLC ESI MS/MS identified 35 spots among the underespressed group and 2 spots among the overespressed. The unidentified are small spots or are spots located in the crowded high molecular weight region where the isoforms separation, especially in preparative gels, is difficult. Among the identified underespressed spots we found contractile and structural proteins, a great number of proteins involved in response to oxidative stress, proteins belonging to metabolic processes (glycolisis, tricarboxylic acid cycle, oxidative phosphorylation and fatty acid metabolism) and proteins related to molecules/ions transport and cell cycle.

These proteins represent the early molecular changes in the proteome of human skeletal muscle after hypoxia exposure suggesting these are first targets of oxidative stress and indicating the metabolic processes involved in the early phase of acclimatization. In our opinion these findings are an important step to understand the origins of both the resulting skeletal muscle damages and the signalling cascade that, eventually, leads to muscle hypoxia adaptation.

Simulated microgravity muscular atrophy investigated by 2D-PAGE and Mass Spectrometry [P109]

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Introduction

Actual and simulated microgravity (bed rest) are known to lead to muscle wasting and weakness. Several factors contribute to this phenomenon, disuse being a primary cause, though recent evidence suggests that microgravity itself has a direct effect on protein synthesis. The loss of muscle strength, however, is not only due to disuse-atrophy, but also to other muscular factors. In fact, the loss of muscle strength induced by these conditions is greater than the decrease in muscle size with a resulting decrease in force per unit cross-sectional area (F/CSA). The molecular mechanisms responsible for this phenomenon are not still understood. Proteomic study was performed on vastus lateralis muscle of human subject before and after 55 day of immobilization to define specific patterns of muscle protein expression after bed rest. The investigation was conducted by DIGE coupled with a dedicated software for the detection of protein expression differences. The differentially expressed spots were automatically gel excised, in gel digested and the peptides were identified by MALDI-ToF and sequenced by ESI MS/MS.

Materials and Methods

The study was performed on 8 healthy subjects (males, age range 24-43) involved in the "Berlin Bed Rest Study" before and after 55 day bed rest. Muscle samples (13-15 mg) from vastus lateralis (a plantar extensor) and soleus were obtained by standard needle biopsy (Bergstrom's technique). After biopsy the samples were solubilized in a lysis buffer containing 7M Urea, 2M tiourea, 4% Chaps and 30 mM Tris, pH 8,5. The solubilized proteins were accurately quantitated and fifty migrograms of muscle lysates, adjusted to pH 8.5, were differentially labelled with CyDye fluors (Cy3 for the internal standard and Cy5 for the samples; GE Healthcare) according to the manufacturer's recomendations. The labelling reaction was stopped by adding 1 mM lysine after incubation at room temperature in the dark for 30 min. The lysates were then incubated for 15 min. on ice with an equal volume of lysis buffer containing 130 mM DTT and 2% ampholine. Protein extracts from each subject were run in triplicate in a non-linear 3-10 pH gradient, 18 cm long (GE Healthcare) and separated on 12% constant concentration polyacrylamide gel. Sample load was 40 μg. The 2DE gels were acquired by a laser scanner (Typhoon 9200, GE Healthcare) and the images analysed with the dedicated software DeCyder (GE Healthcare). Proteins of interest were robotically exicised using Ettan Spot Picker (GE Healthcare); in-gel digestion was carried out with trypsin (Promega) and mass spectrometric analyses of tryptic digest were performed by using MALDI-ToF (GE Healthcare).

Results and conclusions

The differential analysis on vastus lateralis shows both qualitative and quantitative differences after 55 days of bed rest; statistical analysis (Student T-test) revealed the presence of significant differences in 68 spots, 35 were found to be down regulated and 33 up regulated; 42 of them were identified by MALDI ToF. The identified proteins were grouped in functional categories: 23% response to stress; 22% carbohydrate metabolism; 17% transport carrier; 7% structural protein, contraction and development; 7% oxidative phosphorylation; 5% protein translation; 5% signal trasduction; 2% protein synthesis; 12% other. As regard to soleus muscle : 62 spots were differentially changed, 29 were down regulated and 33 were up regulated; 39 of them were identified by mass spectrometry. The identified proteins were grouped in functional categories: 32% structural protein, contraction and development; 23% response to stress; 15% carbohydrate metabolism; 13% transport carrier; 8% oxidative phosphorylation; 3% glycogen biosynthesis; 3% amino acid metabolism; 3% other. The changes in protein expression profile are different in the two muscle types after 55 days of bed rest. In soleus the changes are related to contractile machinery and muscle metabolism while in vastus lateralis a more pronounced change in transport protein is observed.

Thiazolidinediones Induce PPARgamma Activation and Variation of the Protein Expression Profile in HT-29 cells: A Proteomic Approach. [P110]

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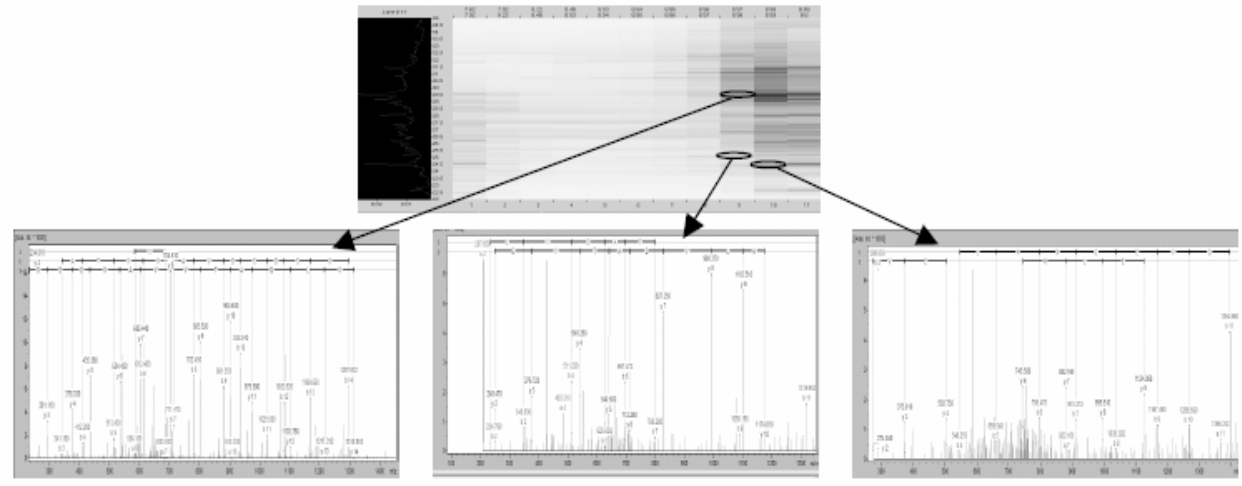
Proliferator Activated Receptors (PPARs) are ligand-induced transcription factors that regulate expression of genes involved in the control of lipid, glucose and energy metabolism. PPARgamma plays a pivotal role in the differentiation of adipose and epithelial cell types. PPARgamma agonists, as the insulin-sensitizers thiazolidinediones (TZD), administered to cancer cells of different origin cause growth arrest and in some cases differentiation. We exploited the possibility of analyzing the variations in the gene expression profile induced by these compounds, using a proteomic approach. We choose the human colorectal cancer HT-29 cells that express PPARgamma and respond to selective stimuli. The proteome profile in basal and TZDtreated cells was then analyzed by the 2-D liquid separation PF2D/mass spectrometry ESI-MS/MS system. This system involves separation by isoelectrophocusing in the first dimension and by a non-porous reverse phase high performance liquid chromatography in the second dimension. The 2D maps obtained in the two experimental conditions were analysed by using DeltaVue software. The peaks corresponding to the differentially expressed proteins were eluted from the last column, trypsin digested and the resulting peptides further separated by HPLC and directly injected into a ESI-MS/MS. This analysis provides peptide masses and sequence information and allows protein identification using Mascot software. Among the proteins showing a differential expression we focused on Cofilin-1, Hsp10 and p54/NonO. Cofilin-1 is a ubiquitous low m.w. actin-modulating protein that is reported to be structurally and functionally conserved in eukaryotes. The function of Cofilin-1 is regulated by phosphorylation and dephosphorylation, thereby it modulates the dynamics of cell division, motility as well as deformation and maintenance of structural architecture of differentiated cells. Hsp10 is a heat shock protein that can be induced when cells are under stress and regulates Bcl-2 family members and mitochondria apoptosis signaling. p54/NonO is a splicing associated factor and serves also as transcription factor of genes involved in cell differentiation. Thus, HT-29 cells respond to PPARgamma activation with a change in the protein profile consistent with a cell proliferation and motility arrest. The data provide further evidence that PPARgamma plays a crucial role in cell-differentiation and protects against cell-transformation.



Rapresentative 2-D liquid protein map of basal cells. The circles mark the differentially expressed proteins. The arrows indicate the aminoacid sequence of one of the identified peptides for each protein.

Topology of the Calmodulin Complex with ATPsynthase Inhibitor Protein IF_1: a Structural Proteomic Analysis [P111]

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IF_1 is the natural inhibitor protein of F_0F_1ATPsynthase able to regulate the ATP hydrolytic activity of both mitochondrial and ecto-F_0F_1ATPsynthase (1). Calmodulin (CaM) is a highly conserved , soluble, ubiquitous 15 kDa Ca^{2+}-binding protein, which is recognized as a major calcium sensor and orchestrator of regulatory events through its interaction with diverse target proteins and peptides. Many CaM-peptide complexes have globular conformations, where CaM's central linker connecting the two domains unwinds, allowing the protein to wrap around a single predominantly á-helical target peptide sequence. However, a growing body of evidence documents that the mode of interaction between CaM and several targets differs from the binding conformations observed in the case of its canonical targets, thus revealing that CaM can also bind to targets in unusual compact or extended conformations. Novel structures of CaM–target complexes have been reported where the conformation of CaM is highly dissimilar to the globular complexes, in some instances with less than a full compliment of bound calcium ions, as well as novel stoichiometries (2, 3).

Protein-protein interaction between IF_1 and Ca^{2+}-CaM, which may be involved in calcium-dependent activation of F_0F_1ATPsynthase, has been analyzed *in vitro* (4), and evidence has been recently provided for co-localization of the two proteins on plasma membrane of cultured cells (5).

IF_1-CaM complex may form on the external surface of cells, with CaM functioning as sequester of ecto-IF_1, thereby modulating the availability of IF_1 to regulate ecto-F_0F_1ATPsynthase.

The stoichiometry and the topology of IF_1-CaM complex were here investigated *in vitro* by a combined structural proteomic strategy which integrates limited proteolysis and cross-linking experiments with mass spectrometric analysis. Our results suggest that IF_1-CaM is a 1:1 complex, with two calcium ions bound. Most interestingly the crosslinking experiments indicate that only the C-terminal lobe of CaM is involved in the formation of the complex.

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Protein expression profiles of ejaculated and capacitated human sperm [P112]

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To become fertilization competent, mammalian sperm undergo complex morphological and biochemical modifications taking place in male and female genital tracts. During epididymal transit spermatozoa are subjected to maturation which results in motile apparatus changes, loss or modifications of preexisting proteins, insertion or adsorption of epididymally expressed protein factors, and changes in lipid composition. Despite all these processes, freshly ejaculated spermatozoa are still unable to fertilize. Sperm capacitation, which in vivo occurs in the female genital tract, represents the final and essential maturation event enabling spermatozoa to bind the oocyte and undergo the acrosome reaction. During capacitation, factors originating from epididymal fluid and seminal plasma are lost or redistributed, membrane lipids and proteins are reorganized, complex signal transduction mechanisms are induced by ion fluxes, and, as recently suggested, ulterior sperm proteins are synthesized. Nonetheless, biochemical bases of capacitation are not yet completely clarified.

This work deals with a proteomic approach to the characterization and comparative analysis of ejaculated and capacitated human spermatozoa obtained from healthy donors. Cellular extracts of ejaculated and capacitated spermatozoa were resolved using 2DE and, according to the image analysis, 144 quantitative and 39 qualitative differences were visualized between the two sperm samples. Among such differences numerous protein spots were in-gel digested with trypsin and identified by MALDI-ToF MS and ESI-Ion trap MS/MS. Our data may provide an important step forward in understanding the molecular mechanisms taking place in sperm capacitation.

* *The first two authors equally contributed to this work*

MALDI Profiling of Normal and Renal Cell Carcinoma Human Samples: Preliminary Results [P113]

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Mass Spectrometry Imaging (MSI) technique, divided in *Profiling* and *Imaging* depending on the level of spatial resolution, consists in the direct analysis of tissue samples using MALDI-MS, giving information about the spatial distribution and abundance of proteins and peptides inside the samples [1]. Profiling technique, due to its low-resolution, is typically used for the discovery of discriminating patterns between different physiological conditions [2, 3], while Imaging is able to maintain the spatial position of protein and peptides, thus providing a real image of the analyzed tissue [4, 5].

Preliminary results of MALDI profiling performed on normal and diseased tissue slices and on primary cell cultures derived from, adjacent normal and Renal Cell Carcinoma (RCC) tissues will be described.

Tissue slices and cells were putted onto conductive glass slides and inserted in the mass spectrometer by an adapted target plate. Matrix was spotted in small droplets directly on the samples and analyzed in a Reflex IV® MALDI-TOF operating in linear mode. The obtained average spectra were analyzed with the ClinProTools® software to find discriminating patterns between normal and pathological condition.

In both cases we were able to find peaks with significantly different intensities between normal and tumour samples, thus providing potential candidates for further investigation.

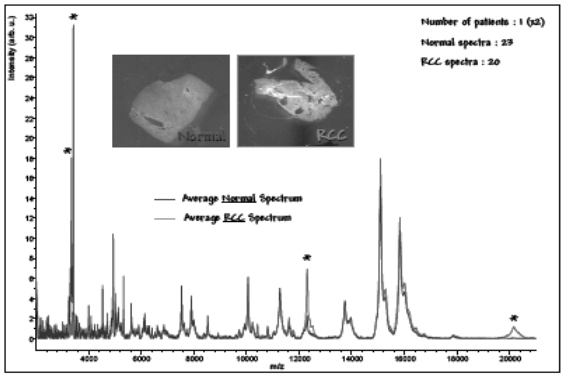

Fig. 1: Average mass spectra obtained from Normal and RCC tissues, some peaks with statistically different intensities are marked with asterisks

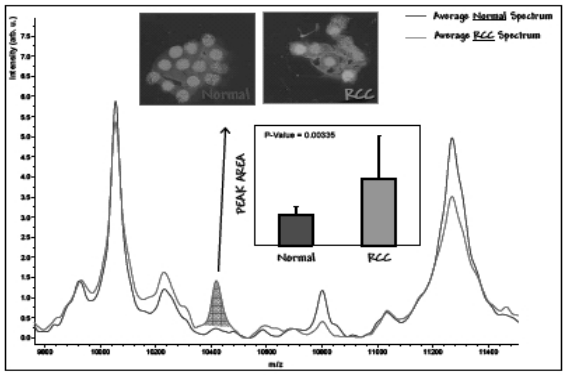

Fig. 2: Example of a statistically different peak between normal and pathological condition

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Serum proteomic profile of melanoma and relation to cancer progression [P114]

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Currently clinical outcome in melanoma is not predictable by known serum biomarkers. The only reliable tool for the diagnosis of this tumor is the histopathological assay after surgical removing. We used a proteomic approach in order to identify novel non-invasive serum biomarkers of melanoma. Serum from 31 individual investigated for suspected melanoma was analyzed by 2D electrophoresis after albumin and IgG depletion. Image analysis revealed the modification of the expression pattern in relation with the presence and progression of the tumor in 5 regions of the proteomic map. Differently expressed spots were identified by MALDI-TOF mass spectrometry after in gel trypsin digestion. Spot corresponded to two isoforms of trasthyretin (TTR), retinol binding protein (RBP), zinc-alpha-2-glycoprotein (ZAG), vitamin D binding protein (DBP), angiotensinogen (AGT), ficolin and several isoforms of haptoglobin. Significant variations of expression were found for an high molecular weight TTR (increased in presence of the tumor), angiotensinogen (increased in stage I melanoma) and DBP (whose expression is decreased in stage III melanoma). While low molecular weight TTR, RBP, ZAG, ficolin and haptoglobins didn't show significant variations after statistical analysis in the group of all melanoma patients. In order to better evaluate protein expression in relation to the effective presence of the malignancy we restricted our analysis to serum samples collected before surgical removal of the tumor (12 controls and 13 melanoma patients). A significant increase of high molecular weight TTR appears as a marker of melanoma in stage I, II and III before surgery. After one month from surgical removal it is still over-expressed only in stage III, but not in early stages. Moreover an acidic low molecular weight TTR significantly increases in stage I melanoma before surgical removal. It appears as an early marker of the tumor since it is low after surgery as well as in advanced stages and controls. TTR involvement in melanoma is of big interest since it is the major carrier of thyroid hormones and retinol binding protein. Thyroid hormones and retinoids acts as modulators of the expression of several genes through the binding of retinoic acid and triiodothronine to the corresponding nuclear receptors. Moreover retinoic acid is an inhibitor factor of cell proliferation, invasion and metastasis and is responsible of melanogenesis in melanoma cell lines. For same reasons the significant over-expression of RBP observed in the stage I and II of melanoma patients before surgery (normal after one month from surgical removal) appears of big interest in the characterization of the tumor progression. Other statistical relevance was found in the expression of spots corresponding to DBP and angiotensinogen in the group of patient analyzed before surgery. In particular AGT, a potent antiangiogenic factor, is over-expressend 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 patient (stage I). DBP spot is reduced in melanoma patients (significantly in stages I and III before surgery) compared to control individuals. We related the decrease of this spot (a glycosylated isoform of DBP from preliminary analysis) to the impaired immune response of cancer patients. In fact cancer cells release an endoglycosidase (alpha-Nacetylgalactosaminidase) that can deglycosylate DBP, whose glycosylated form is a precursor of DBP-MAF mediated macrophage activation cascade, finally leading to immunosuppression in advanced cancer patients. The impaired immune system provides further opportunity for survival and proliferation of neoplastic cells. 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 an drug targeting of this tumor.

Proteome and phosphoproteome modification triggered by HypF-N prefibrillar aggregates in NIH3T3 cells [P115]

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Several human diseases, such as Alzheimer's and Parkinson diseases, spongiform encephalopathies and type II diabetes, are associated with the deposition of stable ordered protein aggregates known as amyloid fibrils. Despite the large differences in the amino acid sequence and structure of the proteins and peptides found aggregated in different amyloidoses, fibrils display common structural features. Several data demonstrated that proteins not involved in amyloidoses, including the N-terminal domain of the prokariotic hydrogenase maturation factor HypF (HypF-N) are able to form in vitro, under appropriate conditions, amyloid fibrils indistinguishable from those formed by disease-related proteins (1). HypF-N early prefibrillar aggregates were shown to induce cell death in different cell lines (2); in particular, NIH3T3 cells exposed for 24h to 10μM prefibrillar aggregates died with necrotic features preceded by early mitochondria membrane depolarization (at 3h) and activation of caspase 9 (at 5h). The mitochondrial injury appeared so severe that the programmed cell death could not be sustained and necrosis was the final outcome. In order to better characterize the effect of cell exposure to HypN-F we performed a differential proteomic analysis of 5 h treated cells *vs* control cells by 2-DE followed by protein identification by MS. Furthermore, since phosphorylation is a key mechanism in many cellular processes including cell death, we also analyzed the phosphoprotein pattern in cells treated for 1 h In this work we report preliminary data about the proteins involved in both processes (fig.1). This double approach discloses the possibility to identify new proteins or new pathways of signal transduction involved in amyloid toxicity.

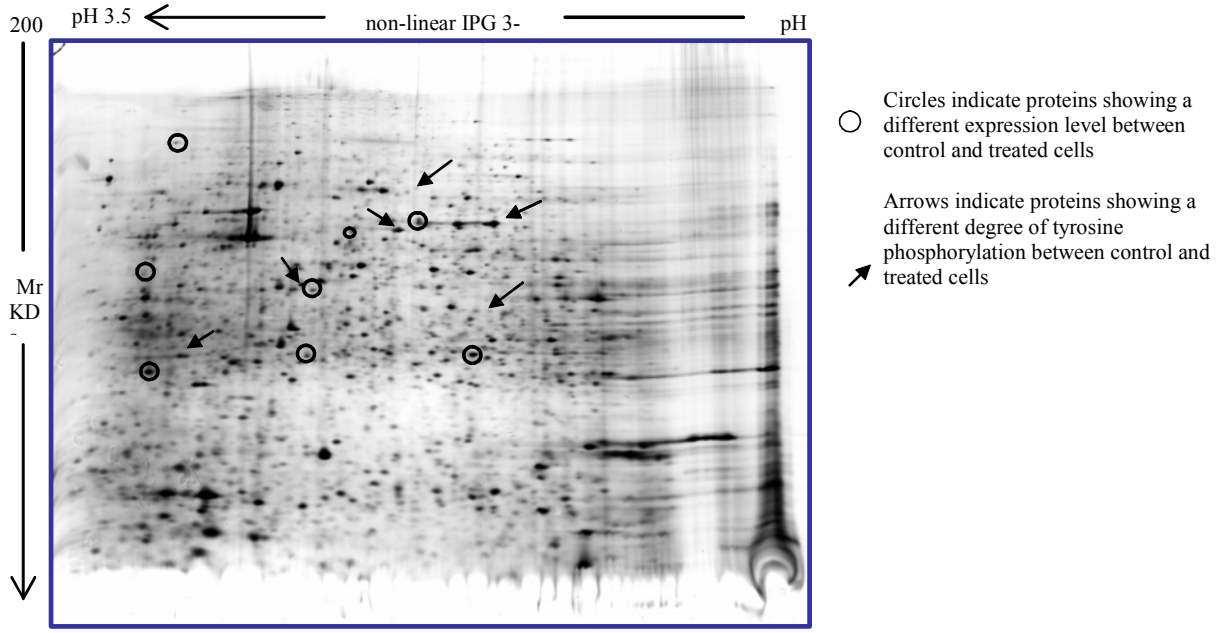

Fig.1

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Proteomic analysis of class III beta tubulin (TUBB3) post-transcriptional modifications [P116]

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TUBB3 has been discovered as a marker of different types of malignant epithelial tumors. TUBB3 seems to be overexpressed in several cancer patients as a marker of poor prognosis (1-5). We identified two main different forms of TUBB3 having a specific electrophoretic profile: a faster migrating and a slow migrating band (Fig. 1A and 1B). We demonstrated that the upper band is glycosylated and phosphorylated and it is mainly localized in the cytoskeleton (Fig.1C). The lower band is instead found exclusively in the mitochondrial compartment (Fig. 1C). The characterization of TUBB3 glycan structure by lectin affinity chromatography (Fig. 2A and 2B) and digestion with Endo H and peptide Nglycosidase F (Fig. 2C) demonstrated that TUBB3 is a glycoprotein with a high mannose and a complex N-linked oligosaccharidic structure. Our data show that, among the beta tubulin isotypes, TUBB3 represents the most glycosylated isoform. Moreover, we observed that the level of phosphorylation and glycosylation of TUBB3 was higher in paclitaxel resistant cells (TC1 and OVCAR-3) compared to paclitaxel responsive cells (A2780wt), in that suggesting that these post-transcriptional modifications have a role on TUBB3 activity on microtubules and are associated with a paclitaxel-resistant phenotype (Fig. 2B and 3A). Since the glycan moiety of TUBB3 was not completely removed after peptide N-glycosidase F treatment and in TC1 cells TUBB3 was resistant to endo-glycosidase cleavage, it is conceivable that a cooperation of glycosylation with other post-transcriptional modifications (probably phosphorylation) and/or proteinprotein interactions may be involved in TUBB3 function. 2D SDS PAGE analysis showed that the lower TUBB3 2D spot migrates at a more basic pH. The modification of beta-tubulin migration in SDS PAGE after treatment with alkylating agents it is already been shown in several cell lines (6). Here we show the existence, under physiological conditions, of an unglycosylated and unphosphorylated form of TUBB3 characterized by a lower molecular weight and a more basic isoelectric point that has probably a role in the mitochondria trafficking. We demonstrated that this change in isoelectric point is not due to carboxymethylation with iodoacetamide during 2D SDS page procedure but it is probably due to another post- transcriptional modification (probably alkylation) that regulates TUBB3 localization in the mitochondria compartment. Thus, even if it is already been shown that mitochondrial tubulin is synthesized in the cytosol, a selection related to inherent properties of tubulin may led to mitochondrial specific distribution and function of tubulin isotypes.

Figures.

Fig. 1: TUBB3 electrophoretic profile is characterized by two main forms: a faster migrating and a slow migrating band. 1A: The two TUBB3 isoforms are found in several and different cell lines. 1B: The faster migrating form of TUBB3 has a more basic isoelectric point. This is a specific characteristic of TUBB3, since TUBB1 and TUBB2 isoforms migrate at the expected isoelectric point. 1C: The two TUBB3 isoforms have a specific cellular distribution.

Fig 2: The slow migrating band corresponds to a glycosylated and phosphorylated TUBB3. 2A: TUBB3 reacts with ConA. 2B: Chromatography affinity analysis using total lysates. 2C: Digestion with endoglycosidases showing that TUBB3 is cleaved by peptide N-glycosidase F.

Fig 3: TUBB3 associated to cytoskeleton is glycosylated (3A) and phosphorylated (3B).

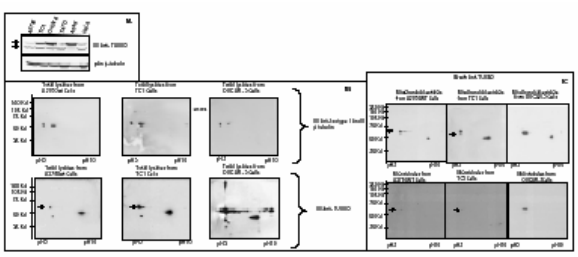

Fig. 1

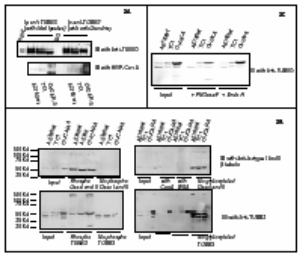

Fig. 2

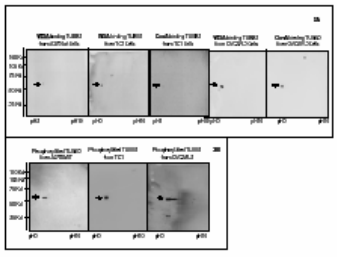

Fig. 3

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Comparison of Different Depletion Strategies for Improving Resolution of Human Urine Proteome [P117]

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During the past few years, proteomics has been extensively applied to different fields of medicine including nephrology. Current applications of renal and urinary proteomics aim to better understand renal physiology and to identify novel biomarkers and new therapeutic targets (1). Urine represents a modified ultrafiltrate of plasma, with protein concentrations about 1000-fold lower than plasma. However, this fluid can be obtained noninvasively and tests of many urinary proteins are well-established in clinical practice. Therefore, it is crucial to use an efficient protocol to isolate/concentrate urinary proteins and to eliminate interfering salts.

Generally, to enhance resolution and recovery of low abundance proteins, several strategies of high-abundance proteins depletion were specifically proposed for human and primate fluids such as serum, plasma and CSF, while very few data from urine are reported in literature (2). In this context, three different immunoaffinity chromatography columns: Genway™ Spin IgY-12 kit, HPLC Agilent Hu-PL7 and a home-made column vs Human Serum Albumin (IAC), able to remove some of the high-abundance proteins, were compared.

To be able to obtain a volume of pooled urine compatible with the capacity of spin and HPLC columns, 20 mL of pooled urine containing ± 500 µg of proteins were centrifuged at 3000g for 5 min at 4° C to precipitate the debris and then concentrated to ± 100 µL using centrifugal filter with 5 kDa molecular weight cut-off (Amicon Ultra-15, Millipore) at 4000g for ± 60 min at 4°C. After measuring proteins concentrations using Bio-Rad protein assay kit, recoveries yield and CV% observed after depletion (bound + unbound fractions) for the three methods ranged from 60-90% and 10.2-15.5% (n=5). To isolate and opportunely desalting unbound fractions for 2D gel electrophoresis, several strategies were investigated. An overnight precipitation at 4°C using a urine-to-solvent ratio of 1:5 of ethanol followed by 90% acetone treatment provided the most simple and reproducible approach.

An equal amount (90 µg) of urinary proteins and depleted samples were analysed by 2-D gel electrophoresis and visualized by Coomassie staining. For spot analysis, gel images, captured with a

ProXPRESS 2D Proteomic Imaging System (Perkin-Elmer), were uploaded into Progenesis PG240 Discovery 2006 image analysis software (Nonlinear Dynamics Ltd, UK) using the analysis wizard.

Proteins spots were detected from triplicate gels. After manual spot editing, number of spots visualized on the gels were 733±22.7, 564±22.5, 433±32.0 and 530±44 (mean±SD) for Geneway, IAC, Agilent and no-depleted urine pool sample, respectively, with a significant difference $p<0.05$ (One Way Analysis of Variance) of Geneway vs Agilent and IAC. Number of spots detected is in agreement with data reported in literature (3). A few spots detected only in all three Geneway gels were trypsin digested, characterized by MALDI-TOF MS and identified as urinary specific proteins (Max-Planck Unified Proteome Database (MAPU) http://proteome.biochem.mpg.de/) demonstrating their relative enrichement in these samples.

These preliminary data suggest the use of depletion approach, particularly of Geneway Spin IgY-12 kit, to improve resolution of 2-D for proteomic analysis of urine.

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Phosphorylation sites identification in single protein and complex mixtures by Metal Oxide Chromatography and Mass Spectrometry [P118]

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Among the several hundred known posttranslational modification, protein phosphorylation is particularly important because it is involved in the control of essentially all biological process and thus in many diseases.

Many techniques have been developed and amongst them the metal oxide chromatography has been successfully applied[1].

To isolate and identify the phosphorylated peptides from single proteins and complex protein mixtures we have used the titanium dioxide microcolumns to enrich the sample in phosphopeptides, coupled to mass spectrometry techniques to identified the modification sites, in particular using a hybrid LTQ/OrbiTrap mass spectrometer.

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Asparagine deamidation in NGR-TNF detected by high resolution mass spectrometry [P119]

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Solid tumors recruit new blood vessels to support the tumor growth, and unique epitopes expressed on tumor endothelial cells can function as targets for cancer therapy. Several lines of evidence suggested that NGR containing peptides target aminopeptidase N (CD13), a marker of angiogenic endothelial cells. In particular cyclic and liner NGR peptides have been used for targeted delivery of chemotherapeutic drugs, virus particles, pro-apoptotic peptides, cytokines and liposomal doxorubicin to tumor blood vessels.

TNF fused to CNGRC peptide (NGR-TNF) improved the therapeutic index of TNF in animal models, either when used alone or in combination with chemotherapeutic agents (1). It is well known that asparagine residues, particularly when followed by glycine residues, may deamidate to form alpha or beta aspartic acid residues. In other words the sequence NGR could be converted into DGR. Since this reaction could take place during storage as well as in vivo, we decided to investigate the stability of NGRpeptides and of NGR-conjugates as well as the kinetics of deamidation in various conditions (buffer, pH, temperature, cyclic constrains etc.).

We found that peptides containing NGR are unstable and rapidly convert to compounds corresponding to DGR, isoDGR, via succinimide intermediate (2).

Deamidation increases molecular mass by only one nominal Dalton (-NH2 —> -OH), so is difficult to determine this chemical reaction by conventional analytical methods like MALDI-TOF-MS and ESI-QTOF, because of the overlapping of the deamidated isotopic envelope with the undeamidated one.

Despite the minimal change in the molecular mass, using a hybrid LTQ/OrbiTrap mass spectrometer we were able to differentiate and clearly separate the isotopic envelopes of NGR- and DGR-containing peptides with high mass resolution (100000) and high mass accuracy (2-5ppm) allowing the simultaneous detection and identification of asparagine containing peptide and of the deamidated one.

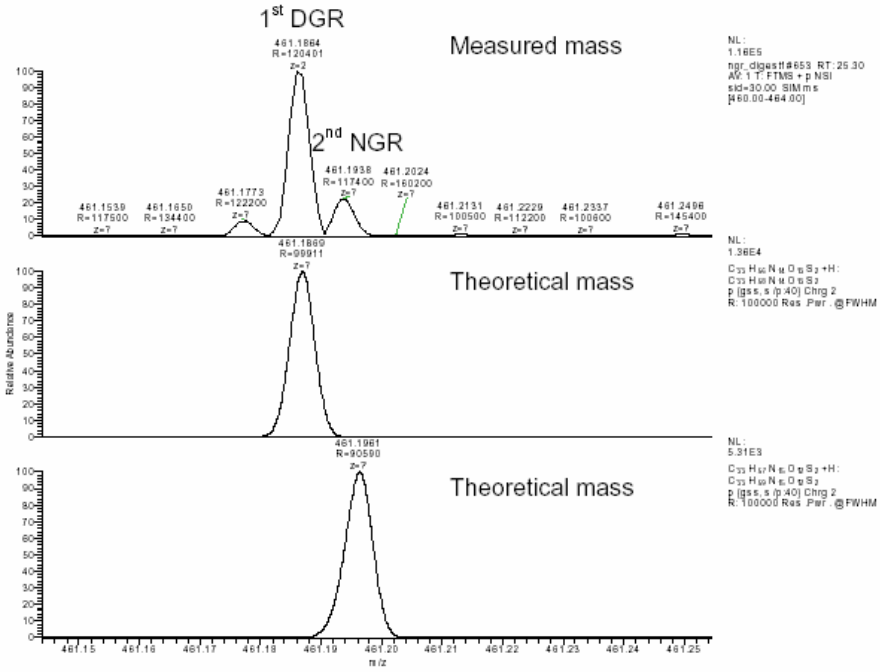

Figure1: spectrum ESI-MS LTQ/OrbiTrap; separation beetween 1st isotopic peak peptide DGR and 2nd isotopic 1 peak peptide NGR.

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Studying a New Coiled-coil Containing Protein that seems to be Involved in Mitosis [P120]

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In a previous study aimed at analyzing rat hippocampal neurons by a proteomic approach, we identified a new coiled-coil containing protein. This protein has been found enriched about three fold in the Synaptosomal (S) fraction compared to High Speed Supernatant (HSS, mainly cell body) fraction of neuronal cells, for this reason we have named it SEMAP: Synaptosome Enriched Microtubule Associating Protein.

SEMAP is a well conserved protein, in fact its mRNA is expressed in rat, mouse, human and primates as indicated in NCBI database; from molecular point of view it is characterized by two coiled-coil regions and a C-term domain similar to the conserved one of ASE1/PRC1/MAP65 family of microtubule bundling proteins. By computer assisted sequence analysis, prediction algorithms showed that SEMAP seems to be cytosolic and it has several putative sites for both Ser/Thr and Tyr phosphorylation.

We have cloned SEMAP and now we are trying to characterize it in order to understand its functions. We have also cloned a truncated form of SEMAP (lacking the microtubule associating C-term domain) used in rabbit immunization so to obtain a SEMAP-specific polyclonal serum, a useful tool in the study of this protein. Our results confirm the ability of SEMAP of interacting with microtubule (Fig. 1) and immunofluorescence analysis in Hela and COS (Fig. 2) cell lines showed that this protein stands near the MTOC region during the interphase while it seems to be involved in mitotic spindle organization in dividing cells.

Our goal is now to understand its function both in dividing (Hela) and in non-dividing (neuronal) cells, where it was identified at the beginning; for this reason we are trying to perform RNAi experiments.

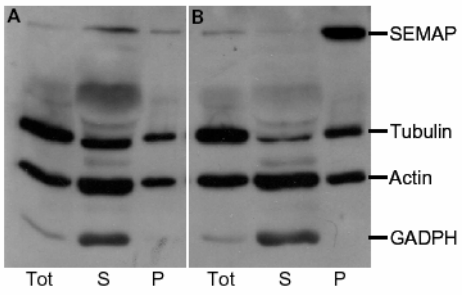
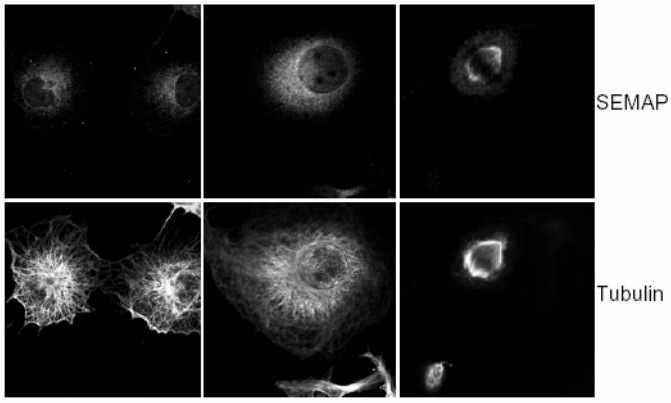


Fig.1 "Cosegregation" of SEMAP with Microtubule

Panel A with Nocodazole (microtubule depolimerization)
Panel B without Nocodazole

Tot	Total Lysate (Hela cells)
S	Supernatant (cytosol,contains monomer of actin and tubulin)
P	Pellet (contains intact cytoskeleton)

Fig.2 Immunofluorescence in COS cells



Comparative analysis of bronchoalveolar lavage fluid (BALf) protein profile in patients with Dermatomyositis or Sistemic Sclerosis with pulmonary fibrosis by 2-DE [P121]

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Background:

Polymyositis (PM) and Dermatomyositis (DM) are inflammatory myopathies in which an interstitial lung disease (ILD) can be frequently detected [1]. In the course of PM/DM, the serologic finding of antibodies against tRNAsynthetases, whose most common is anti-histidyl t-RNA synthetase (Jo1), identifies a disease subset in which patients are more likely than other PM/DM patients to develop ILD and that is classified as Antisynthetase Syndrome (AS) [2].

Prognosis and response to treatment of ILD in AS patients seem different with respect to ILD in the course of classic PM/DM or in other CTDs as Systemic Sclerosis (SSc) [3]. Through bidimensional gel electrophoresis of bronchoalveolar lavage fluids (BALf) [4-6] we compared BALf maps of AS patients with ILD with those of healthy subjects and SSc patients with ILD in order to better define AS patients' peculiarities and factors that could physiopathologically explain the differences observed in term of prognosis and response to treatment.

Patients:

Samples consisted of archived BALF samples acquired between 2004 and 2005 from 5 PM/DM patients, 5 SSc patients and 5 healthy subjects who underwent bronchoscopy for other reasons. All patients satisfied Bohan and Peter's criteria for PM/DM and the preliminary American College of Rheumatology criteria for SSc classification respectively.

PM/DM patients all showed serological reactivity to anti-histidyl t-RNA synthetase by ELISA. Median age was 51 years (range 45-73) with a median disease duration of 90 months (6-150) and a F:M ratio of 4:1 for PM/DM patients, 62 years (range 56-70) with a median diasease duration of 48 months (range 6-156) and a F:M ratio of 5:0 for SSc. All patients were non-smokers and followed up at the outpatient clinics of the Rheumatology Unit of the Policlinico San Matteo, Pavia, Italy. They all gave their informed consent to undergo bronchoscopy.

Methods:

BAL was performed following the standard procedure. To remove the cells and debris, all samples were filtered through gauze and centrifugated, and the supernatant was then divided into aliquots and stored at –20°C for protein analysis. All samples were dialyzed in the presence of protease inhibitor,lyophilized and the protein pellet was resuspended in a solubilization buffer. For each experiment (analytical) of 2-DE the same amount of protein (70 μg) was used; a higher (1 mg) was loaded for the subsequently mass spectrometry identification experiments.

We compared the BALf protein profile obtained from AS patients as compared to SSc patients with pulmonary involvement (SscFib+) and to healthy controls; the total number of spots, non-matched spots and spot parameters were assessed by the PDQuest 7.1 software.

Results:

Only spots which were consistently expressed in a different way in the three groups (>50% difference in intensity or no matched) were taken into consideration. A total of 35 spots were found to significantly differ between the three groups. 30 of those deregulated spots corresponded to 15 identified proteins (among them: transferrin, á1antitrypsin, leucin-rich α2 glycoprotein, SpA, α1acid-glycoprotein Ig light chain κ,λ , Cu-Zn SOD, GSTp, transthyretin), and 5 are still under identification. There are also many spots that are present only in one of the three groups which are being analyzed by mass spectrometry.

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This work is supported by CARIPLO 2006 and by Sovvenzione globale INGENIO.

Identification by mass spectrometry and preliminary in cell characterization of mitotic TCTP phosphorylation sites [122]

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Nerviano Medical Sciences

Translationally controlled tumor protein (TCTP) is a highly conserved protein identified in all eukaryotic organisms.

Several different functions have been suggested for this protein among different species (Sturzenbaum et al., 1998; Haghighat et al., 1992; Li et al., 2001) but the determination of its main biological role remains elusive. In mammalian, TCTP has been recognized as a phosphoprotein, and indeed recombinant TCTP can be phosphorylated in vitro by Pololike kinase 1 (Plk1) as well as by cell lysates obtained from nocodazole treated HeLa cells (Yarm, 2002).

In the present work, we have identified by mass spectrometry Ser46 and Thr65 as Plk1 phosphorylation sites in vitro. The aminoacid sequence surrounding these phosphorylation sites is in agreement with reported consensus phosphorylation for Plk1 (Nakajima et al., 2003).

A specific antibody specific against Ser46 phosphorylation was produced and tested in Western blotting and immunocytochemistry experiments in order to study endogenous TCTP phosphorylation during the cell cycle. In particular A2780 cells were synchronized in pro-metaphase with Nocodazole and complete cell cycle was followed by double Thymidine block and release (fig. 2). Cell cycle distribution of TCTP phosphorylation was analysed through flow cytometry and Western blotting. In order to have more insight into the G2 to M transition, specific markers such as phospho-Histon H3, Cyclin B1, phospho-Nucleophosmin Ser199 and Plk1 were also analysed.

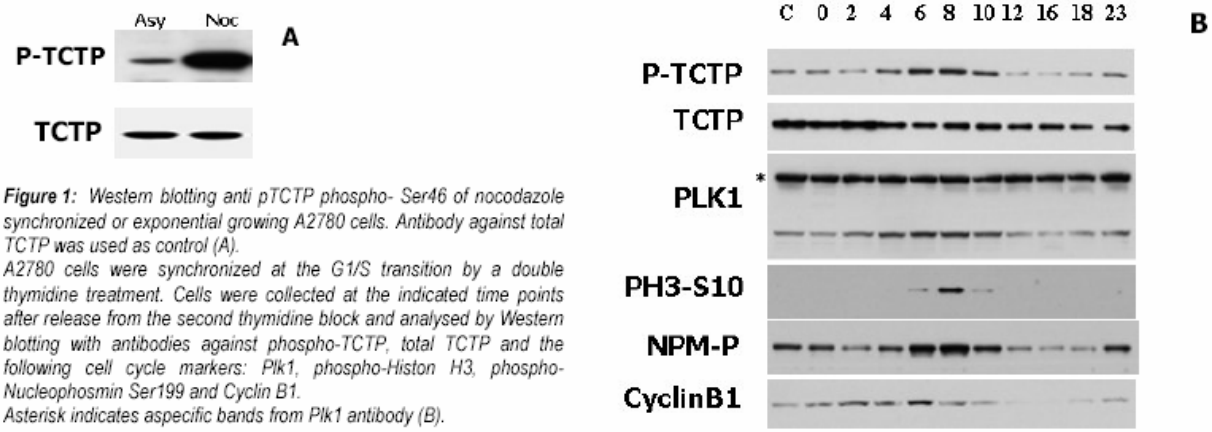

Figure 1: *Western blotting anti pTCTP phospho- Ser46 of nocodazole synchronized or exponential growing A2780 cells. Antibody against total TCTP was used as control (A).*
A2780 cells were synchronized at the G1/S transition by a double thymidine treatment. Cells were collected at the indicated time points after release from the second thymidine block and analysed by Western blotting with antibodies against phospho-TCTP, total TCTP and the following cell cycle markers: Plk1, phospho-Histon H3, phospho-Nucleophosmin Ser199 and Cyclin B1.
Asterisk indicates aspecific bands from Plk1 antibody (B).

Through this approach we have specifically demonstrated that phosphorylation of TCTP at Ser46 peaks from G2 to late M phase, promoting a progressive relocalisation of the protein from the cytoplasm to the nucleus observed by immunocytochemistry.

In vitro analysis clearly indicated that not only Plk1 but also recombinant Plk3, and to a lesser extent Plk2, are able to phosphorylate TCTP and that phosphorylation occurs on Ser46, as resulted from Western blotting experiment. While Plk2 is activated in S phase and is involved in centriole duplication, Plk1 and 3 activities peak during G2/M phase of the cell cycle when TCTP is more phosphorylated. For this reason we evaluated the effect of inhibition of these 2 kinases on phospho- Ser46 TCTP using different tumoral cell lines. We established that the inhibition of only one member of Plk family is not sufficient to significantly decrease TCTP phosphorylation in asynchronous cells while the combined inhibition of Plk1 and 3 with specific inhibitors and RNAi drastically reduces Ser46 phosphorylation, indicating that TCTP is a real substrate of Polo-like kinase family in mitosis.

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Identification of Growth Hormone Isoforms from Different Matrices by Capillary Electrophoresis – UV Spectroscopy and Mass Spectrometry [P123]

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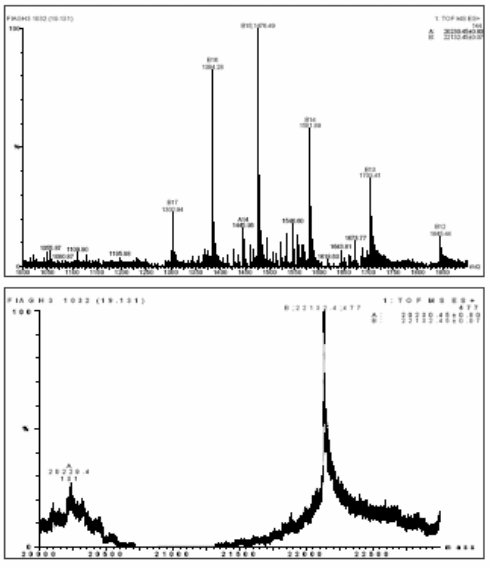
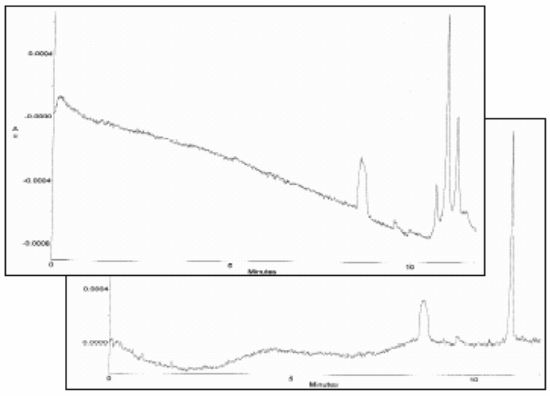
Human growth hormone is a polypeptide hormone released by the hypophysis which stimulates the protein biosynthesis, making it a potential anabolic doping agent. The hypophysis releases two types of hGH, a 22 kDa type, consisting of 191 amino acids (AAs) and a 20 kDa type (176 AAs; AAs 32-46 are missing). GH is one of several pharmaceutical proteins that are currently available from DNA technology (recombinant human growth hormone, r-hGH). Once assumed, r-hGH has been considered to be undetected because it is identical to the 22 kDa fraction of pituitary-derived hGH. Analyses of such products to detect illegal use requires the use of the most powerful tools of analytical protein chemistry. Capillary electrophoresis (CE) is a technology that shows high sensitivity and selectivity and may have promise in this application. Determining the ratio between 22 kDa-hGH, and all isoforms (total hGH) allows the estimation of the relative abundance of 22 kDa-hGH. Administration of r-hGH leads to an increase of the 22 kDa-hGH fraction, thus increasing the cited ratio. This approach should fulfil the criteria of confidence necessary to distinguish between "doping" and naturally occurring variations of GH secretion. We developed a capillary zone electrophoresis technique coupled with a UV detector for separation and quantification of the two most abundant hGH isoforms (22 kDa and 20 kDa) extracted from pituitary gland (Sigma Aldrich product) and from a pharmaceutical preparation (Saizen). Isoforms were separated with uncoated capillaries (ID:50-75ìm). Various analytical conditions were tested (different buffers, applied voltage, ionic strength, pH). Linearity, repeatability, and limits of detection were determined. For qualitative purposes, MS analyses were performed on a QTOF mass spectrometer, that enables automated accurate mass measurements (uncertainties in the parts-per-million range). The electrospray ionization of proteins in positive polarity produces multiply charged ions predominantly by the formation of adducts with several protons. From the multi charged spectra of hGH and r-hGH obtained from infusion of a standard solution into the source of a suitably tuned and calibrated QTOF mass spectrometer, by a "Transform alghoritm" process, we calculated the accurate molecular weights of the two most abundant GH isoforms.



Q/TOF MS spectrum of an infusion of hGH standard 0,4 UI/mL (H2O/AcN 0,2% HCO_2H) □ = 20 µL/min

CE-UV electropherogram of: (a) hGH standard 0,1 UI mL^{-1};(b) Saizen 0,1 UI mL^{-1}.Conditions: Buffer: tris-borate 20 mM; pH= 8,5; pot=10 kv; □= 220 nm



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Biomarkers Identification by Plasma proteomic Profiling in Hereditary Breast Cancer [P124]

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Breast cancer (BC) is a leading cause of death among women. Among the major risk factors, an important role is played by familial history of BC. Germ-line mutations in BRCA1/2 genes account for most of the hereditary breast and/or ovarian cancers. Gene expression profiling studies have disclosed a specific molecular signature for BRCA1/2-related breast tumors as compared to sporadic cases.

Many diseases are correlated with quantitative changes of proteins in body fluids. Plasma potentially carries important information whose knowledge could help to improve early disease detection, prognosis, and response to therapeutic treatments.

In this study, cleavable Isotope-Coded Affinity Tag reagent (cICAT) coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to investigate tumor-specific changes in the plasma proteome of BC patients and healthy family members sharing the same BRCA1 gene founder mutation (5083del19), previously reported by our group, with the aim to identify potentially specific biomarkers.

The characterization and the analysis of reliable plasma disease biomarkers is challenging because of the high presence of proteins such as albumin, immunoglobulins, transferrin, lipoproteins, which constitute ~ 90% of the protein content of serum. These high abundance proteins can interfere with proteomics investigation of signalling proteins. The reduction of sample complexity is thus an essential step in the analysis of the plasma proteome. To this end, we have used an immunoaffinity resin to simultaneously remove Albumin, IgG, Transferrin, Haptaglobin, α1-Antitrypsin, α2-Macroglobulin, IgA, IgM, α1- acid Glycoprotein, Apoliprotein A-I, Apoliprotein A-IV and Fibrinogen II from plasma. 1D-PAGE analysis was performed to evaluate an enrichment of the low abundant component in the immunoaffinity column flow-through fraction compared to the crude plasma.

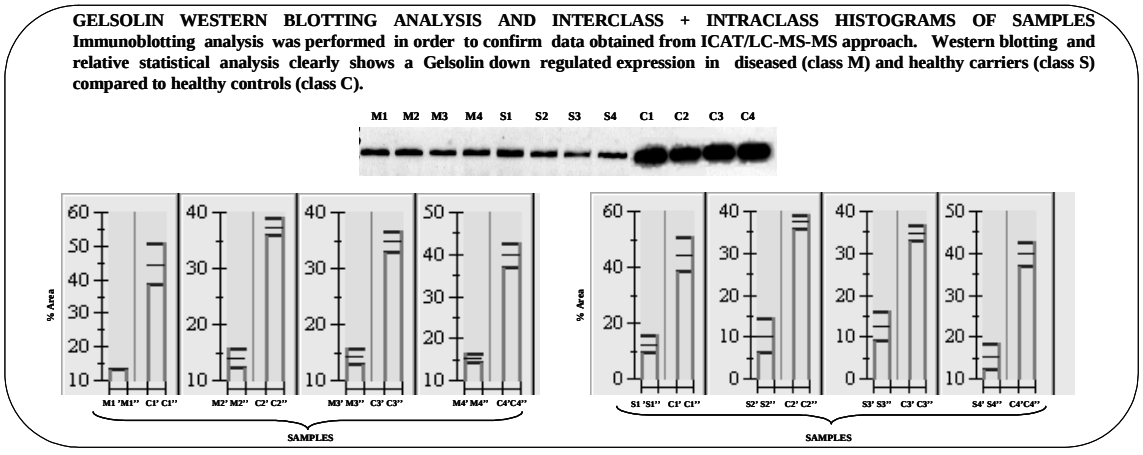
The immunoaffinity column flow-through fraction was subsequently labelled by cICAT and analyzed by LC-MS/MS to produce qualitative and quantitative information of protein expression levels.

Western blotting analysis was performed in order to validate data obtained by ICAT/LC-MS-MS approach.

In this study we compared patients carrying the BRCA1 founder mutation with healthy carriers in order to identify potentially diagnostic biomarkers; an additional analysis on plasma obtained from healthy controls was performed in the attempt to find prognostic markers able to identify, among the healthy carriers, a subset of individuals with high risk of developing BC.

The comparative analysis of the experimental results has led to the identification of interesting BC specific signatures. In the diseased BRCA1 founder mutation carriers compared to healthy controls we found an increased expression of α1 microglobulin, C4 complement factor, α-1B-glycoprotein, and DKFZp686M24218; and a decreased expression of α2-HS-Glycoprotein, Gelsolin, Splicing regulatory protein 129 (SRrp 129) and SERPINC1. On the other side, healthy BRCA1 mutation carriers show decreased levels of a1 microglobulin, C4 complement factor, α-1B-glycoprotein SERPINC1, α2-HS-Glycoprotein compared to healthy people.

These preliminary results are encouraging and can be used in a clinical setting for early diagnosis and follow-up those patients. Moreover, the healthy carrier plasma proteomic analysis might contribute to shed more light on the molecular mechanisms underlying tumorigenesis in a specific (BRCA1-positive) subset of BC patients.



Analysis of differential expression of Notch receptors in gliomas[P125]

DELL'ALBANI P.[1], TRICARICHI E[2]., D'ANTONI S.[1,2], LA CAVA P[2], PELLITTERI R[1], ZAPPIA M[3], PLATANIA N[3], ALBANESE V[1,3], GIUFFRIDA STELLA AM[1,2], ARONICA E[4]. AND CATANIA MV[1]

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Introduction

Gliomas represent the most frequent primary tumours of the CNS. The cascade of molecular events occurring from lower-grade tumors to high-grade ones depends on many different factors such as mutations, amplifications, altered function of molecules both belonging to signal transduction pathways and involved in cellular proliferation and progression (1). Notch proteins are transmembrane receptors widely expressed during embryonic development which play a fundamental role in cell fate decision, proliferation, differentiation and apoptosis. In mammals the Notch family comprises 4 members (Notch1 to Notch4). Recent data indicate that Notch-1 and its ligands are expressed in human gliomas and that Notch-1 down regulation results in inhibition of cell survival and proliferation. In the present study we analyzed the expression of Notch-1, -2, -3 and -4 receptors in four different glioma cell lines (Rat:C6 and 9L and human: U87MG and U373MG) and in primary rat astroglial cell cultures.

Material and Methods

Rat C6 and 9L, human U87-MG and U373-MG glioma cell lines were grown in RPMI 1640 medium plus 10% Fetal Calf Serum (FCS). Primary rat astroglial cell culture were obtained from cerebral hemispheres of newborn rat (2). Notch receptor levels were analyzed by semiquantitative reverse transcription-PCR (RT-PCR) and Western blot analysis. Western blot analysis was performed on total cellular lysates using specific primary antibodies against Notch-1, -2, -3 and -4 receptors and appropriate secondary antibodies linked to horseradish peroxidase (HRP). Immunoreactivity was detected using the Enhanced ChemiLuminescence Method (ECL plus, Amersham Pharmacia Biotech). Autoradiographic signals were evaluated by densitometric analysis. Immunocytochemistry was also carried out on 4% paraformaldehyde fixed cultures with the anti-Notch-1 (Upstate) and anti-Notch-4 (Santa Cruz) antibodies. Signal was detected by using an anti-rabbit-Cy3 secondary antibody and immunofluorescence was analysed by confocal microscopy (LSM 510 Meta, Zeiss). Immunohistochemistry was also carried out on human biopsies. Tumor samples from 18 patients with primary glial tumors [pilocytic astrocytomas, astrocytomas grade II (AstrII), anaplastic astrocytomas (AstrIII) and glioblastoma multiforme(GBM)] who underwent surgical resection were included in the study for immunohistochemical analysis of Notch-1,-4. Control brain tissue (including normal cortex and white matter from the temporal, frontal, and parietal regions) was obtained from 6 age-matched patients who died from a non-neurological disease.

Results

The expression of Notch-1,-2,-3,-4 was studied in four glioma cell lines and primary rat astrocytes at both mRNA and protein levels. RT-PCR and Western blotting analysis of the mRNA and protein obtained from the different cell lines showed differential expression levels of the four Notch receptors. Rat primary astrocytes were characterized by a strong expression of Notch1, but low expression of Notch-2, -3,and -4. Similarly C6 cell line showed a strong expression of Notch1 and low levels of the other Notch receptors. In contrast, 9L, U87-MG and U373-MG were characterized by low levels of Notch-1 and high levels of Notch-2 and Notch-4. Immunohistochemistry for Notch-1, -2, -3, and -4 in primary human glial tumors showed no detectable Notch-1, -2, -3 and -4 labeling in normal white matter. A strong nuclear and cytoplasmic immunoreactivity for Notch-1 was observed in astrocytoma II and in anaplastic astrocytoma, while it was variable and cytoplasmatic in glioblastoma multiform. A weak cytoplasmic immunoreactivity for Notch-2 was observed in AstrII and in AstrIII, while it was stronger and cytoplasmatic in GBM. Notch-3 immunoreactivity was weak and present in cytoplasm in AstrII in AstrIII and in GBM. Notch-4 showed strong nuclear and citoplasmic immunoreactivity in Astro II. The labeling intensity of Notch-4 was increasing from Astro III to GBM

Discussion and Conclusion

Our data show that: 1) Notch-1, -2, -3 -4 showed different mRNA and protein levels expression in the four cell lines analyzed; an expression pattern of strong Notch1and low Notch 2-4 was common to primary astrocytes and C6 cell line, which is the most differentiated among the four glioma cell lines. In contrast, 9L, U87-MG and U373-MG, which are more undifferentiated cells exhibited an expression pattern characterized by low Notch1 and high Notch-2 and Notch-4 expression. -2) Analysis of Notch protein expression in different WHO grade human gliomas suggest that a different pattern of Notch1-4 expression correlate with glioma histological grading. Notch-1 was highly expressed in low grade gliomas to decrease in high grade ones, in contrast Notch-4 was more expressed in grade IV gliomas than in grade II and III ones. It has been reported that Notch-1 and Notch-2 may have opposite effects on cellular proliferation because Notch-1 is expressed in non proliferating cells, while Notch-2 is active in proliferating and differentiating cells (3).

Our data suggest that the differential expression of Notch receptors may play an important role in glioma proliferation and differentiation. A deeper understanding of the different Notch receptors distribution and activity in normal glial cells and gliomas will be important for effective clinical interventions.

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Differential expression of Ceruloplasmin in Amyotrophic Lateral Sclerosis patients [P126]

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Introduction

Amyotrophic Lateral sclerosis (ALS) or MotorNeuron Disease (MND) is a fatal neurodegenerative disease characterized by progressive degeneration of motor neurons in cortex, brainstem and spinal cord.

Actually, the diagnosis in early stages of this pathology has several limitations due to the absence of specific markers.

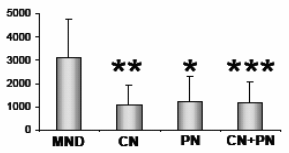
The oxidative stress is one of most important causes of the pathogenesis of ASL, being the condition arising upon imbalance between the production of potentially toxic reactive oxygen species (ROS) and the scavenging activities: these include enzymatic activities (as SOD1), low molecular weight antioxidant species (as vitamin E) plus more complex form of protection such as systems for metal transport. Correct handling of transition metal ions as copper and iron is crucial since these metals are able to undergo alternative redox reaction, during which toxic ROS are generated. One of these "transport system" present in CSF, that seems to be involved in neurodegenerative disorders, is the copper-protein Ceruloplasmin (Cp) that act as ferroxidase oxidizing toxic ferrous iron to non toxic ferric form.

The Cp expressed in the brain likely plays an important role in iron homeostasis and antioxidant defence in the central nervous system (CNS). It is therefore possible that an expression level reduction or alteration/s of Cp may contribute to the neurodegenerative process in ALS by leading to an increase of ferrous iron, which can promote the generation of toxic free radicals.

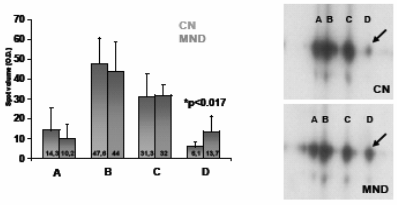
Aim of the Study

We performed a comparative proteomic analysis of Cerebrospinal Fluid (CSF) collected from ALS patients and healthy subjects on Ceruloplasmin expression, in order to investigate different expression level or specific modifications of Cp isoforms occurring in ALS patients as consequence of oxidative stress.

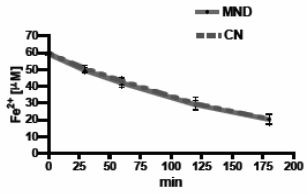
Results



CSF proteins were resolved by SDS-page and Western blot analysis was performed with anti Ceruloplasmin antibody. The total amount of Cp in MND (n= 12), CN (n= 9) and in patients with peripheral neuropathy (PN n= 7) used as further control of neuronal un-related pathology, was evaluated by densitometric analysis.
Data show an increased level of Cp expression in MND versus CN and PN.



On 2D-maps anti-Cp antibody recognizes a group of 4 spots representing different isoforms. In the two different groups (MND and CN) the relative abundance of each isoform has been evaluated.
Among the four A,B,C,D, isoforms, spot D showed an increase (P<0.017) in the MND patients.



The Bathophenanthroline assay was performed to analyze the Ferroxidase activity in CSF, mainly catalyzed by Cp.
No difference between CSF of MND patients (n=6) and CN group (n=6) was found.

Conclusion

The results obtained from Ceruloplasmin analysis indicate the relative abundance of Cp spot D as a possible candidate for ALS marker, in particular because MND patients in this study were at the pathology onset and drug free. Interestingly, the increased level of Cp expression not supported by an increased ferroxidase activity suggests a reduction of Cp functionality that may be associated with biochemical modification/s. Which are the features characterizing the different isoforms of Cp are under investigation.

Human SOCS1 complexes involved in psoriatic pathways [P127]

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Psoriasis is a chronic inflammatory dermatosis characterized by hyperproliferative keratinocytes. The skin lesions are infiltrated by T cells, which secrete gamma interferon (IFN- ã) and are belived to be necessary to maintain the psoriatic phenotype (1). In normal keratinocytes INFgamma is a potent inhibitory cytokine of proliferation. Cytokines comprise a large family of secreted glycoproteins that regulate fundamental biological processes, including immunity and haematopoiesis. Cytokines relay biological information to target cells by binding to receptors on the cell surface. Although cytokine receptors lack intrinsic kinase activity, they are often associated with members of the Janus Kinase (JAK) family of tyrosine kinase (2). SOCS1 belongs to the suppressors of cytokine signalling family comprising at least eight members, SOCS1 to SOCS 7 and CIS. SOCS1 contains a N-Terminal kinase inhibitory region (KIR) costituited of 12 amino acids, a central SH2 domain and a conserved C-terminal motif referred to as the SOCS box (3). SOCS1, protein is undetectable in healthy skin and highly expressed in the epidermis of psoriasis. SOCS1 overexpression in keratinocyte stable clones inhibits IFN-ã-induced phosphorylation of IFN-ã receptor (IFN- ãRá) and activation of STAT1 and STAT3. SOCS1-expressing keratinocytes are resistant to IFN-ã-mediated growth inhibition. Targeting keratinocyte SOCS1 may represent a novel therapeutic approach to IFN-ã-dependent skin diseases (4).

In order to identify the network of proteins involved in IFN-ã- dependent inflammatory responses, induced by SOCS1 overexpression, immunoprecipitation experiments using Myc-tagged SOCS1 will be performed in keratinocytes cell line. The immunoprecipitated complex will be fractionated by SDS-PAGE and individual protein bands will be identified by mass spectrometry. Proteins present in immunoprecipitated and absent in the control will be attributed to SOCS1 partners.

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Sulfatase modifying factor 1 (SUMF1) trafficking through the cells: from the endoplasmic reticulum (ER) to ER [P128]

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The sulfatases are a family of enzymes that catalyze the hydrolysis of sulfate esters (1) after they have been post-translationally activated. A consensus sequence in their catalytic domain contains a cysteine residue that is modified into formylglycine (FGly) within the endoplasmic reticulum (ER). This FGly modification is essential for sulfatase activity (2). The gene that encodes the FGly-generating enzyme is sulfatase modifying factor 1 (*SUMF1)* (3). In patients affected by Multiple Sulfatase Deficiency (MSD), all of their sulfatase activities are reduced because SUMF1 is hampered in its function, and mutations in *SUMF1* have been found in all MSD patients analyzed to date (4). SUMF1 is a glycosylated enzyme and it is an ER-resident protein when it result glycosilated by high mannose moieties. SUMF1 has been shown to be secreted into the cell medium when over-expressed in HT1080 cells, and this secreted form contains a family of complex-type oligosaccharides (5). Endogenous SUMF1 is also secreted. Furthermore both overexpressed and endogenous SUMF1 can be taken up from the medium by different cells and from the plasma in tissues of mice engineered to produce SUMF1 from the liver. Surprisingly, following its uptake by cells, SUMF1 relocates from the plasma membrane to the endoplasmic reticulum. Remarkably, SUMF1 taken-up, once re-localized into the ER, is still active, enhancing the sulfatase activities in both cultured cells and mice tissues. However, the molecular pathways by which SUMF1 is secreted by the cells or is uptaken from the medium are still largely unknown. A functional proteomic approach was exploited with the aims to elucidate the SUMF1 secretion and uptake mechanisms at the molecular level. SUMF1 interacting proteins were isolated by immunoprecipitation approach and separated by SDS-PAGE. The excised protein bands are still under investigation.

Preliminary results suggests that SUMF1 is present in different isoforms when it is secreted in comparison to the intracellular.

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An Insight into DGK-á biological function [P129]

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Diacylglycerol kinases (DGK), which phosphorylate diacylglycerol to generate phosphatidic acid, act as either positive or negative key regulators of cell signalling. Mammalian DGK is known to exist as a large protein family consisting of ten isoforms classified into five subtypes according to their structural features. These subfamilies can be characterized by the presence of a variety of regulatory domains of known and/or predicted functions, clearly indicating their distinct functions and regulatory mechanisms. α-DGK is abundant in the cytosol of T lymphocytes, but it is also expressed in endothelial and epithelial cells, fibroblasts, and oligodendrocytes (1).

Translocation in response to receptor stimulation is rapid, transient, and requires calcium and tyrosine kinase activation. á-DGK-mediated phosphatidic acid generation allows dissociation of the enzyme from the plasma membrane and return to the cytosol (2). We have previously shown that tyrosine phosphorylation by Src mediates growth factorsinduced activation of α-DGK, whose activity is required for cell motility, proliferation and angiogenesis (3). In order to identify the role of α-DGK in cell signaling, immunoprecipitation experiments using myc-tagged α-DGK were performed in SLK cell line. Following pervanadate stimulation, to enrich the protein extract in tyrosine phosphorylated components, the immunoprecipitated complex was fractionated by SDS-PAGE. Individual protein bands were excised and enzymatically hydrolysed in situ and the resulting peptide mixtures analysed by LC-MSMS mass spectrometry. A number of proteins present in the complex and absent in the control were identified and classified according to their functional role.

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Different regulation of Fibrinopeptide A fragments between controls and diabetics with and without nephropathy [P130]

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Type I diabetes (insulin-dependent diabetes mellitus, IDDM) is an autoimmune disease which affect about 6% of the world's population [1]. Diabetic nephropathy (DNP), known to account for nearly 50% of all new cases of end stage renal disease [2], is the major long-term complication of both types of diabetes and retains the highest human, social and economic costs. Thus identifying markers for its early detection is one of major goals in diabetes research.

This study is focused to identify possible candidate biomarkers based on proteomic analysis of serum, plasma and urine of controls (n = 9), IDDM patients (n = 10) and DNP patients (n = 4) by ClinProt technique, an emerging profiling technology based on mass spectrometry. ClinProt allows the selective enrichment of peptides and proteins from biological fluids with functionalized magnetic beads [3]; peptides isolated by the ClinProt technique can be then analyzed by MALDI-ToF MS to evaluate the peptidome/ proteome profile in controls and patients. In a preliminary study we perform purification of serum, plasma and urine using C8 coated magnetic particles, which mainly enrich proteins in range of 1-10 KDa. Statistical analysis with ClinProTools 2.1 software showed several ions differently regulated in serum samples among the three classes but no markers were identified in plasma and urine, probably due to the small number of analysed samples. Nevertheless different patterns of peptides could be selected among three biological fluids by using "Support Vector Machine" and "Genetic" algorithms supplied by the software, which are able to separate the three populations with high sensibility and specificity values (fig.1). Moreover through LC-ESI MS/MS we characterized three fragments of Fibrinopeptide A differently regulated between serum samples of controls and IDDM (fig. 2). Two of these peptides, with mass of 1019.50 and 1205.57 respectively, are down regulated in IDDM while the complete Fibrinopeptide A with mass of 1535.69 seems enhanced. Increasing level of Fib A in serum are in good agreement with results reported in literature for both type of diabetes associated with stroke, cerebrovascular disease, retinopathy and also with diabetic nephropathy[4-7] due to intrarenal coagulation; however down regulation of its fragments is not described. These data suggest a different catabolic pathway of Fibrinopeptide A in diabetes. Moreover these preliminary results show the possibility to identify pattern of peptides allowing to discriminate the three populations and to provide novel information about development of diabetic nephropathy.

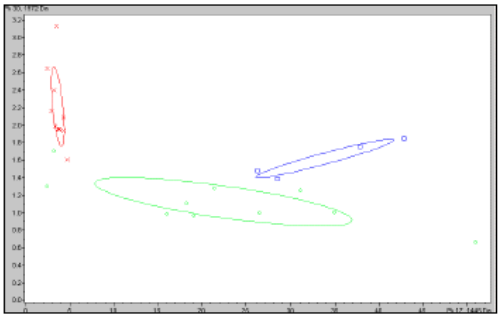

Figure 1. Separation of three clusters using two statistically different peaks. Crosses: controls; circles: IDDM; squares: DNP.

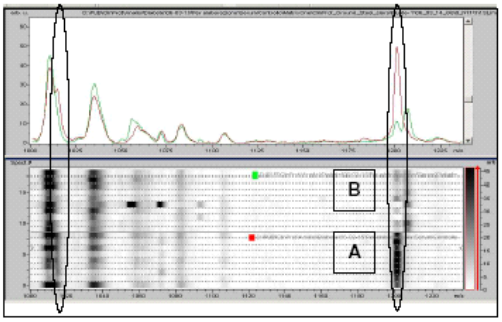

Figure 2. Fibrinopeptide A fragments down regulated in diabetics. Panel A: pseudogel view of controls; panel B: pseudogel view of IDDM.

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