

ABSTRACTS OF THE SECOND INTERNATIONAL CONFERENCE OF THE HELLENIC PROTEOMICS SOCIETY

From Discovery to Application

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SHERLOCK HOLMES AND THE PROTEOME: A DETECTIVE STORY

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'Detective' originates from the Latin *detego* (*detexi*, *detectum*, *detegere*), i.e. to find out, to discover (in fact, to remove the *teges* or *tegmen*, in English slang the cover, therefore to uncover!). Modern proteome analysis is a very complex 'detective story' which might baffle even the most famous investigator, Sherlock Holmes. The reason for this is that in any proteome a few proteins dominate the landscape and often obliterate the signal of the rare ones, so that when the police reach the scene of the crime, the thin thread of evidence remains hidden to the eye. Thus, most scientists lament that in proteome analysis the same set of abundant proteins is seen again and again. A host of pre-fractionation techniques has been described, but all of them, one way or another, are beset by problems in that they are based on a depletion principle, i.e. the removal of unwanted species, often *via* immuno-subtraction (e.g. in sera, by using a set of 6 to 12 antibodies against the most abundant species). It turns out that parasitic co-depletion removes thousands of low-abundance proteins, nullifying any attempt at bringing 'unseen' proteome into the limelight. A revolutionary approach is that of Protein Equalizer Technology, a method enabling the capture of all species present in a proteome, but at much reduced protein concentrations. This comprises a diverse library of combinatorial ligands coupled to spherical porous beads. Considering that 20 different amino acids are used for the synthesis, this means that the library contains a population of linear hexapeptides amounting to 20⁶, i.e. 64 million, different ligands. Such a vastly heterogeneous population of baits means in principle, that an appropriate volume could contain a partner able to interact with just about any protein present in a complex proteome. When these beads come into contact with proteomes, e.g. human urine and sera, egg white or any cell lysate for that matter, of widely differing protein composition and relative abundances, they are able to "equalize" the protein population by sharply reducing the concentration of the most abundant components while simultaneously enhancing the concentration of the most dilute ones. It is felt that this novel method may enable research to take a great step forward in bringing the unseen proteome within the detection capabilities of current proteomics

detection methods. Examples are given of equalization of human urine and sera samples, resulting in the discovery of a host of proteins never reported before. Additionally, these beads can be used to remove host cell proteins from purified recombinant proteins or protein purified from natural sources that are intended for human consumption. These proteins typically reach purities of the order of 98%: higher purities often being prohibitively expensive. Yet, if incubated with these equalizer beads, these last impurities can be effectively removed at a low cost and with minute losses of the valuable main product.

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NITROPROTEOMICS OF THE HUMAN BRAIN

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The nitric oxide-mediated Tyr-nitration of endogenous proteins is associated with several pathological and physiological processes. In order to investigate the

presence and potential roles of Tyr-nitration in the human pituitary and in the formation of pituitary adenomas, 2-dimensional gel electrophoresis (2DGE) and a Western blot against a specific anti-3-nitrotyrosine antibody were used to detect nitroproteins from a human pituitary. The nitroproteins were subjected to in-gel trypsin digestion, and high-sensitivity vMALDI LTQ MS was used to analyze the tryptic peptides. MS/MS data was used to determine the amino acid sequence and the specific nitration site of each tryptic nitropeptide, and were matched to corresponding proteins with Bioworks TuboSEQUENT software. Compared to our previous LCQ^{Deca} study, 16 new nitrotyrosine-immunoreactive positive Western blot spots were found. These data demonstrate that nitric oxide-mediated Tyr-nitration might participate in various biochemical, metabolic and pathological processes in the human pituitary.

We also characterized several endogenous nitroproteins and nitroprotein-protein complexes in a human pituitary nonfunctional adenoma. A nitrotyrosine affinity column (NTAC) was used to preferentially enrich and isolate endogenous nitroproteins and nitroprotein-protein complexes from a tissue homogenate of an adenoma. The endogenous nitroproteins and nitroprotein-protein complexes were subjected to trypsin digestion, desalting and MS/MS analysis. We discovered nine nitroproteins in addition to three proteins in nitroprotein-protein complexes. The nitration site of each nitroprotein was located on the functional domain where nitration occurred, and each nitroprotein was related to a corresponding functional system. These data indicate that protein nitration might be an important molecular event in the formation of a human pituitary non-functional adenoma.

These nitroprotein studies were a part of our long-range studies in which we hypothesize that the proteome differs between human pituitary controls and macroadenomas.

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3 ENDOTOXIN-INDUCED LIVER FAILURE: MORE DAMAGE TO THE ENDOPLASMIC RETICULUM THAN TO MITOCHONDRIA?

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In the present study, protein distribution in subcellular compartments was investigated in a rat model of endotoxic shock. Rats were challenged with lipopolysaccharide (LPS) (at approx. LD₅₀ dose); after 16 hours, blood, liver, microsomal and mitochondrial fractions of liver were collected and analyzed. We observed damage to liver cells resulting in focal necroses and elevated alanine-aminotransferase levels in blood. Simultaneously, we observed a drastic decrease in monooxygenase activity of P450 indicating damage to the endoplasmic reticulum (ER), while the respiratory activity of mitochondria was even better than in controls.

A proteomic approach using 2-dimensional differential gel electrophoresis (2D-DIGE) technology with minimal labelling with CyDyes revealed considerable protein pattern changes in the ER proteome due to LPS challenge. About 16% of the detected spots changed by a factor of 2 at least (in either direction) and 3% by factor of 3 or more. The spots with highest differential regulation were identified by nLC-MS/MS. Endotoxin induced down-regulation of functional proteins involved in protein folding (protein disulfide isomerase A3, a 78 kDa glucose regulated protein) and transport (transitional ER ATPase). Several other differentially regulated proteins were related to oxidative stress (peroxiredoxin-1 and heme oxygenase). Peroxiredoxin-1 was found in several isoforms whose levels changed upon treatment, as confirmed by Western blotting.

Fewer changes were noticed in mitochondria obtained from the same animals. The most marked changes in their protein patterns could be traced back to breakdown of carbamoyl phosphate synthase, a mitochondrial protein providing carbamoyl phosphate to the urea cycle. Another component of the urea cycle, argininosuccinate synthase, was found to be down-regulated in ER. A previous study with highly purified mitochondria has shown even fewer

variations in mitochondrial protein patterns. The main changes were a significant up-regulation of two proteins, mitochondrial superoxide dismutase (SOD) and ATP-synthase α -chain.

In addition, data from transmission electron microscopy examination of livers revealed that damage in the ER is more pronounced than in the mitochondria, despite the fact that levels of reactive oxygen species (ROS) in mitochondria were high (as determined by electron spin resonance spectroscopy).

Our data suggest that mitochondria are protected against oxidative stress, at least in part, by mitochondrial SOD. Thus, the damage to endoplasmic reticula by oxidative stress might mainly be responsible for liver dysfunction induced by endotoxin.

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A LARGE-SCALE PROTEOMIC ANALYSIS OF HUMAN URINARY EXOSOMES

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We are developing techniques for the detection of disease biomarker proteins in urine. Urinary exosomes are the internal vesicles of multivesicular bodies (<100 nm diameter membrane vesicles) that are secreted into the urine from every cell type that is in contact with the urinary space, including renal tubule cells, podocytes and transitional epithelial cells from the renal drainage system. Exosomes can readily be isolated and purified by differential centrifugation, after taking steps to remove Tamm-Horsfall protein complexes through the use of reducing agents. Because exosomal proteins represent only a small subfraction of urinary proteins, exosome isolation can potentially result in the marked enrichment of biomarkers of a subset of diseases involving renal epithelial cells. The purpose of this study is to extend the known proteome of urinary exosomes from normal humans, through the use of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Our previous study of normal human urine identified 304 proteins (1). Here we used a more sensitive mass analyzer (Thermo-Finnegan LTQ), identifying 1,064 proteins despite stringent filtering of spectra to avoid false positives. Specifically, target-decoy analysis was used to adjust filters to a predicted

false-positive identification rate of 1%. Over 30 proteins identified in this study, including many transporters, channels, ion pumps and trafficking proteins, are already known to be associated with specific disease entities. We conclude that urinary exosomes contain an extensive repertoire of proteins including many potential biomarkers. The challenge is to determine the range of variation of the exosomal proteome among normal humans on the basis of factors such as gender, age, diet, and physical activity.

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PROTEOMICS AND SYSTEMS BIOLOGY: THE MODEL ORGANISM HALOBACTERIUM SALINARUM

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The halophilic archaeon *Halobacterium salinarum* is a specialist in adaptation to extreme environments, thriving optimally in 4 M sodium chloride in marine salterns and hypersaline lakes. It has become a focus of intensive research because of its unique bioenergetics and signal transduction network which are attractive subjects for a systems biological approach aimed at modelling parts of a cell.

Proteomics is one of the key approaches for the collection of comprehensive information at the protein level in order to feed the study of systems biology which seeks to integrate experimental and modelling approaches to study biological processes at different levels of complexity. In our department, inventory and quantitative proteomics are combined with guided proteomic approaches for the study of protein maturation, post-translational modifications, and protein-protein interactions in order to answer specific biological questions.

Genome-wide proteomic analysis on *H. salinarum* led to the identification of 69% of its theoretical proteome (1-5). Several methods of quantitative proteomics (SILAC, ICPL, DIGE) were used for the study of the bioenergetics and signal transduction networks of *H. salinarum* under different growth conditions, *i.e.* phototrophic, anaerobic (*via* the TCA cycle) and arginine fermentation. A high number of membrane and cytosolic proteins were found to be differentially regulated under different growth conditions highlighting the ways that the organism uses to adapt to different environmental conditions (6). A large-scale proteomic survey of *N*-terminal protein maturation on archaea revealed a distinct archaeal *N*-terminal acetylation

pattern affecting about 15% of the proteins in Hbt. Salinarum (3, 4). This represents another example where archaeal molecular biology appears to be closer to eukaryotic than bacterial. Study of the post-translational modifications on *H. salinarum*'s proteins and protein-protein interactions is in progress using a combination of shotgun proteomic approaches and specific enrichment protocols. Our emphasis is on the study of protein phosphorylation and methylation on *H. salinarum*.

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6 PROTEOMIC PROFILING OF URINE FOR THE DETECTION OF BLADDER CANCER BIOMARKERS

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Urine is considered a gold mine for biomarker discovery especially for diseases of the urinary tract. Bladder cancer is the second most common type of malignancy of the urinary tract, but is first in mortality. The identification of novel and highly accurate diagnostic and prognostic biomarkers for the disease is a vigorous research field. The

objective of our study is the exploration of the urinary proteome by the use of comprehensive proteomic methodologies so as to unravel novel biomarkers for bladder cancer. Our strategy specifically involves careful collection of random catch or 24 h urine samples from patients with low-grade non-invasive and high-grade invasive bladder cancer, or patients with benign diseases of the urogenital tract. Random catch samples are pooled so as to screen high numbers of samples from each category (>50 each), while the 24 h collected urines are analyzed individually. Our experimental approach involves the application of two-dimensional electrophoresis (2DE) in combination with MALDI-TOF-MS (in case of random catch-pooled samples), or preparative electrophoresis (prep-cell) followed by 2DE and liquid chromatography in combination with mass spectroscopy (in the case of 24-h collected samples). Comparison of the 2DE images corresponding to the pooled samples indicated several differences between the different sample categories including members of the S-100 protein family. Confirmation of these results is underway by the use of western blot analysis. A parallel analysis of 24-h collected urines from patients with invasive disease showed a significant enrichment for low abundance and low molecular weight proteins following sample condensation through membrane filtration and analysis of the prep-cell fractions by 2DE-MALDI-TOF, or in solution trypsinization followed by LC-tandem MS identification. Comparative analysis of additional 24-h collected samples is underway to investigate differences between the different sample categories.

7 PROTEOMICS FOR DISCOVERY AND CLINICAL APPLICATION OF BIOMARKERS OF HEPATOCELLULAR CARCINOMA WITH HEPATITIS C VIRUS INFECTION

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Background: Chronic infection of hepatitis C virus (HCV) is one of the most clearly established risk factors for hepatocellular carcinoma (HCC). Proteomic profiling of HCV-related HCC has been performed to find tumor-associated proteins such as heat shock 70 kDa protein (HSP70) family (1, 2). On the other hand autoantibodies against tumor-associated proteins have been detected in various types of cancer and may be useful for cancer screening, specific diagnosis, determining prognosis, and as potential targets for immunotherapy (3). To detect

autoantibodies which could be diagnostic markers for HCC we performed proteome profiling using the PROTEOMEX technique (4). *Materials and Methods:* Fifteen pairs of tumor tissues and corresponding non-tumor liver tissues were obtained from HCC patients who underwent surgical operation at the Department of Surgery II, Yamaguchi University Hospital with approval of the Institutional Review Board for Human Use of the Yamaguchi University School of Medicine. Normal liver tissues were obtained from five patients without infection with HBV or HCV who underwent hepatic resection for benign liver tumor or metastatic liver tumor due to gastrointestinal cancer. Thirty-five serum samples were used for proteome profiling using the PROTEOMEX technique. Fifteen were autologous sera obtained from each patient whose tumor tissue was analyzed in this study, and twenty were obtained from normal subjects who were negative for both HB antigens and HCV antibody for control. The proteome profiling was performed by two-dimensional gel electrophoresis, immunoblotting with sera, reprobing, and LC-MS/MS. Immunoreactivity of autoantibodies in sera was analyzed by immunoblotting using SDS-PAGE with purified tumor-associated proteins semi-quantitatively. *Results:* In HCC serum samples, the frequency of occurrence of immunoreactivity to the specific four spots, which were identified to be HSP70, glyceraldehyde 3-phosphate dehydrogenase, peroxiredoxin, and manganese superoxide dismutase (Mn-SOD) by LC-MS/MS, was 7/15 (46.7%), 5/15 (33.3%), 5/15 (33.3%), and 6/15 (40.0%), respectively. The immunoblot analysis using commercially available purified proteins showed that the immunoreactivity to each of the proteins was clearly detected in HCC sera, whereas scarcely so in control sera. *Discussion:* In this study, we detected autoantibodies which are specific for HSP70, peroxiredoxin, and Mn-SOD in HCC patients. The raising of autoantibodies against these three proteins has not been reported in any human malignant disease. These three proteins increased in HCC tissues from patients and may be involved in the pathogenesis of HCC. The autoantibodies were predominantly raised in HCC patient sera and were detected by a highly sensitive chemiluminescence reaction, whereas the proteins themselves were not detected in sera. In this study, there was no significant association between the autoantibodies and clinical characteristics of HCC patients. Further analysis with a large number of samples is needed to elucidate the association with clinical characteristics. We have established a novel technique for protein immobilization on a diamond-like coated (DLC) chip substrate (5), which is capable of detecting antibodies against proteins immobilized on the chip in the femtomole range. The combination of the PROTEOMEX technique and a high through-put technique with protein chips may provide a reliable diagnostic system in the near future.

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8 FROM PROTEOMICS BIOMARKER DISCOVERY TO VALIDATION USING MULTIPLEX PROTEIN ASSAYS

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Biomarkers play an important role in the pharmaceutical industry and are assuming an ever greater role in drug discovery and development. Discovery of candidate marker proteins is achieved by high throughput proteomics methods, but the major bottleneck is the validation of these candidates. The potential benefit of biomarkers is to allow earlier, more robust drug safety and efficacy measurements. Generally, a better understanding of the mechanism of disease progression and therapeutic intervention is needed. The major challenge is the selection and validation of biomarkers including clinical end-point validation. This requires extended clinical studies to compare the new biomarkers with clinical end-points determined by other means.

Multiplex assays such as the IMPACT chips are used for the validation of the protein biomarkers coming from the discovery programs. Biomarkers are playing an important role in personalized medicine and several examples in this area will be shown.

9 HIGH PERFORMANCE PROTEOMICS AS A TOOL IN BIOMARKER DISCOVERY

Helmut E. Meyer, Barbara Sitek, Kathy Pfeiffer, Bence Sipos, Ibrahim Alkatout, Sabine Burkert, Birgit Streletzki, Günter Klöppel, Christian Mölleken, Stephan Hahn, Wolfgang Schmiegel and Kai Stühler

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For about 10 years the word proteome catches the attention of more and more researchers in the life sciences field. Over the same period, the term 'high throughput proteome analysis' arose, conveying the intention to analyse all proteins in a complex protein mixture in parallel. Thus, a huge amount of data is produced from a single sample and the subsequent analysis and validation becomes the time-limiting step.

However, the limited number of available biomarkers for diagnosis, status of the disease, therapy control and prediction of the course of the disease demands new efforts of finding new biomarkers. In particular, proteomics raises high expectations in finding new and reliable biomarkers for human diseases.

By applying microdissection combined with saturation differential gel electrophoresis (DIGE) technology and mass spectrometry we succeeded in finding new biomarker candidates for pancreatic carcinoma including samples of six tumour progression stages and liver cirrhosis. This technology allows us to quantitatively analyze the proteome of just 1000 cells from individual patient samples (4 to 9 samples) and to get reproducible proteomics data pointing to about 30 new biomarker candidate proteins for either disease. Validation of these candidate biomarkers is still in progress and quantitative PCR and immunohistochemistry are employed for this purpose. So far, most of the candidate biomarkers tested positive in the further validation steps.

Thus, high performance proteomics is the basic principle for reliable results which allows us to discover new biomarker candidates for pancreatic cancer and liver cirrhosis using minute amounts of patient material. How to reach this goal will be presented in the lecture.

10 QUANTITATIVE PROTEOMICS APPLIED TO BIOMARKER DISCOVERY AND EVALUATION

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In spite of recent advances in technology, plasma proteome analysis remains a very challenging task because of the wide dynamic range of protein expression levels, the sample complexity due to a large number of proteins, and the structural diversity, exemplified by splice variants, mutations, and post-translational modifications (*e.g.* phosphorylation, glycosylation). Three main steps constitute a proteomic analytical workflow: sample preparation, mass spectrometry analysis (both qualitative and quantitative) and the data analysis.

Effective methods are essential to reduce sample complexity (at the protein and/or peptide levels) on one hand, and to enrich for low abundant components on the other. Affinity mediated and solid phase capture methods are valuable means of sample fractionation and enrichment for specific classes of compounds. The *N*-linked glycopeptides were targeted to reduce sample complexity to a level that enabled LC/MS(/MS) analysis in one single run.

Mass spectrometry is pivotal in the workflow, and the latest developments in instrumentation, including higher resolution,

better mass accuracy and increased sensitivity, have brought lower limits of detection and extended the dynamic range while increasing the quality of measurements and confidence in the results. Last but not least, the large volume of data generated in proteomics studies requires sophisticated bioinformatics tools to process and analyze the data, organize the results, and link various analytical platforms.

Our biomarker discovery and evaluation approach includes two stages. The first one focuses on the in-depth identification of peptides in a relative low number of samples (including tissues or proximal fluids). For this task, the analyses were performed on a high performance instrument (FTMS), with emphasis on detecting and sequencing peptides of lower abundance proteins. High quality measurements facilitate the identification and increase the level of confidence.

In a second stage, quantification of a limited number of candidates (discriminating peptides) was carried out on a larger sample set in order to confirm the differences in the level of expression of proteins. The quantitative analyses were performed in MS/MS mode to obtain more precise results and increase selectivity through isolation of one single precursor ion. Analyses were carried out on a quadrupole – linear ion-trap instrument, operated in multiple reaction monitoring mode (MRM), allowing detection and identification of low abundant components with very high sensitivity. The development of an instrument control software exploiting retention times and signal intensities as parameters to trigger measurements enables analysis of a number of peptides in one single LC/MS without compromising on sensitivity.

Furthermore, the use of stable isotope labeling based on tandem mass tags enabled multiplexed analysis of samples and increased throughput, as several samples were analyzed concurrently in one single run. Proof-of-principle was carried out on plasma samples using the 8-plex iTRAQ reagent.

In summary, the combination of an effective sample preparation method, isotope labeling technique and state-of-the art instrumentation results in a platform capable of analyzing large peptide sets in high throughput with increased sensitivity and selectivity.

11 LINKING PROTEOMICS TO SIGNALLING PATHWAYS AND DISEASES

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Molecular signalling pathways are frequently triggered by extracellular molecules binding receptors and activating relay systems inside cells, leading to processes that affects cellular

behaviour and fate. For many genetic disorders, a link between disease and signalling pathways have been established; consequently a systematic analysis of dynamic cellular networks provides an opportunity for pharmaceutical discovery by taking into consideration the complex biological context of drug targets, rather than observing the targets in isolation. Such analyses are, perhaps, ideally suited for a systems biology approach that integrates experimental data with computational modeling with the aims of discovering and validating new drug targets and biomarkers, as well as predicting potential 'off target' effects of drug candidates. Informing, calibrating and validating mathematical models with experimental data is a key component of an applied systems biology investigation and a number of genomic and proteomic techniques can be employed to generate these crucial data sets. These techniques range from LC-MS/MS based discovery of phosphopeptides to genome-wide cDNA and RNAi functional screens.

Recently, we implemented and optimized a proteomics platform based on 'reverse' protein arrays (RPA) that is particularly suitable to monitoring cell signalling events. These arrays are based on the principle that complex protein mixtures or proteomes (such as cell or tissue lysates) are spotted in an array format and probed with selected fluorescent antibodies in a multiplexed manner. To ensure high levels of sensitivity and signal-to-noise ratio of these RPAs, we use planar waveguide technology. The advantage of the evanescent field fluorescence detection ensures that only analyte-bound fluorescent antibodies contribute to the signal. This method makes it feasible to obtain quantitative and kinetic protein expression profiles and signaling information in a wide variety of biospecimens.

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CLINICAL DIAGNOSIS AND THERAPY EVALUATION BASED ON PROTEOME ANALYSIS UTILIZING CAPILLARY ELECTROPHORESIS COUPLED MASS SPECTROMETRY

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All organisms contain thousands of proteins and peptides in their body fluids. A deeper insight into the distribution and the functional relevance of these polypeptides under different physiological and pathophysiological conditions and the resulting discovery of specific biomarkers would greatly enhance both diagnosis and therapy of specific diseases. Proteomic methods can provide means to accomplish this grand medical vision.

We have developed and evaluated a proteome analysis-based approach for clinical applications, such as disease

diagnosis and assessment of response to therapy (1). The stable, robust and reproducible technology, consisting of capillary electrophoresis coupled to time-of-flight mass spectrometers (CE-TOF-MS) and software solutions necessary to evaluate the vast amount of information enable highly reproducible analysis of 1000-5000 polypeptides in one sample within 45-60 min, which hence is applicable as a routine clinical procedure (2).

Urine was chosen as the body fluid of main interest, since it is available in large quantities and should display the health status of the renal, urogenital and vascular systems. However, data on plasma, cerebrospinal fluid and bronchial lavage have also been successfully generated (3, 4). Currently, more than 5000 urine samples from healthy volunteers and patients with a variety of diseases have been analyzed. To obtain highest accuracy, data from FT instruments and from different sequencing approaches were combined with the routine CE-TOF-MS analyses to result in a high resolution 'human urinary proteome map' (5).

We have initially focused on the non-invasive detection of renal diseases, bladder and prostate cancer and arteriosclerosis, monitoring of transplants as well as monitoring of the responses to therapeutic interventions (6-10).

The resulting data indicated that an 'ideal biomarker', a single polypeptide that allows definition of a disease with high accuracy, does not exist. However, a panel of biomarkers with very good discriminatory features can be combined in a specific model in a support-vector-machine based approach. These models are based on 20 to 50 defined and partially sequenced peptides and proteins, and generally show above 90% accuracy in the training set. In addition, the models, due to their high dimensionality, enable assessment of disease progression and display the success of therapeutic intervention (9, 10).

Any approach based on thousands of parameters is at risk of being memorized, hence the diagnostic patterns were validated in several clinical studies involving the blinded assessment of hundreds of samples, in general with 80-100% specificity and sensitivity (7-9).

An additional and essential strength of the technology and the database of thousands of comparable CE-TOF-MS data is the ability to define and validate such diagnostic patterns based not only on cases and controls, but also on an array of samples from patients with similar diseases, enabling the discovery and validation of truly disease-specific biomarkers (8).

CE-MS in combination with the established human urine proteome database today already facilitates the non-invasive and unbiased assessment of several renal, urogenital diseases with high confidence, and hence should certainly be applied before any invasive procedures such as biopsies, which carry a high risk for the patient, are undertaken.

Furthermore, this technology permits the definition of surrogate markers and end-points in (pre)clinical trials, and hence allows evaluation of therapeutic strategies/new drugs on a small number of patients or even animal models at early time points.

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13

ROLE OF CALRETICULIN IN HUMAN FIBROSIS

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Fibrosis is defined as the accumulation of extracellular matrix, leading to structural and functional alterations of several organs. Extracellular matrix overproduction and/or decreased degradation may be assigned to cells of variable origin. Some of them are derived from epithelial cells undergoing epithelial to mesenchymal transition. Fibrosis may be organ-specific and stimulus-specific.

We have used the model of Unilateral Ureteric Obstruction, a well established model for pressure-mediated renal fibrosis. Wistar rats were either sham-operated or ligated and were sacrificed at 2 or 8 days after right ureteric ligation. The development of fibrosis was evaluated by light microscopy using Sirius Red staining. Cortical material was excised and used for proteomic analysis. 2D gels were stained with colloidal Coomassie Brilliant Blue. Gels were scanned and spots that demonstrated quantitative and/or qualitative differences were detected, excised and destained. Following destaining the spots were trypsinised. Tryptic digests were extracted and identified with MALDI-TOF-TOF MS. A total of 264 spots demonstrating differential expression were identified and over 77% of them were characterized. Comparisons were performed between sham-operated and ligated at 8 days, sham-operated and ligated at 2 days, and ligated at 2 *versus* 8 days. Few proteins demonstrated differential expression in all three comparisons. Among the proteins

that were consistently up-regulated during the development of fibrosis was calreticulin.

In order to verify this differential expression of calreticulin, kidney extracts from all groups were analyzed by western blot. Quantification of these results showed that calreticulin is practically undetectable by western blot in sham-operated animals for both time intervals, whereas it is expressed 2 days after ligation and even more at 8 days after ligation. Immunohistochemical analysis further supported these results and showed that calreticulin expression is up-regulated in tubular epithelial cells.

This is the first report demonstrating a correlation between early stages of renal fibrosis and calreticulin. Calreticulin, a Ca²⁺ binding protein, is a major component of the endoplasmic reticulum. Upregulation of calreticulin in cultured MDCK cells has been proposed to promote epithelial to mesenchymal transition.

In order to examine whether calreticulin is involved in other forms of fibrosis, two additional models were examined, both characterized by an acute inflammatory phase preceding tissue fibrosis: the bleomycin model of lung fibrosis and the dilated cardiomyopathy model of the desmin null mice. Calreticulin appeared to be up-regulated in a time-dependent fashion in the bleomycin model, where epithelial to mesenchymal transition occurs. In contrast, no changes in calreticulin expression were observed in the desmin null model, where extended heart fibrosis occurs with no evidence of epithelial to mesenchymal transition.

In conclusion, calreticulin may be a key macromolecule involved in early stages during the development of fibrosis in kidney and in other organs as well, where epithelial to mesenchymal transition contributes considerably to fibrosis. (Supported by funds from IIBEAA, GGET EPAN YB/18, GGET PENED 03ED245, and Philip Morris).

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PROTEOMIC ANALYSIS OF AMNIOTIC FLUID IN PREGNANCIES WITH TURNER SYNDROME FETUSES

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Amniotic fluid (AF), routinely used for prenatal diagnosis, contains large amounts of proteins whose expression reflects

the genotypic constitution of the fetus. An intricate balance of these proteins is required throughout pregnancy, and in cases of fetal genetic abnormalities this balance may be disturbed. Identification therefore of such changes in the balance of proteins may be used to detect a particular type of pathology or to ascertain a genetic disorder.

Analysis of AF supernatant proteins was performed in five pregnancies with Turner syndrome (TS) fetuses, and in normal ones. Samples were obtained in the 17th week of gestation. Gel comparison revealed differences in the two groups. Protein spots with different expression levels were excised and identified by MALDI-TOF-MS. Five proteins, namely serotransferin (TRFE; P02787), lumican (LUM; P51884), plasma retinol binding protein (RETBP; P02753), alpha-1-antitrypsin (A1AT; P01009) and apolipoprotein A-I (ApoA1; P02647) were higher in cases with TS while three, kininogen (KNG1; P01042), prothrombin (THRB; P00734) and apolipoprotein A-IV (ApoA4; P06727) were lower. Additionally, quantitative analysis showed that the total amount of RETBP and lumican was increased by 52% and 30% respectively in cases with TS. These protein differences can possibly be used as biomarkers to identify fetuses with the specific chromosomal abnormality. Furthermore, since differentially expressed proteins are likely to cross the placental barrier and be detected in maternal serum, proteomic analysis has the potential to enhance research in the study of non-invasive prenatal diagnosis (*e-mail: gthtsangaris@bioacademy.gr).

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BIOMARKER DISCOVERY BY HIGH-THROUGHPUT PROTEOMICS: STRATEGIES, POTENTIALS AND LIMITATIONS

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In the last few years, the field of proteomics has expanded greatly in its scope and has become a mainstream strategy to differentially analyze proteins on a global scale. Concomitantly, HPLC and MS instrumentation have become sufficiently cheap, robust and 'easy' to use that even non-expert users can now conduct their own investigations. Elaborate quantification schemes based on stable isotope labeling have shown that mass spectrometry can rival in precision with ELISA assays when it comes to deriving protein abundances in mixture. Finally, a proteomics researcher can now rely on sophisticated software packages so that data analysis can be performed in a much more reliable manner than before.

One would think that the main barriers to conducting proteomics experiments to derive quantitative protein

information from a complex biological set-up have been removed. If our own experience in our laboratory has shown that technical reproducibility and level of precision have indeed increased over the years, biological data, however, must continue to be interpreted cautiously within a strict experimental design. In my presentation, I will show how important it is (still) to ask the proper question(s). I will discuss how high-throughput strategies contribute to improve our confidence to provide the biologically-relevant answer, whether it is applied to a standard two-dimensional electrophoresis-MALDI TOF MS approach or an LC-MS/MS shotgun strategy. Finally, while the use of stable isotope labeling of proteins or peptides provides considerable advantage for quantification purposes, the biological information still needs to be appropriately analyzed within the limitations of the analytical methods. The main progress within the last years, however, has been to relegate instrumentation problems and data analysis issues to the background so that the proteomics researcher can now focus on the much more interesting targeted biochemical problems he has been trained to study.

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PROTEOMICS IN BREAST CANCER RESEARCH

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Proteomics-based analysis of traditional sources of biomarkers, such as serum, plasma, or tissue lysates, has resulted in a wealth of information and the finding of several potential tumor biomarkers. It has become clear that growth and progression of breast tumor cells not only depends on their malignant potential, but also on factors present in the tumour microenvironment. There is compelling evidence demonstrating a role for the breast stroma in mammary gland development, and some studies have suggested the ability of the tumor environment to augment the growth and ability to metastasize of mammary carcinoma cells. In an effort to shed some light on these questions, we carried out a detailed proteomic analysis – using 2D gel-based technology, mass spectrometry, immunoblotting and antibody arrays – of a novel and highly promising source of biomarkers, the tumor interstitial fluid (TIF) that perfuses the breast tumor microenvironment. These studies provided a snapshot of the protein components of the TIF, which we showed consists of more than one thousand proteins – either secreted, shed by membrane vesicles, or externalized due to cell death –

produced by the complex network of cell types that make up the tumor microenvironment. So far, we have identified hundreds of primary high and low abundance translation products including, but not limited to, proteins involved in cell proliferation, invasion, angiogenesis, metastasis, inflammation, protein synthesis, energy metabolism, oxidative stress, cytoskeleton assembly, protein folding and transport. Considering that the protein composition of the TIF reflects the physiological and pathological state of the tissue, it should provide a new and potentially rich resource for diagnostic biomarker discovery and for identifying more selective targets for therapeutic intervention. We will also present our current data concerning the comparative quantitative proteomic profiling of primary breast tumors and their matching axillary lymph node metastasis obtained from the same patient.

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CLINICAL PROTEOMICS: CREATION OF NOVEL BIOMARKERS AND ESTIMATION OF THERAPY EFFICIENCY

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Clinical practice deals daily with two main issues: an early precise diagnosis, and an effective therapy. Regarding diagnosis, the currently used conventional approaches frequently demonstrate some common unfavourable features or even fatal disadvantages, such as the initial detection of a disorder in an advanced stage, the treatment of which is generally extremely problematic or even no longer possible. One of the most impressive examples is in the diagnosis of breast cancer. Application of proteomics in the early diagnosis of breast cancer is mandatory because of the following key features: i) high incidence of breast cancer in the population (10-14 per 100 women worldwide), ii) currently used diagnostic approaches are unreliable and expensive, iii) high probability of metastases in early stages of the disease, iv) early diagnosis is still extremely difficult.

In the past decade, the main emphasis in breast cancer proteome research was on cell culture investigations; the knowledge achieved was extensive and useful. This experience, however, demonstrated that *in vitro* markers were barely applicable in clinical practice. In response to this issue, *ex vivo* blood proteomics technology in conjunction with molecular screening, specific diagnosis and therapeutic monitoring for different forms and stages of breast cancer is now under extensive consideration.

Regarding currently used therapy approaches, particularly, in terms of those of the most fatal cardiovascular and cancer diseases, if at all, only partial or temporary success can be reached in the majority of cases, since corresponding etiology is frequently unclear, and only symptoms secondary to disease become treated. Moreover, some cohorts of patients are currently treated across-the-board without considering their individual predispositions. This is just an acute issue in healthcare of a large and growing diabetic population that represents a serious economical problem in the Western world. Independently of both disease duration and HbA1c-values, some diabetic patients do not develop any secondary complications during their lifespan, whereas others suffer from fatal cardiovascular diseases. Disease proteomics opens new perspectives for risk evaluation of secondary diabetic complications and therapy monitoring. Clinical application of disease proteomics resulted in a creation of a "method for early diagnosis of proliferative diabetic retinopathy", which was patented by O. Golubnitschaja in 2006. The appearance of proliferative diabetic retinopathy is considered as an early indicator for a general predisposition for the development of secondary complications in diabetic patients. The patented technology is based on the central role of activated leukocytes and key enzymes of tissue remodelling in the pathophysiology of diabetic retinopathy. Stable correlations among disease severity, specific alterations in gene expression patterns of circulating leukocytes and activity of tissue remodelling genes in blood plasma have been demonstrated. This diagnostic system has been suggested for the clinical application in diabetic healthcare.

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DISEASE-ASSOCIATED MITOCHONDRIAL PROTEIN CHANGES IN THE SPINAL CORD AND BRAIN OF AN ANIMAL MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

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Amyotrophic lateral sclerosis (ALS) is a late-onset progressive neurodegenerative disorder characterised by the dysfunction and death of motor neurons in the brain and

spinal cord. The only proven cause of ALS is mutation of the gene encoding Cu/Zn superoxide dismutase (*SOD1*), which is detected in 20% of familial and 3% of sporadic ALS cases. Over 116 different human mutations have been identified, mostly missense. Mice transgenically overexpressing eight different *SOD1* mutations develop progressive motor neuron degeneration due to a toxic gain of function with pathology that is strikingly similar to human ALS. The aggregation of mutant *SOD1* within mitochondria has recently been proposed as a possible pathogenic mechanism.

Swelling and vacuolation of mitochondria is one of the earliest and most pronounced changes in the motor neurons of *SOD1*^{G93A} mice and some ALS patients. Native *SOD1* is largely confined to the cytoplasm but mutations appear to enhance its accumulation within mitochondria. In this study, we sought to explore the subcellular distribution of mutant *SOD1* and undertake a comprehensive proteomic analysis of mitochondrial enriched fractions of early symptomatic *SOD1*^{G93A} mice and compare it to non-transgenic littermates and wild type *SOD1* overexpressing controls. Our objective was to identify changes in protein abundance that might lead to mitochondrial dysfunction and ultimately motor neuron degeneration.

Mouse brain and spinal cord mitochondria were isolated by differential centrifugation and purified on a Percoll gradient. The mitochondrial integrity and enrichment within the fractions were assessed by western blotting and transmission electron microscopy. Protein separation was performed using two-dimensional gel electrophoresis (2D-E). Silver staining revealed between 1,000 to 1,400 protein spots. The 2D-E gel images were scanned and the spot position and intensity were analysed using computer software to identify quantitative differences between diseased and control spots. Protein spots that demonstrated greater than two-fold change in abundance and achieved a 95% level of statistical significance were cut out and identified by mass spectrometry. Twenty-five protein spots were identified from the brain and twenty from the spinal cord which displayed altered abundance in *SOD1*^{G93A} mitochondrial fractions against both controls. They include proteins involved in mitochondrial membrane transport, apoptosis, metabolism and energy production (respiratory chain, TCA cycle, glycolysis), axonal outgrowth, molecular chaperones, vesicular pH regulation, and the dynein/dynactin motor protein complex among others. Several proteins have been further investigated by Western blotting and immunohistochemistry in early symptomatic and pre-symptomatic mice determining the time course of their effect. Most of them seem to "preserve" their altered behaviour at the pre-symptomatic stage strengthening the hypothesis that mitochondrial dysfunction may be contributing to motor neurone degeneration in ALS by disrupting many different functional systems.

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CHARACTERIZATION OF NOVEL CANDIDATE BIOMARKERS IN PROSTATE CANCER WITH QUANTITATIVE PROTEOMIC APPROACHES

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Introduction and Objective: The low specificity value of PSA drives the need for more effective biomarkers. Proteomics offers a large scale analysis of protein expression and interactions. The objective of this pilot study, which is based on quantitative proteomic methodologies using iTRAQ™ reagents, is to establish novel biomarkers for prostate cancer (PCA) screening. *Materials and Methods:* Under institutional review and approval, 15 prostate tissue specimens were used to isolate material originating from the following 3 biological states: normal (n=5), benign prostate hyperplasia (BPH) (n=5) and prostate cancer (PCA) (n=5). Specimens were extracted for their protein content and then subjected to proteolytic cleavage using trypsin. The resulting peptides were labeled with iTRAQ™ isobaric tagging reagents followed by fractionation using strong cation exchange-hydrophilic interaction chromatography. The protein extracts were analyzed by liquid chromatography electrospray tandem mass spectrometry using a hybrid quadrupole time-of-flight system. *Results:* The study resulted in the identification of over 1,200 proteins (95% confidence, $p \leq 0.05$) of which approximately 70 proteins exhibited a significant differential expression between the three biological states. The study also resulted in the consistent determination of proteins uniquely occurring in all PCA specimens, including C20orf75, carbonic anhydrase III, HUMMLC2B, myoglobin transcript variant 1, myosin heavy chain IIa, myosin light chain 2, myosin heavy polypeptide 7, titin, TNNT1 protein and 3 unnamed protein products with a protein sequence coverage ranging from 4.9% to 45.4%. Additionally, proteins were uniquely identified in all 5 BPH tissue specimens (including β -microseminoprotein, CAPNS1 protein, integrin-linked kinase, intracellular adhesion molecule-3, keratin 5, keratin 14, mutant β -globin, NG22 protein variant, 1 unknown protein, and 1 unnamed protein product. Finally, proteins common for both PCA and

normal tissue were also identified (including hypothetical protein LOC283284, keratin 7 and pro- α -1(I) collagen). *Conclusion:* Our existing results lead to the comprehensive characterization of a quantitative proteomic profile of normal, BPH and PCA tissue in an effort to discover potential biomarkers with greater discriminatory power than PSA. This ongoing study will incorporate the use of LCM-captured cells from multiple formalin-fixed paraffin-embedded prostate tissue specimens to further validate the above results.

20 TOWARDS BIOMARKERS OF COPPER STATUS

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Copper is an essential trace element used in many important biological functions. It is a component of metalloenzymes in which it acts as an electron donor or acceptor. Cuproenzymes are involved in basic physiological and biochemical processes, such as respiration, maturation of neurotransmitters or antioxidant defense. Because copper is highly reactive, its level must be tightly regulated. When levels are unbalanced, copper produces dramatic effects on health such as in the inherited Menkes's and Wilson's diseases.

Although copper deficiency and copper excess are important health issues, the definition of the normal copper status for individuals still represents a great challenge. Reliable and sensitive indicators of copper levels are still missing. The goal of this project is thus to identify new proteins that respond in their expression level to the copper status of an individual.

In mammals, copper is absorbed at the level of the intestinal mucosa. We thus focused on the brush border membrane (BBM) to observe changes in protein expression in response to insufficient or excessive copper intake. Rats were kept for 39 days on different copper defined diets. Crude duodenal cell lysates were analyzed by 2D gel electrophoresis and purified brush border membranes were analyzed by a shotgun proteomics approach. This combined approach allowed the identification by mass spectrometry of 28 proteins which responded to copper loads. Antibodies to these proteins were raised with synthetic peptides and their differential expression was tested by Western blots. At the end of this screening process, 13 proteins were confirmed to be potentially interesting sensors for copper overload and/or deficiency. Observed differences in protein expression could be confirmed at the mRNA level by quantitative real-time PCR.

Because intestinal biopsy is not a valid approach for patient care issues, we are currently investigating the

expression of our candidates in easy-to-sample biological fluids. Western blotting is being used to check differential expression in the serum. As we are working with intestinal proteins, we are also using real-time PCR to measure mRNA levels in the feces and in mouth scrapings. The validation of our 13 candidates should provide new diagnostic tools to assess copper status in individuals. (**Author for correspondence:* Tel: +41 31 632 3268, Fax: +41 31 632 4997, e-mail: marc.solioz@ikp.unibe.ch)

21 CHARACTERIZATION OF BRUSH BORDER MEMBRANE PROTEINS OF MICE INTESTINAL MUCOSA. EMPHASIS TO THE STUDY OF CHOLESTEROL ABSORPTION

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The brush border membrane (BBM) of intestinal mucosa cells is the initial barrier across which most nutrients pass into the body. There is, therefore, considerable interest to study the mechanisms responsible for the transport of molecules across this specialized membrane. Defects in these mechanisms can cause a variety of pathological conditions, such as disorders in the metabolism of saccharides, amino acids, ions and lipids. An altered cholesterol absorption pathway can also lead to cardiovascular diseases, diabetes and obesity.

There has been an increased interest in the past year to better characterize BBM at the protein level and to understand mechanisms driving cholesterol absorption. Proteomic analyses of these vesicles have been very limited so far since most of the targets of interest are highly hydrophobic and mostly transmembrane proteins. In previous proteomic studies, only cytosolic enzymes (most probably contaminants), cytoskeletal and membrane-associated proteins were reported to belong to the BBM membrane, while only a few of the most abundant transporters were then characterized. In this study, we present an analytical strategy which allowed us to characterize BBM proteins that have been described as participating in the cholesterol absorption. Key points of this strategy consist of an improved protocol to isolate and purify BBM preparations from common contaminants and of a very robust and sensitive instrumentation using capillary liquid chromatography and tandem mass spectrometry. A major challenge of this study was to evaluate the protein degradation that occurs in the BBM

fraction due to the very high abundance of peptidases, such as aminopeptidase N, aminopeptidase P, or dipeptidyl peptidase. A large part of our study consisted of determining the level of degradation and to try to inhibit it so as to increase the level of sensitivity and reproducibility in our proteomic approach.

We believe that this study opens the way to a better understanding of this very important area of cholesterol absorption. A proteomics approach to BBM biology will contribute and complement existing studies about the inhibition of cholesterol absorption that have been focused so far at the gene expression level.

22 APPLICATION OF PROTEOMIC TECHNIQUES IN THE INVESTIGATION OF PLASMA FROM DIABETIC PATIENTS

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Proteomic analysis is widely used for the detection of disease markers. The analysis involves the application of protein separation techniques, such as two-dimensional gel electrophoresis (2-DE) or multi-dimensional chromatography, followed by mass spectrometry (MS) for protein identification. In this study we apply 2DE-MS-based approaches for the identification of biomarkers in the plasma of type 2 diabetes mellitus (T2DM) patients.

Plasma samples from 12 T2DM patients and 7 normal people were used. Samples were analysed by 2-DE on wide range pH 3-10 IPG strips. Comparison of the gel images by the use of image analysis software revealed several differences between plasma from normal and diabetic individuals. These spots were excised from the gels and processed for identification by MALDI-TOF-MS analysis following peptide mass fingerprinting. Twenty-four protein spots either expressed at statistically significantly different levels between the two groups, or presented only in normal plasma samples, were positively identified corresponding to 12 gene products (THRB, KNG1, ALBU, CO3, FIBB, TRFE, CD5L, KAC, APOA1, HPT, APOE and CO7). To increase protein resolution and unravel lower abundance proteins, plasma samples were subjected to albumin and IgG affinity chromatography. HiTrap Blue HP (affinity for albumin) and HiTrap Protein G HP (affinity for IgG) eluates represented 68% and 16% respectively of the total plasma

protein content as determined by 1-D gel electrophoresis and 2-DE. A protein map of the IgG- and albumin-depleted plasma consisting of 350 proteins (50 different gene products) was generated. Comparison of the IgG- and albumin-depleted plasma samples from normal people and diabetic patients revealed the presence of nine protein spots expressed at statistically different levels between the two groups; These protein spots corresponded to four gene products (HPT, K1C10, TTHY, and CAH1). Western blot analysis confirming the differential expression of some of these proteins is presented.

23 QUANTITATIVE LABEL-FREE NANOSCALE LC-MS ANALYSIS OF HIV DRUG-TREATED HUMAN T-CELL CULTURES

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HIV is one of the main health risks in many regions of the world. Most methods of treatment are based on HIV reverse transcriptase and protease inhibition to reduce the rate of HIV replication. There is a need for more drug candidates to effectively treat the disease. Some drugs target the interaction between the virus and T-cells, the primary cell type infected by HIV, with anti-HIV effects, but it remains unclear whether or not the drug candidate may have other consequences on the T-cell itself.

One way of studying the molecular mode of action of a potential next-generation class of anti-HIV compounds, as well as to determine possible side-effects, is to look at the proteome expressed by compound treated T-cells *versus* untreated ones. A promising compound was selected for a feasibility study and a large batch of CD4+ T-cells, a human T-cell line SUPT-1, were grown. Part of the culture was treated with an effective dose of compound. The protein content was comparatively analyzed with that of an equivalent amount of untreated T-cells from the very same culture by nanoscale LC-ESI-MS. The mass spectrometer recorded LC-MS data where the collision energy within the gas cell is continuously switched from low to elevated energy. Data is subsequently normalized to allow quantification and identification of proteins across samples. Identification of putative protein markers was achieved by selecting differentially or uniquely expressed peptides, and searching against a human database. The effect of the treatment was also monitored by utilizing the outcome of

the relative quantification results by looking at the protein composition as a whole. The relative quantification results of the study also provided invaluable input for the investigation on the mechanism of action of the compound on targeted and non-targeted cellular components. The absolute quantification results were used to conduct stoichiometric studies.

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COMPLEX BACTERIOPHAGES AS PROTEIN STANDARDS FOR PROTEOMICS

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There is a current need for protein standards, *i.e.* mixtures of known proteins in defined proportions, for proteomic analyses. The definition of such standards follows certain rules, such as the stability of the proteins making up the standard, the stability of the stoichiometry, a low biohazard, and the versatility required to benchmark several proteomics methods. In addition, the widespread development of proteomics on higher eukaryotes suggests the use of standards derived from prokaryotes or lower eukaryotes, so that spiking of the protein extracts of interest with the standard would not interfere with the analysis of the mammalian or plant extract.

In this context, we have investigated the use of complex bacteriophages (*e.g.* the T bacteriophages infecting *Escherichia coli*) as proteomics standards. In addition to matching the above-mentioned pre-requisites, such phages can be easily propagated in most laboratories and are very easily distributable. The results obtained on bacteriophage T4 with several proteomics methods (including 2D gel electrophoresis and GeLC) are presented and discussed in more detail.

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GATHERING FUNCTIONAL INFORMATION ON PROTEINS FOR BIOMEDICAL APPLICATIONS

Rolf Apweiler

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The availability of hundreds of genomes, many of them of medical importance, has shifted the attention from deciphering the sequence to the identification and characterization of the functional components. Availability of

the ‘parts list’ has fostered the development of experimental approaches to systematically investigate gene function at the genome, transcriptome and proteome levels. Studying gene function at the protein level is vital to the understanding of how cells perform their functions, as variations in protein isoforms and protein quantities underlying a phenotype can often not be deduced from sequence or transcript level genomics experiments alone. The rapid progress in functional genomics and proteomics promises major breakthroughs both in basic and applied science, hopefully leading to a better understanding of biology at a cellular and molecular level and to new biomarkers and new targets for diagnostic and therapeutic interventions. To capture the wealth of biomedical data and to make the data easily usable not only for the classic users of biomolecular databases but also for biomedically oriented researchers leads to new challenges for the producers of biomedical databases. This presentation will show some examples of what has been achieved already and the challenges ahead of us.

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PROTEOMICS DATA ANALYSIS – BEYOND THE T-TEST

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In proteomics experiments, large amounts of data are generated that give excellent opportunities to improve data analysis beyond that which is attainable by pair-wise *t*-tests. We will present approaches that allow estimation of the probability of obtaining a false discovery between sample and control dependent on the detection level of the protein. This approach is powerful as it allows consideration of the higher accuracy of proteomics data at higher protein concentrations while at the same time rejecting many false positive discoveries that occur at small signal-to-noise ratios. We will discuss the procedure’s applicability to many situations, from the analysis of stain coloration on 2D PAGE to the analysis of MALDI PMF data or LC/MS/MS data.

27

MODELING BIOLOGICAL SYSTEMS: DREAMS, MYTHS AND REALITY

Ioannis Xenarios

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Switzerland

The modeling of complex biological systems is the holy grail of computational biologists. For the past years, vast amounts of data have been gathered using various technologies.

However, when one looks at all these data it can be a daunting task to identify the key regulators and the dynamics that is 'encoded' within these biological networks.

How we approach these complex data using various computational and experimental methods will be presented, taking as a case study, the mechanism of induction of tolerogenic dendritic cells and the interplay of several stimuli, such as TNF γ , TGF γ and CD40L.

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ADVANCED TECHNIQUES FOR PRE-PROCESSING OF PROTEOMICS MASS SPECTRA

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Raw mass spectra from MALDI-TOF or SELDI-TOF instruments are unsuitable for statistical analysis, biomarker discovery, or sample classification. Several pre-processing steps are necessary: i) noise reduction, ii) baseline removal and iii) spectral alignment ('warping'). In addition, one may want to apply binning and mass range reduction. These goals can be achieved with innovative statistical techniques. The central theme is to translate each goal into a function that can quickly be optimized.

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GENE COEXPRESSION

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There are two main approaches in the assignment of biological roles to genes using microarrays. In the differential approach, two sets of microarray results are compared and genes implicated can be pinpointed by their differential expression levels. However, unrelated microarray experiments also contain information far beyond the original purposes for which the experiments were performed and can be a valuable predictive tool for gene function and pathway membership. Hence, in the co-expression approach, many microarray experiments from various tissues or developmental stages of the same organism under different experimental conditions are combined using clustering techniques that can group together genes showing correlated expression patterns, implying that those genes are involved in connected biological processes. Coexpression can be quantified using Pearson's correlation coefficient. A 'Co-expression Tool' based on a database containing pair-wise gene correlations,

the newest gene annotations and other text-mining information such as gene description and gene ontology is then developed. Genes are also clustered according to their coexpression patterns (*e-mail: imichalop@bioacademy.gr).

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EXPLORATORY DATA ANALYSIS VIA PCA: A FIRST INSIGHT INTO THE ACCLIMATION OF PLANT STEM CELLS

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The Laboratory of Tropical Crop Improvement (K.U. Leuven, Belgium) hosts, under the authority of Bioversity International, the global *in vitro* collection of banana varieties (*Musa* and *Ensete* spp.). The aim of this international gene bank is to conserve all banana and plantain genetic resources safely and to supply the germplasm to any bona fide users. K.U. Leuven was one of the pioneers to explore the possibilities of storing stem cells in liquid nitrogen (at -196°C). For successful storage at -196°C , cells need to first survive a severe dehydration process prior to freezing and need to stay pluripotent. In general, dehydration tolerance is achieved by an osmotic stress acclimation. However, more than half of the collection consists of varieties that have a nonexistent or low survival rate. Hence, there is a need to unravel the mechanisms behind acclimation and to gain insight into the genotype-specific diversity.

Protein separation *via* two-dimensional gel electrophoresis (2DE) and protein identification *via* tandem mass spectrometry (MS/MS) is the most informative approach for a poorly characterized organism like banana (1, 2). After separation through 2DE several hundred individual protein abundances can be quantified. The datasets contain hundreds of different proteins that are correlated. Proteins fit within the larger entity of networks and interact with each other. Both a good experimental set-up and a valid statistical approach are essential to gain insight from the data and to draw correct conclusions. Our understanding of a biological system is usually rather limited and data may be very heterogeneous and complex. Exploratory multivariate data analysis approaches a biological problem from a different angle and tries to describe patterns, relationships, trends and outlying data. The field of multivariate analysis consists of those statistical techniques that consider multiple related random variables as a single entity and attempts to produce an overall result taking the relationship among the variables into account. It displays the interrelationships between the large number of variables and is able to correlate multiple proteins to a specific experimental group. Principal

Component Analysis (PCA) is one of the multivariate possibilities to perform explorative data analysis. PCA condenses the information contained in a huge data set into a smaller number of artificial factors which explain most of the variance observed.

Here, we are interested in the effects of 3 different specific acclimation treatments over time. Using the DIGE approach we consider 4 different time points. Experiments show that 4 days of acclimation is significantly correlated to the best survival. PCA gives a first insight into the complex dataset. It confirms that 4 days is different from the other sample points, identifies multiple proteins that are correlated to this sample point and shows the correlations among several proteins. The individual differences between the sample points are validated *via* confirmatory ANOVA. This is a nice illustration of how exploratory data analysis performs, indicating correlations but also bringing up candidate markers that would have been missed when using only confirmatory data analysis.

1 Carpentier *et al.*: 2005.

2 Carpentier *et al.*: 2007

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THE IMPACT OF DATA PRE-PROCESSING ON THE INTERPRETATION OF PROTEOMICS DATA: A CASE STUDY ON PEAR

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High throughput two-dimensional gel electrophoresis allows the separation of hundreds of proteins at once, resulting in the generation of large amounts of data which must be properly analyzed in order for the right conclusions to be drawn. Over 60% of published papers use statistics as a 'black box' approach without realizing which assumptions need to be fulfilled prior to performing the appropriate statistical tests.

In this study, we show the effect of the different processing steps prior to statistical analysis on the final selection of the 'most important' proteins. Gel electrophoresis data was collected on 'Conference' pear stored at four different controlled atmosphere storage conditions. The gels obtained were silver stained. After image analysis of the scanned gels using ImageMaster 2D Platinum 6 software, the generated data was exported to MATLAB 7 and Unscrambler 9.6 for the calculation of missing values and subsequent multivariate statistical analysis. Using partial least squares discriminant analysis (PLS-DA) in combination with Variable Important in Projection (VIP), the fifty most important proteins

involved in the distinction of the treatments were selected. The effect of different pre-processing steps such as treatment of missing values (filling with zeroes or applying Bayesian principal component analysis, BPCA), data transformation (none, inverse hyperbolic sine, square root or logarithmic) and outlier treatment (removing data or not) were tested to see how these would affect the final selection of the 50 most important proteins by PLS-DA and VIP. The two most extremes in data processing prior to statistical analysis were: i) raw data with missing values replaced by zeroes with no transformation applied and no outlier data removed and ii) raw data with the missing values calculated using the BCPA method, followed by a log transformation and removal of outliers.

Our results showed that if missing values are replaced by zeroes instead of being calculated using the BCPA method, the final selection of the 50 most important proteins is quite different with only 4 out of the 50 proteins being the same. The raw data required transformation to fulfil the requirement of having a normal distribution. This could be achieved by applying the logarithmic transformation, as was confirmed using Q-Q plots. When one of the other transformations was erroneously applied, different final selections of the 50 most important proteins were obtained. Applying inverse hyperbolic sine, square root or no transformation yielded 19, 35 and 17 proteins, respectively out of the 50 to be the same as those obtained based on the logarithmic transformed data. Not removing outlier gels yielded 44 proteins to be the same as the 50 chosen with outlier gels removed.

In an extreme case, filling the missing data with zeroes without performing any transformation and not removing outlier gels gave only 11 proteins out of 50 to be the same as those selected from the data in which missing values were calculated with the BCPA method where a logarithmic transformation was applied and outlier gels were removed.

In conclusion, applying erroneous processing steps prior to statistical analysis leads to a completely different selection of the 'most important' proteins and as such results in a completely different interpretation of the results, as will be shown in more detail for our case study on pear storage.

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ION MOBILITY SPECTROMETRY COUPLED WITH TIME-OF-FLIGHT MASS SPECTROMETRY FOR DETAILED PROTEIN AND PEPTIDE STRUCTURE STUDIES

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Mass spectrometry has rapidly established itself as the method of choice for detailed studies at the peptide or protein level. The ability to combine molecular mass information upon the intact protein subunit, or even the assembled supra molecular complex, combined with the ability to perform detailed structural studies using tandem MS, typically upon the enzymatically digested proteins has proved highly informative.

Despite the success of mass spectrometry as an analytical tool, there is a clear need to further improve the information content that can be obtained from both top-down (protein-based) tandem MS studies as well as bottom-up (peptide-based) strategies. The hyphenation of different technologies with MS is one such approach to do this and we recently described a novel quadrupole/ TWIMS/oa-TOF mass spectrometer, operated with an electrospray ion source that provides for the separation of ions based both upon their mobility and subsequently their m/z (1). The TWIMS is a stacked-ring ion guide, operated at elevated pressure, with opposite phases of an r.f. voltage applied to adjacent plates to provide radial ion confinement. A continual sequence of d.c. pulses is superimposed on the confining r.f. to provide 'waves' which propel ions through the gas. Protein and peptide species were ionised using nanoelectrospray ionisation and the resulting ions separated based upon their ion mobility, or collision cross section, through the TWIMS device prior to mass analysis using the oa-TOF analyser.

We have investigated the use of the hybrid ion mobility/ time-of-flight system for the analysis of intact proteins and also tryptic peptides. The potential of this configuration for the separation and subsequent mass analysis of multiply charged protein ions and to separate fragments derived from these multiply charged species following CID has been studied. If mobility separation of fragment ions were efficient, this would reduce mass spectral complexity and facilitate detection and subsequent identification. In this study, ion mobility spectrometry (IMS) techniques have been used to separate the protein ion fragments obtained from lysozyme and beta-lactoglobulin. In addition, we have also looked at the potential of this configuration for the analysis of tryptic peptide mixtures and we will show examples of how the IMS-MS and MS/MS approach can be used as a rapid screen of peptide digests. This work will be discussed and further examples of how IMS coupled with MS-TOF can be used for the systematic analysis of protein structure shown.

1 Ruotolo *et al*: Science 310(5754): 1658-1661, 2005.

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RAPID NANOBORE UPLC SEPARATIONS COUPLED WITH ESI MS/MS FOR IMPROVED HIGH-THROUGHPUT PROTEIN IDENTIFICATION

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Mass spectrometry has established itself as the primary technique for identifying proteins due to its unparalleled speed, sensitivity and specificity. Strategies for digestion of the proteins use a specific protease that cleaves at predictable residues along the peptide backbone providing smaller stretches of peptide sequence more amenable to mass spectrometry analysis. When coupled with protein level pre-fractionation strategies, such as 1-dimensional PAGE, thus reducing the complexity of the protein mixture, this approach has proven highly successful in comprehensive protein identification and characterization. The downside of this approach is the number of gel samples, or fractions, to be analysed by the LC-MS/MS system. Typical analytical HPLC run times of 45 min to 1 h, the amount of time required to analyse one top-level sample, can be prohibitive.

Here we describe the use of elevated flow rates combined with nanoscale columns packed with sub 2 μm particles for rapid separations using a nanoUPLC system. Increasing the flow rate to 900 nL/min and running a very rapid gradient over 8 min on a 75 μm x 15 cm column, allows high quality peptide separations to be achieved with a sample-to-sample inject time of ten minutes. This, combined with an orthogonal acceleration time-of-flight mass spectrometer using a newly developed high speed data dependent MS/MS approach fragmenting up to 8 precursor ions per second, allows for the rapid characterisation of simple protein mixtures, such as those obtained from 1D gel bands.

We will present data from standard tryptic digests of known proteins and simple mixtures of protein digest used in the development of this method and data from in-gel digests of 1D gel bands.

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FINDING THE PEPTIDES THAT MATTER: NEW CHEMICAL AND INSTRUMENTAL APPROACHES TO NON-GEL PROTEOMICS

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Proteins are responsible for performing and controlling most of the functions carried out by a cell. Unlike DNA, proteins show a massively diverse array of physicochemical properties that makes it unlikely that a generic high-resolution and throughput separation method will ever be developed. The most effective method up to now has been 2D gel electrophoresis, although it suffers from some limitations. Recently, the trend has been towards reducing the physicochemical diversity of proteins by

digestion into peptides at the expense of an increase in the mixture complexity. Sample preparation can thereby be simplified and does not have to be optimised for each cell or tissue type as is the case for 2D-PAGE. Peptides behave more predictably and are more amenable to automated separation by multi-dimensional chromatography and capillary electrophoresis. The so-called 'shotgun' approach of whole cell digestion has the advantage that all classes of proteins are represented including the traditionally difficult ones such as membrane proteins and ones at the extremes of pI and size.

In response to the success of the genome projects, there has been a movement towards the development of methods and reagents for isotopic labelling to enable protein identification, quantification and the determination of post-translational modifications. The first method to be described, Isotopically Coded Affinity Tags, used isotopically labelled cysteine specific reagents. It provided a means of reducing sample complexity by allowing only the Cys-containing peptides to be selectively isolated and quantified. In recent years, many other isotope labelling approaches have been developed employing chemical reactions by enzymatic digestion or through metabolic incorporation during cell culture. Quantitative labelling experiments have been most extensively studied using pairs of isotopes, until recently, when a new technique employing labelling with isobaric tags enabled multiplexed experiments. The *in vivo* strategy has the advantage of enabling labelled and unlabelled samples to be combined earlier in the experimental process than is the case for chemical labelling strategies.

Despite these advances, the main problems remain the extreme dynamic range of protein expression in the cell with up to over 6 orders of magnitude and the complexity of the peptide digest sample, which is compounded by the limited duty cycle of mass spectrometers currently available. This requires that a reductionist approach be taken, either by enriching for defined subsets of the proteome¹⁶ or by using strategies for labelling and isolating for certain amino-acids as with the ICAT scheme, or alternatively by using the elegant COFRADIC approach which utilises diagonal chromatography of modified peptides for selective proteomics.

We present here a generic approach that allows the selection of only those peptides from proteins that change in expression, post-translational modification levels, for subsequent analysis. We have designed an isotopic label that can be selectively attached to peptide *N*-termini in a digest mixture. The reagent is chemically stable but fragments easily under MS/MS conditions to produce a daughter ion of unique mass, common to all labelled peptides, a so-called signature ion. One subset of proteins is derivatised with the normal 'light' form of the label, while another subset is labelled with the deuterated 'heavy' form of the same reagent, and two types of signature ions are thus obtained. The mass spectrometer is operated in parent (precursor) ion

scanning mode, alternating scans between light and heavy signature ions. A difference spectrum is generated and only those parent peptides showing differences between the two labels are selected for MS/MS analysis. We describe the development and evaluation of a series of reagents and their performance in complex mixtures and the application of other reagents for phosphospecific analysis.

35 NEW IEF GEL-BASED STAINING-FREE METHOD FOR SIMULTANEOUS IDENTIFICATION OF PROTEINS AND DETERMINATION OF THEIR pI VALUES, DATA EVALUATION AND STORAGE

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Proteomic characterization of proteins does not include only identification of a particular isoform of identified proteins, it should include also the distribution of all post-translational forms of the identified protein. In order to fulfill this task, separation methods capable of separating all isoforms need to be applied. Therefore, we developed a new IEF gel-based proteomic staining-free method for simultaneous separation of protein isoforms, identification of proteins and determination of their pI values by using low-molecular mass pI markers. It is based on the separation of proteins in gels by isoelectric focusing in combination with mass spectrometric analysis of both peptides derived by in-gel digestion and low-molecular mass pI markers extracted from the same piece excised from the gel. Extracted pI markers and digested proteins were identified by MALDI-TOF/TOF MS. This procedure allows time-consuming protein staining and destaining procedures to be omitted, which shortens the analysis time. This method was applied to analysis of proteins extracted from barley malt. Several proteins were identified in the gel pieces with the pI markers corresponding to the calculated pI of these proteins; the others in the gel pieces with the pI markers did not correspond to the calculated pI of these proteins. This means that these proteins were either post-translationally modified or the calculated pIs do not correspond to their real values. Several proteins were found in two gel pieces, which shows the presence of PTM. Several glycosylated peptides were found in the digests.

This type of analysis brings more information on PTM, which might be useful for identification of biomarkers. It will also require a new manner of data storage. A new proteomics database is under construction.

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PROTEIN COMPLEX PURIFICATION BY IN VIVO BIOTINYLATION

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Proteomic approaches require simple and efficient protein purification methodologies that are amenable to high throughput. Biotinylation is an attractive approach for protein complex purification due to the very high affinity of avidin/streptavidin for biotinylated templates. We applied *in vivo* biotinylation for single-step purification of STAT5 (signal transducer and activator of transcription 5) factor complexes. STAT5 is implicated in cancer and various stress conditions, transducing signals from activated cell surface receptors to the nucleus. We expressed the bacterial BirA biotin ligase in mammalian cells and demonstrated efficient biotinylation of STAT5 factor bearing a small (23-aa) artificial peptide tag. Using this approach, we isolated the biotin-tagged factor with other known and potentially unknown interacting proteins from crude extracts by direct binding to streptavidin beads. Therefore, BirA-mediated biotinylation of tagged STAT5 provides the basis for the single-step purification of interacting partners from our mammalian cell system, which will provide insights on the molecular mechanisms underlying cancer (*e-mail: ekatsantoni@bioacademy.gr).

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FROM ART-LOVING TO EARTH-LOVING BUGS: A PROTEOMIC CHRONICLE

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In recent years, our group has started to investigate some peculiar bacteria exploitable in bio-restoration interventions. Our first work dealt with the fascinating case of bio-restoration performed on an ancient fresco in a cemetery at Pisa, where a strongly heterotrophic bacterium was used to degrade the highly cross-linked glue layer obscuring the fresco surface (1). Here we present a

new *Stenotrophomonas maltophilia* strain (SeITE02), a γ -proteobacterium capable of resisting high concentrations of selenite (SeO_3^{2-} , Se^{IV}), reducing it to non-toxic elemental selenium under aerobic conditions, a feature that makes it potentially suitable for *in situ* bio-remediation of selenite-contaminated soils. Biochemical and proteomic tools have been utilized for investigating the mechanisms of selenite reduction in this strain. Two enzymatic pathways previously said to be involved in selenite reduction in other bacteria were taken into consideration in this study, namely nitrite-related enzymes and glutathione-based mechanisms. Biochemical analysis demonstrated that: (a) nitrite reductase does not seem to take part in the process of selenite reduction by the bacterial strain SeITE02, although its involvement in this process had been hypothesized in other cases; (b) nitrite strongly interferes with selenite removal when the two oxyanions (NO_2^- and SeO_3^{2-}) are simultaneously present, suggesting that the two reduction/detoxification pathways share a common enzymatic step, probably at the level of cellular transport; (c) selenite reduction, *in vitro*, does not take place in the membrane or periplasmic fractions, but only in the cytoplasm, where maximum activity is exhibited at pH 6.0 in the presence of NADPH; (d) glutathione is involved in the selenite reduction mechanism, since inhibition of its synthesis leads to a considerable delay in the onset of reduction. Moreover a proteomic three-way comparison based on two-dimensional electrophoresis was used to investigate the global bacterial response to the two toxic oxyanions nitrite and selenite. Our results demonstrated that when added to the culture medium 0.2 mM selenite and 16 mM nitrite (which allow for similar growth rates and total cells in the stationary phase) caused a significant modulation (ca. 10%, *i.e.* 96 and 85 protein zones, respectively) of the total proteins visualized in the respective 2D maps. These spots were identified by MS analysis and were found to belong to some common functional classes, among which damaged-protein catabolism, DNA-related proteins and proteins involved in the response to oxidative stress.

1 Proteomics 5: 2453-2459, 2005

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PEPTIDE PROFILING FROM HUMAN URINE BY MULTIDIMENSIONAL LC/MS

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High performance liquid chromatography (HPLC) has many advantages such as high sensitivity, fast analysis time, variable sample size, large number of separating mechanisms, easy to couple on-line to MS and is amenable to automation. The direct injection of biological samples onto a chromatographic column, without any sample preparation, is in most cases problematic and may lead to irreversible contamination of the separation column. This affects not only the selectivity, but also the column performance and efficiency, leading to unacceptable results. A powerful tool to avoid column contamination is to rely on the implementation of restricted access material (RAM) for sample preparation. The RAM, in this case, is characterised by a hydrophilic outer surface, a small, average pore size and an inner surface coated with strong cation-exchange groups. This column extracts positively charged peptides from complex samples by utilising their charge properties.

We used a proteomic technique to determine whether the urine of renal transplant patients undergoing acute allograft rejection had a characteristic profile. Urine has enormous potential for non-invasive detection of protein biomarkers for the diagnosis and clinical monitoring of patient samples. Two dimensional separation with on-line sample clean-up was achieved by a RAM-SCX column and a reversed-phase capillary column coupled to MS. An established system was validated with BSA digest and human urine, in order to obtain valuable data for biomarker analysis. Furthermore, a second identical system was established and cross validated with the same samples. Comparing the systems, Pearson's correlation of peptides abundances was found to be 0.9. Peptide profiles of patient samples have been comprehensively compared.

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REPRODUCIBILITY OF TWO-DIMENSIONAL ELECTROPHORESIS AT DIFFERENT REPLICATION LEVELS

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Reproducibility represents an important issue in two-dimensional electrophoresis (2DE), particularly when the technique is used in differential display experiments to identify quantitative changes in protein expression levels between different types of samples. We investigated the factors that influence spot volume variance on replicate pooled samples run on silver-stained 2DE gels. Spot position (x, y coordinates), spot area, mean spot volume and circularity were used to predict spot volume variability in a linear model including order 2 polynomials of all predictors. All tested parameters except for circularity were found to influence the variance of spot normalised volume.

Furthermore we estimated the amount of variation introduced by the different steps of 2DE: sample preparation, first and second dimension, image acquisition and analysis. For this we compared spot volume variance between data sets representing different levels of replication including biological and technical replicates. All parameters previously shown to explain variance of spot volumes (spot position, area and mean volume) were included as predictors in the models. Our results suggest that the second dimension and the staining are responsible for most of the variation in spot volumes, despite the fact that normalised volumes were used for analysis. Sample preparation explained an extremely low degree of spot volume variance, while the second dimension accounted for as much variation as the biological variation between the pooled samples. Image acquisition also accounted for spot volume variability, mostly in the case of low intensity spots (*e-mail: valcu@wzw.tum.de).

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SPECIFIC DETECTION AND IDENTIFICATION OF CHEMICALLY MODIFIED CITRULLINE CONTAINING PEPTIDES BY TANDEM MASS SPECTROMETRY

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The post-translational deimination of polypeptide-bound arginine residues is known as *citrullination* and is catalyzed by the enzymes of the peptidylarginine deiminase (PAD) family. Immune reactions to those citrulline-containing proteins appear to be central to the immunopathogenesis of rheumatoid arthritis. Consequently there is a need to characterize which proteins in the inflamed joints of rheumatoid arthritis patients contain citrulline residues *in situ*. As a requirement for such studies, we are currently developing methods for the specific enrichment and mass spectrometric detection of citrullinated peptides from heterogeneous mixtures.

Firstly, a technique for the chemical modification of peptide-bound citrulline residues was introduced and validated (1). The reaction, which is based on 2,3-butanedione and antipyrine in acidic conditions, is specific for citrulline residues and results in a mass shift of the modified citrullinated peptide by 238 Da. The structure of the modification product was elucidated by NMR using *N*-butylurea as a model compound for citrulline.

Subsequently we showed that these chemically modified citrulline residues generate specific fragment ions in common tandem mass spectrometry instruments (e.g. ESI-ion trap-, ESI-Q-TOF- and MALDI-TOF/TOF instruments). The observed signature ions allow the specific detection of citrulline-containing peptides present in heterogeneous peptide mixtures when analyzed by LC-MS/MS. To demonstrate the usefulness of this approach, bovine serum albumin was treated with PAD4 and digested with LysC. The digest was subjected to modification conditions and the peptide mixture was analyzed after tailored sample purification by nano-LC coupled to a Q-TOF instrument. Tracing of the fragment ions specific for modified citrulline enabled the rapid identification of eight deiminated peptides including their citrullination sites. In conclusion, the developed methodology combines a highly selective method for chemical labelling of citrullinated peptides with their specific detection and identification by tandem mass spectrometry.

1 Holm *et al.*: Anal Biochem, 2006

41 DISTRIBUTION OF THE PROTEINS IN THE SMALL INTESTINE OF THE RAT INVESTIGATED BY 2D-ELECTROPHORESIS

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The small intestine is an important part of the digestive system of the rat. In this study, the expression and the distribution of its proteins was investigated. In order to examine the expression and distribution of the proteins in the small intestine, which contains a large number of whole proteins, 2D-electrophoresis was used for the homogenates of selenium deficient (Se⁻) and selenium control rats (Se⁺) by means of gels (22x30 cm). After electrophoresis, the protein expression pattern of the (Se⁻)-gel and (Se⁺)-gel were compared. In this way more than 1800 proteins per gel can be detected in the homogenate of the (Se⁻)-gel as well as in the homogenate of the (Se⁺)-gel. In addition more than 20 differences in the expressed proteins were found between

the (Se⁺) and (Se⁻) 2D-gels. Differences were found in various molecular mass ranges and with different isoelectric points (P_i). After the evaluation of the two gels, first results suggest, as expected, that selenium affects the expression of the proteins in the homogenate of the small intestine of the rat, because, as is well known, selenium plays an important role in the metabolism and carcinogenesis of the digestive system. The significance of the protein differences is our current task (*e-mail: Kyriakopoulos@hmi.de).

42 IDENTIFICATION AND LOCALIZATION OF A NEW SELENOCYSTEINE-CONTAINING PROTEIN FOUND IN THE RAT LYSOSOME

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Studies were carried out on rats to obtain information about the distribution of selenoproteins in the lysosomal fraction. By means of analytical and radiochemical techniques, in combination with biochemical methods, a new selenoprotein was found in the lysosomal fraction after labelling of rats *in vivo* with ⁷⁵Se-Selenite and separation of the proteins in the homogenate and in the cellular organelles by electrophoretic methods. Using neutron activation analysis, the concentration of the trace element selenium was determined in the liver, liver homogenate and in the microsomal fraction which contains, among other micro-organelles, lysosomes.

After separation of the proteins in the lysosomal liver fraction, a labelled protein band in the range of 75 kDa was detected besides two further labelled proteins in the range of 60 kDa. By means of two-dimensional electrophoresis the isoelectric point (P_i) of the labelled proteins was determined. The localisation of the new protein in the lysosome indicates the importance of the trace element selenium in these organelles, which are well known as organelles containing most catalytic enzymes especially in eukaryotic cells (*e-mail: Kyriakopoulos@hmi.de).

43 EFFECTS OF DOCOSAHEXAENOIC ACID AND EICOSAPENTAENOIC ACID ON THE DIFFERENTIAL PROTEIN EXPRESSION IN HUMAN HEPATOCELLULAR CARCINOMA CELLS

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Hepatocellular carcinoma (HCC) plays a leading role in causing cancer death due to its high metastatic potential and frequent tumor recurrence rate after treatment. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are two important omega 3-polyunsaturated fatty acids (ω 3-PUFAs) which have been shown to be able to inhibit the growth of hepatocarcinoma in animal models; however, the mechanisms are not fully understood. Adopting a proteomic approach, we investigated the effects of DHA and EPA on the expression of proteins in the human hepatocellular carcinoma cell line PLC with no metastatic potential. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was employed for protein separation in the subcellular fractions prior to fluorescent protein staining by SyproRuby protein gel stain. Comparison of differential protein expression between control and fatty acid treatments and between DHA and EPA treatments was enabled by image analysis and protein spots were identified by mass spectrometry (MALDI-TOF/TOF). Results indicate that DHA (200 μ M) was more effective at causing PLC cell death than EPA (200 μ M). DHA down-regulated the expression of annexin A2, a protein which is closely associated with angiogenesis and metastasis, and placental anticoagulant protein II (PP4-X), which is involved in the morphological diversification and dissemination of human carcinoma. The N-myc downstream regulated gene 1, a metastasis suppressor gene, is up-regulated in EPA-treated cancer cells whereas the ubiquinol-cytochrome c reductase core protein I (UQCRC1) is down-regulated. UQCRC1 is involved in mitochondria-to-nucleus retrograde response in human cancer and is highly expressed in breast and ovarian tumors. These data not only facilitate the identification of differentially expressed proteins that are involved in hepatocellular carcinogenesis, but may also provide candidate biomarkers for the development of therapeutic tools with dietary fatty acids.

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COMPUTATIONAL INTELLIGENCE SOLUTIONS FOR BIOMARKER DISCOVERY IN MASS SPECTROMETRY DATA

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Early detection of cancer is a critical issue for improving patient survival rates. Recent progress in mass spectrometry has shown the promising potential of biomarker discovery in the diagnosis of diseases especially in early stages. In the present study, an alternative approach to feature extraction from mass spectrometry data of prostate cancer is proposed that results in the definition of different biomarkers. The latter provides information-rich features that improve the performance of a PNN classifier in differentiating among datasets with different prostate-specific antigen (PSA) levels of prostate cancer and with no evidence of disease. The prostate cancer dataset was collected from the National Cancer Institute Clinical Proteomics Database. The overall accuracy, in correctly classifying 63 spectra with no evidence of disease (PSA<1) and 43 spectra of prostate cancer with PSA>10 was 98.1%. The high accuracy obtained by the proposed method might lead to informative biomarkers for early stage of prostate cancer diagnosis (*e-mail: bougiouk@upatras.gr).

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A NEW GENERAL APPROACH FOR THE PURIFICATION OF PROTEINS FROM BIOLOGICAL EXTRACTS FOR IDENTIFICATION BY MASS SPECTROMETRY

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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, followed by protein digestion and the classic processes of peptide finger printing and/or peptide sequencing.

Basically the protocol consists of an initial rational selection of a 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 of superimposing sorbents in a cascade manner with the sorbent in charge of capturing 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 is performed by means of mass spectrometry.

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

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UNCONVENTIONAL PREFRACTIONATION METHODS IN PROTEOMICS RESEARCH

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

Classic 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. Here we propose a rational cascade of chromatography media superimposed in several small columns and using the same buffer. As the sample crosses the different adsorbent layers, proteins within are sequentially trapped according to the complementary properties of 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 classic single-chemistry fractionation the proposed approach shows much lower protein overlap between fractions and therefore greater resolution.

The sequence 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 strengths. 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 with the largest specificity. Examples of fractionation will be shown.

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INFLUENCE OF SELENIUM ON METALLOPROTEINS IN THE PROSTATE OF THE RAT

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The prostate gland is a very sensitive organ involved in androgen metabolism. Disorders of the androgen metabolic

process can lead to diseases such as benign prostatic hyperplasia (BHP), prostatitis and prostate cancer, which is currently one of the most common types of cancer of human males. Metals and metalloids have a special role in metabolic processes. Their biological role is associated with their corresponding binding protein and the complex formed develops its biologically active form. Previous studies have shown the relationship of metals/metalloids in the prostate. Some metal-containing proteins were identified by a combination of analytical methods such as SEC and ICP-MS and biochemical techniques. In this way, some metal-containing proteins were identified and localized in the cytosol of the prostate gland. Selenium, a trace element also contained in every tissue of the human body, also plays a very essential role in the metabolism and in redox processes. The selenium status influences the expression of other metallo/metalloid proteins. It is assumed that selenoproteins are necessary for the expression of metallo/metalloid proteins.

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A PROTEOMIC APPROACH TO UNDERSTANDING THE ROLE OF MEMBRANE TRANSPORT PROTEINS IN THE DEGRADATION OF AROMATIC COMPOUNDS

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Large quantities of hydrocarbons are present in the environment, many of which must be removed from contaminated sites because of their toxicity. A huge variety of bacterial strains which have the ability to degrade environmental pollutants into substances harmless to the human body and the environment, are of great importance in achieving the purification of water and soil contaminated by such pollutants (1). These organisms induce the expression of a series of proteins that participate in the metabolic pathway of the degradation of aromatic pollutants in order to use those compounds as the only carbon source for their survival (2, 3). Many bacterial strains have evolved regulated and highly efficient transport processes for aromatic compounds.

Biological membranes form the interface between cells and their environment. Besides the cytoplasmic (inner) membrane, Gram-negative bacteria possess a second lipid layer external to the cell wall, commonly known as the outer membrane (4). The inner or cytoplasmic membrane contains all known active transport systems and many of the

cell envelope enzymes. The outer membrane is distinguished by a unique set of proteins (5). The composition and number of transmembrane spanning helices make these proteins particularly difficult to characterize primarily due to their water insolubility.

Our goal was the study of the membrane proteome of a new strain referred to as *Pseudomonas* sp. strain phDV1, which was isolated in contaminated regions in Denmark and was identified by phenol utilization and 16S rRNA sequence analysis. The strains were grown on aromatic compounds as a sole carbon and energy source. Methods for the fractionation of the membranes were applied which resulted in the separation of the outer from the inner membrane. Electrophoretic methods in combination with mass spectroscopy allowed the identification of the protein change in the presence of the pollutant. Study of the bacterial membrane proteome, though in its early stages, is a field of growing interest in the search for information on nutrient transport and processing and of their implication in bioremediation.

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FISHING IN A RED POND: THE CYTOPLASMIC PROTEOME OF THE ERYTHROCYTE

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When lysing a red blood cell (RBC), haemoglobin (Hb), the major cellular protein, appears to be present at a concentration of *ca.* 97-98%. What the proteome

complement of the remaining 2-3% species is anybody's guess. Recent evidence suggests that there are some other 91 proteins present in the cell sap (1). Even more recent proteomics data suggest that the total number of unique gene products in the minority RBC proteome could be as large as 252 (2). In order to mine deep into the 'hidden' RBC proteome, we have applied a technology based on the use of solid-phase combinatorial peptide ligands to RBC lysates. Packed RBCs from 100 mL of serum, freshly harvested, were washed four times to remove the buffy coat and additionally filtered to minimize the carry-over of lymphocytes (down to negligible amounts). Upon lysis in hypotonic buffer and removal of membrane fragments, a total of 5.5 Hb / 100 mL was filtered through two serially connected columns of packed solid phase libraries (3, 4), each containing 1 mL of pearls. The columns were then independently eluted with 3 mL of the following eluants: TUC (2 M thiourea, 7 M urea, 4% CHAPS; E1 eluate), 9 M urea in citric acid (pH 3.8; E2 eluate) and organic solvent (E3 eluate). The fractions richest in protein species appeared to be E1 and E2. In small dimension 2D maps, E1 and E2, upon silver staining, were found to contain a total of >800 polypeptide chain spots, as opposed to <100 for control, untreated RBC lysates (loaded at 5 times higher concentration), where very heavy trains of α - and β -Hb chains (together with homo- and hetero-dimers) constituted the vast majority of spots.

Upon large-size 2D map analysis (18x18 cm), silvering showed an impressive population of chains, amounting to a grand total of *ca.* 2000 spots. All the eluates are currently under LC-MS/MS analysis to identify gene products. From preliminary data, it would appear that the total number of unique gene products present in a RBC lysate is well over several hundred species.

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TWO-DIMENSIONAL ANALYSIS OF PROTEINASE K-RESISTANT FRAGMENTS PRION PROTEIN IN GSS P102L MUTATION

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The most common Gerstmann-Straussler-Scheinker (GSS) disease phenotype is associated with a point mutation at codon 102, which results in the substitution of proline by leucine.

In P102L mutation, immunoblot analysis of protease-resistant PrP, or PrP^{res}, shows the presence of a C-terminal 21 kDa fragment, in addition to an internal 8 kDa peptide, spanning positions 80-150. Protease-resistant PrP species encountered in P102L mutation are not limited to the above molecules but also include truncated fragments migrating at 17 and 14 kDa. With the exception of the internal 8 kDa fragment, the migration of the 21 kDa core fragment and of truncated PrP species is indistinguishable from that observed in sCJD cases with type 1 PrP^{res}. However, when PrP^{res} species of GSS associated with P102L mutation were analyzed by 2D analysis novel C-terminal PrP^{res} variants were found.

The present findings suggest that the composition of C-terminal variants observed in GSS is different from those found in sCJD and this may account for differences in disease phenotypes.

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SELECTIVE IMMOBILIZATION AND IDENTIFICATION OF PROTEIN COMPLEXES IN ALGINATE BEADS

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Alginate is a polysaccharide consisting of pure or/and mixed groups of the monosaccharides α -L-glucuronate and β -D-mannuronate, linked through β (1, 4) bonds (1, 2). Fast and irreversible binding of polyvalent cations to alginate causes the formation of gel. The ions diffuse from an external solution to the inner of droplets of alginate that fall in it, resulting in the formation of bead-gels able to capture biological material (1). Calcium alginate hydrogels have been used for the entrapment of cells able to produce proteins (e.g. antibodies) (1), yeast cells for more efficient fermentation (5), chloroplasts and mitochondria (6) as well as active enzymes (4, 7). Electronic microscopy has revealed the structure of these hydrogel beads (8), which exhibits a biological affinity for a variety of enzymes, such as pectinase, lipase, phospholipase D, α and β amylases, and glycoamylase. Based on this affinity and the tendency to

'precipitate' in the presence of Ca⁺⁺, alginate has been used for the purification of these enzymes (2-4).

Cytoplasmic proteins from *Pseudomonas* sp. strain pHDV1 were used in this study in order to examine the factors that may determine which proteins are captured. The parameters that were investigated are: i) the alginate concentration, ii) the bivalent cation of the external solution and its concentration in it, iii) the complexity of the initial protein sample and iv) the solution volume and time of washing of the alginate beads that carried the immobilised proteins. The alginate beads were dissolved in (100 mM) sodium phosphate pH 7.4 and the proteins were analysed by polyacrylamide gel electrophoresis. Identification of the proteins that were entrapped in the beads was made by MALDI-TOF MS.

Acknowledgements: The project is supported by the University of Crete and is co-funded by the European Social Fund and National Resources EPEAEKII-PYTHAGORAS.

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PROTEOMIC ANALYSIS OF THROMBI OCCLUDING HUMAN CORONARY ARTERIES

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Percutaneous thrombectomy is an established technique for restoring perfusion through occluded vessels. Aspiration thrombectomy, in which thrombi are removed

by suction with the aid of wide-bore catheters, is used as an adjunct to primary PTCA in acute arterial occlusion. As a by-product, the procedure may provide clinical biochemists with plenty of thrombus samples for thorough characterization.

No comprehensive proteomic analysis has been performed so far on thrombi. In a previous report the immunohistochemical characterization of coronary thrombi in allograft vascular disease using three antibodies (anti-gpIIb-IIIa, anti-fibrin, and anti-endothelium) demonstrated that their composition is related to both the type and age of the thrombus, with platelets as the early and major components of mural microthrombi, and fibrin as the dominant component of occlusive thrombi (1). In another report, *in situ* fibrin degradation was assessed by electrophoretic analysis of thrombi. In acute vascular obstruction, intact, undegraded crosslinked gamma-gamma dimers dominated whereas chronic aortic aneurysms contained extensively degraded fibrin (2).

In this communication, we report on a pilot investigation on a few thrombus samples. We compared solubilization and running conditions and worked out a simple yet effective procedure for proteomic analysis through 2DE electrophoresis. By MS analysis we then identified the main protein components of carotid artery thrombi. The majority of the spots corresponded to *fibrin(ogen) fragments* but several other components could be identified. These included *plasma proteins* other than fibrin(ogen) (albumin, coagulation factor XIII A chain, apolipoprotein A-I), typical *erythrocyte components* (hemoglobin, band 3 anion transport protein, erythrocyte band 7 integral membrane protein, erythrocyte-derived growth-promoting factor), *cytoskeletal proteins* (actin, actinin, myosin, tropomyosin 1 and 4, F-actin capping protein α -1 subunit, tubulin α -1 and α -8 chain, talin, gelsolin and Rho GDP-dissociation inhibitor 2), *enzymes* (involved in *energetic metabolism*: α -enolase, glyceraldehyde-3-phosphate dehydrogenase, L-lactate dehydrogenase B chain, pyruvate kinase, isozymes M1/M2, transketolase, ATP synthase beta chain; in *acid/base equilibrium*: carbonic anhydrase I; in protein processing: proteasome subunit α type 3, protein disulfide-isomerase A3; in redox equilibrium: catalase, peroxiredoxin 2 and 6, superoxide dismutase, also: DJ-1 protein, a redox-sensitive chaperone and as a sensor for oxidative stress) and *signal transduction pathway components* (chloride intracellular channel protein 1, 14-3-3 protein zeta/delta, Ras suppressor protein 1).

A preliminary investigation on 8 thrombus samples detected 3 distinct patterns of fibrin(ogen) proteolysis.

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PROTEOMIC ANALYSIS OF RYE (*SECALE CEREALE*) IN THE PRODUCTION OF BIOETHANOL: STUDY OF PROTEIN RECOVERY

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When dealing with production of biofuels, it is very important to include a number of factors in the cost equation, such as the energy necessary for the production, transformation and transportation of biomasses. Possible re-utilization of by-products in the production cycle may have a prominent impact on the economic viability of these processes. Among the crops utilized for production of bioethanol are maize, wheat, oat and rye, all of which leave important protein residues, amounting to 7.4%, 12.0%, 13.1% and 12% of the total biomass, respectively, with a potential 15000 tons of protein per 100 million litres of bioethanol produced. This proteinaceous material, which can be exploited in both animal feed/human consumption and for the production of non-food products, must be recovered to a maximum extent if the production of biofuel is to be rendered economically favourable. Therefore, a prerequisite to the industrial valorization of seed proteins is that the overall process stages do not irreversibly destroy protein functionality and high quality standards can be reached, as for soybean proteins. We have set up a proteomic investigation aimed at studying the full proteomic asset of rye (*Secale cereale*) and at exploring the feasibility of extracting the waste protein from the by-products of the industrial process *via* an economically attractive procedure. For establishing a comprehensive 2D map of rye proteins, different extraction protocols (2% SDS + 5% 2-mercaptoethanol (2-ME); ethanol + 2-ME; 2% SDS + 60 mM DTT + 20% glycerol in Tris-HCl, pH 8.5; 7M urea + 2M thiourea + 3% CHAPS + 40 mM Tris base + 0.5% Ampholine pH 8-10, TUC) from flours and intermediate wastes were tested. Of all systems tested, the best 2D maps appeared to be the ones obtained with the classical TUC solvent. Different extraction/separation procedures were investigated with emphasis on the use of end product ethanol as extraction solvent for flour or meals obtained after enzymatic saccharification or fermentation. Different water/ethanol ratios were investigated in discontinuous or continuous extraction processes. 2D map analysis of 95% ethanol extract from flour identified only very few polypeptide chains, mostly of small Mr value (10-20 kDa), whereas with 75-60% EtOH the proteins span from Mr 20 to 120 kDa

and pI values were mostly confined to the neutral to basic range (pH 6.5 to 10) (1). This 2D pattern clearly shows a profile belonging mostly to storage proteins (consisting of gamma-75k-secalins, followed by gamma-40k-secalins (about 25%); omega-secalins (16%) and high Mr-secalins (7%) (2). After the saccharification stage basic proteins are mainly lost and extensive degradation of secalins occurs in the fermentation stage (3). Work is in progress to identify the best conditions for recovering proteins, thus ameliorating the extraction procedure.

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CHEMORESISTANCE STUDY OF CANCER CELLS USING PROTEOMELAB PF 2D SYSTEM AND ITRAQ FOR PROTEIN IDENTIFICATION AND QUANTIFICATION

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Synthetic tri-substituted purine derivatives specifically inhibit CDK1 family of kinases and represent a new generation of potential anticancer drugs. In this study, pharmacologically active CDK inhibitors (CDKIs) and a model of human CEM T-lymphoblastic leukaemia cells, a representative of haematological malignancy, were utilised to study the development of chemoresistance. Resistant CEM counterparts (CEM-R) were derived by culturing CEM cells in the presence of sub-lethal doses of CDKI. Proteins from CEM and CEM-R were separated by a two-dimensional liquid chromatography PF 2D system (Beckman Coulter). Candidates for differently regulated proteins were identified and quantified by MALDI-TOF and isobaric tags for relative and absolute quantitation (iTRAQ) coupled to tandem mass spectrometry, respectively. Among them, Rho GDP-dissociation inhibitor 2, vimentin, heat shock cognate 71 kDa protein, proline-rich protein 4 precursor, stress-induced-phosphoprotein 1, 60S ribosomal protein L21 and hnRNP A1 represent proteins associated with development of chemoresistance. Additionally, stress-induced-phosphoprotein 1, which is a direct target of CDK1, deserves further verification by in-

depth studies as a potential biomarker of tumour response. This study was supported by CSF 301/05/0418 research grant, MSMT grant LC07017 and Institutional Research Concept AV0Z50450515.

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METHOD FOR THE ANALYSIS OF NATURALLY OCCURRING PEPTIDES AND SMALL PROTEINS IN BIOLOGICAL FLUIDS USING MASS SPECTROMETRY

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Biological fluids are considered to be valuable biological samples because they are extremely rich in proteins and can be readily obtained from the organism, routinely by invasive methods. Because of the variability of the protein content of biological fluids, it is evident that within this content dwells information relevant to various physiological and pathological states of the organism. Therefore, the identification of various types of protein molecules could offer valuable information relevant to the organism's reactions to various stimuli and states, allowing in depth comprehension of cellular and tissue malfunctions which lead to various diseases and last but not least new therapeutical targets.

Naturally occurring peptides (NOP) are considered as the products of systematic cellular proteolytic activity and protein processing which lie at the core of the cellular metabolism. NOP together with the small proteins (SP) move through tissues and cellular formations easily, diffusing in the biological fluids. Since the NOP and SP are relatively resistant to proteolytic degradation and they have a long half-life which render them as suitable candidates for biological markers. In the present work, we developed a method for the separation and identification of the naturally occurring peptides and small proteins (NOP/SP) present in biological fluids with the use of mass spectrometry. The separation of protein molecules with molecular weight <10 kDa is achieved through mixing of one volume of the biological fluid with an aqueous solution containing acetonitrile and trifluoroacetic acid. As opposed to the methods currently used, the present method does not include complicated and time consuming steps (*e.g.* fractionation, chromatography and ultrafiltration). The NOP/SP analysis with MALDI-TOF method is rapid, subject to automation and quantification. These elements make the method one of high throughput and appropriate for diagnostic methodologies, either through analysis of one or more NOP/SPs as specific biological markers, or through

comparison of normal and pathological patterns/profiles (Patent No.: GR1005304, Appl. No.: EP200060386024, Prior. No.: GR20050100392).

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PROTEOME ALTERATIONS OF TEL/AML1 CONDITIONAL KI MOUSE

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The most common genetic abnormality in pediatric acute lymphocytic leukemia (ALL) is t(12;21)(p13q22), observed at a 25% rate of incidence (diagnosed between ages 2-9 years), compared to the small percentage in adults (1-3%). These patients demonstrate a blockage during the pre- B-cell stage in hematopoietic development, probably as a result of the fusion of two genes, *ETV6* (*TEL*) (chromosome 12) and *RUNX1* (*AML1*) (chromosome 21). The *TEL/AML1* chimeric product consists of the amino-terminal *ETV6* region and almost the entire amino acid *RUNX1* sequence. Both genes encode for transcription factors; *TEL* is a member of the ETS-like transcription factors family, acting as a strong transcriptional repressor, and *AML1* belongs to the Runt Domain (RD) transcription factors family, regulating the expression of hematopoietic genes by acting as a transcriptional regulator (activator or repressor). The chimeric protein of the t(12;21) is believed to convert *AML1* into a strong transcriptional repressor but the data to support the theory are not compelling.

Therefore, the focus of this work is to study the molecular functions and interactions as well as the pathways that link *TEL/AML1* to hematopoietic malignancies. This knowledge will open new perspectives to achieving novel strategies for competent and effective, low-cost, limited side-effect targeted-therapies.

For this purpose, a mouse was generated expressing the *TEL/AML1* conditionally under the endogenous *TEL* promoter. The expression of *TEL/AML1* was additionally regulated by the *Cre/loxP* site-specific recombination system. The animals were screened by PCR and Southern blot analysis for the presence of the DNA 'target' cassette. Adult fibroblasts were isolated from mouse tails and cultured *in vitro*; when Cre was provided, the correct recombination event took place and *TEL/AML1* was expressed, verifying that the mice and the cells deriving from them were indeed expressing *TEL/AML1* in a conditional manner.

Primary embryonic fibroblasts (PEFs) were isolated from embryos (E15.5-E17.5) of *TEL/AML1* conditional knock in and wild-type litter mates. These cells were transduced with viral vectors expressing Cre and puromycin^R or only puromycin^R. After selecting the puro^R clones, RNA was extracted and RT-PCR analysis was performed on the puromycin-resistant cells, demonstrating that the chimeric mRNA was present only when Cre recombinase was provided to the system. Proteins were extracted: i) *TEL/AML1* con.KI PEFs + puro^R, 2) *TEL/AML1* con.KI PEFs + Cre.puro^R, 3) wt PEFs + puro^R, 4) wt PEFs + Cre.puro^R and proteomic analysis was performed. More specifically, the proteins were separated by two-dimensional gel electrophoresis, analyzed by MALDI-MS and identified by peptide mass fingerprinting (PMF). The data from this experiment indicate that several different gene products were identified. The comparison of the four gels indicated that 32 proteins were expressed in the presence of *TEL/AML1* and 6 were absent. We are performing further characterization of these proteins in order to clarify the role of these molecules in the pathogenesis of ALL.

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PROTEOME ALTERATIONS AFTER P21 INDUCTION IN SAOS2 CELL LINE

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p21Cip1 is a protein inhibitor of cyclin-dependent kinases and belongs to the Cip/Kip gene family. Diverse roles of p21 in cellular processes makes it a necessity for the cell in order to proceed in several functions such as replicative senescence, cellular differentiation, apoptosis and the DNA damage response pathway. Thus its expression is regulated by two major pathways: a p53-dependent pathway and a p53-independent pathway (1). In response to DNA-damaging agents, p21 is expressed and accumulated by the p53-dependent pathway to cease the cell cycle in G1/S and G2/M transition so that DNA can be repaired. On the other hand, p21 transcription can be activated in a p53-independent manner by agents that promote differentiation, such as transforming growth factor- β , Ca²⁺ and MyoD, where p21 causes G1 cell cycle arrest.

In order to study the effect of p21 in DNA damage response pathway and carcinogenesis, we used the inducible Saos2-tet ON-p21 system. The human osteosarcoma Saos2 cell line is one of the first generated cell lines, available since

1973, and is broadly used in all biomedical research fields and is derived from the primary osteogenic sarcoma of an 11-year-old Caucasian female. One of the basic characteristics of the Saos2 cell line is the existence of mutations both in the p53 and Rb genes. As a result, these mutations lead to complete absence of p53 and Rb protein, both at mRNA and protein level (2). These two genes are known for their tumor suppressor activity. Furthermore mutations of these two genes are found in various cancers. Absence of p53 expression is the main reason we used the Saos2 cell line to create the inducible Saos2-tet ON-p21 system where p21 expression is under the control of doxycycline (3).

In the present work, we applied proteomic technologies to gain an overview of the changes after p21 induction in Saos2-tet ON-p21 cells. Comparison of the proteomes of Saos2-tet ON-p21 cells before and after the induction of p21 as well as the proteome of the parental Saos2 cell line allowed us to discriminate 95 differentially expressed proteins. Twenty-five proteins were down-regulated and 70 proteins were up-regulated. Further analysis indicated that these proteins belong to 8 groups with major cellular functions. The groups were the 14-3-3 family, the ubiquitin pathway, cytoskeletal proteins, DNA transcription/ translation proteins, adherent proteins, splicing factors and proteins participating in metabolic pathways and the immune system.

Coupling of 2DE proteomics with inducible systems like that of Saos2-tet ON-p21 can provide us with useful information about downstream effectors of a gene product and a screen of proteins involved in cellular functions.

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PROTEOMIC ANALYSIS OF NF-κB-INDUCED ALTERATIONS IN HUMAN LYMPHOMAS

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The Epstein-Barr virus (EBV) is implicated in the pathogenesis of certain human lymphomas such as Burkitt's

lymphoma, lymphomas appearing in immunocompromised individuals, and post-transplant lymphoproliferative disorders, infecting mainly B-lymphocytes. EBV is a member of the herpesvirus family and is carried by the majority of the human population. The transformation of B-lymphocytes by EBV depends upon the activation of NF-κB, which is triggered by the latent membrane protein 1 (LMP1). LMP1 is a transmembrane viral protein, important for EBV-mediated B-cell proliferation that seems to mimic activated tumor necrosis factor receptors.

Nuclear factor κB (NF-κB) is a transcription factor that has crucial roles in inflammation, immunity, cell proliferation and apoptosis. NF-κB exists in a latent state in the cytoplasm and is triggered by a variety of extracellular stimuli. Activated NF-κB can then enter the nucleus and regulate the expression of multiple target genes, after binding to discrete DNA sequences, known as κB binding sites.

We have mainly focused on finding differences in the proteomic profile caused by EBV in Burkitt's lymphoma cell lines. The starting material is total cell lysate or subcellular fractions (nuclear, membranes, cytoplasm) in a chaotropic solution. We use two dimensional (2-D) electrophoresis analysis and the proteins are separated in both wide and narrow range IEF (isoelectric focusing) strips. Narrow range in-solution IEF fractionation is performed as well, in order to reduce sample complexity and improve separation and detection. The differentially expressed proteins are identified by LC-MS/MS. The proteins identified are evaluated for their correlation to NF-κB and EBV-induced lymphomas. We will present preliminary data on some potential EBV-related genes.

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PHOSHOPEPTIDE ANALYSIS – COMPARISON OF IMAC AND TIO₂ ENRICHMENT

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The characterization of post-translational modifications is a focus of molecular biology with special attention to phosphorylation, which plays a key role in signal transduction and in the regulation of numerous biological processes. Despite continuous method and instrumental development in protein mass spectrometry, the determination of the site(s) of phosphorylation still is a challenge.

For years, immobilized metal affinity chromatography (IMAC) dominated phosphopeptide analysis. In this technique, the phosphate group is trapped by a metal ion (usually Fe³⁺ or Ga³⁺) coordinated by an immobilized

chelating agent (IDA or NTA). Binding of acidic nonphosphorylated peptides is eliminated by methyl esterification of the carboxylic groups, which may lead to decreased sensitivity due to the incomplete derivatization. In 2005, phosphopeptide enrichment by TiO₂ was introduced. Under highly acidic conditions phosphopeptides are bound to TiO₂ almost exclusively, then released again upon ammonium hydroxide treatment. Its simplicity and high selectivity made it a very attractive alternative to IMAC.

Comparison of the two enrichment techniques on standard digests, recombinant and *in vivo* samples will be presented.

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REVEALING HIDDEN BIOMARKERS USING
ProteomeLab™ IgY PROTEOME
PARTITIONING SOLUTIONS

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Biomarker discovery is like panning for gold where finding the nuggets gets easier when there is less sand. Within bio-fluid proteomes such as plasma and serum, that 'sand' is comprised of well-characterized proteins. Identifying the potentially golden biomarkers in the presence of these highly abundant proteins has proven to be a serious challenge to current analytical techniques. In serum and plasma, the dynamic range of proteins spans over 10 orders of magnitude, far greater than the measurement capability of current technologies. Additionally, potential biomarkers are entirely masked by the overwhelming abundance of relatively few proteins. Protein depletion has been used for some years to remove most of the albumin and/or IgG from bio-fluids such as plasma and serum prior to analysis, but it is clear that this alone is insufficient to enable progress to be made in biomarker discovery. The presence of highly abundant proteins significantly complicates the discovery process by masking the presence and limiting the detection of low abundance species. The ProteomeLab™ IgY partitioning system addresses this issue by reversibly capturing 12 of the more abundant proteins from human bio-fluids such as plasma and serum, yielding an enriched pool of low abundance proteins for further study. The captured proteins can also be easily recovered for investigation if required, hence the term partitioning rather than depletion. This technology together with the ProteomeLab™ PA 800 Protein Characterisation System may facilitate protein analysis within research and pharmaceutical industry for verifying purity of therapeutics during development and manufacturing processes (*e-mail: sgadher@beckman.com).

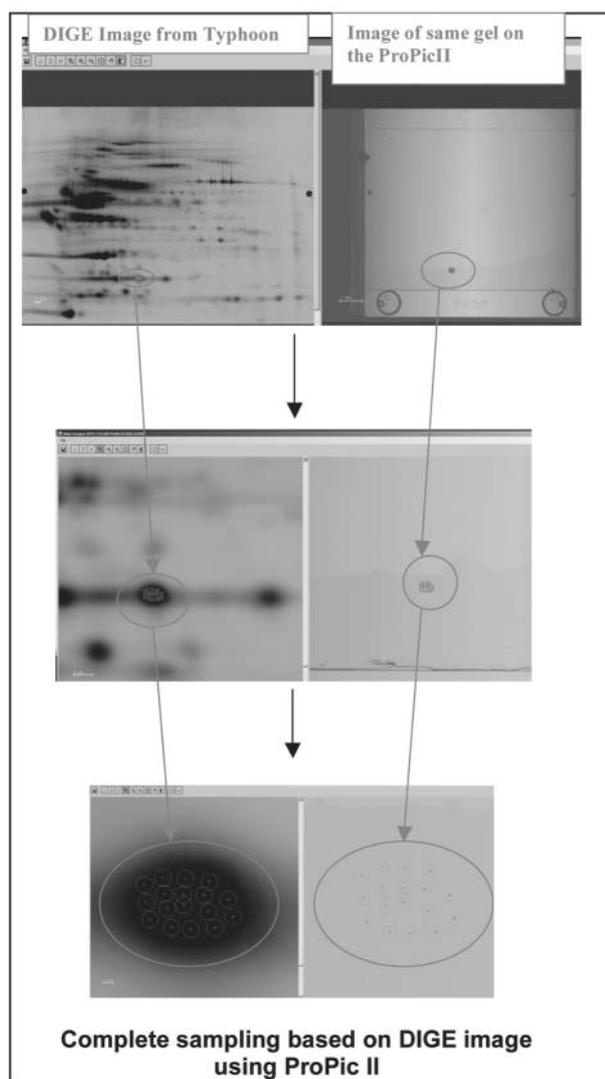
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MAXIMIZING PROTEIN RECOVERY FROM
DIGE-ANALYZED GELS THROUGH EFFICIENT
SPOT EXCISION TO IMPROVE MASS
SPECTROMETRIC ANALYSES

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Proteomics research over the past decade has created a revolution in the way we think and study disease at the protein level. Moreover, the study of proteins in a gel medium (1D or 2D SDS-PAGE), coupled to mass spectrometric characterization, is an indispensable tool in many laboratories



around the world for such tasks. This notwithstanding, challenges still exist in both gel-electrophoresis and mass spectrometry of proteomes. The former analytical method, in conjunction with fluorescent labeling technology (collectively termed differential in-gel electrophoresis, DIGE), can detect low femtomoles of proteins whose expression changes may be biologically important. For the latter, one has to contend with limits of detection which can often vary based on the instrument design, performance and price. When coupled with robotics for protein spot excision off 2D SDS-PAGE gels, sampling of the protein spot also becomes an important issue.

Traditionally, DIGE gels require de-staining and re-staining with a second stain for spot picking. This is costly and time-consuming. The ProPic II has been developed by Genomic Solutions to read DIGE images and allow for spot excision directly from 2D gels using a DeCyder picklist. While the robot provides for unparalleled picking accuracy in this manner, beta software has also been developed for the ProPic II that allows for complete sampling of the protein spot from DIGE gels. In this workflow, the DIGE image and the ProPic II image are aligned and in a click-and-pick fashion, excision points for the picking robot are marked out. This improves sampling of the protein spot area and allows for more protein to be excised from the gel. As such, the amount of peptides recovered following in-gel digestion is maximized, helping bridge the gap between DIGE sensitivity and a mass spectrometer's detection limits. Evidence for this way of picking protein spots over the classic method will be presented.

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PROTEOMIC CHARACTERIZATION OF HUMAN MESENCHYMAL STEM CELLS (MSCS) DERIVED FROM AMNIOTIC FLUID: COMPARISON TO BONE MARROW MSCS

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Human mesenchymal stem cells (hMSCs) constitute a population of multipotent adherent cells able to give rise to many mesenchymal lineages such as osteoblasts, adipocytes or chondrocytes. So far, the most common source of MSCs has been the bone marrow (BM); however BM-MSC harvesting

exhibits major drawbacks. Thus, identification and characterization of alternative sources of MSCs is of great importance. In the present study, we isolated and expanded fetal MSCs of embryonic origin from second trimester amniotic fluid (AF). We systematically tested the immunophenotype of expanded MSCs by flow cytometry analysis using a wide variety of markers. Direct comparison of this phenotype to that derived from BM-MSCs demonstrated that MSCs from both sources exhibited similar expression patterns. Using two dimensional gel electrophoresis and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) approach, we have generated for the first time the protein map of AF-MSCs by identifying 261 proteins and we directly compared it to that of BM-MSCs. We classified the AF-MSC-derived proteins based on their subcellular localization: 41% of proteins were located in the cytoplasm, while others were found in the endoplasmic reticulum (8%), nucleus (13%), mitochondria (12%), ribosomes (1%) and cytoskeleton (6%). The functional pattern of the identified proteins from both sources was classified into several categories related to cell growth and maintenance, cellular and protein metabolism, RNA and DNA regulation, transcription and translation, transport, apoptosis and immune response. Both analyses performed for BM-MSCs-derived proteins exhibited similar results. However, AF-MSCs displayed a number of unique proteins related to proliferation and primitive phenotype, which may explain the distinct features of the two types. Considering the easy access to this new cell source and the yield of expanded MSCs for stem cell research, AF may provide an excellent source of MSCs both for basic research and for potential therapeutic applications.

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DEVELOPMENT, CHROMOSOMAL AND PROTEOMIC CHARACTERIZATION OF A T24-BASED CELL LINE MODEL FOR BLADDER CANCER AGGRESSIVENESS

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Cell culture and animal models of disease are invaluable research tools utilized to increase our knowledge on the molecular mechanisms of tumorigenesis, to identify and

characterize diagnostic and prognostic markers and also pinpoint and evaluate potential drug and therapeutic targets. In the study presented herein we report the development of a bladder cancer cell line model that involves the widely used T24 cell line and the newly developed T24M invasive variant. Besides the characterization of these two cell lines at the chromosomal level, a detailed proteomics analysis is reported.

In brief, for the analysis at the chromosomal level SKY has been conducted. The proteome of the cell lines has been analyzed by two dimensional electrophoresis in combination to MALDI-TOF mass spectrometry. Western blot, immunohistochemical analysis of bladder cancer tissue sections and proteasome activity assays have been conducted to verify the results of the proteomics studies.

The chromosomal analysis demonstrates a) the correlation by lineage of the two cell lines and b) reveals chromosomal differences that may reflect the increase in the aggressiveness in the T24M cells compared to the parental T24. The proteome map of the cell lines has been generated consisting of approximately 250 unique proteins. Comparison of the protein expression levels in the T24 and T24M cells reveals multiple differences in various types of proteins including proteins of the cytoskeleton, heat shock and chaperone proteins, signal transduction molecules as well as proteins involved in proteolysis. In the case of the latter, the proteasome subunit PSB6, is up-regulated whereas Cathepsin B and D appear to be down-regulated in the T24M compared to the T24 cells. The differential expression of these proteins were confirmed in case of Cathepsin B by Western blot analysis in cell lines lysates and immunohistochemical analysis of bladder tissue specimens. In the case of proteasome subunit, a proteasome activity assay was conducted which confirmed the presence of increased proteasomal activity in the aggressive T24M cell line. Collectively, these results lead to the hypothesis that in aggressive bladder tumors there is a selective activation of the ubiquitin/proteasome rather than the cathepsin/ lysosomal pathway. More experiments towards the investigation of this hypothesis may shed more light on the specific role of these two pathways in tumor progression.

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METHOD OPTIMIZATION FOR THE PROTEOMICS ANALYSIS OF PURE POPULATIONS OF KIDNEY CELLS

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It is well established that in several diseases, tissue alterations occur in a focal manner; simply stated, certain areas of tissues develop known pathologies, whereas others in close proximity

and of a similar type do not develop any pathological signs, at least at the morphological level. It is important, in order to better understand the molecular mechanisms underlying these alterations, to be able to isolate and study the areas of the tissue that develop the alterations. In order to achieve this, the application of laser capture microdissection is recommended.

The objective of our study is to establish a methodology for the proteomic analysis of pure populations of kidney cells. The significance of this study stems from the fact that several renal diseases, at least in their early phases, affect either the cortex or the medulla of the kidney. We isolated by laser capture microdissection cortical areas and specifically the tubular compartment from rat renal frozen sections. Extracts from 20,000, 10,000, 5,000 and 1,000 cells were subjected to saturation cysteine labeling and separated on 18 cm 3-10 pH range strips followed by SDS-PAGE. High protein resolution was observed particularly at the 40-80 kD mass range and in the cases of extracts from 20,000 and 10,000 cells; however the protein resolution at the lower (<30 kDa) molecular weight range was significantly reduced. Our current efforts involve further optimization of the technique by the modification of the labeling conditions as well as the of protein extraction protocol.

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AN UNSUPERVISED SPOT DETECTION ALGORITHM FOR TWO-DIMENSIONAL GEL ELECTROPHORESIS IMAGES

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Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis is a well-established technique which analyzes large collections and complex mixtures of proteins simultaneously. The outcome of 2D-PAGE analysis is a two dimensional polyacrylamide gel (2D-PAGE gel) containing distinct spots, each being a separate peptide. Digitization of 2D-PAGE gels produces 2D-PAGE images in which peptide spots appear as spots of various intensities and sizes. The analysis of the 2D-PAGE images is a significant bottleneck in the proteomics research field. In particular, image segmentation, spot detection and spot quantification are plagued by poor resolution, image inhomogeneities, spot overlapping, and the presence of noise. As a result, commercial software packages require manual intervention which can limit the statistical significance of the generated numerical data since subjectivity is introduced.

An original approach to unsupervised protein spot detection in 2D-PAGE images based on a genetic algorithm is presented. The approach is divided into three

main steps: Firstly, the quality of the input images is improved by applying a wavelet-based filtering which reduces the impulse noise. Then the de-noised images are segmented into regions each having a significant possibility to contain a spot. In particular, the de-noised images are segmented around the local maxima of image intensities since local maxima are the most probable candidates for spot centres. Finally, spots are detected and model-based quantified. A genetic algorithm searches within a multi-dimensional parameter space to determine the parameters of multiple diffusion models that optimally fit the characteristics of possible spots. The detection and quantification of the spots is achieved by the superposition of diffusion functions modelling adjacent spots. The optimal spot models are stored in memory and their cross-section with the image plane is depicted, so that the boundaries of the spots become visible.

The proposed method has been tested on a set of real 2D-PAGE images digitized at 2250x3000 pixels and at 16-bit grey level depth. Each gel contains approximately 1500 spots. The performance of the proposed method was compared with the equivalent of the Melanie software. Both methods detected a comparable number of real spots (95.3%) but failed to detect 4.7% of the real spots. However, the proposed method results in a clearly lower spurious spot detection rate; as a matter of fact, the proposed method detected only 3.0% while the Melanie software detected 8.6%.

Concluding, it should be noted that the proposed method has the following advantages: a) it does not require a training phase; b) it is capable of detecting overlapping spots; c) it is capable of detecting spots distorted by imperfect diffusion of the spot substance across the gel medium; d) when compared with the state of the art commercial Melanie software package, it results in a clearly lower spurious spot detection rate. Future work includes further experimentation, optimization and parallelization of the proposed method, and its integration in a complete user-friendly software application.

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QUALITY CONTROL OF PROTEIN EXPRESSION LIBRARIES BASED ON A DEFINED FALSE-POSITIVE RATE (FPR) FOR AUTOMATED PROTEIN IDENTIFICATION

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Introduction: The increasing application of protein biochips for biomarker discovery or target validation generates a need for a huge number of well-characterized proteins. Sources of these proteins are expression libraries which contain ten thousands of cDNA clones from different tissues (Uniclone). Characterization of these libraries is a central task in the

quality control of protein biochip development. In our case, it is achieved by high throughput protein identification by mass spectrometry based on 1D gels. For this purpose a defined false-positive rate (FPR) is required from the point of view of quality control. This gives the opportunity for defining, controlling and realizing certain requirements of quality, e.g. a FPR below 1%, in a fully automated way. *Materials and Methods:* For protein identification we used standard tryptic in-gel digestion with subsequent MALDI mass spectrometry analysis (ultraflex). High throughput was realized by using robots for sample preparation (sp and dp) and a database system for analyzing the acquired MS- and MS/MS spectra (proteinscape). To enable the determination of a false-positive rate, a so-called composite database was used for performing database searches. It consists of two parts, first, a 'normal' amino acid sequence database, e.g. NCBIInr, and second, a reversed or scrambled database. The advantage is the ability to calculate, not only estimate, the false-positive rate based on the obtained search results. The associated significance level is used for automated evaluation of search results. *Preliminary Results:* Actual work is directed on characterization of a protein expression library from lung tissue containing about ten thousand cDNA clones. As a first quality check, the expressed and purified proteins were separated by one-dimensional gel electrophoresis. From these, gels bands were selected for the analysis by MALDI mass spectrometry. To date, about 2000 bands have been processed by tryptic in-gel digestion, and 2,000 MS spectra and about 16,000 MS/MS spectra were acquired. With processing the spectra were imported into proteinscape where the associated database searches were automatically performed against the composite database using the search engine Mascot. Analyzing the search results from the composite database, the significance level for a false-positive rate below 1% was determined and used for automatic evaluation of search results. Using this strategy we identified about 1200 proteins. Working time formerly used for manual inspection of search results was reduced by roughly a factor of ten. We obtained protein identification results with a defined quality standard (FPR below 1%) excluding subjectivity in result evaluation by individual users.

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PHOSPHOPEPTIDE ENRICHMENT AND ANALYSIS USING A COMBINATION OF METAL OXIDE AFFINITY PURIFICATION AND LC-MALDI MS

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Phosphorylation is an important regulator of cell function in eukaryotes. This post translation modification can alter protein localization, regulate protein function and stabilize and mediate their interactions. Due to their associated negative charge, phosphopeptides are often poorly ionized compared to their non-phosphorylated counterpart and their analysis often complicated due to their low cellular abundance. Therefore, it is critical to selectively enrich the phosphopeptides prior to MS analysis.

In this study, we have evaluated a new means of enriching phosphopeptides by using a metal oxide-based solid-phase extraction (SPE) micro-scale device where the eluent is analysed by LC-MALDI MS. This sorbent has a high affinity for phosphopeptides and the problem of acidic peptide

adsorption associated with the immobilized metal-ion affinity chromatography (IMAC), the previous technique, is greatly minimized.

Furthermore, recent developments in MALDI and LC-MALDI spotting devices, allow the coupling of the off-line chromatographic separation step to the subsequent MS analysis. Data will be presented showing the enrichment of phosphopeptides from a digest of a single phospho-protein, beta casein, analyzed by LC-MALDI MS, where enhanced performance was observed in terms of the selectivity of the phosphopeptides. Further analysis of more complex protein mixtures (six and twelve standard proteins) which contain phosphopeptides will be presented demonstrating the selectivity achieved using an aromatic carboxylic acid additive.

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