19.1
Data Acquisition and Integration in the Pilot Phase of the HUPO Plasma Proteome Project

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A major aim of the pilot phase of the HUPO Plasma Proteome Project (PPP) is the comparison of protein identifications and evaluation of combinations of technology platforms from more than 35 participating laboratories in 14 countries. Thus, it was essential to develop an efficient method of data acquisition, storage, analysis, and public access. Data management in such a large scale collaborative project requires a complex infrastructure, prescriptive enough to allow integration of the results from all participants, yet flexible enough to react to changing requirements and new insights gained during the course of the project. We report on the setup of the data management infrastructure, describe successive adaptations, and present the data processing workflow that allows for integration of protein identification results from many disparate sources. We will share many lessons arising from efforts to obtain comparable data from so many different combinations of fractionation, mass spectrometry instruments and search engines, databases, and criteria for “high confidence” and “low confidence” protein identifications. From the PPP experience to date, we have recommendations for future large scale proteomics projects.

19.2
Abundant-Protein Depletion Coupled with Multidimensional Fractionation of the Human Plasma Proteome

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The extremely complex composition of blood plasma consists of blood proteins and those resulting from tissue secretion or leakage. The dynamic range of protein concentration is >10 orders of magnitude, where the ten most abundant proteins comprise 93% of the total mass. This makes it difficult to detect low-abundance proteins that are biomarker candidates. This paper presents the benefits of depleting highly-abundant, plasma proteins prior to a multidimensional approach for proteome profiling. This approach utilizes two-dimensional, liquid chromatography for fractionation of the plasma proteome. Plasma was treated with microbead-conjugated avian IgY antibodies to specifically remove human serum albumin, Haptoglobin, IgG, IgA, IgM, transferrin, fibrinogen, apolipoprotein A-I, apolipoprotein A-II, haptoglobin, α-1 antitrypsin, α-1 acid glycoprotein and α-2 macroglobulin. Treated and untreated plasma were separated by chromatofocusing in the first dimension where proteins are separated by pI over a pH range of 8.5 to 4.0. Fractions, collected based on pH, were separated in a second dimension by high-resolution, reversed-phase chromatography. The proteome profiles were compared before and after IgY gel treatment. Immunoaffinity removal of the twelve abundant proteins increased the number of proteins observed.

19.3
Differential Expression Analysis of the Human Plasma Proteome Following Myocardial Infarction Using Direct LC-MSⁿ Measurement for Protein ID and Quantification

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Novel Aspects: New analytical methodology and computer algorithms for the direct (non isotopically labeled) semi-quantitative differential measurement and identification of plasma proteins in human MI.

Summary: Myocardial infarction (MI) is a major cause of morbidity and mortality worldwide. Proteomics may be used to identify both improved diagnostic markers and pathways triggered during myocardial injury that might serve as therapeutic targets. We therefore studied serial plasma samples from patients having MI to track global changes in the protein profile, implementing a new protein fractionation and bottom-up multidimensional LC-MSⁿ methodology. In this methodology (reviewed elsewhere at this meeting by Richmond et al.) all time points and fractions are analyzed in triplicate and their quantitative changes determined using a new computer algorithm for the chromatographic alignment and relative quantification of LC-MS features without the use of stable-isotope labels. Furthermore, protein identification and PTM mapping is also achieved using multiple database searching and de novo sequencing algorithms. Preliminary results from this model have allowed us to quantify known biomarkers of cardiac disease (e.g. C-reactive protein, CRP), as well as identify several biologically relevant low-level soluble factors with potential applications as biomarkers.
Plasma Proteomics: The Future Diagnostics for Clinical Chemistry

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The future diagnostics for clinical chemistry will be expected to provide more clinically useful information and cost less. Is plasma proteomics the answer? In United States, one in four deaths is caused by cancer. Since cancer is a proteomic disease, the study of cancer proteomics not only allow us to better understand the biology of cancer, but it also provide us the opportunities to identify the dysfunctional protein as potential target for therapy and protein biomarkers for cancer diagnostics. New approaches to identify plasma biomarkers for the detection of diseases are urgently needed. My presentation will focus on the use of surface-enhanced laser desorption-ionization (SELDI) protein chips coupled with time-of-flight mass spectrometry (MALDI-TOF). Because large amounts of data are often generated, the effective and appropriate use of bioinformatics tools become critical to analyze the expression data and to avoid selecting biomarkers whose performances are influenced mostly by non-disease related artifacts in the data. In summary, the combination of proteomics and bioinformatics tools could facilitate the identification of proteins of clinical interest, leading to the development of diagnostics for clinical chemistry. I will present some of our studies at the Johns Hopkins Biomarker Discovery Center and the HUPO plasma proteome initiative.

Immunoaffinity Depletion of High-abundant Proteins from Serum Samples with Spin Cartridges

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Analysis of human serum can provide valuable information for the discovery of new biomarkers and novel drug targets. Serum samples are difficult to characterize due to the high-abundant proteins such as albumin, IgG, transferrin, haptoglobin, IgA and anti-trypsin. These proteins interfere with identification and characterization of the important low-abundant proteins by limiting the dynamic range of mass spectral, electrophoretic and chromatographic analysis. An immunoaffinity spin cartridge has been developed for simultaneously selecting and removing six high-abundant proteins from human serum. Affinity-purified polyclonal antibodies against the six high-abundant proteins were attached to porous polymeric beads individually. The resulting beads were blended in the proportions representative of the proteins found in human serum and packed into a single spin cartridge that provided efficient loading, washing, elution and equilibration (10 minute run) without the need for LC instrumentation. Specificity of the depletion and cartridge reusability were assessed by SDS-PAGE and LC/MS/MS analysis of the flow-through and bound fractions. 1D gel analysis of the flow-through fractions during multiple runs (up to 200 runs) indicates consistency and robustness of depletion. The spin cartridge removes 98–99% six high-abundant human serum proteins indicated by the ELISA of flow-through fractions. Similar performance characteristics were observed for spin cartridges specific for the removal of top 3 high-abundant proteins (albumin, transferrin, IgG) from mouse and rat serum. The efficient and highly specific removal of high-abundant proteins enables loading of 7 to 10 times greater quantities of low-abundant proteins onto an 2D electrophoresis gel or a LC/MS instrumentation, thereby expanding the dynamic range of proteomic analysis.
19.6
Analysis of Proteins Expressed in Rat Plasma Exposed to Benzene Using Two-dimensional Electrophoresis


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Benzene, a ubiquitous environmental contaminant, is an important solvent in the chemical industry and is also known as a constituent of petroleum. It has been reported that benzene is associated with hematotoxicity including leukemia in human and cancer in laboratory animals. To study changes in protein expression levels in rat plasma exposed to benzene, we let the rats inhale benzene at the level of 1, 10, 100 ppm for 6hrs/day and 5 days/week for 6 weeks. The two-dimensional electrophoresis of rat plasma was carried out and approximately 1,000 protein spots were detected. The spots, which showed significantly different expression levels in rat plasma due to benzene exposure, were selected and identified with MALDI TOF-MS and ESI QTOF-MS/MS. Analyzing the spots, there were no correlations between 2-week and 6-week benzene inhaled groups on newly appeared spots: zinc finger protein, TTF. There were several spots which had the changes in the expression volume by benzene inhalation. The expression changes of those spots were confirmed by western blotting.

19.7
Comprehensive Analysis of the Human Plasma Proteome by Multiplex Separation Systems

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We describe the proteome profiling that was obtained from the proteomic analyses of HUPO reference specimens (Caucasian-, African-, and Asian-American) and the Korean serum using various sets of platform technologies such as 2-DE/MALDI-TOF, 1-DE/2D-Nano LC-MS/MS and free flow electrophoresis /1DE/2D-Nano LC-MS/MS. The blood samples were first applied to the Multiple Affinity Removal Column (MARC, Agilent Co.) to deplete six major abundance proteins and then processed through each separation system. We were able to establish a standard reference map of 2DE and a partial comprehensive list of identified proteins from each sample. Our data revealed that resolution, identification and quantitation of low abundance proteins in plasma are heavily dependent on pretreatment of samples. Further, our results also demonstrate that identification of those present in plasma requires very rigorous standard procedures which include mass peak identification, performance of search engine, and establishment of confidence criterion as suggested by HUPO Plasma Proteome Project. We have also constructed Human Plasma Reference Database within YPRC-PDS.

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19.8
Differential Proteomic Serum Pattern of Low Molecular Weight Proteins Expressed by Adenocarcinoma Lung Cancer Patients

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Based on the assumption that proteins can emanate from tumor to serum, we investigated whether serum low molecular weight proteins (LMW) can discriminate lung cancer patients from healthy donors. Pooled sera from 20 lung cancer patients matched in sex (men), histological type (adenocarcinoma) and stage (IIIB and IV) and from 20 healthy donors (men) were submitted to 2-DE coupled to MALDI-TOF peptide mass fingerprinting. To ascertain whether changes in protein pattern could also be detected by a simpler methodology, pooled serum and individual sera from 66 lung cancer patients with different histological types and stages and 44 healthy donors were submitted to Tricine-SDS PAGE. Results of 2-DE/MALDI-TOF showed 5 up regulated proteins (immunoglobulin lambda chain, transthyretin monomer, haptoglobin-2 precursor and two isoforms of serum amyloid protein) and 2 down regulated (apolipoprotein AI and transthyretin multimer) in patients. Tricine-SDS-PAGE results detected serum albumin precursor and haptoglobin-2 precursor only in patient’s pooled sera. By investigating a larger group, serum albumin precursor was more frequently found in patients (80%) than healthy donors (25%) (Fisher P < 0.001); haptoglobin 2-precursor average intensity was 3.5 higher in patients (55.23 ± 31.77) than in healthy donors (15.81 ± 11.02) (Mann Whitney P < 0.0001). In conclusion, proteomic techniques were able to evaluate the presence/absence of some LMW proteins in sera of lung cancer patients and healthy donors.
Quantitative Fractionation and Separation of Human Plasma by Free Flow Electrophoresis (FFE) in Early Stage Sample Prep Gives Reproducible Access to Low Abundance Proteins

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Plasma is a prolific source of potential disease markers and is readily available for clinical analysis. The large dynamic range of proteins present in plasma, as well as lipids, salts, and proteolytic activity limit the consistency and resolution of many analytical techniques to identify biomarkers. As a consequence, sample complexity reduction is becoming widely accepted as an essential first step in protein analysis. Several pre-fractionation strategies using immunodepletion, gel filtration, organic solvent extraction, functionalized magnetic beads, have been described to remove the abundant proteins like albumin and IgGs. Losses caused by unspecific traction, functionalized magnetic beads, have been described to remove other proteins or surfaces limit the effectiveness and reproducibility of these techniques.

As an alternative to depletion, we have used FFE as a pre-fractionation step. FFE is a simple and robust technique for separating complex mixtures into as many as 96 fractions in a standard plate format. Separation occurs under a continuous flow, without any solid-phase matrix. This liquid-only technique gives highly reproducible separation, and has versatility in electrophoretic and buffer conditions for specific applications. The absence of any kind of matrix-like surfaces is directly linked to consistently high sample recoveries of more than 97%, and fast separation times of less than 20 min/run. This approach facilitates continuous separation of plasma proteins, from the mg to the multi gram level.

A demonstration of this method is shown with 1ml plasma fractionated by FFE into 35 fractions. The subsequent separation of the FFE-fractions by 2D-PAGE stained with Coomassie yields to a grand total of more than 4000 protein spots as compared to approximately 800 spots with the unfractionated sample. First results of digested FFE-fractions analyzed by LC-MS/MS showed several 100 protein identifications per fractions. We further demonstrate that FFE can be used for large-scale sample preparation for high throughput proteomic analyses of plasma. In further investigations, we compared different methods of blood collection, like blood bags and evacuated plasma tubes with on-board protease inhibitor cocktails, which immediately limit proteolytic degradation. First results indicate that rapidly induced plasma protein variations, such as protein degradation, is occurring during the blood collection process.

Three-dimensional Separation of Mitochondrial Membrane Proteins from Saccharomyces cerevisiae Using Ion-exchange Chromatography and Gel Electrophoresis

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Enrichment and effective pre-fractionation of membranes are key features for enabling in-depth studies of receptors, transporters, channels and other vital membrane proteins. We present here a strategy that combines ion-exchange chromatography and electrophoresis for identification of mitochondrial membrane proteins from Saccharomyces cerevisiae. By isolating mitochondrial membranes and optimizing pH and detergent/protein ratio using the zwitterionic detergent Zwittergent 3–10, a yield of >90% solubilized membrane proteins was obtained. The solubilized membrane proteins were pre-fractionated by an ion-exchange chromatography step [1]. The protein content in each fraction as well as the non-solubilized proteins was further separated using a two-dimensional gel electrophoresis system (16-BAC/SDS-PAGE) developed for membrane protein applications [2]. This three-dimensional separation strategy resulted in a number of resolved and identified membrane proteins.


Using Multidimensional Liquid Chromatography (MDLC)-MS/MS to Handle the Wide Sample Dynamic Range in Human Plasma Proteomics

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The possibility to screen for presence of tissue leakage proteins in the human plasma could promote the development protein-marker based tools for disease diagnosis and therapeutic monitoring. However, the human plasma proteome is also one of the most challenging proteomes to analyze. Theoretically, the entire human proteome could be present in the plasma leading to an extreme complexity in the digested sample. Moreover, the very wide concentration dynamic range of 10¹⁵ is an analytical challenge.

Thus, there is a need for a reduction of both dynamic range and complexity. In this study, analyzing human plasma including and excluding different types of affinity chromatography to remove the most abundant proteins was tested. The sample was subsequently digested and run on a two dimensional SCX linear-gradient/fractionation MDLC workflow. The sample was fractionated with a linear gradient on a SCX column in the first dimension. 48 fractions were collected and analyzed in the second dimension on 75 μm RPC columns with a high-throughput nano-flow LC system connected to an ion-trap mass spectrometer. The MS/MS spectra of the analyzed peptides were matched against a human protein database.

The results show that low abundant proteins, such as tissues leakage proteins and known biomarkers, can be identified with the selected approach. The results will be discussed in the context of the contribution of the different analytical steps to the overall dynamic range of the method.
Observation of Intrinsic Protease-mediated Protein Degradation in Collected Plasma Samples: SELDI Data, and the Effects of Protease Inhibitors

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Human plasma and derived serum contain a variety of proteins that hold promise as biomarkers for a new generation of diagnostics. These proteins, however, are subject to protease digestion, both during and after blood collection, by proteases intrinsic to blood. We have shown that the MALDI-TOF mass spectra of peptides extracted from plasma are altered, as soon as minutes after sample collection. To further study the effects of post-collection protease damage of blood proteins, we collected plasma from 20 subjects, and used SELDI-TOF MS to monitor the proteins as a function of time after blood draw. EDTA-anticoagulated plasma samples were incubated at room temperature for 0, 24, 48, and 72 hours, and samples were then frozen at –80 °C or in dry ice until SELDI analysis. The results indicate that proteolytic damage of plasma proteins extends with incubation time. To help preserve blood proteins in a more in vivo-representative state, we have developed an evacuated blood collection system with an on-board protease inhibitor (PI) cocktail. Similar time-course SELDI analyses, on inhibited samples drawn at the same time as the EDTA plasma samples, show that these protease inhibitors increase stability of the mass spectra of proteins. It is particularly notable that protease damage happens extremely rapidly in unprotected samples, as demonstrated in our “time zero” comparison. This underscores the importance of in-situ blood-PI mixing during phlebotomy. The implications for these observations on biomarker discovery and proteomic diagnostics will be discussed.

Chinese Plasma Proteome: Comparative Analysis from Male and Female

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Serum, the soluble component of blood, is characterized by owning the most complex human-derived proteome, which contains other tissues proteome as subsets. Plasma proteins have been found to be functionally associated with disease progress and are critical for diagnosis and therapeutic monitoring. That is, besides the classic “plasma proteins”, blood serum contains all tissue proteins (as leakage markers) theoretically. Hence, the proteins in the serum are probably of the greatest biological, medicinal and economic importance. In this study, we adopted shotgun strategy to identify the protein components in sera of Han-nationality Chinese and further analyzed the difference between the two genders. The methodology used encompassed 1) removal of six high abundant proteins, 2) tryptic digestion of low- and high-abundant proteins, 3) separation of peptide-mixture by RP-HPLC followed identification by ESI-MS/MS. In results, total 944 non-redundant proteins were identified under a stringent filter (1.9, 2.2, 3.75, \( \Delta Cn \leq 0.1 \) and \( RSp \leq 4.0 \)) from both male and female sera. Comparing the sera between genders, 594 and 622 non-redundant proteins were identified from male and female ones respectively. Among those proteins, 252 were found in both male and female sera. Comparing the sera between genders, 594 and 622 non-redundant proteins were identified from male and female sera respectively. Among those proteins, 252 were found in both male and female sera, 108 and 119 were uniquely existed in male and female ones. Moreover, some lower abundance serum proteins (ng/ml range) were detected including growth factors, myelin basic protein and tumor necrosis factor. The serum proteins with IPI identifiers confirmed by more than one laboratory were further compared. Of 195 and 46 distinct proteins were found, at least, in more than one and 4 laboratories.
19.14
The Good, the Bad of Albumin Depletion
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Human serum derived from plasma without clotting factors is a vast media containing thousands types of proteins/peptides throughout every organs, in which implicates information of many processes taking place in different tissues and the physiological states of the human body. To entirely identify and characterize the serum proteins/peptides will enrich the knowledge of human proteome and facilitate the disease markers discovery. Unfortunately, the wide dynamic range in abundance of serum proteins (from mg/mL to pg/mL range) gives rise to an analytical challenge to complete characterizing the human serum proteome through current methodology. To better display low abundance proteins, with potential value for disease diagnosis and therapeutic monitoring, depletion of the major high abundant species especially albumin, the large proportion (55%) of serum proteins, is absolutely necessary step prior to analytical strategies currently. Thus, however, to remove the high abundant portion, e.g. albumin, from serum may also result in the specific removal of some low abundant ones of interest, such as cytokines, peptide hormones, and lipoproteins theoretically. In this study human serum albumin (HSA), an important carrier and transporter of serum proteins and some soluble substances, associated proteins were analyzed. After IgG depletion, the HSA associated proteins were immunoprecipitated, enriched especially for some low molecular weight proteins, and then were separated by 1D SDS-PAGE. The protein complexes were profiled by the micropillary reversed-phase liquid chromatography coupled on-line tandem mass spectrometry (LC-MS/MS) identification. Total 56 proteins were identified which contained numerous secreted or low abundant proteins that are critical for signaling cascades and regulatory events.

19.15
Deciphering the Plasma Clot Proteome
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The consequences of vascular thrombosis are a major cause of morbidity and mortality in industrialised countries. Identification of the plasma and cellular proteins that contribute to thrombus formation will aid in our understanding and management of vascular thrombotic disorders. We have focused our proteomic analysis on the plasma and cellular proteins that are actively incorporated into the fibrin clot by forming plasma clots in vitro in the absence and presence of cellular secreted proteins. Washed platelets and erythrocytes were stimulated by the addition of 1 μM ADP/0.5 μM ionophore A23187 respectively. Secreted proteins were isolated by centrifugation to remove cells followed by ultracentrifugation to remove microvesicles. Plasma clots were formed in the presence and absence of 30ng biotinylated cellular protein at 37°C by the addition of thrombin to a final concentration of 1 U/ml at physiological calcium concentration. The clots were washed to remove trapped proteins and then boiled in reducing buffer prior to analysis by SDS-PAGE or 2-DE. Proteins of interest were subjected to MALDI-TOF/MS for identification. Using these techniques, the serum proteins, haptoglobin, apo-A1 and transferrin have been identified as clot-binding proteins, and appear to be substrates for the plasma transglutaminase FXIII. In addition, several secreted cellular proteins have been detected in the fibrin clot, and we are currently working towards their identification. The information obtained from this study will provide new information on the molecular mechanisms underlying thrombus formation and advance the development of therapeutic anti-thrombotic agents.
19.16
The Comparative Proteomics Study on Serum of Normal Mice and the Mice Treated with Corticosterone with Deficiency of the Kidney Yin

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The aim of this study was to confirm the pathological proteins of the syndrome of the kidney yin deficiency by comparing the different proteins in serum of the contrast and the mice with deficiency of the kidney yin, identify the structure of these proteins, and so to make certain the relevance between the kidney in traditional Chinese medicine (TCM) and neuroendocrine immunomodulation (NIM). In this study, twenty female Balb/c mice were divided into two groups, ten per group. Mice were injected subcutaneously with physiological saline and corticosterone respectively, at a dose of 25mg/kg, twice a day for seven consecutive days, and then mice were killed to gather the serum. With two-dimensional electrophoresis (2DE) strategy, the proteins of serum were separated according to the isoelectric point and molecular weight. Relative abundances of the identified proteins were calculated through the image analysis. The interested proteins were digested by trypsin and identified by MALDI-TOF/MS. The results showed that six proteins disappeared and three proteins up-regulated in the serum of the mice with deficiency of the kidney yin. Functional categories of the identified proteins showed that most of the disappeared proteins belonged to monoclonal antibody IgG1 light chain, which involved in immune function. The up-regulated proteins were amyloid A and phospholipase A2, and the latter has been reported involved in chronic neurodegenerative diseases. The results indicated that function of the kidney in TCM was probably related with that of NIM in modern medicine. There may exist similar internal pathological and physiological basis between deficiency of the kidney yin and disturbance of NIM.

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19.17
Distinct Proteome Features of Plasma Microparticles

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Plasma Microparticles (MPs) are spherical cell membrane fragments derived from either apoptotic or activated cells. Characterized by a rich phospholipid moiety and many protein constituents, MPs normally circulate in the blood and contribute to numerous physiological processes. In disease states, microparticles derived from the injured organ likely contain valuable markers for determining the site, type, and extent of disease pathology. However the basic protein characteristics of plasma microparticles have yet to be described. In this study, microparticles from a pooled plasma sample derived from 16 healthy donors, all of group A blood type, were prepared by ultracentrifugation. Flow cytometry confirmed that a majority of these microparticles appear to range in size of less than 1 μm. Factor Xa generation assay revealed the presence of tissue factor activity in these microparticles, confirming MPs’ role in initiating blood coagulation. The MP proteome was analyzed by 2D gel electrophoreses performed in triplicate, and compared with a 2D gel of pooled whole plasma. Overall, plasma microparticles displayed distinct protein features and a greater number of protein spots (1021–1055) than that detected in whole plasma (331–370). Protein spots expressed in high abundance in the microparticle proteome were then excised and submitted for protein identity determination. This process provided protein identification for 169 protein spots and reported their relative protein quantities within the MP proteome. These 169 protein spots represented 83 different proteins and their respective isoforms. Thirty of these proteins have never before been reported in previous proteome analyses of human plasma. These results provide unprecedented information on the microparticle proteome and create a basis for future studies to understand microparticles’ biology and pathophysiology.

19.18
The Human Plasma Proteome Analysis by Multidimensional Chromatography Prefractionation and Linear Ion Trap Mass Spectrometry Identification

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A resurgence of interest in the human plasma proteome has occurred in recent years because it holds great promise of revolution in disease diagnosis and therapeutic monitoring. In this study, proteins from human plasma were prefractionationed by online sequential strong cation exchange and reversed phase chromatography. The resulting 30 samples were individually digested by trypsin, and analyzed by capillary reversed phase liquid chromatography coupled with linear ion trap spectrometry. Spectra from each fraction were searched with the SEQUEST algorithm against the non-redundant human protein database from IPI. The database searching results of each fraction were combined and summarized to be validated using a homemade software build summary, after meeting the following crucial criteria: delta cn >0.1, RSP<4 and cross-correlation score higher than 1.9, 2.2, 3.75, corresponding to singly, doubly and triply charged peptides. At last, a total of 1292 distinct proteins were successfully identified in our work, among which, some proteins known to be present in serum in < 10 ng/ml were identified. Considering our strategy allows high throughput of protein identification in serum, the prefractionation of proteins before MS analysis is a simple and effective method to accelerate human plasma proteome research.
19.19
Proteomic Analysis of Rat Hindlimb Ischemia-Reperfusion Injury
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Purpose: Lower limb ischemia-reperfusion may result in life-threatening injury. Rat hind-limb ischemia-reperfusion injury has demonstrated to have lung, liver, and kidney damage by previous study. Using the proteomics study, we may approach a high-throughput analysis of the post-translational modifications.

Materials and Methods: With the exception of femoral vessels, rat left hindlimb muscle was divided with femoral periosteum stripping. Three groups rat was studies, control group (without ischemia), hindlimb muscle preservation I/R group, and hindlimb muscle reduction I/R group. The I/R groups received a 4 hours ischemia followed by limb reperfusion. The rat blood and visceral organ were taken for proteomics study. The plasma and tissue were studied by IEF/2D gel electrophoresis, in gel digestion, followed by mass spectrometry (MALDI-TOF/TOF) and bioinformatics research for identification of peptides and sequences.

Results and Conclusion: 2D gel electrophoresis demonstrated thousand of proteins. Image analysis revealed differences among samples, thus the spot can be picked up for mass spectrometric study to identify the peptides and sequences, regarding to ischemia-reperfusion injury and the post-translational modifications.

19.20
Preparation and Analysis of a Low Molecular Weight Human Plasma Proteome
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Human plasma is a highly complex fluid containing proteins representative of many cellular processes. Identification of proteins within plasma can provide important information relating to underlying processes in the body and how it responds to different situations. Identification of potential disease biomarkers or biomarker profiles by relatively non-invasive analysis of plasma or serum is considered to be a critical strategy in the early detection of disease, monitoring of disease progression and drug effects and prognostic applications.

Plasma protein concentration is c. 80 mg/ml, with 99% of this being represented by just 22 proteins, albumin accounting for c. 50%. Analysis of plasma is thus hampered by both the complexity of the system, with perhaps 20–30,000 genes being expressed and post-translationally modified in human cells, and by the wide dynamic range of expression (10^9), with abundant proteins masking the underlying proteome. Many current approaches employ depletion methods to remove abundant species prior to analysis, but it is recognised that many proteins interact with these components and are removed, eliminating potentially important information. Known biomarkers for various diseases have been identified in these depleted pools of proteins, many of which are of relatively low MW.

We have investigated various procedures for enriching the low MW (≤50 kDa) proteins for proteomic analysis, without resort to prior depletion of abundant proteins. These include centrifugal ultrafiltration, electrodialysis and preparative electrophoresis. We present data on the suitability of these methods for the preparation of a low MW plasma proteome. We will also describe our current view of the low MW proteome obtained using 2D-E with HUPO test samples and present preliminary data relating to disease processes.
Partial Validation of Biomarker Proteins in Plasma of Human Exposed to Toxic Compounds: Benzene, PAH, TCDD

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The aim of this study is to screen and validate the diagnostic biomarker proteins for toxic compound exposure such as Polycyclic Aromatic Hydrocarbons (PAHs), benzene, and 2, 3, 7, 8-tetrachlorodibenzodioxin (TCDD). To screen the biomarker candidates for toxic compounds, the altered proteins in plasma of human exposed to the toxic compounds were analyzed and identified using 2-DE, MALDI-TOF/MS and ESI-Q-TOF/MS/MS. The 6 proteins in PAH exposure, the 8 proteins in TCDD exposure, and the 23 proteins in benzene exposure were differentially expressed and screened. Among the biomarker candidates, the expression of FKBP, MMP13, TCR β, fibrinogen gamma chain (PAH), alpha-fetoprotein, and fibronectin (TCDD) was reconfirmed by western blotting with commercial antibody, and their expression levels were same as the result of 2DE. Putative capacitative calcium entry channel, fibrinogen γ-A chains, and TCR β were partially validated using lab made antibody of representative peptide fragments, respectively, in PAHs or benzene exposed human plasma samples. The partial validation of biomarker proteins will be required the blind test using random samples in the future work.

Enhanced Sequence Coverage of the Peptide Mass Fingerprint for Nicotinic Acetylcholine Receptor

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The nicotinic acetylcholine receptor (nACHR) is a neurotransmitter-gated ion channel that mediates rapid signaling at the neuromuscular synapse. nACHR shares a high degree of homology to other ligand-gated ion channels such as serotonin receptor, γ-aminobutyric acid receptor and glycine receptor. Upon binding ligand molecules, the receptor adopts a short-lived structural conformation that allows passive flux of ions through the ion channel, thus altering the electrochemical potential across the cellular membrane. Ligand-gated ion channels are important pharmacological targets and dysfunctions associated with these channels include schizophrenia and myasthenia. The binding sites for molecules that regulate ion-channel activity often reside within hydrophobic regions. Mapping of these binding sites by mass spectrometry has been impaired by the typical additives (i.e. salts, detergents) required to maintain these peptides in solution. This study describes results obtained by utilizing novel surfactant blends that are compatible with matrix-associated laser desorption ionization time-of-flight (MALDI-TOF) MS. Peptide mass fingerprints generated in the presence of these surfactant blends yield markedly enhanced sequence coverage and improved statistical scores for database sequence searches. This study suggests a novel method for mapping the binding sites of noncompetitive antagonists for the nACHR.
19.24
Strategies for Improving Protein Identification from Human Plasma
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The human plasma proteome represents an extreme challenge due to the dynamic range of the proteins of interest and the complexity of the proteome. While immunodepletion can be used to remove the high-abundance proteins thus improving detection of low-level proteins, identification of the lower abundance proteins still presents a significant challenge. There are two fundamental strategies for protein identification experiments. The most common is to digest the proteins and separate the peptides. The advantage of this is that peptides are easy to handle than proteins resulting in less losses. In addition, only a few peptides need to be detected from any protein to get an identification. However, this approach creates extremely complex mixtures which are chromatographically challenging. In contrast, protein fractionation can be done first which provides another degree of orthogonality when combined with the 2D separation of the tryptic peptides. However, proteins are more prone to loss than peptides and any losses at the protein fractionation step will result in a missed protein identification. In this work, several multidimensional approaches to identification of proteins in immunodepleted sera are compared based on number of proteins identified and MS analysis time required. In addition, an improved tryptic digestion protocol was compared to standard protocols and found to offer a significant increase in proteins identified. While results show that longer MS acquisition time generally increases the number of protein identifications, separation of proteins prior to digestion and 2D LC/MS offers an even greater increase in protein identifications compared to just 2D LC/MS of digested samples.

19.25
Proteomic Analysis of Human Hemodialysis Fluid
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One of the primary functions of the glomeruli in the kidneys is the removal of low molecular weight molecules including metabolites, polypeptides and proteins from the blood. Thus blood or plasma are normally cleared of smaller proteins and polypeptides quite rapidly. In order to identify smaller proteins that have been missed by proteomic approaches directed at analysis of serum/plasma, we have analyzed the hemodialysis fluid obtained from a patient with renal failure using gel electrophoresis followed by liquid chromatography tandem mass spectrometry (LC-MS/MS). We identified over 270 proteins, half of which were smaller than 30 kDa. Importantly, over two-thirds of the proteins found in this study had not previously been described in serum/plasma. A detailed analysis of the identified peptides allowed us to map 29 N-terminal acetylation sites and several hydroxylated proline residues in proteins. A subset of the peptides identified was non-tryptic, thus providing information previously unknown sites of proteolytic cleavage. Finally, mapping of the peptides identified from large proteins suggests that we identified unique fragments in the hemodialysis fluid that were derived proteolytically processing of larger proteins.

19.26
Ovarian Cancer Marker of 11.7 kDa Detected by Proteomics Is a Serum Amyloid A1
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In order to deplete the major plasma components we use herein a plasma thermostable fractions for ovarian cancer biomarker discovery. An apparent cancer biomarker of 11.7 kDa molecular mass was detected in these fractions by ProteinChip SELDI-TOF mass-spectrometry system. This peak invariably appeared with other close peak of about 11.5 kDa, thereby suggesting that it is a derivative of larger mass. Of 27 cancer plasma specimens, 15 (55.6%) said peak pair, while only 2 specimens of 34 controls (5.8%) were shown to express it with low intensity. Using a method of cysteine modification by 4-vinylpyridine and background data, said peaks were identified by mass-spectrometry as serum amyloid A1 (11.68 kDa) and its N-terminal arginine-truncated form (11.52 kDa).

19.27
Removal of Multiple High-Abundant Proteins from Mouse Plasma Using Immunoaffinity Depletion
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The study of biological samples in disease models, drug therapy or toxicology studies requires the ability to identify proteins, typically present at low concentrations. A significant amount of biomarker research is performed using murine models. The ability to detect low-level proteins in mouse serum is hindered by the presence of albumin, IgG and transferrin. Together, these three proteins can comprise approximately 80% of the total protein mass. The depletion of these three abundant proteins facilitates access to the low-level proteins of interest. An immunoaffinity column for mouse serum addresses this concern by removing three high abundant proteins, including albumin, IgG and transferrin from mouse serum simultaneously and reproducibly using an LC-column format. The protocol with this LC column-based format is easily automated using a conventional HPLC with fraction collector. The column depletes >98–99% of the three target-proteins as determined by ELISA with robust performance for over 200 runs. The immunoaffinity column is based upon polyclonal antibodies, which are purified via a stringent affinity purification method and attached to porous polymeric beads individually. The mouse immunoaffinity column also depletes rat serum with similar efficiency while preserving specificity by removing only the targeted proteins. The depletion results in an improved dynamic range for proteomic analysis. Furthermore, the removal of the high-abundant proteins will improve loading capacity on 2DGE and LC/MS, which simplifies a complex system in the goal of discovering biomarkers.
19.28

Comparative Study on Human Plasma Proteome

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Comparative studies on blood plasma proteome were performed by two-dimensional gel electrophoresis (2-DE) and mass spectrometer (MS) using samples supplied from China and United States by collaborations of Human Plasma Proteome Project of HUPO. The plasma proteins were separated by isoelectric focusing using capillary type polyacrylamide gel (PAG) with carrier ampholytes pH 3–10 in the first dimension and with SDS-PAGE using 12.5% gel in the second dimension. The protein spots were identified by peptide mass fingerprinting using electrospray ionization MS/MS (LC/MSD Trap XCT, Agilent). Over 150 protein spots were subjected to the comparative studies. Substantial difference in the 2-DE patterns of the two samples could not be detected, but a few protein spots such as haptoglobin and vitamin D binding protein could be different between the samples. Furthermore, the 2-DE patterns showed that the samples stored in refrigerator for 3 days would lose inter-alpha-trypsin inhibitor heavy chain H4, and the lyophilized samples would lose IgA heavy chain.

19.29

High Throughput Profiling of Cancer Serum Biomarkers Using Single Capture Antibody Chips

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Profiling biomarkers in complex mixtures such as serum is challenging since the dynamic range of proteins in the serum proteome is broad. We have developed a single capture antibody chip that enables clinical researchers to simultaneously screen large numbers of circulating cancer serum biomarkers from two serum samples. Single capture antibody chips combined with a protein labeling and fluorescent detection system offers a new proteomics capability to measure known serum biomarkers that does not require mass spectrometry instrumentation. Direct labeling of serum proteins with small molecular haptens was performed using chemistries that target multiple amino acids. The abundance of a 120 classical cancer biomarkers, cytokines, and chemokines were measured directly from eight microliters of serum. Biomarker profiling data demonstrate pattern reproducibility from serum obtained from cancer patients compared to age and gender matched control serum samples. We report here that S&S® Serum Biomarker Chip offers clinical cancer researchers a high throughput assay that can discriminate two similar serum samples and identify clusters of over- and under-abundant proteins in test and control serum samples.