Characterization of Degradation Markers in Plasma Used for Transfusion

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Blood transfusion is an essential part of modern health care, and one of the key points of this practice is to ensure the blood transfusion safety. Plasma used for transfusion can be obtained from centrifugation of whole blood or by apheresis procedures. After a blood donation, the plasma is processed using different methods (solvent-detergent, methylene blue...) in order to decrease the infectious risks associated with blood products. Another important and crucial parameter for the quality of the plasma is the delay existing between the donation and the freezing of the product. In France, this delay has to be inferior to 6 hours but the influence of this step during the processing of plasma has yet to be established. So we decided to investigate more thoroughly the evolution of the plasma proteome during this delay. In this aim, plasma was collected by apheresis from different healthy donors, and studied by 2D electrophoresis without any freezing from 1 hour to 24 hours after the donation.

We will present here: (i) our methodology for plasma separation using 2-D electrophoresis; (ii) the optimisation of removal of the more abundant proteins; (iii) our strategy for the fractionation of plasma proteins using multidimensional liquid chromatography; (iv) the detection on 2-D gels of degradation markers of plasma appearing during the delay between blood donation and freezing; (v) and finally the identification of all the degradation markers by mass fingerprint proteomics using MALDI-TOF MS and nano-LC-nano-ESI-MS/MS.

Drilling the Human Plasma Proteome; Novel Advancements in Fractionation and Separation

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Plasma is arguably the largest source of biomarkers, whether for diagnostics or therapeutics. Its vast dynamic range, further complicated by the presence of lipids, salts, and post-translational modifications, as well multiple mechanisms of degradation, present challenges in analytical reproducibility, sensitivity, resolution, and potentially efficacy.

Reduction of sample complexity is becoming increasingly critical in order to drill deeper into the proteome to find new and more specific clinical biomarkers. A multitude of fractionation strategies are available for removal of high abundance proteins, but each has particular limitations, especially losses caused by non-specific protein interactions. In order to ensure assay reproducibility and effectiveness, careful choices of preanalytical tools and methods must be made.

We have developed Free Flow Electrophoresis (FFE) as a novel separation concept that enables greater penetration of the proteome via separation of a wide variety of charged or chargeable analytes, ranging from small molecules to cells. Unlike traditional electrophoretic or chromatographic techniques, FFE provides continuous electrophoretic separation, in the absence of solid phase interactions, providing the simultaneous benefits of reproducibility, speed, a high-resolution fractionation gradient, and high recovery.

Laboratory results demonstrate improved resolution on 2D-PAGE by more than a factor of five, when compared to unfractionated samples. Additionally, digested fractions analyzed by LC-MS/MS yielded hundreds of protein identifications per fraction. A further complementary benefit of this novel technology is the ability to rapidly and efficiently deplete albumin from human plasma during the fractionation process. Additionally, these methods are appropriate for processing large numbers of samples, allowing high sample throughput without sacrificing resolution or protein concentration dynamic range, improving discovery of novel plasma markers.
20.3  
Human Serum Proteome Analysis by Protein 2D Chromatography System; Methodological Aspects

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Screening for differential display of serum proteins between healthy and diseased subjects could significantly improve the detection and identification of disease-related protein markers for diagnosis and monitoring responses to therapy. The wide dynamic range of protein concentration and the structural diversity of the constituent proteins complicate the screening of serum proteomes. There is also the challenge of how to achieve high-throughput analysis of samples, as the classical approach, 2D gel electrophoresis, is not easily amenable to automation and is labour-intensive and time consuming. Furthermore, some inherent features of 2D gel based procedures limit the utility of the technique, e.g. the failure to resolve high and low molecular weight proteins, highly acidic and basic and hydrophobic proteins.

Different non-gel based commercial products have been developed as alternatives to 2D electrophoresis. In this study we have used automated liquid chromatography based on two dimensional protein fractionation (Beckman Coulter ProteomeLab PF 2D) to analyse human serum proteomes. The first dimension separates proteins based on their isoelectric point (chromatofocusing) and subsequently protein fractions are further resolved based on hydrophobicity (reverse phase). The system is provided with dedicated bioinformatic tools for data analysis, enabling comparison between different runs and allowing differential display of protein chromatograms in order to identify differences between samples. Reproducibility and sensitivity of this system have been tested by loading 10 replicates of the same serum sample. After collecting eluted fractions, a further third dimension has been used to separate proteins by molecular weight (SDS-PAGE). Gel bands have been analysed by MALDI-TOF/TOF. Different methodological aspects will be discussed showing the advantages and disadvantages of this approach.

20.4  
Non-denaturating Multidimensional Separation of Serum Proteomes; Improved Method, Automatization, and Applications

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Alternatively to 2-DE, a separation method is introduced that yields liquid fractions with sample constituents conserved in their native states. Size exclusion, anion exchange, and lectin affinity chromatography are used for the first, second, and third separation dimension, respectively. Each separation step may be applied to all fractions of the foregoing one using 96 parallel micro-columns, microplates, and the automated CyBio AG analysis platform. Up to 100 mg of protein can be separated into 3000 - 6000 fractions. 1-D and 2-D protein recovery was 95.3% ± 6.7% (n = 38) and 100.1% ± 29.6% (n = 13), respectively, as quantified by UV-absorbance. Spectrophotometric, enzymatic, and immunological analyses, MALDI-MS, and ESI-MS/MS can be applied to the chromatographic fractions. The method yields evidence of functional protein complexes (interactomes), protein entities, proteolytic fragments, protein glycosylation and glycation, and non-protein constituents, as, e.g., triglycerides and cholesterol.

Low abundance proteins were identified and quantified by enzyme activity determinations (alkaline phosphatases, transaminases, acetylcholinesterase, GPI-phospholipase D) and by immunoassays (CRP). Nearly 800 proteins were identified with mass spectrometry of chromatographic fractions obtained from human serum (www.mti.uni-jena.de/Biochemie/Serum proteins, A. Horn et al., Proteomics 2005, in press). CV values of protein concentrations within homologous fractions of samples of different healthy donors were sufficiently small for proteome comparison using differences and standard errors of concentrations. Proteins with altered concentrations within fractions of pathological sera (Alport syndrome and renal failure) were identified by a semi-quantitative mass spectrometric approach. The multidimensional digitized picture of the proteome comprising all quantitative data obtained allows easy comparison of different functional states.
20.5 Characterization of Seleno-Glycoproteins in the Human Plasma

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By combining methods of trace element analysis, tracer techniques, biochemical and electrophoretical methods information on the characteristics of several selenium-containing proteins were obtained in the human blood. After fractionation of the several blood cell compartments by centrifugation procedures three selenium-containing proteins with the molecular masses of 96 kDa, 84 kDa and 54 kDa were found in the plasma. By affinity chromatography and glycoprotein analysis of the plasma selenium-containing proteins two of them were characterized as glycoproteins. Using micro-characterization techniques such as two-dimensional electrophoresis capillary electrophoresis, RP-HPLC, MALDI-MS, IR-imaging, antibodies and microsequence the selenium-containing proteins could be characterized as already known selenoproteins. The first selenoprotein was characterized as the glycoprotein plasma glutathione peroxidase, the second as the cellular glutathione peroxidase and the 54 kDa protein as the glycoprotein selenoprotein P (Sel P) which is the only selenoprotein that contains 10 selenocysteines. Additionally could be determined that more than 93% of the selenium concentration in plasma is bound in the selenoproteins P and the plasma glutathione peroxidase. Based on these result monoclonal antibodies were produced against the seleno-glycoproteins. By means of the produced antibodies the human selenium status can be determined quickly. In the present study characteristics about the seleno-glycoproteins in the human plasma are presented. Moreover a fast test to determine the human selen status is suggested.

20.6 Proteomic Strategy for the Identification of Low Abundance Proteins and Investigation of Biomarker in Human Plasma

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The huge dynamic range of human plasma proteome issues great challenges for the separation and identification of the low-abundant proteins which have extreme potential as biomarkers. The purpose of this study hereby was to identify more low-abundance proteins both in total plasma proteome and in glycoproteins which take a major part in plasma proteins, then further investigate the biomarkers in them. Our approach involved fractionation of plasma proteins by multiple dimensional liquid chromatography and enrichment of plasma glycoproteins by affinity chromatography, both followed by liquid chromatography tandem mass spectrometry. A total of 3804 distinct proteins were detected by combination of the two methods and 1242 were identified as potential glycoproteins by the affinity method. Such large quantity of dataset generated in this work could allow us to carry out a comprehensive characterization of human plasma to define the plasma proteome. Furthermore, comparison of the complementary information contained in both methods (2D-LC fractionation and lectin affinity purification) could improve the reliabilities of some proteins identified in either method. It is anticipated that, together, these technologies will facilitate the comprehensive study of plasma proteome so that the investigation of low-abundance biomarker can be achieved.

20.7 Erythroid Membrane Proteomics

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Despite the large amount of knowledge regarding red blood cell structure and function, the remarkable phenotypic diversity encountered in common diseases highlights that several aspects remain to be elucidated in order to relate findings at molecular levels to clinical phenotypes. A proteomic approach can reveal proteins important in the pathophysiology of pathologies, and can indicate those that might be an important source of phenotypic variation. Since many red cell pathologies are related to membrane abnormalities, a proteomic map of membrane proteins in abnormal cells in comparison with normal ones can lead to the identification of proteins, that are up- or down-regulated in a disease-specific manner. Although, a first red cell membrane map fingerprint of normal circulating red cells has recently reported, it lacks protein functional evaluation. Analysis of membrane proteins remains a major challenge for proteomic techniques based on 2D electrophoresis. The major weakness comes from the poor efficiency of most commercial detergents to solubilise denatured membrane proteins under the conditions prevailing in 2D electrophoresis. We tested various detergents having different chemical structures. Band III was chosen as a primary model protein to evaluate the efficiency of solubilization. Different methodological strategies allowed us to get a complete coverage of membrane proteins. Our research activity has therefore been focused on hereditary stomatocytosis (HS) as a model of red blood cell disease, associated with abnormalities in cell volume regulation. We are currently running a comparison between 2D electrophoresis maps obtained from lightest and heaviest fractions of red blood cells patients with HS and haematologically healthy controls.

20.8 The Proteomic Responses in Mouse Plasma to Sepsis Generated from CLP Model

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It is estimated that over million individuals develop severe sepsis around the world. Because of problems with dysfunction of vital organs caused by sepsis, people with severe sepsis face 30–35% of mortality. Therefore a key issue for sepsis therapy is how to monitor and to control inflammation at early phase of sepsis. Herein we report an approach to define the candidates of biomarkers for septic process, which are potentially meaningful for clinical diagnosis of sepsis. The mouse model of cecal ligation and puncture (CLP), which mimic the sepsis, was adopted in this study. During septic development, the blood samples were drawn at intervals of 4 and 24 h, respectively. The high abundant albumin was removed from plasma with blue agarose followed by 2DE to separate the plasma proteins. With silver staining, there were averagely 342 (sham) and 372 (CLP) spots detected. These different spots were then in-gel digested and identified by mass spectrometry, resulting in 10 identified proteins which all were up-regulated in 24 h CLP plasma. Importantly, these proteins were typically associated with inflammation and almost existed multiple spots on 2DE, indicating that sepsis process might induce a series of modifications. Furthermore, the analysis of Western blot agreed with the proteomic determinations.
Comparison of Pretreatment Methods for Serum Profiling Using SELDI Proteinchip Technology

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The serum proteome is a valuable source of biomarkers. Several serum pretreatment methods are described that claim to improve reproducibility and number of detectable peaks. The aim was to find the best serum pretreatment method for use in combination with Surface Enhanced Laser Desorption (SELDI). We compared six different methods: 1. untreated sera, 2. denaturation with 20% ACN, 3. denaturation with 9M Urea/2% CHAPS, 4. ultrafiltration with a 50 kDa cut-off filter to remove high abundant proteins, 5. denaturation with 20% ACN followed by ultrafiltration and 6. precipitation with 100% ACN/0.1% TFA to remove high abundant proteins. The pretreated sera were applied in quadruplicate to a CM10 weak cation exchange chip using both CHCA and SPA as matrix. Data were analyzed for the total number of unique peaks (S/N > 3) in the 4 spectra. Reproducibility was determined by calculating the percentage of peaks that were detectable in all 4 spectra and their average CV. Results show that for SPA the most peaks (71) were detected in untreated and ACN precipitated sera. However reproducibility is poor. Reproducibility greatly improves with ACN and urea denaturation at the cost of a lower peak count (47). ACN denaturation followed by ultra-filtration especially improves detection of low molecular weight peptides (~6 kDa). With CHCA the highest peak number (61) and best reproducibility was obtained with the latter method. Urea denaturation in combination with CHCA gave a poor reproducibility. We conclude that ACN or urea denaturation in combination with SPA as matrix and ACN denaturation followed by ultrafiltration in combination with CHCA are complimentary and together best cover the range from 1 to 40 kDa.

A Plasma Protein Reference Set; A Set of Plasma Proteins Identified with Extremely High Confidence through Two Consecutive Stages of Tandem Mass Spectrometry (MS3) and PPM Precursor Mass Accuracy

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An immense diagnostic potential has prompted great interest and effort in cataloging the contents of human plasma, exemplified by the HUPO pilot project. Herewith, we attempt to contribute to the elucidation of the plasma proteome by establishing a high confidence set of reference proteins against which other data sets can be compared. To address the complexity and dynamic protein concentration range of the plasma proteome, different methods of separation at the protein and peptide level were employed, followed by Thermo Finnigan LTQ-FT MS analysis. The LTQ-FT allows the measurement of peptide masses in the low ppm range. Furthermore, we have developed a novel statistical score that allows database peptide identification searching using the products of two consecutive stages of tandem mass spectrometry (MS3). The combination of MS3 with very high mass accuracy in the parent peptide allows peptide identification with orders of magnitude more confidence than that typically achieved. We merged the verified and positively identified proteins into one single non-redundant reference list of human plasma proteins, giving rise to a set of plasma proteins in which we have high confidence due to the instrumentation and validation criteria used. Furthermore, we compared the various separation approaches used and characterized the plasma proteins using cellular localization information and how our list compares to data from other sources.

20.10
Enhanced Detection of Low Abundance Human Plasma Proteins by Optimizing a Multidimensional Protein/Peptide Separation Strategy

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Systematic identification of low abundance proteins in human plasma and serum is complicated by sample complexity, an extremely wide range of protein concentrations, and extensive structural heterogeneity of high abundance proteins. We recently developed a powerful plasma protein profiling method utilizing three orthogonal protein separations (immuno-affinity depletion, microscale solution IEF, and 1-D SDS PAGE) followed by pixelation of the 1-D gels and LC-MS/MS analysis on a linear ion trap mass spectrometer. When human serum was analyzed, more than 2,700 proteins spanning more than nine-orders-of-magnitude were identified using HUPO criteria for high confidence assignments. More importantly, 14 of 20 proteins known to be present in the 1–100 ng/ml range and 2 of 19 proteins in the pg/ml range were identified. To further enhance low abundance protein detection, particularly in the pg/ml range, each step in the method was reevaluated. Two major limitations were identified. First, the limited capacity of 1-D gels severely restricted total plasma and serum volumes that could be analyzed, thereby making identification of proteins in the pg/ml range very difficult. Hence strategies to enhance sample capacities at this step and substitution of alternative separation modes for the 1-D gels, including protein reverse phase separation were evaluated. The second major limitation was the poor capacity of existing commercial software to integrate large LC-MS/MS datasets and to reliably distinguish false positive from true positive protein identifications based on single peptide hits. Alternative database searching strategies, search algorithms and statistical filtering schemes were compared. While statistical filters could remove most false positive identifications, this usually resulted in simultaneous removal of many low abundance protein identifications that were valid.
Baseline Serum Protein Profiles for 40 Normal Individuals and Pooled Subpopulations Using Optimized 2-DE Analysis

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It is important to identify the baseline human serum proteome before undertaking disease diagnosis, prognosis and the monitoring of drug effects. The natural variation of serum protein profiles can make significant biomarkers more difficult to identify. Although there are several protein separation methods that can be used to analyze serum proteins, two-dimensional gel electrophoresis (2-de) remains a versatile method for qualitative and quantitative proteomic analysis. Two major issues in biomarker discovery need to be resolved before disease-related changes in protein expression level, post-translational modifications, and isoforms can be identified: (1) what are the natural fluctuations in “normal” serum?, and (2) are pooled or individual serum samples are best for biomarker discovery? To characterize the baseline variation in a normal population, we compared the protein expression profiles of 40 individual healthy males using our optimized and highly reproducible procedures for serum delipidation, immunoglobulin and serum albumin depletion and subsequent 2-de analysis. Furthermore, we compared individual versus pooled samples using the same 2-de technology. We found that the 2-de protein profiles in “normal” people are quite variable. Our results also suggest that some protein information is lost during the process of sample pooling. Therefore, identifying the natural range of protein changes (expression levels, post-translational modifications and isoforms) in the normal healthy population will help us identify abnormal protein profiles for disease diagnosis, biomarkers or the monitoring of drug effects.

Application of the Evidence® System to Quantify Cytokines in the Stanislas Cohort

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For a long time, scientists focalized their researches on studying one specific molecule in order to understand their action in physiology and/or pathology. Today, there exists a considerable knowledge of different molecules action but simultaneously the notion of metabolic pathways changed the view of researchers and they started to recognize and investigate the complexity of the human metabolism. Moreover, thanks to the big advancements of high throughput technologies, today we can analyze a large panel of molecules in order to better understand their actions not one by one, but in relation to others and interaction between them: the molecular networks. This approach is very useful in understanding inflammation-related disorders, which are the consequence of preferential activation of type 1 (Th1) or type 2 response (Th2) leading to an imbalance in cytokines levels. Studying these levels requires knowledge of the reference values of these molecules in a healthy population. Nevertheless, these values even quantified in physiologic condition, are affected by constitutional, environmental and genetic determinants.

Evidence® is a biochip array analyzer commercialized by Randox, on which are developed several molecule panels. We will describe here how we applied this technology to the quantification of 12 cytokines in a sub-sample of 540 adults and 304 children from a large French supposed healthy population: the Stanislas cohort.

Among molecules quantified, Interleukine-8 (IL-8), Monocyte Chemoattractant Protein-1 (MCP-1), Endothelium Growth Factor (EGF) and Vascular Endothelium Growth Factor (VEGF), are known to be involved in several diseases related to inflammation, vascular remodeling or growth deregulation. In addition, elevation of plasma levels of these proteins has been associated with diagnosis and/or prognosis of theses affections. Given the importance of these molecules, we established their plasma reference values according to age and sex and we looked for their biological determinants and familial resemblance to evaluate part of both genetic and non-genetic determinants.

Information of above molecules could be very useful in the comprehension of the regulation of their concentration in physiological conditions and for the interpretation of laboratory results.
Development and Assessment of a High Abundant Human Plasma Protein Depletion Kit Using Monoclonal Antibodies against Native Serum Proteins

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One of the main limitations of 2-DE is its inability to visualize low-abundant proteins, particularly in the presence of highly abundant proteins. In human plasma, 20 high-abundant proteins, such as albumin, immunoglobulins-lipoproteins-fibrinogen, et al., accounts for approximately 99% of the total protein. Monoclonal antibodies (mAbs) with high affinity and specificity are critical in the development of a depletion kit, which can effectively remove most of target abundant plasma proteins without influencing the low abundant proteins. In the previous studies, we had obtained hundreds of murine mAbs against 20 high-abundant plasma proteins by the approach of subtractive immunization with fractionated human plasma proteins (Ning et al., Molecular & Cellular Proteomics 2004 3:S2). Three different types of human plasma high abundant proteins depletion kit have been development using these mAbs, i.e. depletion kit for human albumin and immunoglobulin, depletion kit for 9 or 15 human plasma high abundant proteins. The kits displayed good capacity and specificity in removing high abundant human plasma proteins in series comprehensive assessment tests including Western-blot, 2-DE and Mass spectrum etc. The depletion kit could significant improve the outcome of 2DE as a large amount of new low-abundant spots were clearly visualized on 2-DE and could be easily cut for use in identification by mass spectrum. We conclude that the depletion kits could effectively and specifically remove most of high abundant serum proteins, and thus could be used in large-scale sample preparation for high-throughput plasma proteomic analysis.