Proteomic Analysis of Liver Diseases: Molecular Mechanisms and Biomarker Discovery

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Abstract: Liver diseases afflict more than 10% of the world population. Although the main risk factors are known and the population at risk is monitored, new biomarkers are urgently needed to allow early diagnosis and hence more effective therapeutic interventions. Here, we revise the contribution of proteomics to the study of liver diseases and its potential impact in the clinical practice is evaluated.

Key Words: Proteomics, mass spectrometry, liver diseases, biomarkers.

INTRODUCTION

The completion of the sequencing of the human genome in 2003 [1, 2], together with hundreds of other organisms, has provided massive information that has changed our interpretation of biological systems. Moreover, new ways have been promoted to study cell biology as well as those alterations involved in the orchestration of adaptive responses or that base the molecular mechanisms involved in the progression of human disorders. DNA microarray-based technologies facilitate the simultaneous analysis and characterization of the more than 20 thousand genes that integrate the human genome on a single experiment. The application of these large-scale procedures has provided genomic profiles that greatly extended our knowledge about the molecular pathogenesis of disease in the last few years and it is expected to have impact on the development of new diagnostic and therapeutic strategies. However, regarding the weak correlation found between transcriptomic and proteomic data [3] and considering that most cellular functions are performed and are regulated by proteins, the study of the cellular proteome is required to unravel the functional message encoded by the genomic information. The term proteome [4, 5] can be defined as the whole proteinaceous component of a cellular system under well defined conditions. Any alteration of the environment might result in proteomic changes leading to the coordination of complex cellular responses. Adaptation to unfavorable conditions and ultimately cell survival is highly dependent on the dynamic nature of the proteome that evolves under different situations. Proteomics is the discipline devoted to the study of the proteome using technologies that allow for large-scale analysis. In comparison with the sequencing of the 3.120.000.000 nucleotides of the human genome, the human proteome is orders of magnitude more complex, and unravelling and mining the human proteome is still an emerging field. A complete description of the proteome must include not only the identification of the polypeptides resulting from the translation of gene transcripts, but also the investigation of their posttranslational modifications, splicing variants, interactive networks with other proteins and cellular components, localization within the cell and tissues, and any other aspect that might confer or modify protein function. Definition of all these aspects is essential to fully understand biological functions, their regulation, and alterations leading to the development of diseases. Especially relevant in biomedicine is comparative proteomics since it allows, through the comparison of normal and diseased samples, the identification of proteins participating in the progression of human diseases as well as for the discovery of new biomarkers. Noteworthy, comparative analysis of biofluids focused on the study of the thousands of circulating peptides and proteins present in serum, plasma, urine, saliva, or cerebrospinal fluid, is only relevant at the proteome level and not at the transcript level. Therefore, proteomics emerges as the most promising approach to identify biomarkers that may promote the development of new clinical applications to improve patient diagnosis, treatment and outcome according to non-invasive methods. However, proteomics is still a novel science in continuous evolution to circumvent current restrictions to deal with the whole complexity of the proteome. Separation, identification and characterization of the hundreds of thousands protein variants integrating the human proteome in a wide dynamic range of concentration, is still challenging due, at least in part, to the limitations imposed by the analytical instrumentation available.

ANALYTICAL STRATEGIES ON PROTEOMICS

In general, comparative proteomic profiling encompasses methods for a comprehensive analysis of whole proteomes by combination of different strategies to resolve complex proteins and peptides mixtures from biological systems with mass spectrometric techniques aimed at the identification and characterization of the proteins of interest. Since different experimental approaches provide with complementary information, it is essential to delineate an appropriate work-

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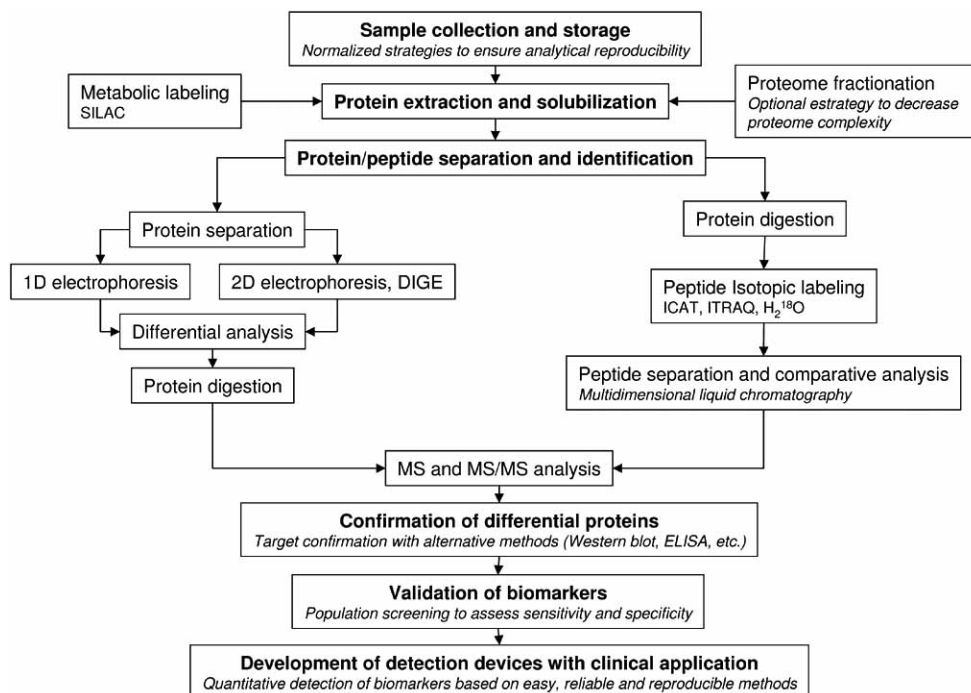
Fig. (1). Schematic representation of a general workflow oriented to the identification of biomarkers of human disease based on proteomics approaches.

flow integrating the ideal combination of analytical procedures to generate consistent answers to the initial objectives of the study, protein cataloguing, molecular mechanisms of cellular functions or biomarker discovery. Sample collection, storage and protein solubilization, protein/peptide separation, protein identification, and bioinformatic analysis are common steps of a typical proteomic analysis. Additionally, after the screening phase leading to the discovery of differential protein targets, subsequent validation as clinically relevant biomarkers is necessary to ensure their efficacy and specificity to detect the onset and state of the particular disease under study (Fig. **1**).

Normalized strategies for sample collection and storage are essential to ensure proteome integrity and analytical reproducibility. This is especially relevant in the case of human samples that should be additionally accompanied by precise diagnostics to permit correlations between clinical phenotypes, histological alterations and molecular parameters. Proteins must be then extracted and solubilized in buffers containing high concentrations of chaotropic agents, reducing agents and detergents. The protocols must be optimized for each type of biological sample to improve the extraction efficiency but minimizing the interaction with the following analytical procedures [6]. Proteome coverage is greatly enhanced by the use of fractionation strategies and the subsequent study of subproteomes. Conventional methods generally based on differential solubilization or centrifugation of sucrose gradient to isolate subcellular organelles, as well as different commercial kits or affinity based selection, are available to obtain fractions enriched on proteins sharing biochemical properties, functions or subcellular location. Although these approaches obviously increase the complexity of the analysis, the limitation imposed by the wide range

of protein abundance and the protein diversity on the detection of less abundant proteins might be partially eluded by the analysis of different subcellular fractions.

After isolation and solubilization of the proteome, constituent proteins must be efficiently separated according to methods allowing identification of isolated polypeptides or comparative analysis. There are numerous methodologies that can be divided into two major categories to achieve this goal. The first one provides protein identification after extensive separation. Up to date, the most widely used method is two-dimensional polyacrylamide gel electrophoresis (2-DE) owing to its resolution, easy availability and abundant accumulated knowledge. Proteins are resolved according to two different biochemical parameters, the isoelectric point (pI) in the first dimension, and the molecular mass (Mr) in the second dimension. Since pioneering studies describe this technique [7], reproducibility has been greatly enhanced by the introduction of immobilized pH gradients (IPG) [8]. Separated proteins are visualized using staining procedures such as coomassie blue, silver staining or fluorescent dyes. More recently, gel-to-gel variation was reduced by the so called difference gel electrophoresis (DIGE) technology where proteins from two different samples to be compared are labeled using different fluorescence dyes (Cy2, Cy3, or Cy5), mixed equally and resolved by 2-DE. Scanning at different wavelengths provides multiple images corresponding to different samples and therefore protein levels can be compared within a single gel reducing variability and improves accuracy in protein semiquantitation [9, 10]. This is a relatively simple method that allows visualization of thousands of proteins, detection of post-translationally modified species, and targeting of protein expression alterations. However, some restrictions are associated to 2-DE preventing a complete descrip-

tion of the proteome. The primary weakness of this method is the limitation for the detection of low abundant proteins, theoretically those at less than 1000 copies per cell [11, 12]. Additionally, hydrophobic proteins and those with extremely acidic or basic pI or Mr less than 10 kDa are hardly observed on 2-DE gels. After separation, spots of interest are excised and incubated with proteases, generally trypsin, and the resulted peptides are analyzed by mass spectrometric methods to allow protein identification. Tryptic digests can be characterized by matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectrometry that provide the *m/z* ratios of peptide fragments, or peptide mass fingerprint (PMF) of the protein that facilitates its identification by the comparison with the theoretical PMFs of genome-wide protein sequence databanks [13]. Alternatively, peptides can be separated by liquid chromatography (nanoLC) and analyzed by tandem mass spectrometry (MS/MS) that provides information relative to the amino acid sequence [14]. The chromatographic column is connected on line with the MS/MS instrument through a nano electrospray source (ESI) that allows ionization of the eluted material. Once ionized, a particular peptide/ion is isolated and fragmented by collisioning with an inert gas generating a fragment spectrum that is compared with the theoretically predicted fragments for all peptide sequences in a database to identify the peptide sequence and, accordingly, the precursor protein.

The second major proteomic approach or shotgun proteomics proposes a gel-free alternative consisting of proteolytic digestion of the protein mixture to produce a large collection of peptides that are then subjected to multidimensional LC and MS/MS analysis. This approach might circumvent some of the typical drawbacks of 2-DE analysis mentioned above and provides means to conduct comparative studies by combination of shotgun peptide sequencing and stable isotope labeling [15, 16]. Peptide mixtures from different samples can be chemically (ICAT, isotope-coded affinity tag [17]; ITRAQ, isobaric multiplexing tagging system [18, 19]), enzymatically (digestion in $H_2^{18}O$) or metabolically (SILAC, stable isotopic labelling with aminoacids in cell culture [20]) labeled using light and heavy isotopes, and then combined equally and analyzed by nanoLC ESI-MS/MS. The relative abundance of peptides is calculated by computer algorithms that calculate the ratio (light versus heavy) for each peptide pair. Differential protein expression profiling, protein interactions or identification of post-translational modifications can be alternatively accomplished by using protein chips [21, 22]. Although protein arrays have a tremendous potential, the development of protein microarrays for high-throughput proteomics investigation is slow due to the complex nature of proteins. Additionally to macro and microarrays or microfluidic and microwell chips, alternative formats such as surface-enhanced laser desorption/ionization (SELDI) and surface plasmon resonance are already available. SELDI, in combination with time-of-flight mass spectrometry (TOF) is the most widely used protein chip format. SELDI TOF combines protein retention on chemically treated supports with specific chromatographic features (protein chip arrays) with TOF-MS analysis to generate specific signature patterns that can be compared to distinguish different samples [23]. Whatever the analytical procedure used, data obtained from high throughput proteomic

analysis must be integrated and processed using appropriate bioinformatic tools to generate valuable biological information from the resulting complex molecular descriptions in formats allowing the validation of data and the exchange among different laboratories.

APPLICATION OF PROTEOMICS TO THE STUDY OF LIVER DISEASES

Fatty Liver Diseases

The liver plays a central role in maintaining the balance of metabolic energy in response to endocrine signals. Long-term imbalance between food intake and energy consumption can induce obesity that is commonly associated with other diseased conditions such as type 2 diabetes mellitus, hypertension, coronary heart disease, or some cancers [24, 25]. Additionally, high fat diets lead to steatosis or fat droplets accumulation in the cytoplasm of hepatocytes [26] resulting from insulin resistance that increases fatty acid synthesis, and mitochondrial dysfunction [27]. In general, fatty liver is a benign condition but association with additional factors or liver lesions might induce development to steatohepatitis with the risk of progression to end-stage maladies like cirrhosis or hepatocellular carcinoma (HCC) [28, 29]. Proteomic-based studies aiming at the identification of potential biomarkers and to depict molecular alterations leading to the development of steatosis and steatohepatitis have been recently conducted on experimental models of these diseases [30]. Marleen *et al.* performed a comprehensive analysis of the hepatic proteome of HcB19 mice with fatty liver and identified novel differential proteins including a reduction of propionyl CoA carboxylase α chain (PCCA) and 3-hydroxyanthranilate 3,4 dioxygenase (3HAAO) [31]. Interestingly, PCCA KO mice develop ketoacidosis and fatty liver [32] and the reduced levels of 3HAAO might be related with the hypersecretion of VLDL reported in these mice through impairment of the synthesis of nicotinic acid [33]. Proteins that regulate generation and consumption of the acetyl-CoA pool as well as others involved in the response to oxidative stress are altered in steatosis [34]. As reported with liver mitochondrial adaptation to chronic alcohol exposure [35], steatosis induces significant changes in liver mitochondrial physiology, indicating the plasticity of the mitochondrial proteome to various physiological or pathophysiological conditions. In agreement to this idea, a chronic deficiency of hepatic S-adenosylmethionine (SAM), a deficiency common to most liver-diseased patients [36], impairs mitochondrial function and generates oxidative stress in liver. The fall of PHB1 leading to a mitochondrial failure, together with abnormal lipid, carbohydrate and amino acid metabolism might explain, at least in part, the pathogenesis of steatohepatitis [37]. Proteomics is also emerging as a useful tool in the toxicology field [38, 39]. One of the main difficulties faced by pharmaceutical companies is the failure of principal compounds in the later stages of development due to unexpected toxicities. Drug-induced steatosis is a severe issue since it could lead to liver failure resulting in pulling out the compound from the market [40]. DIGE in combination with mass spectrometry have been successfully employed to identify alterations at the proteome level occurring before the onset of overt toxicity in response to several compounds under preclinical development. Previous to the alteration of other

biochemical parameters, Meneses-Lorente *et al.* identified changes on rat liver proteome supporting impairment of acetyl-CoA production in early stages of CDA-induced toxicity [41] and perturbation of fatty acid β-oxidation and oxidative phosphorylation [42] in the later stages. The correlation of the differential proteins with clinical and histological data as well as their early occurrence, before other biochemical alterations, might consolidate these signatures as predictive biomarkers of compounds with a propensity to induce liver steatosis.

Liver Fibrosis and Cirrhosis

Liver fibrosis is the abnormal deposition and distribution of extracellular matrix (ECM) in tissue as a consequence of chronic hepatic inflammation promoting clinical sequels such as portal hypertension and encephalopathy. It is characterized by an imbalance of matrix breakdown, mainly forced by matrix metalloproteinases (MMPs) and matrix synthesis. Tissue inhibitor of metalloproteinases-1 (TIMP-1) is up-regulated during hepatic fibrogenesis [43, 44]. Furthermore, hepatic stellate cells, main inductors of fibrogenesis, transform from a quiescent state into an activated myofibroblast like phenotype and produce fibrotic proteins, TIMP-1 and inflammatory cytokines, contributing to the maintenance of fibrogenesis and ECM deposition [45]. Hepatic fibrosis is common to many chronic liver diseases of different etiologies and can lead to cirrhosis and its related complications. In the United States, cirrhosis is the most common non-neoplastic cause of death among hepatobiliary and digestive diseases [46]. Additionally, once cirrhosis is established, the risk of developing a HCC is 1-5% a year [47]. Early detection of patients who may develop liver fibrosis is still hampered by the lack of specific biomarkers [48, 49] and therefore, there is a strong demand for reliable, organ and disease specific, non-invasive biomarkers for fibrosis and fibrogenesis to replace or to complement the invasive method of needle biopsy, which is afflicted with a high degree of sampling error. In contrast to liver biopsy non-invasive tests like transient elastography (Fibroscan®, Echosens) or serum-based tests including Fibrotest [50, 51], APRI score [52] index reported by Forns and coworkers [53] either require further evaluation or display insufficient predictive value [54].

Proteomic technologies have been used to identify protein markers for early detection of fibrosis-associated events. Different studies have applied proteomic profiling to identify differential proteins in the liver of animals with experimental fibrosis. Henkel *et al.* conducted a DIGE-based experiment analyzing liver proteomes of 3 different fibrosis models and identified selenium binding protein 2 as a relevant target for hepatic fibrosis in two mice models [55, 56]. A similar approach allowed identification of altered proteins in the liver of thioacetamide treated rats, a widely used model of liver cirrhosis [57, 58], suggesting down-regulation of fatty acid β-oxidation, branched chain amino acids and methionine breakdown and up-regulation of proteins related to oxidative stress and lipid peroxidation. Interestingly, data were integrated into an overview model that may promote further experimental verification [58]. Comparison of proteomes of disease and control body fluid samples has been done to identify biomarkers of liver fibrosis that might promote the development of non invasive diagnostic tests. Chip-based

SELDI TOF analysis of the thousands of peptides and proteins present in serum, plasma or urine is being applied to the discovery of diagnostic proteomic signatures of various diseases including liver fibrosis. A pioneering study using serum protein profiling by SELDI TOF together with identification of significant serum biomarkers reports an algorithm model to identify a cluster pattern that segregates chemically-induced cirrhosis and bile duct ligation liver fibrosis from normal controls [59]. A predictive artificial neural network model (ANN) based on SELDI serum signatures of 46 patients allowed calculation of a fibrosis index that could differentiate between different stages of fibrosis and predict fibrosis and cirrhosis in chronic HBV infection [60]. More recently a 5 biomarker pattern was identified that allowed discrimination of liver cirrhosis from low fibrosis stages with a sensitivity of 80%, a specificity of 67 % and a positive predictive value of 73% [61]. Besides the unquestionable advantages that SELDI TOF technology provides to the analyst, several drawbacks must be seriously considered such as the limited discovery capacity restricted to several hundreds of individual peaks that in most of the biomarkers identified by serum profiling are in fact fragments of major proteins [62], and the low inter-laboratory reproducibility.

To identify urine proteomic signatures associated with liver fibrosis, we have recently used in our laboratory a combination of two dimensional gel electrophoresis and mass spectrometry. Over 50 ml of first morning urine was collected and stored at –80ºC until use. After centrifugation to remove insoluble materials, all urines were concentrated by ultrafiltration using a 10 kDa cut-off Amicon devices and cleaned by protein precipitation with the ReadyPrep 2D cleanup kit (BioRad). Proteins were then resuspended and resolved by conventional 2-DE electrophoresis. According to this procedure, we obtained 2D maps from urine samples allowing the analysis of about 500 spots. Then, urine 2D maps from 8 controls and 11 patients with severe fibrosis (F4 Metavir score) were compared. Four spots were significantly increased in patients with liver fibrosis and were identified according to their peptide fragment fingerprint acquired by nanoHPLC-ESI/MS/MS analysis as Alpha 1 acid glycoprotein 1 (acute phase reaction protein) Mac 2 binding protein (cell adhesion) and pepsin A and cathepsin A (protease activity) (Fig. **2**). These findings indicate that proteomics combined with protein pattern analysis is a valuable approach for the identification of circulating biomarkers, although it must be emphasize that the available studies are the proof of principle and that further experiments are required to validate the use of the information reported in a prospective manner.

Hepatitis and HCC

The outcome for HCC patients still remains dismal, partly because of our limited knowledge of the molecular pathogenesis and the difficulty in detecting the disease at its early stages. Therefore, studies aiming at the definition of the mechanisms associated to HCC progression and the identification of new biomarkers leading to early diagnosis and more effective therapeutic interventions are urgently needed. In the last few years HCC has been extensively investigated using different proteomic approaches on HCC cell lines, animal models, and human tumor tissues resulting in the
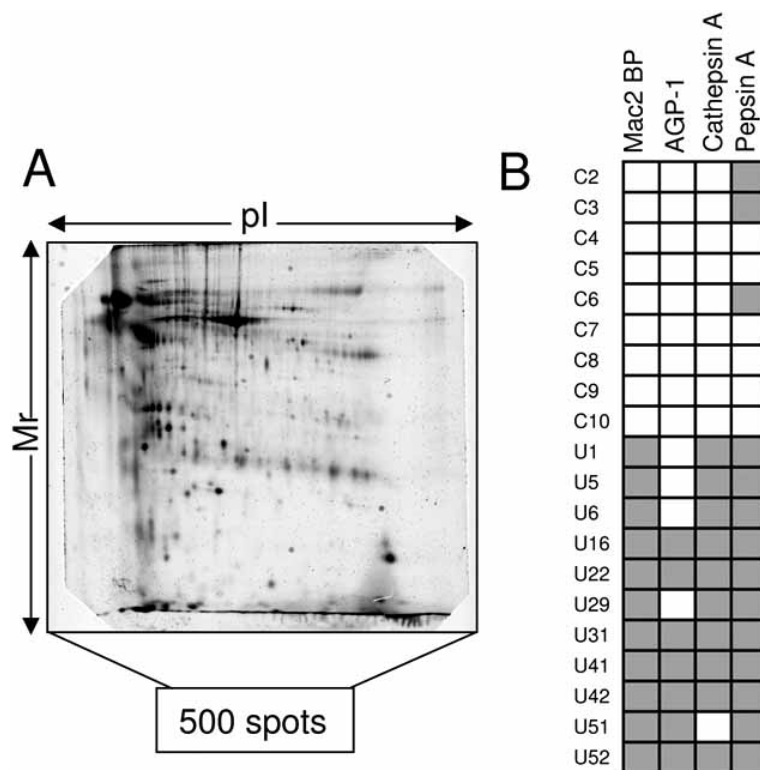
	Mac2 BP	AGP-1	Cathepsin A	Pepsin A
C2				▓
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C7				
C8				
C9				
C10				
U1	▓		▓	▓
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U6	▓		▓	▓
U16	▓	▓	▓	▓
U22	▓	▓	▓	▓
U29	▓		▓	▓
U31	▓	▓	▓	▓
U41	▓	▓	▓	▓
U42	▓	▓	▓	▓
U51	▓	▓		▓
U52	▓	▓	▓	▓

Fig. (2). Urine proteome differential display and liver fibrosis. (A) Representative 2-DE gel allowing analysis of about 500 protein spots after Sypro-Ruby fluorescence staining. (B) Occurrence of differential proteins in controls (C) and patients with liver cirrhosis (U). Shadowed and white squares indicate presence and absence of the corresponding target respectively.

proposal of proteins with great potential regarding its future clinical application [63].

HCC cell lines have been used as *in vitro* models for proteomic studies providing valuable information to recognize changes on cellular pathways that might participate in the development and maintenance of the transformed phenotype, such as metabolism, calcium homeostasis, oxidative stress, reduced tumor suppression capacity and increased cell resistance to apoptosis [64-66]. Additionally, re-expression in HCC of embryonic enzymes has also been reported [67]. The application of subfractionation strategies has extended the capacity to detect HCC differential proteins and allowed recognition of members of heat shock proteins and low Mr annexin families as markers of HCC [68]. Proteomics have also contributed to identify proteins likely supporting mechanisms by which HBV and HCV promote HCC development [69-71], condition the different metastatic potential [72-74], or modify the signaling program of transformed cells [75].

The identification of new diagnostic biomarkers and therapeutic targets for HCC is increasingly approached by comparing protein profiles of diseased and non-diseased human samples, mainly serum and liver tissue. The biological and pathogenic activities of HCV and HBV are different, and, therefore, it is suspected that the process involved in the development of hepatitis and HCC might be distinct for the two viruses [76, 77]. According to this idea, some alterations have been found to be specific of HBV-HCC patients [78], while others are only found in HCV-HCC [79-81]. Addition-

ally, an increasing number of proteins arising from proteomic studies are proposed to be involved in the development of HCC, regardless its aetiology, which might extend our knowledge about the specific properties of the transformed cells [82-86], including the metastatic capacity [87] and the differentiation state of liver tumors [88]. The identification of biomarkers of HCC in biological fluids is especially attractive since comparative inspection of normal and diseased serum proteomes by measuring and characterizing the thousands of individual circulating proteins and peptides might reveal the onset or presence of a disease. Different studies propose circulating protein species that might be used in the diagnosis of HCC [3, 89-92], the evaluation of metastasic potential, aggressiveness, and differentiation state of liver tumors [93] or the response to therapy [94]. The application of SELDI TOF technology has provided HCC specific protein profiles that allow identification of HCC among cirrhosis, hepatitis and less severe liver alterations [62, 95-97]. There is increasing evidence for an immune response to cancer in humans, as indicated by the identification of autoantibodies to tumor antigens. Taking advantage of this biological response, different studies have aimed at the identification of autoantibodies in the serum of patients as markers of HCC. In these studies samples are tested with autologous serum and the immunoreactive proteins are further characterized [98, 99].

Taking together the hundreds of differential HCC associated proteins reported patterns from different groups are barely coincident likely resulting from sample heterogeneity

and dissimilar analytical strategies [100]. The use of experimental models of hepatocarcinogenesis in which the genetic background and the environmental conditions have little impact on proteome variability, and that also facilitate the design of longitudinal studies from preoplastic stages, may have a decisive impact in the identification of HCC associated proteins [101], as has been recently shown with a HBx transgenic mouse [102] and a *MAT1A* knockout mouse with a chronic deficiency of hepatic S-adenosylmethionine [37, 103-105], and hepatitis infected mouse models [106].

Liver Diseases Associated with Heavy Metal Accumulation

Copper is an essential trace element that must be carefully regulated in the cell since its accumulation becomes toxic, as occurs in Wilson disease which is caused by a mutation in the copper transporting P-type ATPase (ATP7B) gene, mainly expressed in the liver. Using 2D gel electrophoresis and MALDI time-of-flight (TOF) MS technology on a sheep model, Simpson *et al.* identified several proteins whose expression pattern was modified after an oral administration of copper, including dehydrogenases, flavin reductase, carbonic anhydrase, and proteins involved in sulfur and glutathione metabolism, suggesting an adaptive response to the oxidative challenge [107]. Additionally, Roelofsen *et al.* using SELDI TOF reported changes in both, intracellular proteome and secretome of HepG2 cells incubated in the presence of a pathological copper concentration although further studies are required to determine the identity of the differential peptides detected [108].

Liver iron overload can be found in hereditary hemochromatosis, chronic liver diseases, or secondary to repeated blood transfusions. The excess iron promotes liver damage including liver fibrosis, cirrhosis and HCC. Using two distinct murine models of iron accumulation, identification of differential proteins supporting alterations of urea cycle, impairment of fatty acid oxidation and changes in the methylation cycle have been reported [109, 110].

SUMMARY CONSIDERATIONS AND FUTURE PERSPECTIVES

The natural history of a disease involves a progression from early stages in which cellular homeostasis is slightly perturbed to more severe phases where the dimension and impact of the pathologic alteration becomes irreversible. A major objective of modern biomedicine is the definition of parameters allowing detection or predisposition to suffer a disease, identification of those patients that might benefit from a particular treatment and prediction of side effects associated with a selected therapeutic strategy in each individual patient. Biomarkers are indicators that would be specific, sensitive, enabling detection at an early stage when treatment is possible, and easily measurable by reproducible and minimally invasive tests. Proteomics is a rapidly expanding discipline with a tremendous potential to extend our understanding of essential aspects about cellular functions, as well as providing information to better define the molecular pathogenesis of human diseases. However, the elucidation of the human proteome is still challenging and several limitations must be circumvented before clinical applications

based on new proteomic discoveries become a reality. Additionally to the exploratory phase aimed at the identification of potential biomarkers using reliable and reproducible assays, further studies are essential to validate the target proteins as biomarkers and assess how close they are to clinical application. Systematic analysis of large cohorts of patients and controls are required to evaluate the capability of biomarkers to differentiate diseased from non-diseased subjects even at an early stage, to detect preclinical disease, and to allow surveillance of the relevant population [111]. Therefore, large prospective cohorts with well-phenotyped patients are needed to ensure correlation between clinical and molecular profiles, technologies have to be improved promoting wider proteome analysis according to normalized and reproducible methods and the enormous amount of data generated must be integrated leading to meaningful functional information that can be easily exchanged and compared among scientists. Consequently, future advances of this young science and its clinical applications require international initiatives encompassing multidisciplinary efforts of experts in different disciplines such as medicine, biology, biochemistry, engineering, bioinformatics, mathematics, chemistry, and physics.

The Human Proteome Organisation (HUPO) was launched in 2001 to "define and promote proteomics through international cooperation and collaborations by fostering the development of new technologies, techniques and training to better understand human disease" (http://www.hupo.org). HUPO has promoted several major initiatives including Human Liver Proteome Project (HLPP) [112] that aims at the establishment of a biological atlas of the liver to extend our understanding of liver functions and to provide tools to develop new diagnostic and therapeutics for liver diseases. The HLPP made great progress in its pilot phase (2003-2005), including identification of close to 5000 proteins and establishment and characterization of more than 1000 murine hybridoma cell lines producing a total of 800 monoclonal antibodies which react with 100 proteins in human liver and plasma [113]. An additional contribution of global initiatives such as the HLPP is the setting of normalized procedures for sample preparation and data analysis. The magnitude of the challenges associated with human samples and validation of candidate biomarkers are highlighted by the declining trend of new FDA-approved biomarkers over the last decade [114]. However, the advances promoted by liver proteome initiatives promises translation of knowledge from current research to future applications in the management of liver-diseased patients permitting early diagnosis, and selection of the appropriate therapeutic intervention for each individual patient.

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