THE PERSPECTIVES OF THE USE OF METABOLOMICS MEASURES IN VIRAL HEPATITIS

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Summary
Metabolomics is one of the '-omic' sciences - beside genomics, transcriptomics and proteomics - belonging to systems biology. It represents an emerging and powerful discipline that provides an explanation of accurate small molecule fingerprints related to the disease. It is an interdisciplinary field of science, which combines analytical chemistry, new technology platforms, mass spectrometry, and NMR spectroscopy with a sophisticated data analysis. In this review we highlight the importance of metabolomics as a potential tool for uncovering metabolic changes in HBV and HCV infections of the liver and for discovering novel biomarkers to improve diagnosis, management and prognosis of the liver diseases.

Key words: metabolomics, hepatitis, biomarkers

CHRONIC VIRAL HEPATITIS

The hepatitis B virus (HBV) infection is still one of the most challenging public health burdens although a safe and effective vaccine has been available since the 80's. It is estimated that nearly one third of the global population has serological evidence of the past or present HBV infection. Approximately 350 million people worldwide are chronically infected with HBV, of whom 1 million die every year of HBV-related liver consequences (1).

The hepatitis C virus (HCV) infection is a leading cause of liver disease worldwide. More than 170 million people are chronically infected with HCV globally and approximately 20% of them develop cirrhosis over a period of 20 years. Patients with established cirrhosis are at high risk for decompensation and development of hepatocellular carcinoma; it is estimated that 350 000 deaths occur every year as a result of these severe complications of the HCV infection (2).

DIAGNOSTICS

These data clearly indicate a crucial importance of an early and accurate diagnosis of liver diseases. Diagnosing liver diseases and assessing the severity of liver injury include biochemical markers, such as activity of serum transaminases (aspartate transaminase AST, alanine transaminase ALT), γ-glutamyl transpeptidase (γ-GTP), alkaline phosphatase (ALP), as well as prothrombin time, serum albumin concentration and blood counts (3). Then, patients are subjected to diagnostic imaging, such as ultrasound and computed tomography (CT). In respect to all patients presenting increased aminotransferase levels, with chronic liver diseases of unclear etiology and with the history of enhanced risk of hepatitis virus transmission HBV and HCV, the diagnostics should be performed.

The diagnosis of HBV infection based on the detection of series serological markers of HBV, such as hepatitis B surface antigen (HBsAg) and antibody (anti-HBs), hepatitis Be antigen (HBeAg) and antibody (anti-HBe) and antibody for hepatitis core antigen (anti-HBc) by serologic assays (radioimmunoassays RIA or enzyme-linked immunoassay EIA). Polymerase chain reaction (PCR) assays allow to directly determine the hepatitis B virus DNA (HBVDNA) in serum (4).

For a diagnosis of the hepatitis C, serologic and nucleic acid-based molecular assays are also available. In clinical practice antibodies against HCV epitopes are detected by 3rd generation EIA test with a high specificity and sensitivity of more than 99%. Positive serologic results require a confirmation by molecular tests for detection of HCVRNA to differentiate between the chronic hepatitis C and a resolved HCV infection. HCVRNA

measurement is routinely performed using PCR. HCV is heterogeneous and until recently six genotypes (1-6) and multiple (a, b, c, d…) subtypes have been characterized. Since HCVRNA was detected, genotyping and HCV RNA load assessment by molecular nucleic acid-based tests is mandatory in every patient who considers antiviral therapy. It determines the treatment duration, a ribavirin (RBV) dose and even success rate (5).

Finally, a liver biopsy is recommended to evaluate the severity of liver diseases for patients with chronic hepatitis. Currently used scoring systems assessed histologic lesions using two separate scores, one for the necroinflammatory stage and the other for the stage of fibrosis (6). Although a liver biopsy remains the reference method for determination of liver fibrosis, it has several disadvantages such as poor patient compliance, sampling error, limited usefulness for dynamic surveillance and follow-up. Alternative non-invasive methods can now be used to assess a disease severity. Liver stiffness measurement and panels of biomarkers of fibrosis can be performed instead of liver biopsy at a safe level of predictability (4, 5).

TREATMENT

All patients with HBsAg positive chronic hepatitis should be considered for antiviral therapy. The decision of treatment initiation is based on the HBVDNA serum level, ALT activity and the severity of liver disease. Two drug classes are available for the treatment of chronic HBV infection, the immune modulator interferon α (recombinant or pegylated pegIFN) and nucleoside or nucleotide analogs. Analogs act as reverse transcriptase inhibitors of the HBV polymerase. Currently, the nucleoside analogs lamivudine (LMV), telbivudine (LdT), entecavir (ETV) and nucleotide analogs adefovir dipivoxil (ADV), tenofovir disoproxil (TDF) are also available (4).

The goal of antiviral therapy of chronic HCV infection is to cure hepatitis C by a sustained virus eradication. It is defined as a negative serum HCVRNA six months after the end of the treatment (sustained virological response SVR). The current therapy recommendation is based on HCV genotype and a viral load before and during the treatment. Standard antiviral regimen consisted of pegIFNα and RBV results in SVR in 40 to 50% patients infected with genotype 1 or 4 and 80% of those infected with genotype 2 or 3 (7). The outcome of a double therapy depends also on host factors, such as age, race, BMI, and a stage of liver fibrosis (8). Recent efforts to improve the rate of SVR have been focused on oral direct-acting antiviral agents. Within this class of drugs, telaprevir and boceprevir are currently available for treating patients infected with genotype 1, with a higher success rate compared to a standard therapy (9).

PLACE OF METABOLOMICS AMONG -OMICS.

With advances in technology and the ability to collect and process enormous amounts of data, the progress of life sciences has seen a change from a reductionist approach towards that provided by systems biology, that is, measuring biological aspects of a whole system and its interaction with its surroundings, rather than targeting one single part of it. In today's research world, -omics techniques such as proteomics, transcriptomics, genomics and metabolomics have become an integral part of systems biology. However, metabolomics is a window that offers a perspective distinct from the lenses of genomics, transcriptomics, and proteomics (10-13).

Metabolomics, an emerging and powerful discipline, reveals homeostatic imbalances in biological systems, and has the capability of providing comprehensive information. It enables the parallel assessment of the levels of a broad range of endogenous and exogenous metabolites, and has been shown to have a great impact on the investigation of physiological status, diagnosing diseases, discovering biomarkers, and identifying perturbed pathways due to disease or treatment. Metabolomics adopts a "top-down" strategy to reflect the function of organisms from terminal symptoms of metabolic network in a holistic context. It can provide a panoramic view of abundance changes of endogenous metabolites in monitoring cellular responses to perturbations (e.g. diseases or drug treatments) (11, 14-18).

Assessing the origin of metabolomics brings us far back to the history of bioanalytical sciences before the terms "metabolome", "metabonomics", and "metabolomics" were finally accepted by the scientific community. Both terms, metabonomics and metabolomics, describe in a broad manner the study of the metabolome, which was first defined as a collective set of metabolites produced or present in a biosystem. The most often cited definition of metabonomics is the one proposed by Nicholson et al.: 'Metabonomics is defined as the quantitative measurement of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification' (19). For metabolomics a very similar definition is often used: 'The study of the quantitative complement of metabolites in a biological system and changes in metabolite concentrations or fluxes related to genetic or environmental perturbations. Studies are typically holistic in nature though targeted studies are also encompassed in the term metabolomics' (20). Then, metabolomics is the study of metabolic changes in biological systems and provides 'the small molecule fingerprints' related to the disease. Importantly, metabolomics' approaches have developed in many areas of biomedical research and recently demonstrated significant potential for example in toxicology studies, nutritional effects, metabolic consequences of genetic modifications, inborn errors of metabolism, diabetes, cancer diagnostics, physiology, diagnostics, functional genomics, pharmacology, toxicology, nutrition and diagnosing of neurological diseases, and etc (example ref.: 10, 11, 15, 16, 18, 20-29).

ANALYTICAL METHODS IN METABOLOMICS

Metabolomics in its practice combines high-throughput analytical chemistry, typically, methodologies based upon mass spectrometry or nuclear magnetic resonance spectroscopy, with multivariate data analysis. There is no single analytical technique that is suited to the precise and accurate identification and quantification of all the metabolites in question. Regardless of the analytical set up, metabolomics studies can be divided into two different types: targeted and non-targeted approaches, depending on at which stage the metabolite identification is performed during the data processing. Non-targeted metabolomics is used for global metabolome analysis, that is, a comprehensive analysis of all the measurable analytes in a sample. In a targeted metabolomics strategy, predefined metabolite-specific signals (by selected reaction monitoring (SRM) by tandem mass spectrometry (MS/MS), or selected ion recording (SIR) by GC-MS) are often used to determine precisely and accurately relative abundancies and concentrations of a limited number of pre-known and expected endogenous metabolites (15, 16).

Generally, rich metabolome data can be collected by the metabolic profiling analysis based on liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS) or nuclear magnetic resonance (NMR) spectroscopy, with all possible modifications or combinations. For the analysis of complex, biological samples like biofluids, these techniques have their advantages and disadvantages. For instance, GC-MS requires derivatization, which lengthens the sample preparation time. In general, LC-MS and GC-MS need more time-consuming sample preparation. On the other hand, GC-MS and LC-MS yield a higher sensitivity than NMR spectroscopy and therefore may detect metabolites that are present in a concentration below the detection limit of NMR. NMR spectroscopy, however, requires a limited sample preparation and is untargeted, quantitative (absolute), non-destructive, reproducible and unbiased. 1H NMR spectroscopy may detect compounds that are too volatile for GC, while metabolites without protons are not detectable by 1H NMR spectroscopy. Nonetheless, the profile of NMR spectra is complex and containing hundreds of information. For an efficient interpretation, the use of statistical tools is necessary, such as the principal components analysis (PCA), partial least squares (PLS), the discriminant analysis (DA) and the simple classification analysis (SIMCA) (13, 15, 16, 19, 20, 25, 30-33).

BIOMARKERS AND METABOLOMICS

Importantly, metabolomics can be used for an analysis of endogenous and exogenous metabolites for a biomarker discovery. According to the Foundation of the National Institutes of Health, a biomarker is defined as 'a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention' (34). Likely, the Food and Drug Administration (FDA) defined a valid biomarker as: 'A biomarker that is measured in an analytical test system with well established performance characteristics and for which there is an established scientific framework or body of evidence that elucidates the physiologic, toxicologic, pharmacologic, or clinical significance of the test results' (35). In general, a biomarker is recognized as a laboratory measurement that is an indicator of diseased processes and also the risk of the appearance.

Today, the improved efficiency and accuracy of biomarkers and early diagnostic technologies have been increasingly used in a clinic setting. It is possible due to a relatively new strategy of FDA that launched Critical Path Initiative, intended to improve the predictability and efficiency of drug development (36). Metabolomics is an FDA-identified Critical Path Opportunity (37) and has the potential to play a vital role in many areas: the development of better biomarkers, safety biomarkers related to kidney, liver, heart, and vascular damage, disease models for lead optimization and toxicity assessment, the identification of safety biomarkers and biomarkers of efficacy and toxicity to facilitate clinical trial designs, patient stratification, diagnostic monitoring of patient response to drug treatment, and many more. As such, metabolomics is fast becoming an important discovery tool for new diagnostic and prognostic biomarkers. It is envisioned that this will provide new avenues towards preventive medicine and prognostic strategies for tailored therapeutic and personalized nutrition management (16, 20).

PRACTICAL ASPECTS OF METABOLOMICS RESEARCH

A metabolomics experiment generally consists of the following steps: a sample collection, an extraction, an analysis of the extract, data reduction and statistics. The results depend heavily upon the very initial steps that involve the sample collection and extraction. Generally, the idea of metabolomics is that the samples to be analyzed contain all the intact metabolites (10, 16, 20).

Sampling for a metabolomics analysis should be fast because of the high rate of metabolome turnover in a living organism or cells and it is clearly very important to collect samples fast and take steps to stop any kind of biochemical activity immediately. Cellular metabolism is a dynamic process and any change in the parameters of the outside-cell environment can alter the metabolic turnover. Rapid inactivation of all biochemical and enzymatic activity in a cell is known as quenching and among methods available we may list: the use of methanol (hot > 80°C or cold < -40°C), which causes a sudden temperature shock and helps

to interrupt biochemical reactions; rapid changes in pH; sudden temperature drops achieved by dipping the sample in liquid nitrogen (10, 16, 17, 20).

Then, the samples can be dried by heating, using ovens, microwaves, hot air or by freeze-drying. The reasons for that are: to facilitate the long-term storage of the samples by further inhibiting enzymatic activity and microbial growth and to remove water, as in some cases water can interfere with the instruments used to analyze the samples. Especially, it is important in the case of nuclear magnetic resonance (NMR) spectroscopy where traces of water in the samples can distort the resolution of the spectra. Finally, to achieve an effective extraction, the samples should be well homogenized before the storage or prior to the analysis, when necessary, with grinding, ultrasonication or freeze–thaw cycles, or a combination of these methods (10, 16, 20).

Importantly, the proper sample collection is crucial for the success of metabolomic measurements. From scientific point of view the best action that could be taken by the clinicians, who are not experienced in metabolomics analyses, is to snap-freeze the human sample at –73°C. Then, the experienced researcher could provide further steps of a sample pretreatment.

METABOLOMICS AND HEPATITIS

Both, HBV and HCV infections, cause metabolic changes within infected hepatocytes that are challenging for diagnostics, since many distinct diseases present similar clinical signs and laboratory findings. As a major organ of metabolism in the body, the liver takes part in many metabolic pathways; it is a source of myriad endogenous metabolites and precursors used by other organs. There can be no other organ where such a plethora of both lipids and water-soluble metabolites are metabolically interchanged. No other organ exceeds the rates of metabolism and energy production and consumption as found in the liver or has such an enormous impact on the systemic state of the organism (6, 14, 38).

The evaluation of liver disease in patients with hepatitis B or C is essential to identify those of them who require antiviral therapy and to determine prognosis. Staging of liver fibrosis and the occurrence of cirrhosis associated with HBV or HCV infection are traditionally done by biopsy, but now there has been a move towards the use of non-invasive biomarkers (3-5, 16, 25). Therefore, metabolomic studies in hepatitis B and C patients are very timely. Considering that HBV and HCV are a major cause for primary liver cancer (HCC), as well as the fact that symptoms for HCC do not appear typically until the last stage of the disease, it is of significant interest to identify infected patients with risk of developing HCC based on the identification of altered metabolites and metabolic pathways (39;40).

Several studies using a variety of analytical techniques have reported a discovery of potential biomarkers in human blood serum or plasma (14, 41-52), urine (17, 27, 33, 35, 53, 54) or liver bioptates (47, 55) of patients with viral hepatitis. Those reports could be divided into a few sub-classes regarding the scope of the study related to the groups of patients compared with metabolomics approach: 1) patients with liver hepatitis and healthy control (14, 33, 50, 52), 2) patients with various liver disorders with underlying viral infection and HCC patients (42, 43, 53-55), 3) patients with viral hepatitis and other liver diseases, but the HCC patients were not included in those studies (44, 48, 51), 4) investigation of progression of metabolic changes in viral hepatitis, including the metabolic response to treatment (31, 45-47, 49, 50).

The potential biomarkers revealed in those studies span a broad range of compound classes, including amino acids, organic acids, nucleosides and nucleotides, purines and their derivatives, amino ketones or ketone bodies, lipid acids and their derivatives and many others. In fact, any analytical technique focused on the detection of a specific class of small molecule weight compounds revealed some minor or major biochemical differences between the samples obtained from different patients. However, all these data could be considered as the preliminary results and therefore the studies with larger patient groups are needed to confirm the clinical value of those compounds. On the other hand, even considered as preliminary, some results are very promising, e.g., the metabolic profiles of the tissues correlated better with HCC diagnosis than the traditional single tumor marker alpha-fetoprotein (AFP) (53).

Importantly, the metabolomics research in viral hepatitis is just a part of the huge area of research on liver diseases mostly oriented towards a discovery of the new biomarkers of a liver cancer, which we only mention in this review. We do not discuss the most recent reports on the studies provided on animal models of viral hepatitis as some of the researchers doubt in a usefulness of such models for research on liver diseases. Especially in metabolomics when the content of the biochemical compounds in the tissues is a resultant of genomic, transcriptomic and proteomic interactions that are different in various species.

CONCLUSIONS

Metabolomics is a rather recent and ambitious concept in bioanalytics that aims to quantify small molecules (molecular weight less than 1500 Daltons) present in any biological system or any specific physiological state. It provides a powerful approach to discover diagnostic and therapeutic biomarkers, but could be regarded as still in its infancy since several issues have not been fully addressed for metabolite profiling to keep pace with genomics technologies. The near causal link between a chronic viral infection and a cancer identifies a patient population whereby biomarkers of cancer risk would be

instrumental in improving current diagnostic and treatment paradigms. Better methods to identify the subset of HBV or HCV patients at higher risk of further progression of liver damage or a higher cancer risk would allow for: 1) the establishment of more intense surveillance mechanisms, 2) the development of chemoprevention protocols, and 3) improved leverage for these patients with respect to liver transplants.

The most valuable, from a clinical point of view, are the measurements obtained from urine and blood plasma/serum, as these tissues are easily accessible and contain a great number of small molecular-weight compounds. The most of those compounds were never considered to be useful in diagnostics but have potential to become the biomarkers of development or progression of liver disease (for reference: see previous subchapter).

The most frequent tools used in metabolomics research on liver diseases are the MS-based platforms probably due to their accessibility at the research institutes: the number of MS-based analytical instruments is significantly higher than NMR tools in any country. Another fact is that the continuous improvements in the MS analysis as well MS-based platforms that combine MS with LC, GC, EC (electrophoresis) or other analytical techniques are much more rapid than in NMR field, which favors them for the clinical use. However, it should be underlined that due to the sophisticated methodology the management of such analytical tools is probably beyond the competence of the clinicians, indicating the need for the close cooperation between medical doctors and the qualified research personnel. On the other hand, the reports on the use of metabolomics approach in the research on liver diseases demonstrate that it is a potential tool for use in medical practice in the near future. □

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