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REVIEW

METABOLOMICS IN MEDICAL SCIENCES – TRENDS, CHALLENGES AND PERSPECTIVES

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Abstract: Metabolomics is the latest of the "omic" technologies that involves comprehensive analysis of small molecule metabolites of an organism or a specific biological sample. Metabolomics provides an insight into the cell status and describes an actual health condition of organisms. Analysis of metabolome offers a unique opportunity to study the influence of genetic variation, disease, applied treatment or diet on endogenous metabolic state of organisms. There are many areas that might benefit from metabolomic research. In the article some applications of this novel "omic" technology in the field of medical sciences are presented. One of the most popular aims of metabolomic studies is biomarker discovery. Despite using the state-of-art analytical techniques along with advanced bioinformatic tools, metabolomic experiments encounter numerous difficulties and pitfalls. Challenges that researchers in the field of analysis of metabolome have to face include i.a., technical limitations, bioinformatic challenges and integration with other "omic" sciences. One of the grand challenges for studies in the field of metabolomics is to tackle the problem of data analysis, which is probably the most time consuming stage of metabolomic workflow and requires close collaboration between analysts, clinicians and experts in chemometric analysis. Implementation of metabolomics into clinical practice will be dependent on establishment of standardized protocols in analytical performance and data analysis and development of fit-for-purpose biomarker method validation. Metabolomics allows to achieve a sophisticated level of information about biological systems and opens up new perspectives in many fields of medicine, especially in oncology. Apart from its extensive cognitive significance, metabolomics manifests also a practical importance as it may lead to design of new non-invasive, sensitive and specific diagnostic techniques and development of new therapies.

Keywords: metabolomics, metabolites, biomarker discovery, omic technologies

The major challenges for the XXI century medicine include better understanding of disease etiology, especially elucidation of the influence of genetic variations and environmental factors on health status, and development of personalized healthcare solutions (therapies tailored to biological state of an individual) (1, 2). In order to face these challenges a new "omic" technology - metabolomics - has been emerged.

Metabolomics involves a comprehensive analysis of small molecule metabolites (< 1 kDa) of an organism or a specific biological sample and is based on a wealth of biochemical knowledge that was developed over many years (3). This latest of the so-called "omic" research fields is focused on intermediates and products of metabolism, which include carbohydrates, fatty acids, amino acids, pigments, nucleotides, organic acids, vitamins, antioxidants and many other classes of compounds. In analogy to the terms "transcriptome" and "proteome", a complete set of metabolites synthesized by a biological system forms its "metabolome" (Fig. 1). Metabolome can be characterized on all levels of biological complexity: organisms, tissues, cells or cell compartments (4). A wide range of living organisms have been investigated using metabolomic approach, from simple microbes to complex biological systems such as mammals.

The number of metabolites in the human body is relatively small in comparison to the number of genes, transcriptomes or proteins (Fig. 1), however, the number of factors influencing the levels of metabolites in biological fluids and tissues is very large. Metabolome is dynamic and susceptible to natural fluctuations and external perturbations and thus metabolomics provides an insight into the cell

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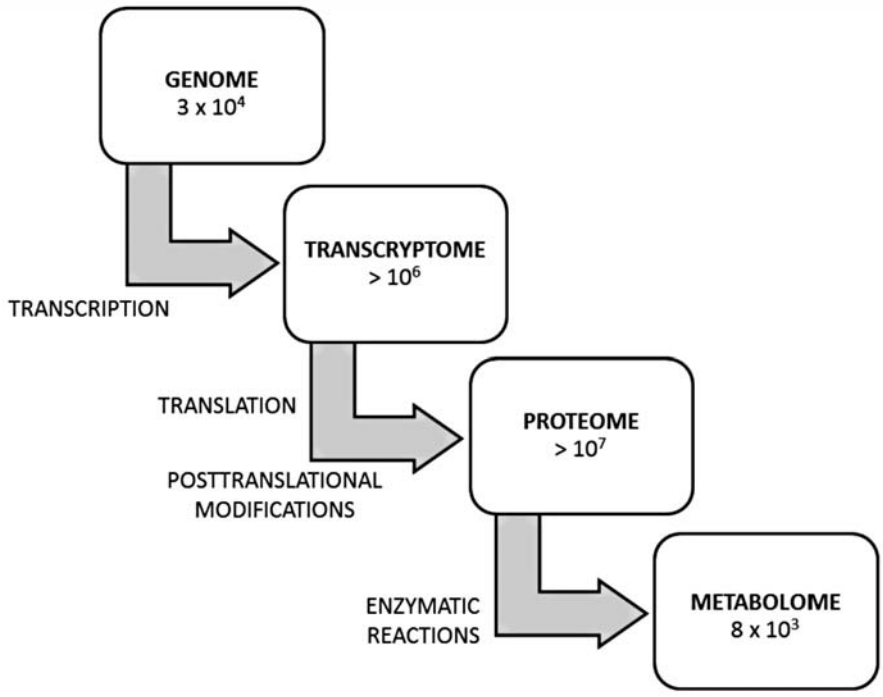

Figure 1. Diagram illustrating "omic" technologies. The flow of information in the human organism begins with genes (3×10^4) and ends with metabolites (8×10^3) (6, 24).

status and describes an actual health condition of organisms (Fig. 2). Metabolite levels can be regarded as the final response of an organism to environmental factors, genetic modifications, changes in gut microflora and altered kinetic activity of enzymes (2, 5). Therefore, this new research platform is most closely related to phenotype among other "omic" technologies (Fig. 2).

Since genomics, transcriptomics and proteomics do not fully explain the complex response of organisms to physiological and pathophysiological stimuli, increased interest of metabolomic research has been observed in recent years. Metabolomics, which attempts to profile all metabolites within a cell or biological system, offers a unique opportunity to study the influence of genetic variation, disease, applied treatment or diet on endogenous metabolic state of organisms (6, 7).

Numerous metabolomic experiments aim to search for metabolites which discriminate samples from individuals who have a disease from those of healthy subjects. According to Food and Drug Administration "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or biological responses to a therapeutic intervention" is called biomarker (8). Searching for specific and sensitive biomarkers that enable to unequivocally detect disease is one of the most crucial goals for metabolomic investigations.

Modern metabolomics dates back last decades. However, it is worth pointing out that the concept of metabolomics, i.e., using chemicals as diagnostic markers, comes from ancient history, regardless of realization or not by ancients the real nature of the concept of biomarkers. The idea that changes in tissues or biological fluids indicate diseases emerged and was present over centuries, i.a., in the work of Hippocrates, who proposed the method for diabetes diagnosis based on the sweet, fruity odor of human breath in the IV century B.C., and of Ullrich Pinder, whose urine wheel enabled to diagnose various diseases based on the urine colors, smells and tastes in 1506. Modern-day metabolomics uses analytical techniques and chemometrics in detection and quantitation of hundreds of metabolites in body fluids or tissues (3, 9).

Analytical techniques used in metabolomics

The most common analytical platforms used in metabolomics involve nuclear magnetic resonance (NMR) spectroscopy, mostly ^{1}H-NMR, and mass spectrometry (MS) (10, 11). Both of these spectro-

scopic techniques enable analysis of a multitude of small molecules coexisting in a sample including metabolite identification and quantitation, but each of them has its own strengths and limitations.

The advantages of NMR spectroscopy include minimal sample preparation and non-reliance on analyte separation. NMR technique does not destroy specimens, thus they can be recovered and used for further studies by other methods. The non-destructive nature of NMR is particularly useful for the metabolite analysis of intact cell or tissue (biopsy samples). In addition, in NMR signal, intensity is less affected by matrix components of the sample than in MS technique and thus NMR is a very appropriate tool for analyses of complex biological samples. The major limitation of NMR remains its low sensitivity (7, 12).

Application of mass spectrometry in metabolomic investigation usually requires separation of metabolites from analyzed biofluids. Thus, mass spectrometry is frequently coupled to high resolution techniques, such as liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis (CE) (11-13). Chromatography-mass spectrometry platforms are often used in metabolomics due to their high sensitivity and

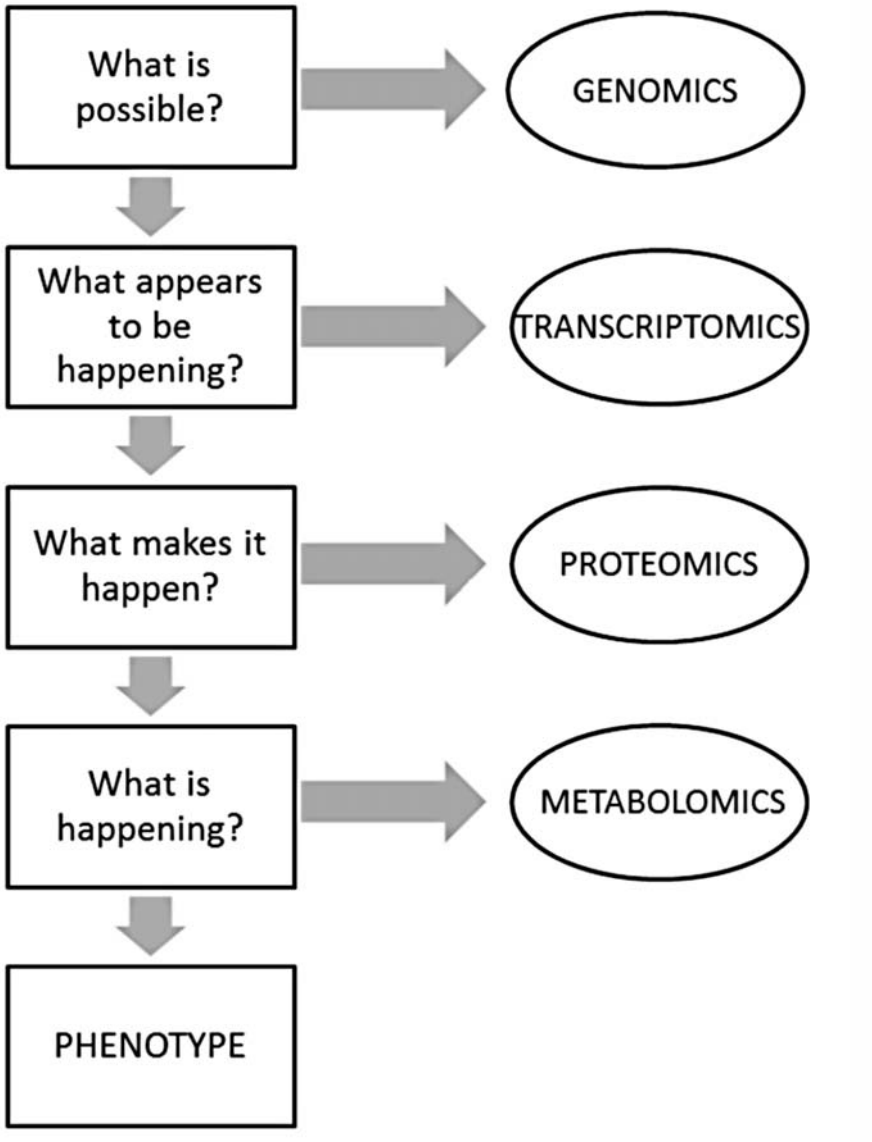

Figure 2. Among „omics", metabolomics is most closely related to a phenotype (24).

repeatability. Although mass spectrometry is a destructive technique, only small amounts of samples (μL) are required for analysis. In GC-MS compounds are heated to the gas state, hence nonvolatile substances cannot be analyzed directly and require derivatization. By selecting the appropriate mobile phases and column, LC-MS provides opportunity for analysis of a wide range of metabolites ranging from hydrophilic to hydrophobic (12, 13). Significant improvement in chromatography has been achieved by releasing ultra-high performance liquid chromatography (UHPLC). UHPLC systems utilize columns with smaller particle sizes, which result in increased peak capacity, higher sensitivity and reduced time of analysis in comparison to HPLC (11, 12). The separation mechanism in capillary electrophoresis is different from that used in liquid and gas chromatography and enables analysis of polar, ionic molecules. Low repeatability is one of the major limitations of CE, whereas excellent separation capacity is regarded as the main advantage of this analytical technique (12-14).

It is observed that biomarkers indicated by NMR and MS techniques are different, which suggests complementary nature of these two analytical platforms (15). It can be concluded that a comprehensive characterization of the metabolome of an organism can be obtained only by a combination of analytical techniques and data from several instruments.

Different approaches in metabolomics

In case of current metabolomics, three different approaches to the research are possible: metabolic fingerprinting, metabolite profiling and targeted metabolomics.

Metabolic fingerprinting is a global, rapid evaluation of reproducible metabolite fingerprint of a biological sample. In this strategy, metabolite identification is not necessary. The fingerprint aims to represent many diverse classes of compounds of potential interest. Compounds (metabolites) are not known a priori in this approach. Metabolic fingerprinting neither requires advanced sample preparation nor ultimate chromatographic resolution techniques. It rather uses techniques which provide less complex and more reproducible data. Metabolic fingerprinting is used mainly for the classification of a sample and not for quantitative analysis. The aim of metabolic fingerprinting is to discriminate between specimens from different biological status, e.g., disease/health, based on unique pattern characterizing a metabolic state in a particular tissue or biological fluid. Being simultaneously applied to a wide range of metabolites metabolic fingerprinting represents a true “omic” approach (16-18).

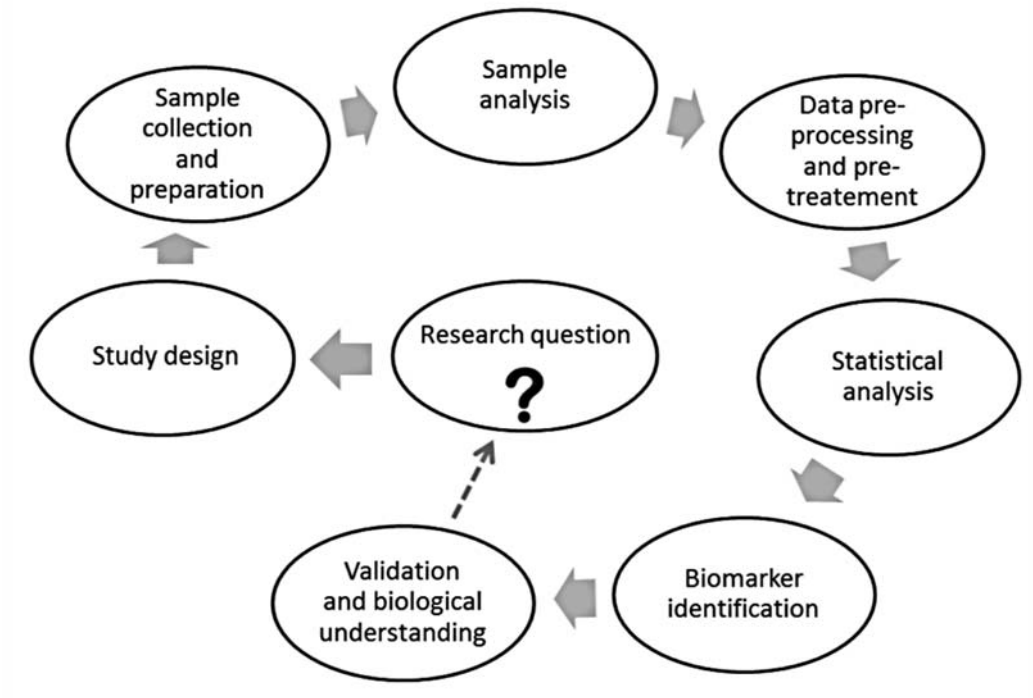

Figure 3. A typical metabolomic experimental workflow for biomarker discovery.

Metabolite profiling is a nontargeted metabolomic approach. It concentrates also on analysis of a wide range of metabolites (for example, amino acids, sugars, lipids, bile acids) and compounds of potential interest are also not known a priori. However, in contrast to metabolic fingerprinting, metabolite profiling aims to identify as many compounds as possible, as well as to quantify them. This involves high-throughput measurement of metabolites, which requires high-resolution chromatographic separation along with mass spectrometry detection. This strategy enables detecting changes in unexpected parts of metabolome. Those changes can be addressed to specific metabolic pathways. Thus, metabolite profiling often leads to formulating of new scientific hypotheses and identification of novel metabolic biomarkers (16, 17, 19).

Targeted metabolomics focuses on monitoring of one or several predefined metabolites, which usually permits their definite identification and precise quantification in a sample. The compounds are selected a priori on the basis of known metabolic pathways or identified biomarkers and are related to a specific reaction in the organism. In case of targeted metabolomics, analytical techniques, including sample preparation and unambiguous detection methods, aim to provide maximum sensitivity and selectivity in order to obtain low detection and quantitation limits of metabolites (16, 17, 19).

Metabolomic workflow

A scheme of a workflow in metabolomic studies is presented in Figure 3. Metabolomic investigation can focus on cells, fluids or tissues. Urine and blood are typical specimens used in metabolomic studies, but saliva, bronchial washes, cerebrospinal fluid, pancreatic juice and other biofluids can be utilized as well (10).

Sample collection, storage and preparation constitute crucial steps, which may determine the success of metabolomic experiment. If the specimens are not taken from patients in the same way or are not stored or processed uniformly, the data obtained from analyses of these samples can be invalid (20). Sample preparation depends both on the type of the sample and on the applied analytical platform and involves such processes as extraction, derivatization or buffering. The next step includes analysis of the samples by one or several analytical techniques. Analytical techniques used in most recent metabolomic studies for metabolite fingerprinting include: direct infusion mass spectrometry and NMR spectroscopy, whereas liquid chromatography or gas chromatography coupled with mass spectrometry are applied for metabolite profiling (19).

The goal of data analysis is to find significant changes (metabolite or set of metabolites) which discriminate patients with a specific disorder and a control group. Before statistical analysis, the output data are subjected to pre-processing (deconvolution, alignment, baseline correction) and pre-treatment steps (scaling, normalization) to make features more comparable (21). Because research in the field of metabolomics generate large amount of data, multivariate statistical analyses are predominantly used (22). There are many pattern recognition methods that can be applied to assess how the spectra of biological samples from individuals who suffer from a disease differ from samples of healthy subjects. Commonly used strategy for identifying significant metabolites include application of unsupervised methods: principal component analysis (PCA), hierarchical cluster analysis (HCA) as well as supervised methods: partial least squares discriminant analysis (PLS-DA), orthogonal projection to latent structure discriminant analysis (OPLS-DA) (12). After determination of metabolite biomarkers, further experiments are needed to validate and test them. Differences in concentration of the discriminating metabolite should be confirmed on a new set of samples (21). The robustness of prediction of supervised analysis can also be checked with internal cross-validated tests to avoid overfitting of the data and confirm reliability of identified biomarkers (23). In the final step of data analysis, the biomarkers can be placed in metabolic networks to enable biological understanding of occurring phenomena.

Applications of metabolomics in medical sciences

There are many areas that might benefit from metabolomic research. This latest "omic" science has a diversity of health applications, including pharmacology, toxicology, new-born screening and clinical chemistry. Metabolomics is currently being used to discover new biomarkers of various diseases and to identify biochemical pathways involved in their pathogenesis (3, 7, 10). The goal of metabolomic research in not only searching for new diagnostic biomarkers that can enable early detection of disease, but also biomarkers useful in selection of the appropriate therapeutic intervention and evaluation of response to the applied treatment. Consequently, metabolomic approach offers a powerful tool in development of personalized medicine. Metabolomic-based research enables assessing levels of multiple metabolites in a single analytical run and thus leads to a better classification of samples as compared to quantifying a single metabolite. That serves for new diagnostic capabilities of meta-

bolomics (5). Because metabolites can be readily measured in non-invasive samples such as urine or blood, metabolites are considered as ideal biomarkers. Moreover, the results of many metabolomic experiments are further broadening the knowledge about complex and heterogeneous nature of disorders, which in turn may contribute to uncover new targets in drug discovery process (3).

One of the major areas of metabolomic studies is searching for novel specific and sensitive cancer biomarkers (5, 7, 10, 24). It is known that cancer cause alterations in cellular metabolism, therefore metabolomics may play a crucial role in early detection and could improve diagnosis of tumors (24). Application of high-throughput and sensitive analytical techniques in metabolomic experiments make them a powerful tool in the field of oncology and aid understanding what is happening in cancer cells.

Numerous groups of scientists has attempted to use metabolic profiling as diagnostic tool in almost every type of carcinoma. For instance, metabolomic approach has been applied to differentiate between urine samples from patients with kidney cancer and healthy individuals. It was discovered that urinary excretion of several acylcarnitines, α-ketoglutarate and quinolinate is altered in patients suffered from kidney cancer as compared to controls (25-27).

Biomarkers for breast cancer have also been extensively investigated *via* metabolomics. Analyses of tissue and serum specimens from breast cancer patients revealed that changes in membrane phospholipids synthesis is associated with the progression of breast tumors (28). Studies proved that urinary metabolomics could be useful for detecting not only early-stage of breast cancer, but ovarian cancer as well (29). Moreover, metabolomic analyses can provide accurate information about the subtype and grade of breast tumors (28).

Many studies have been performed to test metabolomic approach in detection of colorectal cancer (30-32). Analysis of cancer patients' serum samples and controls resulted in identification of four differentiating metabolites: 2-hydroxybutyrate, aspartic acid, kynurenine and cystamine. A colorectal cancer prediction model composed of these four compounds was successfully validated using new sets of patients (30). Other reports indicated a number of colorectal cancer biomarkers, which are associated with perturbation of glycolysis, arginine and proline metabolism, lipid and steroid metabolism (31, 32).

Metabolomic research aiming at detection of altered metabolic pathways in cancer and discovery of novel biomarker candidates also involved other types of tumors. Examples include prostate cancer (33), bladder cancer (34), pancreatic cancer (35) and liver cancer (36). Detection of metastasis, staging of tumor and pharmacometabolomics (the use of metabolomics to evaluate therapeutic efficacy and drug toxicity) should be also mentioned as emerging application of metabolomics in cancer studies (5).

Apart from cancer research, metabolomics has been applied to better understand biochemistry of many other disorders and to improve their diagnosis, prognosis and therapeutic regimens. Conditions investigated by metabolomic approach cover i.a., respiratory, metabolic, neurological and even psychiatric diseases.

Exploration of 400 small molecule metabolites in serum specimens provided evidence for anti-inflammatory changes in tuberculosis (TB) (37). It was shown that 20 metabolites enable robust discrimination of patients with tuberculosis from healthy individuals (37). In addition, metabolomic investigation makes it possible to predict tuberculosis therapy outcome. Based on urine analysis, TB-early treatment response biosignature was defined (38).

Analysis of metabolome is particularly useful in neonatal screening for inborn errors of metabolism (IEM). Inherited diseases of metabolism include i.a., amino acid, fatty acid and organic acid disorders. The purpose of neonatal screening is early diagnosis of the disease and prompt initiation of an appropriate therapy, which allows to avoid serious consequences, such as mental retardation (39). Diagnosis of IEM is often based on finding of the elevated level of a specific metabolite, e.g., phenylalanine in phenylketonuria. Application of mass spectrometry technique in screening for IEM allows the simultaneous determination of multiple markers from one blood sample in a single analysis (40).

The field of metabolomics has provided new insights into the pathogenesis of diabetes as well as methods useful in prediction of the disease onset and has uncovered new potential biomarkers (41-46). Recent epidemiological studies, using metabolomic approach together with statistical analysis to predict incidence of diabetes, have revealed several metabolites which have strong potential as diagnostic biomarkers or highly-significant predictors of future diabetes. Predictors of diabetes include branched-chain and aromatic amino acids, such as leucine, isoleucine, tyrosine, phenylalanine and valine (43). Subsequent research proved that these diabetes-predictive amino acids are also novel markers for development of cardiovascular disease and constitute link between diabetes and susceptibility to car-

diovascular disease (44). Moreover, it was indicated that elevated concentrations of abovementioned amino acids correlate with obesity in children and adolescents and may be regarded as independent predictors of future insulin resistance (45, 46).

Based on analyses of plasma samples from patients suffering from Alzheimer's disease (AD) several putative biomarkers of this condition were found and identified (e.g., lysophosphatidylcholines, sphingosine and tryptophan) (47). In addition, in order to increase knowledge of the dysregulated metabolic pathways associated with progression of Alzheimer's disease, metabolic profiling of AD brains was performed (48).

Despite significant advances in medicine, diagnosis and treatment of psychiatric disorders remain problematic. Since there are no biomarkers of major depressive disorder (MDD), metabolomic approach was employed to identify urinary metabolite markers for this disease. The study revealed a panel of five metabolite biomarkers, which can aid in the development of urinary diagnostic test for depression (49). Analysis of metabolome was also used to predict and assess response of patients with MDD to treatment with sertraline (50, 51). Other reports illustrated that metabolomic investigations can be helpful in diagnosis of schizophrenia. Orešič et al. (52) concluded that proline-related metabolic alterations and insulin secretion-related abnormalities constitute two shizophrenia-related pathways, whereas He et al. (53) stated that metabolites with significant changes in schizophrenia include four amino acids and one lipid. It was also demonstrated that measurements of steroid levels may improve diagnosis of schizophrenia (54). Characterization of metabolic perturbations linked to the pathophysiology and drug treatment of psychiatric conditions, i.a., MDD, schizophrenia and bipolar disorder, can help to develop laboratory tests in psychiatry and improve treatment of mental diseases (55-57).

Metabolomic studies have been increasingly important in drug development process and pharmaceutical research (3, 58-60). By identifying alterations in biochemical pathways, metabolomics leads to discovery of new targets for therapeutic interventions (58). The first chemicals used in cancer treatment were called "antimetabolites", because they replace specific metabolites or compete with them and thereby interfere with the metabolism of tumor cells (3). Till now, there is a lot of interest in development of new chemotherapeutic strategies that are based on targeting the aberrant metabolism observed in cancer (58).

One of the most important stages in drug development is safety assessment of the new drug candidates. The application of metabolomics in evaluating drug toxicity was estimated by the association between Imperial College London and five pharmaceutical companies named COMET (Consortium for Metabonomic Toxicology). Based on NMR spectra of blood and urine samples taken from rodent that had received a range of toxins, a model, which successfully predicts hepatotoxicity and nephrotoxicity of drug candidates, was created (3). Analysis of metabolome can be useful in characterization of mechanisms of drug toxicity as well as identifying early toxicity biomarkers (59, 60).

Challenges

Although metabolomics is now a rapidly growing area of scientific research, as the latest of the "omic" technologies is still in its infancy (10). Challenges and pitfalls that researchers in the field of analysis of metabolome have to face include i.a., technical limitations, bioinformatic challenges and integration with other "omic" sciences (Fig. 4). Heedful consideration of methods employed for sample preparation, data acquiring and data mining is needed (3). Implementation of metabolomics into clinical practice will be dependent on establishment of standardized protocols in analytical performance and data analysis and development of fit-for-purpose biomarker method validation (61). These requirements are needed to help scientists draw valuable conclusions based on performed metabolomic experiments.

Apart from typical problems associated with analytical performance, metabolomic experiments encounter additional challenges. Metabolome is a set of huge amounts of low molecular weight compounds, which are derivatives of amino acids, lipids, carbohydrates, nucleotides, hormones etc. The analysis of metabolome is particularly demanding due to tremendous chemical diversity of metabolites. Furthermore, metabolites vary dramatically in concentration ranging between 0.5 nmol/L for estradiol and 5 mmol/L for cholesterol, which equals seven orders of magnitude (6). Difficulties in measurements of metabolites include complexity of samples and differences in analyte polarity (9). Complex nature of analyzed biological specimens forces to utilize multiple analytical methods and platforms in order to investigate a wide range of metabolites. Another challenge is related to the fact that metabolome is a dynamic system affected by environmental influences, such as diet, and rapidly changing in a short period of time (19). Additionally, in untargeted approach reliable identification of metabolites is a major challenge.

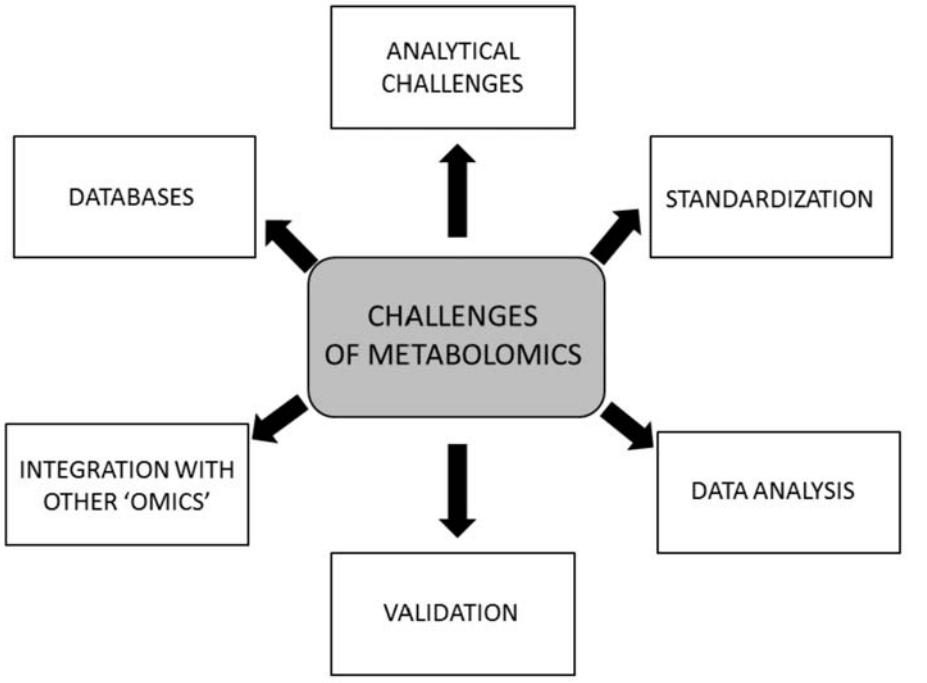

Figure 4. Challenges of metabolomics for the following years.

Although progress has been made in development of analytical techniques, we are still far from obtaining a complete metabolome map of any organism due to both great complexity and dynamic range of metabolites. Currently, the most commonly utilized system for LC-MS based metabolic profiling is high efficiency UHPLC, using reverse-phase (RP) or hydrophilic interaction chromatography (HILIC), combined with high resolution mass spectrometry. Anticipated improvements in analytical strategies include greater use of miniaturized systems, chromatography at elevated temperature, supercritical fluid chromatography (SFC) and liquid chromatography-ion mobility-mass spectrometry (11, 62, 63). However, despite development of chromatographic methods such as HILIC, analysis of highly polar constituents of metabolome remains a challenge (11).

Clinical applications of metabolomics are limited by the lack of standardization of metabolomic procedures. Establishment of commonly accepted standardized protocols in the range of methodology, data mining and data reporting is necessary (5, 9, 11). Standardization of sample collecting, handling and storing constitutes an essential condition to preserve sample stability and obtain reproducible results. It should be taken into account that differences in sample handling and acquisition can dramatically impact outcomes from metabolomic studies. It is especially important in case of biopsy samples, since during collection of this kind of specimens large and fast changes in intracellular levels of metabolites are possible (5, 61). Reduction in variability at each step of metabolomic workflow from collection to data processing is crucial to ensure reliable and consistent measurement at biomarker assays (61). Development of minimum standards for metabolomic experiments is an aim of The Metabolomics Standards Initiative (64). This group has published in recent years a series of articles, which propose the minimum requirements related to chemical analysis (64), NMR-based studies (65) and data analysis (21) in metabolomic research. Protocols for metabolic profiling for long-term and large-scale studies has been described by Dunn et al. (66).

The application of quality control measures and method validation are regarded as prerequisite for acquiring comparable and reproducible results inter different sample batches, experiments or laboratories. Although biomarker method validation differs from pharmacokinetic and routine laboratory validation, there is little regulatory guidance on biomarker assay validation (61). The goals of incorporation of quality control (QC) samples involve proving good performance of entire methodology and

reliability of obtained metabolomic data. QC samples should be used in making decisions on rejection or acceptance of an analytical run. Moreover, adding standardized QC samples during data acquisition can help evaluate inter-laboratory variability and establish bias. QC samples allow to perform signal correction in order to minimize analytical variation (19, 20, 67).

Bias is one of the most serious difficulties in every "omic" science. It is the most important threat to validity of clinical research and can be explained as an unintentional systematic misassociation of some variable level with one of the studied groups, for example with the cancer or control group. It occurs when samples representing two groups are handled in different ways, for example when they were collected, transported or stored differently. Moreover, it can occur when groups vary, for example in terms of diet or age. Such differences in specimen origin or handling can introduce a particular signal to one of the groups. As a result, the identified biomarker may be false because discrimination between the two groups is associated with sample handling differences, and not on the basis of real cancer presence/absence (23, 68). The need to identify non-invasive and specific diagnostic biomarker for conditions for which there is no non-invasive test available put pressure on researchers to publish results of their experiments quickly. Some notable outcomes in cancer marker research have not been reproduced and confirmed and bias has been indicated as a source of the problem (9, 23). Elimination of bias is a difficult task, because bias cannot be reduced by simply enlargement of the sample set. Bias can be minimized only by careful planning of the experiments (including careful age, gender, smoking-status etc., matching), using quality control measures, sample blinding and randomization, including randomization in order and in time of the analyses themselves (9, 23).

A grand challenge for research in the field of metabolomics is to tackle the problem of data analysis. Data analysis is probably the most time consuming stage of metabolomic workflow and require close collaboration between analysts, clinicians and experts in chemometric analysis (9, 11). Similar to the other high-throughput technologies, such as proteomics and transcriptomics, metabolomic investigations generate huge amount of data, which can contain many experimental artefacts. Due to the biological variation in metabolism between individuals and the influence of environmental factors on metabolism, large sample numbers are necessary to obtain the appropriate statistical power essential to the meaningful interpretation of output data (69). The problem is not only large data volume, but also its complexity and diversity (9, 11, 16). Usage of multi-dimensional data sets for searching for subtle and complex relationships occurring in living organisms cause additional problems. Since during metabolomic studies large number of parameters is being measured and often limited number of specimens is analyzed, the problem of false positives and irreproducible findings occurs (9). In order to avoid these pitfalls statistician should be involved in metabolomic studies from their outset, that is from the design of the experiment. For large volume of complex data the statistical analysis may be very complicated, even if the applied analytical techniques are perfect. Metabolomic data require specialized statistical tools and has specific bioinformatics needs, which are caused by utilizing multiple analytical platforms and necessity of extensive data pre-processing. According to Blekherman et al. (16), development in data analysis techniques is critical to progress of metabolomics and areas requiring further advancement include i.a., raw analytical data processing, data mining, data integration and metabolic network modelling.

If the chemometric treatment indicates a meaningful correlation between metabolomic and biological status, then a validation step should be performed (5, 11, 21). The term "validation" refers not only to analytical methodology, but is defined as "all activities aimed at assuring the quality of the conclusions drawn from the data analysis" (21). Validation of the findings represents a strongly recommended stage, which allows to eliminate statistically unsound reports and unreliable results. Hypotheses derived by metabolomic approaches need to be verified by analysis of an additional set of samples in order to discriminate false positives from clinically relevant biomarkers.

An example indicating that more widespread clinical trials are needed in order to provide validation for new identified biomarker is sarcosine, which was proposed as a marker of prostate cancer (33, 70-72). In 2009, Sreekumar et al. (33) announced that the metabolite which was significantly elevated in prostate tumor samples and can be detected in urine is sarcosine. However, the validation study using a new set of samples found that urinary sarcosine level does not result in better differentiation between prostate tumor patients and the control group as compared to currently utilized screening test for prostate cancer (PSA test) (70). Several other groups of investigators made also attempts to appraise the value of sarcosine concen-

tration in serum as a putative marker of prostate cancer. Struys et al. (71) found that serum sarcosine level manifests no differences between healthy individuals and patients with prostate tumor, whereas Lucarelli et al. (72) documented diagnostic value of sarcosine in sera of patients with low PSA level, amounting less than 4 ng/mL. Moreover, Bohm et al. (73) stated that serum sarcosine concentration cannot differentiate the early and the advanced stages of a prostate tumor. Controversy about sarcosine may be due to lack of standardization along with samples collection procedures and different approaches used. For instance, Sreekumar et al. (33) measured sarcosine to alanine ratios in urine sediment, while Jentzmik et al. (70) determined urinary supernatant sarcosine to creatinine ratios.

Nowadays, many of metabolomic research tend towards small scale studies, in which the number of sample ranges from tens to hundreds. It is anticipated that the next trend will be much larger epidemiological studies covering analysis of thousands specimens (11). Moving from a small sample number to a large clinical studies will demand not only widely accepted quality control and standardization, which make it possible to integrate data obtained from multiple instruments in different laboratories, but also web-accessible databases. Storing, organizing and interpretation of metabolomic data require specialized and well-designed databases (16, 20). Databases enable to better elucidate and understand output data and provide valuable information about current state of knowledge on analysis of metabolome. One of the most popular and most completed databases is Human Metabolome Database (HMDB), a comprehensive and online available database that gathers quantitative data about thousands of human metabolites (74, 75).

Another challenge of metabolomic research is integration with other "omic" technologies. It cannot be forget that metabolomics does not exist in vacuum, but is a part of the so-called systems biology. Integration of proteomics, transcriptomics, genomics and metabolomics provides a holistic perspective on complex interactions occurring in living organisms. By combination of "omic" sciences it will be possible to get a more complete picture of the functioning of biological systems (3, 5, 6). The problem involving each of the "omics" is that they are highly complicated and refer to interactions of thousands of genes, proteins, metabolites and other compounds. In order to simplify them, computing power and new bioinformatic algorithms have to be developed. Till now, we are still far from explaining complex systemic questions, because scientists should first deal with problem of different timescales of processes occurring in organisms and various quantitative dimensions of compounds of "omics" interest. Each level of organization and control in living organisms (gene expression, protein expression and metabolism) operates on noticeably different timescales and thus finding linkages between results of metabolomic studies and other "omic" research fields is problematic. In addition, environmental and lifestyle factors have a great impact on metabolism, making it difficult to discriminate their effects from gene-related influence (3, 9).

Perspectives

Newly emerging field of metabolomic research enables deep investigation of metabolism of organisms. By using the state-of-art analytical techniques along with advanced bioinformatic tools it is regarded as a powerful approach which allows researchers to scrutinize fluctuations in metabolite profiles. Because samples of biofluids can be collected quite easily, the variations of metabolites resulting from occurring genetic alterations, disease or environmental factors can be studied in detail (3).

Metabolomics opens up new perspectives in many fields of medicine, especially in oncology. Apart from its extensive cognitive significance, metabolomics manifests also a practical importance, as it may lead to design of new non-invasive, rapid, economic, sensitive and specific diagnostic techniques. There is an urgent need to identify reliable cancer biomarkers, which will be useful in cancer diagnosis and treatment. Introduction of new biomarkers to the panel of the currently used diagnostic indicators will allow to evaluate morbid risk linked to tumors, select the appropriate treatment and monitor response to the applied therapy. What is the most important issue, comprehensive analysis of metabolome offers the possibility of diagnosis of cancer in its early stage, when it is still amenable to cure, which in turn increases chance of recovery (10). However, the metabolites that discriminate cancerous and non-cancerous samples are in many cases common for different types of cancer, because of commonality of dysfunctional metabolism occurring in all cancers. Further investigations are needed to estimate how useful such a biomarker could be. It will also turn out whether changes in metabolic profiles will be strong enough to be detected in non-invasive biofluid samples and applicable to clinical practice and management of patients (5).

Metabolomics has also a great impact on development of other fields of medicine and pave the way for new healthcare strategies. It is envisioned that this "omic" technology, by providing clinically relevant

biomarkers, enables to predict developing of many disorders and contribute to improvement in undertaking therapeutic decisions (3). Undoubtedly, metabolomics will play in the future a major role in selecting an appropriate therapy tailored to an individual patients and identifying molecular targets for new treatment strategies. An example illustrating the potential of metabolomics is the study of Wang et al. (43). They identified five metabolites (five amino acids) which fasting concentrations predicted development of diabetes many years before the onset of this disease and before any changes in insulin action can be measured by standard biochemical assays. It should be noted that these findings were proved using two prospective cohorts, each consists of more than 3000 individuals.

The combination of metabolic fingerprinting and profiling will contribute to the realization of the aims of metabolomics. First, alterations in fingerprinting will be associated to a physiological state of organism. The next step will focus on identification and quantitation of discriminating metabolites. This workflow will allow to achieve the primary goal of metabolomics, which is translation of metabolomic data into deeper biological understanding. Development of bioinformatic tools and databases will be helpful in biological interpretation of obtained data. Integration of multiple "omic" results seems to be a way to obtain answers to unsolved questions and has a great promise to improve medical practice in the future, especially diagnosis and management of patients (76).

Summary

Metabolomics represents a rapidly expanding and interdisciplinary field of science by combination of biochemistry, analytical chemistry, bioinformatics, medicine etc. This "omic" science provides fresh insight into pathogenesis of diseases, effects of diet or applied drugs. One of the most popular aim of metabolomic study is biomarker discovery. Metabolomics allows to achieve a sophisticated level of information about biological systems and hold great promise for development of novel diagnostic tests and therapies including personalized medicine. Despite powerful analytical and computational systems integration of multiple "omic" results, it remains a challenge.

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