Metabolomics: A New Frontier for Research in Pediatrics

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SYSTEMS BIOLOGY AND 'OMIC' SCIENCES

The study of molecular biology has traditionally been based on a reductionist approach: cells are separated into their components, which are further separated into smaller components (genes, RNA, proteins) to be studied.[1] This approach is fundamental to describing the components of a biological system one by one.

The need has emerged in recent years to move toward the comprehension of the system as a whole (systems biology approach) to understand not only the functioning of the individual components but also how a system works altogether.[1] Systems biology is defined as the quantitative analysis of the dynamic interaction between several components of a biological system through the combination of mathematical modeling and experimental biology.[1]

Systems biology considers the interactions between DNA, RNA, proteins, and metabolites and studies the network of relationships in which they are involved, characterizing the flow of information at each level of biomolecular organization.[2] This approach views the organism as a whole picture, leading to an integrated comprehension of its functioning in health and disease.

The strength of systems biology lies in a comprehensive investigation of the "omic cascade," with a combination of transcriptomics, proteomics, and metabolomics (Table), in which these "-omic" terms have been formulated to define approaches capable of describing the entire set of proteins, mRNAs, and metabolites in a given organism.[2,3]

Transcriptomics is defined as the study of gene expression. *Transcriptome* is the term used to describe the full set of mRNA present in a cell or tissue at any one time. Similarly, the study of protein translation is called *proteomics,* and a *proteome* is the complete set of proteins expressed in a cell or tissue at any one time. Transcriptomics and proteomics are therefore functional analysis at mRNA and protein level, respectively. Integrating the resulting information can contribute substantially to our understanding of the molecular processes involved in physiologic mechanisms and their disruption due to disease.

The transcriptome represents the template on which the proteome is created, though the correlation between mRNA and protein abundance may be not so strong because of biological differences between transcription and translation processes and experimental differences in the production of transcriptomic and proteomic data.[4] Information from these two approaches may be pooled but still cannot give us the whole picture of how an organism functions.

Metabolomics is the most recent of the "omic" sciences. Nicholson defines metabolomics as the quantitative analysis of all the metabolites of a biological sample,[5] and metabonomics as the quantitative measurements of the multiparametric metabolic response of a living system to pathophysiological stimuli or genetic modifications.[5] Indeed, the terms metabolomics and metabonomics are often used interchangeably; in this review the term metabolomics will be used.

Metabolomics takes a non-selective approach, potentially enabling the identification and quantification of all the metabolites (small molecules <1 kDa) in a biological system (metabolome).[6] As far as metabolomics is concerned, metabolites are not the static end-product of a unidirectional linear flow of information, but they form a dynamic network of biocompounds continuously interacting with one another.[7] A metabolic profile consists of the set of metabolites in a cell or tissue, reflecting enzyme expression and activity, and includes the building blocks and breakdown products of the DNA, RNA, proteins, and cell membrane cellular components.[3] Although the metabolic profile can be seen as the ultimate expression of the information contained in the genetic code, it is also affected by several factors unrelated to the genome, such as interactions with commensal microorganisms (eg, microbial metabolites from the intestinal microflora can be detected in human serum and urine), nutritional factors, environmental agents, and any exposure to drugs or toxic substances resulting in discordance between genotype and phenotype.[2,8]

CSF	Cerebrospinal fluid	MS	Mass spectrometry
HPLC	High-performance liquid chromatography	MSI	Metabolomics standard initiative
IBD	Inflammatory bowel disease	NMR	Nuclear magnetic resonance
LC-MS	Liquid chromatography–mass spectrometry	UPLC	Ultra performance liquid chromatography

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Table. Glossary

Systems biology	Systematic study of the complex interactions between the single components of a biological system (DNA, RNA, proteins, metabolites) and characterization of the flux of information at each level of biomolecular organization through the combination of mathematical modeling and experimental biology.
Transcriptomics	The study of the transcriptome, which is the complete set of RNA transcripts produced by the genome at any time. RNA formation, structure, and function are studied.
Proteomics	The study of the proteome. which is the complete set of proteins expressed in a cell or in a tissue at any time. Protein structure and modifications, expression, and reciprocal interaction are studied.
Metabolomics	The analysis and interpretation of global metabolic data of a complex biological system. These metabolic data result from the information enclosed in the genetic code as well as from the effect of physiological factors (eg, diet, age, commensal microorganisms), pathological conditions, environmental agents, and exposure to drugs or toxic agents.

(Figure 1). Metabolomics is therefore considered the "omic" science that comes closest to phenotype expression offering the possibility to look at genotype-phenotype as well as genotype-envirotype relationships.

Moreover, as recently highlighted by the FDA (www.fda.gov/nctr/science/centers/metabolomics), characterizing an individual's metabolomic profile may have a significant role in predicting the course of disease and the response to treatment.

In line with the growing interest in the potential applications of this approach, the intent of this review is to provide an update on the emerging research area of metabolomics.

DIFFERENT APPROACHES TO STUDY METABOLITES

Metabolite *targeting* is the most direct approach to metabolite analyses, aiming to identify a specific metabolite, the product of an enzymatic pathway or derived from the degradation of a drug or toxic agent.[7,9]

Metabolite *profiling* aims to identify and quantify a particular set of metabolites belonging to a specific metabolic pathway or a class of compounds.[6] This approach extends from seminal studies of inborn errors of metabolism in infants.[10] In metabolite profiling the metabolites are sought on the base of the questions asked: there is no chance of coming across unknown metabolites in the sample.[9]

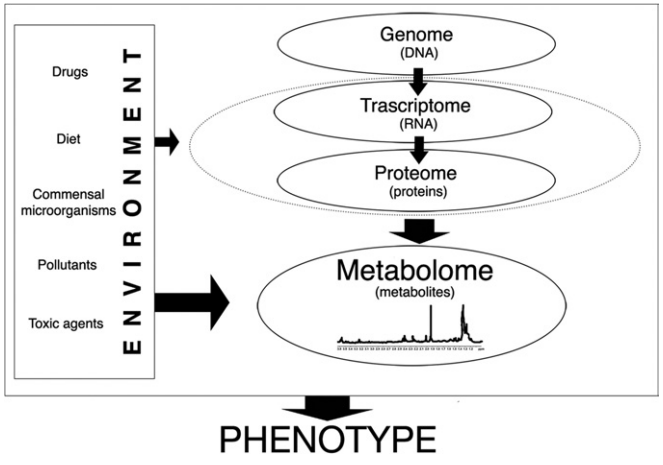

Figure 1. "Omics" sciences and their relationship with environment and phenotype.

Metabolite *fingerprinting*, on the other hand, is a genuine "-omic" approach, a truly comprehensive methodology that aims to classify samples without any a priori hypothesis, to identify metabolite patterns associated with a given pathological condition, or exposure to a given toxic agent or drug, or environmental or genetic change.[9] Metabolic fingerprinting does not necessarily involve identifying each metabolite, but tries to detect the metabolic characteristics that discriminate between groups of subjects. The search for these metabolic patterns is not driven by a researcher's hypothesis, so it is open to new findings. Unexpected or even unknown metabolites may turn out to be important in characterizing specific groups of subjects so new pathophysiological hypotheses may be formulated.

The exact number of human metabolites is not known and the last version of Human Metabolome Database reports more than 6500 entries (version 2.0; November 1, 2008; www.hmdb.ca).[11] Targeted metabolic analysis can be used to measure a limited number of these metabolites. Metabolomics can be interpreted as an extension of targeted metabolite profiling in as much as it enables the study of many more metabolites.

Once a metabolic pattern typical of a given condition has been characterized, in fact, the analysis may go on to identify single biomarkers relevant to sample clustering.[9] The metabolomic approach may thus become a tool for moving from studying single biomarkers (which can hardly reflect a whole pathological process) to identifying patterns of biomarkers capable of distinguishing between health and disease and characterizing different disease subphenotypes.

The metabolomic approach has its limits, however, due mainly to the analytical performance of available instruments and to the sample variability introduced by factors such as diet and drugs.

METABOLOMICS: ANALYTICAL PLATFORMS AND PATTERN RECOGNITION METHODS

Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) (often combined with chromatographic separation) are widely used platforms for performing

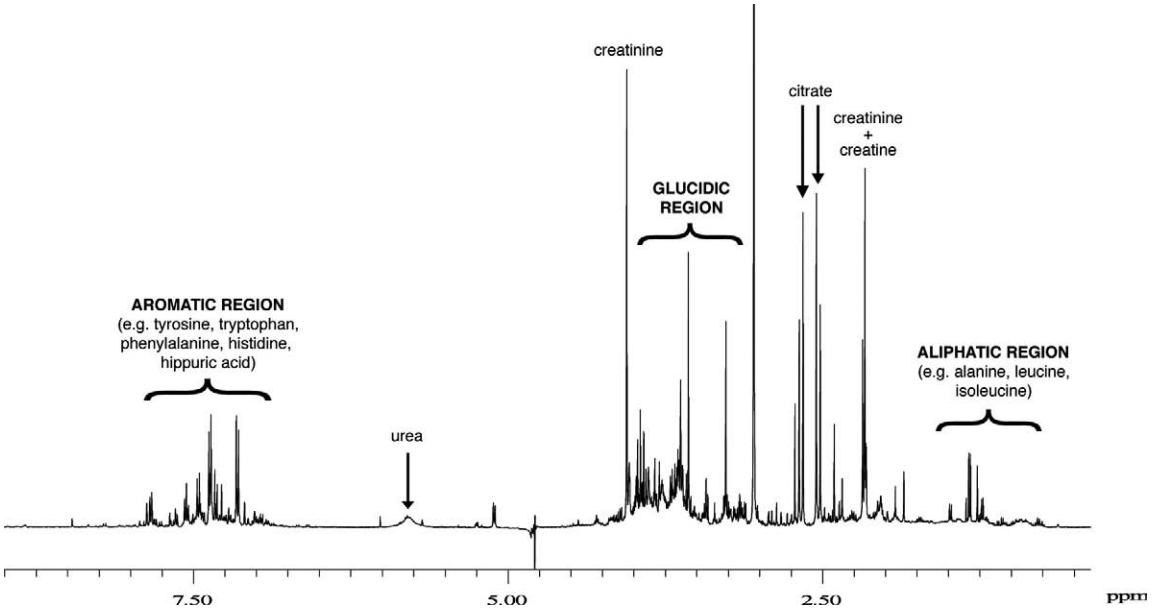

Figure 2. Example of a 500-MHz 1H-NMR spectrum of human urine. Experimental conditions: 200 μL urine in 400 μL d-phosphate buffer (pH 7); inverse broad band probe; 5-minute experimental time.

metabolomic analyses and profiling.[12] These two spectroscopic techniques are complementary and play a central part in the "omic" world.

^{1}H-NMR spectroscopy enables the detection of proton-containing metabolites in a sample, different molecules producing different signals in the NMR spectrum[13] (Figure 2).

NMR spectroscopy provides detailed information on molecular structure of compounds, both pure and in complex mixtures. Recent technological improvements have led to the availability of stronger magnetic fields as well as the development of cryogenic probes, increasing sensitivity and spectrum dispersion. These improvements have been useful in studies based on small-molecule analysis, as in the case of metabolomics.

Some of the advantages of this technique are that it is non-selective, fast and usually demands no sample preparation. Furthermore, a technique called high-resolution magic angle spinning (MAS) NMR spectroscopy can be used to acquire high-resolution NMR data from small pieces of intact tissues, with no pretreatment.[13]

MS is a powerful method for identifying and quantifying metabolites, and it is considered more sensitive than ^{1}H-NMR.[9] The technique generates a spectrum in which biocompounds are represented according to their masses (m/z).

MS is often applied using tandem-MS methods. A necessary preliminary step is usually the separation of the complex mixture sample using chromatography.

Recent applications of MS in metabolomics are based on instruments like Quadrupole Time-of-Flight (Q-ToF), and Fourier Transform Mass Spectrometry (FT-MS).

NMR and MS analysis can be performed on very small samples: for NMR spectroscopy a minimum of 20 μL in a capillary tube and 300 μL in a flow system; for MS less than 20 μL are needed (for reviews of metabolomics technologies, see References 9 and 13).

Both NMR and MS are powerful spectroscopic methods for generating multivariate datasets: NMR and MS spectra are highly complex and the biological information they contain can only be extracted using appropriate multivariate statistical approaches—the so-called *pattern recognition* methods. These are computer-based procedures that can be classified as unsupervised or supervised.[14] The unsupervised methods, such as principal component analysis (PCA), reduce the complexity of the data contained in the spectra and represent them by means of plots that the human eye can interpret. This approach helps to identify any intrinsic sample clustering, to see whether different groups of subjects (eg, healthy vs ill) can be discriminated by their spectra characteristics.[14] The supervised methods, such as partial least squares–discriminant analysis (PLS-DA), use a training set of samples (of known classification) to create a mathematical model that is then used to test an independent dataset. Unlike the unsupervised methods, the supervised methods enable us to predict which group a new sample belongs to on the strength of its spectra characteristics.[14]

These bioinformatic methods can therefore be used to identify signals that enable discrimination between groups. The next crucial step is the structural identification of the single metabolites involved in the sample classification.

Identifying biomarkers can involve many analytical physical and chemistry disciplines: NMR and MS spectroscopies play a very important part, but no single technique fulfills all requirements for fully elucidating metabolite structure and multiple approaches need to be integrated.

Fundamental support for molecular identification comes from various on-line databases. The most comprehensive

available database of human metabolites is the Human Metabolomic Database.[11] Other important databases are KEGG,[15] BioCyc,[16] Reactome,[17] and Metlin.[18]

METABOLOMICS: ITS ROLE IN PATHOLOGICAL CONDITIONS

Recently, a number of studies have investigated whether the metabolomic analysis of biofluids (eg, blood, urine) can be usefully applied in the diagnosis of certain diseases.

In the field of cardiovascular diseases, the NMR-based metabolomic analysis of blood samples has been successful in the discrimination between subjects with hypertension and normal blood pressure.[19] A recent study has also demonstrated that the urinary biomarker formate, identified through an NMR-based metabolomic approach, correlates inversely with blood pressure in human populations.[20] Metabolomic analysis based on HPLC-MS proved capable of characterizing patients who develop myocardial ischemia after an exercise test.[21] It has been demonstrated that NMR-based metabolomic analysis of blood samples can distinguish between people with and without coronary heart disease.[22,23] The analysis is more effective than the conventional risk factors (age, sex, lipoprotein levels, smoking habit, blood pressure) in establishing the severity of the disease.[22] By means of a targeted MS-based metabolomic platform, Lewis et al[24] recently demonstrated significant metabolite abnormalities in blood samples as soon as 10 minutes after a myocardial infarction, even though presently used indicators of myocardial injury are reliably detected only a few hours after the infarction.

Individuals with type 1 diabetes reportedly have a characteristic serum NMR-based metabolomic profile that seems as good as a full set of biochemical variables in diagnosing diabetic nephropathy.[25] In diabetic patients, characteristic serum metabolomic features have also been shown to be associated with vascular complications and premature death.[26] Characteristic metabolomic profiles have likewise been described in patients with type 2 diabetes.[27,28]

Some attempts have been made to apply metabolomics as a diagnostic tool in clinical investigations in the field of neurology. NMR-based metabolomic analysis of cerebrospinal fluid (CSF) samples was capable of discriminating between patients with bacterial meningitis, cases of viral meningitis and controls.[29] Patients who had already been given antimicrobial therapy before sample collection for metabolomic analysis (and who consequently had a negative CSF culture) were still correctly assigned to the bacterial meningitis group using the metabolomic approach.[29] Dunne et al evaluated the ability of the NMR-based metabolomic analysis of CSF to characterize patients with subarachnoid hemorrhage and identify those with the worst outcome due to prolonged vasospasm.[30] Ghauri et al demonstrated a biochemical profile typical of patients who died of Alzheimer's disease in samples collected post mortem.[31]

Combined NMR spectroscopy and pattern recognition methods have been proposed for analyzing fecal extracts from patients with inflammatory bowel disease (IBD)[32]. The preliminary study was unable to reveal any significant differences in the metabolomic profiles of patients with Crohn's disease versus those with ulcerative colitis; however, the authors did demonstrate that metabolomics can clearly discriminate IBD patients from healthy subjects.[32]

A few studies have assessed the potential role of metabolomics in following up patients who have received an organ transplant. The urinary NMR-based metabolomic profile can distinguish between kidney recipients with normally functioning versus nonfunctioning grafts.[33,34] Another study of a patient who had 2 liver transplantations, identified a characteristic early metabolomic profile (obtained by NMR-spectroscopic analysis of blood samples) that was associated with the failure of the first graft but was no longer seen after the second transplant, when the organ was functioning properly.[35]

A promising application for metabolomics is in cancer screening and early diagnosis. In particular, there may be a role for NMR-based metabolomic analysis of blood samples in the early identification of women with epithelial ovarian cancer.[36] HPLC-based metabolomics has been applied successfully to urine samples to distinguish patients with liver cancer from those with cirrhosis or hepatitis.[37] Finally, a recent study was able to discriminate, with high level of sensitivity and specificity, between patients with and without bladder cancer by HPLC-MS–based metabolomic analysis of urine samples, suggesting a potential role for such analysis as a screening tool for bladder cancer.[38] In the oncological field, metabolomics can also be used to analyze tissue samples,[39-42] enabling the tumor's metabolic profile to be characterized and different types of cancer to be distinguished. Being a nondestructive technique, NMR spectroscopy is particularly suitable for analyzing tissues that can subsequently be used for histopathologic analyses.

METABOLOMICS: PEDIATRIC STUDIES

In one publication, the untargeted metabolomic approach was applied to studying disorders due to inborn errors of metabolism.[43] This study used untargeted metabolomic analyses on plasma samples. Investigating disorders of propionate metabolism (methylmalonic acidemia [MMA] and propionic acidemia [PA]) the authors demonstrated that the most important metabolite for the discrimination between healthy and ill subjects was propionyl carnitine, which is, indeed, the target compound for screening newborn for MMA and PA by tandem-MS. This result validates the role of untargeted metabolomic analysis in identifying biomarkers of disease. Moreover, the untargeted metabolomic analysis showed that many other compounds are important in differentiating both between healthy and ill subjects and between MMA and PA subjects.[43]

Another study described a characteristic metabolomic profile in the CSF of children with influenza-associated encephalopathy, suggesting that it might be possible to identify

specific biomarkers useful for the early diagnosis of this disease.[44]

Metabolomics can be effective in identifying asthmatic children.[45] The metabolomic approach was applied to analyzing exhaled breath condensate (breathomics): this is a biofluid collected in a totally non-invasive way by cooling down the exhaled air and its composition is believed to mirror the composition of airway lining fluid.[46] The authors demonstrated that NMR-based metabolomic analyses of exhaled breath condensate can clearly discriminate between asthmatic and healthy children. On submitting the spectra to partial least square analysis, they achieved an approximately 95% success rate in their classification (Figure 3). Many authors believe that asthma should no longer be considered a single disease and that efforts should be made to identify the different biochemical-inflammatory profiles underlying asthma symptoms in order to treat them with specifically targeted therapies.[47] The metabolomic approach may have a role in characterizing asthma subphenotypes, potentially leading to more tailored therapies. The metabolomic analysis of exhaled breath condensate has been also applied to the study of respiratory diseases in adults.[48]

A study has assessed the role of metabolomics in identifying early urinary biomarkers of acute kidney injury after cardiopulmonary bypass surgery.[49] Using ultra performance liquid chromatography (UPLC)/MS–based metabolomic-analyses on urine samples collected 4 and then 12 hours after surgery, the authors were able to identify the children who were to develop acute kidney injury over the next 3 days. From a clinical standpoint, identifying early metabolic biomarkers may improve our understanding of the pathophysiological mechanisms involved in acute kidney injury and enable a timely diagnosis of this disease.

METABOLOMICS IN NUTRITION SCIENCE

In numerous studies metabolomic analysis has been used to assess the effects of diet on metabolism. Bertram et al compared the metabolomic profiles of 2 groups of children receiving a diet rich in milk or meat proteins. They succeeded in separating the children according to their diet and identified a significantly greater urinary excretion of creatine in the children on a diet rich in meat.[50] A study conducted in adults demonstrated that the urinary metabolomic profile of meat eaters differs considerably from that of vegetarians.[51] Solanky et al[52] described consistent metabolomic plasma profiles in a group of healthy premenopausal women adopting specific soy-rich dietary measures. Walsh et al[53] demonstrated that changes in dietary intake of phytochemicals induce modifications in an individual's metabolomic urinary profile. Law et al[54] showed that the metabolomic approach can be used to provide a comprehensive picture of the metabolic changes induced by ingestion of green tea.

Nutritionists are now considering the metabolomic approach with a view to establishing individual nutritional phenotypes—or, in other words, how diet interacts with an individual's metabolism—to provide a comprehensive definition of nutritional status and predicting health and disease outcomes.[55]

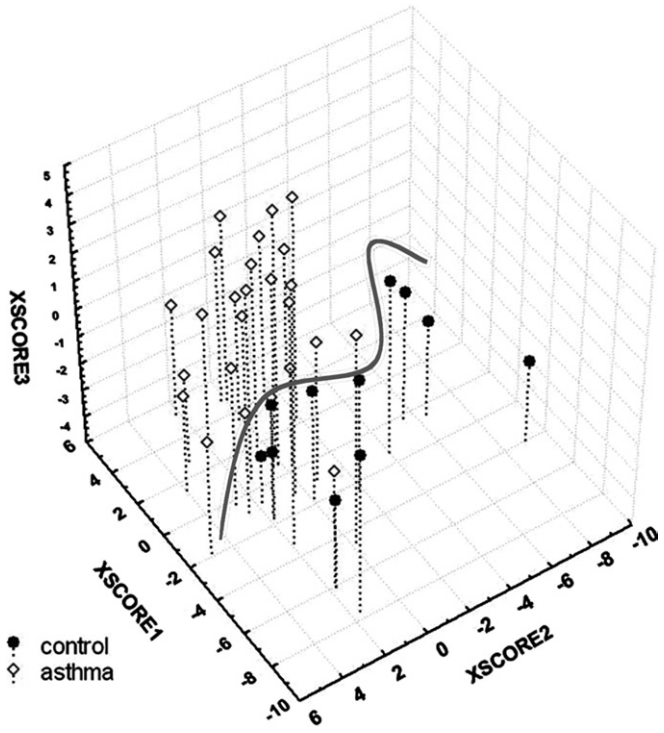

Figure 3. Sample discrimination obtained by means of pattern recognition methods in childhood asthma (modified from Reference 45).

It is worth mentioning that in studying the metabolic effects of dietary interventions, other methodologies play an important role, such as the in vivo labeling of stable isotopes. For example, the effects of enteral nutrition on protein balance and kinetics have been studied in children with Crohn's disease, and the effect of parenteral nutrition was investigated in infants undergoing abdominal surgery.[56,57]

METABOLOMICS IN PHARMACOLOGY AND TOXICOLOGY

NMR- and MS-based metabolomics are widely used to test drug toxicity.[58] In preclinical candidate drug studies, metabolomics can be useful in describing how a drug affects an individual's metabolism, providing information on the site and mechanism of toxicity, and revealing the time course of its metabolic effects.[59]

Knowing the metabolomic profile associated with a given disease may also help us to monitor biological and metabolic responses to treatment.[58] For example, patients with type 2 diabetes mellitus reportedly have an early characteristic metabolomic profile in response to treatment with thiazolidinediones.[60] Among AIDS patients, NMR-based metabolomic analysis can distinguish cases treated with antiretroviral therapy from HIV-negative subjects, suggesting that the technique may become a tool for monitoring the metabolic effects of the therapy and possible side effects.[61]

Another promising opportunity afforded by metabolo-

mic analysis concerns studying xenobiotic effects on an organism—potentially enabling us to predict the effectiveness or toxicity of a drug on the basis of the individual's pretreatment metabolomic characteristics, and helping us to understand the mechanisms behind idiosyncratic toxicity.[58] This branch of metabolomics is called pharmacometabolomics. An elegant study conducted in mice demonstrated that the hepatotoxic effects of the analgesic acetaminophen could be predicted on the basis of the pretreatment urinary profiles.[62] This report was later confirmed by a study showing that the baseline metabolite profiles could be used to predict the response to different xenobiotic measures in experimental animals.[63] These studies have introduced the concept that an individual's response to a xenobiotic is determined not only by genetic characteristics, but also by other factors such as diet, aging, gut microbiota and physiological diurnal variation.[63] Pharmacometabolomics appears to be an extremely promising branch of metabolomics for screening human populations, implying the concrete possibility of a genuinely customized approach to treatment.

STANDARDIZATION IN METABOLOMICS

The metabolomic profile of a biological sample is affected by several physiological factors, including age, sex, diet, and circadian variations (Figure 1), and the related changes in human metabolic profiles have been investigated.[64,65] As an example, a study conducted on healthy subjects adopting a strict diet and particular lifestyle showed a relatively small intersubject and intrasubject variability.[66] Another study in which no dietary restrictions were applied found a significant degree of inter-individual variability in urinary metabolomic profile.[67] This variability is reduced if a standard diet is followed on the day before urine collection.[68]

In metabolomics, more than in the other "-omic" sciences, it is therefore crucially important to collect background data on the individual concerned to help interpret the results.

In addition, for metabolomic experiments, it is important to develop standard protocols regarding sample collection and storage, chemical analyses, data processing and the exchange of information.[69]

The Metabolomics Standard Initiative (MSI) was created, with the support of the Metabolomic Society (www.metabolomicssociety.org), to recommend standard protocols for use in all aspects of metabolomic research.[70] Such standardization will facilitate the future development of applications of the metabolomic approach.

CONCLUSIONS

Although it has been used in only a limited number of clinical studies so far, metabolomics seems to have promising applications. This approach carries the promise of a more individualized medicine thanks to the possibility of predicting the course of disease and the response to treatment on the basis of an individual's metabolomic profile.

Metabolomics has potential applications in both the diagnosis of diseases and the development of treatment strategies, with the advantage of the analysis being performed on biofluid samples that are collected noninvasively (eg, urine or exhaled breath condensate) or by only minimally invasive procedures (eg, blood drawing). Metabolomics can lead to the discovery of new noninvasive metabolic biomarkers that could be translated, in the near future, into clinical tools for diagnosing diseases and developing treatment strategies, paving the way toward a medicine tailored to the patient.

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