

Metabonomics: a Revolution in Progress*

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Abstract Metabonomics is the branch of science concerned with the quantitative understandings of the metabolite complement of integrated living systems and its dynamic responses to the changes of both endogenous factors (such as physiology and development) and exogenous factors (such as environmental factors and xenobiotics). As a holistic approach, metabonomics detects, quantifies and catalogues the time related metabolic processes of an integrated biological system, ultimately, relates such processes to the trajectories of the pathophysiological events. Ever since its birth in 1999, metabonomics has already been described in more than 800 scientific papers and half dozen patents, amongst which almost 700 papers were experimental articles. Now, metabonomics has been established as an extremely powerful analytical tool and hence found successful applications in many research areas including molecular pathology and physiology, drug efficacy and toxicity, gene modifications and functional genomics, and environmental sciences. This holistic approach has thus become an important part of systems biology and has now evolved to be a unique part in global systems biology. The essence of metabonomics and some of the present applications were reviewed to illustrate the rapid development of this extremely exciting new frontier.

Key words metabonomics/metabolomics, NMR, multivariate data analysis, metabolites, systems biology

1 General introduction

With the era of systems biology bursting into reality, the analysis of the whole biological systems whether they are cells, tissues, organs or whole organisms, has now become the norm of biological research^[1~6]. This indicates a shift of research philosophy from “reductionism” to “holism” as well as from “individual” science to real “interdisciplinary” science, which attracted attentions and interests from biologists, chemists, physicists and mathematicians, leading to an explosive occurrence of “omes” and “omics” sciences. In essence, however, these “omes” and “omics” are aimed to understand the biological systems in the levels of genes (DNA), transcripts (mRNA), proteins and metabolites as a whole rather than the sum of individuals (as shown in Figure 1). Therefore, not only the information at the levels of genes, transcripts, proteins and metabolites are important to retrieve and be understood but also the correlations/interactions between these levels have to be established and understood (ideally quantitatively). To

achieve these, new technologies are required to enable the high density information to be retrieved, archived,

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analysed in an integrated fashion and interpreted in the biologically meaningful ways, in which scientists including physicists, chemists and engineers all have some vital roles to play. Ultimately, the purpose of the systemic approach is to have the quantitative, universal, integrated and predictive (QUIP) understandings of biological systems. For this, proper integration of information in genes, transcripts, proteins and metabolites will be necessary *via* appropriate mathematical modelling.

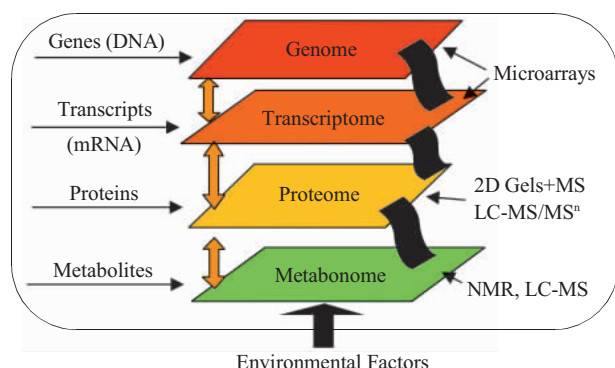


Fig. 1 Schematic representation of the tasks for global systems biology

Amongst these analyses of biological systems, the metabolite analysis itself has always been an essential component in life science research for all organisms since the nature of metabolites and, in particular, the changes of them carries rich information in the metabolism level as well as in the gene expression and protein functioning^[5,7~11]. Such importance has already been highlighted by numerous Nobel Prizes awarded to scientists for their works to understandings to metabolisms (<http://www.nobel.se>). These previous efforts, essentially based on a “reductionism” philosophy, have made tremendous contributions to the detailed understandings of the biochemical pathways. Because of the complexity of living organisms and, in particular, the underlying molecular mechanisms of their biological processes, the conventional “reductionism” approach has faced ever greater challenges. Based on these aforementioned progresses and aided with new technology development, an era of “omes” and “omics” explosion has been with us for a number of years. For example, more than 100 so-called “omes” and “omics” have, so far, been coined and such development is still proceeding with a breathtaking pace. From biology point of view, nevertheless, these “omes” and “omics” can be

broadly classified into four groups, namely, genome/genomics, transcriptome/transcriptomics, proteome/proteomics and metabome/metabonomics (or metabolome/metabolomics), to enable us to understand an integrated organism in the levels of genes, transcripts, proteins and metabolites respectively. The well known and high profile human genome project^[12,13] represents a gigantic step forward and huge attempt, perhaps eagerness, to reveal the secret of human biology. Currently, some international programmes are in progress in the area of the cancer genomes to use the human genome sequence and high throughput mutation detection techniques to identify somatically acquired sequence variants or mutations so as to identify genes critical in the development of human cancers (<http://www.sanger.ac.uk>, <http://cancergenome.nih.gov>) for the similar purposes. Some human proteome (<http://www.hupo.org>) and metabome (<http://www.metabolomics.ca>) programmes are also in various stages with aims to understand how human system functions in an integrated fashion and how to prevent diseases.

In the metabolism level specifically, the adoption of “holism” philosophy has led to the development of the concepts of metabolome^[14,15], metabonomics^[5,8,9] and metabolomics^[10,16] (see Table 1 for details), which will undoubtedly be vital to gain insights into life processes and to understand the complexity of the whole organisms and their interactions with environment in greater depth. This is because the metabolism represents not only the near-end point products of biological processes but what really has happened in contrast to genomes, transcriptomes and proteomes which provided the material foundation for what might happen in a biological system (that may or may not happen).

2 What are metabome and metabonomics?

The word “Metabonomics” originated from Greek “*meta*”, meaning changes or adjacent, and “*nomos*”, meaning rules or laws (e.g. as in economics)^[17]. The concept of metabonomics was initially defined as^[8] “the quantitative measurement of the multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modifications”. A more general definition can probably be established as: “metabonomics is the branch of science concerned with the quantitative understandings of the metabolite complement of integrated living systems and its dynamic responses to the changes of both endogenous

factors (such as physiology and development) and exogenous factors (such as environmental factors and xenobiotics)". Another related term "metabolomics" though having a number of definitions has essentially been defined as "the quantitative measurement of all low molecular weight metabolites in an organism's cells at a specified time under specific environmental conditions" ([http://](http://www.jic.bbsrc.ac.uk/)

www.jic.bbsrc.ac.uk/). Although, for the time being, the terms "metabolome and metabolomics" are used when plant and microbial systems are concerned whereas the terms "metabonome and metabonomics" are used in animal models, these expressions, nevertheless, do have different meanings scientifically (Table 1).

Table 1 Some useful definitions

Metabolism: the total chemistry of living cells
Metabolites: (bio)chemical reaction products or intermediates
Metabonome: the total metabolite complement of an integrated living system and its dynamic responses to the changes of both endogenous and exogenous factors
Metabonomics: the quantitative measurement of the multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modifications
Metabolome: total metabolite complement of a living system at a given time
Metabolomics: the quantitative measurement of all low molecular mass metabolites in an organism's cells at a specified time under specific environmental conditions

By definition (Table 1), metabolomics is concerned with the snap-shots of cellular metabolome (total metabolites complement)^[14] at a given time, thus it is analytical by nature and static by default. In contrast, metabonomics measures not only the metabolite complement of integrated biological systems, whether they are cellular models, tissues, functioning organs or whole organisms, but also the dynamic changes of such metabolite complement in response to the internal or external stimuli. It is therefore reasonable to consider the metabolome and metabolomics as part of metabonome and metabonomics respectively^[17]. In fact, just measuring

the metabolite complement alone has limited usefulness since life process is a dynamic and integrated one. Nevertheless, it is noteworthy that, with the emergence of concept of dynamic metabolomics (<http://www.nih.gov/roadmaps/>), the definition of metabolomics is evolving rapidly towards its "brother" —metabonomics. It is conceivable that as an infant in science at this stage, metabonomics/metabolomics will foresee much more development and will probably converge given enough time and discussions (see the brief history of metabolomics and metabonomics in Table 2).

Table 2 Brief history of metabonomics

• 1983 ¹ H NMR of blood plasma (Nicholson J K <i>et al</i> , <i>Biochem J</i> , 211 : 605~615)
• 1984 ¹ H NMR of urine (Bales J R <i>et al</i> , <i>Clin Chem</i> , 30 : 426~432)
• 1991 Pattern recognition combined with ¹ H NMR spectroscopy of urine (Gartland K P R <i>et al</i> , <i>Mol Pharmacol</i> , 39 : 629~642)
• 1998 Metabolome was defined as the "total metabolite pool" (Tweeddale H <i>et al</i> , <i>J Bacteriol</i> , 180 : 5109~5116)
• 1999 Metabonomics was defined by Nicholson J K, <i>et al</i> (<i>Xenobiotica</i> , 1999, 29 : 1181~1189)
• 2000 "Metabolomics" was first coined (Fiehn O <i>et al</i> , <i>Nature Biotechnol</i> , 2000, 18 : 1157~1161)

It is now clear that understandings in just genome or transcriptome or proteome or metabonome alone will hardly be possible to facilitate complete understandings of a given biological system. In human, for instance, the community of the gut micro-organisms (microbiota) and the host (human) together form a co-functioning symbiotic system due to their billions of years of co-evolution^[18]. Therefore, in a functioning human body, the functioning genome

consists of both human genome and microbiota genomes (microbiome), in terms of the genome size, human genome alone only represents about 10% of the total genomes for the functioning human body^[19] (without microbiome, human cannot survive!). For such systems, metabonomics approach will offer unique global information (at least in the metabolism level) since metabonomic analysis provides metabolism information from both hosts (e.g., human),

microbiota and their interactive co-metabolisms. The ultimate goal is to correlate the metabonomic data with the proteomic and transcriptomic ones to have a complete view about what are happening in a biological process in different levels. This, however, requires one to take into consideration that these data represent processes preceding on different time scales.

Ever since its birth, metabonomics has shown a rapid development and widespread applications. Scientific publications and patents related to metabonomics have experienced an exponential increase on yearly basis (Figure 2). Amongst them, NMR-based metabonomics, in particular, appears enjoying a more rapid progress in both method development and applications although both chromatographic and mass spectrometry (MS) based metabonomics methods are increasingly making good progress and contributions as well.

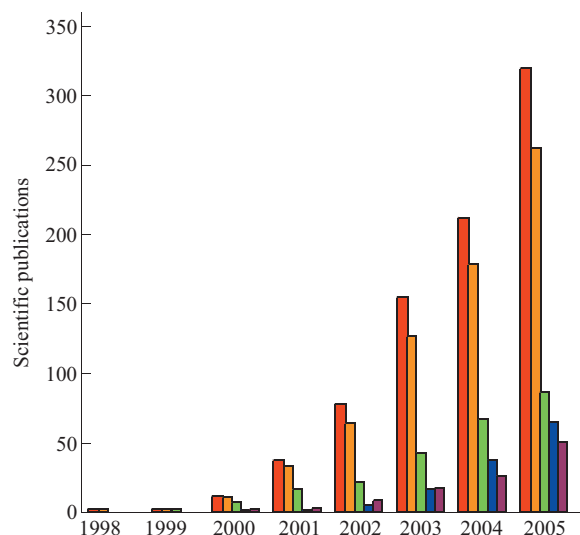


Fig. 2 Scientific publications on metabonomics/metabolomics

■: Total; ■: Experimental papers; ■: NMR-based; ■: MS-based; ■: Chromatography-based.

Unlike in the case of genomes, the transcriptome, proteome and metabome measurements are all limited by the detection sensitivity. The detected signals are often related to compositions and concentrations, which are not the only factors related to the biological processes. In the case of proteins, for example, their biological significance is not only related to their structure and concentration but also their activities, locations and compartmentation. The current proteome measurements, in most instances, can only provide information on the identities of some of the proteome and their concentration. It is, however,

difficult for their activities and compartmentation to be established with the existing proteomics technologies although this may change in the future. Moreover, both transcriptome and proteome measurements currently suffer from some bottlenecks such as throughput and high costs though this may also change in the future. In contrast, the metabome measurements are possible to provide information about what already happened in the biological events together with the identity, concentration and compartmentation of metabolites especially when the NMR-based metabonomics technologies are employed appropriately. The compartmentation information is particularly important for the multicellular biological systems where important events include both metabolism in a single cell and the inter-cellular metabolite exchanges. In the case of mammals, metabolite exchanges and co-metabolism between the gut microbiota and hosts often have functional significance related to health^[20~23] and disease^[24,25]. Metabonomics concurrently measures the metabomes and exchanges of the whole system together with the effects of other environmental factors, thus provides useful “global” information for the systems biology studies with complementary information to that from genes, transcripts and proteins. Furthermore, although our present understandings of biochemical pathways are pretty comprehensive already, it is, nonetheless, by no means complete or absolutely accurate. As an excellent biomarker discovery tool, metabonomics measures the metabolism without pre-conditions or pre-knowledge, therefore, is likely to tackle problems related to such incompleteness and inaccuracy.

2.1 Metabonomics technologies

Currently, there are a number of metabonomics techniques in use as shown in Figure 3. However, these technologies can be broadly classified as

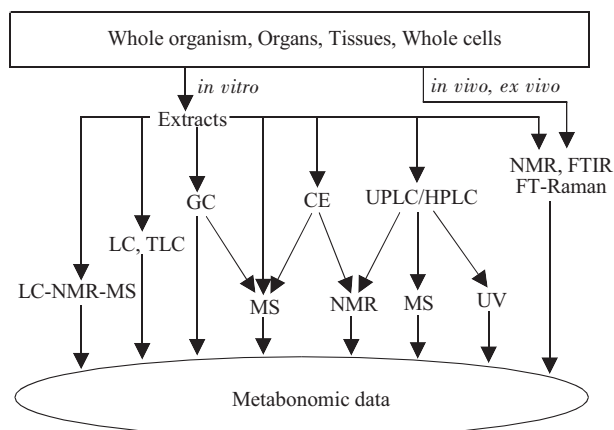


Fig. 3 Techniques used in metabonomics studies

chromatography-based, MS-based and NMR-based according to the major detection methods used (see Table 3 for details). With so many techniques, which technique should one choose? Before answering this question, it is necessary to have a detailed evaluation of these detection methods. It is conceivable that an ideal detection method for metabonomics ought to (1) be objective (thus user independent), (2) have high sensitivity, good signal resolution and reproducibility, (3) not require pre-knowledge to assist biomarker discovery, (4) have good quantification capability for complex mixtures, (5) be able to provide rich molecular information, such as structure,

concentration, dynamics, interactions, pH and compartmentation, (6) be holistic rather than selective (or biased towards certain analytes), (7) be inexpensive and have high throughput (and not labour intensive), (8) requires little/no sample preparation (separation, derivatisation *et al*), (9) be non-invasive, non-destructive to facilitate *in vivo*, *in situ* studies, (10) have low recurrent expenditure (to reduce costs and improve efficiency). In reality, no technique will meet all these requirements and a good realistic metabonomics technique ought to meet as many criteria as possible.

Table 3 Pros and Cons of the currently used metabonomics methods

	Chromatography-based methods	Mass spectrometry based methods	NMR-based methods
Objective	Yes	Yes	Yes
Reproducibility	Poor	Good	Good
Resolution	Fair	Fair	Good
Sensitivity	Selective	Selective	Fair
Unbiased detection (simultaneous)	No	No	Yes
Holistic	No	No	Yes
Molecular information	Poor	Fair	Rich
Sample preparation	Extensive	Extensive	Little or no
Pre-knowledge requirements	Yes	Yes	No
<i>in vivo/in situ</i>	Impossible	Almost impossible	Yes
Throughput	Fair	High	High
Recurrent expenditure	High	High	Low
Labour intensiveness	Fair	Fair	Low
Cost per sample	Fair/low	Fair	Low

For all three currently used technologies, they are well established analytical tools and have good objectiveness. In terms of sensitivity, although both chromatography and mass spectrometry based methods enjoy excellent intrinsic sensitivity, they are not holistic or simultaneous detection but selective detection methods especially when they are used to analyse mixtures; they are biased for some metabolites but against others when they are used to analyse the biological metabonomes. In other words, their sensitivities are not uniform for all metabolites and can be pretty low for certain analytes under given analysis conditions. For instance, when reverse-phased chromatographic methods are used to analyse the metabonome (or metabolite compositions) of blood plasma, urine and indeed extracts of other biological tissues, the polar metabolites such as sugars, some amino acids (e.g., glutamine and glutamate), hydroxyl carboxylic acids (e.g., those in TCA cycle) and ketone

bodies are biased against relative to, for example, phenylalanine or tyrosine even though these polar metabolites may be extremely important. In the case of MS, the ionisation efficiency differences between different metabolites and ion suppressions also result in some “invisible” high concentration metabolites. As for reproducibility, chromatographic methods are generally poor, though GC methods are better, whereas the MS-based methods are highly reproducible. For metabonomics purposes, both chromatography and MS methods suffer from some common disadvantages in terms of metabolite identification and quantification. Both techniques require fairly extensive sample preparations and are invasive and destructive (to samples), hence are not suitable for *in vivo* or *in situ* studies. Both of these techniques require pre-knowledge about samples and have high recurrent expenditure though chromatographic methods are reasonably inexpensive.

Both require substantial improvements to obtain rich molecular information (as discussed earlier) to ascertain the identities and properties of biomarkers. For MS-based methods, equipment can also be fairly expensive (e.g., FTMS). It is impossible to obtain compartmentation information with chromatography and MS based methods even though such information is extremely important. For both techniques, although quantification can be done by constructing *in situ* standard calibration curves, this will require enormous amounts of work because, in many cases, metabonomes consist of hundreds of metabolites and their standards are often not readily available. Even more importantly, new biomarkers, by default, are often unknown metabolites. Some of the disadvantages can be overcome to some extent by their hyphenation. For example, GC-MS has shown great promise^[10,26,27] since GC is well established and reproducibility is reasonably high. However, the remaining problems are still associated with the aspects of quantification, selective (biased) detection, poor molecular information, time consuming sample preparation procedures and invasive/destructive nature. Recently, hyphenated ultrahigh performance liquid chromatography and mass spectrometry (UPLC-MS)^[28~30] has been developed, which substantially improved chromatographic resolution and reproducibility, showing great potential in metabonomic applications. It may be possible that, with some more improvements in both facilities and column stationary phases, UPLC-MS will play even greater roles in the future metabonomics research. This technique and GC-MS will probably become the main stream technology especially when *in vivo*/*in situ* studies are not strictly required and the compartmentation information is not critically vital. However, some urgent attentions are needed to comprehensively deal with the problems of “known unknown markers” for both chromatography-MS based technologies.

In contrast, the NMR-based method is user independent, requires little or no sample preparation (e.g., for biofluids of mammals), and has excellent resolution and reproducibility. In addition, NMR method offers rich molecular information including metabolite structure, concentration, molecular dynamics, interactions, pH and compartmentation when diffusion-editing techniques are employed. Furthermore NMR is non-invasive and non-destructive to samples, making it possible to facilitate *in vivo* and *in situ* studies. For example, recently developed high

resolution magic-angle spinning (HRMAS) NMR methods remove the line broadening factors resulting from residual dipole-dipole interaction, chemical shift anisotropy and susceptibility, achieving high resolution spectroscopy for tissues which is comparable to that in the liquid state NMR^[31~38]. The introduction of this method makes it possible to directly investigate tissues and even whole living organisms^[39] without any destruction of samples. Moreover, NMR is a quantitative and simultaneous detection method thus not biased for any molecules. To quantify the metabolites, NMR methods only require a known concentration compound or an electronic signal as reference. The NMR-based method is also high throughput and can measure up to 400 samples per 24 h with flow-injection technology^[40]. With the rich information obtained, such methods have low recurrent expenditure so that the analysis cost is usually low on cost-per-sample basis even though NMR spectrometers are expensive. In fact, NMR method is the only current metabonomics detection method which can be used to undertake *in vivo* and *in situ* studies at the levels of cells, multicellular tissues, organs and whole organisms. NMR-based methods can also be employed to separate signals without separate samples *via* spectral editing techniques by taking advantages of differences in relaxation properties and diffusivities of metabolites^[41~43], further favouring the spectral simplification and signal assignments. The major disadvantage for the conventional NMR is its low intrinsic sensitivity. However, there are two ways to improve NMR sensitivity, namely, improving signal-to-noise ratio by reducing electronic noise and chemical noise levels. In order to reduce electronic noise levels, cryogenic probe technology has been introduced for a number of years in which the temperature of the electronic detection circuits was reduced to about 20K, hence increased detection sensitivity by about four folds. Chemical noises resulting from signal overlapping can be reduced by increasing the magnetic field strength. This will also increase detection sensitivity and spectral resolution. Currently the standard 600 MHz spectrometer equipped with optimised cryogenic probe technology can detect metabolites at nanogram (10^{-9} g) level. Since the polarisation rate in NMR obeys Boltzmann distribution law and such rate is extremely low under normal circumstances, methods are also currently under development to increase the polarisation rate by using dynamic nuclear polarisation techniques. It is thus conceivable that further developments of this

technique will make the “low sensitivity” nature of NMR rapidly become a history.

It is clear that the best choice at present may lie at the combination of a number of existing techniques. The hyphenation of chromatography, MS and NMR (LC-NMR-MS) played an important role already in the metabolite identifications. It is particularly exciting to note that a statistical heterospectroscopy method has been reported^[44] which takes advantages of both NMR and UPLC-MS. It is predictable that such correlation techniques will become increasingly important in the future.

2.2 Samples for metabonomics studies

In theory, any biological samples including biofluids, tissues and even whole organisms can be used for metabonomics studies. In practice, however, the sample requirements are dependent on the purpose of studies and methods used. For both chromatography and MS based methods, the samples have to be in the liquid form and often require removal of proteins or extractions. For NMR-based methods, on the other hand, both biofluids (e.g. urine and blood plasma) and tissues (e.g., liver, kidney and brain biopsies) are generally employed directly without much sample preparation or extractions, so that *in vivo* and *in situ* information is readily available. In such cases, only as little as 2 μ l biofluids^[45] and about 10 mg tissues are required to obtain information in the manners of either *in vivo*, *ex vivo* or *in situ*. Information on the metabolite compartmentation is also readily retrievable using diffusion-edited NMR methods^[46~49]. Furthermore, with such a small volume of biofluids required, the time course of metabonomic changes can also be monitored with minimum or no invasion to the subjects^[45]. Such studies can also be carried out directly on whole animals^[50].

For mammalian models, the most commonly used biofluids are blood plasma and urine. Blood plasma carries the snap shot information about the given physiological and pathological processes and how well the homeostasis is maintained; urine samples, on the other hand, contains information about the metabolites excreted in a period of time, which may reflect the states of physiology, biological age, possibility of diseases including the inborn errors and environmentally induced pathology. Tissues' metabonome provides a unique opportunity for detecting molecular information of an organ where the biochemical processes occur, hence the underlying mechanism of its biochemical response to stimuli can be understood. For some specific purposes, other

special biofluids such as CSF, milk and dialysates can also be important. By comparing with the metabonome of the control samples, the effects of internal and external stimuli on the subjects can be easily analysed in a holistic and quantitative way using statistical tools.

For plant systems, most currently used methods are based on solvent extracts thus are *in vitro* by nature. This is owing to that the plant “biofluids” are not as readily available as in the case of animals. However, tissues and sometimes whole organism studies are possible by employing high resolution magic-angle spinning (HRMAS) NMR. This method can also be applied to microbiota and cultured cells, in which both cells and media are important carriers for metabonomic information. The analysis of media is also important and often referred to as cell metabolism “foot-printings”.

2.3 How to do metabonomics studies?

There are three general steps for conducting metabonomics studies, namely, sample preparation, data acquisition and data mining in order to extract the biological significances. However, for such a complex subject, extra care has to be taken in each step. For example, the sample collections and preparation step is fundamentally important. Many factors affecting metabonomes have to be carefully thought through such as variations in species, strains, gender, age, diet, acclimatisation, timing of samples and the strength of stimuli. In the case of using extracts, the extraction methods (completeness of extraction) have to be optimised so that maximum metabonomic information is available. To acquire data for the metabolite complement, one can use chromatography, mass spectrometry or NMR spectroscopy to obtain digital information on the metabonome composition and responses to the exerted stimuli. The choice of detection methods has to be appropriate for the purposes of studies. The data analysis and interpretation is the most crucial step, in which appropriate justifications and validations have to be carefully considered especially when “supervised” data analysis methods are employed. Biological meaningfulness is the ultimate aim for metabonomics studies which can be clearly illustrated in the following applications.

3 Applications of metabonomics

Metabolite analysis is important because any perturbation to a living system, whether it is physiological or pathological in nature, will cause perturbations of concentration or flux of endogenous

metabolites. Most of metabolism pathways are functioning not in isolated ways but in an interactive manner with some other metabolism processes thus changes of multiple metabolites can occur. Measurements of a single or only a few limited metabolites naturally have limited values. Metabonomics, by default, measures the metabolic changes in a holistic fashion, therefore, has become a successful research technology and found widespread applications in molecular phenotyping, functional genomics, drug toxicity/efficacy, molecular pathology and disease diagnosis/prognosis, and environmental sciences.

3.1 Toxicology

NMR techniques have been applied to toxicological studies since 1980s and there are vast amount of data reported already covering both drug toxicity^[9,17,51] and environmental effects^[52~57]. The early work was mostly performed by using ^1H NMR to analyse the mammalian urine and plasma^[58,59] samples. More recently, ^1H high resolution magic angle spinning (HRMAS) NMR methods have been developed to enable *in situ*, *ex vivo* and even *in vivo* studies, which have potential to provide molecular and compartmentation information correlating with histopathology results^[60~62]. Such studies have now been extended to non-drug toxicity fields. In the heavy metal toxicity studies, for example, high resolution NMR spectroscopy of urine, blood plasma and ^1H HRMAS NMR of tissues have been analysed to assess the renal toxicity of $\text{Cd}(\text{II})$ ^[54], HgCl_2 ^[63,64], $\text{La}(\text{NO}_3)_3$ ^[65~67] and $\text{Ce}(\text{NO}_3)_3$ ^[68] to discover the relevant biomarkers and underlying mechanisms of toxicity. In a ^1H NMR study, for example, the biochemical effects of $\text{La}(\text{NO}_3)_3$ on rat metabonome have been investigated^[65~67] by analysing their urine, plasma and tissue samples, revealing the observable liver and renal damages upon long term oral administration. The most comprehensive metabonomic toxicity studies, so far, have been carried out within the frame of the Consortium for Metabonomic and Toxicology (COMET) project^[69,70] (<http://bc-comet.sk.med.ic.ac.uk/>), which was formed by Imperial College London and six high profile international pharmaceutical companies, namely, Bristol-Myers-Squibb, Eli Lilly & Co, Hoffmann-La Roche, Novo Nordisk, Pfizer Inc. and The Pharmacia Corporation (now part of Pfizer). Within the frame of this project, NMR-based metabonomics approach has been employed to carry out studies on about 150 known toxins, involving tens of thousands animal models. The objectives of the

COMET project were to evaluate the utilities of metabonomics comprehensively, to discover biomarkers related to xenobiotic toxicity and to establish the machine learning expert systems empowered with abilities of predicting the organ specific toxicity^[70]. Although it was aimed mainly towards some renal toxins and hepatotoxins, the project, in fact, covered much more extensive factors, including physiological stresses, pancreas, testicular, bladder and multiple organ targets, representing the most comprehensive evaluation of the biochemistry of xenobiotic toxicity ever^[70]. The second phase of this project will be extended into more detailed mechanistic studies to advance our understandings of molecular mechanisms of the toxicology (<http://bc-comet.sk.med.ic.ac.uk/>). Based on such understandings, it will be probable to establish some quantitative, universal, integrated and predictive (QUIP) expert systems, and to provide a powerful tool for future drug discovery and environmental toxicology research.

Metabonomics has also been applied to study the side effects of clinically employed drugs. This is particularly important in the case of cancer treatments where most chemotherapeutic drugs have toxic side effects, resulting in nephrotoxicity, hepatotoxicity and in some cases neurotoxicity. Ifosfamide and *cis*-platin, for example, are widely used to treat cancers in both children and adults, both drugs induce nephrotoxicity. Metabonomics studies of tamoxifen^[71,72], a drug widely used for treatment of the estrogen-dependent breast cancer, has shown that this drug caused the elevation of lipid and glucose levels in rat livers accompanied with the decrease of glycine and serine levels even though it is uncertain whether such effects are related to liver tumour promotion or not^[72]. Such drug toxicity though unavoidable at present causes considerable problems for the patient treatment and NMR-based metabonomics will have some important roles to play in discovering the early side effects and underlying mechanisms.

3.2 Gene functions

The functions of genes are often manifested in the regulation (mRNA) and expression levels (proteins). This certainly results in changes at the metabolic level as the metabolites are near end-products of the relevant biological processes. As a holistic metabolite measurement approach, therefore, metabonomics is the tool of choice to be employed to study the gene functions, which is exemplified in a number of functional genomics studies. GC/MS has been used^[10,73]

to quantify more than 300 compounds from *Arabidopsis thaliana* leaf extracts though half of them were not assigned structurally. By comparison, four genotypes were characterised based on metabolic features, providing a possible tool for gene function studies. In another case, a functional analysis by co-responses in yeast (FANCY) has been employed^[16] to search for the gene functions of *Saccharomyces cerevisiae* by measuring the mutation-induced metabonomic changes. FTIR and MS have also been employed to analyse the metabolites in bacterial medium (or footprints) to study the metabonomic changes upon mutation^[74]. Metabonomics methods have been used in the functional genomics studies of plants^[75-78], micro-organisms^[79] and animals^[80,81], most of which have been comprehensively reviewed already. Amongst many functional genomics studies, the “Biological Atlas for Insulin Resistance (BAIR)” project funded by the Wellcome Trust in UK and started in 2002 is worth mentioning. This project consists of 18 research groups and 60 contributors with expertise in insulin signalling, rodent gene targeting, human genetics, metabonomics, proteomics, transcriptomics, bioinformatics, and structural biology (<http://www.bair.org.uk>). The main objective of this project is to generate a comprehensive description of well-defined states of insulin resistance in rodent models. It is conceivable that similar studies will soon become much more widespread given the amount of attentions that systems biology has received.

3.3 Phytomedicines and traditional Chinese medicines

Natural medicines from plants (phytomedicines) have been used for thousand years throughout the world and have made tremendous contributions to human health care. In recent centuries, many important drugs were developed from the natural medicines or stemmed from herbal remedies. For example, the cardiac drug, digoxin, was originally extracted from the leaves of *Digitalis lanata* almost two hundred years ago. A group of similar drugs called “digitalis glycosides” with in common specific effects on myocardium have been subsequently isolated from a number of plants. One of the world's oldest and most common drugs, acetylsalicylic acid also known as aspirin, used as a non-steroidal anti-inflammatory drug, was also derived from the ancient idea of using willow barks as pain-relief medicine. Other well-known examples in this category include the pain-management drug, morphine, from *Papaver somniferum*, anti-malaria drug, quinine, from the

leaves of *Cinchona officinalis* and L-dopa from seedling of *Vicia faba* beans for treatment of Parkinson's disease. More recently, paclitaxel or taxol was isolated from pacific yew tree (*Taxus brevifolia*) and found applications in treating a broad range of cancers, artemisinin, a sesquiterpene lactone, has been isolated from the plant *Artemisia annua* L (sweet wormwood) and it together with its derivatives have been rapidly developed into a unique family of anti-malaria drugs. In fact, the idea of using *Artemisia annua* L (qinghao or 青蒿 in Chinese) to treat malaria and haemorrhoids is more than 150 years old in traditional Chinese medicines. Some antibiotics can also be considered as natural medicines such as so-called “miracle drug” penicillin. Therefore, natural medicines represent some important sources for new drug discovery and development.

In China, the traditional Chinese medicines (TCM) has been practiced for thousands of years and the written documents showed sophisticated theories for TCM even in its early forms although these theories were established on philosophical basis and clinical experiences rather than science perhaps owing to the fact that it had been developed well before modern science was born. Like almost all other traditional medicines, the practice of TCM suffers, at present, from insufficient modern scientific research. For example, many aspects of TCM are still practicing in its original form and although it has been effective in treating many conditions especially chronic ones, it lacks necessary well-defined molecular mechanism and sometimes even molecular basis. For instance, in TCM, the concept of “main and collateral channels” has not been firmly backed up by anatomical evidences. Prescribed medicines in TCM are normally mixtures from a number of plants and their composition is not well defined. In fact, the composition of most of the single herb itself is not fully characterized, thus, there is insufficient information on the mode of action in molecular levels for single herbs and mixtures. From reproducibility point of view, a number of species are often named under the same medicinal herb and they grow at different locations with different climate and harvested at different time. The raw materials for medicines are often inconsistent in chemical composition. To prepare mixed medicines used for clinical use, each medicinal material is often required to be processed in certain ways, which is often not subjected to modern quality control management either. Consequently, the inconsistency of raw materials and processing is

transferred and sometimes manifested into the resultant clinically used medicines. The prescriptions are often liable to many complications and lack quantitative description. Therefore, there is clear urgency for scientific research in terms of quality control, clinical efficacy, molecular mechanism for the traditional medicines and patient responses to a treatment in order to modernise the traditional medicines, which undoubtedly demands significant amount of analytical power and efforts.

There have been many recent attempts to address these issues but most of them were still based on the “reductionism” philosophy. These approaches effectively followed the routes of the chemical drug discovery, consisting of isolation, active component discovery and chemical modifications. These attempts have, so far, made some progress, for example, in discovering the anti-malaria drug, artemisinin. However, such approaches have fairly limited throughput and appeared inefficient when compared with the vast amount of traditional medicines to be investigated. This approach will also not be able to effectively address such questions as whether some synergistic effects of mixture are important. Moreover, the existing experiences of the TCM were not fully taken into consideration. Since TCM is based on “holism” philosophy instead of “reductionism” as in the case of “omics” theories, it is conceivable philosophically that the metabonomics techniques ought to play some roles in providing molecular information to the TCM in the integrated manner.

For authentication and quality control, most of herb/natural medicines were traditionally assessed by practitioner's experience and, at best, by measuring a few known compounds (as “markers”) in the mixture using HPLC/TLC methods. However, such assessments often fail to sufficiently address whether these measured components are relevant in terms of the quality control or bioactivity (e.g., treatment/adverse effects). Metabonomic characteristics ought to be the good “standard” for good manufacturing practice purpose regardless which components are active. For this purpose, some common herbs have already been subjected to metabonomics studies as the proof of concept. The NMR-based metabonomic analysis was shown effective for chemotaxonomic analysis and authentication purposes in a study of three *Ephedra* species and some commercial materials^[82]. A broad range of metabolites in the *Ephedra* extracts were identified without any chromatographic separation and

the major differences in *Ephedra sinica*, *E. intermedia* and *E. distachya* var. *distachya* were found due to benzoic acid analogs in the aqueous fraction and ephedrine-type alkaloids in the organic fraction. Based on this metabonomic recognition, one of nine commercial *Ephedra* materials evaluated was shown to be a mixture of *Ephedra* species^[82]. Similar techniques applied to 12 *Cannabis sativa* cultivars also showed^[83] powerfulness in discriminating those samples, revealing that D9-tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA) were important metabolites to differentiate the cultivars from each other. Moreover, the discrimination of the cultivars could be obtained from a water extract based on the concentration of carbohydrates and amino acids. Sucrose, glucose, asparagine, and glutamic acid were the major discriminating metabolites^[83]. Such chemotaxonomic analysis was also exemplified in a study of 11 *Ilex* species, showing clear discrimination of those samples based on the metabolites present in the organic and aqueous extracts^[84]. The major discrimination metabolites include arbutin, caffeine, phenylpropanoids, and theobromine, amongst which arbutin was not previously reported in *Ilex* species but was found^[84] to be a biomarker for *I. argentina*, *I. brasiliensis*, *I. brevicuspis*, *I. integririma*, *I. microdonta*, *I. pseudobuxus*, *I. taubertiana*, and *I. theezans*. The metabonomic analysis for seeds, leaves, stem bark, collar bark and root bark of three *Strychnos* species^[85], *S. nux-vomica*, *S. ignatii*, and *S. icaja*, showed a good discrimination between all samples; the key compounds responsible for the discrimination include brucine, loganin, fatty acids, and *Strychnos icaja* alkaloids such as icajine and sungucine. The method was also applied^[85] to classify several “false angostura” samples, revealing, as expected, their identities as *S. nux-vomica*.

Multiple component analysis based on the combination of high resolution NMR spectroscopy with pattern recognition (i.e., common metabonomics approach) has been employed to investigate batches of 14 commercially available feverfew samples. The results showed^[86] that amongst them, two batches are significantly different in chemical composition from the others, the remaining twelve batches can also be classified into discrete groups by principal component analysis on the basis of minor differences in overall chemical composition. The powerfulness of such methods is also highlighted in quality control of phytomedicines. In another study, chamomile (*Matricaria recutita*) flowers were chosen as an

example of phytomedicines since this herb is usually marketed in Europe as beverage and for medicinal uses either administered as a tea or a compress for external healing or a bath for babies. It has been commonly claimed that chamomile has many effects including soothing and relaxing at bedtime, relieving restlessness, teething problems, and colic in children, relieving allergies, much as an antihistamine would, aiding digestion when taken as a tea after meals, relieving morning sickness during pregnancy, speeding healing of skin ulcers, wounds, or burns and treating gastritis and ulcerative colitis. Although some studies showed that chamomile had antioxidant activity^[87,88], and the “essential oil” extracted from the chamomile flowers possessed antimicrobial activity^[89,90], its previous quality control often, as usual, relied on the practitioner’s experiences and only monitoring limited “markers” using TLC and HPLC methods. When metabonomics methods were employed to profile its global composition^[91], clear differentiation (see Figure 4 for details) was achieved between samples from Northern Africa (Egypt) and Eastern Europe (Hungary and Slovak Republic). It was particularly encouraging to distinguish samples from Hungary and Slovak Republic based on their metabonomic compositions though they were from close geographic locations^[91]. Furthermore, this method was effective for measuring the “purity” of chamomile samples, such as the percentage of stalks^[91], and distinguishing samples extracted with different methods. The methods were further employed to investigate the effects of chamomile ingestion on human biochemistry^[92]. The results showed that chamomile ingestion had some mild effects on human

biochemistry, such as reducing the oxidative stress and altering the state of gut microflora, which was not completely recoverable within the period of a week time^[92] (Figure 5). It is, however, unknown what significance of this slow recovery has at present. In another example, metabonomics studies have been carried out on the extracts of *Artemisia annua* to discriminate samples from different sources and classify them according to their anti-plasmodial activity without pre-knowledge of this activity^[93]. The use of partial least squares (PLS) analysis also allowed the predictions of actual values of such activities for some independent samples that were not used in model construction.

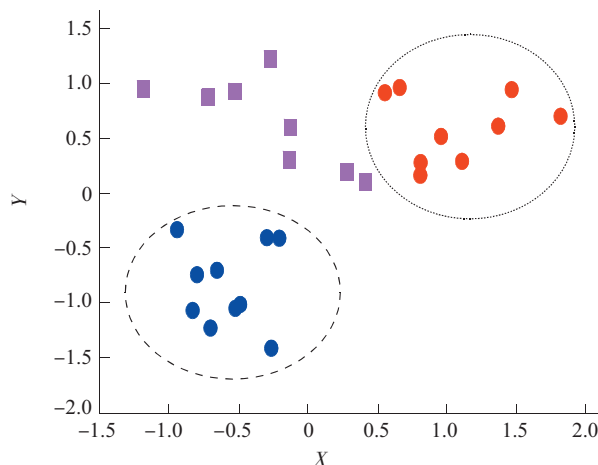


Fig. 5 Trajectory of the effects of chamomile ingestion on human urinary metabonome

● : pre-dose; ● : during dose; ■ : post-dose.

3.4 Disease studies

In most cases, the clinically diseased states are recognized by phenotypic characteristics and diagnosed by monitoring and analyzing the morphological phenotypic developments. For most of the diseases, however, there is a phenotype development phase in the pre-clinical stage, during which perturbations to biological chemistry of a given system initiate and propagate to fulfill the quantitative accumulation. Until such accumulation reached the critical point can the disease phenotypes appear morphologically (e.g., under microscope). However, when most diseases become histopathologically visible, the irreversible damage to human body in the levels of tissues, organs and sometimes even functions of whole body has already occurred. What doctors can only do in these cases is to “save life”, rather than to achieve an intrinsic “recovery”. It is conceivable

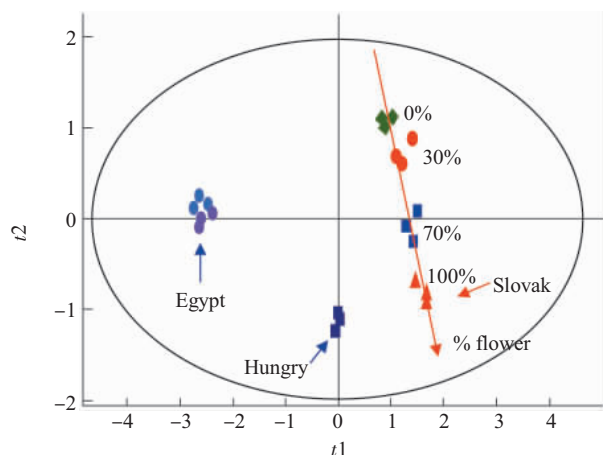


Fig. 4 Metabonomic characteristics of chamomile samples from different locations and Slovak samples with different purity (percentage of flower weight)

that disease development even in the early stages will cause some perturbations to human biochemistry thus some changes in the levels of regulations, protein expressions and especially metabolism. Therefore, capturing the early stages of a disease event is more important from the disease prevention point of view, which can be achieved by measuring the systemic transcriptome, proteome and metabonome.

Since metabolism results represent what has already happened in the level of genes and proteins, it is possible that the development towards disease phenotypes should result in metabolic changes in the either whole systems, organs, tissues or cellular levels. It is now well-known that metabonomics techniques are sensitive even to the perturbations induced by moderate changes in physiological^[17, 94~97] or environmental factors^[98,99]. From phenotyping point of view, it is perhaps not surprising that ¹H NMR data from “healthy” rats, rabbits, mouse and human urine samples have shown marked differences in urinary metabolite complement due to different species^[100]. However, similar results also showed that metabonome differences are present between Han Wistar (HW) and Sprague Dawley (SD) rat strains^[101] even though they were perceived to be similar genetically and metabolically. Moreover, metabonomics techniques have been successful to differentiate morphologically indistinguishable species of earthworm^[102] by analyzing their coelomic fluid and tissue extracts with ¹H NMR spectroscopy and multivariate data analysis. It is particularly interesting that the inter-individual metabonomic differences have been recorded using NMR methods. More usefully, the “metabolite clusters” (or biomarker clusters) related to these aforementioned studies have also been established. Metabonome is found sensitive to food and water restriction, diurnal and temperature effects and some mild stress induced biochemistry changes of rodents. Therefore, on the way towards most (morphological) disease phenotypes, molecular phenotypes (i.e., metabotypes) should be developed and such molecular phenotypes may be present well before morphological states. It is now clear that metabonomics is an extremely useful tool for detecting molecular phenotyping caused by disease processes.

Some good examples have already been reported, including detecting inborn errors, diagnosis of coronary heart diseases, schistosomiasis, and applications in organ transplantations. Inborn errors of metabolism have been studied for hundreds of years and the conventional analysis includes specific enzyme

assays and GC-MS methods, which are generally sensitive. However, the limitations of such methods include that (1) one has to know the nature of such specific inborn error, (2) the methods require considerable sample preparation hence time consuming, and (3) the methods are not capable of discovering the unknown inborn errors. ¹H NMR spectroscopy has now been widely used in inborn error studies which can be conducted by analyzing urine and blood plasma samples^[103~107]. It is particularly noteworthy that the NMR methods, requiring little/no sample preparations, are extremely powerful in discovering the unknown inborn errors and these studies have already been comprehensively reviewed else where^[108].

One of the most important examples for disease diagnosis using metabonomics techniques has been reported for coronary heart disease (CHD)^[109]. As a major cause of mortality especially in the developed world, CHD diagnosis is traditionally done by visualizing the coronary arteries using X-ray angiography, which is invasive, expensive and not suitable for early disease screening. The metabonomics methods, with less than half milliliter blood plasma, have not only classified CHD patients effectively but also the severity of such diseases^[109]. The potential of this approach is now being explored through a clinical trial (www.med.cam.ac.uk/magicad). More recently, metabonomics approaches have been used to study the effects of *S. mansoni* and *S. japonicum* infection on the rodent urinary metabonome^[110,111]. The results showed that schistosomiasis in both cases led to comprehensive suppression of the host's TCA cycles, disruption of the amino acid metabolism, liver injuries, and the metabolism of the gut microbiota^[110,111]. A recent study has shown that metabonomics technology is useful for diagnosis of prions diseases such as transmissible spongiform encephalopathy (TSE)^[112]. LC-MS methods have also shown its potential as a disease screening tool^[113,114] though complete identification of biomarkers remains to be a difficult issue. For organ transplantations, there is always a need to rapidly evaluate the state of crafts before transplantation, to assess compatibility and monitor the acceptors' body responses during the post-transplantation period. A preliminary study showed that metabonomics is potentially a useful tool^[115] to address these aspects in the case of liver transplantation. Metabonomics technology will make more contributions in understandings to disease processes although there is still a great deal of

foundation work to be done before the metabonomics approach becomes clinically useful.

To sum up, metabonomics measures the metabolite complement and metabolic response to the internal and external stimuli, therefore, is an extremely powerful platform for studies associated with the biological metabolisms. So far many successful applications of metabonomics have been reported for a wide range studies including fundamental biology, xenobiotic effects such as drug efficacy and toxicity, gene functions and disease processes. Some applications have also been extended to the environmental toxicology and human screening studies. It is anticipated that metabonomics will become a more and more important global systems biology tool and be applied in other research areas such as nutritional science, authenticity, and product quality controls (such as GAP and cGMP).

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代谢组学: 一个迅速发展的新兴学科^{*}

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摘要 对代谢组学的含义, 中心任务, 研究方法, 样品要求, 应用及其发展方向进行了简要综述. 系统生物学概念的诞生标志着研究哲学由“还原论”向“整体论”的变化. 系统生物学的中心任务就是要针对生物系统整体 (无论它是生物细胞, 多细胞组织, 器官还是生物整体), 建立定量, 普适, 整体和可预测 (QUIP) 的认知. 具体而言, 系统生物学研究就是要将给定生物系统的基因, 转录, 蛋白质和代谢水平所发生的事件, 相关性及其对所涉及生物过程的意义进行整体性认识. 从而出现了许多的“组”和“组学”的新概念. 但是现已提出的一百多个“组”和“组学”, 可以大体归纳为“基因组”/“基因组学”, “转录组”/“转录组学”, “蛋白质组”/“蛋白质组学”和“代谢组”/“代谢组学”四个方面. 显而易见, DNA, mRNA 以及蛋白质的存在为生物过程的发生提供了物质基础 (但这个过程中有可能不发生!), 而代谢物质所反映的是已经发生了的生物事件. 因此代谢组学是对一个生物系统进行全面认识的不可缺少的一部分, 是全局系统生物学 (global systems biology) 的重要基础, 也是系统生物学的一个重要组成部分. 在现有的英文表述中, 代谢组学同时存在两个不同的词汇和概念, 即 *metabonomics* 和 *metabolomics*. 尽管前者多用在动物系统而后者多用于植物和微生物系统, 但这些概念的本质从他们的定义中能够得到较细致的了解. *Metabonomics* 的最初定义是就生物系统对生理和病理刺激以及基因改变的代谢应答的定量测定 (“the quantitative measurement of the multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modifications”). 我们认为这个定义现在可以更广泛地表述为: 代谢组学是关于定量描述生物内源性代谢物质的整体及其对内因和外因变化应答规律的科学 (“*Metabonomics is the branch of science concerned with the quantitative understandings of the metabolite complement of integrated living systems and its dynamic responses to the changes of both endogenous factors (such as physiology and development) and exogenous factors (such as environmental factors and xenobiotics).*”). 其中心任务包括 (1) 对内源性代谢物质的整体及其动态变化规律进行检测, 量化和编录, (2) 确定此变化规律和生物过程的有机联系. *Metabolomics* 存在多个定义, 但其精髓是: 对一个生物系统的细胞在给定时间和给定条件下所有小分子代谢物质的定量分析 (the quantitative measurement of all low molecular weight metabolites in an organism's cells at a specified time under specific environmental conditions). 因此, *metabolomics* 可以译作“代谢物组学”. 不难看出, 前者是对生物系统进行的整体和动态的认识 (不仅关心代谢物质的整体也关注其动态变化规律), 而后者强调分析而且是个静态的认识概念. 因此可以认为, *metabolomics* 是 *metabonomics* 的一个组成部分 (参看定义). 近年又有人提出了 “dynamic metabolomics” 的概念, 这个概念所表达的含义十分接近 “metabonomics” 本身的含义. 所以, 可以预见, 随着这门新兴学科的发展和更深入讨论, 这两个概念必将趋向一致. 因此我们建议, 在中文表述中将“代谢组学”一词和英文中的 *metabonomics* 相对应, 以避免不必要的混淆和争议. 就细胞系统而言, 不仅存在细胞自身的代谢物质组成问题, 存在细胞之间代谢物质交换的问题, 也存在代谢过程所发生的位点问题. 因此, 简单地分析代谢物质的总组成 (即代谢组) 缺乏“整体论”所要求的全面性, 其意义有一定局限. 代谢组学属于全局系统生物学 (Global systems biology) 研究方法, 便于对复杂体系的整体进行认识. 譬如, 一个正常工作的人体包括“人体”本身和与之共同进化而来且共生的消化道微生物群体 (或称菌群), 孤立地研究“人体”本身的基因, 转录子以及蛋白质当然可以为人们认识人体生物学提供重要信息, 但无法提供使人体正常工作不可缺少的菌群的信息. 人体血液和尿液的代谢组却携带着包括菌群在内的每一个细胞的信息, 因此代谢组学方法对研究如人体这样复杂的进化杂合体十分有效. 正因如此, 代谢组学已经广泛地应用到了包括药物研发, 分子生理学, 分子病理学, 基因功能组学, 营养学, 环境科学等重要领域. 在代谢组学诞生的过去 6 年里, 有关代谢组学的研究论文和专利以指数的形式逐年增长. 可以预见, 这门新兴学科将应用到更为广泛的领域.

关键词 代谢组学, 核磁共振, 代谢物, 系统生物学

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