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Metabolomics in Medicinal Chemistry

Metabonomics in the preclinical and environmental toxicity field

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Preclinical studies assess both efficacy and safety of new drugs through a series of assays used to identify potential target organs and determine safety thresholds. However, despite these efforts, too many drugs prove toxic to humans during clinical phases or later on the market. This paper reviews how metabonomics, one of the key players in systems biology, should be able to assist toxicologists in better predicting the adverse effects of xenobiotics.

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Introduction

From the identification of a lead molecule presenting a reasonable affinity for a receptor target to its approval for marketing by regulatory bodies, drug goes through a complex and highly controlled process over an averaged period of 10 years [1].

After selection of the most effective chemical scaffolds capable of interacting with the target, hundreds of representative molecules progress to the preclinical phase to deliver the 'drug candidate' that will enter the clinical phase. During the preclinical phase, surrogate animal models are used to assess the safety of the drugs, to identify potential target organs and to determine safety thresholds, such as the 'no adverse effect level' (NOAEL) under which adverse effects are unlike to occur in animals. Based on the pharmacological dose and the NOAEL determined in animals, safety margins are calculated to minimize the risks associated with early human exposures in subsequent clinical phases.

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Challenges in preclinical studies

According to the principles of the 3Rs (Replacement, Reduction and Refinement), the academic and industrial sectors are conducting exploratory and preclinical studies with more emphasize on developing alternative approaches to the use of animals. Better models and tools are needed to more closely reflect human biology and predict the efficacy and safety of new medicines. For example, human cell cultures and *in vitro* assays (hERG or '*ether-a-gogo*' related gene and Ames assays for instances) are now currently used to assess the toxicity of a drug candidate (for review, see [2]).

Systems biology integrates data concerning genes (genomics), proteins (proteomics), and small molecules (metabolomics) sharing common signaling pathways and how their interactions can be perturbed by stress factors such as diseases or exposures to xenobiotics [3]. In this respect, the 'omics' technologies should be able to assist toxicologists in better understanding and predicting the adverse effects of drugs and other xenobiotics (for example chemicals and environmental pollutants), especially when the adverse effects emerge unexpectedly in humans. According to experts, metabolic network understanding is essential to the future of drug development [4], and metalonomics is especially well suited for this purpose (Fig. 1). 'Metabolomics' is the study of the complete collection of metabolites present in a cell or tissue under a particular set of conditions (= the metabolome) generating a biochemical profile. There is another term used in the

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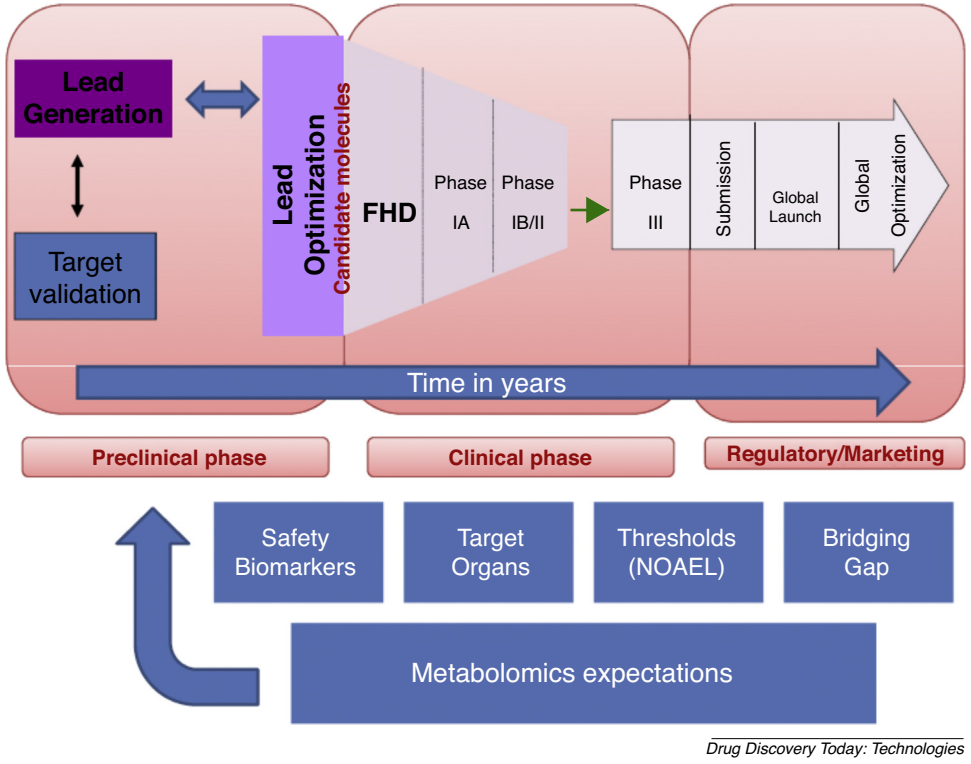

Figure 1. Expected impacts of metabonomics in the preclinical phase during drug development.

literature, 'metabonomics', which is usually applied only to studies of responses to drugs or disease, and compares profiles without identifying individual compounds. Hence, this terminology 'metanonomics' will be used for the rest of this review.

This recently introduced discipline assesses endogenous and exogenous metabolites, identified and quantified in various types of biofluids (urine, blood, saliva, intracellular fluids, ...) and biopsies. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and mass spectrometry (MS) are the main metabonomics analytical platforms. The technological developments in the field of NMR spectroscopy have enabled the identification and quantitative measurement of the many metabolites in a single sample of biofluids in a non-targeted and non-destructive manner. Combination of NMR spectra of biofluids and pattern recognition methods has driven forward the application of metabonomics in the field of biomarker discovery [5]. But what is the contribution of metabonomics to the drug development so far?

It has been almost a decade since the potential of metabonomics in the evaluation of xenobiotic toxicity has been evaluated by the Consortium for Metabonomic in Toxicology (COMET) [6,7]. Since then, metabonomics has been seen as a promising technology to address the upcoming challenges of toxicology [8].

The present review aims at presenting recent advances in the use of metabonomics in the safety assessment of xenobiotics, with a special focus on the interpretation on the possible biological links between metabolic changes and tissue damages.

Metabonomics, target organs, and safety biomarkers

The metabolic signatures obtained by spectroscopic analysis of biofluids are complex, but they allow inference in biochemical pathways and cellular mechanisms which provide evidences for the identification of target organs. Then, safety biomarkers can be isolated from those organ-specific profiles (Fig. 2), as exemplified in the following section reviewing recent applications of metabonomics in the evaluation of liver and renal toxicities.

A recent metabolomic investigation using mass spectrometry [9] unveiled the perturbations of bile acid homeostasis in groups of rats exposed to various prototypical hepatotoxins known to cause different types of DILI (drug induced liver injury). Necrosis, cholestasis, and steatosis were the main induced DILI as confirmed by clinical chemistry and histopathology. Regarding the metabonomics findings, changes in bile acids levels in plasma, liver, and urine samples as well as alterations in the biosyntheses of liver glutathione and taurine were the most important reported alterations. Urinary excretion of cholate was significantly increased by all hepatotoxins in the necrosis and cholestasis categories. Based on those results, the authors proposed that a small panel of metabolic biomarkers (plasma glycine and taurine

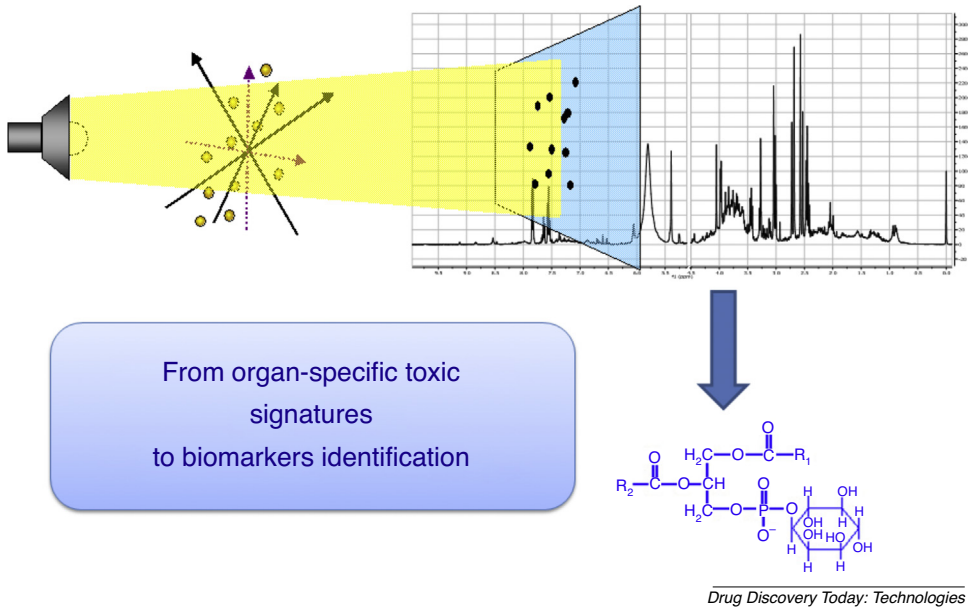

Figure 2. This figures shows how spectroscopic analysis of biofluids combined to and chemometric methods supply metabolic profiles characteristic of organ toxicity from which safety biomarkers can be identified.

conjugated primary bile acids and urinary cholate) may have the potential for early and sensitive detection of DILI in rats.

Cirrhosis and fibrosis are two potential adverse effects of DILI. Unfortunately, assessment of liver fibrosis is generally based on liver biopsy. So, metabonomic analyses provide a potential non-invasive tool for fibrosis evaluation. Wei *et al.* [10] explored metabolic changes during chronic exposure to low doses of thioacetamide to produce hepatotoxicity in rats and to establish a model of liver fibrosis and cirrhosis. Metabonomics revealed several potential biomarkers for liver fibrosis (2-hydroxybutyrate, 3-hydroxybutyrate and adipate in urine, as well as phenylalanine, *N*,*N*-dimethylglycine, *O*-acetyl glycoprotein, *N*-acetyl glycoprotein and choline in serum).

The toxicity of monocrotaline (MCT), a prototypical molecule of drug-induced hepatic sinusoidal obstruction syndrome (SOS), was evaluated in rats using a NMR-based metabonomics approach [11]. SOS is difficult to diagnose due to large inter-individual fluctuations in key clinical parameters and histopathological examination of liver biopsy remains the gold standard diagnostic tool of SOS. Two successive phases of alterations in the metabolic urine profiles of MCT-exposed animals were revealed. During the early phase, protective mechanisms were activated as demonstrated by increased synthesis of glutathione (GSH) and the recruitment of liver cell osmolytes (taurine and betaine). The subsequent late phase was characterized by a disturbance of the urea cycle (increased ornithine and urea reduction), leading to the depletion of nitric oxide (NO). Finally, a default in the energy metabolism is a key player in SOS and it was clearly demonstrated by a decreased activity of the Krebs (TCA-) cycle, the change in glucose utilization, and the increased urine levels of acetate.

Urine and blood are not the only biofluids which can inform on liver toxicity. For example, bile samples were investigated to evaluate the effects of a co-therapy with rifampicin (RIF) and isoniazid (INH), known to cause liver injury in humans [12]. The authors used a pregnane X receptor (PXR)-humanized mouse model (hPXR). PXR is a ligand-dependent transcription factor that regulates a gene network involved in the metabolism of xenobiotics and endobiotics and RIF is a human PXR-specific activator. Co-exposures of hPXR mice to RIF and INH caused the accumulation of the endogenous hepatotoxin protoporphyrin IX in the liver through PXR-mediated alteration of the heme biosynthesis pathway. Interestingly, this was the first animal model that showed reproducible results which were consistent with the human disease.

In an effort of compliance with the 3R's rule, but also due to an increasing demand for mechanistic understanding in toxicology, new models combining *in vitro* cell lines and metabonomics have been developed which could be of interest in the pre-clinical drug development. As an example, the metabolic response of HepG2/C3a, a human hepatocarcinoma cell line, exposed to flutamide, an antiandrogenic pro-drug used in prostate cancer treatment was investigated in a microfluidic biochip [13]. ^{1}H NMR spectroscopy analysis of culture medium revealed that exposure to flutamide did cause a disruption of glucose homeostasis and significant mitochondrial dysfunctions, as indicated by a reduction of the extracellular glucose and fructose consumptions and a general reduction of the Krebs cycle activity. A reduction in

glutamine consumption associated with glutamate accumulation was also observed and lactate kept released despite a reduction of the extracellular glucose consumption. Those metabolic features were characteristic of the Warburg effect described in cancer cells. Thus, the entire set of results contributed to extract specific mechanistic toxic signatures and their relation to hepatotoxicity, which appeared consistent with literature reports.

In conventional toxicity testing, sub-toxic effects are difficult to identify but may eventually cause severe and costly long-term problems such as idiosyncratic hepatotoxicity. Using a metabolic flux analysis, Niklas *et al.* [14] evaluated three compounds (amiodarone, diclofenac and tacrine), all known for their hepatic mitochondrial toxicity, on the human hepatoma cell line Hep G2. The mode of action of those three drugs uses an uncoupling of the oxidative phosphorylation. Consecutively to the exposure to either diclofenac or tacrine, an increase in TCA-cycle activity was noticed, suggesting an adaptation of the cellular metabolism to an uncoupling of the oxidative phosphorylation. This effect however was not observed with amiodarone, which could indicate that the uncoupling of the oxidative phosphorylation is not a subtoxic effect of amiodarone and occurs only upon treatment with higher concentrations of this compound.

Acetaminophen-induced toxicity was also assessed in the same cell line (hepG2/C3a) cultivated inside a microfluidic biochip [15]. Acetaminophen injury is related to glutathione consumption and depletion via the formation of NAPQI. Higher levels of 2-hydroxybutyrate production and consumption of cysteine, histidine, and methionine were seen by NMR spectroscopy of culture medium. Such changes were directly linked to acetaminophen detoxification mechanism via conjugation to glutathione, and confirmed by others in animals and in humans [16,17]. According to these authors, 2-hydroxybutyrate is released, under metabolic stress, as a by-product when cystathionine is cleaved to cysteine before its incorporation into glutathione. Moreover, increased levels of 3-hydroxybutyrate in the acetaminophen treated biochip cultures demonstrated an intense lipid metabolism through the ketone degradation pathway.

Besides liver, kidney is the other major target organ for acute toxicity. Tyagi *et al.* [18] investigated the changes that occur in the kidney metabolic profile following intraperitoneal administration of $NiCl_2$ in a rodent model. The major endogenous metabolites modified for kidney homogenates contained products of glycolysis (glucose, lactate), amino acids, organic osmolytes (e.g. betaine, myo-inositol, taurine and trimethylamine N-oxide, TMAO), membrane metabolites (choline, phosphorylethanolamine) and creatine. The authors interpreted the decrease in amino acids as a severe damage to the tubules, and the reduction in osmolytes such as myo-inositol, betaine and TMAO as disturbed renal homeostasis. Oxidative stress affects mitochondrial energy metabolism, resulting in down-regulation of Krebs cycle metabolites.

Liang *et al.* [19] investigated the long-term metabolic effect of pyrethroid insecticides by ^{1}H NMR-based metabonomics of urine and serum samples from exposed rats. Concomitant higher urine excretions of acetate and dimethylamines suggested the induction of renal papillary lesions. Being lipophilic, most pyrethroid pesticides interact with living organisms through lipid-rich biomembranes and then induce the generation of free radicals and oxidation. The rise in the serum concentrations of choline after pyrethroid treatment denoted the disruption of membrane fluidity caused by lipid peroxidation and the increase in serum ketone bodies supported the hypothesis of an impaired fatty acid β-oxidation in the liver.

Hanna *et al.* [20] assessed the value of metabonomics approach for detection of gentamicin induced toxic injury in the developing kidney in neonatal rats. Some of the metabolic alterations demonstrated proximal tubular kidney injury. Gentamicin has been shown to decrease DNA and protein synthesis in the proximal tubules and to increase mitochondrial energy metabolism by enhancing the oxidative phosphorylation in the cortical mitochondria. The present study confirmed these effects as evidenced by the decrease in succinate and the increase in uridine in the urine of gentamicin injected rats compared to controls. Tryptophan was also significantly increased and is involved in two metabolic pathways: serotonin and kynurenine pathways. Kynurenic acid is one of the few known endogenous excitatory amino acid receptor blockers with a broad spectrum of antagonistic properties in supraphysiological concentrations. The lower levels of kynurenic acid that were noted in the urine of gentamicin injected rats, coinciding with higher levels of tryptophan, suggest a degrading effect of gentamicin toxicity on tryptophan metabolism pathway.

Cultured human renal epithelial cells were exposed to Cyclosporine A (CsA), an immunosuppressive agent used to prevent transplanted organ rejection for 14 days [21] and ^{1}H NMR spectra from cell lysates were recorded wile supernatant medium was analyzed by LC–MS. A large increase in γ-glutamylcysteine and a marked induction of glutathione synthesis were the most prominent changes caused by CsA. In addition, fumarate was decreased while citrate, NADH, and lactate were elevated, suggesting a negative effect on the mitochondrial oxidative phosphorylation.

Metabonomics and developmental toxicity

Besides target organs toxicity, effect on the development is another important endpoint for which animal models are not necessarily transposable to the human situation. A new alternative to discover biomarkers of developmental toxicity has been recently proposed based on an *in vitro* method that

combines human embryonic stem (hES) cells with metabonomics [22]. HES cells were exposed to several drugs of known teratogenicity. A great correlation between teratogenicity and changes of greater than 10% in the ratio of arginine to asymmetric dimethylarginine (ADMA) levels was observed. Indeed, this metabolite decreased in response to several strong teratogens, including valproic acid. ADMA is an inhibitor of nitric oxide synthase (NOS), an enzyme that converts L-arginine to L-citrulline, which is necessary for neural tube closure. Valproate is known to cause neural tube defects while nitric oxide synthase activity is essential for neural tube closure and nitric oxide has been shown to induce neural tube defects in rat embryos. ADMA might be a good candidate biomarker for neural tube defects. Interestingly, this model was subsequently tested for its predictive accuracy in two blinded studies using eight drugs of known teratogenicity, where it correctly predicted the teratogenicity for seven of the eight drugs.

Metabonomics and environmental pollutants

Humans and animals are more and more exposed to a variety of chemical substances with very different uses (domestic, industrial, agricultural, ...). For example, medicinal and aromatic plants have always played an important role in the world of medicine, the composition of perfumes and food preparation. Some of these plants have been used for generations as traditional remedies, including Traditional Chinese Medicines (TCM). Recently, it was reported that various plant extracts used in TCM elicit similar metabolomic responses in rats [23–25]. Changes in some key metabolites (for example up-regulations of betaine and down-regulation of phosphatidylcholine) were detected in the serum and/or urine samples of TCM-treated groups in a dose-dependent manner. Not only the authors highlighted such metabolites as sensitive biomarkers of TCM toxicity, but they were able to establish a safe therapeutic range in the clinical use of those particular TCM, with promising predictions of possible drug toxicity.

Polyacetylenes are widely distributed in food plants and medicinal herbs, which have been shown to have highly but still unclear neurotoxic effects [26]. Bupleurotoxin (BETX), a representative polyacetylene, was tested in male BALB/c mice. Serum samples were screened using a metabonomics approach that revealed 17 metabolites perturbed after BETX treatment. A strong correlation between the GABA receptor signaling pathway and these metabolites was demonstrated. A validation test using a rat hippocampal neuron cell line was performed, and the results confirmed that BETX inhibited GABA-induced currents in a competitive manner. Severe morphological damages in the brain hippocampus were seen by histopathology. The metabonomics analysis was thus able to unveil the molecular mechanism of the toxicity of polyacetylenes.

The potential adverse effects of some *Thymus vulgaris* L. extracts were investigated in male rats by metabonomics [27]. Although no histopathological changes were found in the liver and kidney of rats treated with both extracts of thyme, ^{1}H NMR-based metabonomic analysis of urine samples revealed alterations in several urine metabolites involved in the energy metabolism in liver mitochondria: increases in lactate and ketone bodies, and decreases in citrate, α-ketoglutarate, creatinine, hippurate, dimethylglycine, and dimethyalanine. Impairment of mitochondrial function was suggested as a possible mode of action.

Considering the previous studies in preclinical models, it seems as if untargeted metabolomic approaches offer new opportunities for a better understanding of the mode of action of toxic compounds at the cellular and molecular levels. However, it remains important for a technology and for the arising biomarkers to be transposable to humans. The two following studies give evidences that metabonomics is undoubtedly a promising tool to translate biological findings from preclinical to clinical phases. First, a metabolomic investigation of biochemical alterations occurring in urine as a result of dioxin toxicity was performed, using urine samples collected from Czech chemical workers submitted to severe dioxin occupational exposure in a herbicide production plant in the late 1960s [28]. The results supported the hypothesis of liver damage and oxidative stress for long-term dioxin toxicity. The metabolomic strategy presented in this work allowed the determination of metabolic patterns related to dioxin effects in human and the discovery of highly predictive subsets of biologically meaningful and clinically relevant compounds. These results are expected to provide valuable information for a deeper understanding of the molecular events related to dioxin toxicity.

The second case concerns the Balkan Endemic Nephropathy or BEN, a chronic renal disease mainly affecting rural populations in the valleys of the Danube. Bread poisoning with aristolochic acids is now widely accepted as the causal agent. The source of this toxic substance is considered to be *Aristolochia clematitis*, a perennial plant that invades farming fields. The poisoning with aristolochic acids was suggested when clinical and histopathological changes similar to those observed in the Balkan patients were reported in several cases of nephropathy in Belgian patients unintentionally exposed to aristolochic acids during a Chinese herbs diet. Those clinical and histopathological features were then reproduced in laboratory experimental models. Using NMR-based metabonomics, Duquesne *et al.* [29] evaluated early signs of renal toxicity from urine samples collected in a rat model of intoxication with aristolochic acids. Changes in urine composition were consistent with a proximal tubular damage, most probably initiated by a mitochondrial default and an inappropriate response to oxidative stress. The same metabonomic approach was applied to surplus of urine samples

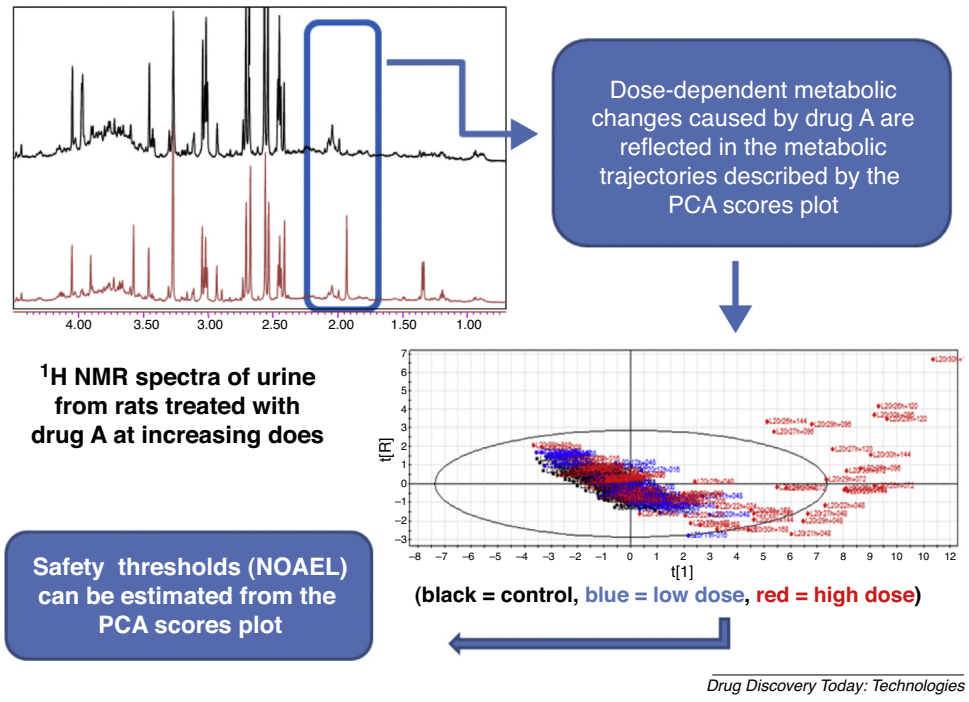

Figure 3. Dose-related metabolic changes observed in spectral data are converted into dose–response separations in multivariate data analysis, from which safety thresholds (i.e., NOAEL) can be extrapolated.

collected from Belgian and Croatian patients in clinical and epidemiological studies, respectively. It allowed a clear discrimination of the Belgian patients from a database of healthy volunteers. On the other hand, a trend to discrimination was noticed when comparing urine samples collected from individuals living in Croatian endemic regions as compared to Croatian non-endemic villages. Finally, when included in the same analysis, both Belgian and Croatian patients displayed similar urine metabolic signatures, suggesting a common etiology of both diseases. Similar observations were provided by Tsai *et al.* [30], comparing the toxicities of various sources of aristolochic acids in BALB/c mice. Detection of increased glucose, lactate, and creatine in urine suggested injuries in proximal renal tubules as well as in the renal papillary of rats AA-exposed.

Finally, in their recent review of the prominent metabonomics methodologies employed in data acquisition and analysis of natural products and disease-related biomarkers, Cox *et al.* [31] highlighted unequivocal value of metabonomics in natural products discovery, gene-function analysis, systems biology and diagnostic platforms.

Metabonomics and safety thresholds determination

The severity of changes in the metabonomics profiles may be used to assess dose–response effects (Fig. 3). Consequently, metabonomics can also appreciate safety thresholds below which no adverse effect are seen (NOAEL). For instance, the toxicity of various organophosphate (OP) pesticides was evaluated in rats using metabonomic technology at their corresponding NOAELs [32]. The results show that a single pesticide did not elicit a toxic response. The joint toxic action of four pesticides (at their corresponding NOAELs) was then evaluated by metabolomic analysis of rat plasma under experimental conditions similar to those of the four single OP pesticides [33]. The mixture of four pesticides showed a joint toxic action at the NOAELs of each pesticide as demonstrated by significant increases in 19 metabolites including a series of lysophospholipids, 4-pyridoxic acid, glutamic acid, glycocholic acid, and arachidonic acid, as well as decreases in sphinganines, tryptophan, and iodotyrosine in rat plasma. The results indicate that the mixture of OP pesticides induced oxidative stress, liver and renal dysfunction, disturbed the metabolism of lipids and amino acids, and interfered with the function of the thyroid gland.

Such data highlight the importance of the NOAEL determination in toxicology studies. More generally, the question was raised on how sensitive the new 'omics' technologies, including metabonomics, are relative to classical regulatory toxicity parameters [34]. To this end, the metabolome database MetaMap®Tox was created based on metabolomic data for more than 500 reference compounds. The metabonomics profile of rats exposed in the context of 28-day studies was recorded for chemicals ($n = 104$) for which a toxicological NOAEL was obtained at either high or mid-dose level. When comparing metabonomics versus conventional parameters, comparable sensitivities were noted in 75% of the cases,

increased sensitivity of metabonomics in 8%, and decreased sensitivity in 18% of the cases. The authors conclude that metabonomics profiling has a similar sensitivity to the classical toxicological study design.

General conclusions

During drug development, conventional toxicology studies are time consuming, they request a highly qualified staff and, despite all the efforts made during the risk assessment, too many marketed molecules still cause adverse effects in patients. According to experts, the identification of robust biomarkers which could be transposed from animal studies to clinics as well as the refinement of toxicity threshold could be seen as possible solutions. Recently, experts from academia, industry, and regulatory bodies discussed and reported the current status of applied metabonomics and its potential in risk assessment of drugs [35]. Since its first steps into the world of drug development with the Consortium COMET back in 2000, Metabonomics has rapidly grown in research field involving quantitative and qualitative metabolite assessment within biological systems. The technology is now applied to natural products discovery, to understanding the impact of gene expression or suppression, or as a diagnostic platform. Metabonomics can also be used to appreciate the role of epigenetics on the overall drug response. In the field of biomarker discovery, recent studies suggest that the metabolome opens new opportunity for discovering biomarkers, with the advantage over conventional methods that a full set of biomarkers can be identified from one single technological platform. Using metabolic signatures instead of individual markers might address the lack of robustness and the gap between animal models to humans which are alleged against conventional parameters. Now, regarding the refinement of toxicity threshold, recent publications do support the fact that metabonomics can be as sensitive as classical regulatory toxicity parameters, even better in some cases [34]. Finally, in a constant search for alternative methods to animal testing and more predictive tools, combining 'omics' sciences to human cell cultures and humanized mice seems to be a very promising approach.

Conflict of interest

The author has no conflict of interest to declare.

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