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Confidence in metabolite identification dictates the applicability of metabolomics to regulatory toxicology

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Abstract

The strength of omics technologies in toxicology is their ability to identify pathways of toxicity. Although pathway discovery is not required for chemical risk assessment, it can generate molecular biomarkers that accelerate the use of molecular assays in regulatory toxicology. For metabolomics, this application is limited by the challenge of metabolite identification. Here, we construct a framework around the confidence in identification of metabolites and molecular pathways and map multiple toxicometabolomics studies to this framework. This reveals that few studies achieve the highest level of confidence defined by the Metabolomics Standards Initiative. However, we argue that the level of confidence required is dependent on the regulatory application. For some, such as chemical grouping, current practices can suffice, whereas other applications require a more rigorous approach to metabolite identification.

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Keywords

Metabolite annotation, Mass spectrometry, NMR spectroscopy, Biomarker discovery, Molecular key event, Chemical risk assessment.

Introduction

In 2007, the U.S. National Research Council published Toxicity Testing in the 21st century: A Vision and a Strategy [1,2], marking the start of a paradigm shift in toxicity testing from observational studies in vertebrate animals towards high-throughput predictive approaches incorporating mechanistic information from *in vitro* studies [3,4]. This shift has in part been driven by the growing backlog of chemicals requiring safety assessments and the recognition that animal testing is slow, costly and ethically inappropriate [5,6]. In consequence, novel higher throughput, higher information-content approaches are needed in regulatory toxicology to better assess the potential hazards of industrial chemicals and biocides [7,8]. Omics technologies, including transcriptomics, proteomics, metabolomics and lipidomics, have been highlighted as promising tools for this purpose, most recently by regulatory bodies [9–11]. The strength of omics technologies lies in their ability to provide mechanistic information to help identify pathways of toxicity [5]. Ultimately, the discovery and incorporation of molecular mechanistic information into regulatory toxicology, while not a requirement for setting hazard thresholds, could have multiple benefits: improved chemical grouping (based on similarities of molecular responses to chemicals), accelerated use of pathway-based approaches (e.g. adverse outcome pathways) and reduced costs [5,12]. Importantly, it could also help reduce vertebrate animal testing because mechanistic information could in part be derived through *in vitro* testing and extrapolated to humans through modelling approaches [13].

During workshops organised by the European Chemicals Agency in April 2016 [10] and European Food Safety Authority in April 2018 [9], attention was drawn to the contributions that metabolomics has and could increasingly make to regulatory toxicology. Metabolomics refers to the application of analytical and bioinformatic tools to enable an untargeted characterisation of the low-molecular-weight biochemicals (typically <1500 Da) that are present in biological fluids, cells and/or tissues [14,15]. Undoubtedly, a growing number of researchers in academia and industry are employing this omic technology to investigate the effects of chemicals in a variety of species [16–20], but its uptake into regulatory toxicology is still minimal compared with the application of transcriptomics (or gene expression profiling). This review addresses the question as to how effective metabolomics is for the challenges associated with toxicity testing in the twenty-first century, with a focus on metabolite identification. Other factors to improve its utility to discover molecular mechanisms, but beyond the scope of this article, include the need to improve analytical sensitivity and throughput, as well as to study time courses of metabolic perturbations induced by chemicals. One of the principal motivations for its use in toxicology is that metabolomics measures — with high information content — the most

downstream molecular phenotype of a cell or organism, that is, metabolomics provides a molecular readout that can be most directly associated with more traditional adverse outcomes (or 'apical endpoints') used in regulatory toxicology. Consequently, adverse perturbations in the metabolome (i.e. 'metabolic biomarkers') can in principle be more directly interpreted as functional observations of toxicity, while also providing insights into the mode of action of a chemical [12,21].

Practically, an optimal workflow that uses omics technologies to characterise pathways of toxicity would include an exposure study, omics data generation and analysis, extensive and robust identification of the molecular entities (genes expressed, metabolites perturbed, etc.), pathway enrichment analysis of these molecular perturbations and then using knowledge bases to help associate the perturbed molecular pathways to downstream adverse outcomes such as carcinogenesis, mutagenesis or reproductive dysfunction. Transcriptomics studies in human health, with robust gene identification coupled with extensive systems biology knowledge bases (such as Ingenuity Pathway Analysis® and MetaCore™), are increasingly achieving this optimal workflow [22–24]. This is not yet the case for metabolomics, however, for which definitive metabolite identification of thousands of heterogeneous metabolites remains a significant bottleneck [25,26]. Consequently, the development of new strategies for metabolite identification, including a diverse range of hyphenated analytical platforms using gas chromatography, liquid chromatography, mass spectrometry and/or nuclear magnetic resonance spectroscopy, is an active research field [27,28]. Given the central position of 'robust identification of the molecular entities' in the optimal workflow introduced previously, it should immediately be evident that this represents a potentially serious impediment to the application of metabolomics data to regulatory toxicology, which we explore in the following sections.

Levels of confidence in identification of metabolites and pathways

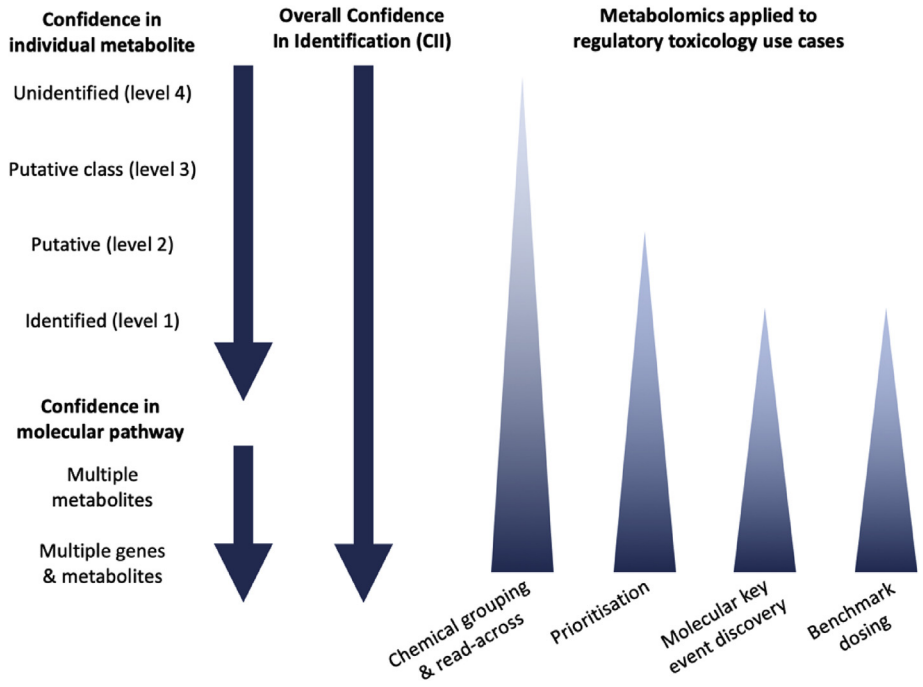
The importance of defining and reporting the confidence in metabolite identification has been widely recognised by the metabolomics community for over a decade, with the international Metabolomics Standards Initiative (MSI) originally proposing 4 levels of confidence (Table 1) [29]. Amendments to these levels of confidence have since been proposed [30–32]. The confidence in identifying pathways of toxicity using metabolomics increases from MSI levels 4 through 1 and could be further increased by identifying several metabolites within the perturbed biochemical pathway (effectively a weight-of-evidence approach) and increased still further by identifying both metabolites and genes in that pathway (Figure 1). Such a multiomics 'triangulation' of both upstream gene expression and downstream function of metabolites and lipids would not only help to substantiate the identities of the molecular entities (i.e. correlated changes in GSH and *GSH synthetase* gene expression) but would provide greater confidence in the importance of a particular biochemical pathway in response to a chemical.

Given this gradient from unknown metabolite to the optimal of known identification of genes and metabolites within a pathway, the question must be asked as to what level of confidence is required for a metabolomics data set to address a specific regulatory need in toxicology. Arguably, even without definitive identification

Table 1 Levels of confidence in metabolite identification as originally defined by the international Metabolomics Standards Initiative [29].

MSI level	Descriptor	Criteria
1	Identified metabolite	Requires that two or more orthogonal properties (e.g. chromatographic retention time, accurate *m/z*, mass fragmentation pattern and/or NMR chemical shift) of the experimentally measured metabolite match those of an authentic chemical standard, both in the same laboratory and using the same method of data acquisition. Documented spectral evidence to validate the metabolite identification should be provided.
2	Putatively annotated metabolite	Experimentally measured metabolite matches characteristic physicochemical properties of a known metabolite and/or has similar spectra to those of a known metabolite within public/commercial libraries and does not require a chemical reference standard. Documented evidence should be provided.
3	Putatively characterised metabolite class	Experimentally measured metabolite matches characteristic physicochemical properties of a known chemical class of metabolites, and/or has similar spectra to those of a known chemical class of metabolites within public/commercial libraries and does not require a chemical reference standard. Documented evidence should be provided.
4	Unknown metabolite	Metabolites that fall outside the thresholds required for levels 1–3 yet can still be differentiated and (relatively) quantified based on spectral data.

Figure 1



Confidence-in-identification (CII) framework spanning the identification of individual metabolites (according to the original Metabolomics Standards Initiative levels 1–4) and molecular pathways. We propose the confidence levels required when metabolomics is applied to several regulatory toxicology use cases.

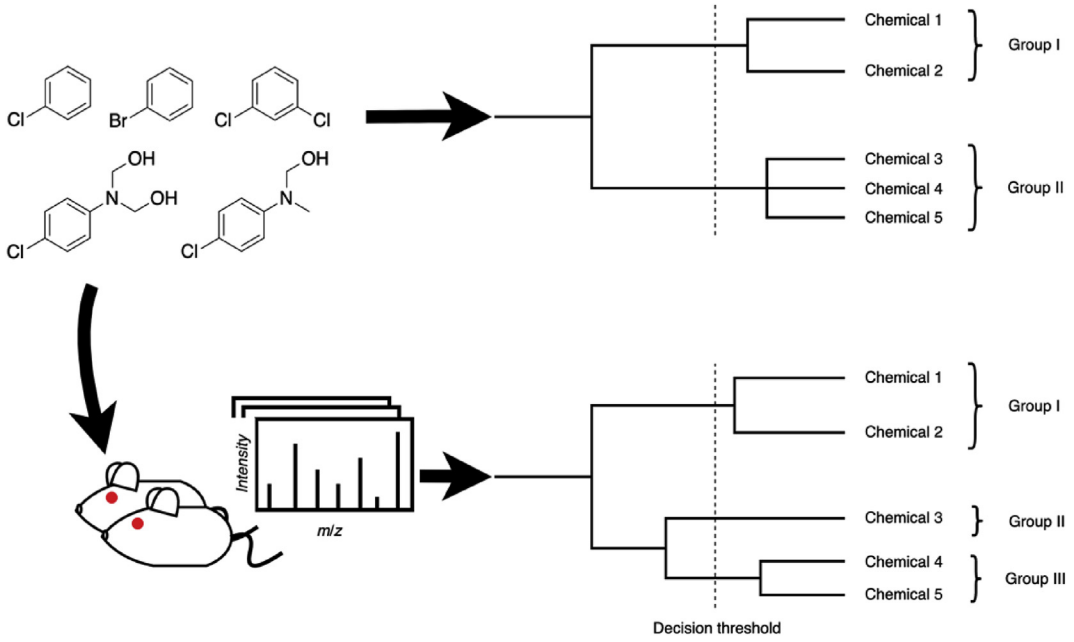
of the molecular entities, omics technologies can contribute (albeit with less confidence) to the discovery of mode(s) of action (MoA) of a chemical, for example, by pattern matching the response to a new chemical to a library of responses to known chemicals (discuss in the following sections). Here, we construct a framework around this confidence-in-identification (CII) axis (Figure 1) that maps the CII to particular regulatory applications. We also map existing metabolomics studies in toxicology to this framework. This exercise highlights where existing metabolomics methods can contribute to regulatory applications now and which applications are reliant on the development of more robust metabolite identification and pathway synthesis tools.

Mapping CII to regulatory applications in toxicology

Regulatory applications within toxicology that may benefit from the application of metabolomics (and metabolic biomarkers) include chemical grouping and read-across, screening (or prioritisation) of chemicals for extensive hazard testing, derivation of benchmark doses and health thresholds and cross-species extrapolation of toxicity pathways (or 'biological read-across'), as recently identified by the MEtabolomics standaRds Initiative in Toxicology (MERIT) consortium [33]. Here, we critically examine these and related use cases in the context of the minimal requirements for metabolite identification, drawing upon the recent literature.

Chemical grouping is based on a notion that chemicals with similar structure and/or physicochemical properties have similar (eco)toxicological properties. Consequently, if a chemical is 'data-poor' (i.e. minimal or no toxicity measurements have been made), it is possible to interpolate or extrapolate toxicity data from structurally similar data-rich substances to fill information gaps; this approach is known as 'chemical read-across' (Figure 2). Even though this approach is widely used, it seems imprudent to ignore the existence of so-called 'activity cliffs' for which the imputed toxicity data are grossly in error and lead to inaccurate safety assessments [34]. It is increasingly acknowledged that grouping of chemicals not only on their structure but also incorporating biological response data could be an effective strategy for chemical read-across [34,35]. Van Ravenzwaay et al. [35,36] have incorporated biological information, derived from metabolomics studies, into chemical grouping and read-across by building a library of metabolic responses to chemicals (BASF MetaMap® Tox). The proprietary library houses the plasma metabolic responses of rats exposed to several hundred individual chemicals, many with known MoA. Metabolite profiles from data-poor chemicals can be screened against this library to determine which library chemicals

Figure 2



Two approaches for forming groups (or categories) from a series of individual chemicals. At the top of the figure, the grouping is based on the similarity of the chemical's molecular structures or other physicochemical properties. At the bottom, biologically based chemical grouping is based on the similarities of the molecular responses to chemical exposure. In the hypothetical example, two groups are formed (dependent on the decision threshold) for structure-based grouping, whereas three groups are formed based on the similarity of the metabolic responses (identified to MSI level 4 or higher). Group membership can then be used to read-across toxicity (e.g. an adverse outcome) from one chemical in the group to all other chemicals.

they most resemble, forming a chemical group for read-across of apical toxicity endpoints. Their approach also offers a possibility to refine and reduce animal testing.

With regards to the CII required for chemical grouping, the initial process used by van Ravenzwaay et al. [35] does not require identification of each metabolite to demonstrate similarities in toxicological responses. Each plasma metabolite profile is compared with metabolic profiles of chemicals in the library in a pairwise manner. However, in subsequent processing, metabolites with high confidence identifications ('anchor metabolites') can be compared to ensure that they change intensity in the same direction in response to the test chemical and library chemical(s). Anchor metabolites are considered essential components of a particular biochemical pathway and MoA. This identifies where on the CII axis this application of metabolomics currently sits, namely a combination of MSI level 4 for profile matching and level 1 to anchor to a MoA. Arguably, if the regulatory need was solely to group chemicals according to their biological responses, MSI level 4 descriptions of the metabolites should suffice. This still requires that the spectral data provide (relative) quantification of the unidentified metabolites, where those feature intensities (i.e. peak intensities) are then used to assess the similarities of the metabolic responses across chemicals (Figure 2). Even though it is not (yet) necessary to identify significantly changed features to group chemicals, reporting the confidence levels of the metabolite identification is essential [33].

While there has been steady progress in applying metabolomics to chemical grouping and read-across, its application to screening, cross-species extrapolation of toxicity pathways and deriving benchmark doses (BMDs) to set health thresholds is only now beginning. In terms of screening applications, while in principle an intensity change in an unidentified feature in a metabolomics spectrum could be used to trigger more extensive toxicity testing, it is unlikely that such a decision (with financial and ethical considerations) would be taken; that is, if a single metabolic biomarker was being used as a screening alert, we argue that an MSI level 1 identification would be needed. However, if a panel of metabolic biomarkers were used as a trigger for additional testing, then a range of confidence levels could be acceptable, although some MSI level 1 identifications would be mandatory. For the application of metabolomics to derive points of departure (PoDs) and consequently dose thresholds for regulation, strict criteria for metabolite identification should be applied. While there are no published examples of applying BMDs to metabolomics data (such studies are underway in the authors' laboratory), there are reports on the application of BMD to transcriptomics [37]. Given

the approach includes using individual genes (and potentially metabolites) to indicate PoDs from baseline biochemistry, robust identification will be essential within a regulatory context. Hence, we argue that individual metabolites for which dose–response relationships are measured and used as indicators of toxicological perturbation should be identified to MSI level 1, or ideally higher, on the CII axis. Indeed, this plays to one of the advantages of using metabolic biomarkers, namely to causally associate these downstream molecular changes with the apical endpoints that are traditionally used in regulatory toxicology.

The discovery of which molecular toxicity pathways are perturbed by a chemical is not a requirement of current risk assessment practices. However, the discovery of molecular pathways and associated biomarkers (or 'key events') promises to fuel the application of molecular assays in regulatory toxicology because these biomarkers could be used for chemical grouping, screening, BMD modelling, etc. We then survey the CII associated with published metabolomics studies that have discovered MoA(s) and putative metabolic biomarkers. Early-response metabolic biomarkers predicting reproductive fitness in *Daphnia magna* were reported by Taylor et al. [12] after exposure to cadmium, 2,4-dinitrophenol and propranolol. The authors discovered a panel of 49 biomarkers specific to reproductive fitness, with the identification of the individual markers spanning MSI levels 1–4; these confidence levels were explicitly reported. Ortiz-Villanueva et al. [38] investigated the effects of three endocrine disrupting chemicals, bisphenol A, perfluorooctane sulfonate and tributyltin, on zebrafish (*Danio rerio*) embryos. They proposed potential biomarkers with "unequivocal metabolite identification" by comparison to online database resources, although according to the MSI, this is only to level 2. Others discovered metabolic biomarkers that predicted the energetic fitness (termed scope for growth) of marine mussels (*Mytilus edulis*) exposed to copper and pentachlorophenol with different MoA and resulting in different metabolic signatures [39]. In this study, a single biomarker predictive of pentachlorophenol-induced decrease in scope for growth was identified at MSI level 1 by comparison with an authentic standard. Considering applications of metabolomics in human toxicology, Cuykx et al. [40] discovered metabolic biomarkers associated with non-alcoholic fatty liver disease induced by sodium valproate (a reference toxicant) in a metabolically competent cell line (HepaRG). Noteworthily, these authors reported the level of identification for each metabolic feature (using an amended scale of 1–5 [31]). Although it is clear that the potential of metabolomics to discover MoAs and/or metabolic biomarkers in (eco)toxicology has been recognised by the academic community, utilisation of this approach in regulatory science will require a more consistent and rigorous approach with respect to metabolite identification and its reporting, including a push towards more level 1 identified biomarkers.

Next steps and recommendations

Extensive level 1 and above (i.e. pathway discovery) metabolite identification is very challenging. Indeed, the requirement to match experimental spectral data to an authentic chemical standard is only achievable if the metabolite standard is available (Table 1). Yet, it is estimated that less than 20% of the metabolome is commercially available as standards, and custom synthesis is a specialism and typically expensive. It is important that regulatory scientists recognise these facts and, at least currently, should not assume that lack of level 1 identification is an indication of complacency. That said, there is much that the metabolomics community can do to increase scores on the CII axis (Figure 1). We must continue to expand spectral databases of authentic chemical standards, including building MS/MS and MS^n libraries of thousands of metabolites, in part to facilitate the development of semi-targeted metabolomics (achieving MSI level 1). Toxicologists have a role to play in prioritising metabolites and metabolic pathways of highest toxicological importance for spectral data generation and potentially *de novo* synthesis of commercially unavailable metabolites. Furthermore, we should build on the philosophy of the international Metabolomics Society's Model Organism Metabolomes task group, which is to focus initially on characterising the metabolomes of a few model organisms to leverage the critical mass of research activity and existing knowledge for such species [28,41]. Again, toxicologists have a role to play in defining the priority model species and systems for metabolome annotation, for example, strain(s) of rat (*Rattus norvegicus*) [42], water flea (*D. magna*) for ecotoxicology, HepaRG and HepG2 as widely used human hepatocyte models, etc. Finally, scientists applying metabolomics to regulatory toxicology must strive towards high confidence in metabolite identification and provide documented spectral evidence to validate the metabolite identifications that they report.

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Conflict of interest statement

Nothing declared.

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* of special interest
** of outstanding interest

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