METABOLOMICS IN CHEMICAL RISK ANALYSIS: A FOOD SAFETY PERSPECTIVE

M. Hernández-Mesa*, B. Le Bizec, G. Dervilly*

LABERCA, Oniris, INRAE, Nantes, 44307, France

* Corresponding authors:
Maykel Hernández-Mesa (maykel.hernandez-mesa@oniris-nantes.fr; laberca@oniris-nantes.fr)
Gaud Dervilly (gaud.dervilly@oniris-nantes.fr; laberca@oniris-nantes.fr)

ABSTRACT

Food safety has become a major issue worldwide and, in particular, detecting the presence of toxins, contaminants or residues of chemical substances along the food chain and *in fine* in foods constitutes a strong consumers demand. In general, all these substances and the corresponding metabolites of interest are analyzed using efficient targeted methodologies. However, in some cases these targeted approaches do not allow the detection of emerging compounds or practices, and therefore new approaches and strategies are required. Thus, the study of physiological perturbations induced by exposure to a given chemical substance has emerged as an interesting alternative approach to apply in chemical food safety.

This review focuses on describing significant applications of metabolomics in the field of risk analysis from a chemical food safety perspective. The different risk assessment steps, including hazard identification, dose-response assessment and exposure assessment, and risk management are addressed through various examples to illustrate that such an approach is fit-for-purpose and meets the expectations and requirements of chemical risk analysis. It can be considered as an innovative tool for predicting the probable occurrence and nature of risks, while addressing the current challenges of chemical risk analysis (e.g. replacement, reduction and refinement (3R) of animal testing, effects of exposure to chemical mixtures at low doses, etc.), and with the aim of responding to global food safety issues and anticipating human health problems.

Keywords: chemical hazards, risk assessment, risk management, biomarkers, mode of action, exposomics

1- INTRODUCTION

Food is one of the main routes of exposure to potentially hazardous chemicals that can enter at many points in the food chain [1]. Chemicals are present in food due to their use to increase efficiency and yield in food production (e.g. pesticides, veterinary drugs), their addition for technological purposes during food processing, transport and storage, as well as to confer specific organoleptic properties to food commodities (i.e. food additives such as emulsifiers, preservatives, sweeteners, colorants, etc.), and their generation during food treatments such as heating or as a consequence of storage conditions (e.g. acrylamide, chloropropanols, nitrosamines, polycyclic aromatic hydrocarbons (PAHs), biogenic amines, natural toxins, etc.). A wide range of anthropogenic chemicals can also end up in food due to contamination from different sources, including materials used in food containers (e.g. phthalates, bisphenols, etc.) and environmental contaminants (e.g. polychlorinated biphenyls (PCBs), per- and polyfluoroalkyl substances (PFAS), flame retardants, etc.). The presence of these chemicals in food represents a risk to human health and is a major concern for a significant part of the population [2], who may look for 'safe food' involving zero risks. Unfortunately, the zero-risk framework is not feasible in most cases [3], and government bodies are continuously adopting measures, including regulations, control plans and creation of competent agencies in the field, to protect consumers' health from the risks associated with chemical, biological and physical hazards [4,5].

In this context, it is necessary to distinguish between 'hazards' and 'risks' because both terms are often used interchangeably, but they refer to different concepts. According to Codex Alimentarius Commission (CAC), a hazard is 'a biological, chemical or physical agent in, or condition of, food with the potential to cause an adverse health effect', while a risk represents the probability of suffering an adverse effect on health and the severity of that effect as a consequence of being exposed to a hazard [6]. Therefore, pesticide and veterinary drug residues, food additives and contaminants occurring along the food supply chain can be considered chemical hazards and the risk they pose to public health depends on the chemical substance, the duration, frequency and level of exposure [2,3]. In the framework of risk assessment, a hazard may also be related to the property of the agent instead of the agent itself since it could represent multiple hazards (e.g. carcinogen, endocrine disruptor, etc.) [7], but the former CAC definition for hazard is applied in this manuscript.

Risk analysis is a process to systematically and transparently collect, analyze and evaluate scientific and non-scientific evidence and/or information about chemical, biological or physical hazards present in food with the aim of selecting the best option to manage that risk based on the various alternatives identified [8]. From a food safety perspective, it supports decision-making and must ensure the perfect balance between minimizing potential risks to human health and the environment and maximizing the benefits to society (e.g. nutritional benefit, physical availability of food, economic and physical access to food, utilization of food, and stability of the food supply) [3]. Risk analysis is constituted by three main components: risk assessment, risk management and risk communication (**Figure 1**). Although they can often be viewed as separate processes, they complement each other and are integrated by risk managers to successfully accomplish risk analysis [6,9]. Risk assessment represents the scientific-based element of risk analysis and provides knowledge about the potential adverse effect of human exposure to food-borne hazards. It consists of the following four steps: (1) hazard identification (i.e. identification of agents which may cause adverse health effects and be present in food); (2) hazard characterization (i.e. qualitative and/or quantitative evaluation of the adverse health effects associated with hazards and involving a dose-response assessment); (3) exposure assessment (i.e. qualitative and/or quantitative evaluation of the likely intake of hazard agents *via* food and other relevant sources of exposure); (4) risk characterization (i.e. integration of previous steps of risk assessment to estimate qualitatively and/or quantitatively, including attendant uncertainties, the probability of occurrence and severity of known or potential adverse health effects of a hazard agent in a (sub)population under defined exposure conditions) [6,7]. Risk assessment feeds into the risk management process to develop policies for the protection of consumer health and the promotion of fair trade practices. Risk management not only takes into account the scientific information provided by risk assessors (i.e. risk characterization), but economic, social, cultural and ethical factors are also weighed to select appropriate prevention and control options [10]. Finally, risk communication constitutes an interactive exchange of information and opinions about hazards, risks, risk-related factors and risk perceptions, among risk assessors, risk managers, consumers, industry, the academic community and other interested parties, and includes discussion about risk assessment findings and the basis of risk management decisions [6].

In recent years, ‘omics techniques (i.e. genomics, transcriptomics, proteomics, and metabolomics) have emerged as plausible approaches for conducting risk analysis and addressing its current challenges, specifically those related to risk assessment [11,12]. In general, transcriptomics has been the ‘omics technology most

frequently used in risk assessment studies [13], but there is a growing interest in other tools such as metabolomics and proteomics for the toxicological and epidemiological evaluation of hazardous chemicals. Although metabolomics has been widely applied in various scientific fields in the last two decades, it is still considered an emerging approach in the area of risk analysis. This review presents an overview of the current needs and challenges of risk analysis for food safety and how metabolomics can be a powerful tool to address them. This is not a comprehensive overview of all metabolomics applications in the field, but relevant applications have been selected to illustrate how metabolomics can be applied in the different steps of the risk analysis workflow, specifically in risk assessment but also including the perspective of risk management. The current state of the art of analytical platforms in metabolomics is also covered, since the development and implementation of 'omics approaches in different fields of application, including risk analysis, is closely related to advances in analytical techniques and tools.

2- CURRENT CHALLENGES IN CHEMICAL RISK ANALYSIS FOR FOOD SAFETY

Global demand for food and international food trade are expected to double in the coming decades, leading to an increase in foodborne diseases with a negative impact on consumers' health and also on the economy, trade and industries of affected countries [14]. It is of great interest to consumers, food producers and suppliers, and governments to ensure food security and safety. This is not always an easy task because the food sector is constantly evolving. Food production and supply chain must feed a growing population but ensuring the sustainability of the environment, new food technologies and products appear in the market (e.g. insects consumption), consumer demands change over time (e.g. related to population aging, interest in bio-based products, etc.), new scientific evidence warns about emerging hazards and risks (e.g. ciguatoxins, chlorinated paraffins, mineral oils, etc.); therefore, food safety standards, including control measures, need to be continuously developed and updated. Consequently, risk analysis is a continuous and dynamic process that must respond to new challenges in food safety.

Risk analysis is a complex and laborious task due to the large number of chemicals that can be present in food. The European Union (EU) has regulated up to 8,000 chemical substances in food, but the actual number of chemical hazards that can compromise food safety is unknown, as more than 100,000 chemicals have been reported in man-made products [15]. Furthermore, of the approximately 30,000 chemicals used commercially in

the United States (US), less than 5% of substances have a risk assessment published in the Integrated Risk Information System (IRIS) database of the US Environmental Protection Agency (EPA), or have been evaluated by the Office of Pesticides Programs (OPP) [16]. Risk analysis faces the following priorities, challenges and constraints within the current food safety framework.

2.1 Unravelling the mode of action and the adverse outcome pathway of chemicals

There is current shift in the way chemical risk analysis is carried out, from the investigation of apical endpoints (i.e. empirically verifiable outcomes of exposure such as carcinogenicity, mutagenicity, hepatotoxicity, endocrine disruption, developmental toxicity, etc.) towards understanding the mechanistic action of chemicals. The 'mode of action (MoA)' and the 'adverse outcome pathway (AOP)' are relatively new concepts in the field of risk analysis that represent pragmatic simplifications of complex biological pathways to carry out risk assessment [17]. Both MoA and AOP refer to the mechanistic processes that occur at different levels of biological organization as a consequence of exposure to chemical hazards; however, there are subtle differences between these concepts. The MoA of chemicals is the biologically plausible sequence of key events in an organism that follows the interaction of the compound with biological targets and leads to an observed effect. It does not imply complete understanding of mechanism of action at the molecular level, but it is commonly supported by robust experimental observations and mechanistic data [23]. Key events are empirically observable steps, or their biological markers, that constitute the necessary elements of the MoA. They must be measurable and reproducible and are connected to each other through key event relationships. Unlike MoA, which takes into account the toxicokinetics of chemicals (i.e. kinetic processes of absorption, distribution, metabolism and excretion of a chemical hazard in an organism), AOP does not consider the metabolism and only focuses on the toxicodynamics (i.e. the mechanisms by which a chemical hazard concentration at the action site causes adverse effects on target tissue(s), organ(s), or the organism) [18]. The AOP is the biological cascade of key events resulting from the exposure of an individual or population. It is triggered by a molecular initiating event (MIE), in which the chemical hazard interacts with the biological target(s) to cause the perturbation, and produces measurable adverse outcomes [12,17]. Although conceptually the MoA includes the AOP, the elucidation of both mechanisms/pathways provides relevant and additional information for the risk assessment of chemicals. MoA analysis provides insight into the dose- and time-dependency of the key events that relates the initial interaction

with a chemical to a specific toxic effect [18,19], while the AOP is aimed at identifying the MIE and the early key events of an adverse outcome regardless of the chemical stressor [20]. Knowledge of the MoA and AOP of toxicologically important chemicals is expected to contribute to establishing exposure thresholds for adverse effects and to identifying vulnerable population groups.

2.2 Adverse effects caused by real exposure scenarios

In traditional toxicology studies, hazard characterization is generally based on the no-observed-adverse-effect level (NOAEL) approach (or more recently also on the benchmark dose (BMD) approach), which represents the highest dose tested in dose-response experiments without significantly causing any effect. The NOAEL approach, which depends critically on the sensitivity of the toxicity test, assumes that an exposure to greater doses of chemicals is associated with an increase in the body's response and that there is no adverse effect below a certain level of exposition (NOEL). This monotonic dose-response assumption has recently been questioned for endocrine disrupting compounds (EDCs) which may show non-monotonic dose-response curves and, consequently, cause adverse effects to health at very low doses [21]. Furthermore, traditional chemical risk assessment applies the chemical-by-chemical approach (i.e. toxicity studies for each chemical separately) and involves animal toxicological studies where a relatively high dosage is needed to observe toxicological effects. These conditions do not represent the chemical risk of the actual exposure scenario. In real-life scenarios, the population is subjected to long-term exposures of low-dose chemical mixtures with different mechanisms of action, which may show non-monotonic dose responses and be liable to bioaccumulate [22].

Exposure to chemical mixtures has become a major concern because humans are continuously exposed to multiple hazards from different sources [12,23]. The effect of chemical mixtures, the so-called 'cocktail' effect, may occur although the same chemical substances do not show any effect when individually present at the same concentration level [22,24]. A 'cocktail' effect can also be more complex than a simple additive effect, which is a widely applied approach in risk assessment, but which may underestimate or overestimate the risk posed by the mixture because of interactive effects (i.e. potentiation, synergism and antagonism) can also take place [25,26]. Chemical legislation is primarily based on assessments conducted for individual chemical substances, so it may not be protective enough in the event of exposure to multiple chemicals with the same toxic effect. Co-exposures

to chemicals regulated by different pieces of legislation are totally neglected, even though some compounds show cumulative and synergistic effects [27].

2.3 Replacement, reduction and refinement (3R) of animal testing

Hazard characterization using the NOAEL approach is generally performed on animal models. The results are ultimately transferred to humans applying a safety factor to cover the variability in extrapolation between species and the entire population including sensitive groups [6]. However, animal experimentation raises ethical and economic concerns [28], whereas it is not guaranteed that animal models are always valid for predicting the toxicity of chemical hazards in humans, since different toxicokinetics and toxicodynamics may occur in different species [29,30]. Further investigation in human and animal toxicokinetics, and also in toxicodynamics, is recommended to reveal differences in dose-response between individuals and species. Toxicokinetics and toxicodynamics data also gives a new perspective to toxicology studies because, instead of relying on manifestation of toxic effects or apical endpoints, it proves sufficient exposure and provides information on how the effect is induced [i.e. MoA/AOP at different levels of biological organisation (organism, organ, cellular and molecular level)]. These earlier and more fundamental indications of human health problems allow for a more accurate estimate of risk [31,32].

2.4 Need for holistic exposure assessment

Epidemiological studies have traditionally focused on the precise measurement of single or few environmental exposures with adverse health effects. However, the targeted analysis of few exposures as typically carried out in human biomonitoring (HBM) plans is a simplification of real exposure scenarios. The effects on human health of co-exposure to different chemical hazards can go unnoticed, especially if they occur separately over time but undergo bioaccumulation. Therefore, a better understanding of relevant exposure scenarios is essential, including the nature of the active chemicals and their number [33]. In addition, traditional biomonitoring do not take continuous measures, thereby limiting detection of short-lived chemicals as well as suspected chemicals of concern are less likely to be detected [34]. An improvement of exposure assessment approaches is needed to prioritize chemicals for hazard assessment.

2.5 Identification of emerging chemical risks

European Food Safety Authority (EFSA) defines an emerging risk as 'a risk resulting from a newly identified hazard to which a significant exposure may occur, or from an unexpected new or increased significant exposure and/or susceptibility to a known hazard' [35]. Emerging risks may be associated with traditional concerns (e.g. pesticides) but related to new discoveries (e.g. pesticide transformation products) [36,37], emerging concerns such as EDCs, microplastics, etc. [31,38], new food products and technologies (e.g. nanomaterials, including food contact materials) [39], new food consumption habits (e.g. organic, vegan, alternative proteins such as insects, etc.), or other factors such as climate change (e.g. increase of mycotoxin contamination [40]), globalized food trade, or even derived from the global movement of marine transport and intensive tourism to (sub)tropical areas (e.g. appearance of ciguatoxins in Canary Islands [41]).

2.6 Harmonization in risk analysis

The harmonization of food safety and quality standards, which has a high impact on risk management, is one of the main challenges for food control authorities [6]. In the current context of globalized trading environment, local food safety problems can jeopardize public health at international level, as occurred with the bovine spongiform encephalopathy (BSE) outbreak (UK, 1998) and the melamine incident (China, 2008) [42]. The adoption of internationally agreed standards may also favor food exports and provide a solution to trade barriers related, *inter alia*, to the EU ban on growth-promoting hormones in meat production while their use is authorized in US or Canada, or to different maximum residues limits (MLRs) of pesticides in food established by each country [43,44].

2.7 Complying with consumer's demands

On basis of the above, risk analysis faces a challenging panorama to properly carry out risk assessment and management of chemical hazards in food, which is even more complex if it is taken under consideration consumer demands. Consumers are more concerned than ever about food safety, with a part of the population developing a fear to chemicals, namely 'chemophobia'; although this feeling can also be the result of the biased perception that 'natural is better' and 'chemicals are harmful' [2,45]. The gap between the general public perception and expert opinion on chemical hazards and risks may be due to a compendium of factors, such as consumer subjectivity, incompressible or confusing scientific terminology for non-experts, or even contradictory

messages from risk assessors and risk managers [46]. Beyond health promotion, it is also a challenge of risk analysis, trough risk communication, to assure consumers' confidence in the safety of food supply [6].

3- 'OMICS TECHNOLOGIES FOR BIOMARKERS DISCOVERY IN RISK ANALYSIS

Although recent, 'omics technologies have already demonstrated a valuable contribution at all levels of risk assessment; however they are more established in the hazard identification and characterization steps [47,48]. Unlike traditional methods based on dose-response approaches in which perturbations of a particular biological or clinical parameter are measured (e.g. hormones changes), 'omics studies generally involve non-targeted analysis and are not hypothesis-driven; therefore, they provide a holistic overview of the effects caused by chemical exposures. In general, 'omics approaches give insight into MoA-related biochemical alterations and AOP of chemical hazards at the level of deoxyribonucleic acid (DNA)/ribonucleic acid (RNA) (transcriptomics), proteins (proteomics) and the metabolome (metabolomics). These perturbations are related to biomarkers that are objective indicators of the organism state and can be measured accurately and reproducibly. They can be classified as exposure biomarkers (i.e. exogenous substances, their metabolites or interaction measures which reflect an exposure), effect biomarkers (i.e. endogenous substances whose changes in the concentration levels are linked to an early health effect) and susceptibility biomarkers (i.e. inherent or acquired abilities of an organism to respond to a specific exposure) [49,50]. Although exposure biomarkers can be detected, the main objective of 'omics studies in risk analysis is mainly focused on the identification of effect biomarkers that may later be associated with a chemical exposure to understand its possible adverse impact on human health. Furthermore, biomarkers related to dose-response modeling can be identified on both humans and animals, showing interspecies differences and their relevance in humans. In this sense, *in vitro* models can also be applied in 'omics studies to investigate the AOP of chemical hazards, moving towards predictive modeling and reducing animal experimentation [51], which is fully in line with the resolution of some of the current challenges of risk analysis listed above.

The identification of biomarkers of effect in the metabolome is highly relevant because it reflects all the information of expression and modulation processes occurring upstream (i.e. in the proteome, transcriptome and genome), thus providing a highly integrated profile of the biological status (**Figure 2**) [52]. The metabolome is the biological layer closest to the phenotype and the exposure environment where adverse perturbations are directly

related to functional observations of toxicity or the adaptation of an organism, while allowing the identification of the MoA of chemicals [53,54]. The metabolome covers all the molecular changes, formed by a large network of metabolites and metabolic reactions, where outputs from one biochemical reaction are inputs to other reactions [55]. Metabolome perturbations are the last changes that occur in an organism before classical toxicological effects manifest.

Metabolomics is the profiling and fingerprinting of small molecules or metabolites involved in the metabolome, with low or medium molecular weight (30-1500 Da approximately), of endogenous origin present in biological fluids, cells, tissues or organisms, and which can also include the detection of exogenous compounds [53,56]. It provides the most complete information on the responses in the organism due to chemical exposures because metabolites cover a significant part of the internal chemical milieu of the organisms, and the variation of their concentration reflects dynamic and rapid changes in the phenotype of the system in study. The investigation of metabolites as biomarkers represents a novel approach to support the biological plausibility of chemicals in risk assessment because, in contrast to genes and genetic risk scores used to predict what might happen, metabolites describe what is happening in the biological system [51,53,57]. Ultimately, up- or down-regulated concentration levels of certain metabolites can inform on biological pathways disruptions, providing a link between chemical exposures with adverse health effects and disease development [58,59].

It is still unclear whether the biomarkers identified in 'omics studies provide more useful information on toxicity than traditional toxicological endpoints, taking into account that the sensitivity of methodologies employed in 'omics studies may lead to the identification of biomarkers without any biological or toxicological relevance [48,51]. Although biomarkers detected in toxicological or epidemiological studies may be associated with adverse effects arising from chemical exposure, they may simply represent a physiological adaptation of the organism without any impact at the health level. In this context, the application of 'omics technologies in risk analysis is in its early years and 'omics workflows can still result cumbersome and complicated, requiring advanced molecular and analytical techniques, highly specialized staff, and sophisticated bioinformatics tools to analyze large datasets. 'Omics studies must also be well-designed and restricted to well-known reference substances to be able to correlate the large datasets with standardized endpoints (e.g. clinical chemistry, histophathological endpoints).

4- ANALYTICAL APPROACHES IN METABOLOMICS FOR THE DISCOVERY OF BIOMARKERS

Advances in metabolomics have been closely associated with improvements in analytical techniques and the development of bioinformatics tools, including databases, for data processing and metabolite annotation or identification, and which currently remains the main bottleneck of metabolomics [52,60,61,62]. From an analytical perspective, metabolomics studies have traditionally been classified mainly into targeted and non-targeted methods; although semi-targeted metabolomics, also referred as suspect screening, has emerged in recent years as a third metabolomics approach (**Figure 3**).

Targeted metabolomics involves the analysis of a small specific group of compounds that are associated with hypothesis-driven studies. The entire analytical workflow including sample preparation is optimized to enhance the detection of specific metabolites, providing high analytical specificity and quantitative reproducibility. Targeted methods are limited to the analysis of a few dozens of compounds, thereby reducing the information obtained on the metabolome. In contrast, non-targeted metabolomics pursues the broad characterization of the metabolome to reveal unexpected changes in metabolites concentration due, for example, to exposure to chemical hazards, and to associate them with biochemical perturbations in metabolic pathways. As a result, analytical conditions in non-targeted metabolomics are very generic to extract a wide range of compounds of different chemical classes from biological samples and detect them without any bias against certain classes of metabolites (i.e. metabolic fingerprinting), thus leading to the determination of hundreds to thousands of compounds [56]. Non-targeted approaches are applied for broad coverage of the metabolome, but data processing can be very tedious and time-consuming due to the extensive datasets generated. Furthermore, the identification of relevant biomarkers is not always accomplished. The combination of advanced bioinformatics tools for feature (i.e. each analytical signal) detection, peak alignment, etc., and chemometrics (mainly multivariate analysis) has favored the implementation of non-targeted metabolomics [60]. Nevertheless, considerable progress still needs to be made in the development and improvement of software and databases that allow the identification of a greater number of metabolites with great certainty [61]. Finally, semi-targeted metabolomics is considered an alternative strategy that falls between targeted and non-targeted approaches. This hypothesis-driven approach aims to quantify hundreds of metabolites whose identity is known or suspected (‘known-unknowns’) before data acquisition and is

intended for the analysis of metabolites belonging to the same chemical family or metabolic pathway (i.e. metabolic profiling) [62].

Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are the leading analytical platforms in metabolomics. Metabolomics studies based on NMR spectroscopy usually involve one-dimensional (1D) ^{1}H (proton) NMR, although other NMR spectra such ^{13}C, ^{15}N and ^{31}P as well as two-dimensional (2D) approaches [e.g. ^{1}H-^{1}H COSY (correlated spectroscopy), ^{1}H–^{1}H TOCSY (total correlation spectroscopy), and ^{1}H–^{13}C HSQC (heteronuclear single-quantum correlation)] can also be used [63]. NMR offers several advantages because it is a non-destructive technique, allowing the analysis of living samples. It is not restricted to the analysis of biofluids, and solid or semi-solid samples (e.g. intact organs) can be studied by NMR. It also provides a high level of experimental reproducibility and allows the quantification of metabolite levels [64]. Nevertheless, the low sensitivity attributed to NMR spectroscopy compared to MS, which limits its application to the determination of major metabolites, has made MS the gold-standard technology of metabolomics studies.

MS improvements in terms of sensitivity, acquisition speed and, specially, resolution and accuracy, have contributed to the rapid development and implementation of metabolomics [65,66]. High resolution mass spectrometers such as time-of-flight (ToF) and Orbitrap mass analyzers with electrospray ionization (ESI) are the most widely used MS technologies in metabolomics. Fourier transform mass spectrometers provide the highest resolution power and mass accuracy, but their implementation in routine laboratories has been limited by the high investment cost required. On the other hand, ionization sources such atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) have been used as alternative to ESI, but to a lesser extent [67,68]. Lately, there is a growing interest in direct MS metabolomics analysis, and especially in matrix-assisted laser desorption/ionization (MALDI)-MS imaging approaches, but despite the high sample throughput of direct MS, its applicability is hampered by ion suppression. Consequently, low signal sensitivity is observed and metabolites are only detected if they show high ionization and/or are at high concentration levels. Moreover, the differentiation of isomers and isobars cannot be achieved in direct MS analysis, which is one of its main drawbacks [69,70]. Therefore, MS platforms are generally coupled with chromatographic or other related techniques to extend the coverage of the metabolome. Reversed-phase liquid chromatography (RPLC) coupled to MS is the technique of choice for metabolomics studies, although the application of complementary approaches (e.g. hydrophilic interaction liquid chromatography (HILIC)-MS for the analysis of highly polar

metabolites) is recommended to obtain more complete information on the metabolome. Gas chromatography (GC), supercritical fluid chromatography (SFC) and capillary electrophoresis (CE) coupled to MS are also mature techniques in metabolomics and can be used as complementary approaches to liquid chromatography (LC)-MS [71,72].

From a technical point of view, recent advances in MS-based metabolomics includes: (1) hyphenation of mass analyzers; (2) 2D chromatography separations; (3) ion mobility spectrometry (IMS)-MS hyphenation; (4) ambient mass spectrometry (AMS) and MS imaging. Hybrid MS instruments (e.g. quadrupole (Q)-ToF, Q-Orbitrap, linear ion trap (LTQ)-Orbitrap, etc.) have increased the possibilities for fragmentation studies (from MS^2 to MS^n experiments) and data acquisition (i.e. data dependent acquisition (DDA) and data independent acquisition (DIA) modes), thus providing more information on metabolite structure to enhance feature annotation and reduce the identification of false negatives [66,73]. On the other hand, biological samples are very complex and co-elution of metabolites can make their identification difficult or almost impossible, especially in the case of isobaric and isomeric compounds. The assignation of *m/z* signals to specific metabolites can be laborious and incorrect due to their co-elution and the complexity of mass spectra, which is even more complex in the case of fragmentation experiments. 2D-LC and 2D-GC approaches improve peak resolution, allowing the detection of a greater number of features, enhancing the detection sensitivity of minor metabolites and, consequently, increasing the number of characterized metabolites [74,75]. IMS also introduces an extra separation dimension into LC-MS and GC-MS workflows that especially favors the separation of isobars and isomers. Certain IMS technologies also provide the collision cross section which is new information on ion structure and, in addition to retention indexes (e.g. retention time, migration time, etc.) and mass spectra, contributes to feature annotation and the identification of metabolites [76,77]. In addition, the development of new AMS or direct mass spectrometry approaches, such as direct analysis in real time mass spectrometry (DART-MS) or rapid evaporative ionization mass spectrometry (REIMS), among others [78], as well as recent advances in MS imaging [79] are creating a great expectation within the metabolomics community to carry out high throughput metabolomics studies involving reduced analysis time. First AMS metabolomics applications for food safety have already been reported [80]. Nevertheless, improvements in measurement reproducibility, in combination with software developments to facilitate signal processing, are expected to further implement these approaches as a general tool in metabolomics. Finally, MS imaging and in particular the widely applied MALDI-MS, has traditionally been used for the analysis of high

molecular weight compounds such as proteins. However, new matrices have recently been proposed to extend this technique to the analysis of low molecular weight compounds [81], providing new alternatives for carrying out metabolomics studies and, most importantly, obtaining information on the distribution of relevant metabolic biomarkers in the sample.

It cannot be overlooked that all developments in analytical approaches applied in metabolomics must be associated with advances in bioinformatics and chemometrics for the processing, interpretation and storage of the large datasets generated in metabolomics studies. As discussed in more detail in Section 6, this is one of the main challenges of risk management in the context of risk analysis. Within the framework of metabolomics data acquired by different platforms mentioned above, it is not only necessary to develop and apply powerful multivariate tools to analyze and obtain relevant information from large metabolomics datasets. It is also required to adequately merge the information from different sources to avoid redundant information that can lead to an incorrect interpretation of the data due to overrepresentation, and at the same time that complementary information can be obtained to improve data interpretation and facilitate the biological explanation arisen by the metabolomics study [82].

5- METABOLOMICS IN CHEMICAL RISK ASSESSMENT

Traditional *in vivo* methods applied in toxicological risk assessment raise ethical and economic concerns. For this reason, new approach methodologies (NAMs) such as *in vitro* testing using human or animal cells, tissues or organs and *in silico* studies are currently promoted to comply with the 3R principles [28]. Metabolomics and other 'omics approaches applied to *in vivo* and *in vitro* models, as well as *in silico* tools (e.g. (quantitative) structure–activity relationships (QSARs), structural alerts, read-across, etc.), have recently been developed to investigate the toxicokinetics and toxicodynamics of chemicals and, consequently, to understand the biological mechanisms of their toxicity [51]. These new strategies provide a deeper understanding of the adverse effects caused by chemical hazards in real-life scenarios and beyond the toxic effects observed in animals exposed to inappropriate high doses within the current risk assessment framework, and at the same time that the use of animals in toxicological research is reduced [10,19,31]. Quantitative predictions of *in vivo* kinetics using non-animal data is viewed as a great opportunity to reduce uncertainty in human risk assessments and a real alternative to animal testing [30].

'Omics tools are also called to unravel the MoA/APO of chemicals as a new strategy to address the aforementioned challenges of risk assessment (e.g. providing knowledge on the adverse effects of exposure to chemical mixtures at low doses) [83,84]. Understanding the MoA of chemicals, typically applying *in vitro* approaches, contribute to the classification of chemical mixtures for better risk assessment to predict their adverse effects [83]. *In vitro* metabolomics is also useful to identify the MIE related to the APO of chemicals, which can be applied as prioritization tool to select chemicals that require *in vivo* testing to validate their toxic effects [18,20]. *In vitro* studies to elucidate the MoA/APO of chemicals not only reduce animal testing, but also overcome the drawbacks related to the transfer of results between species. Animal-based data may fail to predict toxicity for complex human endpoints, providing a poor predictability rate (60-70%) [85]. At least as a first approach, risk assessment can be carried on human cells or tissues.

Despite being a new methodology in risk analysis, metabolomics has already been shown to provide relevant information in chemical risk assessment, especially in hazard identification and hazard characterization [13]. Moreover, metabolomics plays an important role in the new trend in exposure assessment, which is directed towards a more holistic concept of exposure [34]. This section does not cover risk characterization, as its mission is to integrate information from hazard characterization and exposure assessment into appropriate advice for use in decision-making or risk management [86], and metabolomics is not yet directly involved in it.

5.1 Hazard identification

Hazard identification is the first step to be taken in the risk analysis process when raising a potential food safety concern. It is intended to provide evidence on chemical hazards capable of causing adverse health effects and that may be present in a particular food or group of foods [6]. Hazard identification typically applies the weight-of-evidence approach, in which 'all the evidence considered relevant for risk assessment are evaluated and weighted', and involves data from different lines of evidence, such as epidemiological studies, animal-based toxicology, *in vitro* tests and information generated by *in silico* methodologies [87]. *In vivo*, *in vitro* and *in silico* observations support the identification of adverse health outcomes, as well as their nature, since knowledge of both aspects is required to initiate risk assessment. A chemical can only be considered a hazard when adverse effects resulting from its exposure are identified, so hazard identification studies generally focus on unraveling the nature of any adverse effect and consider biomarkers of effect as the endpoints to be used for this purpose [88].

In this context, metabolomics provides relevant information for hazard identification because it can allow the detection of chemical hazards *per se* (i.e. exposure biomarkers) when the workflow is applied in an exposomics framework, but above all, it allows to highlight the perturbations or effects that chemical hazards produce in the organism (i.e. effect biomarkers). Furthermore, the combination of metabolomics with *in vitro* and *in silico* approaches is beginning to attract the attention of researchers for chemical risk assessment in food with the aim of achieving the objectives established by the 3R principles [51]. Metabolomics is playing an important role in the shift from animal data to *in vitro* data-based models and more accurate *in silico* approaches [13].

Given the strict definition of hazard identification, the identification of chemicals with potential hazardous properties in routine food analysis cannot be included as a part of hazard identification step. However, the application to food analysis of analytical workflows and data processing tools traditionally applied in metabolomics can lead to the identification of exposure biomarkers of unknown substances, allowing the early detection of emerging risks that may require risk analysis [89]. For example, a non-targeted LC-MS approach has revealed the presence of dioctyl phthalate (DEHP), which is a widely used plasticizer, in treated/recirculated drinking water samples at concentration levels five times greater than in raw water samples [90]. This fact indicated a possible addition of this chemical to the drinking water during its treatment process. Therefore, metabolomics-based workflows and specifically non-targeted methods can be applied to detect unexpected food contaminants, including new chemical formulations, by-products or transformation products which may be more toxic than the parent species [91,92]. One of the advantages of analyzing food and other exposure sources such environmental samples (e.g. water) using suspect screening and/or non-targeted metabolomics approaches is that they allow retrospective analysis of the data. It brings the possibility of exploiting historical data to identify chemicals of emerging concern (CEC) which potentially represent a hazard to human health, but are not typically included in monitoring programs (e.g. industrial chemicals such as bisphenol S (BPS) or surfactants such as polyethylene glycol) [93].

Some illustrative examples of the application of metabolomics in different lines of evidence for hazard identification are described below. In general, the studies listed refer directly to an environmental exposure since metabolomics for risk analysis in food safety has been applied to a lesser extent. However, the chosen examples are equally valid in the field of food safety, as they assess the effects of chemical hazards that can also be found in food.

5.1.1 Human metabolomics for hazard identification

Although toxicological data based on animal experiments is normally associated with the main line of evidence for hazard identification, in several cases epidemiological data serve as the first observable evidence of the effects caused by exposure to certain chemical hazards. HBM (i.e. measurement of chemical concentrations in human biological samples such as serum or urine) provides useful information to identify chemical hazards or their metabolites, which can also pose risk concerns. Xenobiotics can undergo bioactivation processes in human metabolism, leading to metabolites that can be an equal or even a greater health risk than the original parent compounds [94]. The identification of exposure biomarkers in human biological samples is typically associated with exposure assessment rather than hazard identification, but biomonitoring data represents an option to detect effect biomarkers, carry out hazard identification and, ultimately, be applicable to dose-response assessment in hazard characterization [95]. Exposure biomarkers and effect biomarkers are not generally analyzed simultaneously since the concentration of exogenous chemicals in biological samples is at lower concentration levels than endogenous compounds (~10^{-7}-10^{0} µM *vs.* ~10^{-5}-10^{3} µM) [96], and analytical instrumentation used in metabolomics such as MS does not have a dynamic range capable of covering such a large range of concentrations.

Targeted methods are generally applied to the detection of exposure biomarkers to achieve accurate quantification of chemicals, while both targeted and non-targeted approaches can be used to identify effect biomarkers. The identification of effect biomarkers in targeted methods is hypothesis-driven, so the selection of metabolites to investigate is related to the pathways that can be potentially affected by a specific chemical exposure. As many chemical hazards show endocrine-disrupting properties, targeted metabolomics of the steroid biosynthesis pathway is a possibility to study the effects of these chemicals on the 'steroidome' and, consequently, on human health. As a result, sample preparation and analytical method can be optimized to obtain a more complete picture of changes in the metabolic profile of this class of compounds. For example, in a previous study, Jeanneret *et al.* applied a non-targeted metabolomics approach using an ultra-high pressure liquid chromatography (UHPLC)-Q-ToF-MS method to identify urinary biomarkers in workers undergoing severe occupational exposure to EDCs, specifically dioxins [97]. Based on prior knowledge, data dimension consisting of 3,682 unidentified features was reduced to 284 variables by applying *m/z* filters related to endogenous steroid

sulfates and glucuronides. Finally, 24 steroid-related metabolites were identified as biomarkers of acute dioxin exposure. In a subsequent study, these biomarkers were investigated applying a targeted approach (i.e. selective solid phase extraction (SPE) as sample treatment and selective extraction and integration of targeted *m/z* signals from UHPLC-Q-ToF-MS analysis) to characterize a chronic environmental exposure to dioxins [98]. Exposure to dioxins was confirmed to cause dysregulation of urinary steroids and bile acids in humans.

Targeted methods are powerful tools to investigate certain metabolic pathways in detail but the application of non-targeted metabolomics is generally preferred to identify effect biomarkers. As a non-driven approach, it is intended to cover the entire metabolome without prior hypothesis, providing more comprehensive information on the MoA and consequent adverse effects of chemicals. Although still limited, several epidemiological studies, mainly focused on occupational and environmental exposures, have reported the discovery of effect biomarkers that are relevant in hazard identification through the application of non-targeted metabolomics [99]. Duan *et al.* have found elevated levels of hypoxanthine in young patients with nephrolithiasis as a consequence of exposure to melamine [100]. Melamine is considered a food contaminant that has also been used for milk adulteration and is associated with renal disease. Urine samples from healthy children (n = 74) and from children diagnosed with nephrolithiasis and a history of melamine exposure (n = 40; melamine-induced nephrolithiasis was not directly associated with the consumption of formula milk) were analyzed by UHPLC-Q-ToF-MS, and a partial least-squares discriminant analysis (PLS-DA) was applied for group separation. Up to eleven compounds were identified or putatively annotated as effect biomarkers that differentiated control and melamine-exposed groups (e.g. hypoxanthine, uric acid, proline, etc.). To validate the results, urine samples from young patients with nephrolithiasis and a negative history of melamine exposure (n = 33) were also analyzed. Four of the eleven biomarkers were common in children with nephrolithiasis and seven of them were finally identified as specific biomarkers for melamine-induced renal disease (e.g. hypoxanthine). This intermediate metabolite was identified as being responsible for causing disorders of purine metabolism and, in combination with the action of uric acid, for the formation of stones in the renal tract.

Another illustrative example of a non-targeted metabolomics approach using biomonitoring data has covered the investigation of effect biomarkers associated with exposure to various persistent organic pollutants (POPs), in which the consumption of animal fat is attributed as the main route of exposure [101]. Various PCB congeners (i.e. organic chlorine compounds widely used in electrical equipment in the past and which may currently be

present in food as environmental contaminants), hexachlorobenzene (HCB) and β-hexachlorocyclohexane (β-HCH) (i.e. organochloride pesticides), and p,p′-dichlorodiphenyldichloroethylene (p,p′-DDE), which is the main exposure biomarker of the pesticide dichlorodiphenyltrichloroethane (DDT), were quantified in serum samples applying a targeted GC-MS approach, and different levels of these exposure biomarkers were found in each individual. The individuals who participated in the study were classified into two groups according to their low or high exposure to each of the selected POPs, since almost the entire population is exposed to these chemicals. Subsequently, and for each POP, the serum samples were analyzed by UHPLC-Q-ToF-MS following a non-targeted approach, and both groups were differentiated by means of an orthogonal partial last-squares discriminant analysis (OPLS-DA) (**Figure 4**). In total, 40 compounds were found to be potential effect biomarkers of POPs exposure, but only 10 metabolites were putatively annotated (or presumptively identified). This fact outlines the main drawback of non-targeted metabolomics, which is metabolite annotation or identification. Glycerophospholipids, specifically glycerophosphocholines and glycerophosphoethanolamines, were finally identified as biomarkers of effect of exposure to p,p′-DDE, HCB and PBCs, revealing that these POPs may have a similar MoA. These biomarkers indicated a perturbation of the lipid metabolism and regulation.

5.1.2 Animal *in vivo* metabolomics

Despite epidemiological studies provide evidence for hazard identification, the associated information remains limited due to inadequate characterization of chemical exposure (e.g. the number of analyzed chemical substances may be restricted by the cost, etc.) or the difficulty in controlling confounding factors. In fact, humans are exposed to multiple chemicals presenting similar MoA, so if chemical confounding is not adequately addressed, such confounding can hinder the identification of the effect of a specific chemical [102]. In contrast, exposure to a single chemical or chemical mixture can be controlled in animal-based toxicology; therefore, it remains as the predominant line of evidence for hazard identification. Although still rare, several *in vivo* metabolomics studies have been conducted to characterize the MoA and plausible adverse effects of chemicals, especially in the field of environmental toxicology [103,104,105,106]. From the point of view of human risk assessment, the main limitation of most of these studies is the high exposure concentrations considered in them, which are generally not nearby actual exposures [13]. The potential of metabolomics to perform the risk

assessment of actual exposure scenarios, including low dose exposures, is discussed in the next section (i.e. hazard characterization) in more detail.

Recently, Faeste *et al.* have studied effects of chronic dietary exposure to mycotoxin deoxynivalenol (DON) at the NOAEL level (i.e. 100 µg DON/kg body weight (bw)/day), and which serves as an example of the potential of metabolomics for hazard identification at low exposure levels [107]. C57BL/6J mice (males and females) were chosen as animal model and exposure was carried out for two weeks. Serum and brain samples were analyzed by UHPLC-Q-Orbitrap-MS for the detection of exposure biomarkers (only DON was detected in serum samples) and effect biomarkers. The separation of both control and exposed groups was observed by PLS-DA analysis, but the relevant metabolites were not annotated. Although further investigation is still required to explain the MoA of DON by metabolomics, it is clear that low exposure doses to this food contaminant may cause a perturbation of the metabolic pathways involved in neuronal activity. In this study, it was also indicated that DON doses close to the NOAEL level may be responsible for psychological disorders, as concluded from the monitoring brain activation by c-Fos protein expression and behavioral experiments.

Bearing in mind that current *in vivo* studies involving high exposure levels may provide limited information for hazard identification, the use of metabolomics to identify effect biomarkers and deciphering the MoA of chemicals has increased considerably in recent years. As in traditional toxicology, rodents have generally been selected as animal model to assess the adverse effects of exposure to a wide range of chemicals, including pesticides [108], inorganic contaminants such as arsenic [109], or food contact materials such as BPS [110]. As an example, perturbations of amino acids (i.e. tyrosine, phenylalanine, leucine, isoleucine, tryptophan, and the derivative *N*-acetyl glutamate) and phospholipids (i.e. lysophosphatidyl choline (16:0) (LysoPC (16:0)) and LysoPC (18:0)) levels have been observed in different groups of Sprague–Dawley rats exposed to 0.1 µg toxic equivalence quotient (TEQ)/kg diet of pure 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), Aroclor 1254 (i.e. a mixture of PCBs) and soot dioxins from a hospital incinerator, respectively [111]. Although various biomarkers such as leucine, isoleucine, tyrosine and tryptophan were related to all types of dioxin exposure, the phenylalanine biomarker was characteristic of exposure to Aroclor 1254 and to soot dioxins. It did not represent a biomarker of effect to exposure to pure TCDD, and thus was shown to be relevant in actual exposure scenarios.

In addition to rodents, other animals such as dog and pigs are accepted for risk assessment in the context of human toxicology. Targeted metabolomics has been applied to study the MoA of hydroxylated PCBs (OH-PCBs),

which are PCB phase I metabolites and exert toxic effects, and their adverse outcomes in the brain of dogs (*Canis lupus familiaris*) [112]. Low doses of OH-PCBs suppress 3,5,5'-triiodothyronine (T3)-induced transcriptional activation of thyroid hormone (TH) receptors and can cause disruption of neurodevelopment. Within this framework, brain samples from beagle dogs treated with a mixture of twelve PCB congeners (i.e. one-day administration of the mixture CB18, 28, 70, 77, 99, 101, 118, 138, 153, 180, 187, and 202 at 0.5 mg/kg) were analyzed by CE-ToF-MS. A list of approximately 900 ionic compounds from the major metabolic pathways (i.e. glycolysis, gluconeogenesis, pentose-phosphate pathway, citric acid and urea cycles, purine and pyrimidine metabolism, tricarboxylic acid cycle, and amino-acid and nucleotide metabolism) was selected as metabolites to be targeted. In total, 198 metabolites were detected in brain samples. OPLS-DA analysis unveiled differences for 33 metabolites between control and PCBs-exposed groups, which were identified as effect biomarkers and related to the urea cycle and adenosine triphosphate (ATP) biosynthesis (**Figure 5**). A decrease in ATP levels in dogs exposed to PCBs was associated with the down-regulation of the urea cycle, which can cause hyperammonemia and, consequently, neurologic disorders. Furthermore, 4-OH-CB202 and 4-OH-CB107 were found by GC-MS analysis as the main exposure biomarkers in brain.

Non-targeted metabolomics using UHPLC-ToF-MS and subsequent OPLS-DA analysis has also provided insight into the toxic mechanisms of 5-nitroimidazoles (i.e. metronidazole, dimetridazole, and ronidazole) in pigs (crossbreed large white/landrace) [113]. These antibiotics, especially metronidazole, are used in human medicine but are currently banned by the EU in food producing-animals, either as feed additives or veterinary drugs because these substances pose a risk to consumers' health. A total of 27 up-regulated ions and 15 down-regulated ions were found to be significantly different between control and exposed groups, and 7 of them were finally annotated (i.e. 5-oxoproline, riboflavin, guanosine and four bile acids). These biomarkers demonstrated the hepatotoxicity of 5-nitroimidazoles that was related to perturbations of the bile acid metabolism. It is worth mentioning the discovery of riboflavin as biomarker of exposure to 5-nitroimidazoles because the riboflavin biosynthesis is only possible by plants and some bacteria. However, in this and in previous studies, this effect biomarker has been associated with protein degradation promoted by hepatotoxic chemicals and due to carbolic states.

Furthermore, the zebrafish (*Danio rerio*) has recently emerged as the alternative *in vivo* model, albeit not new and still an animal, to be implemented in this field of research. It is now accepted that this vertebrate non-human

model is useful for understanding developmental toxicity pathways in humans due to the similarity of its metabolism with that of mammals. In addition, the use of the zebrafish model is more appropriate according to 3R principles, as well as involves lower cost and is easier handling than other animal models [114]. Elie *et al.* has evaluated the zebrafish as *in vivo* model in combination with non-targeted metabolomics to study the developmental toxicity of benz[a]anthracene (BAA) and benz[a]anthracene-7,12-dione (BAQ), which are a PAH and an oxygenated-PAH derivative (Oxy-PAH), respectively [115]. Exposure concentration was set at 4 μM in both experiments, and zebrafish larvae were subsequently subjected to HPLC-Q-ToF-MS analysis. Finally, the dataset was examined by both principal component analysis with discriminant analysis (PCA-DA) and PLS-DA. In total, 63 metabolites were identified as relevant to BAA or BAQ exposures, or both of them, showing perturbations of various metabolic pathways (i.e. glutathione metabolism; glycine, serine and threonine metabolism; cysteine and methionine metabolism; purine metabolism; phenylalanine metabolism; phenylalanine, tyrosine and tryptophan metabolism; and aminoacyl-tRNA biosynthesis). Purine metabolism was the pathway most affected by exposures to BAA and BAQ. It was associated with the fact that PHAs induce mitochondrial oxidative damage and reduce ATP levels, so in response, purine catabolism increases to finally restore nucleotide levels, including ATP.

5.1.3 *In vitro* metabolomics

Animal models have traditionally been used in toxicological studies because they are expected to show a similar response to chemical exposure as humans. However, there are several examples in which the toxic effects of chemicals observed in laboratory animals and humans are qualitatively and/or quantitatively different [10]. *In vitro* studies overcome this drawback of species extrapolation, since toxicity assessment can be carried out directly on human cell cultures, and pathways affected *in vitro* by exposure to chemicals are also normally affected *in vivo* [13]. In this regard, physiologically-based pharmacokinetic (PBPK) models correctly address the main challenge of *in vitro* to *in vivo* extrapolation to realistically reflect human physiology and metabolism [51]. PBPK models are mathematical descriptions that cover the organs and tissues of body and their connections by the cardiovascular system, and are applied to explain the toxicokinetics of chemicals [116]. More complex physiologically-based models also allow linking toxicokinetics and toxicodynamics [51].

The human body is made up of a wide range of different cell types, so it is crucial to select the appropriate cell type to perform *in vitro* assays using human-derived cells. The liver is actively involved in the metabolism of xenobiotics and a large percentage of them have shown hepatotoxic properties. Consequently, human liver cells have traditionally been selected to assess toxicity using *in vitro* models [117].HepG2 cell line, which consists of human liver carcinoma cells, and HepaRG cell line, which is an original human hepatoma cell line, have been the most widely used *in vitro* models in the hazard identification and characterization of various chemicals, such as organophosphate and halogenated flame retardants [118,119,120]. HepG2 cell is not recommended for the detection of hepatotoxicity due to the low endogenous expression of cytochromes, but is the model of choice for studying mitochondrial toxicity due to their high content of organelles and mitochondrial DNA (mtDNA). HepaRG cells show a metabolic capacity similar to normal hepatic metabolic function, making it the recommended model in studies of xenobiotic metabolism, hepatotoxicology, and hepatocyte differentiation [117].

Since *in vitro* experiments are more affordable in terms of cost and easy manipulation than *in vivo* testing, *in vitro* studies generally investigate several exposure concentrations; therefore, most articles involving *in vitro* metabolomics for risk assessment found in the literature are classified primarily in the context of hazard characterization rather than hazard identification. The metabolomics study on exposure to nine organophosphate flame retardants in Hep G2 cells represents an example of hazard identification using *in vitro* models [119]. Different cell cultures were exposed to one of the organophosphate flame retardants at a concentration of 10^{-8} M and the aqueous intracellular metabolites were analyzed by ^{1}H NMR. Cells were classified by PLS-DA into three groups distinct from the control group. Cluster 1 encompassed cells exposed to tris(methylphenyl)phosphate (TMPP), tris(2,3-dibromopropyl) phosphate (TDBPP) and tris(phenyl) phosphate (TPHP). Cluster 2 consisted of cells exposed to tris(2-ethylhexyl) phosphate (TEHP), tris(2-butoxyethyl) phosphate (TBOEP), tris(2-chloroisopropyl) phosphate (TCIPP) and tris(chloroethyl)phosphate (TCEP). Finally, cluster 3 was constituted by cells exposed to tri-n-butyl phosphate (TNBP) and tris(1,3-dichloro-2-propyl) phosphate (TDCIPP). Using the "Pathway Analysis module" included in the web-tool MetaboAnalyst [121], all organophosphate flame retardants were shown to alter glutathione biosynthesis and histidine degradation pathways, while compounds classified in specific PLS-DA groups also disrupted γ-aminobutyrate (GABA) shunt and ornithine biosynthesis pathways. Oxidative stress was found as the common effect of exposure to organophosphate flame retardants.

In addition to liver cells, other cell lines can be selected to assess toxicity mechanisms that lead to other adverse effects rather than hepatotoxicity. EDCs like bisphenol A (BPA) and its analogs (e.g. bisphenol F (BPF), BPS, etc.) have been shown to disrupt normal mammary development and even cause cancer. Within this framework, metabolomics has been applied to study the toxic mechanisms of BPS in human breast epithelial MCF-10A cells, which are immortalized and not tumorigenic cells and are widely used as *in vitro* breast model [122]. Cells were exposed to 1 μM of BPS and subsequently studied using an integrated non-targeted metabolomics and proteomics approach. Metabolites were analyzed by LC-Q-Orbitrap-MS, showing that 35 of them were down/up-regulated due to BPS exposure. In combination with the differential expressed proteins (DEPs) found, these biomarkers of effect revealed significant disturbances of tricarboxylic acid (TCA) cycle, purine metabolism, glycosylphosphatidylinositol (GPI)-anchor biosynthesis, pyruvate metabolism and alanine, aspartate and glutamate metabolism, and which were ultimately related to sustaining cell proliferation and cellular signal transduction. Multi-omics approaches such as this example show how a more complete and clear view on the MoA of chemicals can be obtained when combining metabolome and proteome investigation, since both involve closely related biological processes.

5.1.4 *In silico* approaches applying metabolomics

In silico methods have been developed to estimate the toxicity of chemicals in the absence of experimental data and as an attempt to refine and reduce animal testing, while contributing to the rationalization of *in vivo* and *in vitro* studies in the context of weight-of-evidence assessment generally applied in hazard identification [51]. Within *in silico* methods, the application of QSAR approaches is widespread in the field of toxicology. QSAR methodology consists of mathematical models that link the biological/toxicological activity of chemicals with their physicochemical property-based descriptors, electronic and topological descriptors, and/or chemical structure-based descriptors. Models are created with a training set of chemicals of known toxicity and are used to predict the toxicity of test compounds with biological and/or chemical properties similar to those of training compounds (e.g. using matching learning approaches). As a result, chemicals can be grouped into toxicity categories based on their structural similarities. QSAR models are very useful for quickly grouping toxicants based on their physicochemical properties and their correlation within large datasets, but they neglect specificity and complexity of molecular interactions, which introduces significant uncertainty into the effective prediction of toxicity [123].

Chemical similarity does not necessarily imply a toxicological similarity. Thus, better prediction ability and chemical grouping can be achieved by incorporating biological information into QSAR models, extending the application of QSAR models to hazards that belong to different chemical families and leading to quantitative biological activity relationships (QBAR) models as described by Ravenzwaay *et al.* [124]. Biological information can come from metabolomics studies that effectively contribute to grouping chemicals with similar toxicity endpoints according to specific patterns of metabolites up- or down-regulated as result of their MoA. In this sense, the industry has led during the last decade the initiative to demonstrate the added value of metabolomics in toxicity assessment, particularly when *in silico* approaches are used, and with special relevance in the shift of chemical groping concept (from one based on the similarity of chemical structures to one more efficient based on the similarity of metabolic fingerprints) [125]. Metabolomics-based QBAR models have not only demonstrated that chemically similar substances, as expected, are also toxicologically similar (e.g. 2-methyl-4-chlorophenoxyacetic acid (MCPA), which is a phenoxy herbicide, matches structurally and toxicologically with other phenoxy-herbicides [i.e. dichlorprop (2,4-DP), 2,4-dichlorophenoxyacetic acid (2,4-D) and mecoprop (MCPP)]), but have also revealed toxicological dissimilarities of compounds with slight structural differences [124].

Since the predictive ability of QSAR models is limited when applied to chemicals belonging to different classes, their combination with other *in silico* methods (e.g. read-across extrapolations) and other lines of evidence (e.g. *in vivo*, *in vitro* data) is recommended to provide more reliable results for hazard identification [126]. Chemical grouping and subsequent read-across from data-rich chemicals that belong to the same group is considered as the most efficient *in silico* approach to reduce animal testing in risk assessment [124,127]. Read-across extrapolations use relevant data from source substances to predict the toxicity of target compounds that have a structural or toxicological relationship according to a similar plausible MoA [128]. Like QSAR models, read-across can benefit from taking biological data into account rather than relying solely on structural similarities to functionally support a read-across case. In general, read-across approaches are not accepted as weight-of-evidence by, for example, the European Chemicals Agency (ECHA) due to their inherent uncertainty; however, metabolomics data provide sufficient confidence regarding mechanistic similarity to support toxicity assessment applying read-across approaches and to fulfil data gaps on non-experimentally evaluated toxicity endpoints [127,129]. As a first proof of concept, Ravenzwaay *et al.* applied a read-across approach using metabolomics data to assess the toxicity of MCPP and selecting 2,4-DP as the best source substance (based on metabolic

profiling similarities and compared to MCPA) [127]. Metabolomics was performed on blood samples from a 28-day exposure experiment in Wistar rats, and read across predictions were comparable to a 90-day rat toxicity study of MCPP, with the liver and kidney being the target organs for toxicity. Therefore, this latter experimentation can be avoided and replaced by predictions based on read-across in combination with metabolomics data from 28-day studies and information from a 90-day toxicity study of 2,4-DP.

5.2 Hazard characterization

Hazard characterization is conducted within the chemical risk assessment process to establish a relationship between chemical exposure dose levels and observed adverse effects (i.e. dose-response relationship). Dose-response relationships contribute to the estimation by risk assessors of toxicological reference values [e.g. TDI (tolerable daily intake) and acceptable daily intake (ADI)], which is the main outcome of the risk characterization step and subsequently support risk managers' decision-making to set maximum exposure levels for each chemical hazard (e.g. MLRs of pesticides in food). In this context, metabolomics applied in *in vivo* and *in vitro* studies has demonstrated to be a powerful approach to differentiate groups exposed to different concentrations of chemicals, such as PCBs [130,131], flame retardants [120,132,133], BPA [134], PFAs [135], organotin compounds [134], PAHs [136], or pesticides [137,138,139]. *In vitro* metabolomics approaches are of special interest because they allow testing a greater number of dose levels and a larger set of chemicals at lower cost and execution time than *in vivo* studies, and in compliance with the 3R principles [140]. Information resulting from *in vivo* metabolomics has generally been limited by the low number of data points due to ethical concerns and the high cost of animal experimentation, leading to poor quantitative data on dose-response relationships [85]. Two or three dose levels (low, medium and high exposure levels) have often been considered to assess dose-response relationships in *in vivo* studies [132,135,137]. This reduced number of assayed exposure levels may not be sufficient to accurately estimate the risk of chemical hazards, especially at low exposure levels as in the case of hormesis or non-monotonic dose response such as EDCs [19,21]. Another limitation of the *in vivo* metabolomics studies reported so far is that, in general, they have not investigated doses close to actual exposure levels, nor have they provided information on the dose of exposure at which the first adverse effects are induced [13], and what is crucial to understand the developmental toxicity pathway of chemicals. As in traditional toxicological studies, high dosage has been applied to observe toxicity effects. This fact must be taken into account and

changed in future metabolomics investigations to properly integrate the resulting data into the hazard characterization of chemicals [85].

The sensitivity of metabolomics to identify changes caused by chemical exposure in an organism and its ability to demonstrate the absence of an effect are the main strengths of metabolomics to apply this technology in the prediction of effects and the establishment of a no observed effect level or NOEL [127]. Although the increased sensitivity of metabolomics compared to classical NOAEL approaches has been recently questioned [141], it is necessary to differentiate between the identification of sensitive biomarkers of effect and early biomarkers of effect. Sensitive biomarkers refer to visible effects that only occur at higher doses, whereas early biomarkers are related to the first changes in the organism before visible toxicity [13]. Metabolomics has the potential to detect early biomarkers of effect, thus identifying the MIE caused by specific chemical hazards in the organism and accelerating the construction of AOPs [125]. This strength makes metabolomics the ideal technology to investigate the toxicity of chemicals at low doses that correspond to real exposure scenarios.

5.2.1 Metabolomics to address low-dose effects

'Low-dose' effects refer to the reported effects on chemical exposure at concentration levels below the doses used in traditional toxicological studies for risk assessment [142], and which are normally below the currently accepted NOAEL. In this regard, risk assessment should generally address exposure to these low-dose levels because they represent the real exposure scenarios that humans experience. Although still limited, metabolomics studies are interested in unveiling the low-dose effects of chemicals, with a special focus on EDCs and substances with possible endocrine disruption properties, such as BPA or short-chain chlorinated paraffin (SCCP) plasticizers, respectively [143,144]. The low-dose effects related to BPA exposure have been investigated by metabolomics using ^{1}H NMR as analytical tool [143], and it represents one of the few *in vivo* metabolomics approaches that has truly addressed the challenge of low-dose effects in the context of human hazard characterization. Despite the TDI for BPA is currently set at 4 µg/kg bw/day within the EU according to the EFSA, this threshold was set at 50 µg/kg bw/day at the time of the study [142]. Cabato *et al.* exposed fetuses and neonates of CD-1 mice to low doses of BPA (0.025, 0.25, or 25 µg BPA/kg bw/day) by administering BPA to their mothers from day of gestation 8 to day of lactation 16 [143]. PLS-DA showed greater differences between the group affected by perinatal exposure to the 25 µg dose of BPA and the other offspring groups when postnatal day

21 serum samples were analyzed. However, greater differences in brain samples were observed for the group exposed to a 0.025 µg dose of BPA. Glucose, pyruvate, some amino acids, and neurotransmitters (i.e. GABA and glutamate), lipids (i.e. phosphatidylcholine and glycerophosphocholine), among others, were found as effect biomarkers, suggesting a disruption of energy metabolism and brain function, and outlining adverse health outcomes at BPA levels at which exposure was tolerable. The metabolic effects of BPA at low and very low doses (i.e. 10^{-6}, 10^{-9} and 10^{-12} M) has also been demonstrated in *in vitro* tests using the HepG2 cell line [145]. Metabolomics studies were carried out by means of ^{1}H NMR analysis, and ^{1}H-^{1}H COSY and ^{1}H–^{13}C HSQC analysis determined the structure of metabolites of interest. Metabolic sub-networks were generated from the metabolic fingerprints to elucidate the MoA of BPA at low doses. The obtained results confirmed that exposure to low doses of BPA induce perturbations in the energy metabolism, as observed in previous *in vivo* metabolomics study [143], and more specifically in the first steps of the Krebs cycle. The detected biomarkers of effect (i.e. amino acids) also appointed to the possible obesogenic properties of BPA.

Although other *in vivo* metabolomics studies have not adequately addressed low-dose effects as has been done for BPA, they have tended to consider environmental exposure levels as at least the lowest data point in dose-response assessment. For example, hazard characterization of SCCPs has been carried out in Sprague-Dawley rats exposed at different dose levels (i.e. 0, 0.01, 1 and 100 mg/kg bw/day) for 28 days [144], in which 0.01 mg/kg bw/day represented daily human exposure levels to SCCPs and 100 mg/kg bw/day was related to the lowest-observed adverse effect level (LOAEL) reported in rats experiments. Metabolomics analysis by LC-MS of liver samples, and further analysis of the data by PLS-DA, showed a separation of the control and the three-exposed groups (**Figure 6**). Furthermore, the metabolic effect level index (MELI) exhibited a significant dose-dependent increase for SCCPs exposure (**Figure 6B**). Integrated metabolomics and transcriptomics data suggested two major MoAs for SCCPs, namely inhibition of energy metabolism and activation of peroxisome proliferator-activated receptor α (PPARα), which has functions on lipid metabolism and peroxisome proliferation. This study not only showed the plausible hepatotoxicity of SCCPs, but also demonstrated that environmental doses (or low doses) of SCCPs may cause relevant adverse health effects. PPARα activation was clearly observed for the high dose tested, but an acceleration of fatty acid metabolism attributed to the SCCP-induced activation of a small amount of PPARα was also identified for exposure to environmentally relevant doses.

5.2.2 Decoding the effects of chemical mixtures

In a step forward to address actual exposure scenarios, metabolomics studies are also beginning to provide risk analysis information on exposure to chemical mixtures and their possible additive, potentiating, synergistic or even antagonistic effects. Although they have primarily addressed this challenge from a hazard identification perspective (e.g. for mixtures of pesticides [146,147] or mycotoxins [148]), some of these studies have also covered the hazard characterization of chemical mixtures, using either *in vivo* or *in vitro* models [149,150,151,152]. Targeted *in vitro* metabolomics has been applied to carry out the hazard characterization of POPs mixtures, including chlorinated (i.e. PBCs, and organochlorine pesticides), brominated (i.e. hexabromocyclododecane and polybrominated diphenyl ethers), and fluorinated substances (i.e. perfluoroalkyl acids) [150]. Since POPs have endocrine disrupting properties and the adrenal cortex is one of the major target organs affected by their action, the H295R adrenocortical cell line was selected to investigate the effects of different combination of POPs mixtures (e.g. fluorinated; fluorinated and chlorinated; fluorinated, chlorinated, and brominated compounds; etc.) on steroidogenesis. H295R cells stimulated with forskolin to promote steroidogenesis and unstimulated cells were exposed for 48 hours to four different concentrations of POPs mixtures, corresponding to 1, 10, 100 and 1000 times the estimated concentrations in human blood. The combination of brominated and fluorinated mixtures showed the most severe perturbations in the steroidome, while a non-additive effect was observed for the combination of chlorinated and fluorinated mixtures at the highest dose level compared to the single effects of each mixture. This result represents just one of many that challenges the dose addition approach widely applied to predict the toxic effects of chemical mixtures with a similar MoA, and which considers the sum of the toxicities of the individual components equal to the toxicity of the chemical mixture [26]. Furthermore, it also reflects the need for further research on the characterization of the interaction of chemicals at doses related to environmental and dietary exposures, but in the low-dose region of the dose-response curve with data points at or below the NOAEL for individual components of the mixture rather than unrealistic exposure doses [12].

5.2.3 Metabolomics for actual exposure scenarios: effects of low-dose chemical mixtures

The assumption that chemical hazards do not pose a risk at levels below the NOAEL should be reviewed as evidence shows that joint exposure at these low-dose levels can cause adverse effects [33]. NOAELs are

generally established by risk assessment of individual chemicals, and it is assumed that there is no risk for chemical mixtures whose individual components are at exposure levels below these thresholds [26]. Actually, the joint action of chemicals at levels below their NOAEL can lead to additive or interactive effects and endanger human health [153]. Therefore, improving risk assessment seems crucial to cover actual exposure scenarios in which hazard characterization must address long-term low-dose exposure to chemical mixtures. However, simulating actual exposure scenarios raises several questions, such as how chemical mixtures should be selected for risk assessment or what dose levels should be examined to appropriately represent environmental and dietary exposures and obtain useful information for risk assessment. Although this discussion is beyond this review, it is interesting to mention that, in the framework of risk assessment for food safety, TDI or ADI values can be applied as reference to choose data points for dose-response curves of chemical mixtures, as recently proposed by Tsatsakis *et al.* [154]. While the middle dose may be established at the ADI of each chemical in the mixture, the lower doses should investigate the effects of exposure levels currently considered 'safe' (e.g. 0.25×ADI).

Environmental and dietary exposure levels or NOAELs are usually included as the lowest data point of dose-response curves when carrying out human health hazard characterization of chemical mixtures by metabolomics approaches [150,152,153]; therefore, it cannot be considered that the effects of low doses of chemical mixtures have been studied by metabolomics in detail. More importantly, when investigating possible 'cocktail' effects by metabolomics, it should be relevant to include groups exposed to individual chemicals in order to correctly conclude that the metabolome disruption is a consequence of additive or interactive effects of the mixture rather than an observation resulting from increased sensitivity of the metabolomics approach compared to the toxicity test used for the establishment of the individual NOAELs. This consideration is not common in toxicological metabolomics studies that address exposure to chemical mixtures.

Simply as an illustrative example, metabolomics has been applied in the hazard characterization of a mixture of pesticides (i.e. dichlorvos, acephate, dimethoate, and phorate) and the NOAEL corresponding to each substance (2.4, 0.5, 0.04, and 0.05 mg/kg bw/day, respectively) was established as the lowest data point [153]. Other data points included 3 and 9-times the NOAEL of each pesticide as middle and high doses. Wister rats were exposed to the chemical mixture and PCA of urine fingerprints already showed differences between the control and exposed groups after 12 weeks from the beginning of the exposure. The high-dose group was clearly

differentiated from the other groups after 24 weeks of exposure, and only a partial overlap in the PCA was observed for low- and middle-dose groups. In this context, perturbations of various metabolites were detected in the low-exposure group after 24 weeks of treatment compared to the control group ($p < 0.01$, analysis of covariance), including biomarkers of exposure (e.g. dimethylphosphate) and effect (e.g. uric acid, citric acid, cholic acid, etc.). These results associated exposure to low doses of this pesticide mixture with several possible health outcomes such as oxidative stress, impaired lipid metabolism, and interference with the TCA cycle. Evidence like this highlights the need to conduct an adequate risk assessment of chemical mixtures at levels below individual NOAELs to better understand potential 'cocktail' effects, as well as the requirement of legal mandates to carry out further research and move forward on this topic [155].

5.3 Exposure assessment

Exposure assessment is a crucial step in risk assessment because there is no risk without exposure. Therefore, its main objective is to define the agents, sources, and routes of exposure and involves estimating or measuring the levels, frequency and duration of the exposure. Exposure assessment can be performed using direct (i.e. point-of-contact and biological biomonitoring) or indirect (i.e. exposure scenarios estimation) approaches [156], and the determination of specific and sensitive biomarkers of exposure is a method to carry out chemical exposure assessment [102]. Data treatment and analytical tools similar to those used in metabolomics are applied to identify exposure biomarkers, either in exposure sources such as food (i.e. generation of point-of-contact data) [157], or directly in human samples where exposures from all routes are integrated (i.e. HBM data) [158]. Due to the multiple sources and scenarios of exposure, biomonitoring data is the best choice for a proper exposure assessment [159]. HBM, in combination with biomarkers discovered by metabolomics, also contributes to human health risk assessment by providing information on uptake, bioavailability and bioactivation of chemicals in humans. In a risk assessment framework, chemical hazards are generally analyzed in first contact tissues (e.g. lung lining fluid), but there is a current trend to investigate the metabolites of these substances in biological fluids (e.g. urine, blood, etc.) and their interaction with tissues [37]. However, the pharmacokinetics of absorption, metabolism, and excretion, as well as the time between exposure and sample collection, must be known when considering biomarkers of exposure in these biological fluids to accurately measure exposure [156].

Although the exclusive determination of exposure biomarkers in HBM studies goes beyond the purposes of metabolomics studies, the same or similar data processing and analytical tools are used in both cases. In this regard, the current state of the art of HBM for exposure assessment is briefly discussed in this section. It serves as an introduction to the new trend in exposure assessment (i.e. exposomics) that does not only intend to identify chemical exposures, but also to use the resulting epidemiological data to link chemical exposures with adverse health outcomes. This fact implies that the same epidemiological study can respond to both exposure assessment and hazard identification steps (even to hazard characterization step) by means of a different interrogation of the data. Exposomics studies require information obtained from, among others, 'omics approaches including metabolomics in order, through the identification of biomarkers of effect, to be able to link chemical exposures to the development of disease. At the same time, it provides a new approach to carry out exposure assessment through the identification of metabolic fingerprints that can be related to exposure to specific chemical hazards, and in which metabolomics plays a relevant role.

5.3.1 Human biomonitoring

HBM methods are generally intended for the determination of compounds of the same chemical family and typically apply targeted approaches involving LC-QqQ-MS analysis. Targeted biomonitoring provides quantitative information on the internal dose of chemicals and their prevalence in the population [160], ensuring that population is not exposed to concentration levels of chemicals at which bioactivity has been observed in *in vitro* or *in vivo* studies. The main limitation of traditionally targeted biomonitoring programs for exposure assessment is the small number of known substances that are monitored [160], which currently comprises around 250 chemicals [158] and do not cover actual exposure scenarios. As an attempt to improve the current risk assessment scenario, more comprehensive exposure assessments are pursued by implementing suspect screening and non-targeted HBM approaches [161]. These strategies, which are based partially (e.g. sample treatment, analysis, data mining, etc.) or completely on workflows similar to those of metabolomics, provide a more complete fingerprint of chemical exposure to discover new exposure biomarkers.

Suspect screening and non-targeted do not only contribute to the evolution of HBM from targeted analysis of a few chemicals towards more holistic approaches in the context of exposure assessment, but overall they are also impacting exposure and epidemiological studies. In this vein, the study of the exposome using non-targeted

'omics is generating high enthusiasm and expectation among researchers to understand the adverse effects of exposure to multiple chemicals over the entire human lifetime [34,161]. Nevertheless, the ability of these methods to support exposure and health studies beyond the identification of CECs remains to be demonstrated due, for example, to the high false negatives rate [162].

5.3.2 Exposomics

There is a clear shift in the way toxicological studies are conducted to address the current challenges of risk assessment (i.e. from identifying apical endpoints of toxicity to understanding the mechanisms of toxicity), and that it is also impacting epidemiological research. Exposure studies are evolving from empirical observations to a molecular epidemiology paradigm that incorporates exposure and pathogenesis [85]. This trend is part of the global change that risk assessment is undergoing as a whole. It involves a shift from hazard-driven to exposure-driven approaches based on the integration of relevant exposure data (i.e. external dose) into an internal dose (i.e. toxicokinetics) to further relate it with a MoA or AOP (toxicodynamics) [51].

The term 'exposome', originally coined by C. Wild [163], emerged to support the shift in the current risk assessment model to a more holistic and integrated approach that investigates all sources of environmental exposure (e.g. chemical agents, biological agents, radiation, psychosocial components) [58,59]. Specifically, the chemical exposome comprises any exogenous exposure and endogenous chemical exposures caused in response to external stressors [164]. The study of the exposome is a step beyond traditional HBM-based exposure assessment because it aims to capture all exposures that occur from conception onwards and, at the same time, linking these non-genetic factors with adverse health outcomes (i.e. exposomics). Exposomics, through among others, the application of 'omics approaches such as metabolomics, is called upon to unravel disease mechanisms by discovering effect biomarkers that explain the connections between environmental exposure and health outcomes [165,166]. As a consequence, the population groups most sensitive to such exposure can also be identified and critical exposure concentrations can be established to support risk management and the enforcement of health prevention actions.

Measuring all exposures in a long-term approach is a Herculean task, so early exposome research focuses (or is recommended to do so) primarily on critical periods of life when chemical exposure may have a high incidence (e.g. *in utero* exposome) and/or on priority chemicals [167,168]. In this sense, the characterization of

the exposome in terms of cumulative measure of environment and biological responses should not necessarily measure exposure during the entire individual's lifespan, since some risk factors related to specific exposures can already be defined by detecting specific biomarkers of effect [59]. In fact, the effect biomarkers represent one of the two pillars of the exposome studies as they allow refinement of exposure assessment due to their ability to provide information on MoA and dose-response relationships [58].

Within this framework, non-targeted metabolomics is called to play an active and important role in exposome research [169], because it provides extensive information on the metabolome to feed 'environment-wide association studies' (EWASs). Based on the application of different statistical methodologies, EWASs allow the categorization of chemical exposures and establish relationships between them and a health outcome [59,170]. ^{1}H NMR metabolomics analysis of urine samples from pregnant women and subsequent EWAS have shown, for example, specific associations between exposure to heavy metals (i.e. thallium, cesium, copper and lead) and steroid hormones in the third trimester of pregnancy or strong associations between phthalates exposure and kreb's cycle metabolites in the first trimester [171]. Furthermore, mercury was associated with decreased levels of estrogen metabolites, whereas no association was found for chlorinated pesticides or BPA. The observed associations reflected differences in the *in utero* environment that may affect fetal development and child health.

The implementation of the exposome approach faces great challenges, such as difficulties in identifying significant associations from high-dimensional exposomics data that must also integrate information from all biological layers to account for their interactions [172], or correlations among all exposures that may hinder the identification of the directionality of the potential causal relationship between exposures and outcomes [167,173]. The first findings applying the exposomics approach to link exposure with health effects have been reported recently [174] and, while promising, it is still too early to determine whether the exposome concept manages to turn into a real application with impact on risk assessment [175].

5.3.3 Metabolic fingerprints for exposure assessment

It is expected that a more comprehensive understanding of the relationship between exposure to certain chemical hazards and the changes they produce in the metabolism may lead to the identification of specific metabolic fingerprints, which may be directly related to specific chemical exposures. As a consequence, a new approach could be implemented to carry out exposure assessment, as information could be obtained from the

measurement of exposure biomarkers (as usually done) or from metabolic fingerprints that reflect effect biomarkers. In this sense, epidemiological data from metabolomics studies of cohorts could be evaluated not only for hazard identification (or hazard characterization if dose-response relationships are established), but also for exposure assessment if specific effect biomarkers have been accepted as intrinsically related to a specific chemical hazard (or mixture of chemical hazards).

This approach is in line with new advances in the science of exposomics for exposure assessment, and it is too early to predict its efficacy to meet the objectives of exposure assessment. It will not be enough to identify effect biomarkers for a wide range of chemical exposures (and mixture of chemical exposures), which already requires a breakthrough in the identification of metabolites. It will also require the identification of different but still specific metabolic fingerprints for exposures to the same hazard (or mixture of hazards) at different concentration levels and taking into account the exposure period. In this last sense, longitudinal studies must be carried out to evaluate the evolution of the metabolic fingerprint according to short- and long-term chemical exposures. Overall, this new approach to exposure assessment is a resource intensive process, not only from a technical point of view, but also for the storage of metabolic fingerprints associated with a large number of exposures.

Current metabolomics studies using epidemiological data such as those described in Section 5.1.1 already provide insight into the metabolic fingerprints associated with certain chemical hazards, although their specificity has yet to be demonstrated. Several examples can be found in the literature on effect biomarkers discovered by human metabolomics that may be related to metabolic fingerprints characteristic of specific chemical hazards such as lead [176] or TCDD [97], or more generally, with exposure to complex pesticide mixtures [177]. Associations between lipid-related metabolites and exposure to organochlorine pesticides (i.e. HCB and p,p'-DDE) have been highlighted in a human cohort of 965 samples [178]. However, specific effect biomarkers in serum were related to each pesticide, suggesting that exposure to each of these compounds leads to different metabolic profiles in humans. Specifically, p,p'-DDE was associated with decreased levels of lysophosphatidylcholine congeners (18:1, 18:2/0:0, 0:0/18:2, 18:3), which have been linked to coronary heart disease and diabetes, with increased levels of monoacylglycerol, with fatty acids and related compounds (i.e. oleamide, linolenic aldehyde, and arachidonic acid ethyl ester), and with flavone. The latter metabolite and its association with p,p'-DDE was attributed to some common dietary source or the perturbation of flavonoid metabolism by p,p'-DDE exposure. On the other hand, HCB exposure was negatively associated with

lysophosphatidylethanolamine (18:1) and lysophosphatidylethanolamine (18:2), and positively associated with lysophosphatidylethanolamine (18:1p/0:0), docosahexaenoic acid, and a cinnamic acid related metabolite.

Finally, toxicological data can also be used to elucidate and establish specific metabolic fingerprints related to exposure to chemical hazards as observed for different pesticides [147] or the mycotoxin DON [179], although it is mandatory to demonstrate their analogy in humans to be applied for exposure assessment purposes.

6- METABOLOMICS IN CHEMICAL RISK MANAGEMENT

Perhaps complying with regulatory toxicology requirements represents one of the biggest challenges facing metabolomics in risk analysis. If metabolomics is used only to generate a weight-of-evidence on the MoA/AOP of chemicals, working in a Good Laboratory Practice (GLP) environment may be secondary. On the contrary, the implementation of GLP conditions is mandatory to enhance regulatory use of 'omics data [180]. The harmonization of risk analysis tools is an important concern of risk managers because they must provide solid scientific evidence of risks for decision-making. However, it is considered that 'omics technologies do not yet meet the standardization and validation criteria required for regulatory toxicology [181]. The lack of best practice guidelines, including performance standards, minimal reporting standards and quality control practices, is viewed as the Achilles' heel of metabolomics for its implementation in regulatory toxicology, limiting its application in risk analysis [51,125,162]. The metabolomics community is making a great effort to overcome this drawback and, as a result, the Metabolomics Standard Initiative (MSI) proposes reporting standards guidelines for all the stages of metabolomics (i.e. experimental design, biological context, chemical analysis and data processing) since 2007 [182]. Furthermore, advances in the harmonization of quality control standards in metabolomics [i.e. quality assurance (QA) and quality control (QC)] are expected to be achieved in the coming years [183]. In this context, the MEtabolomics standaRds Initiative in Toxicology (MERIT) has recently been launched to implement metabolomics approaches in regulatory toxicology by overcoming the abovementioned limitations [125]. It is a European project supported by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) that pursues two main objectives. First, it aims to develop best practice guidelines to harmonize laboratory protocols for the acquisition, processing and analysis of toxicological data within a regulatory framework. Second, its goal is also to develop minimal reporting standards, including QA and QC in metabolomics workflows, to be

followed by regulatory agencies (http://www.ecetoc.org/topics/standardisation-metabolomics-assays-regulatory-toxicology/).

The difficulties encountered in assigning an identity to biomarkers found in metabolomics studies also represent a disadvantage for the implementation of metabolomics in regulatory toxicology, since most of the detected MS features remain unidentified. Furthermore, although it would also be desirable to achieve an identification level of 1 according to the MSI for the annotation or identification of metabolites (i.e. unambiguous identification of the metabolite by two or more orthogonal techniques and additional validation in the laboratory with an authentic chemical standard), risk managers and regulation agencies should be aware that commercially available standards cover less than the 20% of the metabolome [54]. Thus, an annotation level of 2 based on information from widely accepted and accessible databases, also generated *in silico*, provides high confidence for metabolite annotation without implying complacency. Nowadays there are a large number of mass spectral databases for the identification of metabolites in non-targeted metabolomics applying LC-MS and GC-MS approaches [60,184], and they are continuously growing in terms of metabolite entries. Recently, the International Agency for Research on Cancer (IARC) has created the 'Exposome-Explorer database' that contains information on exposure biomarkers associated with environmental risk factors for diseases [175]. Several of these databases and software include information on fragmentation mass spectra (i.e. MS/MS spectra), which provides high specificity for metabolite annotation. Great efforts are also being made to make peak annotation an accessible process enriched by a systematic transfer of knowledge between laboratories. Consequently, initiatives such as the Global Natural Products Social Molecular Networking (GNPS) platform have emerged to support the storage, analysis and knowledge dissemination of MS/MS spectra [185]. GNPS also supports molecular networking to correlate sets of MS/MS spectra of related molecules that, while not consistent with known compounds in MS/MS libraries, can be putatively identified.

Other important aspects currently hampering the implementation of metabolomics in regulatory toxicology include a relative lack of training opportunities, as well as limited accessibility to the analytical and computational tools required for data generation, curation and processing [125]. With regard to the latter, bioinformatics tools such as Galaxy or MetaboAnalyst, among others, are in continuous development to provide automated and standardized operational pipelines for data pre-processing (e.g. data alignment, filtering, normalization), statistical approaches (i.e. univariate and multivariate analysis), metabolite annotation and metabolic network [60,121,186].

On the other hand, there is also a recent debate on how data storage and sharing should be managed within a regulatory toxicology framework, and how desirable it is to make data publicly accessible to ensure the transparency of chemical safety regulations [125]. Regulatory toxicology can benefit from the current trend in data management aimed at making metabolomics data Findable, Accessible, Interoperable and Reusable (FAIR) [187]. Several metabolomics repositories are currently available to support the FAIR data objective, such as MetaboLights (European initiative), Metabolomics Workbench (North American initiative) and MetabolomeXchange (developed by an international consortium to aggregate metabolomics data from different repositories). Standardization will undoubtedly be the key for establishing a data sharing framework where data can be successfully re-used and reproduced [188].

Finally, from a regulatory point of view, it is required to set essential decision criteria such as a metabolomics NOAEL that reflects a metabolic perturbation correlated with an observable adverse effect rather than an adaptation of the organism to the exposure. These findings linking toxicological effects with changes in the metabolome will also need validation to be accepted for regulatory purposes [189]. The validation of 'omics studies will give confidence to risk managers in the reliability, robustness, repeatability and reproducibility of the data generated in the risk assessment process. Although still scarce, some metabolomics studies have begun to tackle validation aspects, including robustness over time [190] and inter-laboratory comparisons [191]. Despite the lack of specific guidelines for the validation of non-targeted metabolomics methods, the recognition of this type of approach has been reflected by the ISO 17025 accreditation recently awarded to a non-targeted metabolomics method applied in the screening of illegal administration or exposure to β-agonists in cattle and implemented in the French monitoring and control plans for food safety [192], fulfilling regulatory requirements in terms of expected performances.

7- CONCLUSIONS

As a new NAM in chemical risk analysis, metabolomics has already demonstrated to be a fit-for-purpose approach to meet the steps of hazard identification, hazard characterization and exposure assessment steps by identifying biomarkers of exposure and effect. These biomarkers reveal the earliest mechanisms caused by chemicals and provide insight into their toxicokinetics and toxicodynamics, thus contributing to deciphering the MoA and AOP of such hazards. This represents a shift from current toxicology and epidemiology paradigms

towards a molecular framework in which the consequences of exposure can be predicted even before adverse effects are observed. However, evolution is still required to prove that the discovery of such biomarkers provides a benefit to the assessment frameworks currently used in the context of regulatory toxicology [47]. It remains a challenge to unequivocally associate the biomarkers found by metabolomics with real adverse health outcomes rather than showing an adaption of the organism to chemical exposure; therefore, it would be desirable to establish a NOAEL for metabolomics. Furthermore, and from a risk management perspective, metabolomics studies have yet to comply with a standardized framework and undergo validation to be accepted for regulatory toxicology.

Metabolomics studies reported so far, however, have some limitations for risk assessment due to high dosage generally applied in *in vivo* experiments, and the low number of data points when assessing dose-response relationships. Nevertheless, this methodology has the potential to successfully overcome the current challenges of risk analysis, with special focus on food safety. Metabolomics in combination with *in vitro* and *in silico* models provides relevant evidence for chemical risk analysis, reducing animal testing in accordance with the 3R principles as well as the uncertainty related to animal experiments traditionally carried out in toxicology for human risk assessment. On the other hand, the high sensitivity of metabolomics can reveal the effects of chemicals at low concentration levels, which will have an important impact on understanding the risks associated with exposure to low doses of compounds showing, for example, non-monotonic dose-response curves. In a step forward in addressing actual exposure scenarios, linking metabolic fingerprints to the MoA of chemicals will favor their grouping to better study additive or interactive effects related to exposures to low-dose chemical mixtures.

Within the framework of actual exposure scenarios and as a part of the new exposome paradigm, both suspect and non-targeted screening provide a holistic exposure assessment for a more compressive understanding of human exposure to chemicals throughout the human lifespan and, as a consequence, to establish a link between such exposure and adverse health outcomes. The inclusion of the biological response in exposure assessment leads to the integration of the AOP into the exposome paradigm. The exposome-AOP tandem can be applied to group chemicals with convergent AOPs, while AOP networking contributes to assessing complex mixtures of the exposome [164]. Furthermore, the role of metabolomics can go beyond the measurement of exposure biomarkers to assess internal exposure and effect biomarkers to identify the early events of chemical AOPs. The application of metabolomics to investigate the source of exposure, in this case

food, allows the establishment of aggregate exposure pathways (AEP) to link external exposure to complex chemical mixtures with concentrations at the internal target site and subsequent AOP, gaining a more complete understanding of the exposome to support risk assessment [193]. Analysis of both the dietary source and population groups applying non-targeted metabolomics, and further validation using targeted metabolomics methods, also implies a new approach to detect new chemical hazards in food, thus identifying new risks that must be addressed.

From a technical point of view, LC-MS and, to a lesser extent, ^{1}H NMR have been shown to be the main analytical techniques used in metabolomics for risk analysis. Although both, and especially LC-MS, provide extensive coverage of the metabolome, the application of other methodologies such as GC-MS, CE-MS, SFC-MS or other novel methodologies (e.g. IMS-MS, LC×LC-MS, etc.) can improve knowledge about metabolic perturbations caused by chemical hazards. DDA and especially DIA experiments give more information on relevant metabolites for their unequivocal identification. However, extended information on the metabolome comes at a data cost, leading to difficulties in the manipulation, interpretation and storage of metabolomics data. Bioinformatics tools, databases for metabolite annotation and biological interpretation, and repositories for data storage are under continuous development, favoring the execution of metabolomics studies and their implementation in the field of risk analysis.

Finally, although chemical exposure inevitably leads to metabolic pathway perturbations that support the use of metabolomics for risk assessment, more complete information on the MoA/AOP of chemicals and the consequences of their exposure on human health is obtained by combining metabolomics data with data from other 'omics studies (i.e. epigenomics, transcriptomics or proteomics). A single 'omics dataset is generally not enough to identify the MIE of certain hazards or adequately reflect the link between the MIE and the pathway perturbation. Systems biology promotes the integration of information from different biological layers to fill the gaps in toxicity mechanisms. Nevertheless, it involves the integration of data obtained by different 'omics methodologies, which is viewed as an emerging challenge for their integrated implementation in toxicology. In this vein, systems toxicology intends to benefit from computational advances in the field of systems biology field to build comprehensive digital mechanisms that integrate different 'omics datasets, different pieces of evidence (*in vivo*, *in vitro* and/or *in silico* studies), and even different toxicological pathways to more accurately predict the toxicity of hazards and better understand the relationship between chemical exposure and adverse health

outcomes [194]. Definitely, the combination of 'omics technologies (toxicogenomics) and different toxicological methodologies (*in vivo*, *in vitro, in silico*) constitutes a new integrated approach to testing and assessment (IATA) in risk analysis to reduce the existing gap between the number of chemical substances currently in use and the number of chemicals already subjected to risk analysis.

ACKNOWLEDGMENTS

We thank G. Cano-Sancho for his critical review of this manuscript. **Funding:** This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement HAZARDOmics No 795946.

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Figure captions

Figure 1. Risk analysis framework.

Figure 2. 'Omics cascade' showing that the metabolomics layer is the closest to exposure sources. Figure created in BioRender.com.

Figure 3. A) Global overview of metabolic pathways, B) Bisphenol degradation pathway, C) Bisphenol A. Metabolic pathways have been extracted from the Kyoto Encyclopedia of Genes and Genomes (KEGG) with permission [55].

Figure 4. (A) Chromatogram of the polar extract of a serum sample analyzed in ESI+ mode. (B) The scores plot displaying the separation between the two sample groups (i.e. low and high DDE exposure). Explained variance (R2) was 94%, predictive ability (Q2) was 85%, and root-mean-square error of validation (RMSEV) was 18%. (C) The loadings plots that correspond to the scores in B. Metabolites that significantly differ between groups are indicated in red and blue, and are related to high and to low DDE exposure, respectively. These metabolites are further emphasized by the S-line-plot (D) and their relative variable importance to the model. Figure reprinted with permission from [101]. Copyright (2017) Elsevier.

Figure 5. Proposed pathway of inhibition of ATP biosynthesis by disruption of oxidative phosphorylation (OXPHOS) derived from the uncoupling action by OH-PCBs in beagle brain and inactivation of the urea cycle with reduced ATP production. Black thick arrows indicate the metabolites with decreased concentrations. Figure reprinted with permission from [112]. Copyright (2019) Elsevier.

Figure 6. Changes in metabolic profile of Sprague-Dawley rat liver after 28-day oral administration of SCCPs. (A) PLS-DA score plot of liver metabolites after exposed to SCCPs at various doses. (B) MELI of liver metabolism fingerprint in the control and SCCP-treated groups. (C) Metabolic correlation networks of the differential metabolites and related pathways. SM: sphingomyelin; PC: phosphatidyl choline; LysoPC: lysophosphatidyl choline; PE: phosphatidyl ethanolamine; LysoPE: lysophosphatidyl ethanolamine; PA: phosphatidic acid; CoA: coenzyme A; GSSH: oxidized glutathione; ADP: Adenosine diphosphate. (D) Hierarchical clustering based on the differential metabolites with p-value < 0.05 based on one-way ANOVA and VIP value > 1, PEA: palmitoylethanolamide; AEA: anandamide; Leu-pro: leucylproline; MG: monoacyl glycerol; DG: diacyl glycerols. *, p-value < 0.05; **, p-value < 0.01. Figure reprinted with permission from [144]. Copyright (2019) Elsevier.

Figure 1.

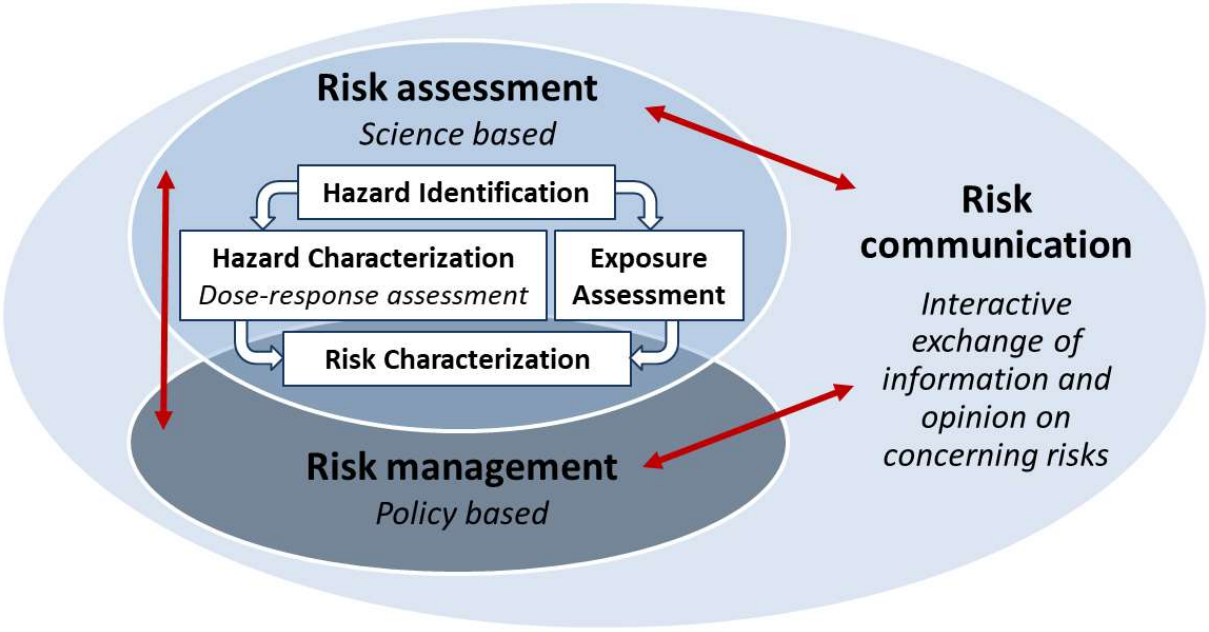

Risk assessment
Science based
Hazard Identification
Hazard Characterization
Dose-response assessment
Exposure Assessment
Risk Characterization
Risk management
Policy based
Risk communication
Interactive exchange of information and opinion on concerning risks

Figure 2.

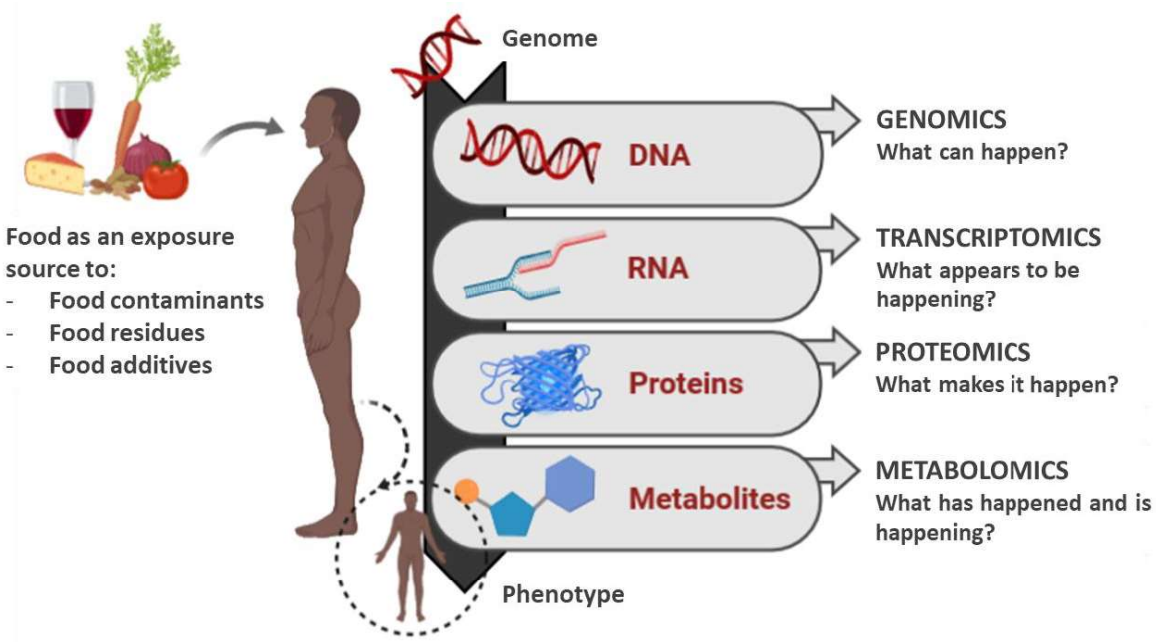

Genome
DNA
GENOMICS
What can happen?
RNA
TRANSCRIPTOMICS
What appears to be happening?
Proteins
PROTEOMICS
What makes it happen?
Metabolites
METABOLOMICS
What has happened and is happening?
Phenotype
Food as an exposure source to:
- Food contaminants
- Food residues
- Food additives

Figure 3.

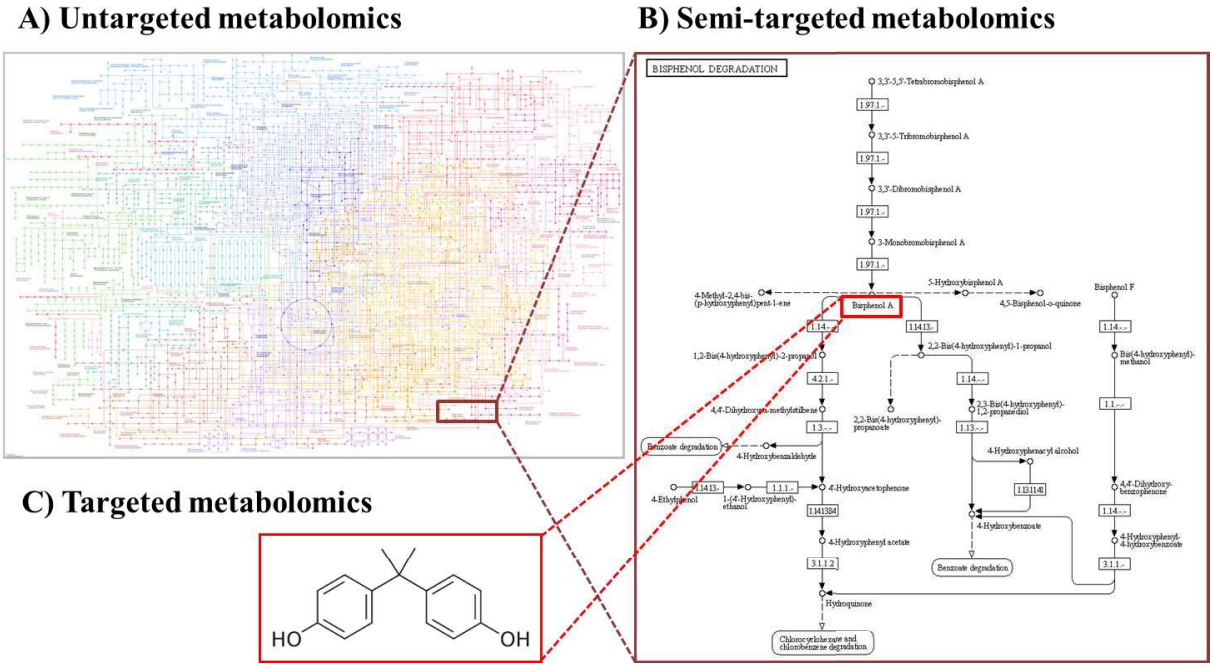
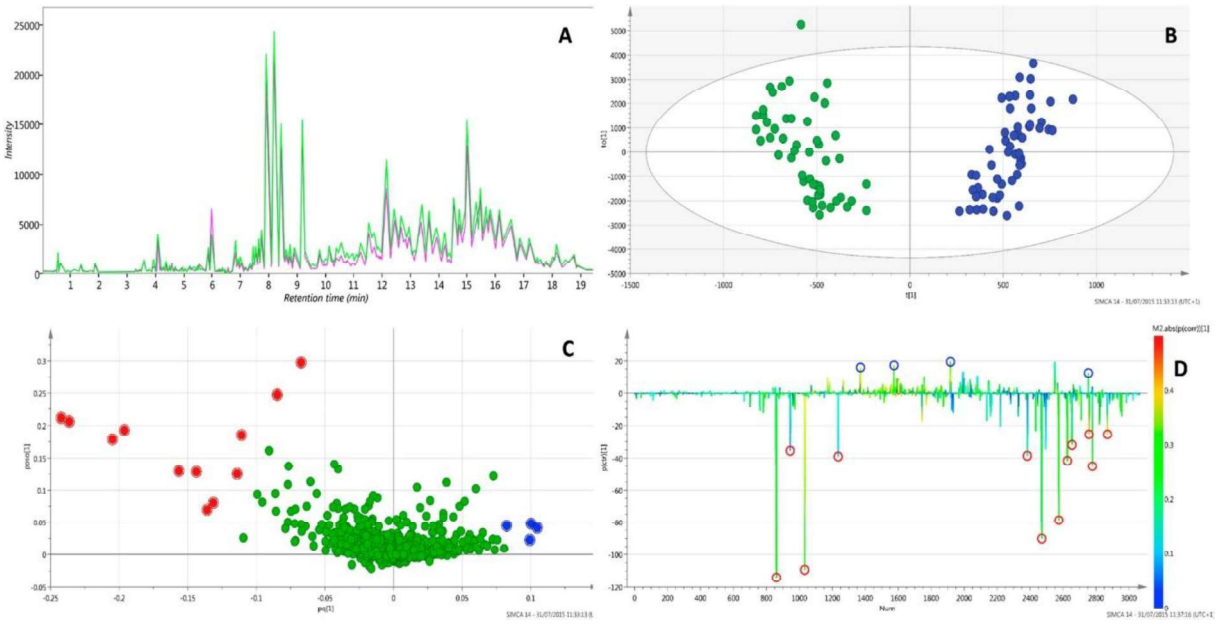

A) Untargeted metabolomics
B) Semi-targeted metabolomics
C) Targeted metabolomics
BISPHENOL DEGRADATION
Bisphenol A
Benzoate degradation
Chlorocyclohexane and chlorobenzene degradation
HO
OH

Figure 4.



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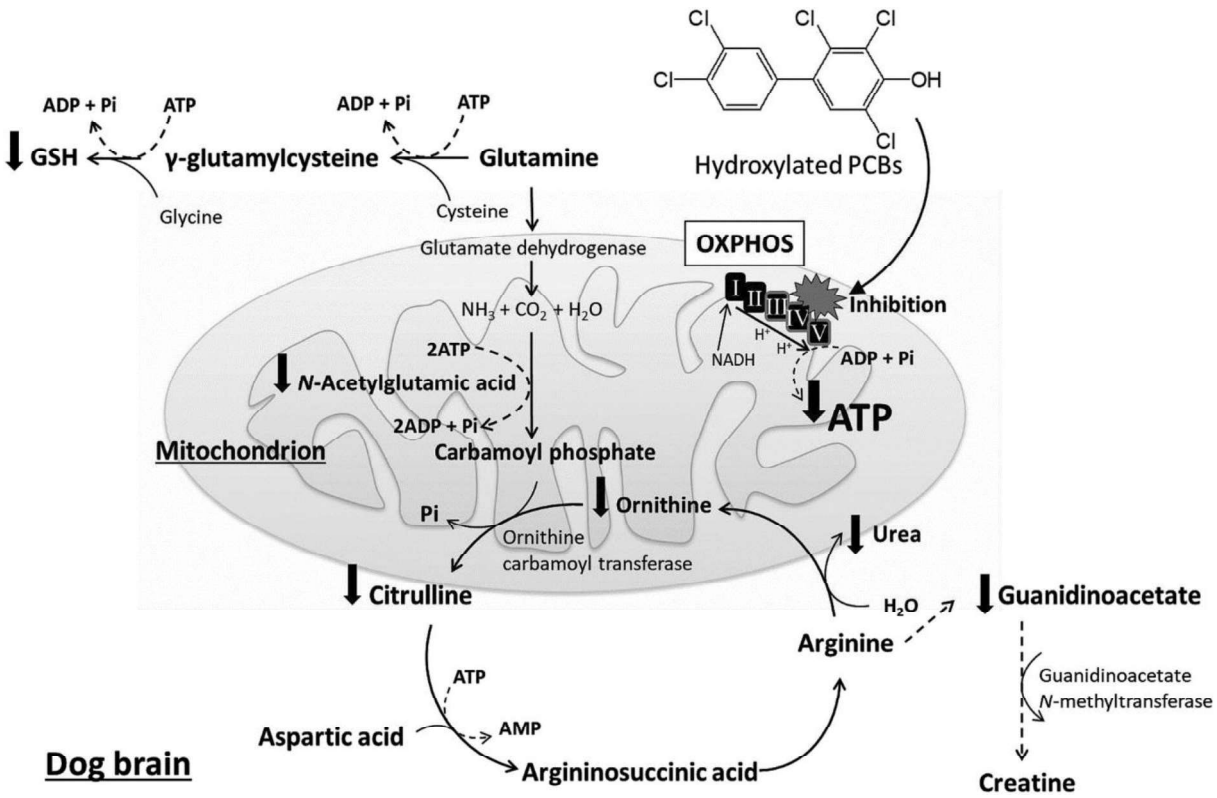
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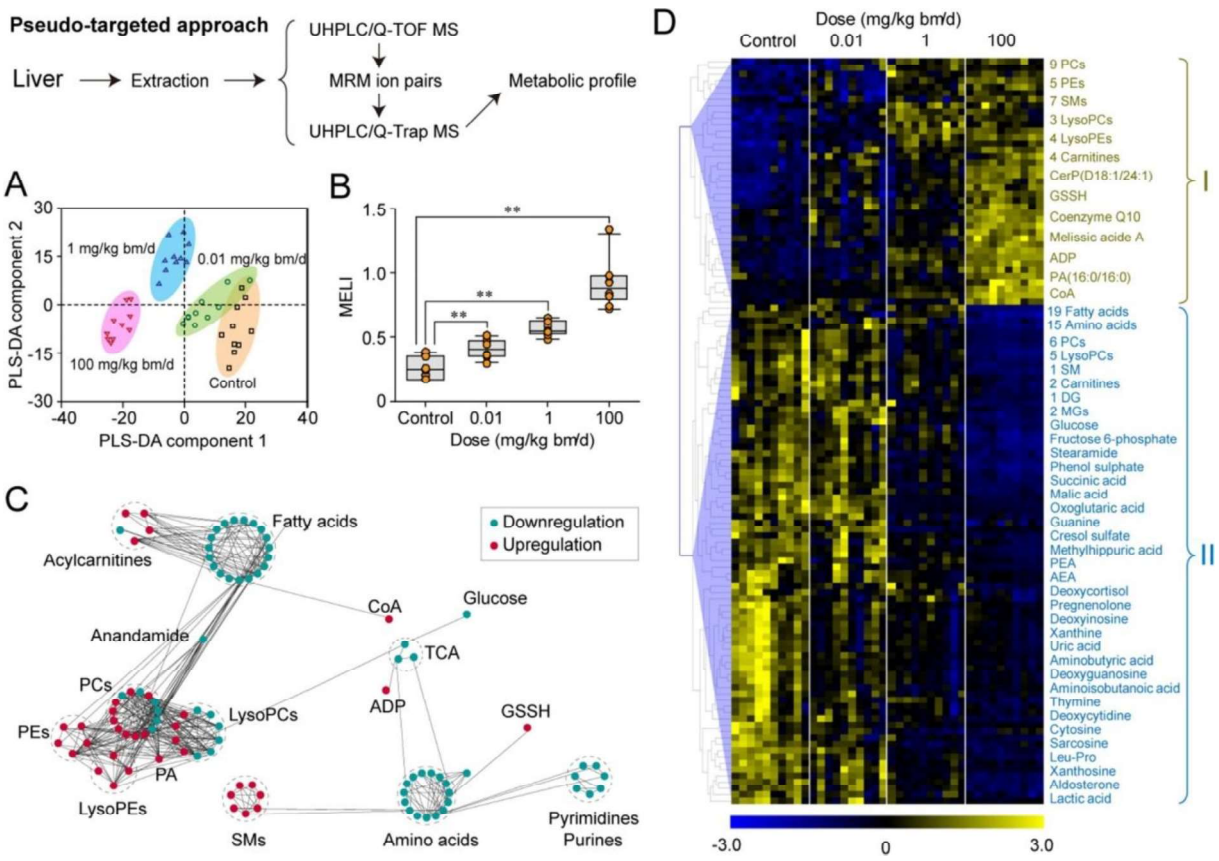
Cl
OH
Cl
Hydroxylated PCBs
ADP + Pi
ATP
GSH
γ-glutamylcysteine
ADP + Pi
ATP
Glutamine
Glycine
Cysteine
Glutamate dehydrogenase
OXPHOS
I
II
III
IV
V
Inhibition
NADH
H+
H+
ADP + Pi
ATP
NH3 + CO2 + H2O
2ATP
N-Acetylglutamic acid
2ADP + Pi
Mitochondrion
Carbamoyl phosphate
Pi
Ornithine
Ornithine carbamoyl transferase
Urea
Citrulline
H2O
Guanidinoacetate
Arginine
ATP
Aspartic acid
AMP
Argininosuccinic acid
Guanidinoacetate N-methyltransferase
Creatine
Dog brain

Figure 6.


Pseudo-targeted approach
Liver
Extraction
UHPLC/Q-TOF MS
MRM ion pairs
UHPLC/Q-Trap MS
Metabolic profile
A
1 mg/kg bm/d
0.01 mg/kg bm/d
100 mg/kg bm/d
Control
PLS-DA component 2
PLS-DA component 1
B
MELI
Control
0.01
1
100
Dose (mg/kg bm/d)
C
Downregulation
Upregulation
Fatty acids
Acylcarnitines
Anandamide
PCs
PEs
LysoPCs
PA
LysoPEs
SMs
CoA
Glucose
TCA
ADP
GSSH
Amino acids
Pyrimidines
Purines
D
Dose (mg/kg bm/d)
Control
0.01
1
100
9 PCs
5 PEs
7 SMs
3 LysoPCs
4 LysoPEs
4 Carnitines
CerP(D18:1/24:1)
GSSH
Coenzyme Q10
Melissic acide A
ADP
PA(16:0/16:0)
CoA
19 Fatty acids
15 Amino acids
6 PCs
5 LysoPCs
1 SM
2 Carnitines
1 DG
2 MGs
Glucose
Fructose 6-phosphate
Stearamide
Phenol sulphate
Succinic acid
Malic acid
Oxoglutaric acid
Guanine
Cresol sulfate
Methylhippuric acid
PEA
AEA
Deoxycortisol
Pregnenolone
Deoxyinosine
Xanthine
Uric acid
Aminobutyric acid
Deoxyguanosine
Aminoisobutanoic acid
Thymine
Deoxycytidine
Cytosine
Sarcosine
Leu-Pro
Xanthosine
Aldosterone
Lactic acid
I
II
-3.0
0
3.0

***Declaration of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Figure

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Figure 1

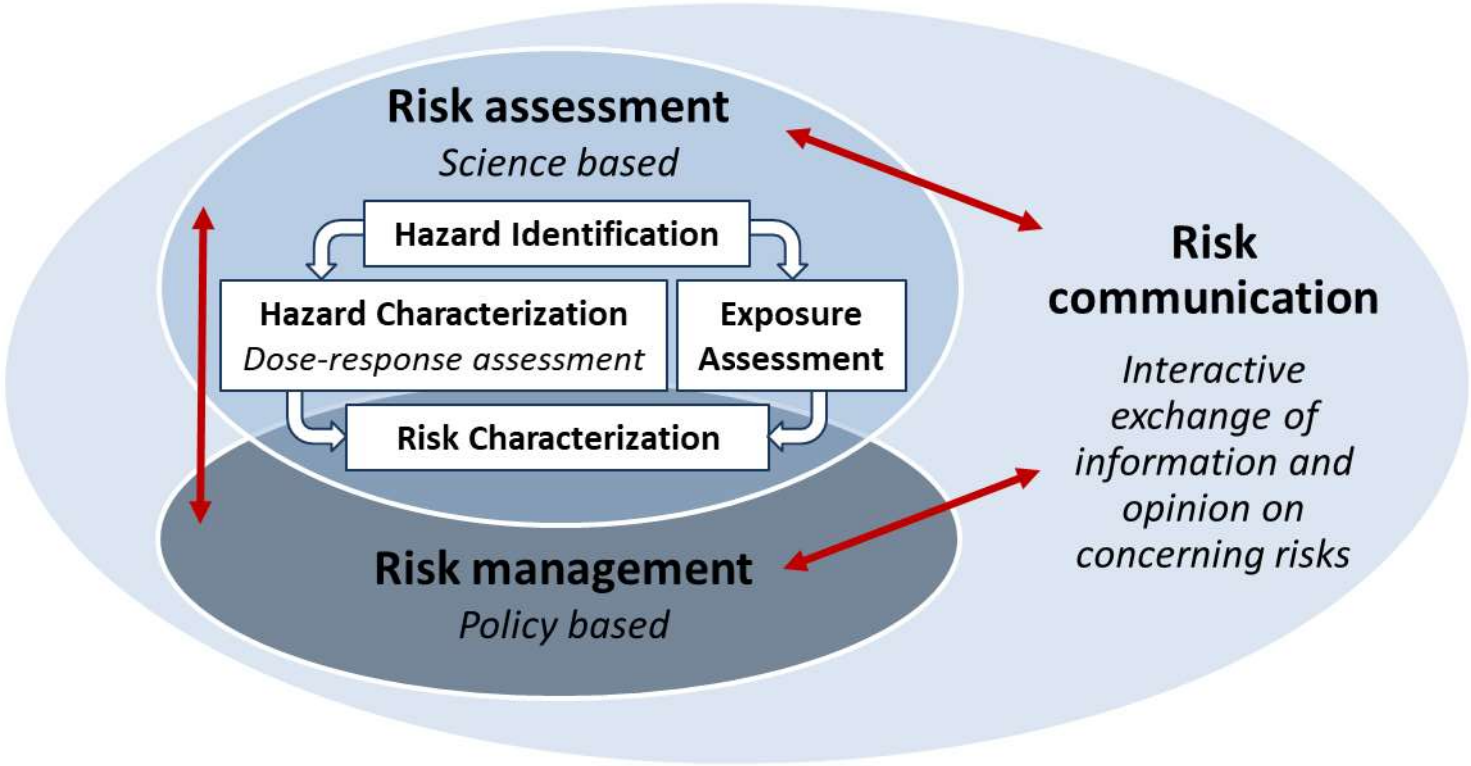
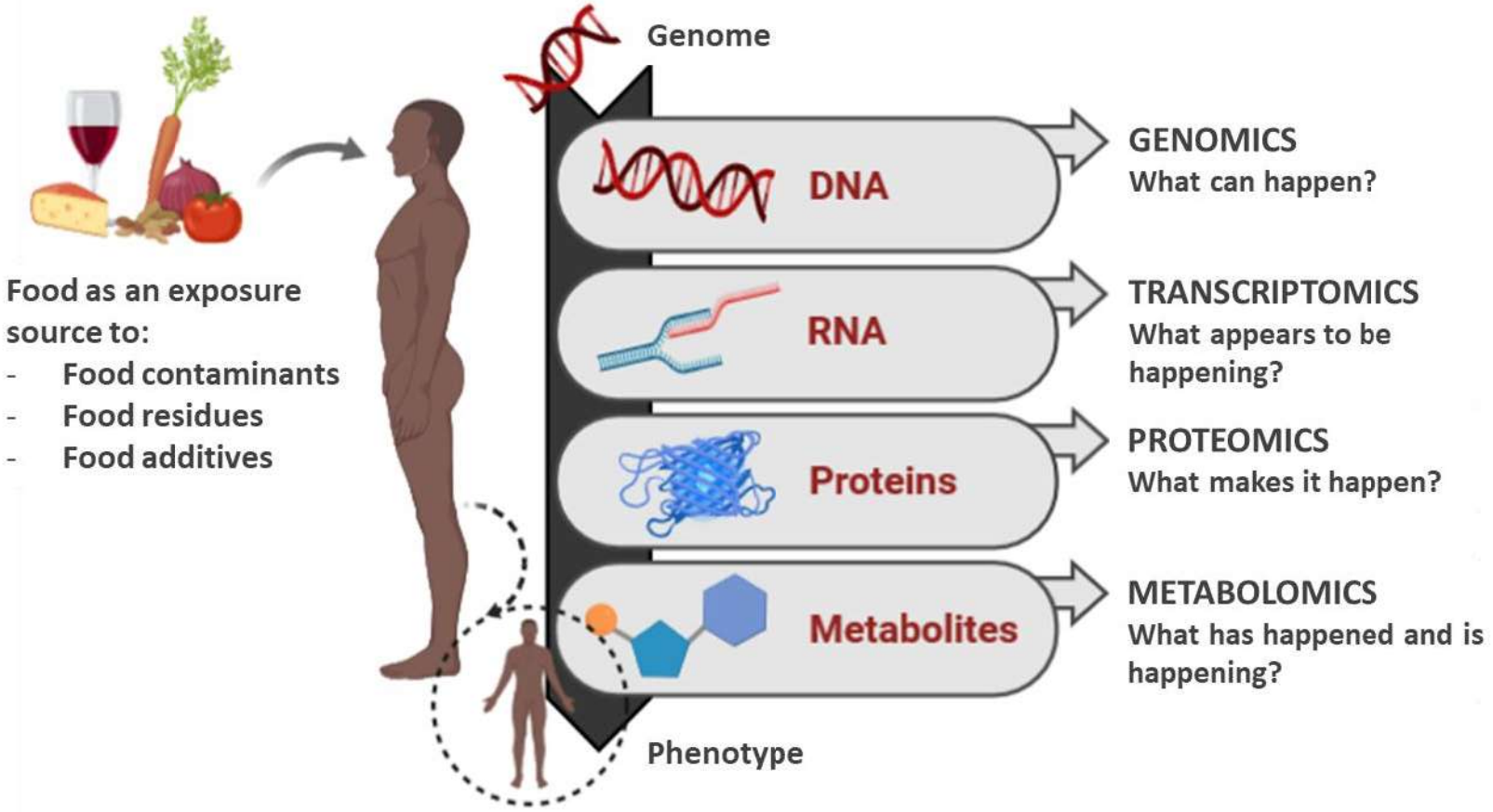


Figure 2


Genome
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- Food contaminants
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- Food additives
DNA
RNA
Proteins
Metabolites
Phenotype
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Figure 3

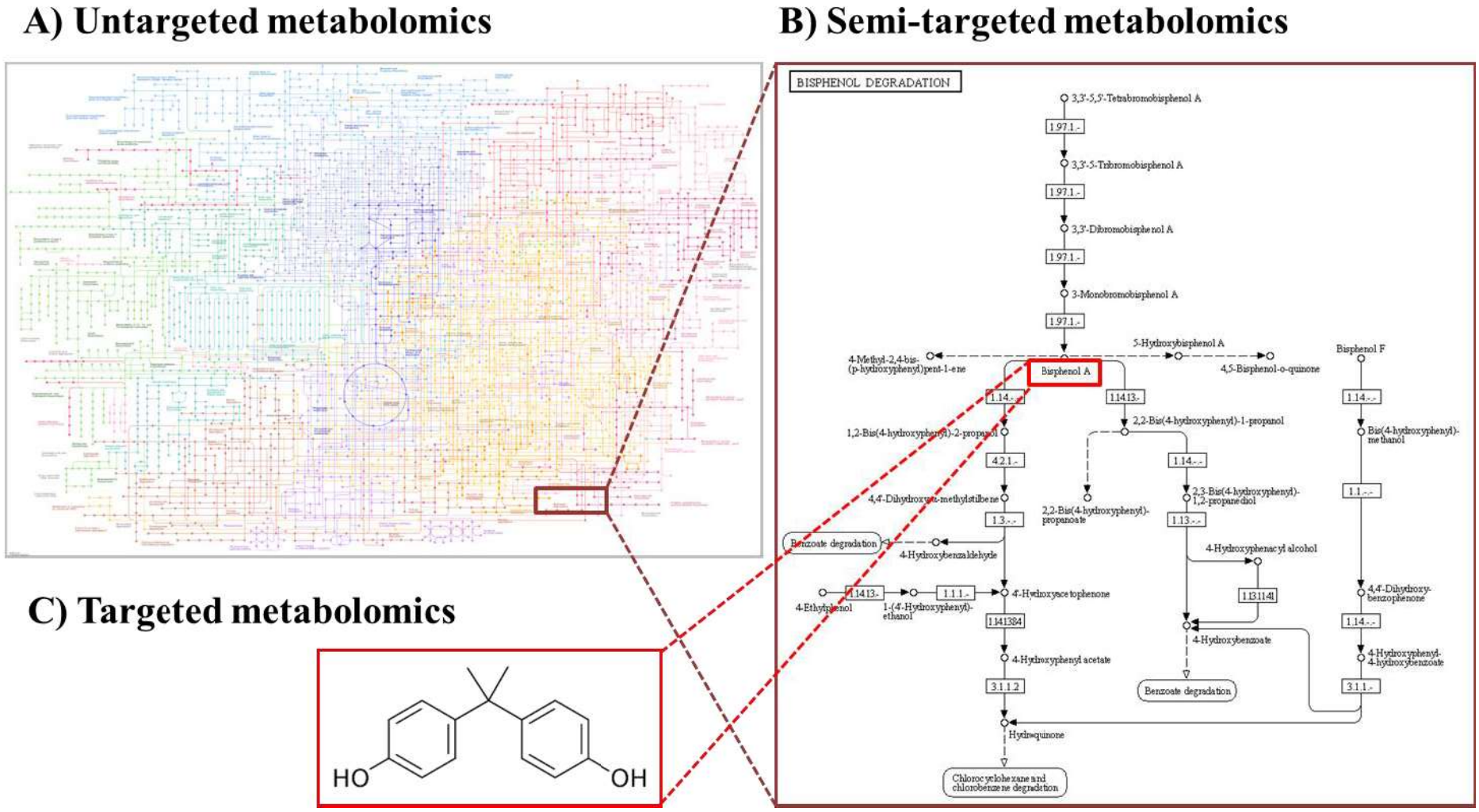
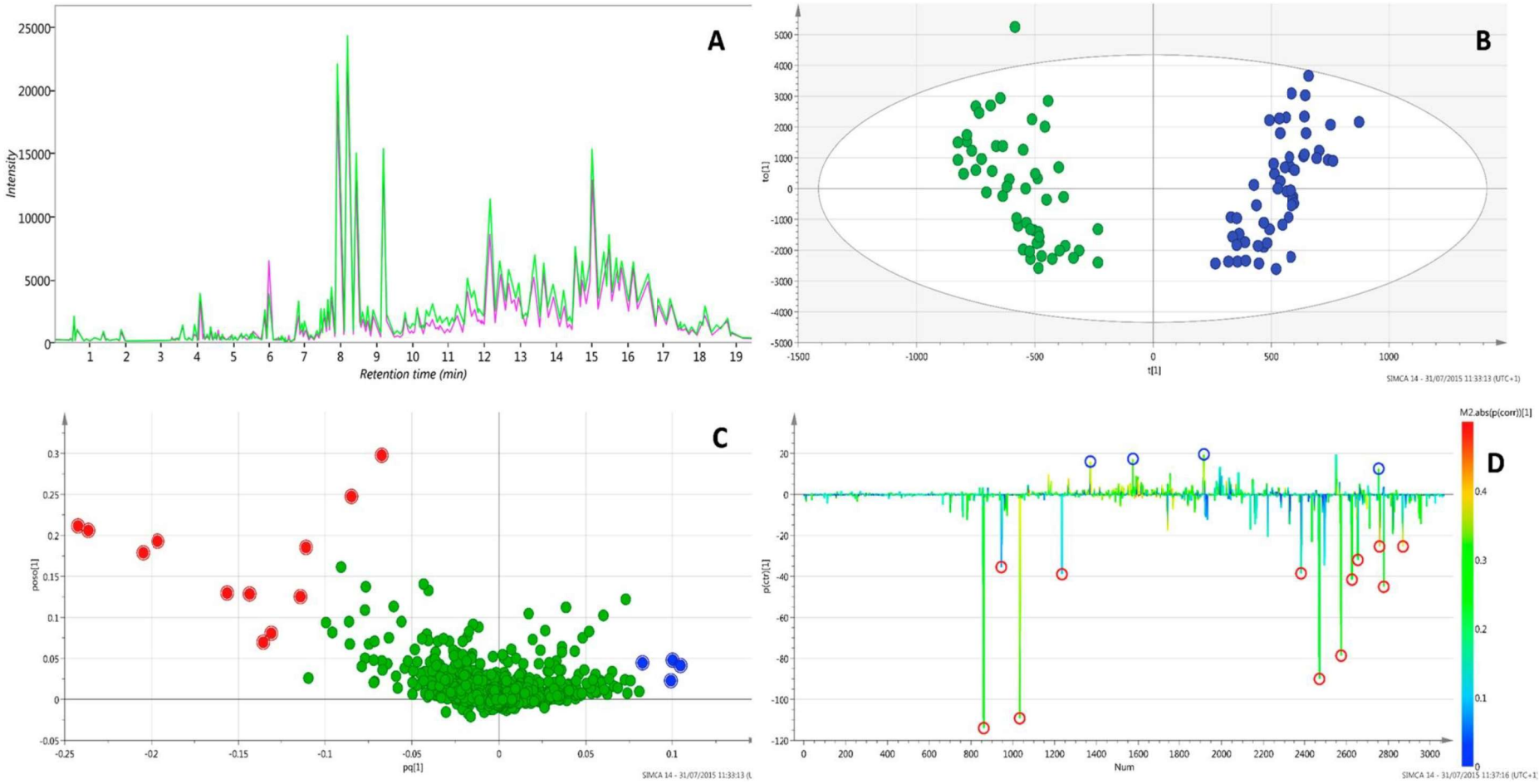

A) Untargeted metabolomics
B) Semi-targeted metabolomics
BISPHENOL DEGRADATION
3,3'-5,5'-Tetrabromobisphenol A
1.97.1.-
3,3'-5-Tribromobisphenol A
1.97.1.-
3,3'-Dibromobisphenol A
1.97.1.-
3-Monobromobisphenol A
1.97.1.-
5-Hydroxybisphenol A
4-Methyl-2,4-bis-(p-hydroxyphenyl)pent-1-ene
Bisphenol A
4,5-Bisphenol-o-quinone
Bisphenol F
1.14.-.-
1.14.13.-
1,2-Bis(4-hydroxyphenyl)-2-propanol
2,2-Bis(4-hydroxyphenyl)-1-propanol
Bis(4-hydroxyphenyl)-methanol
4.2.1.-
1.14.-.-
1.1.-.-
4,4'-Dihydroxy-α-methylstilbene
2,2-Bis(4-hydroxyphenyl)-propanoate
2,3-Bis(4-hydroxyphenyl)-1,2-propanediol
1.3.-.-
1.13.-.-
Benzoate degradation
4-Hydroxybenzaldehyde
4-Hydroxyphenacyl alcohol
4-Ethylphenol
1.14.13.-
1.1.1.-
4'-Hydroxyacetophenone
1-(4'-Hydroxyphenyl)-ethanol
1.13.11.41
4,4'-Dihydroxy-benzophenone
1.14.13.84
1.14.-.-
4-Hydroxybenzoate
4-Hydroxyphenyl acetate
4-Hydroxyphenyl-4-hydroxybenzoate
3.1.1.2
Benzoate degradation
3.1.1.-
Hydroquinone
Chlorocyclohexane and chlorobenzene degradation
C) Targeted metabolomics
HO
OH

Figure 4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Retention time (min)
B
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poso[1]
pq[1]
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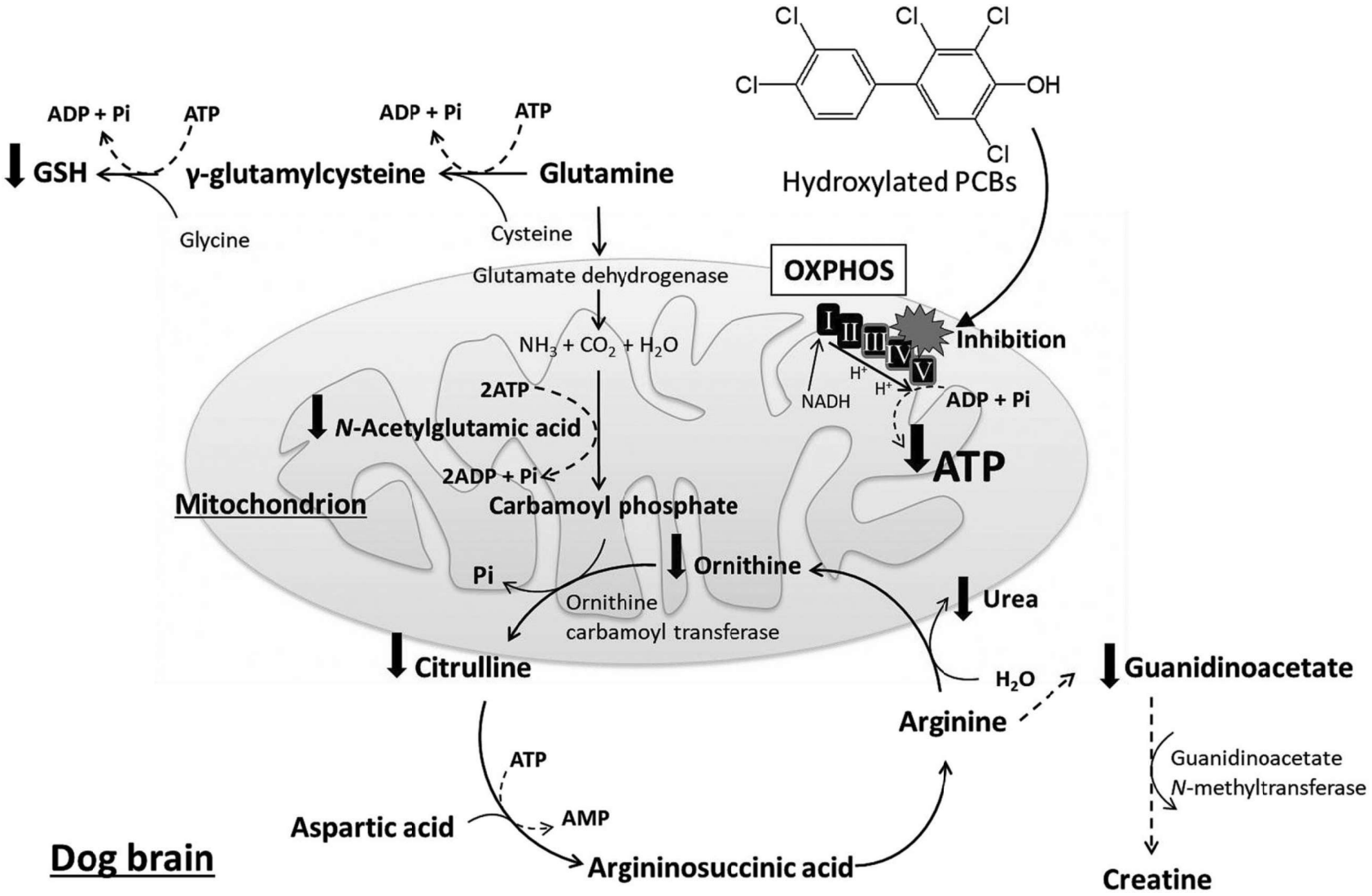
Num

Figure 5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OH
Cl
Hydroxylated PCBs
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GSH
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Glycine
ADP + Pi
ATP
Glutamine
Cysteine
Glutamate dehydrogenase
OXPHOS
I
II
III
IV
V
Inhibition
NADH
H+
H+
ADP + Pi
ATP
NH3 + CO2 + H2O
2ATP
N-Acetylglutamic acid
2ADP + Pi
Mitochondrion
Carbamoyl phosphate
Ornithine
Pi
Ornithine carbamoyl transferase
Urea
Citrulline
H2O
Guanidinoacetate
Arginine
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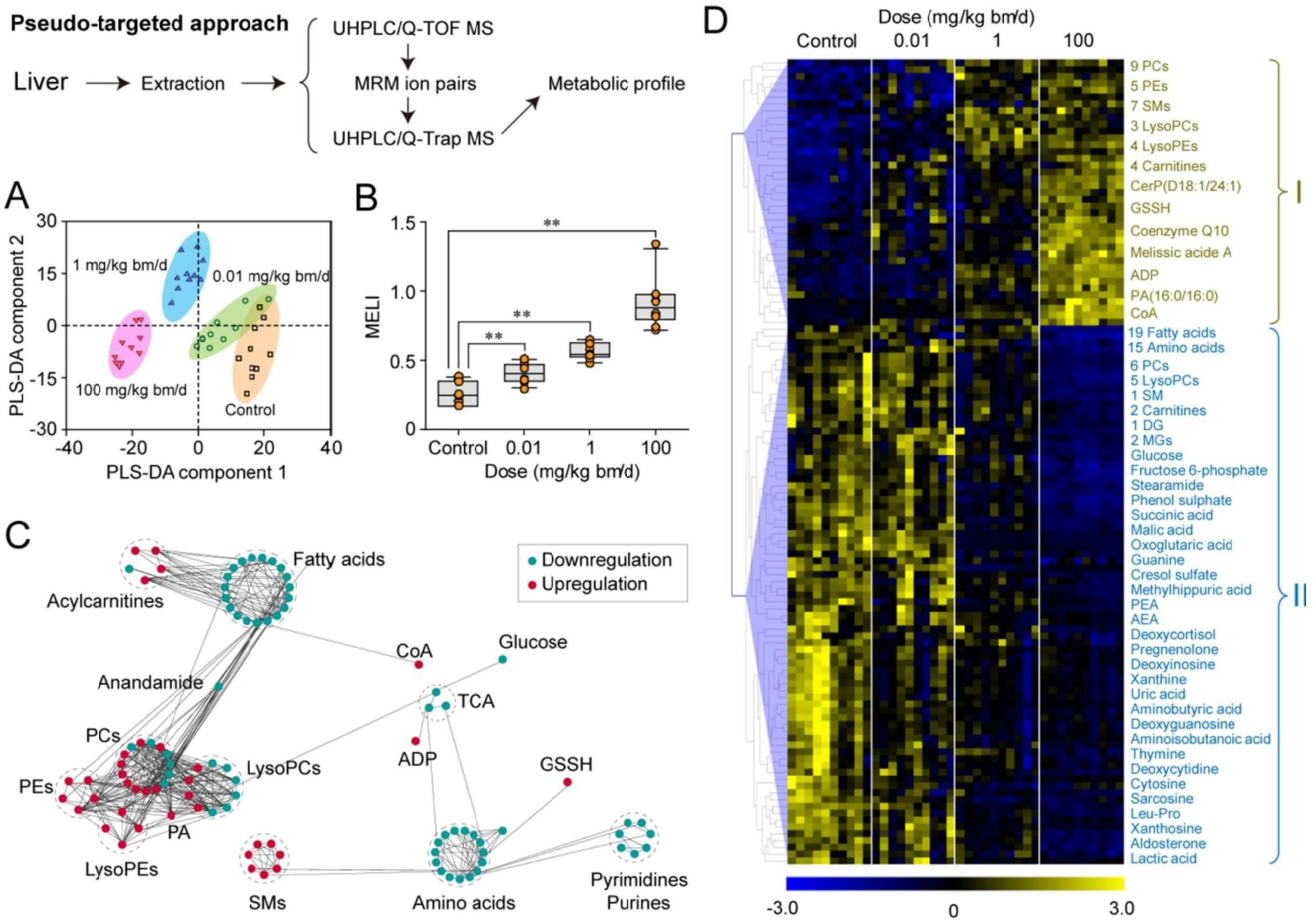
Aspartic acid
AMP
Argininosuccinic acid
Guanidinoacetate N-methyltransferase
Creatine
Dog brain

Figure 6


Pseudo-targeted approach
Liver
Extraction
UHPLC/Q-TOF MS
MRM ion pairs
UHPLC/Q-Trap MS
Metabolic profile
A
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PLS-DA component 1
1 mg/kg bm/d
0.01 mg/kg bm/d
100 mg/kg bm/d
Control
B
MELI
Dose (mg/kg bm/d)
Control
0.01
1
100
C
Downregulation
Upregulation
Fatty acids
Acylcarnitines
CoA
Glucose
Anandamide
TCA
PCs
ADP
GSSH
PEs
LysoPCs
PA
LysoPEs
SMs
Amino acids
Pyrimidines
Purines
D
Dose (mg/kg bm/d)
Control
0.01
1
100
9 PCs
5 PEs
7 SMs
3 LysoPCs
4 LysoPEs
4 Carnitines
CerP(D18:1/24:1)
GSSH
Coenzyme Q10
Melissic acide A
ADP
PA(16:0/16:0)
CoA
I
19 Fatty acids
15 Amino acids
6 PCs
5 LysoPCs
1 SM
2 Carnitines
1 DG
2 MGs
Glucose
Fructose 6-phosphate
Stearamide
Phenol sulphate
Succinic acid
Malic acid
Oxoglutaric acid
Guanine
Cresol sulfate
Methylhippuric acid
PEA
AEA
Deoxycortisol
Pregnenolone
Deoxyinosine
Xanthine
Uric acid
Aminobutyric acid
Deoxyguanosine
Aminoisobutanoic acid
Thymine
Deoxycytidine
Cytosine
Sarcosine
Leu-Pro
Xanthosine
Aldosterone
Lactic acid
II
-3.0
0
3.0

***Author Biography (one per author)**

Dr Maykel Hernández-Mesa obtained his PhD in Chemistry in the laboratory of Prof. García-Campaña at the University of Granada (Spain) in 2016 (PhD Extraordinary Award in Sciences-UGR). He then joined LABERCA (ONIRIS; France) with a postdoctoral fellowship ('Fundación Ramón Areces'; Spain) to investigate the potential of ion mobility spectrometry (IMS)-mass spectrometry (MS) for the characterization of steroids. He is currently a Marie Skłodowska-Curie fellow at LABERCA. His HAZARDOmics project aims to apply metabolomics approaches in hazard identification of bisphenol A and polychlorinated biphenyls. His current research interests are IMS-MS, liquid chromatography-MS and capillary electrophoresis-MS and their application in food safety and risk analysis.

***Author Biography (one per author)**

Dr Bruno LE BIZEC, is professor in Public health, director of French National Reference Laboratory and head of INRAE research unit within the National Veterinary College of Nantes, France. He has more than 30 years of expertise in analytical chemistry. He is the head of the CONTAM panel at ANSES. As EFSA member, he has been involved in the data collection working group for the identification of emerging risks related to food and feed (DACO). He is also JECFA expert (FAO member).

***Author Biography (one per author)**

Dr Gaud Dervilly is head deputy of INRAE research unit within the National Veterinary College of Nantes, France. For 20 years, her research activity has been devoted to Chemical Food Safety issues. She is responsible for the management of research projects related to the modelling of contaminants transfer along the food chain and the evaluation of consumer's chemical exposure. Her competences range from targeted mass spectrometric approaches to more global and non-targeted strategies, such as metabolomics, to study the effects of chemical exposure and related biomarkers discovery, in a risk assessment perspective.

***Author Image (one per author)**

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