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Review

Human exposure to chemical mixtures: Challenges for the integration of toxicology with epidemiology data in risk assessment

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ABSTRACT

Little is known about the potential adverse effects from longterm exposure to complex mixtures at low doses, close to health-based reference values. Traditional chemical-specific risk assessment based on animal testing may be insufficient and the lack of toxicological studies on chemical mixtures remains a major regulatory challenge. Hence, new methodologies on cumulative risk assessment are being developed but still present major limitations. Evaluation of chemical mixture effects requires an integrated and systematic approach and close collaboration across different scientific fields, particularly toxicology, epidemiology, exposure science, risk assessment and statistics for a proper integration of data from all these disciplines. Well designed and conducted epidemiological studies can take advantage of this new paradigm and can provide insight to support the correlation between humans low-dose exposures and diseases, thus avoiding the uncertainty associated with extrapolation across species. In this regard, human epidemiology studies may play a significant role in the new vision of toxicity testing. However, this type of information has not been fully considered in risk assessment, mainly due to the inherent limitations of epidemiologic studies. An integrated approach of *in vivo*, *in vitro* and *in silico* data, together with systematic reviews or meta-analysis of high quality epidemiological studies will improve the robustness of risk assessment of chemical mixtures and will provide a stronger basis for regulatory decisions. The ultimate goal is that experimental and mechanistic data can lend support and biological plausibility to the human epidemiological observations.

Contents

1. Exposure to chemical mixtures

The presence of chemical mixtures in the food or the environment constitutes a major health challenge deserving due attention. The scientific community has shown a great interest in assessing,

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and if possible quantifying, how combined exposures to chemicals may affect the human health (Tsatsakis et al., 2016, 2017). As shown by human biomonitoring studies, populations (including children) from distinct geographic areas are exposed to a large number of chemicals throughout their lives, with these exposures occurring at intermittent and inconsistent doses instead of at a consistent rate and dose magnitude (Nachman et al., 2011). Although not all these exposures are potentially harmful, information is needed on how to regulate and screen chemical mixtures since they might threaten the human health. Regulatory requirements for risk assessment of these mixtures are rare, except for intentional mixtures such as formulated products. Until now, little is known about the adverse effects from long-term exposure to complex mixtures at low doses, close to regulatory reference values in humans (health-based guidance values) and animals (NOAELs, used as points of departure used for derivation of guidance values). By studying the toxicity of chemical mixtures, instead of their individual constituents, it may be possible to clarify their potential role in the development of chronic diseases, particularly those with long latency periods. If so, tailored public health interventions could be implemented to prevent the potential impact of these exposures (Taylor et al., 2016; Tsatsakis et al., 2016).

2. Testing the safety of chemical mixtures for cumulative risk assessment

Prior to market entry, chemicals such as pesticides and biocides undergo a process of scientific assessment to ensure their safety for humans, animals and the environment. Despite a comprehensive and rigorous battery of regulatory toxicological studies to evaluate the safety of individual chemicals, there are growing concerns about their potential long-term effects and the appropriateness of experimental models to adequately predict human health risks. A number of methodologies have been developed for assessing the combined effects of chemicals on humans and the environment, and the most widely used for human risk assessment are based on the framework proposed by the WHO/IPCS (Meek et al., 2011), which provides tiered approaches for screening level assessments and further refinements.

Nowadays, the international scientific community as well as international regulatory authorities have started to realise the need for a cumulative risk assessment and new methodologies are being developed (EFSA, 2013a; US-EPA, 2006). In particular, EFSA started to give special attention to cumulative risks from exposure to pesticides that produce common adverse outcomes on the same target organ/system (EFSA, 2013b). However, the lack of data from toxicological studies investigating chemical mixtures represents one of the major regulatory challenges. In the European Union, the Regulation on the classification, labelling and packaging (CLP) of substances and mixtures (Regulation (EC) No. 1272/2008) transferred the responsibility of performing animal testing of commercial mixtures to industry as a last resort to prove a toxicological hazard; however, no regulatory provision has been taken for non-commercial artificial mixtures that represent the real scenario of real life exposure.

Exposure scenarios simulating real life is a complex issue because exposure to multiple chemicals may lead to a web of interactions with a wide array of underlying mechanisms that ultimately may result in diverse health outcomes (Fig. 1). In this respect, linear −monomodal, but also nonlinear, effects can be seen in the range of low and/or high concentrations of exposures (Hernández et al., 2013a). Special concerns are related to different types of toxicity, such as neurotoxicity (Baltazar et al., 2014; Dardiotis et al., 2013; Zaganas et al., 2013), cardiotoxicity (Posnack, 2014; Zafiropoulos et al., 2014), nephrotoxicity (Vardavas et al., 2016), genotoxicity (Stivaktakis et al., 2016; Tsitsimpikou et al., 2013), hepatotoxicity (Hernández et al., 2013b) and endocrine disruption (Bergman et al., 2013; Ihde et al., 2015; Mrema et al., 2013).

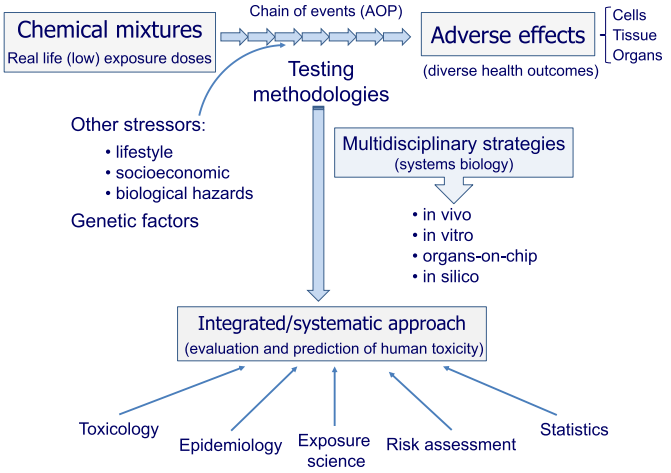

Fig. 1. Multiple hazard approach for long-term health outcomes and integrated approach of multiple lines of evidence for toxicity testing and prediction (AOP: adverse outcome pathways).

Evaluation of chemical mixture effects is considered a multifactorial task that needs an integrated and systematic approach not only for long-term scenarios but often for acute or subchronic exposures. The need for a new experimental methodology for mixture testing is intended to answer to multiple questions on health concerns related to exposure to low realistic doses that raised the attention of researchers in the field (Tsatsakis et al., 2016; Tsatsakis and Lash, 2017).

Prior papers (Docea et al., 2016; Tsatsakis et al., 2017) reported animal protocols to evaluate the cumulative toxicity of different chemical mixtures by using realistic doses following long term exposure. These experimental methodologies have the ambition to provide at one strike multi-answers to multi-questions, e.g. to study long term toxicity of non-commercial chemical mixtures consisting of common everyday life chemicals (pesticides, food additives, components of lifestyle products) at low and realistic dose levels around the human regulatory limits with the simultaneous investigation of several key endpoints like target organ toxicity and non-organ directed toxicity such as genotoxicity, endocrine disruption and oxidative stress.

Understanding exposure to real-world concentrations of chemical mixtures and their associated health effects require close collaboration across different scientific fields, particularly toxicology, epidemiology, exposure science, risk assessment and statistics for a proper integration of data from each of these disciplines (Carlin et al., 2013) (Fig. 1). Besides, there is a need to develop novel statistical approaches for the evaluation and prediction of effects associated with exposure to chemical mixtures (Taylor et al., 2016; Tsatsakis et al., 2016). However, the joint action of multiple environmental exposures is not limited to chemicals since other stressors may act also as determinants of diseases (e.g., socioeconomic status, risky behaviours including lifestyle, biological agents, etc.).

If the hypothesis of an increased hazard from cumulative exposure to chemicals around regulatory reference levels were shown to be true, this will encourage public authorities and the scientific community to shift from the single-compound risk assessment to the era of cumulative risk assessment. The next step in cumulative risk assessment will be to evaluate whether any type

of mixture effects, based either on the concept of additivity or departures from additivity (interaction), can appear among the chemicals in a mixture and how this will influence the final effect of the mixture even if its components show different adverse outcomes pathways (AOP) and act on different target organ/system (Tsatsakis et al., 2016, 2017).

3. Modern toxicity testing paradigm

Dose-dependent and time-dependent toxic responses at biochemical level involve interactions of specific chemicals with intra- and extra-cellular targets in sensitive tissues and organs. These interactions may trigger the chain of events that threaten cells integrity potentially leading to abnormalities in tissue and organ function. Diverse -omics tools can be used to assess these interactions at the molecular level, which allows for a better the understanding of toxicity mechanisms in complex biological systems. The biologically meaningful omics signatures identified by performing omics-exposure and omics-health association studies provide useful data for advanced risk assessment. This approach supports moving away from apical toxicity endpoints towards earlier key events in the toxicity pathway resulting from chemical-induced perturbation of molecular/cellular responses (NRC, 2007). Shedding light to chemically-related adverse effects on human health entails multidisciplinary strategies that encompass global systems biology aimed at developing and describing toxicity pathways based on a cascade of key events that may lead to disease states through linear cause-effect relationships. Developments in knowledge of molecular biology, cellular pathways, genetics and computer-supported modelling have resulted in a better delineation of the molecular pathways that can be disturbed after exposure to chemicals (Raunio, 2011). Thus, a better understanding of chemical-specific or common networks or nodes of interaction and key events under the AOP conceptual framework can provide the basis upon which organ-specific effects could be investigated. Novel biomarkers are indispensable tools for assessing, validating and deciphering chemical-induced processes deviating from normal cellular/tissue function and eventually leading to diseased states.

Both *in vivo* and *in vitro* experimental methodologies contribute to identifying and understanding the molecular and cellular mechanisms involved in chemical toxicity. Data from *in vivo* animal experiments are the basis of current toxicity testing of chemicals that are then used for human risk assessment. However, animal data not always parallel the human toxicokinetics and/or toxicodynamic of chemicals which hampers the extrapolation and prediction of their toxicity to humans. Animal surrogates of human diseases are being challenged by their scientific validity and translatability to humans, and the lack of correlation often found between animal data and human outcomes can be attributed to the substantial interspecies differences in disease pathways and disease-induced changes in gene expression profiles (Esch et al., 2015). *In vitro* methods (including -omics technologies such as transcriptomics, proteomics and/or metabolomics), organs-on-chips and computational toxicology are approaches that enable understanding complex biological systems and complement traditional *in vivo* studies for the identification and characterization of chemical mechanisms in humans, thereby representing the modern testing paradigm (Hernández et al., 2016a). *In vitro* systems allow examining changes at the molecular level for multiple interacting systems and may provide a means for predicting toxicity in less time than classical toxicological studies taking advantage of high-throughput screening. The disadvantage of the *in vitro* procedures is that they are mostly performed on cancerous cell lines that have a substantially abnormal function that, along with the absence of biokinetics in *in vitro* methods, may lead to a misinterpretation of the data. Another important drawback is the difficulty in the extrapolation of the *in vitro* effects to the human (Saeidnia et al., 2015).

The organs-on-chips technologies are microchips lined by living human cells that reproduce the specific dynamic microenvironments of human tissues and organs in microfluidic cell culture systems. These emerging methodologies have greater potential for studying biological mechanisms in specific tissues and organs and can anticipate toxicities at various levels of biological complexity (subcellular, cellular, tissue and organ levels). Thus, the development of organs-on-chips can predict the toxicity of chemicals in the human body more accurately than animal models and detect unanticipated off-target toxicities (Esch et al., 2015). However, there exist scientific and technological challenges for the success of organs-on-chips as these technologies require further scientific validation and characterization to define their capability for practical biomedical applications and limitations in predicting human responses to chemicals (Esch et al., 2015). Mathematical models are being developed to correlate data from organs-on-chips and *in vivo* experiments to extrapolate data to humans, including toxicokinetics and toxicodynamic models (Yum et al., 2014).

In silico toxicity assessment integrates computational methods and information technology with molecular biology to analyze, simulate and predict the toxicity of chemicals such that not only complement current toxicity tests but also allow chemicals to be prioritised for further toxicity testing (Raies and Bajic, 2016). Currently these computational methods also provide mechanistic information to explain underlying systems. As *in silico* approaches are non-testing methods, they should be followed by *in vitro* and *in vivo* data to confirm the biological activities. However, the uncertainties surrounding current *in silico* data do not preclude the use of these methods in the context of a weight of evidence approach in which additional information compensates those uncertainties. Additional drawbacks of *in silico* methods include that toxicokinetic features are not taken into account, their applicability domain are sometimes not clear, and the lack of carcinogenicity prediction for non-genotoxic compounds (Raunio, 2011; Saeidnia et al., 2015).

These various types of data can be integrated for an improved risk assessment methodology (Fig. 1), although in the future it is expected a gradual replacement of some classical *in vivo* tests by *in vitro* and *in silico* methods.

4. The use of epidemiological studies for risk assessment

Animal data have received much more attention than human data in regulatory risk assessment since regulators have clear guidance on acceptable animal toxicity studies carried out using high doses of single chemicals. However, traditional chemical-specific risk assessment may be inadequate or insufficient because humans are exposed to a large number of chemicals from environmental, dietary and occupational sources, either individually (leading to aggregated exposure) or in combination. In addition, humans may experience exposure to non-chemical stressors simultaneously, whereas most animal studies involve a single chemical stressor. This multi-chemical exposure in epidemiological studies makes it difficult to attribute health outcomes to a single compound (US-EPA, 2016).

Besides environmental factors, different lifestyles and genetic factors in the populations introduce variability in the epidemiological results, adding more difficulties and uncertainties to their interpretation (Fig. 1). Genetics may affect susceptibility to chronic disease among the subgroup of people exposed to certain chemicals. Although sustained chemical exposure over the course of

decades could initiate or accelerate the underlying disease process, if concurrent genetic risk factors are lacking the disease may not necessarily develop. This means that most cases are likely due to gene-environment interactions, which may explain why despite the large number of people regularly exposed to chemicals in the environment or though the diet, not everyone develops the disease but only those carrying a genetic vulnerability. Currently, most studies addressing gene-environment interactions are limited by the small sample size and recall bias inherent to case-control designs (Liu et al., 2012).

Epidemiological studies have shown associations between repeated occupational and environmental exposure to chemicals and specific health outcomes, including those affecting children following gestational or postnatal exposures. Despite the large volume of available research data, firm conclusions cannot be drawn for the majority of the health outcomes putatively induced by chemicals because many epidemiological studies have important weakness to substantiate causal relationships (Fig. 2). The quality of many observational studies on environmental chemicals is suboptimal as they suffer from many limitations, errors/bias and large heterogeneity of data, reflecting variability in exposure assessment, health outcomes or populations. Limitations include the lack of an accurate exposure estimate (from both a qualitative and quantitative standpoint), little information on dose-response relationship (which is difficult to achieve for diseases with long latency periods) and lack of temporal concordance (most studies are case-control or cross-sectional in design). Moreover, the inconsistency of findings across similar studies may in part be due to the problem that only few studies are adequately sized and/or have sufficiently detailed information on exposure to allow associations to be observed (Hernández et al., 2016b). For these reasons, epidemiological studies need to undergo a quality assessment to ascertain whether they meet the quality criteria required for the epidemiological review process prior to risk assessment (Fig. 2).

Despite the aforementioned limitations inherent to epidemiological studies, these are highly relevant as they provide insight into the true range of human exposures in the real world and may support the correlation between humans low-dose exposures and diseases, thus avoiding the uncertainty associated with extrapolation across species. Human epidemiology studies are considered to play a significant role in the new vision of toxicity testing proposed by the NRC (2007). However, until now the available information from epidemiological studies on chemical exposures and health effects has not been fully considered in the regulatory practices of risk assessment (EFSA, 2015).

In the last few years, regulatory agencies have started to incorporate observational epidemiological studies on long-term adverse health effects from chemical exposures in the risk assessment process. These studies, in particular systematic reviews and meta-analysis of observational studies, provide information that strengthen the understanding of the potential hazards of chemicals, exposure-response characterization, exposure scenarios and methods for assessing exposure, and ultimately risk characterization (van den Brandt et al., 2002) (Fig. 2). The application of a systematic review approach for epidemiological data assessment is needed along with defined criteria and guidelines for statistical analysis of epidemiological data. When the evidence is extensive, meta-analysis can be particularly useful in summarising the data and providing more precise estimates of effects or parameters with enhanced statistical power than individual studies. Conversely, if the evidence is scarce, systematic reviews allow knowledge gaps to be identified (EFSA, 2010).

Current systematic reviews can be used only for problem formulation and hazard identification. For this stage of risk assessment, quantified exposure data is not required. Meta-analyses are relevant for risk assessment because they increase the statistical power and precision for the effect of interest by combining the results of all individual studies available. As meta-analyses determine the size of association averaged over the considered studies, they provide a stronger basis for hazard identification. Meta-analyses would be more informative for risk assessment if they present odds ratios for a given change in the continuous variable of exposure (or per a given percentile change in exposure) as this could be informative to derive health-based reference values (Hernández et al., 2016b). In addition, this type of evidence is well suited to complement, or to integrate with, data from experimental laboratory animal studies.

While many epidemiological studies have shown associations between chemical exposures and chronic diseases, complementary experimental research is needed to overcome their limitations. The ultimate goal is that experimental and mechanistic data lend support and biological plausibility to the human epidemiological observations. However, the majority of findings identifying associations between chemicals and chronic diseases comes from studies examining health risks arising from a single chemical and the toxic mechanisms underlying these risks. In contrast, human exposures are much more dynamic and involve numerous risk modifiers including simultaneous or sequential exposure to multiple chemicals with a wide range of structures and mechanisms of toxicity, although not necessarily all of them contribute to the development of the disease. This represents an additional complexity in the understanding of to what extent epidemiological studies can contribute to risk assessment of real life exposure to numerous different chemicals through diverse pathways at generally low concentrations across the lifespan. To this end, a number of challenges remain to be addressed, such as whether an appropriate and quantitative exposure measurement to all chemicals of potential health significance can be assessed by exposomic approaches and/or traditional biomonitoring methods (with particular focus on repeat sampling to capture non-persistent chemicals). The use of broad coverage techniques such as statistical assessment of increasingly complex datasets and bioinformatics requirements for these types of data will provide greater insight on the contribution of individual components of chemical mixtures on long-term health outcomes. These approaches will also enable to link external exposures to internal biochemical perturbations (Dennis et al., 2016).

The multi-hit hypothesis supporting complex chronic diseases is based on that exposure to different chemicals may initiate a

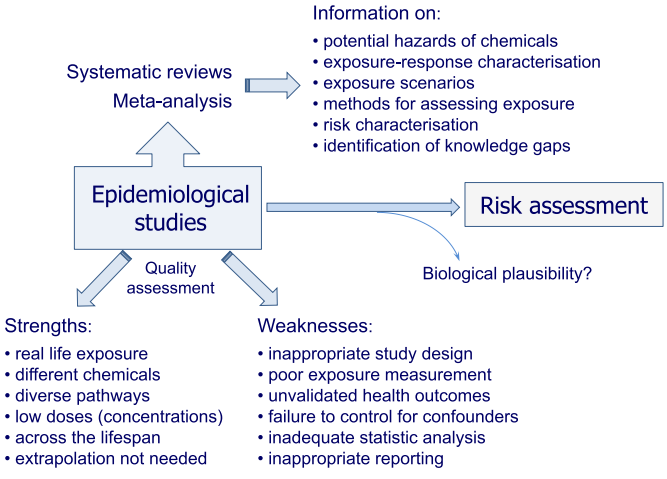

Fig. 2. Use of epidemiological studies for risk assessment.

number of toxic mechanisms that may converge later in a chain of linked events eventually leading to the final adverse outcome (EFSA, 2017). Therefore, the effect of chemical mixtures has to be considered for risk assessment (Tsatsakis et al., 2016; Tsatsakis and Lash, 2017).

5. Integrated approach of multiple lines of evidence

A number of regulatory agencies (EFSA, US-EPA, among others) have begun to develop and evaluate a new framework for testing the safety of individual chemicals, or chemical mixtures to better protect the human health and the environment.

Historically, toxicology has played a significant role in verifying conclusions drawn on the basis of hypothesis or associations generated from epidemiological findings. Thus, chemicals suggested to be involved in human diseases have been tested in animals to firmly establish a causative link. With the advent of toxicity testing guidelines and protocols, toxicology acquired a preeminent role to anticipate or predict potential adverse effects in humans, whereas epidemiology contributed to verifying or refuting these toxicological predictions (Adami et al., 2011). Because of increasing concerns about the appropriateness of experimental models to adequately predict long-term effects on human health, the coupled role of toxicology and epidemiology in discerning human toxicity of chemical agents is compelling. However, currently there is no systematic and transparent way to bring the data and analysis of the two disciplines together in a way that provides a unified view on the adverse causal relationship between exposure to chemical agents and particular diseases.

The limitations of epidemiologic observational studies (see section 4 and Fig. 2) have been often used to dismiss or reduce a potentially useful body of information. Conversely, animal data are considered a critical component of any well-conducted risk assessment, although experiments are usually conducted at unrealistic high doses of chemicals following internationally accepted testing guidelines. Human and animal data should be considered as complementary, with one stemming from controlled exposures (usually to a single substance) using experimental study design and a relatively homogeneous surrogate population, and the other reflecting the changes observed in a heterogeneous target population from mixed (and varying) exposure conditions using non-experimental study design (ECETOC, 2009).

A harmonised framework is needed for the combined assessment of epidemiologic and toxicological data. Once the best available data across multiple lines of evidence are compiled and evaluated, including *in vitro*, *in vivo* and *in silico* data, the next step is to weigh the evidence of these data using modified Bradford Hill criteria to evaluate biological plausibility (US-EPA, 2016) (Fig. 3). Establishing biological plausibility as part of the interpretation of epidemiological studies is relevant and should take advantage of modern technologies and approaches (see section 3). In this context, the AOP framework can be used as a tool for systematically organizing and integrating complex information from different sources to investigate the biological mechanisms underlying toxic outcomes and to inform the causal nature of links observed in both experimental and observational studies (EFSA, 2017; Villeneuve et al., 2014). The key events identified in AOPs can also serve as a basis for developing toxicity assays that could contribute to build-up an AOP-informed Integrated Approach for Testing and Assessment (IATA) (Fig. 3). This can be further applied for screening and prioritization of chemicals for further testing, hazard characterization or even risk assessment when combined with information on exposure and toxicokinetic data (EFSA, 2017).

The integration of epidemiologic data with experimental findings needs to comply with a number of requirements: a) epidemiologic studies should be assessed with consideration of the potential role of random and systematic errors (bias), and Bradford-Hill criteria should be applied for the evaluation of causality; b) *in vivo* and *in vitro* studies should be critically analyzed for assessing the reliability of methods and the evaluation of findings, and c) integrative evaluation of the epidemiological findings with experimental toxicology and mechanistic data in complex and multifactorial chronic diseases (Fig. 3). Once the quality, reliability and suitability of all sources of evidence have been assessed, it would be possible to proceed to the integration of such data to improve human health risk assessment of chemical substances.

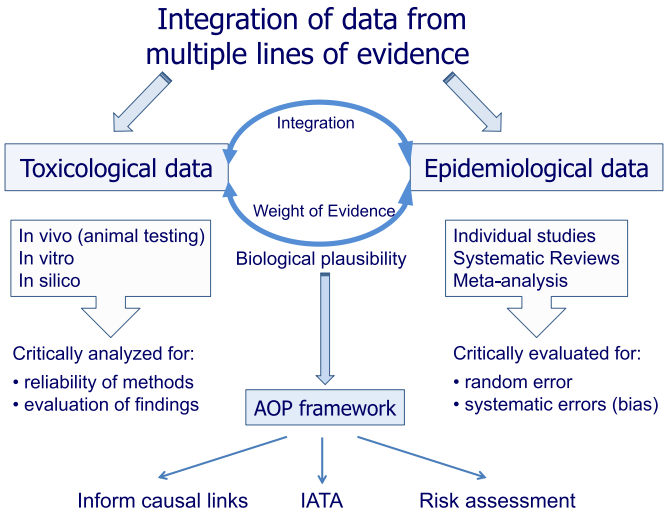

Fig. 3. Harmonised framework for risk assessment of chemical mixtures (AOP: adverse outcome pathways; IATA: integrated approach on testing and assessment).

The comparative interpretation of human and animal findings should be based on two principles: a) the stronger evidence should have precedence, irrespective of this evidence comes from human or animal data; b) where human data of high quality are available, these should take precedence over animal data for risk assessment (ECETOC, 2009). Although both lines of evidence should be considered for formal risk assessment, they must be subjected to a quality assessment and then weighted according to their concordance/discordance. Where human and animal data have equivalent quality, both dataset can be used, particularly when they are concordant as one supports another. Conversely, if human and animal data with similar equivalent quality are not concordant, the approach should be based on each stage of risk assessment, i.e. for hazard identification data suggesting a hazard should take precedence, whereas for dose-response assessment data indicating a lower safety level should take precedence. In any case, a protective approach should be followed (ECETOC, 2009).

6. Conclusion

The advances in toxicological science, molecular biology and computational methodologies have generated new and meaningful data on the mechanisms underlying chemical mixture effects that enable a better understanding of toxicity pathways and to predict the adverse effects of chemical mixtures. On this ground, the incorporation of the epidemiological perspective, which recognizes the involvement of multiple determinants in most of the adverse health outcomes, affords a high potential of being applied within a renewed risk assessment framework to better inform risk managers in the decision making process. Therefore, an integrated approach of *in vivo*, *in vitro* and *in silico* data, together with

systematic reviews or meta-analysis of high quality epidemiological studies will improve the utility and robustness of risk assessment of chemical mixtures and will provide a strong basis for regulatory decisions. The AOP framework will facilitate the development of integrated testing strategies for these purposes.

Conflict of interest

The authors declare no conflict of interest with this paper.

Transparency document

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