

European partnership on the assessment of risks from chemicals (PARC): Focus on new approach methodologies (NAMs) in risk assessment

Edited by

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European partnership on the assessment of risks from chemicals (PARC): Focus on new approach methodologies (NAMs) in risk assessment

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Editorial: European partnership on the assessment of risks from chemicals (PARC): focus on new approach methodologies (NAMs) in risk assessment

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KEYWORDS

PARC, risk assessment, NGRA, chemical safety, new approach methodologies (NAMs), alternative methods, toxicology

Editorial on the Research Topic

European partnership on the assessment of risks from chemicals (PARC):
focus on new approach methodologies (NAMs) in risk assessment

Numerous systems are in place for assessing the potential of chemical substances to cause harm to environmental or human health. However, current risk assessment approaches underlying regulatory decision-making are not adequate for dealing with the ever-increasing number of chemicals and products introduced on the market. Thus, there is an international push to innovate chemical risk assessment through integrated exposure and hazard assessment models and implementing new approach methodologies (NAMs). This next-generation risk assessment (NGRA) paradigm requires integrating and assembling elementary data, with levels of uncertainty, into robust predictions for human risk; an exercise which has proven challenging. To address this challenge, the European Union launched the European Partnership on the Assessment of Risks from Chemicals (PARC) project in 2022 (Marx-Stoelting et al., 2023, and <https://www.eu-parc.eu/>) to enhance the protection of human health and the environment through a sustainable research and innovation programme.

The PARC project, set to run for 7 years, is divided into several work packages (WPs), with WP5 focusing on hazard assessment for human and environmental health, including more than 80 partners across Europe. WP5 aims to fill data gaps for specified chemical substances of concern (e.g., bisphenol alternatives), improve NGRA approaches as well as adverse outcome pathways (AOPs) and to develop or improve NAMs for chemical hazard assessment. This Research Topic highlights the first activities of a WP5 sub-section specifically relating to the development of NAMs to improve chemical risk assessment for human health. It includes five major research projects that address immunotoxicity, non-genotoxic carcinogenicity, neurotoxicity, and endocrine disruption pertaining to metabolic disorders and the thyroid hormone system.

The first paper in this Research Topic, a minireview by De Castelbajac et al., provides an overview of the objectives and overall structure of the PARC project, with a specific focus on

WP5. It introduces the five projects, frames the context of endpoint selection, and discusses how the projects will develop NAMs and their potential regulatory impact.

The paper by [Snapkow et al.](#) describes a project aiming to develop NAMs to enhance the risk assessment of immunotoxic chemicals. Traditional methods rely heavily on animal models, such as the T-cell-dependent antibody response (TDAR) and local lymph node assays (LLNA) ([Anderson et al., 2011](#); [Lebrec et al., 2014](#)). This project aims to create new *in silico* and *in vitro* tests such as *in vitro* substitutions for the whole blood cytokine release assays (WBCRA), avoiding the need for *ex vivo* material, and an integrated testing strategy for respiratory sensitization potential. Additionally, a review by [Hargitai et al.](#) examines the current mechanistic understanding of chemical respiratory sensitization, highlighting knowledge gaps crucial for future NAMs advancements.

The next paper in the Research Topic describes a project dealing with non-genotoxic carcinogens (NGTxCs) ([Audebert et al.](#)). Unlike genotoxic substances, which induce DNA damage, NGTxCs cause toxic effects through a broad array of mechanisms, complicating the development of alternative test methods. Long-term animal studies remain necessary but have limited predictivity for human cancer risk. This project aims to develop and improve NAMs for identifying and characterizing NGTxCs and establish a reliable, efficient testing strategy for a safety assessment toolbox.

The third project ([Tal et al.](#)) focuses on developing NAMs for developmental (DNT) and adult neurotoxicity (ANT). Chemical exposures can adversely impact the development and function of the nervous system across all stages of life ([Grandjean and Landrigan, 2006](#); [Costa et al., 2004](#)). Currently, DNT/ANT data requirements in the EU typically come from *in vivo* studies under OECD test guidelines, with *in vitro* data used for Weight of Evidence assessment for classification and labelling, to trigger further DNT tests, or to support grouping and read-across from similar substances. This project aims to build on an existing DNT *in vitro* test battery that covers several cellular neurodevelopmental processes vital for normal brain development, refining the existing assays, and generating new NAMs to cover essential gaps in the battery, thereby increasing overall cost efficiency and endpoint coverage.

The last two projects aim to develop NAMs to improve the identification of endocrine-disrupting chemicals. The first addresses thyroid hormone system disruptors ([Ramhøj et al.](#)). The thyroid hormone signaling axis is a complex network of endocrine regulation, offering numerous entry points for endocrine-disrupting substances to interfere with biomolecules and cause adverse health outcomes ([Gilbert et al., 2020](#)). This project focuses on perinatal life as a sensitive window for endocrine disruption and aims to develop a battery of NAMs for human health-relevant risk assessment of thyroid hormone system disruptors. It includes activities to translate or validate assays, leveraging data from zebrafish embryos to inform human health and *in vitro* to *in vivo* extrapolations.

The second endocrine disruptor project focuses on metabolic disorders, specifically developing NAMs to identify endocrine metabolic disruptors ([Braeuning et al.](#)). Recognizing that endocrine disruption can occur through mechanisms other than the classic estrogenic, androgenic, thyroid, and steroidogenic

(EATS) modalities, this project aims to develop NAMs for non-EATS modalities, such as signaling pathways involved in cellular energy metabolism ([Heindel et al., 2017](#)). Disruptions in these pathways are thought to contribute to metabolic disorders like type II diabetes and non-alcoholic fatty liver disease. The project will enhance testing capacity by establishing methods using human-relevant stem cell models, incorporating serum-free conditions and 3D culturing.

With the focus on developing and enhancing NAMs for chemical hazard identification and risk assessment, most of the described projects also link their work to the Adverse Outcome Pathway (AOP) framework ([Ankley et al., 2010](#)). The AOP framework organizes existing biological knowledge for humans and wildlife from the perturbation by a stressor leading to an adverse effect in a structured manner, facilitating predictive toxicology capacities. The main repository for AOPs is the AOP-Wiki, a freely available open-access repository supported by the OECD. The last paper in this Research Topic ([Jaylet et al.](#)) presents a way to identify well-defined biological areas, as well as gaps within the AOP-Wiki. This approach aids in pinpointing under- and over-represented adverse outcomes to better guide prioritization for further research and development efforts.

This Research Topic of papers highlights ongoing activities within the extensive PARC partnership, specifically focusing on projects aiming to develop NAMs to enhance our capacity to identify harmful chemicals with less reliance on animals for toxicity testing. Importantly, with PARC being a 7-year project, these five projects will be finalized before the end of PARC, but with additional projects being added to the program around halfway through. These will be selected based on identified regulatory needs within the EU.

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New approach methods to improve human health risk assessment of thyroid hormone system disruption—a PARC project

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Current test strategies to identify thyroid hormone (TH) system disruptors are inadequate for conducting robust chemical risk assessment required for regulation. The tests rely heavily on histopathological changes in rodent thyroid glands or measuring changes in systemic TH levels, but they lack specific new approach methodologies (NAMs) that can adequately detect TH-mediated effects. Such alternative test methods are needed to infer a causal relationship between molecular initiating events and adverse outcomes such as perturbed brain development. Although some NAMs that are relevant for TH system disruption are available—and are currently in the process of regulatory validation—there is still a need to develop more extensive alternative test batteries to cover the range of potential key events along the causal pathway between initial chemical disruption and adverse outcomes in humans. This project, funded under the Partnership for the Assessment of Risk from Chemicals (PARC) initiative, aims to facilitate the development of NAMs that are specific for TH system disruption by characterizing *in vivo* mechanisms of action that can be targeted by *in embryo/in vitro/in silico/in chemico* testing strategies. We will develop and improve

human-relevant *in vitro* test systems to capture effects on important areas of the TH system. Furthermore, we will elaborate on important species differences in TH system disruption by incorporating non-mammalian vertebrate test species alongside classical laboratory rat species and human-derived *in vitro* assays.

KEYWORDS

PARC, endocrine disruption, thyroid disruption, non-animal test methods, regulatory toxicology, adverse outcome pathways, chemicals

1 Introduction

Within current European regulatory testing frameworks, chemical compounds that can cause adverse effects *in vivo* through thyroid hormone (TH) system disruption are primarily assessed in laboratory rodents. Assessments typically focus on gross effects on the thyroid gland itself, including changes in weight or histopathology, or by measuring levels of circulating THs (ECHA/EFSA, 2018). Although these measurements are informative, they are inadequate when it comes to identifying the broad suite of potential TH system disruptors (Noyes et al., 2019; Couderq et al., 2020; Kortenkamp et al., 2020). For instance, it is recognized that many potential TH system disruptors can act via a number of different mechanisms that are not detected by analyzing the thyroid gland itself, nor by measuring systemic levels of the THs triiodothyronine (T3) and thyroxine (T4) (Noyes et al., 2019; Gilbert et al., 2020). This is because changes in, for instance, deiodinase activity or membrane transport of THs, will not necessarily affect circulating levels of TH, but could still have a large impact on TH availability and action in target tissues (Landers and Richard, 2017; Gilbert et al., 2020; Köhrle and Frädrich, 2022). Lastly, TH-mediated adverse effects in target tissues peripheral to the thyroid gland are not addressed by current test methods, including TH-mediated developmental neurotoxicity.

By early 2023, more than 22,000 unique industrial chemicals were registered under the European REACH regulation (Registration, Evaluation, Authorisation and restriction of Chemicals) (Directorate-General for Environment, 2023). The REACH standard information requirements are based on the tonnage level of combined import and production per registrant, but the same chemical can be imported or produced by several different registrants. Based on today's information requirements, this means that *in vivo* tests that aim to determine whether chemicals possess TH system disrupting properties in mammals are not performed for chemicals at the 1–10 tonnes/year level (per registrant). From 10 tonnes and above, endpoints and biomarkers such as TH concentrations and thyroid histopathology are included in some rodent tests but, as already discussed, these are insufficient in identifying all TH system disruptors. Although pesticides and biocides (under the plant protection products or biocidal products regulations) are subject to much stronger testing requirements despite low tonnage levels, the tests, biomarkers, and endpoints are the same as for REACH registered chemicals. There is thus a pressing need to establish additional measurable endpoints or biomarkers for testing purposes including new approach methods.

Decades of research into the hypothalamic-pituitary-thyroid (HPT) axis, and the biological functions of the THs, has identified a complex network of endocrine regulation and highlighted multiple entry points for possible disruption by chemical substances. These include TH synthesis, transport and

receptor binding, as well as local tissue uptake and metabolism (Figure 1) (OECD, 2014; Noyes et al., 2019; Gilbert et al., 2020). The potential molecular initiating events (MIEs) for TH system disruptors can be tested for by various new approach methodologies (NAMs), including *in silico* approaches, *in chemico* and *in vitro* assays as well as information from chemical exposure for hazard assessment (European Chemicals Agency, 2016). Indeed, a number of newly developed NAMs have already shown great promise for future implementation in international test guideline programmes and hence are currently in the process of regulatory validation within the framework of the Organization for Economic Co-operation and Development (OECD) (Joint Research Centre, 2023). However, even with these new additions to a broader testing battery of methods relevant for assessing TH system disruption, not all critical steps between a MIE and an adverse outcome (AO) in adverse outcome pathways (AOPs) are covered.

A major challenge in developing new NAMs for TH system disruption is the lack of fundamental biological knowledge of key aspects of TH-mediated developmental processes. Thus, current efforts aimed at improving our capacity to safeguard human and ecological health from TH system disrupting chemicals should include efforts to better understand the complex biology of toxicological effects *in vivo*. And these efforts should leverage the knowledge we already possess to devise a broad NAM-based screening battery capable of identifying key events (KEs) of TH-mediated causal pathways. In this project, that we are performing under the European Union (EU)-funded Partnership for the Assessment of Risk from Chemicals (PARC, 2023) programme (Marx-Stoelting et al., 2023; PARC), we aim to provide both novel biological knowledge of how THs may regulate development and how TH system disruptors can perturb these processes, in order to establish and develop NAMs to be incorporated in regulatory decision making for the identification of TH system disruptors.

2 Background

2.1 Thyroid hormone system disruption and the developing organism

THs are involved in the differentiation, growth, and function of virtually all vertebrate tissues and organs (Yen, 2001). They play distinct roles throughout life and exert specific actions in a spatiotemporal manner through both systemic and local regulation of TH signaling. This is achieved by a fine-tuned regulation of an intricate network of TH synthesis, feedback mechanisms, serum distribution, membrane transporters, metabolizing enzymes, receptors and more (Zoeller et al., 2007; Ortega-Carvalho et al., 2016). The different components all work in concert to deliver THs to target cells where they regulate

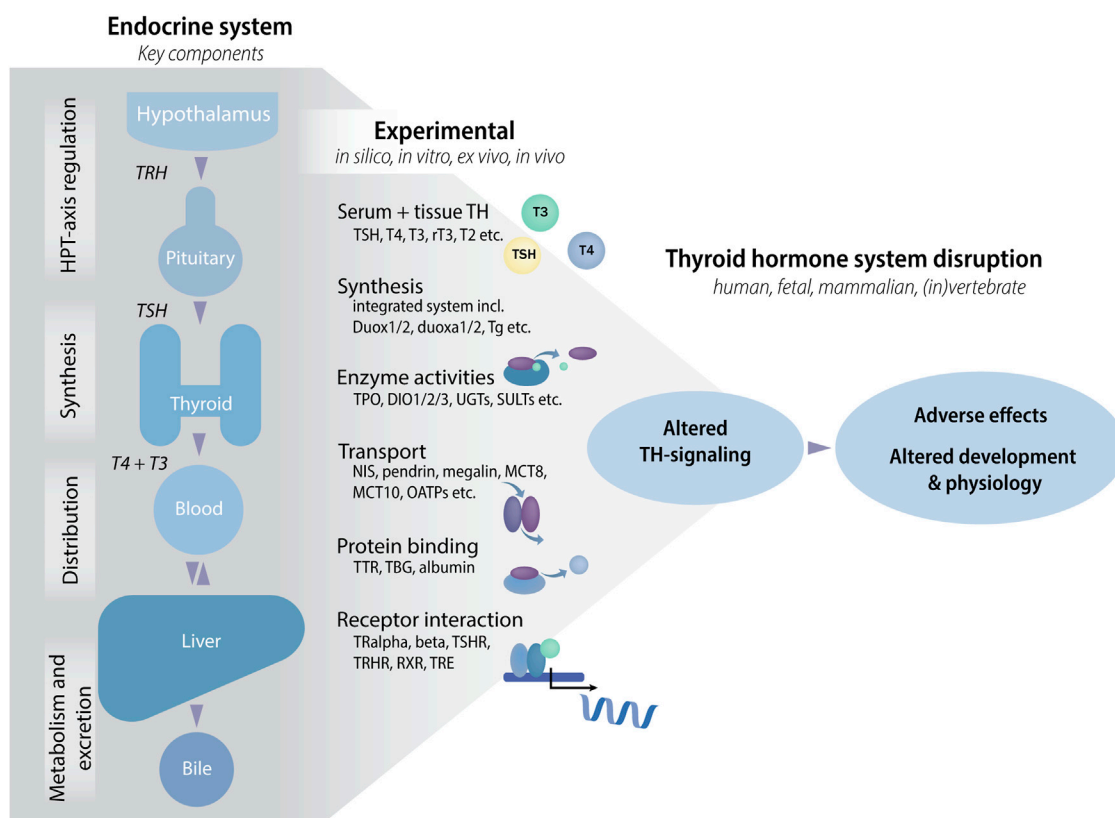


FIGURE 1

The thyroid hormone (TH) system and tests to assess chemical effects that may lead to altered TH-signaling and adverse effects. The TH system has many components and molecules that can be targeted by environmental chemicals in one and/or all tissues. Targets can be grouped into classes according to the type of assay that can be used to test for potential effects of chemicals, e.g., by developing enzyme activity assays where each assay tests the activity of one enzyme, TPO, DIO1, DIO2, DEHAL1 etc. *In vivo*, each interaction may lead to altered TH-signaling with potential adverse effects on development and physiology. Abbreviations: DEHAL1: iodothyronine dehalogenase, DIO: deiodinase, HPT: hypothalamic-pituitary-thyroid, MCT: monocarboxylate transporter, NIS: Sodium/Iodide Symporter, OATPs: organic-anion-transporting polypeptides, rT3: reverse T3, RXR: retinoid X receptor, SULTs: sulfotransferases, T2: 3,5-diiodo-L-thyronine, T3: triiodothyronine, T4: thyroxine, TBG: thyroxine-binding globulin, Tg: thyroglobulin, TH: thyroid hormone, TPO: thyroperoxidase, TR: thyroid receptor, TRE: thyroid response element, TRH: thyrotropin releasing hormone, TSH: thyroid stimulating hormone, TSHR: thyroid stimulating hormone receptor, TTR: transthyretin, UGTs: Uridine 5'-diphospho-glucuronosyltransferases.

gene expression through interactions with nuclear TH receptors (TRs) or through non-genomic mechanisms (Ortiga-Carvalho et al., 2016; Taylor and Heyland, 2022).

TH action is critical for the development of the central nervous system where THs help regulate crucial events such as neuronal differentiation, migration, synaptogenesis and myelination. Deficiency in THs during these events can thus have profound and irreversible effects on the developing brain. Worryingly, many environmental chemicals have been shown to act as TH system disruptors and can thus potentially affect brain development with significant consequences for life-long cognition (Gilbert et al., 2020; Marty et al., 2022). In this context, it is also important to consider the regulatory networks that finetune TH action in complex organisms. For instance, THs can act directly on the developing liver and control processes such as lipid metabolism, but the liver itself may also modify systemic TH regulation. Deciphering these mechanisms of effects and function will be critical if we are to faithfully capture potential TH system disrupting chemicals by use of alternative test method batteries.

2.2 Pregnant women are uniquely sensitive to thyroid hormone system disruption

The developing mammalian fetus does not synthesize THs during early stages of gestation and is therefore dependent on maternal supply at critical stages of neuronal development (Figure 2). In humans, the fetal thyroid gland starts producing THs by the second trimester, but maternal supply continues to be important throughout gestation until birth (Morreale de Escobar et al., 2004). Consequently, fetal brain development is dependent on maternal thyroid function, something that is evident from numerous epidemiological studies linking low maternal T4 levels to child neurodevelopmental effects (Henrichs et al., 2010; Román et al., 2013; Ghassabian et al., 2014; Modesto et al., 2015; Gyllenberg et al., 2016; Korevaar et al., 2016). From the second trimester of pregnancy, a correct iodine supply from the mother to the fetus is also essential, so that the fetus can produce its own THs (De La Vieja and Santisteban, 2018). Therefore, amongst other things, the presence of membrane transporters of both iodide and THs in the

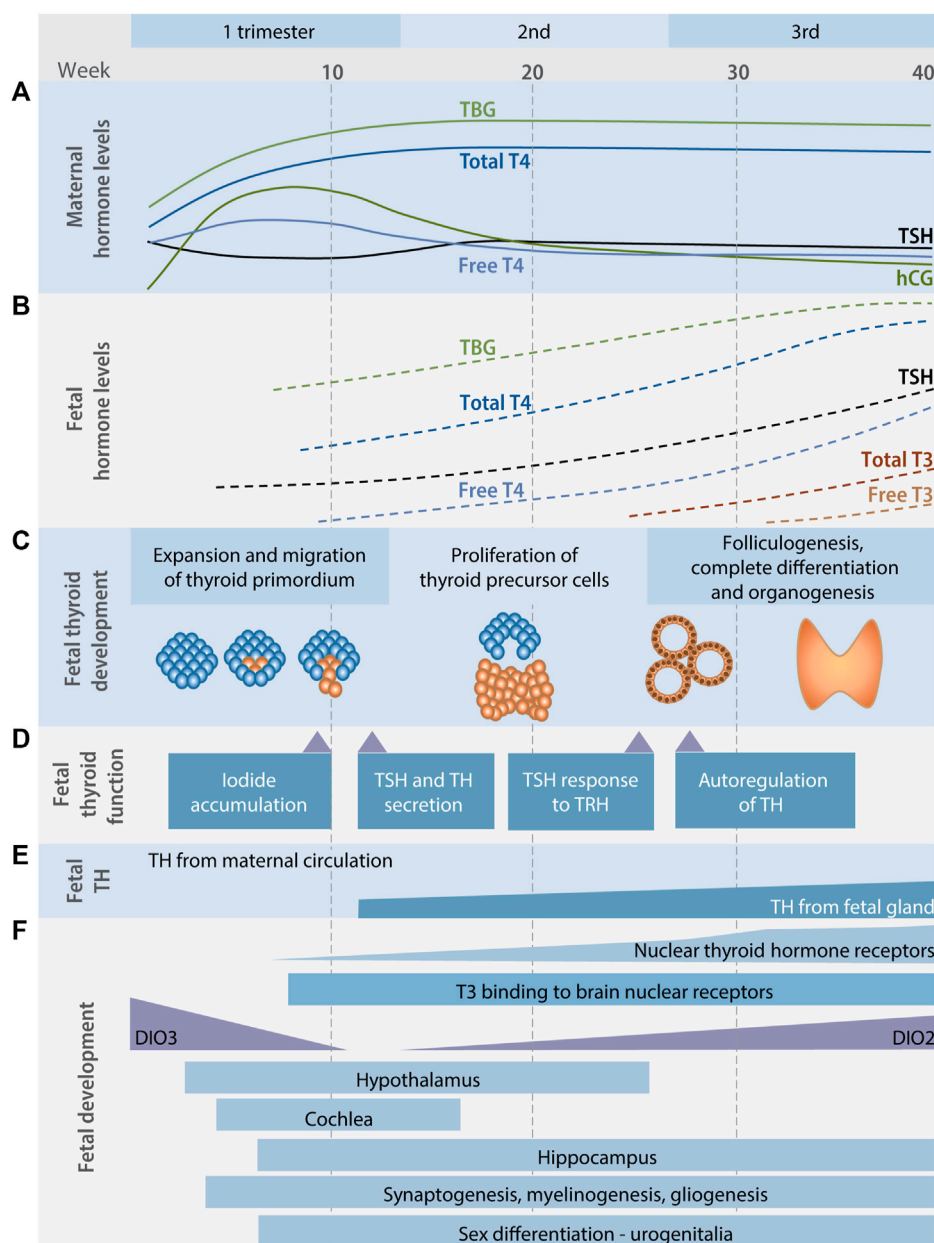


FIGURE 2

The thyroid hormone system and fetal development. Maternal (A) and fetal (B) TH levels during human pregnancy. In this period the requirement for iodine in the mother's diet increases considerably, as does the net demand for TH production by the maternal thyroid gland. Before the onset of fetal thyroid function the fetus is critically dependent on the maternal supply of TH. Timing of ontogeny (C), function and autoregulation (D) of the fetal thyroid gland during pregnancy. (E) Maternal TH fully supply fetal requirements until fetal TH secretion begins at around 15 weeks of gestation after which fetal TH levels come from both the maternal and the fetal thyroid gland. (F) Timing of some TH mediated neurodevelopmental processes in the fetal brain. Early neuronal proliferation and migration is dependent on maternal TH in the first trimester of pregnancy. Also in the first trimester, inactivating type 3 deiodinase (DIO3) enzyme expression drops and development of the thyroid gland begins. In the second and third trimesters, the brain continues to develop, now increasingly relying on T4 produced by both the fetus and the mother. As the fetal hypothalamic-pituitary axis develops, a surge in thyroid-stimulating hormone (TSH) secretion occurs, initiating fetal TH production, expression of the activating enzyme deiodinase type 2 (DIO2), and increasing thyroid hormone nuclear receptors occupancy by T3. DIO: deiodinase, hCG: human chorionic gonadotropin, T3: triiodothyronine, T4: thyroxine, TBG: thyroxine-binding globulin, TH: thyroid hormone, TRH: thyrotropin releasing hormone, TSH: thyroid stimulating hormone, (Burrow et al., 1994; Howdeshell, 2002; López-Márquez et al., 2021).

placenta throughout pregnancy is essential to maintain the correct balance of mother-fetus TH levels.

During pregnancy, both total and free T4 levels increase during the first trimester to reach a steady state that is

maintained for the remainder of the gestational period. Although these absolute changes to TH levels are relatively small, there is a net loss of THs and an increase in hormone production by the thyroid gland. This is because there is a net

increase in plasma volume as the fetus grows, so that more TH must be produced to keep steady state conditions, but also due to an increase in renal excretion of iodine that potentially reduces available iodine in the thyroid gland. Furthermore, during the first trimester, human chorionic gonadotropin (hCG) stimulates TH production in the thyroid gland and the presence of estrogen causes an increase in thyroxine-binding globulin (TBG). Later in gestation there are also changes to peripheral TH metabolism (Howdeshell, 2002; Morreale de Escobar et al., 2004). Combined, all these factors increase the net demand for TH synthesis in the thyroid gland; demands that can be met by a healthy and robust TH system, but not necessarily by a compromised TH system.

2.3 Identification of thyroid hormone system disrupting compounds

Current chemical legislation in the EU mandate the testing of potential endocrine disrupting properties for pesticides, biocides and REACH-regulated chemicals produced at high tonnage levels. These assessments typically include evaluation of estrogenic, androgenic, thyroid, and steroidogenesis (EATS) modalities using various test assays and animal toxicity studies in a tiered process (ECHA/EFSA, 2018; OECD, 2018). Of the five tiers, the first two can be performed animal-free, whereas the last three tiers require animal testing. In many ways, the EATS modalities have, to a large extent, defined the types of assays that are used to test for endocrine disruption. As already discussed, a challenge with respect to the thyroid (T) modality is that current OECD test methods remain inadequate when it comes to identifying TH system disruption and its adverse effects on the developing organism (Gilbert et al., 2020; Kortenkamp et al., 2020); not only because we do not have an adequate battery of NAMs to capture all potential MIEs and KEs, and lack sensitive *in vivo* tests for TH-mediated adversity, but also because current regulation requires *in vivo* evidence to support indicative NAMs data. Notably, the REACH §44 (1) risk concept mentions explicitly structural similarity and the relevance of transformation products, thus pointing to *in silico* (e.g., read-across, ligand-receptor docking) and *in chemico* (compound transformation to active or inactive metabolites) approaches to support compound evaluation, but the regulatory frameworks still have a way to go in incorporating non-animal test data for chemical regulation.

2.4 Current state of AOPs for thyroid hormone system disruption

TH system disruption has been identified as one of the priority areas for AOP and integrated approaches for testing and assessment (IATA) development within PARC. In this context, there is close interaction with the work on AOP development for endocrine and metabolic disruption (PARC Task 5.3.2) and the development of IATAs for endocrine disruption (PARC Task 6.1.1). All experimental data on TH system disruption that are generated in PARC are being mapped to the AOP network, either for improving or expanding existing AOPs, or for the development of new AOPs. The PARC

endocrine disruption AOP and IATA development processes are closely aligned to ensure that the methods that are considered for inclusion in the IATAs are supported by KE descriptions of well-developed AOPs. The development of TH system disruption IATAs for both human and environmental health has been initiated, and since these IATAs are based on the conservation of pathways across vertebrates, envisioned to share a number of early KE, the work on assay development and cross-species comparison and extrapolation will be highly relevant to support these activities.

At time of writing this report, there are around 30 AOP pages for TH system disruption already available in the AOP-Wiki, together including 50 linear AOPs. A limited number of AOPs have been developed to the level where they have been endorsed by the OECD Working Party on Hazard Assessment and the Working Group of National Coordinators of the Test Guidelines Programme (WPHA/WNT). This includes the AOP initiated by inhibition of thyroperoxidase (TPO) and leading to adverse neurodevelopmental outcomes in mammals (Crofton et al., 2019) and the AOP leading from inhibition of the Na⁺/I⁻ symporter (NIS) to learning and memory impairment in mammals (Rolaki et al., 2019). Five AOPs linking TPO and deiodinase inhibition to impaired swim bladder inflation in fish have recently been endorsed (Vergauwen et al., 2022a; Vergauwen et al., 2022b; Vergauwen et al., 2022c; Vergauwen et al., 2022d; Vergauwen et al., 2022e). A multitude of other AOPs describing TH system disruption with applicability to different taxa are in varying stages of development. When combining these AOPs in one large AOP network, a cross-species AOP network emerges (Knäpen et al., 2018; Noyes et al., 2019; Knäpen et al., 2020).

As a first step in using this AOP network to support cross-species extrapolation and sharing of knowledge between human and environmental health, the taxonomic domain of applicability of the MIEs and the AOs in the network was investigated in the context of altered TH levels. This effort, which was part of the Horizon 2020 ERGO project (Holbech et al., 2020), was based on both an analysis of target conservation (for the MIEs) using the US Environmental Protection Agency's Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS) tool, and empirical evidence from the literature for the MIEs and AOs. While most AOPs typically are initially developed for one species or taxonomic group, evidence was found that multiple molecular initiating events and adverse outcomes are in fact applicable across different vertebrate taxa. This analysis provides a basis for developing case studies to investigate responses to TH system disrupting chemicals across species, and the development of approaches for AOP network-based cross-species extrapolation.

3 The project: to improve and develop NAMs for human health-relevant risk assessment of thyroid hormone system disruptors

Despite previous activities at the OECD level and in various EU initiatives on the identification, development and evaluation of *in vitro* methods addressing the thyroid hormone system (OECD, 2014), we still lack key knowledge about the many MIEs and downstream KEs that can lead to TH-mediated AOs. To address

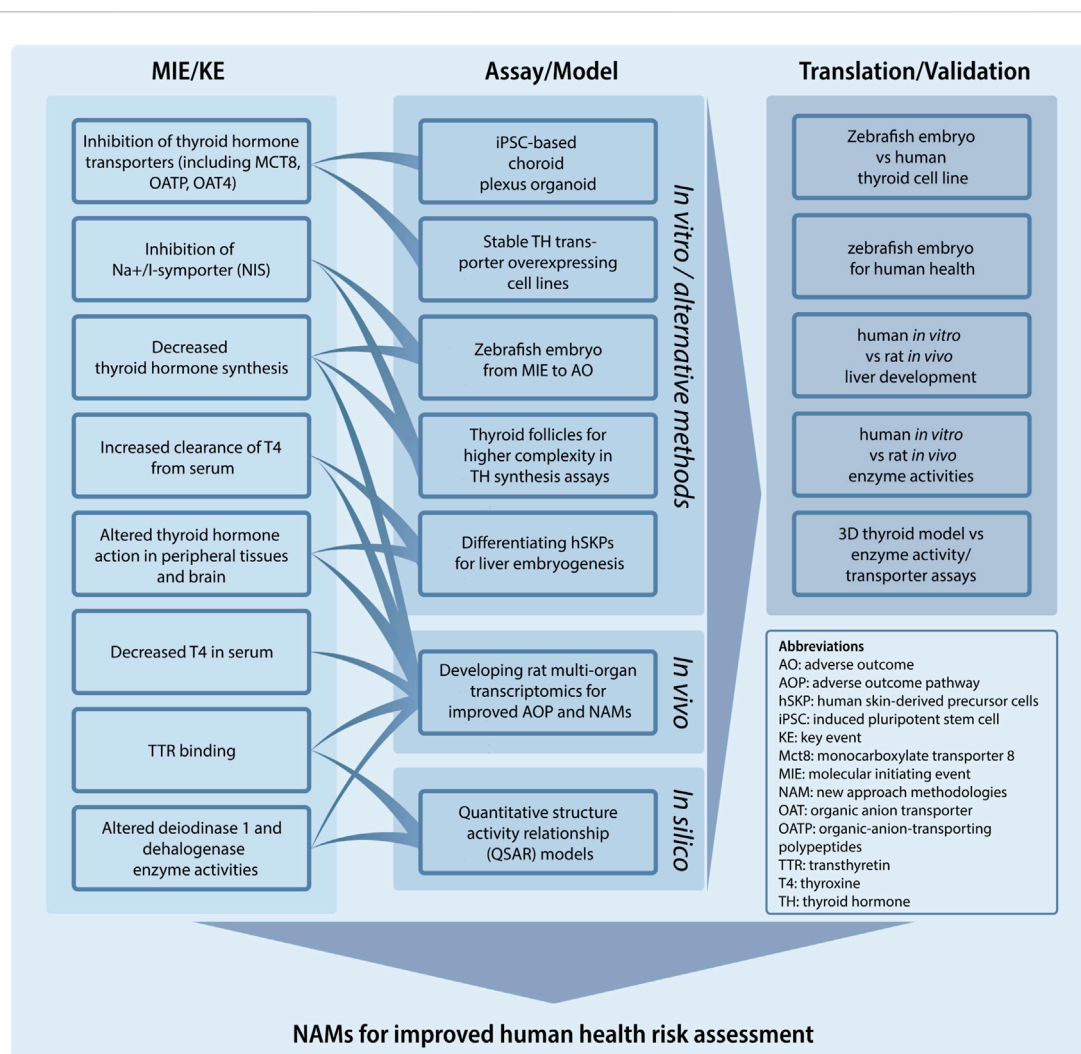


FIGURE 3

The project: New approach methodologies (NAMs) for improved human health risk assessment. Building upon the adverse outcome pathway (AOP)-framework the project develops NAMs relating to specific molecular initiating events (MIE) and key events (KE) for thyroid hormone system disruption. Some of these relations are shown. The project also includes a number of activities to translate and/or validate assays, including leveraging data from zebrafish embryos to inform on human health and *in vitro* to *in vivo* translation.

this challenge, this specific project under the PARC programme, aims to provide new mechanistic knowledge that will facilitate NAM development (Figure 3). Specifically, we will address key characteristics of TH system disruption such as TH system regulation and specific points of vulnerability to chemical disruption. This will include direct effects on the developing brain by diminished TH levels, but also effects mediated through other targets such as the liver. Thus, direct versus indirect perturbation of the TH axis by exogenous chemical substances will be scrutinized by use of sophisticated NAMs that build on, for instance, human stem cell-based *in vitro* assays. We will develop and mature *in vitro* and *in silico* tests to capture effects on key components of the TH system, such as TH transport across physiologic barriers. Importantly, we will include the characterization of evolutionary conservation of the TH system axis between rats and humans, but also mammalian and non-mammalian vertebrates (e.g., fish and amphibians) and conserved

elements of the thyroid-like system that exist in some invertebrate phyla (e.g., mollusks) to allow for additional NAMs that can inform on human health hazards. The project will focus on the following three areas and align activities with the AOP framework to fill essential data gaps necessary for improved risk assessment regimens for TH system disruptors.

3.1 From *in vivo* rodent toxicogenomics to human-relevant NAMs

We will address considerable knowledge gaps about how TH system disruptors act in the developing organism. It still remains poorly understood how different MIEs cause effects throughout the organism. This is partly because THs exert specific spatiotemporal functions during development; functions that are not well characterized. Another uncertainty relates to how chemical

exposure, TH signaling, and developmental processes interact both systemically and locally. It is recognized that disrupted hormone concentrations at the systemic level can cause varying effects locally, also for TH system disruption, as recently exemplified in rats exposed to the perfluorinated compound PFOS (Davidson et al., 2022). This means that we need to carefully consider the mechanisms or effects that we are targeting with NAMs when designing non-animal test methods for predictive toxicology purposes. We will leverage *in vivo* rat toxicity studies with multi-organ RNA-seq approaches (e.g., brain, liver, thyroid gland, heart) to decipher modes of action and characterize relationships between different MIEs, their downstream KEs and ultimately AOs. This will provide knowledge needed to pinpoint future NAMs that are necessary to cover more completely the various MIEs, pathways and effects that TH system disruption may have on the intact organism.

3.2 In vitro assays and *in silico* predictions for human-relevant risk assessment

For the *in vitro* assessment of TH system disruptors, additional focus will be on the liver. This is an important area to consider because a large proportion of chemical compounds may cause TH system disruption through effects in the liver (extrathyroidal mode of action), including per- and polyfluoroalkyl substances (PFAS), flame retardants, pesticides and food additives. With attention on the developing liver, a human-relevant stem cell-based *in vitro* model will be used to assess TH-mediated liver toxicity relevant for the early phases of development. Multipotent human skin-derived precursor cells (hSKPs) will be differentiated towards “hepatic progenitor cells” (hSKP-HPC), mimicking human liver embryogenesis (De Kock et al., 2009; Snykers et al., 2009). With this assay we aim to develop a method to characterize effects of chemicals on the developing liver, TH-signaling and its downstream effects (e.g., lipid metabolism), but also how chemicals may change the TH system in the liver (including deiodination and sulfation, nuclear receptor activation, TH distribution and transmembrane transport) and their downstream consequences for the organism. To achieve this, we will for instance use transcriptomics approaches to enable comparisons with effects observed in *in vivo* toxicity studies to scrutinize potential similarities and differences between rats and humans. Finally, through synergistic activities across PARC projects, physiological-based kinetics (PBK) modelling will further aid in the quantitative understanding of causal relationships leading to adverse effect outcomes.

In addition to the *in vitro* assays, we will develop new quantitative structure–activity relationship (QSAR) models for *in silico* prediction of chemical substances’ potential TH disrupting properties. They will be developed to expand on the existing publicly available battery of endpoints relevant for TH system disruption (see e.g., www.qsar.food.dtu.dk). QSAR models that are already finalized or under development by the PARC partner include TPO, NIS, TRs, deiodinases (DIOs), pregnane X receptor (PXR), constitutive androstane receptor (CAR), and aryl hydrocarbon receptor (AhR). This project will contribute with models for TTR binding (expansion/remodeling with other technologies of previously published model (Zhang et al., 2015)) and, to our knowledge, the

first models for monocarboxylate transporter 8 (MCT8) and iodothyrosine dehalogenase (DEHAL1) activity, and possibly more. Generation of additional experimental data to challenge and expand the predictive capacity of certain QSAR models is also planned as collaborative activities across the PARC partnership.

To address some of the complexities of the TH system, we will explore the use of a 3D cell culture to model the complex process of TH synthesis and develop assays to test for potential chemical interference with TH transport across biological barriers. We will explore a thyroid follicle model (human or animal) for its inherent characteristics, ability for TH synthesis and its predictive capacity for TH system disruption by comparison with results from various NAMs, e.g., covering NIS and TPO activities. We will develop *in vitro* bioassays to study the inhibition of TH transport across physiological barriers with a focus on the blood-cerebrospinal fluid-barrier. This is crucial since TH action depends on TH being actively transported into both cells, the fetus and the brain. To this end, an induced pluripotent stem cell (iPSC)-based choroid plexus organoid model capable of excreting a cerebrospinal (CSF)-like fluid (Pellegrini et al., 2020) will be developed and used to study TH transport across the blood-cerebrospinal fluid-barrier and to identify new TH transmembrane transporters. Then, relevant transporters will be selected to expand the battery of stable TH transmembrane transporter-overexpressing cell lines, where MCT8 currently exist and organic-anion-transporting polypeptides 1C1 (OATP1C1) and organic anion transporter 4 (OAT4) are under development. These cell lines can be used to screen compounds for their capacity to inhibit TH transmembrane transporters that not only play a role in TH transport across the blood-cerebrospinal fluid-barrier, but also across the placenta and the blood-brain-barrier.

3.3 Non-mammalian assays for human health-relevant assessment of chemicals

To further facilitate the implementation of more NAMs for human-relevant chemical risk assessment, we will assess the benefit of including non-mammalian test species such as fish embryos and invertebrates in IATAs tailored for human and ecological health parameters. Fish embryos are not protected under the current EU legislation for the use of laboratory animals until the free-feeding stage, which corresponds to 5 days post fertilization (dpf) for zebrafish kept at 28°C (European Commission, 2020). This represents an opportunity for reducing animal testing. The TH system is highly conserved across vertebrate taxa and using data from, for instance, fish to inform on potential risks to human health would greatly facilitate the transition away from high reliance on mammalian animal toxicity testing. Development of the TH system in zebrafish covers the earliest phases of embryonic development that rely on maternally transferred THs, but also the development of the entire HPT axis, which parallels human development. During the first 5 dpf the thyroid and HPT-axis develops into a fully functional organ and, importantly, is susceptible to TPO inhibition (Opitz et al., 2011; Stinckens et al., 2016; Persani and Marelli, 2017; Vergauwen et al., 2018; Walter et al., 2019). Furthermore, elements of a vertebrate-like TH signaling pathway (that is; TRs, retinoid X receptor (RXR) and enzymes involved in

vertebrate TH synthesis) have been identified in some invertebrate phyla (Taylor and Heyland, 2017) including mollusks, and both TRs and TH (of endogenous and/or exogenous origin) appear to regulate aspects of larval metamorphosis in some mollusk species (Morthorst et al., 2023). Although the exact mechanisms are largely unknown, disruption of TH signaling and metamorphosis in invertebrates may inform on potential effects in vertebrates.

We will establish new assays in zebrafish to determine the function of iodide and TH transporters. We will apply genomic and proteomic approaches to zebrafish toxicity studies to decipher the modes of action and characterize the relationships between the different MIEs and their key downstream events. These molecular analyses will be correlated to behavioral assays using zebrafish larvae to assess how potential TH system disruption affects brain development and alters behavior, for instance tail coiling to assess early motor innervation (Saint-Amant and Drapeau, 1998; de Oliveira et al., 2021), locomotor response to changes in light condition to assess the integration of the central nervous system with the peripheral nervous system and sensory organs (Berg et al., 2018), anxiety-like behaviors to assess serotonergic, dopaminergic, and adrenergic system function (Markin et al., 2021), and a habituation assay in response to auditory and light stimuli to assess non-associative learning (Roberts et al., 2013). This integration will link MIEs and KEs with AOs at the level of an intact organism. Finally, functional and proteomics data from zebrafish will be compared to data from human-based *in vitro* assays to validate cross-species extrapolation capacity.

To further investigate the potential of using data from e.g., fish to inform on potential risks to human health, zebrafish embryo assay data will be used in cross-species extrapolation case studies. We will collect TH system disruption data from different vertebrate taxa (i.e., fish, amphibians and mammals), both from the literature as well as data becoming available in PARC (see work described above), over the next years. This work will use the AOP framework, and specifically the TH system disruption cross-species AOP network and the associated evaluation of the taxonomic domain of applicability, to anchor observations across species to common toxicological pathways. This will aid the development of strategies for cross-species extrapolation, and sharing knowledge between human and environmental health evaluation.

4 Concluding remarks

As we move towards greater reliance on NAMs for chemical risk assessment, we need to close existing knowledge gaps to ensure adequate test and non-test evaluation of TH system disrupting properties. By focusing on *in vivo* rodent toxicogenomics, advanced stem cell-based *in vitro* bioassays, transporter and

zebrafish embryo investigations, concomitant with cross-species and *in silico* studies, this project, under the PARC framework, will close important gaps in knowledge and available tests necessary to facilitate further 3R approaches for a testing framework capable of adequately identifying TH system disruptors. In other words, this project aims to improve human health risk assessment of TH system disrupting chemicals to better safeguard human health and the environment.

Author contributions

LR and TS wrote the first draft of the manuscript. LR, TS and ADV made graphics. All authors contributed to conception, design of the study, and wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Development of new approach methods for the identification and characterization of endocrine metabolic disruptors—a PARC project

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In past times, the analysis of endocrine disrupting properties of chemicals has mainly been focused on (anti-)estrogenic or (anti-)androgenic properties, as well as on aspects of steroidogenesis and the modulation of thyroid signaling. More recently, disruption of energy metabolism and related signaling pathways by exogenous substances, so-called metabolism-disrupting chemicals (MDCs) have come into focus. While general effects such as body and organ weight changes are routinely monitored in animal studies, there is a clear lack of mechanistic test systems to determine and characterize the metabolism-disrupting potential of chemicals. In order to contribute to filling this gap, one of the project within EU-funded Partnership for the Assessment of Risks of Chemicals (PARC) aims at developing novel *in vitro* methods for the detection of endocrine metabolic disruptors. Efforts will comprise projects related to specific signaling pathways, for example, involving mTOR or xenobiotic-sensing nuclear receptors, studies on hepatocytes, adipocytes and pancreatic beta cells covering metabolic and morphological endpoints, as well as metabolism-related zebrafish-based tests as an alternative to classic rodent bioassays. This paper provides an overview of the approaches and methods of these PARC projects and how this will contribute to the improvement of the toxicological toolbox to identify substances with endocrine disrupting properties and to decipher their mechanisms of action.

KEYWORDS

endocrine metabolic disruption, energy metabolism, obesogens, nuclear receptors, adipocytes, liver

1 Introduction

Endocrine-disrupting chemicals (EDCs) are defined as compounds, which cause an adverse effect in an intact organism, by a toxicological mechanism involving the disturbance of parts of the endocrine system. These characteristics are widely accepted (Solecki et al., 2017) and have been adopted for defining regulatory criteria for endocrine disruptors (European Commission, 2017; 2018). In general, the adverse effects of EDCs can be detected using classic rodent *in vivo* studies, whereas validated mechanistic assays to unequivocally demonstrate an endocrine mechanism of action as the underlying cause of an observed adverse effect *in vivo* are sparse. Current validated methods to identify EDCs are mainly focused on (anti-)estrogenic and (anti-)androgenic mechanisms, as well as on interference with thyroid signaling or steroidogenesis. These endpoints are often referred to as “EATS.”

Recently, it has been recognized that in addition to EATS modalities, other mechanisms of endocrine disruption may occur. For example, one specific main adverse effect of a number of some EDCs is their capability to induce a lasting disruption of cellular pathways essential for the synthesis or degradation of endogenous compounds and metabolites. These compounds are therefore termed endocrine metabolism-disrupting chemicals (MDCs; also referred to as metabolic EDCs) and may affect, for example, signaling pathways related to cellular energy metabolism (Heindel et al., 2017). MDCs are suspected to contribute to the incidence of obesity and related metabolic disorders, such as type II diabetes and non-alcoholic fatty liver disease, in association with genetic factors, nutrition and lifestyle. Presently, over 50 million people in Europe suffer from metabolic disorders and the potential role of environmental stressors such as MDCs (either man-made or natural chemicals) is being increasingly recognized. Substantial research efforts have recently been made by the European Union to promote the screening, testing, and research on MDCs and their modes of action, for example, within the EURION cluster of EU H2020 projects. However, no suitable *in vivo* or *in vitro* tests fit for regulatory testing of metabolism disrupting effects of chemicals are presently available (OECD, 2018). Since the absence of suitable specific and mechanistic test methods prohibits hazard and risk assessment of chemicals to determine their potential for metabolic disruption, the development of such test methods has been internationally recognized as a high priority (OECD, 2012; Bopp et al., 2017; Manibusan and Touart, 2017; OECD, 2018; Audouze et al., 2020; Browne et al., 2020; Kublbeck et al., 2020; Legler et al., 2020). Screening assays for relevant nuclear receptors, which bind certain MDCs and therefore constitute an early event in the molecular pathogenesis of some metabolic disruption related pathways, are underway (Manibusan and Touart, 2017), but there is also a need for *in vitro* methods that can cover endpoints corresponding to adverse outcomes of metabolic disruption with relevance to human health. Recent endeavors in the development of such assays are part of the ongoing H2020 EU projects EDCMET, OBERON and GOLIATH, addressing important

organs, such as liver, pancreas and adipocytes. However, these assays are at different readiness levels from pre-development towards the (pre)- validation stage for some assays. In PARC, we aim to further develop these assays and complement them by new test systems addressing additional endpoints. Taken together, the chosen assays and endpoints will constitute a big step forward in the testing for metabolism-disrupting properties of chemical substances. Figure 1 provides an overview of the different sub-projects and assays and how they fit together to improve future toxicological testing strategies.

2 Stable reporter cell lines to determine the potency and efficacy of chemicals on human nuclear receptors

Environmental chemicals including bisphenols (BPA, BPS, BPF), halogenated bisphenols (TBBPA, TCBPA), chemicals used as bisphenol substitutes (BPS-IP, Pergafast 201), and their metabolites will be characterized for their activities at the peroxisome proliferator-activated receptor gamma (PPAR γ), the pregnane-X-receptor (PXR) and the constitutive androstane receptor (CAR). To this end, we will use reporter cell lines expressing the ligand binding domain (LBD) of hPPAR γ , hPXR or hCAR fused to the yeast GAL4 DNA-binding domain (DBD) (Riu et al., 2011; Grimaldi et al., 2019; Toporova et al., 2020). To characterize their retinoid-X-receptor alpha (RXR α) activities, we will use a retinoic acid receptor beta (RAR β) reporter cell line where both RAR β and RXR α ligands are active (Ren et al., 2023). These reporter cell lines were generated by a two-step transfection procedure. First, stable cell lines expressing the reporter gene were developed (Table 1). Then, the cells were transfected with the different receptor genes. These cell lines stably express chimeric nuclear receptors containing the yeast transactivator GAL4 DBD fused to LBD regions (PPAR γ , PXR and CAR reporter cell lines) or in which the DBD was replaced by the estrogen receptor alpha DBD (RAR β reporter cell line) (Table 1). In addition, the HG5LN parental reporter cell line was used as negative control. These nuclear receptor reporter cell lines are powerful tools to characterize the nuclear receptor activity of MDCs in a standardized, high-throughput screening technique. During the project, we will analyze the PPAR γ , PXR, CAR or RXR α selectivity and activity of test chemicals. Active MDCs will be compared to reference compounds (Table 1).

A precise knowledge of the binding mode of MDCs to nuclear receptors is crucial to deeply understand their activity, predict the activity of related compounds and guide the rational development of safer substitutes. To achieve this goal, we will use X-ray crystallography to solve the high-resolution structures of MDCs in complex with their receptor targets. Using this experimental approach, our past work has increased our knowledge of the mechanisms by which various MDCs can substitute for endogenous ligands and alter the normal functions of nuclear receptors, including RXR α (le Maire et al., 2009; Ren et al., 2023),

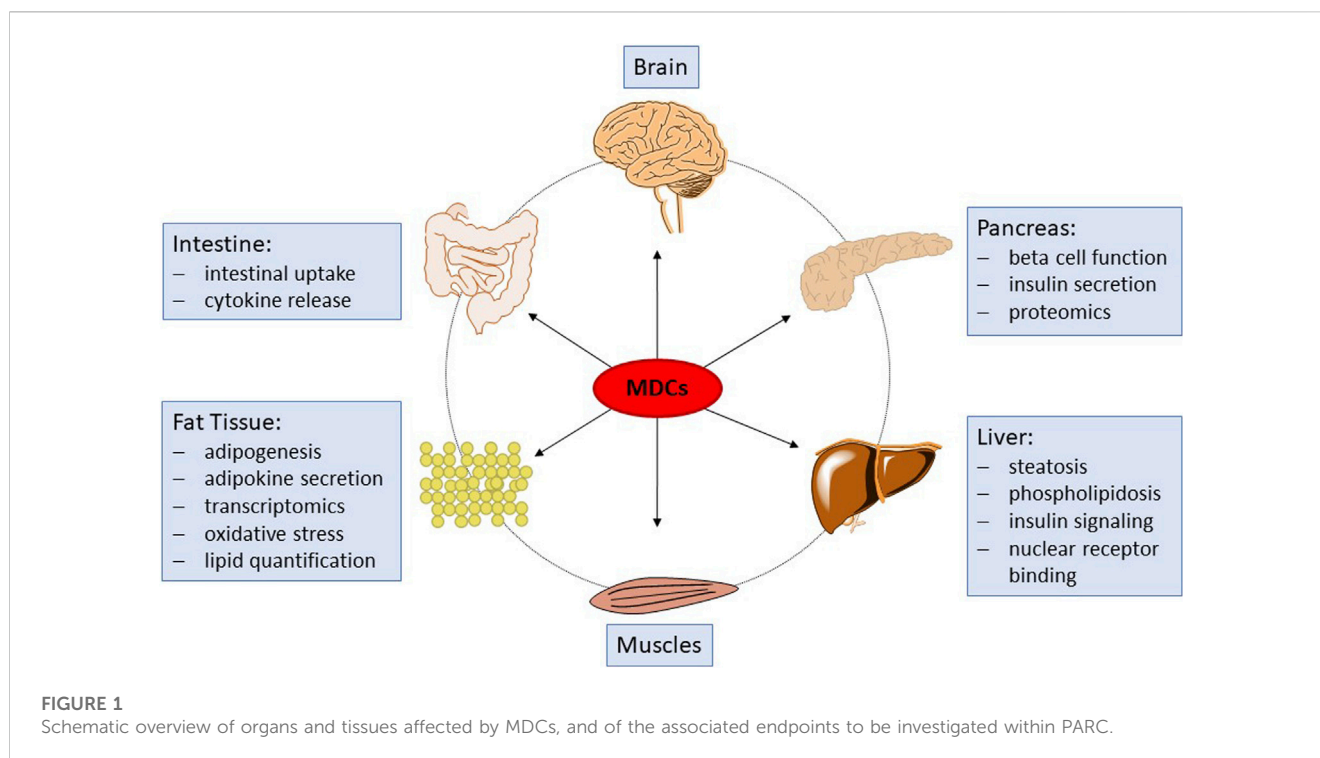


TABLE 1 Nuclear receptor reporter cell lines and model agonists/antagonists.

Reporter cell line	Reporter gene	Nuclear receptor	Reference agonist	Reference antagonist
HG5LN	GAL4RE ₅ -βGlobin-Luc-neomycin			
HG5LN PPAR _γ	GAL4RE ₅ -βGlobin-Luc-neomycin	GAL4-PPAR _γ -puromycin	Rosiglitazone	T 0070907
HG5LN PXR	GAL4RE ₅ -βGlobin-Luc-neomycin	GAL4-PXR-puromycin	SR12813	SPA70
HG5LN CAR	GAL4RE ₅ -βGlobin-Luc-neomycin	GAL4-CAR-puromycin	CITCO	PK11195
HELN	ERE-βGlobin-Luc-neomycin			
HELN RARβ (ERα DBD)	ERE-βGlobin-Luc-neomycin	RARβ-puromycin (RXRα endogenous)	CD3254	UV3003

PPAR_γ (Riu et al., 2011) or PXR (Delfosse et al., 2021). In the PARC project, we will crystallize the purified LBDs of hPXR, hCAR, hPPAR_γ and hRXRα bound to the most active chemicals identified in our cell-based assays, and elucidate the structures of the corresponding complexes with the aim to establish comprehensive structure-activity relationships.

3 Analysis of MDC effects on lipid metabolism in human liver cells

As the central metabolic organ of the body, the liver is highly exposed to MDCs and is one of their main targets. MDCs may interfere with energy, lipid and glucose metabolism pathways, leading to different manifestations of metabolic dysfunction in the liver, for example, to an accumulation of triglycerides or to phospholipidosis, an intracellular lysosomal accumulation of phospholipids with a largely unknown mechanism (Alonso-Magdalena et al., 2015).

Standardized methods to identify and characterize MDCs as well as analysis regarding metabolic functions and endpoints are still lacking. As part of the EU-funded research project EDCMET (www.uef.fi/edcmet; Kublbeck et al., 2020), a test method for measuring hepatic triglyceride accumulation (Lichtenstein et al., 2020) has been optimized in human HepaRG liver cells and is currently on its way towards formal assay validation with the PEPPER platform (Grignard et al., 2022). In order to complement this previous work, it is planned to establish a phospholipidosis assay with differentiated HepaRG cells to screen MDCs for effects on another lipid metabolism-related endpoint. The screening will focus on several substance groups such as pesticides, food additives, perfluorinated compounds, bisphenols, and MDC mixtures. As the liver also plays a major role in glucose metabolism, we want to proceed with a similar approach for establishing a glucose uptake assay in human liver cells. To understand the molecular mechanism leading to phospholipidosis, the mode of action of the most potent MDCs will be analyzed by multi-omics approaches (including

transcriptomics and proteomics) to cover key signaling pathways of cellular metabolism. The identification of essential key events by means of bioinformatics data mining approaches will help to close data gaps, to provide mechanistic insights into adverse outcome pathways and to improve the prediction of adverse effects.

Recent research indicates that 3-dimensional cell culture models and the presence of extracellular matrix components improve metabolic activity and physiological behavior (Wang et al., 2021). Moreover, in the context of metabolic disruption, it is useful to not only research isolated cell or organ types, but also to analyze organ-to-organ communication and effects related to an interplay between different tissues. Therefore, an additional aim in PARC is the development of *in vitro* models enabling cells to organize in a 3-dimensional structure which will allow the study of organ interplay in co-cultures of different cell types. Such models will be used to mimic an *in vivo*-like substance uptake and to investigate effects on transport, compound metabolism, and disruption of organ interactions.

4 Disruption of pancreatic beta cell function by MDCs

The pancreas is a crucial organ in maintaining metabolism homeostasis through the production of digestion enzymes and hormones by its exocrine and endocrine cells, respectively (Citro and Ott, 2018). Pancreatic beta cells are endocrine cells important for maintaining glucose homeostasis as they produce, store and secrete the hormone insulin, a key regulator in glucose levels (Seino et al., 2010; Marchetti et al., 2017). An impairment in pancreatic beta cell function is a characteristic of both type 1 and type 2 diabetes mellitus (Eizirik et al., 2020). Studies have shown that MDCs can destroy beta cells, thereby promoting the development of type 1 diabetes mellitus. MDCs also interfere with beta cell signaling and function, resulting in either an increase or decrease in glucose-stimulated insulin secretion. Influence on beta cell division and death have also been attributed to MDCs (Heindel et al., 2017; Legler et al., 2020). Therefore, we want to develop testing methods, which will be able to screen for MDCs' ability to affect the function of pancreatic beta cells. We have selected the Endoc-βH1 and Endoc-βH5 cell lines as a starting point. Endoc-βH1 have been shown to be a reliable human pancreatic beta cell line capable of secreting insulin as a response to glucose and expresses beta cell marker expression (Tsonkova et al., 2018). We will conduct omics analyses of gene and protein expression to elucidate the specific pathways and processes that are disrupted by MDCs by means of bioinformatics data mining. As MDCs may not only cause effects as a single substance, focus will also be placed on mixture effects. Mixture effects can alter the toxicity of substances, including but not limited to plant protection products (Großkopf et al., 2021; Feiertag et al., 2023) and can be generally grouped into additive, antagonistic, or synergistic effects.

In a whole organism, several organs are responsible for the homeostasis of glucose, including the intestine, which absorbs glucose, and also the liver, which plays a role in the regulation of glucose (Heindel et al., 2017). Therefore, we plan to create a co-culture with the Endoc-βH1 cells and liver cell lines to simulate the organ to organ communication present in *in vivo* systems.

5 Measures for obesogenic effects of MDCs

Epidemiological studies have indicated that MDCs may promote the development of obesity and metabolic syndrome, including type 2 diabetes (Heindel et al., 2017). Despite an increasing number of studies focusing on adipogenesis as an endpoint, a mechanistic understanding of chemically modulated adipogenesis and adipocyte function is still lacking, especially data gaps on a) the suitability of models for adipogenesis and b) the molecular mode of actions. These need to be filled in to develop testing strategies.

Recently, it was demonstrated that BPA and some of its alternatives bind to PPAR γ , one master regulator of human adipogenesis. Instead of activating PPAR γ , the BPA alternatives studied had an inhibitory effect on human adipogenesis in SGBS cells, which was promoted by inhibiting PPAR γ (Schaffert et al., 2021). The exposed adipocytes exhibited an inflammation-like state and dysfunctional properties, e.g., disturbed insulin receptor signaling and altered adipokine release. The BPA substitutes caused similar metabolic dysfunction to BPA and, thus, may promote metabolic dysfunction of adipose tissue, possibly involved in the development of type 2 diabetes. In contrast, in human bone marrow-derived mesenchymal stem cells (hMSCs), BPA alternatives induced adipogenesis (Norgren et al., 2022), stressing the importance to research mechanisms and variable exposure scenarios.

Previously obtained data gained mechanistic insights into how activation of nuclear receptors, like PPAR γ or RXR α promote adipogenesis, impair adipose tissue homeostasis and function, potentially resulting in the development of obesity and metabolic syndrome that increases the populations morbidity and mortality (Heindel et al., 2017). However, adverse effects mediated by stressors like bisphenols or other chemicals still could not reveal whether PPAR γ is the main molecular initiation event. Disturbed insulin receptor signaling, mTOR signaling, or glucose metabolism were observed to be altered. However, it is still not entirely understood which essential key events are triggered to mediate the adverse effect. In PARC different models for adipogenesis will be employed to fulfil the need for new or improved testing strategies for MDCs (OECD, 2012). During adipogenesis, such compounds can either directly activate or interfere with key processes such as via the activation of PPAR γ , or via less researched pathways involving for instance insulin, the mTOR pathway or glucose metabolism. Established adipogenesis models have been used to research MDCs, such as the widely used murine 3T3-L1 model (Kamstra et al., 2014; Kassotis et al., 2021), the human SGBS cell line, or primary human bone marrow-derived human mesenchymal stem cells (hMSCs) (Chamorro-García et al., 2012; Liu et al., 2019; Norgren et al., 2022). The 3T3-L1 model has been tested for robustness in previous initiatives (Christopher et al., 2021) and the hMSC model is being characterized within the EU Horizon 2020 project GOLIATH (Legler et al., 2020). Although these models have been developed using conventional culture methods, research indicates that the use of other culture methods such as spheroids might be more physiologically relevant as these models resemble *in vivo* physiology more closely (Kassotis et al., 2022).

Furthermore, present cell cultures use fetal bovine serum containing medium, sometimes up to 15%, which is not in line with the 3R principle.

In PARC, we aim to improve the current testing methods by directly employing the human-relevant SGBS and hMSCs cell models. Using hMSCs we will establish methods for culturing in 3D and in serum free conditions. This model will be fully characterized with transcriptomics analysis, and tested for reproducibility with different known MDCs. A hallmark of unhealthy obesity is inflammation of adipose tissue, often visceral adipose tissue (Kawai et al., 2021). In order to mimic this, we will also combine the model with an intestinal transwell barrier model and investigate the combination of chemical exposures and released cytokines to the health of mature adipocytes. We furthermore aim to assess the effects of BPA alternatives, their potential biotransformation products and mixtures in SGBS adipocytes and on white adipose tissue functionality (secretion of adipokines and adipogenesis) *in vivo*. Using a combined approach with phenotyping, interactome studies to decipher the molecular modes of action (Türkowsky et al., 2019), and multi-omics data to identify relevant signaling pathways affected (Großkopf et al., 2021). Data for AOP elaboration and quantitative *in vitro-in vivo* extrapolation will be delivered. Furthermore, detailed analyses of the mTOR signaling pathway, a key regulator of cellular metabolism, will be conducted to decipher the exact modes of action by which MDCs affect this pathway and contribute to the observed adverse outcome.

Our studies will enable the provision of biomarkers to support human biomonitoring, and the work carried out in these projects will initially support hazard assessment and ultimately integrated risk assessment of chemicals, and will include adipogenesis and obesity as further endpoints.

6 Zebrafish-based assays to detect metabolism-disrupting chemicals

In this project, different zebrafish life stages are explored as a vertebrate model for metabolic disruption, allowing extrapolation to human health effects. Many endocrine pathways/axes, including receptors, targets, feedback loops, etc. are well conserved across vertebrate taxa. Although there are some differences between zebrafish and humans that are relevant in a metabolic context, such as absence of certain nuclear receptors (RAR β , LXR β and CAR) (Schaaf, 2017), the zebrafish is considered a valuable model for metabolic disruption in human health (Zang et al., 2018). Several studies have used different life stages of zebrafish as a model to study the impact of chemical exposure on the development of metabolic disorders (Tingaud-Sequeira et al., 2011; Riu et al., 2014; den Broeder et al., 2017; Sun et al., 2019; Martinez et al., 2020; Nakayama et al., 2020; Tian et al., 2021). At present, the zebrafish obesogenic test (ZOT) is the most advanced test method for investigating metabolic disruption in the zebrafish model (Tingaud-Sequeira et al., 2011). In this test, larvae are raised on control diet until they reach a length between 7.5 and 9 mm (3–4 weeks post fertilization). The experimental treatment consists of 1 day of feeding with a high-fat diet, followed by 1 day

of starvation, and 1 day of aquatic exposure to the compound of interest. The endpoint of this test is a whole-body adiposity measurement using Nile Red staining. Among others, this test has been shown to be responsive to known PPAR-modulating compounds (Riu et al., 2014). In general, more insight into the mechanisms and effects of metabolic disruptors can be gained when different life stages, exposure scenarios and endpoints are used.

We will focus specifically on zebrafish early-life stages in order to explore different potential strategies to assess mechanisms and effects of metabolic disruptors. We will investigate responses to metabolic disruption in two different life stages, applying different exposure strategies and measuring a set of relevant endpoints. First, zebrafish embryos will be used. They are not protected under the current EU legislation on the use of laboratory animals until the age of free-feeding, which is at 5 days post fertilization when kept at 28°C (European Commission, 2012). On one hand, this is an advantage in terms of reducing animal testing and on the other hand the absence of exogenous feeding prohibits dietary intervention. There is, however, an added layer of complexity related to the fact that the liver and digestive system develop during the exposure period. Secondly, larval assays like the ZOT allow for dietary interventions, including diets with altered composition as well as for dietary exposure to compounds. We aim to expand upon the ZOT's basic principles in terms of life stage used, exposure duration, and endpoint complexity. Custom fish feeds such as a western diet (high fat, high cholesterol, high carbohydrate content including fructose) are under development based on previous experiences (Gabriels et al., 2019). Endpoints with relevance to human health will be explored, such as the condition factor (corresponding to body mass index), whole organism lipid staining and quantification, oxidative stress and transcription of genes related to endocrine disruption and energy metabolism. A case study with exposure to bisphenol A and some of its alternatives is envisioned, based on PARC priority areas.

An embryonic zebrafish metabolic disruption test would be very valuable since it would combine the advantages of a whole organism assay with the facts that it is considered an alternative to animal testing and it is a new approach methodology (NAM). Larval feeding trials such as the ZOT are animal tests. However, as also suggested by Tingaud-Sequeira (2011) in relation to the ZOT, zebrafish early-life stage metabolic disruption testing could be an intermediate step in a tiered approach between *in vitro* assays and rodent tests. As such, this approach would aid in reducing mammalian testing.

7 Summary and outlook

As detailed above, PARC will substantially contribute to the field of metabolic disruption by chemicals. This will especially comprise the collection of data for important groups of endocrine active compounds, e.g., bisphenols, as well as the development and implementation of new *in vitro* or non-rodent *in vivo* test methods to identify and characterize MDCs. Moreover, the metabolic disruption-related projects in PARC

will elucidate molecular mechanisms of toxicity and thus contribute to the improvement of AOP networks.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

AB: overall coordination and manuscript finalization. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor TV declared a shared research group PARC PROJECT: European Partnership on the Assessment of Risks from Chemicals (PARC) with the authors at the time of review.

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Corrigendum: Development of new approach methods for the identification and characterization of endocrine metabolic disruptors—a PARC project

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A Corrigendum on

Development of new approach methods for the identification and characterization of endocrine metabolic disruptors—a PARC project

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In the published article, there was an error. In **Section 5** an erroneous entry was introduced, indicating an opposite effect of what was shown in the reference referring to that entry.

A correction has been made to ‘5 *Measures for obesogenic effects of MDCs*’, *paragraph 2*. This sentence previously started:

“In contrast, in human bone marrow-derived mesenchymal stem cells (hMSCs), BPA alternatives attenuated adipogenesis (Norgren et al., 2022), stressing the importance to research mechanisms and variable exposure scenarios.”

The corrected sentence appears below:

“In contrast, in human bone marrow-derived mesenchymal stem cells (hMSCs), BPA alternatives induced adipogenesis (Norgren et al., 2022), stressing the importance to research mechanisms and variable exposure scenarios.”

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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New approach methodologies to facilitate and improve the hazard assessment of non-genotoxic carcinogens—a PARC project

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Carcinogenic chemicals, or their metabolites, can be classified as genotoxic or non-genotoxic carcinogens (NGTxCs). Genotoxic compounds induce DNA damage, which can be detected by an established *in vitro* and *in vivo* battery of genotoxicity assays. For NGTxCs, DNA is not the primary target, and the possible modes of action (MoA) of NGTxCs are much more diverse than those of genotoxic compounds, and there is no specific *in vitro* assay for detecting NGTxCs. Therefore, the evaluation of the carcinogenic potential is still dependent on long-term studies in rodents. This 2-year bioassay, mainly applied for testing agrochemicals and pharmaceuticals, is time-consuming, costly and requires very high numbers of animals. More importantly, its relevance for human risk assessment is questionable due to the limited predictivity for human cancer risk, especially with regard to NGTxCs. Thus, there is an urgent need for a transition to new approach methodologies (NAMs), integrating human-relevant *in vitro* assays and *in silico* tools that better exploit the current knowledge of the multiple processes involved in carcinogenesis into a modern safety assessment

toolbox. Here, we describe an integrative project that aims to use a variety of novel approaches to detect the carcinogenic potential of NGTxCs based on different mechanisms and pathways involved in carcinogenesis. The aim of this project is to contribute suitable assays for the safety assessment toolbox for an efficient and improved, internationally recognized hazard assessment of NGTxCs, and ultimately to contribute to reliable mechanism-based next-generation risk assessment for chemical carcinogens.

KEYWORDS

non-genotoxic carcinogens, NGTxC, new approach methodologies, NAM, PARC

Introduction

Cancer is one of the leading causes of death in Europe, with nearly 3 million new cases and 1.3 million deaths per year (www.ecri.jrc.ec.europa.eu) (Dyba et al., 2021). The importance of cancer prevention is recognized in the EU's Chemicals Strategy for Sustainability Towards a Toxic-Free Environment, as well as in Europe's Beating Cancer Plan (EC, 2023). These ambitious programs are challenged by the fact that cancer is a highly diverse and complex disease that affects various organs and involves multiple steps and mechanisms of progression that vary with cancer type and tissue affected. This complexity is also reflected in the different functional capabilities that cancer cells must acquire during malignant transformation, as described by the concept of "hallmarks of cancer" (Hanahan, 2022). Moreover, the vast majority of cancer-related deaths occurs at the late stages of carcinogenesis (i.e., invasive growth and metastasis) (Lyden et al., 2022).

The multiple causes of cancer can be divided into endogenous risk factors, comprising genetic predisposition, aging, hormone levels, stem cell division and DNA replication infidelity, and exogenous risk factors like (natural) radiation, lifestyle, infections and exposure to environmental chemicals. Environmental chemicals causing cancer can be grouped into genotoxic and non-genotoxic substances (NGTxCs). In a recent report by the European Environment Agency, it is estimated that such exogenous risk factors are responsible for a not negligible part of cancer cases (EEA, 2022). Interestingly, the International Agency for Research on Cancer (IARC) estimates that 10%–20% of human carcinogens classified as Class 1 may act as NGTxCs (Hernandez et al., 2009). A comprehensive knowledge of the carcinogenic potential of substances is urgently needed to minimize exposure and thus the risk of cancer. Genotoxic chemicals interact directly with DNA or interfere with the cellular machinery that maintains genomic integrity, leading to mutations and/or chromosomal alterations. In contrast, NGTxCs promote tumor formation through a variety of mechanisms and pathways without evidence of genotoxicity (Jacobs et al., 2020). To classify carcinogenic chemicals, the concepts of key characteristics (Smith et al., 2016; Guyton et al., 2018; Smith et al., 2020; Madia et al., 2021) or the hallmarks of environmental insults (Peters et al., 2021) have been proposed to link carcinogenic modes of action and the hallmarks of cancer concept.

In the different regulations, carcinogenicity is evaluated in long-term (2-year) exposure studies with rodents, in combination with *in vitro* and *in vivo* genotoxicity testing (OECD, 2016a; OECD, 2016b; OECD, 2018a; OECD, 2018b; Madia et al., 2019; OECD,

2022a; OECD, 2022b). However, long-term exposure rodent bioassays are time-consuming, costly, ethically questionable, and considered of limited relevance for humans. Indeed, some mechanisms induced by NGTxCs are potentially confounding biological and physiological species differences (Heusinkveld et al., 2020a). Since less than 5% of chemicals registered worldwide have been tested in long-term carcinogenicity rodent studies (Doe et al., 2019), there is an urgent need for a transition to new approach methodologies (NAMs). This allows for the inclusion of human-relevant *in vitro* and *in silico* methods that enable using recent advances in our understanding of diseases and employ the latest non-animal methods available in a modern safety assessment toolbox.

NGTxCs may have multiple modes of action, including epigenetic alterations, endocrine modulation, immune suppression, or continuous stimulation of inflammatory responses, sustained proliferation and migration (Hernandez et al., 2009). However, various carcinogenic processes are not exclusively cancer related, since they are also involved in non-cancer processes. In addition, tissue-specific effects must also be considered (Bianchi et al., 2020; Madia et al., 2021; Arner and Rathmell, 2022; Doe et al., 2022).

In 2020, the European Commission adopted its Chemicals Strategy for Sustainability (EC, 2020) with the main objective of promoting innovation for safe and sustainable chemicals, and improving the protection of human health and the environment from hazardous chemicals. This led to the establishment of the EU-funded Partnership for the Assessment of Risk from Chemicals (PARC) (Marx-Stoelting et al., 2023). This partnership consists of several sub-projects and sub-tasks. In the current sub-task project, we aim to provide appropriate assays for efficient and improved hazard assessment of NGTxCs, in order to support a mechanism-based next-generation risk assessment (NGRA) of chemical carcinogens.

To achieve this goal, a consortium of 13 partners across Europe has been established within the PARC project. Each partner contributes with its own expertise and knowledge in assay development that are compiled into a comprehensive strategy to identify and predict the carcinogenic potential of NGTxCs. The selected assays address a range of endpoints relevant to human carcinogenesis, and cover a broad spectrum of MoA that fit into the concept of hallmarks of cancer.

The objective: improve and develop NAMs for the identification and the characterization of NGTxCs and the establishment of a reliable and efficient testing strategy for development of a safety assessment toolbox for NGRA.

TABLE 1 Overview of the different cell types, complexity levels, exposure scenarios and endpoints addressed by the various partners of the NGTxC project. A = acute, R = repeat.

Partner	Cell/Tissue models	2D/3D	Treatment	Endpoints								
				Genomic instability	Proliferation	Cytotoxicity	Oxidative stress	NR-activation	Migration	Phenotypic changes	Inflammation	Epigenetic changes
ANSES	Liver	2D/3D	A/R		•	•	•	•		•	•	•
BfR	Breast, Liver, Colon	2D	A/R		•	•		•	•	•		
INRAE	Multicellular liver	3D	A/R	•	•	•	•			•	•	
INSERM	Breast, Adipocytes	2D/co-culture	A		•	•			•	•		
IRFMN	NA	NA	NA	•				•				
NIB	Liver	3D	R	•	•	•	•	•		•		•
NILU	Breast	2D/3D	A		•	•	•				•	
RECETOX	Liver	2D	A		•	•						
RIVM	Zebrafish	NA	A/R		•		•					
RPTU	Colon	2D	A/R	•	•	•	•		•			
UL-LACDR	Breast, Liver	2D	A		•	•	•	•	•	•	•	
UNAV	Liver	2D/3D	A/R	•		•	•					

ANSES: french agency for food, Environmental and Occupational Health and Safety, BfR: german federal institute for risk assessment, INRAE: national research institute for agriculture, Food and the Environment, France INSERM: national institute for health and medical research, France, IRFMN: istituto di ricerche farmacologiche mario negri, Italy, NIB: national institute of biology, Slovenia, NILU, the climate and environmental research institute, Health Effects Laboratory, RECETOX: research centre for toxic compounds in the environment czech republic, RIVM: national institute for public health and the environment, the Netherlands, RPTU: Rheinland-Pfälzische Technische Universität, Germany, UL-LACDR: leiden academic centre for drug research, the Netherlands, UNAV: University of Navarra, Spain.

In light of the limitations of the 2-year rodent bioassay, which under some regulations is the required test for the identification and characterization of carcinogens, there is an urgent need for an efficient and reliable *in silico* and *in vitro* testing battery. Several past and ongoing European research initiatives, not exclusively dedicated to NGTxCs detection, aim to develop new *in vitro* toxicity test methods. These include the EU-ToxRisk and RISK-HUNT3R projects as well as the ASPIS (<https://aspis-cluster.eu/>) and EURION (<https://eurion-cluster.eu/>) project clusters. Over the last years, a number of new and improved methods have become available that might play a pivotal role in the development of a regulatory acceptable integrated approaches for testing and assessment (IATA) for NGTxCs. In 2020, an OECD expert group developed an IATA for NGTxCs and published a consensus paper describing the overarching IATA, with the molecular initiating events (MIE) of cellular metabolism and receptor interactions, followed by the early key events (KEs) of inflammation and immune dysfunction, mitotic signaling, and cell injury, leading to (sustained) proliferation, morphological transformation and tumor formation (Jacobs et al., 2020). Several assay evaluations and reviews have been conducted and published in relation to epigenetics (Desaulniers et al., 2021), cell transformation (Colacci et al., 2023), gap junction (Sovadinova et al., 2021), gene signaling (Oku et al., 2022), cell proliferation and oxidative stress (Veltman et al., 2023). These publications provide details on the next steps needed to facilitate developments that will have regulatory relevance for the safety assessment of NGTxCs.

Our project aims to provide a comparison of the different selected methods to check how they could be applied and how they may complement in the context of an IATA. In this context, the project can also help to improve knowledge on the relationships between specific toxicity mechanisms and adverse outcome pathways (AOPs) leading to cancer. AOPs correspond to chains of events starting from a MIE, then describing all the steps involving KEs at different biological levels (cellular, tissue, organism) leading to an adverse outcome (toxic impact or pathology). For the *in vitro* identification of NGTxCs, it is mandatory that the developed methods cover MIEs and/or KEs associated with hallmarks of cancer. In this context, AOPs can be a useful framework for mapping aspects of these complex relationships of carcinogenesis.

In this project, the partners provide a wide variety of methods using model systems of increasing complexity, from 2D human cell lines to advanced 3D human systems and zebrafish embryos models. These innovative human-relevant NAMs focus on organs (liver, breast and colon) commonly affected by chemical carcinogens, and include state-of-the-art transcriptomic approaches, cell painting, high-content image analysis and high-throughput-compatible reporter systems. The assays address well established and relevant mechanisms involved in carcinogenesis, (Table 1). The challenge here is to sufficiently capture the high complexity of NGTxCs triggered carcinogenesis with a manageable testing battery covering key features of human cancer (Table 1) (Hanahan, 2022). Moreover, factors supporting tumor initiation and progression as oxidative stress, activation of nuclear receptors and (tissue-specific) cytotoxicity leading to continuous regenerative proliferation are important mechanisms, but do not count to the hallmarks of cancer themselves. Excessive oxidative stress is known to play an important role in tumorigenesis by

causing oxidative DNA damage that can directly lead to mutations. On the other hand, oxidative stress can also stimulate proliferation and epithelial-mesenchymal transition (EMT), leading to cancer progression through metastatic processes, and can be associated with inflammatory responses and cytotoxicity (Heusinkveld et al., 2020a; Hayes et al., 2020).

Interestingly, methods based on Nrf2 gene reporter cell lines, a key mediator of oxidative stress response, as well as a direct assay for measuring reactive oxygen species (ROS) content have already been accepted by the OECD as test guideline (TG) TG 442D and TG 495, with a focus on skin sensitization and phototoxicity, respectively. Estrogen receptor activation is a common therapeutic target, closely linked to the proliferation of endometrial, colorectal, prostate and breast cancer, but also associated with metastasis, EMT and epigenetic changes (Garcia-Martinez et al., 2021). Similarly, the role of AhR-mediated functions in cancer development is not limited to the generation of carcinogenic metabolites, but also feeds into various other cancer-related processes, including cell migration, cell transformation, inflammation, and epigenetic processes (Larigot et al., 2018; Larigot et al., 2022). Finally, sustained cytotoxicity has been shown to be an important mechanism of action for carcinogenic chemicals, particularly inducing regenerative proliferation (Hernandez et al., 2009). Thus, the endpoints addressed by the consortium partners are related to several hallmarks of cancer. As the same endpoint is addressed by different methods, their respective applicability will be established by comparing the responses of appropriate reference substances. Moreover, the comparison will also investigate the relevance of cell models and type of exposure methods in effects of carcinogenic chemicals. The variety of models and methods in this project should facilitate the development of an efficient and reliable test battery to identify NGTxCs and analyze their responses. Experimental approaches will be complemented by the optimization of *in silico* tools for the identification of NGTxCs.

A large set of reference compounds (positive or negative for carcinogenicity), including some prioritized PARC compounds (toxins and bisphenol analogues), will be tested, allowing chemical specific comparison of the different methods. The number and chemical classes of reference chemicals to be tested for the various mode of actions will depend upon the availability of reliable human-relevant data, including rodent bioassay data. The sensitivity and specificity of the various systems as well as their complementarity in detecting the different mechanisms involved in non-genotoxic carcinogenicity will be supplied.

Next, we will describe in more detail the methods applied in the project by the individual consortium partners.

ANSES: High content analysis of oxidative stress, nuclear receptor translocation, epigenetic markers, inflammatory responses, and mitochondrial metabolism in liver cell models

High-content analysis (HCA) is the application of automated high-resolution microscopy combined with quantitative image analysis for cell-based or organoid-based assays. This multiparametric image-based approach is capable of quantifying

a large number of features at single cell and population levels. In addition, the high throughput nature of the approach enables a large number of chemicals or endpoints to be tested in a single experiment. Furthermore, HCA can be used for the analysis of live and fixed cells, which makes this approach an important *in vitro* method for toxicological and mechanistic studies needed to predict NGTxCs (Li and Xia, 2019). Through the use of antibodies or fluorescent probes specific for cellular processes, this system is well suited for the detection of different hallmarks of cancer, including oxidative stress, nuclear receptor translocation, epigenetic markers, inflammatory responses, and mitochondrial metabolism. HCA therefore represents a promising *in vitro* approach to detect NGTxCs. In this context, a HCA approach to assess potential mode of action of NGTxC compounds using the human bipotent progenitor HepaRG cell line in 2D and more complex 3D spheroid models will be applied (Marion et al., 2010).

BfR: Identification of NGTxCs by phenotypic screening for carcinogenic effects on cell adhesion, migration, proliferation, and mitochondrial dysfunction

The BfR will apply phenotypic screening methods in order to investigate and group compounds according to their modes of action. The first model, called E-morph assay, is based on ER- α expressing MCF7 breast cancer cells and evaluates the changes in adhesion junction (AJ) organization as an indicator for anti-estrogenic or estrogenic effects (Kornhuber et al., 2021). The Estrogen-dependent changes in the AJ directly influence cell stiffness and motility, two functions that are highly relevant for cancer progression, in particular in respect to progression from benign to malignant tumors or metastasis and address the relevant endpoints of phenotypic changes-migration and nuclear receptor activation at the same time. By applying a cytoplasmic stain on the parental cells or by using an E-Cadherin-EGFP-expressing reporter cell line (de Beco et al., 2009; Klutzny et al., 2022), the appearance of the plasma membrane can be quantified by high content imaging. Depending on the assay set-up specific competitive compounds can be included allowing to screen for (anti-) estrogenic or (anti-) glucocorticoid compounds.

In our second model, cell-painting techniques (Bray et al., 2016) will be applied in order to generate compound-specific morphological profiles. In this assay, the breast cancer cell line MCF7 and the non-tumor cell line hTERT-HME1 are labeled with 6 different stains, followed by high content imaging and computational image analysis. The extraction of up to 50 features (e.g., size, shape, intensity, texture) allows the generation of compound-specific data. The direct comparison of a rather normal, immortalized cell line with a cancer cell line might also facilitate the detection of specific effects on the process of tumor progression, also in respect to proliferation, oxidative stress and cytotoxicity. Based on the assumption that compounds with similar profiles share common modes of action (Fetz et al., 2016), the applicability to identify carcinogenic compounds will be evaluated. Within this project, it will also be tested if the classical cell painting set up or another dye-set allowing the detection of different cellular structures can contribute best in the discrimination of such

compounds. In parallel, the effects on the composition of the extracellular matrix (ECM) will be analyzed. Changes in the composition of the extracellular matrix have long been known to play an important role in the activation of “silent” neoplasia (Bissell and Hines, 2011) and, thus, a thorough analysis of chemically induced changes in ECM composition will also provide a better mechanistic insight into the effects of potential carcinogens on cellular migration and metastasis.

INRAE: Distinguishing genotoxic carcinogens from NGTxCs

In the EU, carcinogenicity studies are required for new active substances in the pesticides (1107/2009) and biocide (528/2012) regulations, whereas they are only required for high tonnage industrial chemicals under REACH (1907/2006). They can be waived if no evidence for hyperplasia or pre-neoplastic lesions are detected in repeated-dose studies and the substance is found non-mutagenic in genetic toxicity tests. Cancer has long been known to be induced by mutations, a molecular process that led to the development of the classical battery of genotoxicity tests available to detect chemicals that induce gene mutations (OECD TG 471, 476, 490) and chromosomal aberrations (OECD TG 473, 484). Although the sensitivity of this battery of the current regular genotoxicity assays is high, the specificity is rather low leading to a high false-positive rate compared with *in vivo* data, especially with the mammalian cell-based assays.

The methodology proposed by the INRAE team allows an evaluation of genotoxicity using two biomarkers, phosphorylated histones H2AX (γ H2AX) and H3 (pH3), specific for clastogenic and aneugenic chemicals, respectively. The γ H2AX biomarker is also related to genomic instability, is detected in cancerous cells and clinically used in cancer biology (Palla et al., 2017). The γ H2AX/pH3 method was developed and pre-validated on 2D cell models and demonstrated a predictivity higher than 90% (Kopp et al., 2019). Moreover, some NGTxCs chemicals were detected with this method, indicating that this assay may permit to detect genotoxicity of some NGTxCs not revealed previously through the regular OECD genotoxicity assays (Kopp et al., 2019). In the PARC project, the assay will be further developed with a 3D multicellular liver model. The spheroids will include different cell types: the HepaRG hepatoma cell line, Kupffer cells and hepatic stellate cells (Yan et al., 2021). Repeated chemical treatment over numerous days will be used to take into account the real human exposure more efficiently. Additional specific biomarkers of cytotoxicity, oxidative stress, proliferation and inflammation will be included to discern some specific chemical toxic MoA. In the same idea, cell painting experiments will be performed on this 3D model to detect phenotypic changes induced by the compounds.

INSERM: Analysis of cell migration

The methodology proposed by the METATOX team (Inserm T3S) allows the real-time monitoring of proliferative, morphologically and migratory capacities of breast cancer cells in a context that mimics the tumor microenvironment. Additionally, a change in the cellular

morphology provides a qualitative indication of potential cytotoxicity (to be then confirmed using other specific tests). The system is based on the co-culture of MCF7 human breast cancer cells and hMADS human pre-adipocytes in multi-well plates equipped with a permeable membrane insert that allows communication between the two cell types. We already demonstrated that by exposing this model to Seveso dioxin or a mixture extracted from cigarette smoke, the phenotype of the cancer cells was modified, with the setting up of a process suggesting an epithelial-mesenchymal transition (EMT), the first step in the formation of metastasis (Koual et al., 2021; Benoit et al., 2023). Changes in cell phenotype (morphology and migration) can be followed in real-time using the xCELLigence system. In case of modification upon exposure to a chemical, morphological changes can be further characterized using microscopy and expression profile analysis for biomarkers of tumor progression taking proliferation and cytotoxicity into account. This model has the great advantage of mimicking the early stages of metastatic cell formation and appears relevant to study the influence of NGTxCs that may promote metastasis, which is involved in up to 90% of cancer mortality.

IRFMN: In silico identification of NGTxCs

At the basis of the *in silico* models there is the possibility of anticipating the activity of a compound starting from its chemical structure. It is known that, in fact, certain physico-chemical properties, such as the melting point, are related to the chemical structure. Similarly, certain types of toxicities are predicted with QSAR (Quantitative Structure-Activity Relationship) models, too. As initial steps, we collect information on the feasible *in silico* models related to carcinogenicity (Benigni et al., 2008; Fjodorova et al., 2010; Benfenati and Gini, 2013; Benigni et al., 2013; Golbamaki et al., 2016; Toma et al., 2020) and associated endpoints, such as genotoxicity (Roncaglioni et al., 2008; Baderna et al., 2020; Van Bossuyt et al., 2020) and nuclear receptor activity. Indeed, there are many QSAR models freely available, for instance within the platform of *in silico* tools, VEGAHUB (<https://www.vegahub.eu/>). These tools can be used on the selected compounds, to evaluate the carcinogenicity potential and the binding to specific nuclear receptors (<https://www.vegahub.eu/portfolio-item/vega-nrmea/>), for instance. The analysis of the predictivity of the *in silico* approach towards this kind of biological activity will be examined in depth developing new *ad hoc* models starting from big sets of data (e.g., ICE-Integrated Chemical Environmental of NTP) related to the key characteristics of carcinogens. An additional activity is using the tools for the so called read-across approach (<https://www.vegahub.eu/portfolio-item/vera/>) (Vigano et al., 2022). In this case, the model explores the similarity between the target substance and related compounds with experimental data.

NIB: High Content analysis of genotoxicity, epigenetic changes, AhR activation, stress response and cell proliferation in liver spheroids

A multi-labeling approach using specific antibodies and stains and the use of flow cytometry to simultaneously detect multiple

endpoints in the same cell and cell population represents a HCA tool. The effects of chemicals, including NGTxCs, on cell proliferation will be studied by detecting fluorochrome signals for proliferation markers (e.g., Ki67, PCNA) and by analyzing the distribution of cells in the cell cycle. In addition, other hallmarks of carcinogenesis will be investigated simultaneously, including genomic instability by detecting DNA damage (γ H2AX marker) reflecting clastogenic activity, and mitotic cells (histone H3-positive cells) reflecting aneugenic activity, oxidative stress, nuclear receptor activation (AhR), and epigenetic modifications of histones (e.g., H3K27me3, H3K9ac, and H3K9me2) that serve as predictors of carcinogenicity (Stampar et al., 2022). The effects on cell growth and proliferation will be assessed using the MTS assay.

Toxicogenomics, the application of gene expression analysis techniques in toxicological studies, identifies global changes in gene expression associated with a toxicological outcome and contributes to the refinement of toxicity and carcinogenicity testing. It provides supporting mechanistically based data on the molecular pathways underlying the apical effects (Ellinger-Ziegelbauer et al., 2005). Recently, gene expression profiling has been proposed as a useful tool for hazard identification and human health risk assessment, providing not only qualitative, but also quantitative information related to relevant mechanisms of action induced by the stressors (Corvi and Madia, 2017). It is well known that stressors with similar biological activities (e.g., oxidative stress inducers, genotoxic carcinogens/NGTxCs) can deregulate the expression of specific genes, which can therefore be used to distinguish different molecular mechanisms of action of the stressors under study (biomarkers of exposure or effect). Using a combination of multi-labeling and toxicogenomics approaches, the mechanisms of action of NGTxCs will be investigated in a hepatic *in vitro* 3D cell model (spheroids) developed from human hepatocellular carcinoma (HepG2) cells. The advantage of spheroids over traditional monolayer cultures is that they more closely resemble tissue cell organization and therefore better mimic the *in vivo* microenvironment and provide more predictive data for human exposure (Stampar et al., 2020).

NILU: *In vitro* cytotoxicity, oxidative stress (ROS), proliferation and inflammatory markers

Breast cancer (BC) is one of the most common cancer type in the world and the second leading cause of cancer deaths among women (Bray et al., 2018). Exposure to environmental chemicals and pollutants have been linked to BC incidence (Kortenkamp, 2006; Bonefeld-Jorgensen et al., 2011). BC mortality is associated mainly with the development of metastases (Ruscitto et al., 2022). Therefore, identification of the mechanisms involved in metastasis formation is of high importance. The signaling pathways involved in metastatic tumor cells emergence and progression are increasingly linked to exposure to environmental chemicals and pollutants (Koual et al., 2020; Gabet et al., 2021; Kay et al., 2022). Oxidative stress is one of the best documented mechanisms for carcinogenesis, together with impacts on cell cycle progression (Martínez-Reyes and Chandel, 2021; Dharshini et al., 2023). Environmental pollutants may induce cell damage by

induction of ROS and oxidative stress, which can trigger an inflammatory response which may also evolve into a chronic inflammation (Dharshini et al., 2023). Inflammation has been linked to various steps in tumorigenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis. NILU's contribution to this project will focus on breast cancer and breast cancer cell models. We will study oxidative stress and ROS production as an early KE in carcinogenesis, as well as induction of relevant pro-inflammatory cytokines (ELISA) (Esquivel-Velazquez et al., 2015). Further, we will study cell proliferation. One of the promising advanced *in vitro* cellular models which mimic more closely the *in vivo* situation, is the 3D spheroids models. Different cell lines with features representing different BC subtypes, JIMT-1, MDA-MB-231 and T-47D have been used to establish advanced 3D multicellular aggregates. NILU will focus on one of these cell lines and apply the above mentioned endpoints. Both 2D and 3D models of JIMT-1 cells will be used, and their sensitivity compared. In addition to the use of cancer cell lines, the use of normal cell lines should also be considered, e.g., the epithelial MCF-10A cell line. These cells are subline of spontaneously immortalized human breast epithelial cells derived from human fibrocystic mammary tissue with characteristics of normal breast epithelium, which make the MCF-10A a valuable *in vitro* model to study normal breast cell function and determine the potential of environmental chemicals, including NGTxCs, to induce tumor transformation.

RIVM: Analysis of oxidative stress responses in zebrafish

RIVM's contribution to this project is focused on oxidative stress. Oxidative stress, resulting from an imbalance between the generation of oxidants and their scavenging by antioxidants, may lead to sustained cytotoxicity and regenerative proliferation and is therefore a MoA relevant for carcinogenesis (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011; Leuthold et al., 2019; Hanahan, 2022). Oxidative stress can be induced through endogenous processes, but also upon exposure to chemical substances (Heusinkveld et al., 2020b; Veltman et al., 2023). This is known to promote carcinogenesis through DNA damage and impaired repair, as well as through indirect actions influencing homeostasis and signaling (Klaunig, 2018). Additionally, ROS play a role in numerous stages of the multistep carcinogenic process (Weinberg et al., 2019).

RIVM will use zebrafish embryos as whole-organism model for measuring oxidative stress for a diverse set of substances. The added value of this model for toxicity testing of both single substances and chemical mixtures, as well as MoA identification has been shown by us and others (Leuthold et al., 2019; Heusinkveld et al., 2020b; Bauer et al., 2021; Van Der Ven et al., 2022). Various parameters for oxidative stress will be measured, including ROS levels, lipid peroxidation and changes in gene expression. For this purpose, both fluorophore-based techniques in wild-type zebrafish and transgenic reporter lines are available, responsive to activation of the Nrf2 pathway allowing for microscopic determination of oxidative stress on a multi-organ level in live embryos. The rationale for using zebrafish embryos is to generate data in a

more complex test system, complementary to other models available to the project. In particular, the zebrafish can provide data for higher-tier key events in AOP approaches (organ-organism levels) that are not covered by (complex) cellular models.

RECETOX: Analysis of intercellular communication

The RECETOX team will explore the applicability of the scrape loading-dye transfer (SL-DT) assay (Dydowiczova et al., 2020) assessing gap junction intercellular communication (GJIC) *in vitro* using rodent and human liver cells to distinguish between known NGTxCs and non-carcinogens. GJIC is a vital process in multicellular animals, facilitating the exchange of ions and molecules between cells and coordinating tissue actions (Nielsen et al., 2012; Zefferino et al., 2019). Disruption of GJIC has been a well-recognized but overlooked hallmark of NGTxCs for a long time (Trosko et al., 2004; Aasen et al., 2016; Jacobs et al., 2016; Zefferino et al., 2019; Jacobs et al., 2020). In the recently proposed IATA for identification of NGTxCs, GJIC has been linked to pivotal key events of mitogenic signaling and cell injury (Jacobs et al., 2020), thus related to the endpoints of cell proliferation and cytotoxicity. However, there are no validated methods for GJIC assessment. While several methods can be used to evaluate the dysregulation of GJIC, many have limited throughput and require specialized equipment (Sovadinova et al., 2021). The SL-DT assay is the most commonly used *in vitro* method for evaluating the impact of toxicants or carcinogens on GJIC with a proven potential for high-content analysis and screening (HCA/HCS) (Dydowiczova et al., 2020; Sovadinova et al., 2021). The SL-DT assay has been widely employed to study alterations in GJIC caused by hundreds of chemicals, including NGTxC. Overall, the sensitivity of the SL-DT assay to predict IARC carcinogens (Group 1, 2A, or 2B) is 77% (Sovadinova et al., 2021). However, most of the published data were generated using a rat liver epithelial cell line (WB-F344), and negative compounds (non-carcinogens) have not been widely assessed. These issues will be addressed in this project by testing a common set of reference compounds using the multiparametric SL-DT assay allowing assessment of GJIC along with cell proliferation and cytotoxicity (Dydowiczova et al., 2020). The results obtained in WB-F344 cells will be validated the results using human cells. This will help evaluate the potential of the multiparametric SL-DT assay as a screening/testing tool for NGTxCs within integrated testing approaches.

RPTU: Identification of NGTxCs using human colon cell models

Non-malignant human colonic epithelial cells (HCEC) (Roig et al., 2010) and HCT116 human colorectal cancer (CRC) cells (Ahmed et al., 2013) have already being used to study dietary chemical carcinogens, bacterial toxins as well as drug candidates in the context of CRC (Mimmeler et al., 2016; Seiwert et al., 2017; Dorsam et al., 2018; Seiwert et al., 2020; Arnold et al., 2022). To identify NGTxCs, a test battery covering the following endpoints will be applied: cell proliferation, formation of ROS, EMT, cell

migration and genotoxicity. The latter endpoint is based upon the sensitive detection of γ H2AX as established DNA damage marker (Kinner et al., 2008) using InCell Western or immunofluorescence methodology, which will help to distinguish genotoxic from non-genotoxic compounds. Increased cell proliferation is a well-described hallmark of cancer (Hanahan, 2022) and known to be triggered by various NGTxCs. Effects on cell growth and proliferation will be first assessed using the Alamar Blue cell viability assay (Vieira-da-Silva and Castanho, 2023), allowing for extensive concentration-range finding studies in a high-throughput manner. This will be complemented by the 5-ethynyl-2'-deoxyuridine (EdU) assay, which measures the incorporation of EdU into nascent DNA during replication (Salic and Mitchison, 2008) and thus correlates with cell proliferation. EdU incorporation is visualized via Click chemistry using fluorescent azides, such as 6-fluorescein azide, followed by fluorescence microscopy or flow cytometry in low-to-medium throughput.

The induction of oxidative stress will be analyzed as a further endpoint using the cell-permeable probe CM-H₂DCFDA, which is converted intracellularly by ROS into a fluorescent dye (Kalyanaraman et al., 2012). This can be measured in a multi-well plate reader using an additional Hoechst staining for normalization to cell counts. Depending on its level, spatiotemporal formation and chemical nature, oxidative stress contributes to carcinogenesis via both non-genotoxic and genotoxic mechanisms (Sies and Jones, 2020). Another important endpoint in the context of metastasis is EMT. This process involves the activation of specific transcription factors, leading to the reorganization of the actin cytoskeleton and degradation of extracellular matrix as a prerequisite for migration and invasion (Kalluri and Weinberg, 2009). Here, the F-actin will be stained using a fluorescent phalloidin probe, which will be combined with the detection of E-cadherin as a marker of epithelial morphology that decreases upon EMT. Both markers are then visualized by fluorescence microscopy, but may alternatively be measured using a multi-well plate reader to adjust to more samples.

Finally, cell migration as pivotal step during the metastasis of cancer cells will be studied using the so-called wound healing assay that is also termed scratch assay (Vang Mouritzen and Jenssen, 2018). To this end, confluent cells with a scratch/gap are exposed to the compound of interest and cell migration is then monitored as gap closure via microscopy (endpoint measurement), thus somewhat limiting the number of samples to be analyzed.

UKHSA: Augmentation of the CYP induction test method for use in the NGTxC IATA and validation management for the cell transformation Assay using transcriptomics: the transformics assay

UKHSA will provide input on the further development of the HepaRG CYP induction test method (Bernasconi et al., 2019) (Jacobs et al., 2022), to support the NGTxC IATA (Jacobs et al., 2020) and will be implicated for the (pre)validation of promising test methods that address the outstanding needs as identified in the work of the OECD NGTxC IATA expert group, specifically here in relation to cell transformation (Colacci et al., 2023) and gap

junction (Sovadinova et al., 2021) in the first instance. A regulatory framework for the IATA for NGTxC developed with ECHA will also be provided (Louekari et al. paper in prep), this will assist in supporting the regulatory targeted assay development.

UL-LACDR: High-Throughput screening for oxidative stress as well as mitogenic and inflammatory signaling

UL-LACDR focuses on quantitative, mechanism-based, and human-relevant hazard characterization, employing an AOP/AON conceptual thinking in direct relation to the hallmarks of cancer concepts and taking advantage of high-throughput modular assays using automated confocal microscopy and high throughput transcriptomics. This allows for testing redundancy and validation of methodologies whilst permitting constant improvement and innovation of integrated testing strategies. The approach will follow a tiered testing strategy that includes high-content 2D and 3D cell-based imaging platforms for next-generation risk assessment of NGTxCs. In the context of this project, the focus will be on mammary gland and liver, two of the most critical target organs for chemically induced carcinogenesis in 2-year rodent cancer bioassays (Sistare et al., 2011). Pivotal key events in the proposed integrated approach to testing and assessment (IATA) of NGTxCs (Jacobs et al., 2020) will be addressed, in particular oxidative stress, inflammatory signaling and mitogenic signaling. Since several known human NGTxCs target estrogen receptor signaling, fluorescent BAC reporters for ER α -induced proliferation and cell cycle progression in human ER α -positive MCF7 breast cancer cells will be included (Duijndam et al., 2021; Duijndam et al., 2022), as well as a panel of CRISPR-based endogenously tagged fluorescent human induced pluripotent stem cell reporter lines that can be differentiated to relevant lineages including progenitor cells of mammary epithelial cells and hepatocytes. These reporters cover diverse critical cellular signaling responses that are essential in cancer development. Using validation sets of chemical compounds with known toxic effects, the reporter lines will be evaluated with high content live cell confocal imaging for sensitivity and specificity in the detection of specific types of stress-response in different target organ lineages, in 2D or 3D. This includes the analysis of general markers of cytotoxicity and effects on proliferation, migration and other cellular phenotypes. The imaging-based approaches will be complemented by high throughput targeted mRNA sequencing as well as high throughput single-cell sequencing using TempO-Seq (<https://www.biospyder.com>) or BART-seq (Uzbas et al., 2019), to get broader insight in AOP activation and thresholds of carcinogenic events.

UNAV: Detection of repairable DNA lesions induced by NGTxCs

Genotoxic carcinogens are compounds that give a positive response in classical batteries of genotoxicity testing, which includes *in vitro* and *in vivo* assays that mainly detect point mutations or chromosomal aberrations [e.g., ICH (ICH, 2008)

and EFSA (EFSA, 2011)], and in the *in vivo* 2-years carcinogenicity studies. Compounds that induce DNA lesions that are completely repaired may not be detected by the genotoxicity battery.

On the other hand, NGTxCs are compounds that induce tumors in the *in vivo* carcinogenicity studies but are negative in the aforementioned genotoxicity testing batteries. However, the current regulatory genotoxicity testing strategies are mainly focused on point mutations or chromosomal aberrations. A key question is whether the presence of repairable DNA lesions could be a marker for some NGTxCs. To explore this option the detection of DNA lesions, also called pre-mutagenic lesions, that cause genomic instability, will be carried out *in vitro* using different versions of the alkaline comet assay.

The standard version of the alkaline comet assay detects DNA strand breaks and alkali labile sites (ALS), such as abasic sites, at a single cell level (Collins et al., 2023). The assay is used in genotoxicity testing, in both *in vitro* and *in vivo* models. The *in vivo* version is included in the ICH and EFSA genotoxicity testing strategies (ICH, 2008; EFSA, 2011); its OECD test guideline was published in 2014 and reviewed in 2016 (OECD, 2016c). Indeed, this is the only assay included in the standard testing strategies that does not detect point mutations or chromosomal aberrations, but DNA strand breaks and ALS.

On the other hand, the comet assay has been modified to detect specific DNA lesions (Collins et al., 2023) such as altered bases and it is widely used for the detection of oxidized bases, a marker of oxidative stress. Oxidized DNA bases can be induced directly by a compound or indirectly through a physiological toxic response. In PARC the alkaline comet assay will be used in its standard version but also combined with the enzyme Fpg for the detection of oxidized purines and with the enzyme hAAG for the detection of alkylated DNA bases (Muruzabal et al., 2020; Muruzabal et al., 2021). These three versions of the assay will be applied in 2D and 3D HepG2 human liver cells after acute and repeated exposure. Cytotoxicity will be also measured in the same exposure conditions.

Concluding remarks

In the course of this project, a wide variety of already established methods will be optimized to test the effects of NGTxCs on different KEs known to be involved in carcinogenesis. In addition, new methods and NAMs will be developed and tested to complement existing methods with respect to the various modes of action of NGTxCs. Other work packages from PARC focus on the development of IATAs and AOPs for genotoxic and NGTxCs. It will be important to align this work with the efforts of the OECD that has established an expert group to evaluate the currently available NAMs suitable for the published NGTxC IATA (Jacobs et al., 2020). Most importantly, several PARC projects are addressing toxicological endpoints that are directly related to (non-) genotoxic carcinogenesis, in particular immunotoxicity, inflammation and metabolic endocrine disruption. In the future, NAMs developed in these specific PARC projects as well as new methods currently being developed in EU Horizon 2020 projects, in particular in the EURION and ASPIS clusters, but also methods

that are already established and are undergoing validation, will be taken into account. It is important to ensure that there is sufficient overlap in the selection of compounds tested to allow, at a later stage, a comprehensive analysis of various combinations of these methods in testing strategies. In combination with the assays developed in additional PARC and Horizon 2020 projects, test methods for several of the defined hallmarks of cancer will be (further) developed. Comprehensive coverage of all the hallmarks of cancer will not be possible in the PARC project alone or even in cooperation with ongoing EU projects, including processes such as de- or trans-differentiation or the influence of the microbiome.

This project provides a unique opportunity to directly compare a wide variety of methods and approaches, and the results will feed into other projects and work packages to support the development of AOPs and IATAs, in close cooperation with parallel activities in Europe and the OECD. This work will facilitate the development of more efficient and human-relevant assessment of chemicals for potential non-genotoxic carcinogenic activities by developing NAMs for identification of NGTxCs, mapping KEs for further developing AOPs and IATA for carcinogenicity, and contributing to the safety assessment toolbox to support NGRA of carcinogens (Bischoff et al., 2020).

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Innovative tools and methods for toxicity testing within PARC work package 5 on hazard assessment

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New approach methodologies (NAMs) have the potential to become a major component of regulatory risk assessment, however, their actual implementation is challenging. The European Partnership for the Assessment of Risks from Chemicals (PARC) was designed to address many of the challenges that exist for the development and implementation of NAMs in modern chemical risk assessment. PARC's proximity to national and European regulatory agencies is envisioned to ensure that all the research and innovation projects that are initiated within PARC agree with actual regulatory needs. One of the main aims of PARC is to develop innovative methodologies that will directly aid chemical hazard identification, risk assessment, and regulation/policy. This will facilitate the development of NAMs for use in risk assessment, as well as the transition from an endpoint-based animal testing strategy to a more mechanistic-based NAMs testing strategy, as foreseen by the Tox21 and the EU Chemical's Strategy for Sustainability. This work falls under work package 5 (WP5) of the PARC initiative. There are three different tasks within WP5, and this paper is a general overview of the five main projects in the Task 5.2 'Innovative Tools and methods for Toxicity Testing,' with a focus on Human Health. This task will bridge essential regulatory data gaps pertaining to the assessment of toxicological prioritized endpoints such as non-genotoxic carcinogenicity, immunotoxicity, endocrine disruption (mainly thyroid), metabolic disruption, and (developmental and adult) neurotoxicity, thereby leveraging OECD's and PARC's AOP frameworks. This is intended to provide regulatory risk assessors and industry stakeholders with relevant, affordable and reliable assessment tools that will ultimately contribute to the application of next-generation risk assessment (NGRA) in Europe and worldwide.

KEYWORDS

PARC, NGRA, NAMs, hazard assessment, human health

1 Introduction

The current chemical risk assessment system is challenged by increasingly complex regulatory needs. Emerging challenges include an ever-increasing number of chemicals requiring safety assessment, changes in materials and types of chemicals being produced, as well as complex health effects and aggregate/mixture exposure. These issues, alongside growing ethical concerns related to animal testing, have prompted a shift within chemical risk assessment towards a more mechanism-based predictive paradigm. In this context, Next-Generation Risk Assessment (NGRA) (“the concept of using data from New Approach Methodologies (NAMs) for chemical risk assessment,” Marx-Stoelting et al, 2022) is seen as a promising alternative to conventional risk assessment although implementing innovating hazard and exposure assessment approaches in accordance with regulatory needs has proven to be challenging. The European Partnership for the Assessment of Risks from Chemicals (PARC) has been established specifically to address many of these difficulties. A central idea behind this partnership is that a combined effort from risk assessors, authorities and the scientific community will make real headway towards implementing much needed innovations in testing and assessment for regulatory purposes. The PARC vision is to advance research and share knowledge within the broad field of chemical risk assessment and, by so doing, support the European Union’s Chemicals Strategy for Sustainability and “zero pollution” ambition of the European Green Deal (COM/2019/640 final) (Marx-Stoelting et al, 2022).

The overarching objective of PARC is to consolidate and strengthen the EU’s Research and Innovation (R&I) capacity for chemical risk assessment. This includes safeguarding both human and environmental health. Being a project of significant size, including 200 partner institutions across 28 countries, PARC is structured around nine work packages (WPs). Of these, four are scientific WPs (WP4—Monitoring and exposure, WP5—Hazard assessment, WP6—Innovation in regulatory risk assessment, WP8—Concepts and toolboxes) and five support WPs (WP1—Partnership management and coordination, WP2—A common science-policy agenda, WP3—synergies, collaborations and awareness, WP7—FAIR data, WP9—Building infrastructural and human capacities). The four scientific WPs are interconnected to ensure combined efforts to reach the following three specific objectives (SOs):

SO1—EU, national risk assessors and regulatory entities come together with the scientific community in a cross-disciplinary network to set priorities for R&I in chemical risk assessment;

SO2—European and national risk assessment entities and their scientific networks carry out a joint research and innovation program to respond to the agreed priorities in chemical risk assessment; and.

SO3—European risk assessors, their scientific network and the wider stakeholder community have access to the research and innovation capacities required to implement innovative chemical risk assessment.

Within PARC, WP5 will develop NAMs for hazard assessment, provide data to fill gaps in knowledge on poorly characterized contaminants or new emerging hazards, and promote the use of innovative methods and tools to contribute to the integration of new technologies. WP5 is divided into three overarching tasks and 12 projects (Figure 1). One of these tasks is Task 5.2 ‘Innovative Tools and methods for Toxicity Testing’, which aims to improve the current hazard characterization by establishing comprehensive testing

strategies that logically combine novel methods with well-established approaches, preferably in a tiered manner. Task 5.2 is closely linked to the task ‘Quantitative systems toxicology and development of new AOPs’, which addresses the “physiology-toxicology crosstalk” (Heindel et al, 2017). In the following, we provide an overview of the activities under this Task 5.2, with a focus on Human Health, to promote open science and engage the broader scientific community to foster increased collaborations. Each project will be described in detail in separate papers in the same special issue of Frontiers <https://www.frontiersin.org/research-topics/48691/european-partnership-on-the-assessment-of-risks-from-chemicals-parc-focus-on-new-approach-methodologies-nams-in-risk-assessment#articles>.

Activities under this task of WP5 will provide innovative methods (individual assays) and methodologies (such as IATAs) and thereby directly aid a wide range of EU regulations, such as REACH (EC) No 1907/2006, the Food Contact Materials regulation (EC) No 1935/2004, the Plant Protection Products regulation (EC) No 1107/2009b, the Cosmetics regulation (EC) No 1223/2009, the Classification, labelling and packaging (CLP) regulation (EC) No 1272/2008, the Biocides regulation (EC) No 528/2012, the EU chemicals strategy for sustainability (COM/2020/667 final), and the European Green Deal (COM/2019/640 final). The stated aims will be achieved by evaluating the relevance of a suit of technologies ranging from genomics, transcriptomics, and proteomics, to state-of-the-art *in vitro* assays and human stem cell technologies, with an initial focus on five prioritized endpoints.

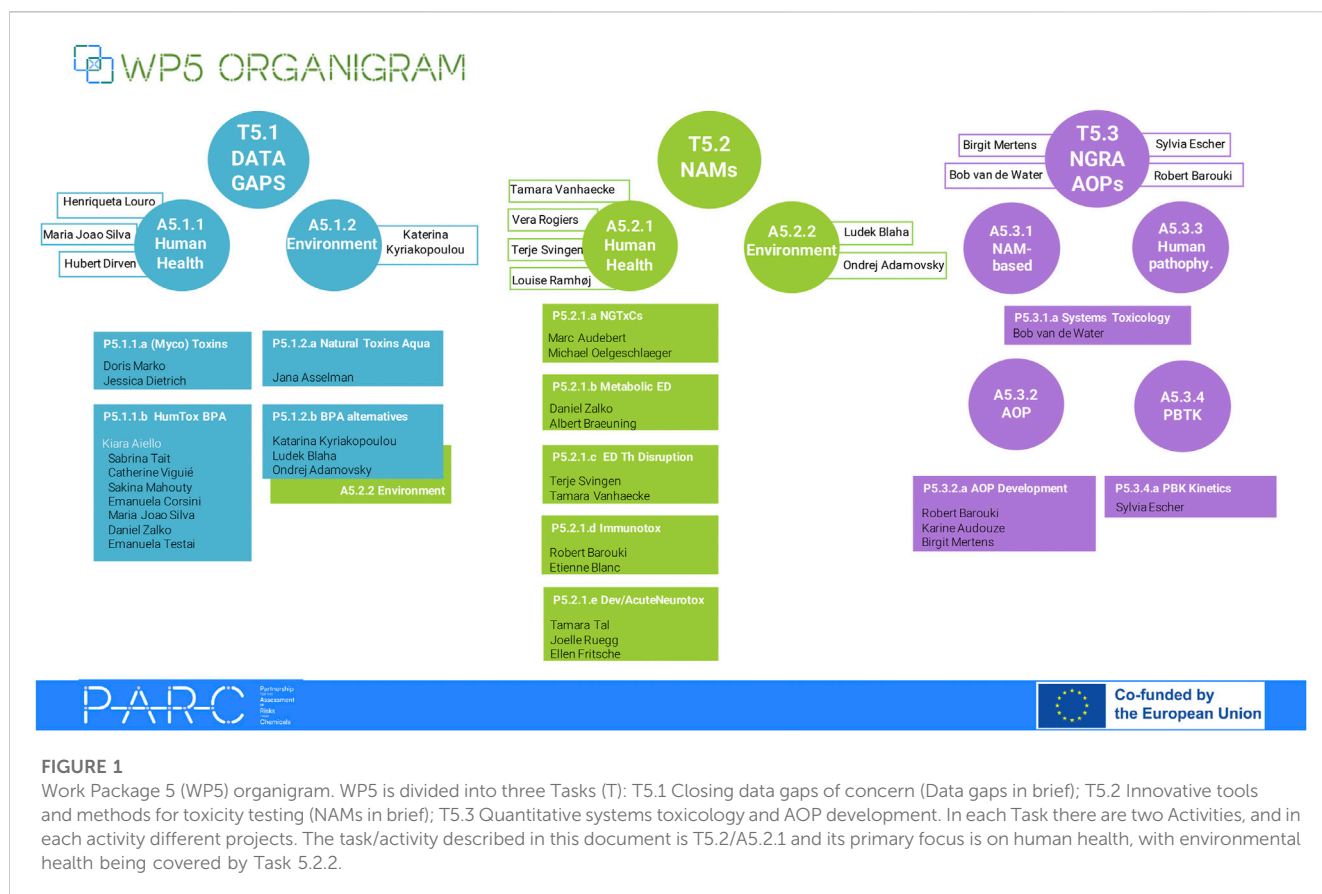
1. Non-genotoxic carcinogenicity;
2. Metabolic endocrine disruption;
3. Endocrine disruption (thyroid);
4. Immunotoxicity; and
5. (developmental and adult) neurotoxicity.

Additionally, tools and methods will be developed to identify toxicity drivers in mixtures and to support the grouping of chemicals, including the application of read-across. Predictive *in vitro* and *in silico* methods will be developed and refined, to ultimately be used for identifying and characterizing specific hazards. This will facilitate one of the overall objectives of the PARC initiative—to develop tools and strategies in accordance with regulatory needs—by establishing comprehensive testing strategies based on new and improved NAMs for human-relevant predictive toxicology. A clear vision is to increase overall scientific and regulatory confidence in NAMs.

2 Non genotoxic carcinogens

2.1 Context

Some substances may induce cancer without being detectable in regulatory genotoxicity assays, for instance by acting as tumor promoters, modulators of nuclear receptors or as inducers of tumor progression and metastasis or tissue-specific toxicity, as well as immune and inflammatory responses. Currently, the assessment of a potential hazard from chemical exposure that might lead to cancer relies on long-term rodent-based bioassays, which are carried out mainly in accordance with OECD TG451 and TG453. However, these standards require a great number of animals and their human



relevance has been questioned (Elcombe et al, 2014; Felter et al, 2018; Holsapple et al, 2006). There is therefore an urgent need for a transition to *in vitro* and more human-relevant approaches to exploit our current understanding of cancer and use the latest non-animal methods available in a modern safety assessment toolbox. The OECD and other international regulatory authorities have acknowledged the lack of such methods in carcinogenicity testing (Jacobs et al, 2020).

2.2 How?

Human relevant-NAMs (transcriptomic approaches, high-content analysis cell painting, among others) and *in silico* tools will be used and optimized to investigate a range of systems (liver, breast, colon, adipocytes) and develop a non genotoxic carcinogens (NGTxCs) integrated approaches to testing and assessment (IATAs). Physiological and toxicological relevant information pertaining to the adversity of potential carcinogenic effects will be integrated using increasingly complex methods (2D cell culture up to 3D spheroids and zebrafish as a simple vertebrate model system).

2.3 Innovation and regulatory impact

The added value of this project will be significant. From a risk assessor's perspective this project will, for the first time, allow for a rapid mechanistic NGTxCs hazard identification of a high number of substances, as well as the screening of mixtures.

3 Metabolic endocrine disruption

3.1 Context

There are over 50 million people in Europe suffering from metabolic disorders, and latest estimates from the WHO for European Union countries indicate that 30%–70% of adults are overweight and 10%–30% obese (World Health Organization, 2020). Recent research into endocrine disrupting chemicals (EDCs) has demonstrated that several EDC, referred to as “metabolism disrupting chemicals” (MDC) can induce a lasting disruption of endogenous metabolism. MDCs are suspected of being linked to obesity and related metabolic disorders such as type II diabetes and non-alcoholic fatty liver disease (Lind et al, 2016). Despite this potential major role in the worldwide epidemics of metabolic disorders, there are currently no specific regulatory *in vivo* or *in vitro* tests allowing to identify their adverse effects. The need for testing developments in the field of metabolic disorders has been highlighted in recent work by EURION cluster projects (Audouze et al, 2020; Küblbeck et al, 2020; Legler et al, 2020).

3.2 How?

A comprehensive exploration of nuclear receptor-driven effects will be carried out using stably transfected cell lines, expanding on the on-going pre-validation of transient cell lines already in Horizon-2020 EURION cluster of projects. Specific compounds

will be selected from the PARC priority list (BPA alternatives), the EURION cluster, or data gaps identified by EU agencies such as ECHA and EFSA. The assessed substances include major metabolites and breakdown molecules, allowing further MoA exploration, as well as adverse outcome pathway (AOP) and read-across development. NAMs encompassing hepatic and non-hepatic *in vitro* systems, including multi-tissue cross-talk, will be developed, opening for a refined assessment of the obesogenic effects of MDC. Ultimately, this project will bridge major data gaps in the field of metabolic disruption through the development of accurate NAMs based on human as well as non-human models.

3.3 Innovation and regulatory impact

New methods will be developed for identifying MDCs with outcomes expected to be of great use for risk assessors as there are currently no specific regulatory *in vivo* or *in vitro* tests allowing to identify their adverse effects, and the role of environmental stressors in metabolic disorders is being increasingly recognized. The regulatory robustness of some promising methods developed in EURION projects will be increased, and research on key-pathways involved in metabolic disruption will be conducted.

4 Endocrine disruptors—Thyroid hormone system disruption

4.1 Context

Despite recent regulatory advances such as the revised OECD guidance document 150 adopted by ECHA, current testing strategies and regulations are widely regarded as inadequate when it comes to identifying thyroid hormone system disruptors (THSDs) (Courderq et al, 2020; Gilbert et al, 2020; Kortenkamp et al, 2020; Noyes et al, 2019). Although some *in vitro* assays (non-OECD TGs) for certain molecular initiation events relevant for THSD are available, and are currently in the process of regulatory validation within the OECD, there remains a pressing need to develop more extensive test batteries of NAMs that cover a broader range of mechanisms linking exposure with adverse outcomes in humans and animals.

4.2 How?

To facilitate the development of thyroid hormone system specific NAMs, we will use a variety of approaches. State-of-the-art multi-organ RNA-sequencing (toxicogenomics) will be used to characterize *in vivo* mechanisms of action for targeted NAMs development. Human-relevant *in vitro* test systems will be characterized and developed for key events of relevant toxicological pathways, including elaboration of species-specific differences by combining human-derived *in vitro* assays, *in silico*, zebrafish and rats as well as an effort to leverage data from non-mammalian vertebrates to inform on hazards for human health.

4.3 Innovation and regulatory impact

The project will deliver valuable contributions to chemical safety assessors by improving and developing NAMs for detection of THSDs. By filling critical knowledge gaps on the effects and mechanisms of THSDs in developing organisms, as well as interpretations in the AOP framework, the aim is to enable an improved assessment of THSD properties. This will contribute to the detection of hazardous substances with focus on EDCs and THSDs, thus adhering to the zero-pollution ambition of the European Green Deal (COM/2019/640 final), as well as the Chemicals Strategy for Sustainability (COM/2020/667 final) set out by the European Commission.

5 Immunotoxicity

5.1 Context

Immune dysregulation ranges from acute uncontrolled inflammation, chronic inflammation, allergic to sensitization reactions, autoimmunity, and immunosuppression. Since exposure to certain chemicals can cause such dysregulations, it is critical to develop tools and test that distinguish between normal adaptive conditions and immune dysregulations, for regulatory purposes. As such, this project aims to characterize NAMs addressing three major endpoints in immunotoxicity: organ-related immunotoxicity with a focus on respiratory toxicity and sensitization; immunosuppression, in particular through response to vaccination; characterizing new models for cellular immunotoxicity assessing key events. The major outcome will be the development of NAMs in these immunotoxicity areas.

5.2 How?

This project will deliver a set of *in vitro* systems to investigate key events in the immune system function or dysfunction. New parameters will be developed to better explore the immune system cellular content and activity, cytokine and antibody production and release, as well as the effect of signaling. Different primary cell systems and cell lines as well as epithelial and immune cell co-cultures will be characterized. Reference chemicals will be used and once the NAMs are developed, PARC priority substances will be tested when relevant.

5.3 Innovation and regulatory impact

This project will aid risk assessors by providing innovative methods to allow for rapid mechanistic hazard identification of a high number of substances, contributing to the detection of hazardous substances. Additionally, the methods will enable the screening of mixtures, and thereby supporting the many EU strategies referred to in the Introduction.

6 Neurotoxicity

6.1 Context

Developmental (DNT) and Adult (ANT) Neurotoxicity guideline studies are extraordinarily resource-intensive and therefore not suited for studying the adverse effects of a large number of chemicals. In REACH, DNT guideline studies are not mandatory and ANT testing is not performed for low-tonnage compounds. There is international consensus, strongly supported by EFSA and the OECD (EFSA PPR Panel et al, 2021), that DNT testing must be made faster and more human-relevant by implementing an alternative DNT testing battery for regulatory purposes. The current state-of-the-art is the use of a DNT *in vitro* test battery (DNT IVB) comprised of several human- and rat-based *in vitro* test methods covering key neurodevelopmental processes which include neural progenitor differentiation, proliferation, neurite outgrowth in CNS neurons and neural crest cells, neural crest cell migration, and oligodendrocyte differentiation (Masjosthusmann et al, 2020). While this represents a step forward towards establishing an alternative DNT testing regime, there are significant gaps in the DNT IVB including human synaptogenesis, human neural network formation, myelination, and correlates for the rodent *in vivo* studies including endpoints that measure behavior, sensory function, learning, and memory.

6.2 How?

The key strategy is to provide a comprehensive battery of NAMs that fills the gaps in the current DNT *in vitro* battery (IVB) and, for ANT, to set up such a battery. In both cases, NAMs will be added that cover missing endpoints and key DNT/ANT modes-of-action (e.g., endocrine disruption, epigenomic alterations). For *in vitro* NAM development, the focus lies solely on human cells which circumvent the problem of species specificity. In addition, zebrafish embryos are used as an alternative, whole organism, low-cost screening tool with integrated nervous system functions capable of detecting chemical effects on missing, complex endpoints including behavior, sensory function, learning, and memory. As there are few neurotoxicity adverse outcome pathways (AOP) available, new AOPs will be generated. Newly established or optimized methods in this sub-project will undergo test method set-up and mechanistic validation.

6.3 Innovation and regulatory impact

Additional IVB NAMs developed for DNT/ANT will provide risk assessors with rapid mechanistic hazard analysis. This will contribute to, along with mixture screening in these NAMs, the European Green Deal (COM/2019/640 final), as well as the Chemicals Strategy for Sustainability (COM/2020/667 final).

7 Concluding remarks

This paper gives a brief overview of Task 5.2 of PARC, named Innovative Tools and Methods for Toxicity Testing. Research and innovation towards NAMs and, more broadly, NGRA, is one of the major objectives of the PARC initiative. It aims to review current approaches and provide novel practices and methods, foster NAMs regulatory uptake, all whilst following the 3 R (Reduction, Refinement and Replacement) principles. With its many activities and interactions across Tasks and WPs, it is beyond the scope of this short report to outline all the many facets of the project; rather, the focus is on providing a brief overview of the Task's mandate and activities to promote the underlying mission of PARC, which is to engage a broad range of professionals from various fields to develop innovative solutions to modern chemical testing strategies in an open, collaborative manner. We have highlighted the five main endpoints that are in focus for the initial few years of the 7-year project duration. The vision is to complete some of these activities within 3–4 years as well as include additional endpoints as the project progresses. For these updates and additional information on the PARC project, we would like to refer to the open website, <https://www.eu-parc.eu/>.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Comprehensive mapping of the AOP-Wiki database: identifying biological and disease gaps

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Introduction: The Adverse Outcome Pathway (AOP) concept facilitates rapid hazard assessment for human health risks. AOPs are constantly evolving, their number is growing, and they are referenced in the AOP-Wiki database, which is supported by the OECD. Here, we present a study that aims at identifying well-defined biological areas, as well as gaps within the AOP-Wiki for future research needs. It does not intend to provide a systematic and comprehensive summary of the available literature on AOPs but summarizes and maps biological knowledge and diseases represented by the already developed AOPs (with OECD endorsed status or under validation).

Methods: Knowledge from the AOP-Wiki database were extracted and prepared for analysis using a multi-step procedure. An automatic mapping of the existing information on AOPs (i.e., genes/proteins and diseases) was performed using bioinformatics tools (i.e., overrepresentation analysis using Gene Ontology and DisGeNET), allowing both the classification of AOPs and the development of AOP networks (AOPN).

Results: AOPs related to diseases of the genitourinary system, neoplasms and developmental anomalies are the most frequently investigated on the AOP-Wiki. An evaluation of the three priority cases (i.e., immunotoxicity and non-genotoxic carcinogenesis, endocrine and metabolic disruption, and developmental and adult neurotoxicity) of the EU-funded PARC project (Partnership for the Risk Assessment of Chemicals) are presented. These were used to highlight under- and over-represented adverse outcomes and to identify and prioritize gaps for further research.

Discussion: These results contribute to a more comprehensive understanding of the adverse effects associated with the molecular events in AOPs, and aid in refining risk assessment for stressors and mitigation strategies. Moreover, the FAIRness (i.e., data which meets principles of findability, accessibility, interoperability, and reusability (FAIR)) of the AOPs appears to be an important consideration for further development.

KEYWORDS

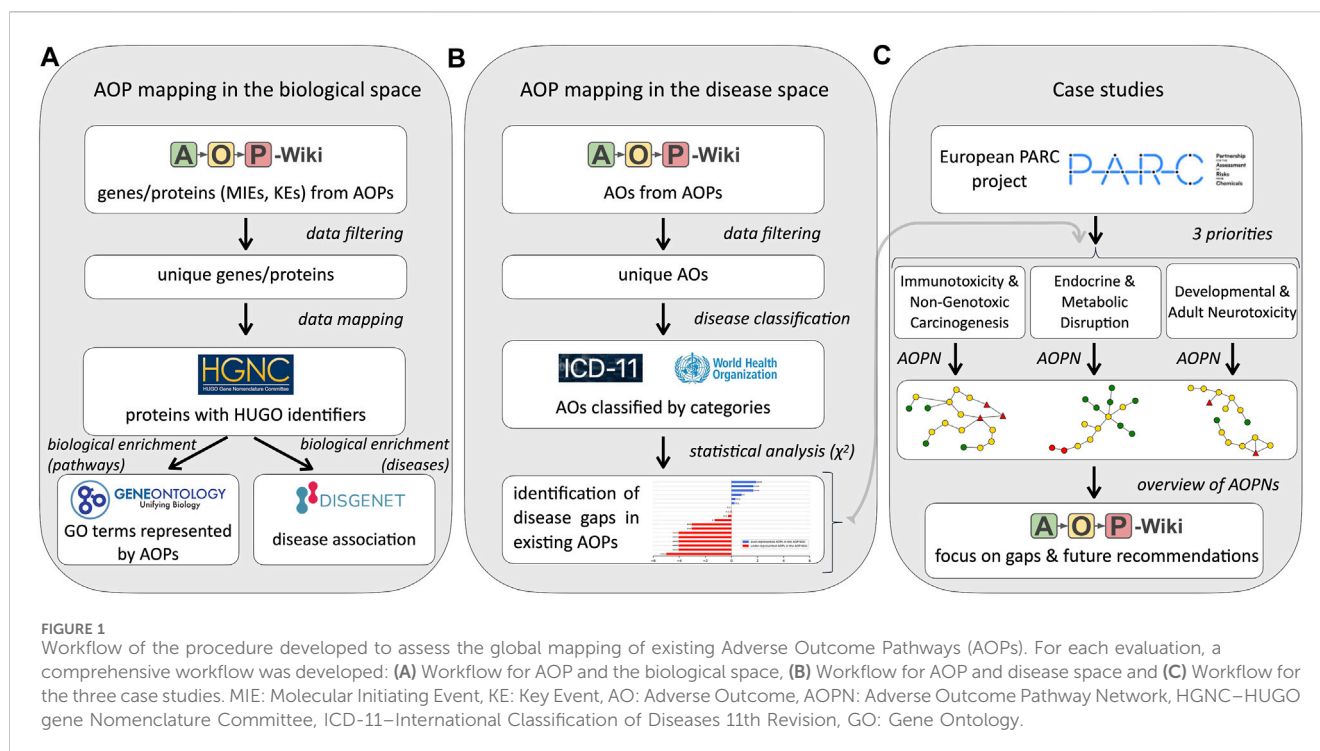
adverse outcome pathway, AOP network, immunotoxicity, neurotoxicity, nongenotoxic carcinogenesis, diseases, PARC

1 Introduction

In 2007, the US National Research Council (NRC) published a review ‘Toxicity testing in the 21st century: a vision and a strategy’, highlighting the ongoing paradigm shift in toxicity testing (Krewski et al., 2020). Indeed, existing risk assessment methodologies and toxicological tests are not in line with the large number of not sufficiently tested existing and the rapidly growing number of novel substances, including metabolites produced in the environment by non-humans, that need to be evaluated. Consequently, the potential toxicity of many substances is poorly characterized. Moreover, the diversity of the organ systems being targeted by contaminants or pollutants, increases the need to implement new tests. Therefore, in this study, authors suggested recommendations to improve and accelerate chemical testing, and emphasized the concept of ‘toxicity pathways’ (Ankley et al., 2010). The development and use of New Approach Methodologies (NAMs), which include animal and non-animal-based methods, have increased to assess specific endpoints that indicate human and ecotoxicological risks from exposure to environmental substances. NAMs are developed to identify a toxicologically relevant response at different biological levels (molecular, cellular, organ, organism) provoked by a chemical exposure (Vinken et al., 2017). NAMs include *in cellulo*, *in vitro* and *in chemico* screening, computational methods as well as high throughput multi-omics and exposomics (Babin et al., 2023; Maitre et al., 2023) to mention but a few methodologies. Recently, a study explored the progress in this ‘next-generation’ of risk assessment methodologies, and highlighted a low, but still growing evolution and use of NAMs in toxicity testing and risk assessment (Krewski et al., 2020). Moreover, limitations of the use of animals in regulatory studies are also an important component to take into consideration, as animal OECD test guideline studies are time- and cost-intensive. Furthermore, it is increasingly ethically questionable (testing one substance may require several thousand animals) in addition to the uncertainties in methodologies, evaluation, regulation, and extrapolation. Although still assumed to be the most protective types of study, *in vivo* animal study predictivity for the protection of human health is sometimes insufficient given the differences in organ function/complexity, exposure, developmental timing, toxicokinetics and

toxicodynamics between humans and other mammals (Tsuji and Crofton, 2012). In the recent assessment of Extended One-Generation Reproductive Toxicity Studies (EOGRTS; OECD TG 443) conducted with 55 industrial chemical substances under REACH (European Chemicals Agency, 2023), methodological issues were presented concerning the developmental immuno- and neurotoxicity cohorts that appear to be particularly demanding in terms of proficiency, i.e., deficiencies were frequently found that hindered the interpretation of the results. This, again, highlights the need for methods’ development to support risk assessment of chemicals by considering human biology and providing a deep mechanistic understanding of the toxicological events leading to adverse effects.

To facilitate the use of NAMs in a regulatory context, a new concept was proposed by Ankley in 2010, named Adverse Outcome Pathways (AOP) (Ankley et al., 2010). The AOP framework describes and organizes existing biological knowledge for humans and wildlife from the perturbation by a stressor leading to an adverse effect, in a structured manner. An AOP starts with a Molecular Initiating Event (MIE) (initiated by a prototypical stressor, the latter not being part of the AOP), linked to a sequence of biological Key Events (KE) that ultimately lead to an Adverse Outcome (AO) at the level of an individual, population or ecosystem. One main objective is to support chemical risk assessment based on mechanistic reasoning and regulatory toxicology. In 2013, the Organization for Economic Co-operation and Development (OECD) started an initiative for the formalization of AOP development, resulting in guidance documents (OECD, 2017; OECD, 2018). Today, AOPs are becoming more and more acceptable as a framework to help chemical safety assessment and regulatory toxicology by supporting a systematic way of predicting AOs based on accumulated mechanistic knowledge (Svingen et al., 2021). To follow the regulatory framework proposed by the European Union (EU) to reduce levels of environmental stressors (e.g., Endocrine Disrupting Chemicals (EDC)), research-driven approaches need to be developed, which include AOP-informed Integrated Approaches for Testing and Assessment (IATA) as an imperative feature of the risk assessment process. The European Cluster to Improve Identification of Endocrine Disruptors (EURION, <https://eurion-cluster.eu>) (Street et al., 2021) as



initiated a few years ago with the aim to propose new and effective methods for EDC testing, related to several health outcomes such as metabolic disorders (Audouze et al., 2020) or developmental neurotoxicity (DNT) (Lupu et al., 2020). Likewise, the ASPIS cluster (<https://aspis-cluster.eu/>) uses AOP-based approaches for implementation of novel strategies for animal-free safety assessment of (non-endocrine) chemicals. Furthermore, the Partnership for the Assessment of Risks from Chemicals initiative (PARC) has the ambition to develop a robust risk assessment of new generation chemicals to better protect health and the environment (<https://www.eu-parc.eu/>) (Marx-Stoelting et al., 2023). The development of AOPs is a key feature of PARC. Previously, several studies have described both the development of AOPs and their potential uses for risk assessment (Villeneuve et al., 2014; Leist et al., 2017; Villeneuve et al., 2018a; Knapen et al., 2018; Vinken, 2018; Bajard et al., 2023). Evidence-based approaches (Audouze et al., 2021) and innovative tools have been proposed to help in the construction of AOPs such as the AOP-helpFinder tool that is based on artificial intelligence (<http://aop-helpfinder.u-paris-sciences.fr/>) (Carvaillo et al., 2019; Jornod et al., 2022; Jaylet et al., 2023). AOP-helpFinder automatically screens the available literature and provides a very important source of knowledge that can be used for AOP development (Benoit et al., 2022; Jaylet et al., 2022). Since 2014, all developed AOPs are stored in the AOP-Wiki database (<https://aopwiki.org/>) that is part of the AOP-knowledge-base (AOP-KB; <https://aopkb.oecd.org>) set up by the OECD. For each proposed AOP, KEs should be measurable, meaning that associated test methods should need to be mentioned if they exist, or developed if they do not. Therefore, the development of AOPs follows the current availability of test methods. For example, a recent study focusing on EDCs, highlighted that validated *in vitro* and *in vivo* tests used by the EU to identify the potential toxicity of EDCs do not assess all the endocrine pathways (Zgheib et al., 2021). From these

findings, an online tool has been developed to evaluate the status of their tests under development before submission to the OECD for endorsement (<https://readedtest.u-paris-sciences.fr/>).

The current study aims in a holistic approach across the whole content of the AOP-Wiki to identify gaps in the current AOPs within the AOP-Wiki database for future research needs. It does not intend to provide a systematic and comprehensive summary of the available literature, but rather summarizes and maps the biological knowledge and diseases represented by the already developed AOPs (OECD endorsed status or still under validation).

2 Material and methods

The proposed approach, to explore information related to AOPs from the AOP-Wiki (i.e., at the gene/protein levels and the disease level), is a multi-step procedure illustrated in Figure 1.

2.1 Compilation and preparation of AOP information from the AOP-Wiki database

For both mapping approaches (genes/proteins and adverse outcomes), the AOP-Wiki database was used. The AOP-Wiki compiles data of all existing AOPs submitted by researchers and are either under development or have been endorsed by the OECD. By the time of our analysis, it contained 403 unique AOPs, but continues to increase rapidly due to global efforts based on crowdsourced collaboration. However, until now only 29 AOPs have been endorsed by the OECD, the others being under development or under evaluation. In this study, all information related to AOPs and any key event (MIE, KE, AO) were downloaded from the AOP-Wiki database on the 22nd of May 2023 (version 2.6; https://aopwiki.org/info_pages/5).

2.1.1 Data set for the gene/protein space exploration

To extract the complete set of genes and proteins involved in existing AOPs, we performed a comprehensive evaluation of the 1371 biological events (MIEs, KEs and AOs) derived from the 403 AOPs contained in the AOP-Wiki database. Considering the inter-species characteristics of AOPs and the collaborative nature of the AOP-Wiki, it was crucial to consider possible redundancies in gene and protein annotations including synonyms and orthologs. To ensure the uniqueness of biological entities and eliminate synonyms, a meticulous manual verification process was conducted (e.g., the NR1I3 receptor is labeled as Androstane receptor, CAR, or NR1I3 depending on the AOP authors). Then, to facilitate data integration and interpretation, as well as to avoid potential conflicts arising from inter-species genes during subsequent enrichment steps, we decided to retain only human genes and map them to their corresponding HGNC gene nomenclature (<https://www.genenames.org/>) (Seal et al., 2023). For non-human genes, we performed a search for the presence of human orthologs using the HGNC Comparison of Ortholog Predictions (HCOP) tool (<https://www.genenames.org/tools/hcop/>), aiming to preserve maximum information for subsequent analyses. The complete list of extracted genes and proteins is presented in [Supplementary Table S1](#). Genes mapped to their HGNC symbols were subsequently used for the exploration of biological pathways using Gene Ontology (GO) and for disease associations using DisGeNET (as detailed in [Sections 2.2.1 and 2.2.2](#), respectively).

2.1.2 Data set for the disease space exploration

From the 403 existing AOPs, we extracted 194 unique AOs. In the AOP-Wiki database, some events can be categorized differently depending on the AOP they are involved in, according to the developer the same event can be labeled as either an MIE, a KE or an AO (e.g., event ID 759 '*Increased, Kidney failure*' was labeled as an AO in the AOP ID 33 and 447 while it was labeled as a KE in the AOP ID 377 and 413). No curation was done to assess and count the actual number of AOs scattered in all the AOPs, i.e., data related to AOP-Wiki events is represented as it was in the AOP-Wiki, so that the event ID 759 '*Increased, K. failure*' was counted as occurring twice as an AO. Then, each compiled AO was manually checked by experts to avoid redundancy of information as some may be synonyms. For example, the AO ID 1458 '*Pulmonary fibrosis*' and AO ID 1276 '*lung fibrosis*' were merged into one unique AO. The name chosen among all the synonyms of an AO was arbitrary, for instance, we kept '*lung fibrosis*' in the above example, so that '*lung fibrosis*' encloses the number of occurrences of AO ID 1458 and AO ID 1276. After merging synonyms, a total of 149 AOs were kept for the classification ([Supplementary Table S2](#) sheet 1 *ICD_11 classification* and sheet 6 *synonyms*). These AOs were involved in 379 AOPs among the 403 currently present in the AOP-Wiki, meaning that 24 AOPs did not have any AO events listed.

2.2 Mapping the AOPs to the biological space using gene/protein information

2.2.1 Gene and pathways

An overrepresentation analysis (ORA) was performed on 22 May 2023, using the Gene Ontology (GO) database

(Ashburner et al., 2000), which classifies genes based on their Cellular Component (CC), Molecular Function (MF), and Biological Process (BP) annotations. The analysis was conducted with the g:Profiler tool (version e109_e.g.,56_p17_1d3191d) (Raudvere et al., 2019) using a Fisher's one-tailed test (or hypergeometric test). The Benjamini-Hochberg False Discovery Rate (FDR) method was applied to adjust the *p*-values, and an adjusted *p*-value threshold of 0.05 was used to select enriched GO terms. The results for the BP, CC, and MF categories are provided in [Supplementary Table S3](#). To simplify the visualization and interpretation of the ORA results, the ReviGO tool was used (Supek et al., 2011). By applying a clustering algorithm that incorporates semantic similarity measures, ReviGO reduced data redundancy while facilitating the graphical representation of the most relevant terms through multidimensional scaling (MDS).

2.2.2 Genes and diseases

Each selected HGNC symbol was investigated for disease associations using the DisGeNET database, a comprehensive platform containing one of the largest publicly available collections of genes and variants associated with human diseases (Piñero et al., 2020). The purpose of this analysis was to identify potential disease associations and gain further insights into the biological implications of our gene list. We utilized the *disease_enrichment* function from the *disgenet2r* R package to search for disease associations (Piñero et al., 2020). The results were filtered based on a False Discovery Rate (FDR) threshold of 0.05 to select statistically significant associations. In addition to the disease enrichment analysis, a second analysis was performed using the *gene2disease* function from the *disgenet2r* R package, with the list of genes from the AOP and the curated DisGeNET database. This analysis allowed us to explore the relationship between the genes and their corresponding Medical Subject Headings (MeSH) disease classes. A visualization was created to display the associations between the genes and the MeSH disease classes, using a chord diagram to better understand these relationships. To enhance clarity and focus on the most relevant associations, only relationships with greater than 60% involvement were kept in the final visualization.

2.3 Mapping of the AOP to the disease space using the adverse outcome information

2.3.1 Disease enrichment

To get an overview of existing data obtained from the AOP-Wiki and to facilitate further analysis, all selected AOs from 2.1.2 were automatically classified using the 11th International Classification of Diseases system (ICD-11) provided by the World Health Organization (<https://icd.who.int/browse11/l-m/en>). The classification outcome was further checked and curated by experts within the field of human toxicology. ICD-11 version was launched 1st January 2022 and contains a total of 24 disease categories. To have the most complete overview of the diseases present in the different AOPs already developed, the unclassified AOs using ICD-11 were checked by experts, and, if needed, new classes were created.

2.3.2 Identification of gaps

To avoid any interpretation bias concerning AOPs that have been over- or insufficiently appraised, we carried out a formal statistical analysis to investigate the biological areas that are most studied, and those where research needs to fill in the gaps. With no preconceived assumptions regarding the AOPs distribution from the AOP-Wiki database, ICD-11 categories that were statistically too common or too rare within the AOP space were investigated. Therefore, we first performed a Chi-Square Goodness of Fit (χ^2 GoF) test (Pearson, 1900). Particularly, we tested whether the distribution of the ICD-11 categories was uniform (H_0), i.e., whether all categories were equally distributed across existing AOPs in the AOP-Wiki. If there is no discrepancy (i.e., all classes are studied in the same proportion), the full distribution should be uniform. To avoid bias in the χ^2 GoF test, AOPs belonging to the ‘unclassified’ home-made family class and those belonging to six categories including ‘Conditions related to sexual health’, ‘Factors influencing health status or contact with health services’, ‘Symptoms, signs or clinical findings, not elsewhere classified’, ‘Injury, poisoning or certain other consequences of external causes’, ‘External causes of morbidity or mortality’ and ‘Decrease, Population growth rate’ were removed. Such categories are imprecise and may include terms that are too divergent from each other, in addition to hosting non-humans AOs. They are also not expected to be equally distributed compared to specific categories of a particular disease type. Therefore, the distribution was tested on 304 AOPs spread over 18 classes. Subsequently, we conducted a *post hoc* test to investigate which classes were over- or under-represented. In particular, we computed Haberman’s residuals to determine which classes have contributed the most to the rejection of H_0 , leading to a non-uniform distribution of ICD-11 categories in AOP-Wiki (Agresti, 2007; Sharpe, 2015). These so-called standardized residuals r_i were computed for each category according to the following formula (Eq. 1):

$$r_i = \frac{(\text{observed}_i - \text{expected}_i)}{\sqrt{N \times p_i \times (1 - p_i)}} \quad (1)$$

where ‘observed_{*i*}’ is the actual number of observed AOPs belonging to a specific class *i*, ‘expected_{*i*}’ the number of expected AOPs belonging to the class *i* under the null hypothesis (expected_{*i*} = 304/18 ≈ 17 $\forall i$ since H_0 states that the distribution should be uniform), *N* the number of AOPs considered in the test (*N* = 304) and *p_i* the probability that an AOP drawn at random from the 304 considered for the test belongs to the class *i* under the null hypothesis (*p_i* = 1/18 $\forall i$ since H_0 states that the distribution should be uniform). The larger the residual in absolute value, the greater the contribution of the category to the magnitude of the χ^2 value obtained and the more the category was over- or under-studied (the residual sign indicates the direction of the gap).

2.4 Case studies

The three case studies that were prioritized within the PARC project were evaluated in depth by presenting AOPN from existing data in the AOP-Wiki. This identifies the state-of-the-art for chosen cases, also giving an overview in which areas future development should take place.

These three cases correspond to Immunotoxicity and Non-Genotoxic Carcinogenesis (Case 1), Endocrine and Metabolic Disruption (Case 2), and Developmental and Adult Neurotoxicity (Case 3).

The AOP-DB RDF tool (Martens et al., 2022) was used to extract the data for the subcases with customized queries for each one. Furthermore, key events common to all subcases were connected, when possible, followed by a graphical visualization of the AOP network with Cytoscape v3.10.0 (Shannon et al., 2003).

3 Results

3.1 Extraction and preparation of the AOP information from the AOP-Wiki database

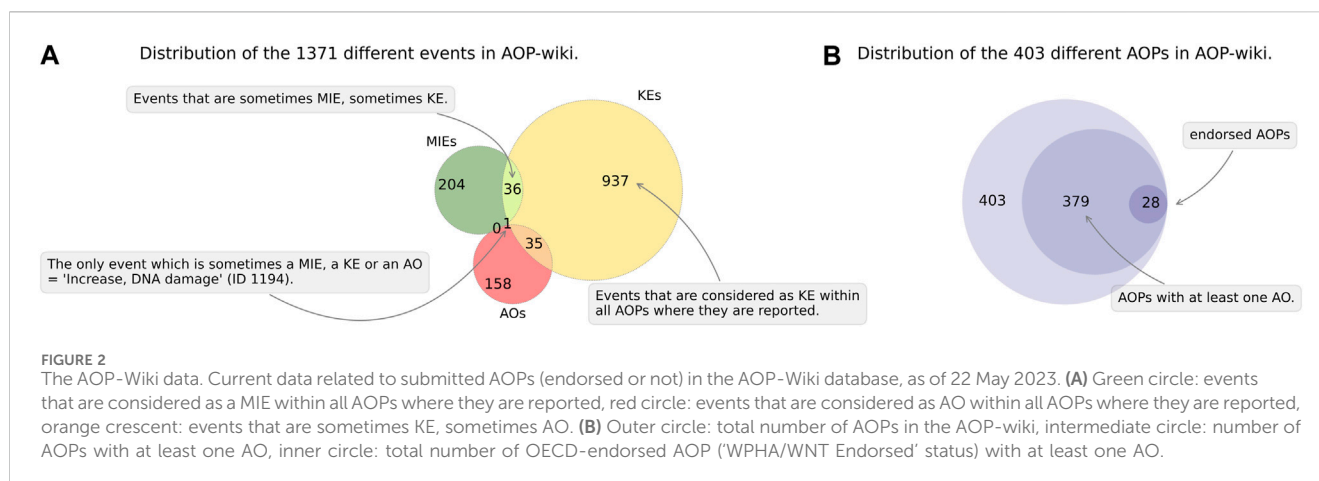
From the AOP-Wiki database, we extracted information for 403 different AOPs, that include 1371 unique ID events (Figure 2).

Of the 1371 unique ID events, 204 were labeled as MIEs (i.e., events which are always referenced as MIEs within all AOPs), 937 were labeled as KEs and 158 as AOs. Some other events belonged to several types, as they are defined depending on the AOP they are involved in and were sometimes a MIE or KE (*n* = 36), KE or AO (*n* = 35), and even an MIE, KE or AO in one case (e.g., ID 1194 ‘Increase, DNA damage’ which is an MIE in the AOP ID 293, a KE in AOP ID 200 and an AO in the AOP ID 294) (Figure 2A). The existence of such events with different statuses depending on the AOP is a way of extending the concept of AOPs to AOPNs (Knapen et al., 2018). The interconnection between the same event belonging to several AOPs is one of the pillars of the AOP-Wiki philosophy, which seeks to connect AOPs. For example, the event ID 1194 is used about 11 times, which allows it to be frequently cited (all states KE, MIE, AO- Rank 10). Out of the 403 AOPs surveyed, 379 have at least one AO. Among the 379 AOPs with at least 1 AO, only 28 have the ‘WPHA/WNT Endorsed’ status (~7%) (Figure 2B). The AOP ID 21 ‘Aryl hydrocarbon receptor activation leading to early life stage mortality, via increased COX-2’ has this status but did not have any AO reported, bringing the total number of OECD-endorsed AOPs to 29.

3.2 Global mapping of the AOP to the biological space using gene information

3.2.1 Data set for the biological space exploration

A total of 245 genes or proteins were manually extracted from the 1371 events contained within the 403 AOPs. After removing duplicates resulting from the collaborative nature of the AOP-Wiki (e.g., see 2.1.1 for NR1I3), a total of 220 unique entities were kept. Then, all 220 genes were mapped to their corresponding symbols to facilitate GO and DisGeNET enrichment analysis. Among these, 149 genes were directly associated with a HGNC gene symbol (represented in green in Supplementary Table S1). Additional 14 entities were manually verified by experts (e.g., when isoform information was missing) and successfully mapped (represented in orange in Supplementary Table S1). However, 57 entities could not be mapped (represented in red in Supplementary Table S1). Some of these genes did not correspond to human genes and lacked a human ortholog (e.g., Vitellogenin), resulting in their exclusion from the



enrichment analysis. On the other hand, some genes could not be mapped due to insufficient precision during the AOP development. For instance, among the most frequently unmapped entities, protein families such as Cytokines or Caspases were found in 7 and 6 AOPs, respectively.

Consequently, the total number of genes properly associated with their HGNC symbols has been raised to 163, being the starting point for both GO and DisGeNET analysis.

3.2.2 Gene and pathways exploration

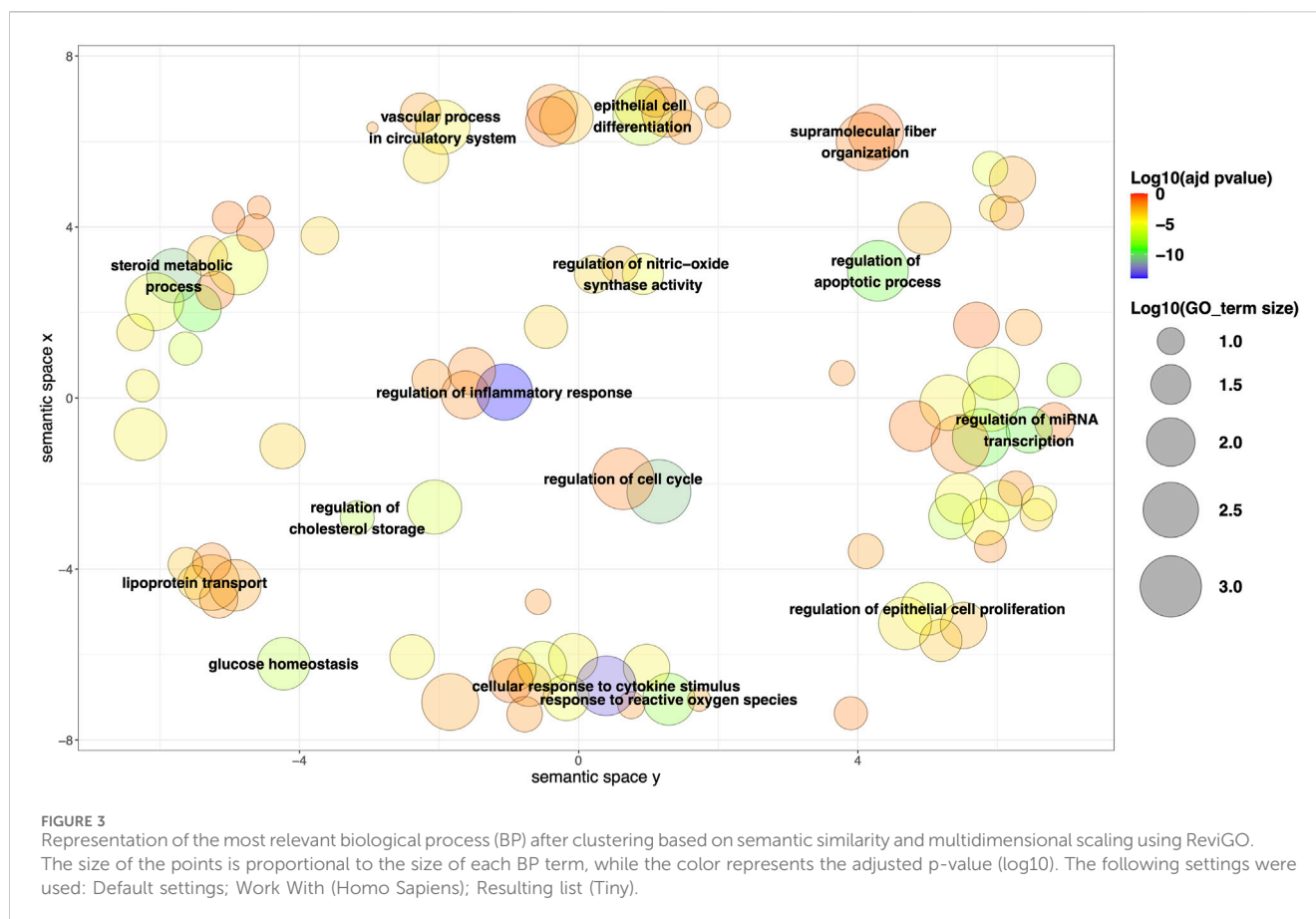
Among the genes prominently represented in our final list, ($n = 163$), the Angiotensin Converting Enzyme 2 (ACE2) and Aryl Hydrocarbon Receptor (AHR or AhR) are the most frequently mentioned in the AOP-Wiki, appearing in more than 17 distinct AOPs (Supplementary Figure S1). The prominent presence of these proteins in numerous toxicity pathways can be explained by their important biological roles. For example, ACE2, being a multifunctional protein (Turner, 2015), is found in various AOPs related to diverse organs (e.g., pulmonary fibrosis in AOP ID 319, renal dysfunction in AOP ID 384, and cardiac dysfunction in AOP ID 427). Moreover, a wide range of AOPs including ACE2 are related to the SARS-CoV-2 virus (COVID-19) (<https://www.ciao-covid.net/aops>), which has been extensively studied in recent years (AOP IDs 379, 426, 430, 468), as the virus uses ACE2 as a cellular entry point for infection (Beyerstedt et al., 2021). The AhR is a cytosolic transcription factor that plays a significant role in various cellular processes, including xenobiotic metabolism. It is primarily associated with environmental pollutants, such as dioxins and polycyclic aromatic hydrocarbons. Activation of the AhR is linked to metabolic disorders (e.g., AOP ID 57) and the progression of specific cancer types, which explains its presence in various cancer related AOPs (AOP IDs 416, 417, 420, and 439) (Wang et al., 2020). However, the AhR is also regulated by endogenous compounds including tryptophan metabolites, by microorganisms or ultraviolet light (Fritsche et al., 2007; Rannug, 2022). This will likely increase further the number of AOPs in which it is involved.

After the GO enrichment of the 163 mapped genes, a total of 963 significant terms (adjusted p -value < 0.05) were obtained for BP, 87 for MF, and 60 for CC (Supplementary Table S3). Among the most relevant BPs identified using ReviGO, a substantial representation of metabolism-related processes was observed (e.g., glucose homeostasis, regulation of cholesterol storage, steroid metabolic process, lipoprotein

transport). Additionally, there is a notable presence of BPs commonly associated with cancer, such as cellular regulation (e.g., cell differentiation, regulation of apoptotic process, regulation of miRNA transcription, regulation of cell proliferation), as well as processes related to oxidative stress and inflammation (Figure 3). Among the most frequently observed genes in metabolism-related processes, examples include AhR and LXR in AOPs associated with steatosis (e.g., AOP IDs 34, 57, 58 and 232), PPAR α in AOPs describing lipid metabolism alterations (e.g., AOP ID 166), and ER α in an AOP related to obesity (AOP ID 493). For processes involved in tumor progression (cellular regulation, oxidative stress, inflammation, etc.), numerous genes are implicated in tumor progression (e.g., ERs contribute to disruptions in proliferation, apoptosis, and/or oxidative stress in AOP IDs 167 and 200). AhR activation leads to increased inflammation and/or apoptosis in AOP IDs 419 and 439, disruptions in cell proliferation in AOP ID 420, and the accumulation of ROS in AOP IDs 418 and 420). This trend was confirmed by the results obtained for MF. Among the most relevant enrichment results, we particularly found 'cholesterol binding', 'lipid binding', 'cytokine receptor binding', 'oxidoreductase activity', and 'molecular function regulator activity', which align with the identified BPs related to metabolism and cellular regulation (Supplementary Figure S2). Additionally, there are results such as 'nuclear receptor activity' and 'signaling receptor activator activity', which correspond to genes and proteins frequently found across all AOPs, such as steroid receptors (androgens and estrogens), LXRs, PXR, CAR, and others.

3.2.3 Gene and disease exploration

To complement the work on biological pathways performed above, a disease enrichment analysis was conducted using the DisGeNET database, based on the 163 mapped genes (Supplementary Table S1). The goal of this analysis was to gain further insight into the biological implications of the gene list to expand our understanding of disease associations with AOPs. For the first analysis, a disease enrichment analysis was performed, and a total of 1215 significantly enriched diseases (FDR < 0.05) were identified. These 1215 diseases group into 165 unique DisGeNET disease classes, based on the MeSH hierarchy. The complete list of significantly enriched diseases can be found in Supplementary Table S4, and Supplementary Figure S3. In the second analysis, the *gene2disease* function was used to explore gene-disease



associations and visualize the relationships between the genes in the list and the MeSH disease classes they are associated with. Gene-disease associations were computed individually, yielding percentages to measure gene involvement within specific disease classes relative to overall disease associations. Subsequent investigation focused on associations where gene involvement surpassed a 60% threshold, emphasizing pronounced connections between genes and specific diseases. The resulting chord diagram (Supplementary Figure S4) effectively illustrates the associations between DisGeNET diseases and HGNC genes. Interestingly, the genes that are strongly connected with the DisGeNET disease classes are different from those that are most frequently found in the AOPs. This suggests that many of the DisGeNET disease classes represented in the gene-disease associations are not adequately captured by the existing AOPs, highlighting potential areas where the AOP framework could benefit from further development. One reason for this might be taxa specificities. For example, AOP ID 41 ‘Sustained AhR Activation leading to Rodent Liver Tumours’ is a very well defined AOP established in rodents which is not directly transferable to the human system. After applying the 60% threshold filter, only 8 genes were related to 6 disease classes (Supplementary Figure S4). This highlights the value of performing a disease enrichment analysis to uncover novel associations and better understand the links between the AOPs and various diseases. By identifying diseases that are significantly enriched in relation to the genes involved in AOPs, we gain insights into potential AOs that may not have been previously considered. This information can

contribute to a more comprehensive understanding of the adverse effects associated with the molecular events in AOPs and aid in refining risk assessment and mitigation strategies.

3.3 Global mapping of the AOP to the disease space using AO information

3.3.1 Preliminary feedback for MIEs, KEs and AOs

The most studied MIEs are both the activation of the AhR (ID 18) and a deposition of energy (ID 1686) with 17 occurrences each, followed by ‘Binding to ACE2’ (15 occurrences) (Supplementary Figure S5). As AhR and ACE2 are proteins, this was consistent with the results looking at the most studied proteins (see 3.2.2), suggesting here that the majority of MIEs involve a deregulation of a protein (through activation or inhibition). Indeed, among the 30 most common MIEs identified, 21 that were directly protein-based.

For the KEs, the event ‘Thyroxine (T4) in serum, Decreased’ (ID 281) is the most studied, with 24 occurrences followed by ‘Thyroid hormone synthesis, Decreased’ (ID 277; 18 occurrences) and ‘Cell injury/death’ (ID 55; 17 occurrences) (Supplementary Figure S6). Unsurprisingly, there was a greater disparity of events which can be understood because an AOP is supposed to have only one (or two or even three) MIE(s) but several KEs at various organizational levels. ACE2-related events such as ‘SARS-Cov-2 cell entry’ (ninth place, 10 occurrences) can still be found among the top 30 KEs as well as cancer-related and inflammation-related

events such as ‘Inadequate DNA repair’ (ninth place, 10 occurrences), ‘Increase, Cell Proliferation’ (11th place, 8 occurrences) or ‘Induction, Epithelial Mesenchymal Transition’ (12th place, 7 occurrences) and ‘Increased, secretion of proinflammatory mediators’ (eighth place, 11 occurrences) or ‘Increased, recruitment of inflammatory cells’ (11th place, 8 occurrences) respectively.

The distribution of the 30 most common AOs is shown in [Supplementary Figure S7](#) (see [Supplementary Table S2](#) sheet 4 *full_distribution AOs* for the complete list). It appears that the event ID 360 ‘Decrease Population Growth Rate’ is the most represented in the AOP-Wiki (57 occurrences), followed by ‘Increase Mortality’ (37 occurrences) and ‘Death/Failure, Colony’ (21 occurrences), thus reflecting the field of ecotoxicology. In non-mammalian species, decreases in population growth rates are strongly causally linked with disorders related to reproduction since 27 out of 57 AOPs hosting this AO co-mentioned terms such as ‘Impairment of reproductive capacity’, ‘Decreased fecundity’ or ‘Reduction, Cumulative fecundity or spawning’. That the AOP concept was initially established for ecotoxicological questions ([Ankley et al., 2010](#)) might explain the overrepresentation of ecotoxicological AOs. Among the top 30 AOs, we also saw 2 terms related to cancer (‘Lung cancer’, 5 occurrences; ‘N/A, Breast Cancer’, 4 occurrences) which represent the two most common cancers worldwide ([Mattiuzzi and Lippi, 2019](#)). We observed the presence of diseases related to metabolism (‘Increased, Liver Steatosis’, 9 occurrences; ‘Occurrence, Kidney toxicity’, 5 occurrences) or neurological type (‘Cognitive Function, Decreased’, 12 occurrences; ‘Occurrence, Epileptic seizure’, 8 occurrences). These most represented AOs are in line with the GO enrichment of the 163 mapped genes since they fit with BP related to cancer and metabolism.

Here we would like to bring to the reader’s attention that the frequency distribution of KEs published in the AOP-Wiki, similar to the general published literature, does not represent their importance for human diseases due to funding and

publication bias caused by the ‘streetlight effect’ ([Newquist et al., 2015](#); [Evans, 2020](#)).

3.3.2 Mapping the AOPs to the disease space

Among the 149 AOs identified in [Section 2.1.2](#), we were able to classify only 127 AOs using the ICD-11 classification. To have the most complete overview of the diseases present in the different AOPs already developed, the 22 unclassified AOs using ICD-11 were checked by experts, and when needed new classes were created. A total of four new classes were proposed: i) one related to reproduction, ii) one related to population, iii) one for cell damages and iv) a last one for all the AOs that were not fitting within all these classes ([Figure 4](#)) ([Supplementary Table S2](#) sheet 1 *ICD_11 classification*). We noticed that some AOPs were connected to several AOs. This led to a total of 480 AOs disseminated in 379 AOPs and AOPs with more than one AO were classified into several categories. For example, the AOP ID 402 had 2 AOs including ‘Periventricular heterotopia formation’ and ‘Occurrence, Epileptic seizure’ and has been thus classified in the ‘Developmental anomalies’ and ‘Diseases of the nervous system’ ICD-11 categories ([Supplementary Table S2](#) sheet 5 *AOs_in_AOPs*). In the present study, AOPs without any AO (n = 24) were not integrated in the classification.

It appears that diseases of the genitourinary system are the most studied, followed by the AO ‘Decrease, Population Growth Rate’, neoplasms and developmental anomalies. The AO ‘Decrease, Population Growth Rate’ is so widely studied that it is present in more AOPs than any of the ICD-11 classes (excluding ‘Diseases of the genitourinary system’) that yet group together several AOs. It is noticeable that only 7 AOPs refer to diseases of the immune system but 2 out of 7 of them are OECD-endorsed while 21 AOPs refer to diseases of the respiratory system but 0 out of 21 are OECD-endorsed ([Figure 4](#)).

As statistical tests are carried out on human diseases, the pseudo-category ‘Decrease, Population Growth Rate’ (n =

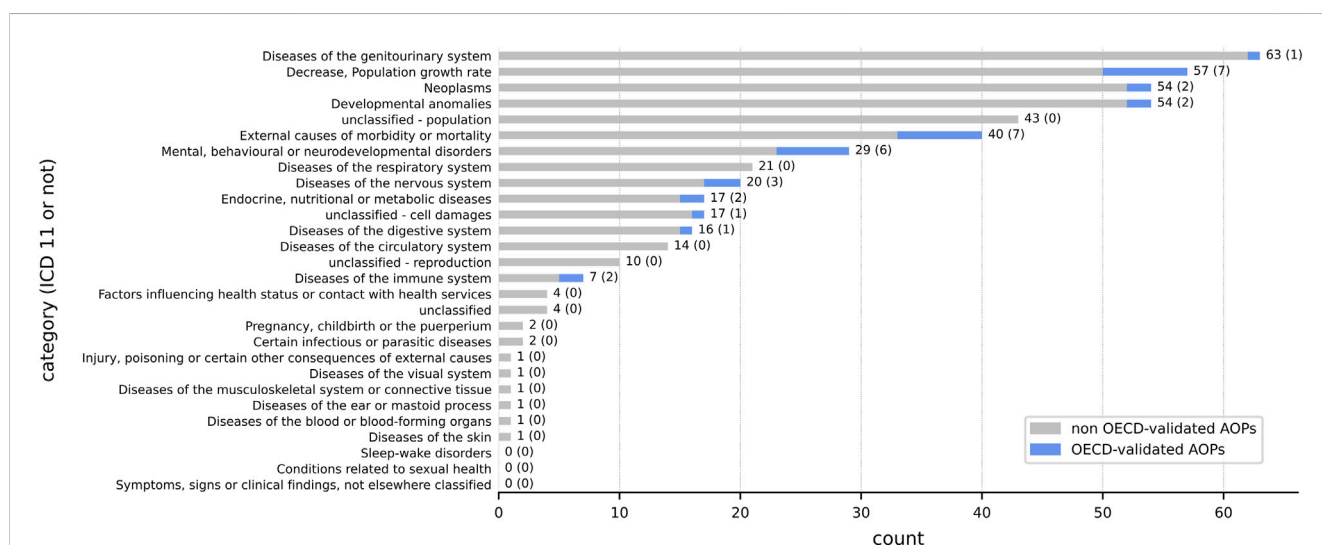


FIGURE 4

Classification of the 379 AOPs that mention at least one AO using the ICD-11 system. The classification was performed using the AO information associated with the existing AOP in the AOP-Wiki database. The number at the end of each row represents the total number of AOPs (endorsed or not) while the number in brackets represents the number of AOPs with the ‘WPHA/WNT Endorsed’ status. The sum does not equal 379 because an AOP can be classified into several categories (1 AOP \neq 1 AO). The associated OECD-endorsed AOP IDs are listed in [Supplementary Table S2](#) sheet 3 *clustering_AOPs*.

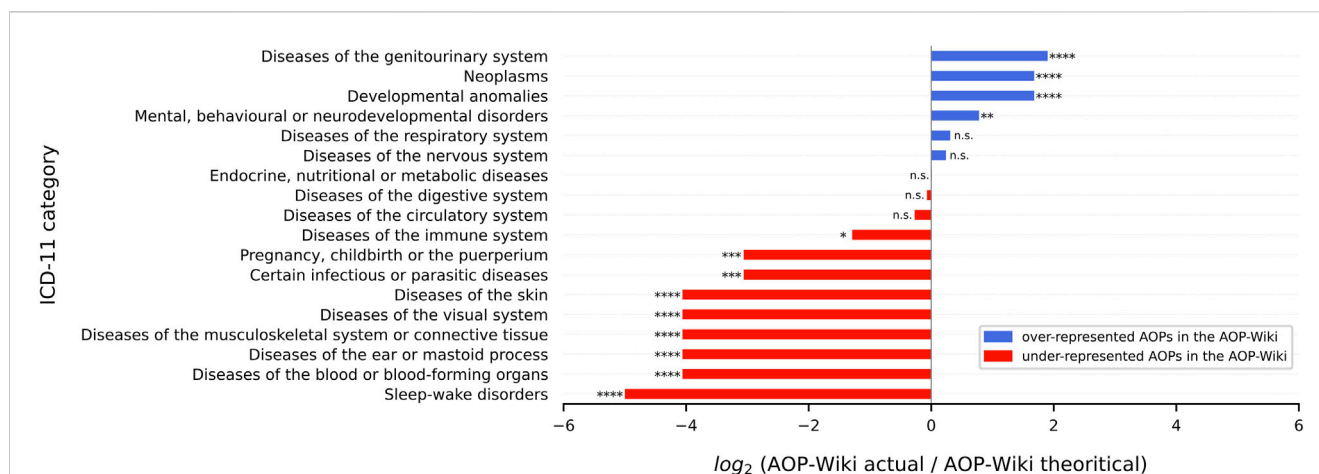


FIGURE 5

Imbalance in the representation of the AOPs in AOP-Wiki using the ICD-11 disease classification. The quantity plotted here is the binary logarithm of the ratio between the actual number of AOPs and theoretical AOPs in AOP-Wiki. This representation was chosen for convenience as it is clearer than the residuals which are more subtle. For instance, the value of $1.90 \approx 2$ for diseases of the genitourinary system indicates that there are about $2^2 = 4$ as many AOPs in this category as there should be if the distribution of all categories was uniform. A value of 0 means that the ratio is equal to 1, i.e., the number of actual AOPs perfectly matches the number of theoretical AOPs. When there were exactly 0 AOPs in a category ('Sleep-wake disorders'), we arbitrarily set the log₂ value to -5 to avoid forbidden values, as log₂(0) is not defined. However, the p-value computed is associated with the residuals and not with the log₂ value of the ratio. n. s. = $p \geq 0.05$, * = $0.01 \leq p < 0.05$, ** = $0.001 \leq p < 0.01$, *** = $0.0001 \leq p < 0.001$, **** = $p < 0.0001$.

57 AOPs) has been subsequently ruled out, in addition to the considerations set out in point 2.3.2. Moreover, AOPs belonging to classes 'unclassified' ($n = 74$), 'Conditions related to sexual health' ($n = 0$), 'Factors influencing health status or contact with health services' ($n = 4$), 'Symptoms, signs or clinical findings, not elsewhere classified' ($n = 0$), 'Injury, poisoning or certain other consequences of external causes' ($n = 1$) and 'External causes of morbidity or mortality' ($n = 40$) have also been removed in line with the hypothesis adopted (2.3.2). Finally, statistical tests were carried out on the 304 AOPs spread over 18 classes that fit the assumptions.

3.4 Gaps

To identify gaps (i.e., AOPs that were not yet developed or poorly developed for some disease categories), statistical analysis was performed using the previous classification with ICD-11. A χ^2 GoF test followed by a *post hoc* test (residual analysis) was performed to investigate which classes were over- or under-represented (Figure 5; Supplementary Table S2 sheet 7 gaps) according to the presumed distribution (2.3.2).

The distribution of the 18 ICD-11 categories in AOP-Wiki considered for the χ^2 GoF test was widely skewed ($\chi^2 = 423.46$; $p = 2.28 \times 10^{-79}$). On the one hand, the *post hoc* test (residual analysis) highlighted that 3 classes were largely overstudied including 'diseases of the genitourinary system', 'neoplasms' and 'developmental anomalies' ($p < 0.0001$). On the other hand, it highlighted that most of the other classes were under-studied, which reflects the large disparity in the study of AOPs. Currently, there are only 5 categories ($p > 0.05$) that are correctly distributed (under the null hypothesis H_0 that the distribution should be uniform). For instance, the residual value of the 'Endocrine, nutritional, and metabolic diseases' class is, following the Eq. 1 in 2.3.2:

$$r_{\text{endocrine, nutritional or metabolic disorders}} = \frac{(17 - 16.89)}{\sqrt{304 \times \frac{1}{18} \times \left(1 - \frac{1}{18}\right)}} \approx 0.03$$

where 17 is the actual number of observed AOPs belonging to the 'Endocrine, nutritional and metabolic diseases' class, 16.89 the number of expected AOPs belonging to this class under the null hypothesis ($= 304/18 \approx 16.89$ since H_0 states that the distribution should be uniform), 304 the number of AOPs considered in the test and $1/18$ the probability that an AOP drawn at random from the 304 considered for the test belongs to the 'Endocrine, nutritional and metabolic diseases' class under the null hypothesis.

Therefore, the highest value in the dataset showed that the 'diseases of the genitourinary system' class contributed the most to the rejection of H_0 and is the furthest from the uniform distribution. Particularly, $r = 11.54 > 0$ ($p = 8.29 \times 10^{-31}$) so this class was over-represented in the AOP-Wiki. Moreover, results showed a concordance with the 25 most significant diseases found with the curated DisGeNET database (Supplementary Figure S3), especially regarding cancer (neoplasms) terms. Not only does cancer have a prominent place in the AOP-Wiki database with the highest number of AOs referring to it ($n = 25$, Supplementary Figure S8, Supplementary Table S2 sheet 2 clustering_AOs) and the second class the most studied ($r = 9.29$; $p = 1.54 \times 10^{-20}$), but it is also the field that gathers the highest number of terms (MIE, KE or AO) as shown by the analysis with the CURATED DisGeNET database (Supplementary Figure S3) in addition to the GO enrichment analysis (see 3.2.2).

However, a parent category that fits to its theoretical number of AOPs may have a non-uniform distribution within its daughter categories. As an example, for the 'Endocrine, nutritional, and metabolic diseases' class which is neither over- nor under-studied ($p = 0.98$), we noticed that within it the distribution was not uniform (Supplementary Figure S9). The most studied pathologies are those related to the liver such as 'liver steatosis' and

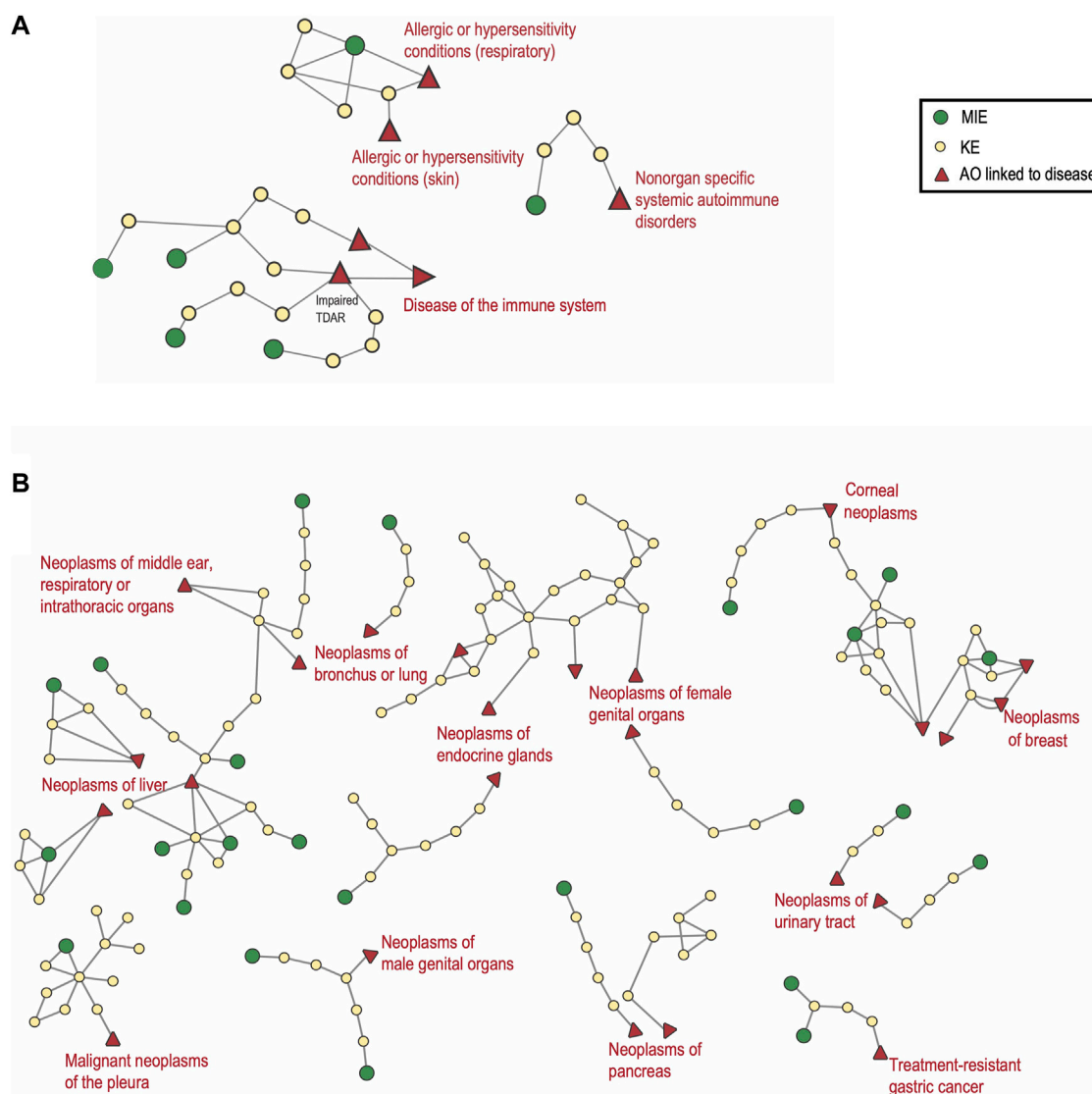


FIGURE 6

Adverse Outcome Pathway Networks for Immunotoxicity and for Non-Genotoxic Carcinogens. **(A)** covers AOPs related to immunosuppression, sensitization and autoimmunity made up of 4, 2 and one AOP respectively. For the AO Impaired T-cell dependent antibody response “TDAR”, the three AO synonyms were merged. **(B)** 31 AOPs linked to non-genotoxic carcinogenesis are displayed in this AOPN. In both A and B, the relevant ICD-11 disease category is annotated in red. Non-mammalian and empty AOPs were not included.

‘steatohepatitis’ which represent almost two-thirds of the parent class. Besides, there are only a few AOPs that cover ‘Endocrine, nutritional, and metabolic diseases’ excluding the liver. Particularly, only a minority of AOPs report diseases such as obesity and diabetes which contrasts the numerous studies in the literature outside the AOP-Wiki framework that deals with the presence of obesogenic and diabetogenic substances in our environment (Wolf et al., 2019; Lam et al., 2023).

3.5 Cases studies

To shed light on how different AOPs are represented within the AOP-Wiki, we explored more precisely three case studies that were prioritized within the PARC project. These three cases correspond to

Immunotoxicity and Non-Genotoxic Carcinogenesis (Case 1), Endocrine and Metabolic Disruption (Case 2), and Developmental and Adult Neurotoxicity (Case 3).

3.5.1 Case study 1: Representation of immunotoxicity and non-genotoxic carcinogenesis AOPs in the AOP-Wiki and their links to human health

In the context of immunotoxicity, human health is influenced by four principal processes: hypersensitivity, autoimmunity, immunostimulation, and immunosuppression. Immunotoxic compounds may directly trigger responses towards themselves, suppress, promote and/or aggravate responses to self-antigens/allergens/infectious antigens, and modify disease risk. Actions may be mediated by affecting the intensity of immune responses

(insufficient or exaggerated response or duration) or by modulating the immune system leading to inappropriate responses. As mentioned above, AOPs representing immunotoxicity endpoints are currently under-represented in AOP-Wiki ($r = -2.48$, $p = 0.013$, Figure 5). Nine AOPs representing hypersensitivity, autoimmunity and immunosuppression were identified in the AOP-Wiki. Two AOPs were related to hypersensitivity, one to autoimmunity and six to immunosuppression. Two of the six immunosuppressive AOPs (AOP IDs 84 and 85) have honeybee colony failure as their AOs. Therefore, an AOPN based on the seven AOPs with taxonomic applicability to mammals was constructed (Figure 6A).

For immunosuppression, three complete and one less complete AOP were identified in the AOP-Wiki (AOP IDs 315, 154, 277 and 14, respectively), of which one is endorsed (AOP ID 154; *Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response*). Four different MIEs are represented (*Inhibition of calcineurin activity*; *Inhibition of JAK3*; *Impaired IL-1R1 signaling*; *Activation of Glucocorticoid Receptor*) all acting at the level of the T-cell. Two of the AOPs have inhibition of NF- κ B as a common KE and three of the AOPs converge on the same AO, Impaired T-cell dependent antibody response ('TDAR') for which there are three AO-synonyms used (Supplementary Table S2 sheet 6 synonyms). Immunosuppression can be linked to an increased risk of infection as well as an increased risk for certain cancers (World Health Organization and Safety, 2012). In the regulatory setting, the TDAR *in vivo* assay is used as an indicator of immunotoxicity, e.g., in the extended one-generation reproductive study (EOGRS, OECD TG 443) in which a developmental immunotoxicity cohort may be included if triggered by a concern. TDAR assays provide a functional readout reflecting the joint activities of innate and adaptive cells (Plitnick and Herzyk, 2010). An impaired TDAR is used as a functional marker of immunosuppression. All four AOPs have similar late KEs addressing suppression of lymphocyte/T-cell activity and they all represent well-characterized mechanisms of actions (MIEs and KEs) for different classes of immunosuppressive drugs. The available AOPs for immunosuppression address effects on adaptive immunity and can be mapped to the main ICD-11 category 'Diseases of the immune system'. None of the AOPs for immunosuppression include KEs related to the involvement of the innate immune system, cell-mediated immunity or direct effects on B-cell populations. There is also a need for further development of AOP ID 14, which covers Glucocorticoid Receptor Activation, and the further inclusion of additional MIEs of relevance for environmental factors such as the suppression mediated by AhR or by mTOR inhibitors. The immunosuppressive effects mediated by AhR signaling and the interaction with estrogen have also been identified as a cross-cutting priority for further work in case study 2.

Immune stimulation is most understood to involve exaggerated or prolonged inflammation and/or tissue damage, which underlies a wide range of pathological processes and is linked to numerous diseases, including states of hypersensitivity/allergies and autoimmunity. Many immune system-related diseases arising in nonlymphoid tissues are categorized in the ICD-11 based on the target tissue (e.g., skin, lung) rather than as a 'disease of the immune system'. One example is AOP ID 313 '*Stimulation of TLR7/8 in dendritic cells leading to Psoriatic skin disease*', an inflammatory skin

disorder categorized under 'Diseases of the skin'. Autoimmune diseases result from a harmful immune response directed against the body's own organs, tissues, and cells. More than 80 autoimmune diseases are recognized, being either systemic or affecting specific organs. Among the more common are type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease and autoimmune thyroid diseases. Environmental factors are linked to autoimmune disease etiology (Parks et al., 2014; Conrad et al., 2023). However, the specific mechanisms leading to autoimmune diseases and the effects of environmental exposures remain for the most part poorly understood. In the AOP-Wiki, one AOP for autoimmune disease was identified; the AOP ID 314 '*Binding to estrogen receptor (ER)- α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)*'. SLE is categorized in the ICD-11 under 'Diseases of the immune system 04' and the subcategory 'Nonorgan specific systemic autoimmune disorders'. Several autoimmune diseases are increasing in incidence, like type 1 diabetes, but are currently not represented in the AOP-Wiki.

Two AOPs related to the immune system disease subcategory allergic, or hypersensitivity conditions are represented in AOP-Wiki: AOP ID 40 '*Covalent Protein binding leading to Skin Sensitisation*' and AOP ID 39 '*Covalent Binding, Protein, leading to Increase, Allergic Respiratory Hypersensitivity Response*'. The status of the latter is still under development, while AOP ID 40 is considered in chemical regulation (OECD, 2027). Both AOP IDs 39 and 40 describe pathways for responses directed towards the compound itself and share the MIE '*Covalent Binding, Protein*' (MIE ID 396) as well as the 2 KEs: '*Activation, Dendritic Cells*' (KE ID 398) and '*Activation/Proliferation, T-cells*' (KE ID 272) (Supplementary Table S5 sheet 1 AOP IDs 39 & 40). Certainly, there are organ-specific differences to consider in the sensitization processes. However, aspects like the secretion of inflammatory mediators and activation of epithelial cells play an important role in both disease processes, thus there may be more mechanistic overlap than can be extracted from a comparison based only on the terminology of the events. Furthermore, there is increasing evidence that the route of exposure does not necessarily restrict itself to the same target organ, with abnormal regulation of immune cell responses playing an important role (Kimber et al., 2011). No AOPs for adjuvant or allergy-promoting processes were identified.

Considerably more work will need to be done to cover immunotoxicity endpoints in sufficient depth (develop AOPs fully) as well as breadth. In particular, developmental immunotoxicity (DIT), which is a regulatory toxicity endpoint of high concern, is currently not represented in the AOP-Wiki. AOPs and (non-animal) test methods targeting the sensitivities of the developing immune system need to be developed to specifically support DIT evaluation as well as other immunotoxicity evaluations. In addition, the important linkages between impaired immune function and the two other focus areas of PARC, endocrine and metabolic disruption, and neurotoxicity, are currently poorly represented in the AOP-Wiki.

Chronic, low-grade inflammation is a major driver and mode of action of non-genotoxic carcinogens (NGTxC). Notably, immune evasion of cancer cells and inflammation make up the two immune-related events that are part of the well-established 10 hallmarks of cancer, described by Hanahan and Weinberg (2011). Within the

AOP-Wiki, 45 AOPs, 13 of which remain poorly developed, have been identified that can be linked to NGTxC (Supplementary Table S5 sheet 2 NGTxC AOPs). These include AOPs with and without inflammation as a key event and are linked to the ICD-11 category 'Neoplasms (02)'. As shown in Figure 5, neoplasms feature prominently in the AOP-Wiki ($r = 9.29$; $p = 1.54 \times 10^{-20}$). In Figure 6B, 32 of the more developed AOPs have been represented in an AOPN, with their links to the relevant ICD-11 disease subcategory. Lung, liver, and breast cancers are the ones represented by the highest number of AOPs and AhR activation is the most prevalent MIE. This is also reflected by the findings in section 3.2.2, above, which indicates that AhR signaling appears in more than 17 AOPs overall, which alongside ACE2, makes them the most frequent genes/proteins in the AOP-Wiki. Three hub key events have been proposed for the representation of inflammation in AOP networks; 'tissue resident cell activation', 'increased pro-inflammatory mediators', and 'leukocyte recruitment/activation' (Villeneuve et al., 2018b). Eight of the NGTxC AOPs included in the AOPN include inflammation as a KE or a modifying factor.

As cancer has a high contribution to the global burden of disease, further developing AOPs for NGTxC is needed to better predict the contribution of chemical and physical factors to cancer risks. Beyond further developing AOPs, Audebert et al. (2023) also describe the new experimental methods and NAMs that are being developed within the PARC project to map KEs and identify NGTxCs. Although cancer as an AO is well represented in the AOP-Wiki, several of the neoplasms with a presumed environmental or lifestyle component in the human population are not well represented, e.g., testicular seminomas, prostate cancer, colorectal cancer and skin cancer.

Several ongoing projects, including the Aspis cluster (<https://aspis-cluster.eu/>), the Eurion cluster (<https://eurion-cluster.eu/>), the EHEN network (<https://www.humanexposome.eu/>), the CAAT-led working group for alternatives to *in vivo* DIT Testing (<https://caat.jhsph.edu/programs/DIT/>), as well as PARC, are likely to deliver data and/or test methods (NAMs) that will be suitable to support further development of AOPs for immunotoxicity and NGTxC including the role of lifestyle and environmentally induced chronic inflammation in the promotion of disease.

3.5.2 Case study 2: Representation of AOPs leading to endocrine and metabolic diseases in the AOP-Wiki

This case study focuses on the five current priority areas for AOP development related to endocrine and metabolic disruption in PARC: (i) thyroid hormone system disruption (THSD), (ii) disrupted androgen receptor (AR) signaling, (iii) disrupted estrogen receptor (ER) signaling, (iv) disrupted steroidogenesis, and (v) metabolic disruption. Across these priority areas, 70 AOPs are currently available in the AOP-Wiki. Ten of those AOPs have been endorsed by the OECD WNT/WPHA: AOP ID 6 describing antagonist binding to PPAR α leading to body-weight loss, AOP IDs 23 and 25 linking androgen receptor agonism and aromatase inhibition respectively to reproductive dysfunction, AOP ID 42 linking thyroperoxidase (TPO) inhibition to decreased cognitive function, AOP ID 54 linking Na⁺/I⁻ symporter (NIS) inhibition to learning and memory impairment, and AOP IDs

155–159 linking thyroperoxidase and deiodinase inhibition to impaired swim bladder inflation in fish. The latter 7 AOPs are all related to THSD, reflecting a recent and strong international research focus in that area. AOP IDs 42 and 54 lead to developmental neurotoxicity and are relevant in the context of Case study 3 as well.

An AOPN was constructed that includes all 70 AOPs (Figure 7). Since in this area efforts are being made to bridge the gap between environmental and human health and investigate whether non-mammalian models can be used for informing on human health, we opted to include both AOPs that were initially developed for human health and those initially developed for environmental health. Also, because many of these AOPs had already been curated, we were able to include both complete and incomplete AOPs in this case study to provide a broader picture. Figure 7 shows that most AOPs together form one large AOP network connecting different areas of endocrine and metabolic disruption. While the THSD AOPN has recently been curated to improve the connectivity among AOPs (Haigis et al., 2023), curation may be needed in other parts of the AOP network to reduce duplication and increase connectivity, e.g., to reflect crosstalk between endocrine axes. In the THSD area of the AOP network, hub key events, mostly representing alterations in hormone levels, can be identified where AOPs with multiple MIEs converge and subsequently diverge towards various AOs. In order to investigate the current coverage of endocrine and metabolic diseases by existing AOPs, the ICD-11 category 'Endocrine, nutritional or metabolic diseases' was identified as the most relevant category for this case study in a first step. Fifteen AOPs have been identified that lead to AOs mapped to diseases in ICD-11, including liver steatosis, steatohepatitis, obesity, decreased body weight and diabetes. Statistically, this disease category was found to be not over- nor under-represented in the AOP-Wiki ($r = 0.03$; $p = 0.976$; Figure 5). However, as discussed in section 3.4, the focus of AOP developers on diseases such as obesity and diabetes has been relatively limited in general, while the scientific evidence supporting the diabetogenic and obesogenic potential of, for example, pesticides has significantly grown in recent years (Heindel and Blumberg, 2019; Wei et al., 2023). In the broader perspective of diseases related to an organism's energy metabolism, addressing this gap in AOP development is relevant for both human and environmental health: in humans, adverse health effects largely depend on the dietary context (e.g., a high-caloric, high-fat and high-sugar western diet) while in wildlife, where caloric intake is typically limited, any disruption of energy metabolism may have important consequences for supporting survival, growth and reproduction.

In a second step, which in general was aimed at manually searching for additional AOPs leading to endocrine diseases (i.e., diseases of endocrine organs) that are not captured in the ICD-11 category, we therefore explicitly included AOPs related to metabolic diseases as well. AOPs that have an EDC mechanism but do not have an endocrine or metabolic disease identified as the adverse outcome (e.g., developmental neurotoxicity, impaired fertility, impaired development, hepatocellular carcinoma, endometrial carcinoma, or breast cancer) were not selected for our analysis. Manual identification of those adverse outcomes in the AOPN that could be mapped to the ICD classification system showed that in fact 23 out of 70 AOPs were linked to endocrine

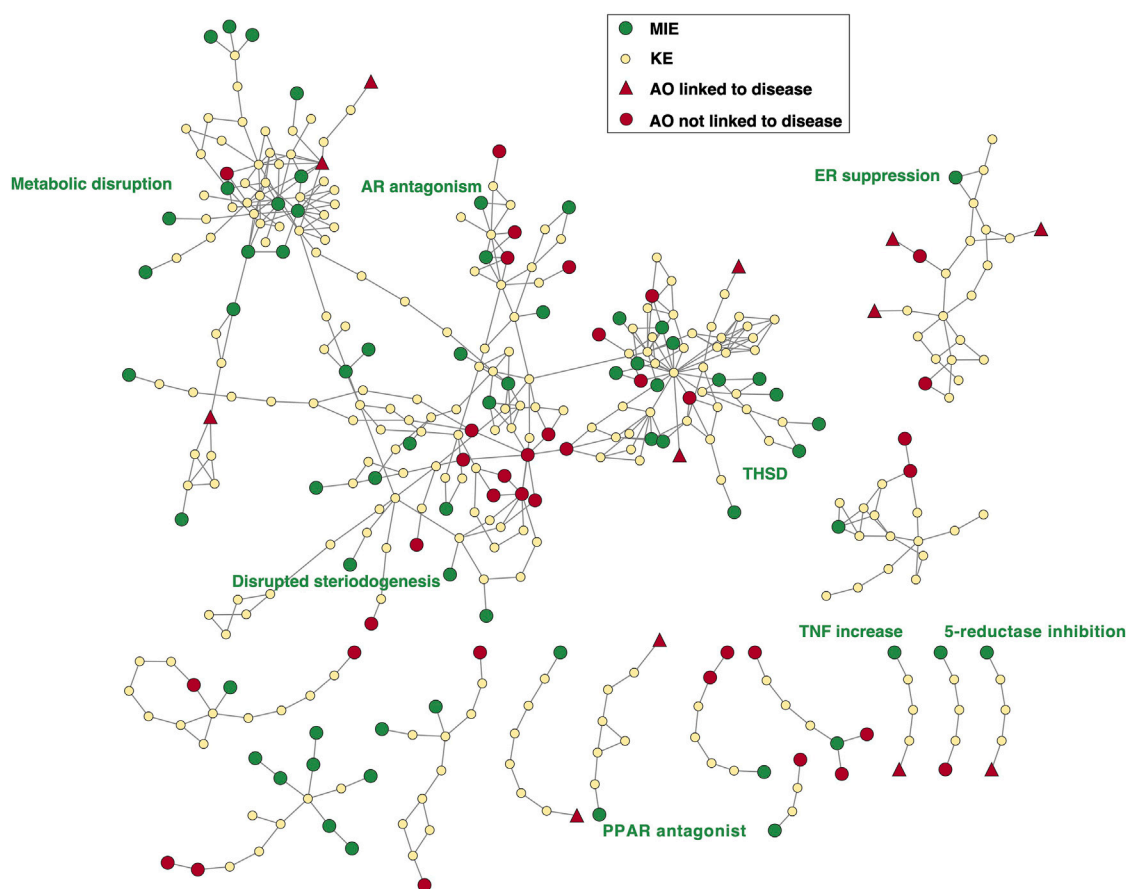


FIGURE 7

Adverse Outcome Pathway Network consisting of 70 AOPs describing endocrine and metabolic disruption. For each AO linked to a disease, the ICD disease subcategory is shown as a label in red. Labels in green are used to situate the broad areas of endocrine and metabolic disruption in the AOPN. THSD = thyroid hormone system disruption.

diseases (See [Supplementary Table S6](#)). Many of these additionally identified AOPs lead to neoplasms of endocrine-active organs (e.g., thyroid, male and female genital organs) which are part of the disease class ‘Neoplasms’ which is over-represented in the AOP-Wiki (See [section 3.4](#)), presumably due to a historical focus on cancers with a toxicological basis. These paths are also relevant in the context of Case study 1.

Because hormones are involved in regulating almost all aspects of normal development and physiology, Case study 2 differs from the other two case studies in the fact that endocrine and metabolic disruption in an AOP context could be considered as encompassing all pathways leading to endocrine and metabolic diseases on the one hand, or all endocrine mechanisms leading to any adverse health effects (i.e., not restricted to endocrine diseases) on the other hand. As described above, we opted to only focus on endocrine and metabolic diseases for the purpose of the present study. There are, however, some gray areas with respect to linkage between EDC exposure, EDC mechanisms and endocrine diseases. One example is impaired male fertility where the Testicular Dysgenesis Syndrome (TDS) links male reproductive disorders like cryptorchidism, hypospadias and some types of testicular cancer (at least partly) to chemical exposure during sensitive developmental stages ([Skakkebaek et al., 2016](#); [Skakkebaek et al., 2022](#)).

Although important advances have been made in the development of AOPs for endocrine and metabolic disruption and a considerable number of AOPs describe mechanisms leading to endocrine and metabolic diseases, several gaps remain to be addressed. From a regulatory perspective, an important consideration in this context is whether the currently available AOPs and AOP network provides sufficient coverage of the overall toxicological space that is relevant to the assessment of endocrine disrupting chemicals, or whether certain important toxicological mechanisms are not yet captured at all. In this context, several AOP development priorities for endocrine and metabolic disruption have been identified in the PARC project. A priority relates to linkages between THSD and metabolic disruption. It is recognized that while some metabolic outcomes such as liver steatosis have received considerable attention, linkages to hormone imbalance and especially thyroid hormone imbalance have been investigated ([Brenta, 2011](#); [Zhang et al., 2017](#); [Gao et al., 2021](#)), and merit additional AOP development efforts. The recently developed AOP ID 457 linking succinate dehydrogenase inhibition to increased insulin resistance through reduction in circulating thyroxine could be used as a basis to expand on this. A second priority is related to MIEs that are currently missing in the AOP network, such as thyroid hormone transporter inhibition ([Noyes](#)

et al., 2019; Di Cosmo et al., 2022). The addition of such MIEs will improve the description of paths leading to downstream AOs/diseases. A third priority focuses on the molecular mechanism of crosstalk between estrogen and AhR signaling pathways leading to immunosuppression (Groestlinger et al., 2022), and linking to the work in Case study 1. Overall, the linkage between endocrine disruption and impaired immune function is an important area where AOPs are currently missing. A fourth priority focuses on expanding the available AOPs linking THSD to DNT. So far, focus in this area has been on impaired function of the hippocampus, and inclusion of impaired development of the hypothalamus leading to impaired motoric activity or social responses has been identified as a priority. For example, Harder et al. (2018) found that maternal thyroid hormone is required for parvalbumin neuron development in the anterior hypothalamic area. While such AOPs are not considered to lead to endocrine diseases *per se* and the existing AOPs leading to DNT (e.g., AOP IDs 42 and 54) have therefore not been included in the list of AOPs linking to diseases in Case study 2, they are addressed in detail in Case study 3. Across all AOP development efforts, attention will go to the comparison of pathways in mammalian and non-mammalian species including invertebrates to on the one hand advance the use of alternatives to animal testing such as fish embryos and invertebrate models for informing on human health, and on the other hand improve the use of mammalian data such as those of human *in vitro* systems for informing on environmental health.

3.5.3 Case study 3: Neurotoxicity AOPs in the AOP-Wiki and their human health relevance

Neurotoxicity encompasses the study of adverse effects on the structure or function of the developing or adult (mature or aging) nervous system following acute or chronic exposure to chemical, biological, or physical agents (EPA, 1998; FDA, 2000). The distinction between developmental and adult neurotoxicity is fundamental due to the higher and/or different susceptibility of the highly plastic developing *versus* the poorly regenerative adult brain. Also, the role of signaling molecules guiding brain development differs as a function of the neurodevelopmental window. This distinction between developmental and adult neurotoxicity is also reflected in the AOPs, and the two case studies are discussed separately here.

3.5.3.1 Developmental neurotoxicity (DNT)

In total, 12 AOPs were identified for DNT in the AOP-Wiki, as listed in Supplementary Table S7 (sheet 1 DNT AOPs). According to ICD-11, AOP IDs 12, 13, 17, 42, 54, 134, 152, 300 and 442 can be classified under '06 Mental and Behavioural Disorders; neurocognitive disorders', which address impairment of learning and memory, and decreased cognitive function. Furthermore, AOP ID 12 "*Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development leads to neurodegeneration with impairment in learning and memory in aging*" can also be classified under "*Diseases of the nervous system*" since early life disturbances may lead to adverse effects later in life. Although some cognitive disorders are covered by the above-mentioned AOPs in the AOP-Wiki, motor-function-related disorders (ICD-11 6A04 Developmental motor coordination disorder) represent one of the AOP-Wiki AO gaps. Such AOPs

were previously hypothesized in the literature focusing, e.g., on the MDMA-induced decreased motor function in children (Barenys et al., 2020) and would merit further development and upload in the AOP-Wiki.

After exclusion of duplicate MIEs (Supplementary Table S7 sheet 2 DNT MIEs) and KEs (Supplementary Table S7 sheet 3 DNT KEs), a total of 10 MIEs and 41 KEs have been identified in the DNT context. Several of these KEs also visualized in Figure 8 constitute well-described factors influencing the onset and progression of neurological and neurodevelopmental disorder (NDD) pathologies. A large group of KEs (KE IDs 277, 281, 280, 425, 958, 959, 960, 1656, and 279) related to thyroid disruption and presents a direct link with chemically-induced congenital hypothyroidism (ICD-11 5A00) (Korevaar et al., 2016; Sun et al., 2022). KE IDs 1502 and 1503 characterizing defective histone acetylation were established as key regulators of processes leading to neural tube defect cases such as spina bifida (ICD-11 LA02) and anencephaly (ICD-11 LA00) (Tamkeen et al., 2021; Takla et al., 2023). Most importantly, KE ID 381 represents the brain-derived neurotrophic factor (BDNF), a key molecule necessary for healthy brain development and notably its learning and memory function. The dysregulation of BDNF was linked with numerous NDDs such as Autism Spectrum Disorder (ASD, ICD-11 6A02) (Liu et al., 2021), dyslexia (ICD-11 6A03) (Abdelraouf et al., 2023) and intellectual disability (ICD-11 6A00) (Esnafoglu and Adigüzel, 2021), but not with Attention Deficit Hyperactivity Disorder (ADHD, ICD-11 6A05) (Mei et al., 2022; de Lucca et al., 2023). ADHD was, on the other hand, associated with impaired synaptogenesis (KE ID 385) (Dark et al., 2018; Halperin et al., 2021). At last, the downstream KE 386 of the decreased neural network function is a hallmark of many cognitive disorders including fetal alcohol syndrome, ASD or intellectual disability amongst others (Uhlhaas and Singer, 2006; Frega et al., 2020; Adams et al., 2022; McCready et al., 2022). Mapping neurodevelopmental KE from AOPs to human NDD will increase certainty for their regulatory application. For this purpose, human-relevant *in vitro* assays like the ones that are present in (Crofton and Mundy, 2021) or currently developed for the DNT IVB are valuable tools for filling the large data gap on DNT modes-of-action for large numbers of so far for DNT untested chemicals (Sachana et al., 2021).

Despite the fact that diseases of the nervous system are not underrepresented in the AOP-Wiki ($r = 0.78$; $p = 0.435$; Figure 5), it is well established that there are challenges in developing AOPs for neurotoxicity following neurodevelopmental exposure (Bal-Price et al., 2017), e.g., the lack of understanding of the MIEs that are causally responsible for triggering downstream KEs resulting in cognitive defects. One reason for this poor knowledge specifically when it comes to MIEs relevant for human NDD is the inability to study such events in humans and the poor representation of the spectrum of features of NDD in rodents (Paparella et al., 2020). Moreover, there are considerable comorbidities among NDDs (Surén et al., 2012) as well as several phenotypic similarities such as problems with language, cognitive function, social interaction, and attention across different NDD. Also here human-relevant, multicellular *in vitro* assays provide an exceptional opportunity for gaining information on the broad spectrum of MIEs that have the ability to trigger NDD in humans (Hogberg and Smirnova, 2022; Klose et al., 2022).

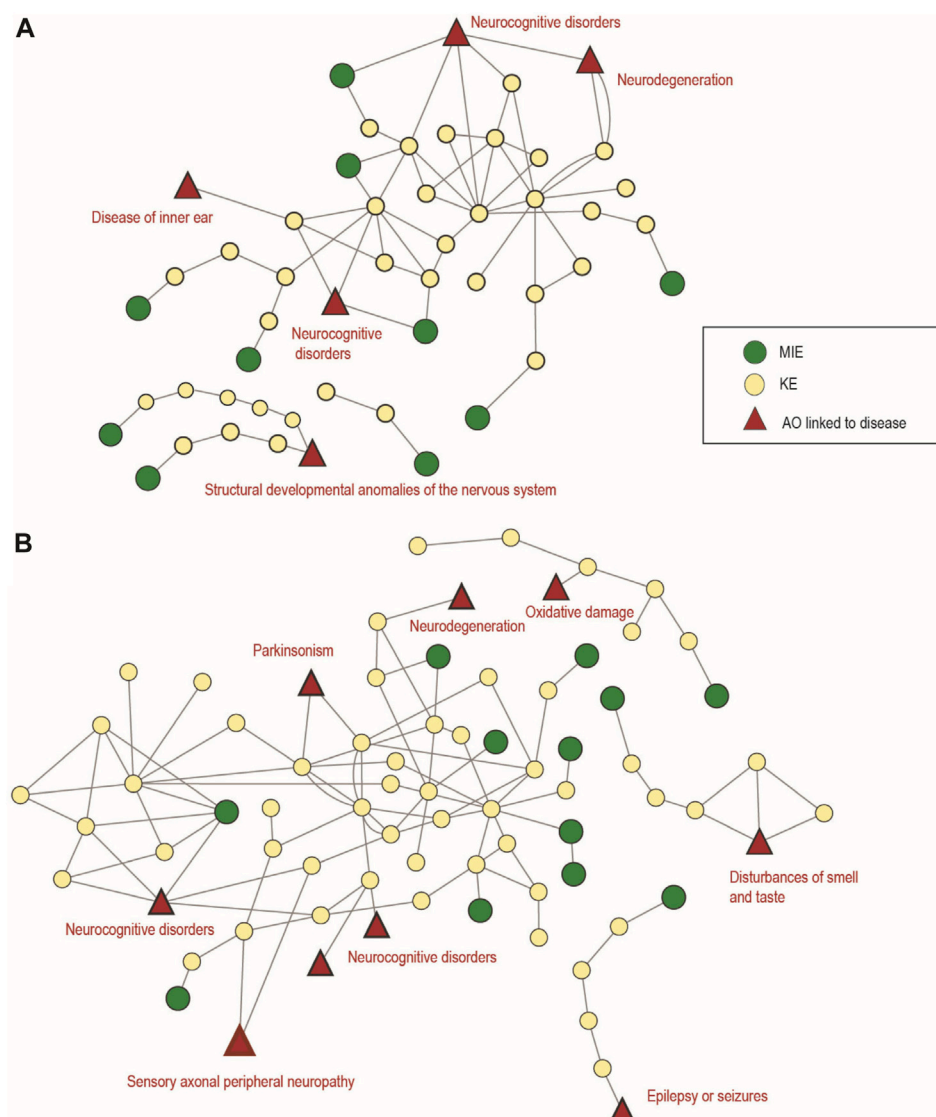


FIGURE 8

Adverse Outcome Pathway Networks (AOPN) for developmental and adult neurotoxicity. **(A)** AOPN for 12 AOPs linked to developmental neurotoxicity, identified in the AOP-Wiki. **(B)** AOPN for 14 AOPs linked to adult neurotoxicity, identified in AOP-Wiki. In both (A) and (B) Figures, the relevant ICD-11 disease category is annotated in red. Non-human relevant and empty AOPs have been excluded.

The onset of these disorders stems from alterations in the precisely orchestrated processes at different stages of human brain development. Despite its complexity, key neurodevelopmental processes that are vital for normal brain development have been identified and a majority of them are assessed in the DNT *in vitro* battery (IVB) (Crofton and Mundy, 2021). Although well established as KEs in brain development and DNT processes as well, several of these key neurodevelopmental processes (e.g., migration, neurite outgrowth, neuronal maturation, glia cell and neuronal differentiation) are currently not described in the AOP-Wiki and therefore illustrates major gaps that need filling. The following KEs that are so far not represented in the AOP-Wiki will enable AOP mapping of gene-environmentally induced NDDs such as microcephaly (ICD-11 classified LD20.2) caused by decreased NPC-proliferation KE (de Groot et al., 2005), ASD (ICD-11 6A02) linked to synaptic pruning KE (Pagani et al.,

2021), as well as disorders linked to deficient autophagy KE (Deng et al., 2021). Mapping molecular and/or cellular features of human NDD to AOPs will strongly increase confidence in their regulatory application.

Although dysfunction of glial cells contributes to the pathogenesis of NDDs, KEs linked to their impaired development are mostly lacking from the AOP-Wiki. Thus, a KE of deficient astrocyte development, that is currently deficient in the AOP-Wiki, could be linked to intellectual disabilities (ICD-11 6A00) (Cresto et al., 2019), KE of radial glia development leading to simplified gyral appearance and reduced cortical surface area could be associated with Down syndrome (ICD-11 LD40.0) (Baburamani et al., 2020), and KE of impaired oligodendrocyte development with subsequent hypomyelination KE could be connected to perinatal white matter injury (ICD-11 KB02) (Murphy et al., 2007; Volpe et al., 2011; Motavaf and Piao, 2021). Furthermore, endocrine disruption-

related KEs and MIEs absent from the DNT-related AOPs in the AOP-Wiki (e.g., binding to estrogen, androgen, liver x, and retinoic acid receptors) represent another major data gap preventing the linkage of cognitive disorders observed in children after prenatal exposure to endocrine disruptors (Lupu et al., 2020; Cediell-Ulloa et al., 2022) or fetal alcohol spectrum disorder (ICD-11 6A0Y) (Petrelli et al., 2019). By filling these data gaps in the future, we aim at providing a solid basis of scientific confidence to regulatory agencies that allows application of NAMs for DNT in a regulatory context. Considering that currently testing chemicals for DNT is not mandatory, an AOP-based NAM approach to broad-based substance testing is being pursued to protect the highly sensitive brains of our future generations.

3.5.3.2 Adult neurotoxicity (ANT)

The development of an AOPN from all currently available linear ANT AOPs in the AOP-Wiki requires a thorough analysis and expert knowledge to clean up and harmonize the definition of KEs and even AOs, as often different terminologies are used for the same (or very similar) KE or AO description, which can be merged. In this case study, the only curation performed was to consider only fully or partially developed AOPs as part of the network, excluding empty AOPs (Figure 8B). As a result, 14 AOPs (11 partially drafted and 3 completed) referred to ANT in the AOP-Wiki database.

Five AOPs are included in the OECD work plan (AOP IDs 3, 10, 48, 394 and 475) and 3 of these are endorsed (AOP IDs 3, 10 and 48). According to the general category identified in the ICD -11, 4 AOPs (AOP IDs 48, 405, 475, 483) can be classified under '*Mental and Behavioural Disorders*' with particular reference to '*Neurocognitive Disorders (6D71)*', which address learning and memory impairments and refer to more cognitive domains that represent a decline from the individual's previous level of functioning (Figure 8B). Eight AOPs can be classified in '*Diseases of the nervous system*' with reference to Parkinson's disease (8A00) (AOP IDs 3 and 464), Alzheimer's disease (8A20) (AOP ID 429), epilepsy or seizures (AOP IDs 10 and 281), disorders of nerve roots, plexus or peripheral nerves, covering peripheral neuropathy of sensory neurons (AOP IDs 279 and 450) or disorders of olfactory nerves (AOP ID 394) (Figure 8B). Three AOPs (AOP IDs 26, 260 and 281) do not refer to specific disease of the nervous system since addressing general neurotoxic effects such as oxidative damage or neurodegeneration (necrosis or apoptosis) (Figure 8B).

Motor deficit disorders, some of which are coded in ICD-11, such as ataxia (8A03), dystonia (8A02), myoclonus (8A06), choreoathetosis (8A01) and weakness, flaccid/spastic paralysis in addition to delayed neuropathy, psychosis and emotional disturbance, visual impairment (9D90) and hearing loss are among the adult nervous system dysfunctions resulting from exposure to toxic substances (Klaassen, 2018) that are not currently developed in the AOP-Wiki database.

A systematic review conducted in 2017, covering 27 years of literature, identified a set of key endpoint categories induced by human neurotoxicants and associated with ANT (Masjosthusmann et al., 2018). These categories relate to neurotransmission (cholinergic, GABAergic, glycinergic, glutamatergic, adrenergic, serotonergic, dopaminergic, neurotransmission in general), ion

channels/receptors (sodium channels, potassium channels, calcium channels, chloride channels, other receptors), cellular endpoints (mitochondrial dysfunction/oxidative stress/apoptosis, redox cycling, altered calcium signaling, cytoskeletal changes, neuroinflammation, axonopathies, myelin toxicity, delayed neuropathy, enzyme inhibition) (Masjosthusmann et al., 2018). MIEs and KEs associated with AOPs that are fully or partially described in the AOP-Wiki database were organized according to these and related categories (Supplementary Table S7 sheet 4 'ANT endpoint categories' and sheet 5 'Additional ANT endpoints'). It is clear from these tables that domains such as 'ion channels' and part of the neurotransmitter system targeted by neurotoxicants are completely neglected (Supplementary Table S7 sheet 6 ANT endpoints not covered), although some of these, e.g., increase/inhibition of dopaminergic neurotransmission, are described in AOPs linked to obesity (ID 72) epithelial tumors (ID 170), malignant neoplasms of female genital organs (ID 112) and diseases of the genital system (ID 73) (Supplementary Table S2). According to the OECD AOPs Developer's Handbook (OECD, 2018), KEs should be described as single isolated measurable events in order to be modular and to be used in other AOPs. These KEs can thus be reused to develop AOPs that address ANT.

Another aspect that emerges is the focus of AOPs on neurons. It is now well accepted that glial cells are key players in the control of nervous system homeostasis and that dysfunction of this cell group plays a role in neurological disorders (Jäkel and Dimou, 2017; von Bernhardt et al., 2016). With the sole exception of neuroinflammation, AOPs targeting glial cell toxicity (i.e., astrocytes, microglia, oligodendrocytes/Schwann cells) in the context of ANT are lacking.

From a regulatory point of view, a major challenge for ANT is the long-term health effects arising from repeated low-level exposure. For example, meta-analyses suggest an association between pesticide exposure and neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (Malek et al., 2012; EFSA, 2013; Ntzani et al., 2013; Yan et al., 2016; Shrestha et al., 2020; Andrew et al., 2021). Thus, although epidemiological studies do not prove causality, they raise concerns and questions about the adequacy of *in vivo* regulatory studies to provide information on complex human health outcomes. Parkinson's disease is already addressed by AOP IDs 3 (endorsed) and 464. In this context, to facilitate the functional understanding of complex biological systems, the curation of the ANT network and the development of AOPs addressing key steps leading to Alzheimer's disease, with particular attention to the data gaps described, have been identified as priorities in PARC.

3.5.4 Adverse outcome pathway network of the three prioritized endpoints of PARC

The three case studies were developed in the context of three priorities that have been set in the PARC project for AOP development in the areas of immunotoxicity and non-genotoxic carcinogenesis, endocrine and metabolic disruption, and neurotoxicity. Figure 9 shows an AOP network combining all AOPs that have been identified as related to immunotoxicity and non-genotoxic carcinogenesis (yellow in Figure 9, related to Case study 1), endocrine and metabolic disruption (pink, related to Case

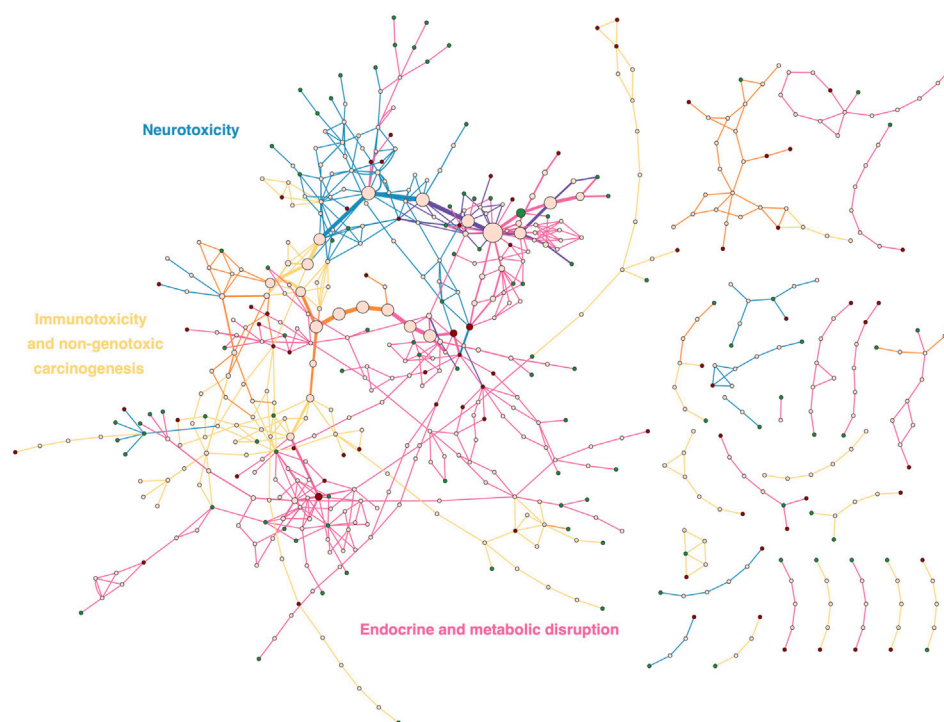


FIGURE 9

AOP network consisting of all AOPs inventoried in the areas of the three prioritized endpoints of PARC, corresponding to the three case studies. KERs related to immunotoxicity and non-genotoxic carcinogenesis (Case study 1) are shown in yellow, KERs related to endocrine and metabolic disruption (Case study 2) are shown in pink and KERs related to neurotoxicity (case study 3) are shown in blue. KERs that are shared between immunotoxicity and non-genotoxic carcinogenesis and endocrine and metabolic disruption are shown in orange. KERs that are shared between neurotoxicity and endocrine and metabolic disruption are shown in purple. MIEs are shown in green and AOs are shown in red. Node size shows directed stress and edge width shows directed betweenness on uncured subnetworks, calculated using the Cytoscape plugin CentiScaPe.

study 2) and neurotoxicity (blue, related to Case study 3). There is a particularly well-connected area in the network where both the directed stress of the KEs (shown as larger node size in Figure 9) and the directed betweenness of the KERs (shown as larger edge width) are higher. Stress and betweenness consider the number of shortest paths that must pass through the KE/KER in question (Scardoni et al., 2009; Villeneuve et al., 2018a). This area connects the AOPs available in the AOP-Wiki in the three PARC priority areas. Neurotoxicity is strongly connected to endocrine and metabolic disruption through reduced thyroxine levels leading to reduced levels of BDNF. Immunotoxicity and non-genotoxic carcinogenesis is strongly connected to endocrine and metabolic disruption through DNA damage linked to increased activation of NF- κ B and estrogen receptor antagonism, eventually resulting in metastatic breast cancer (AOP ID 443). As is the case for the three case studies separately, this overarching AOP network analysis is limited to those AOPs that have been entered into the AOP-Wiki by the community and to the level of curation and overall quality that is currently available in the AOP-Wiki (See Conclusion section for more details on related challenges). As such, this analysis provides a state of the art of the available AOPs and especially of connections between these areas that have been described so far. It should be noted that the linkages between the three case studies that can be discerned at this point are not necessarily the most important linkages, from a biological and toxicological point of view, among these research areas. Additional linkages may be

investigated in the future, such as a link between thyroid dysfunction and Alzheimer's disease (Kim et al., 2022).

4 Discussion/conclusion

The AOP framework supports a better description of evidence-based pathways leading to diseases, and it provides robust knowledge on critical key events and relationships that can lead to the development of novel tests and relevant biomarkers. In this study, we provide an overview of the existing AOPs available in the AOP-Wiki, generalized by overrepresentation analysis, highlighting their significance in advancing our understanding of toxicological mechanisms and informing risk assessment practices (Mortensen et al., 2022). We have presented the general characteristics of over- and under-represented AOPs in the AOP-Wiki and established possible connections to existing diseases (defined by ICD-11 categories) through a combination of computational tools and expert curation. In addition, we supplemented details about three case studies, prioritized within the PARC project, to shed light on the findings from this computational exercise.

One of the main outcomes of this study is that there is a large difference between the number of AOPs mapped to certain diseases as compared to other ones. There may be trivial reasons for that such as the focus of the groups who first developed AOPs. Yet, some critical diseases such as cardiovascular, blood, skin diseases or gut

diseases appear to be underrepresented in the AOP-Wiki despite their considerable contribution to mortality and morbidity. This does not mean that the effort concerning well represented diseases such as genitourinary diseases or neoplasms should decrease as we are still far from a full description of pathways in these diseases, but rather that more effort should be devoted to underrepresented or neglected diseases. A stronger interaction between scientists involved in these diseases and those involved in AOP development should be helpful.

Another reason for explaining the disparity in AOPs may stem from the assumption we made regarding the theoretical distribution of the disease classes. Indeed, we assumed that the distribution would be uniform, i.e., that all categories would be strictly equal in terms of the AOPs that make them up. However, as AOPs are a framework strongly associated with toxicology and the environment surrounding us, since each MIE arises from a stressful exposure, it may be conceivable that the unknown set of all existing stressors disrupts only a limited number of biological functions, unevenly, as a subset of all existing pathologies. In this case, the distribution of ICD-11 classes should not be uniform, rather an asymmetrical distribution, to reflect the situation more accurately. For instance, this hypothesis would not attribute such a high negative weight to the sleep wake disorders class, which is currently significantly under-represented. Accordingly, it could have a more moderate weight because this physiological function is less disturbed by stressors. On the other hand, it might be expected that genitourinary diseases, neoplasms, and developmental diseases are genuinely over-represented since stressors lead to these pathologies more than any others. Moreover, the unequal distribution of AOPs with respect to human diseases may also be due to the fact that some biological spaces are over-represented, such as fundamental function which appear to be common and involved in various processes. A bias in coverage of diseases, originating from a database used, its versions or granularity could also be expected. Here we used the latest WHO database, that is a world-wide recognized, curated database of human diseases but other existing databases with human diseases definition could potentially depict slightly different patterns, reflecting a historical aspect of naming and characterization of human diseases.

Further, our study revealed that the genes that are strongly connected with the disease classes differ from those that are most frequently found in the AOPs. There are several explanations for this observation, but it does suggest that some important disease pathways in current AOPs are missing and that efforts should be devoted to developing additional AOPs involving genes that are highly connected to diseases. It can be further explained by the fact that the structure of ICD-11 and disease categories consider different ontology and semantics compared to AOP-Wiki (ICD-11 is more related to genetic phenotypes and clinical data and AOP-Wiki more focused on adverse outcomes).

Relative to the AOP framework itself, our analysis illustrates a number of aspects and challenges that are important in the context of the currently ongoing effort to increase the FAIRness (Findability, Accessibility, Interoperability, and Reusability) of the AOP-Wiki (Wittwehr et al., 2023) and of toxicological data in general as emphasized by the recently established European intergovernmental ELIXIR toxicology

community (Martens et al., 2021). For example, for efforts such as the present analysis where AOPs are collected through the AOP-Wiki and assembled into AOP networks to be used for meta-analyses, a strong need for AOP (network) curation arises, such as the merging of synonymous KEs to reduce redundancy, the grouping of related but not necessarily identical KEs, and the unambiguous description and identification of KERs. In this context the use of ontological annotations in the AOP-Wiki is becoming increasingly important (Ives et al., 2017; Wittwehr et al., 2023) and is envisioned as one of the cornerstones of the data model that is being developed for the AOP-Wiki version 3.0, which aims to address many of these challenges. This will increase the interoperability with other databases and platforms and facilitate analyses such as the present one.

Also, the development of large AOP networks results in the emergence of new linear AOPs by re-using building blocks from user defined AOPs (Pollesch et al., 2019). An important challenge lies in inventorying and assessing the quality of such emergent AOPs. New tools to address this challenge are underway, which will allow the prioritization of emergent AOPs for further manual assessment and consideration. Another aspect that becomes especially important when assembling AOPs into AOP networks is how to deal with domain of applicability (DOA) descriptions (currently limited to taxonomic group, life stage and sex, but potentially also including other components such as tissue and organ type) across interconnecting AOPs. The DOA has often not been thoroughly investigated for many users defined AOPs, and determining the DOA becomes even more complex in the case of emergent AOPs. For example, an AOP may be established in animals but its relevance to humans may not have been explored. For that reason, not only documenting evidence of conservation across species, but also including evidence that a certain KE/KER is not conserved in a particular taxon may become an important tool for analyzing large AOP networks. A deeper analysis of the KER would also be interesting for further exploration in terms of relevance to human diseases, and to gain a better understanding of the biological space.

Finally, the AOP concept serves as a powerful tool for the systematic classification of knowledge, offering immense potential within the regulatory landscape. By providing a structured framework to understand complex pathways leading to AOs that we can connect to diseases, AOPs will enable more informed decision-making and risk assessment. Furthermore, the emphasis on identifying gaps in our understanding paves the way for the targeted development of assays that address these areas. This dynamic approach encourages innovative thinking, allowing us to explore uncharted territories in toxicology. As we harness the full scope of AOPs, we not only enhance our comprehension of biological interactions but also shape a future where predictive toxicology is both accurate and comprehensive.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

TJ: Investigation, Writing–original draft, Data curation, Methodology, Visualization, Formal Analysis. TC: Data curation, Investigation, Methodology, Visualization, Writing–original draft, Formal Analysis. NS: Investigation, Methodology, Visualization, Writing–review and editing. BV: Investigation, Writing–review and editing, Methodology, Writing–original draft. BL: Investigation, Writing–review and editing, Methodology, Visualization. LV: Investigation, Writing–review and editing, Visualization, Methodology. OM: Investigation, Writing–review and editing. NY: Writing–review and editing, Methodology, Software, Visualization. JG: Writing–review and editing, Investigation, Writing–original draft. PM-L: Writing–review and editing, Formal Analysis, Visualization. FJ: Writing–review and editing. HH: Investigation, Writing–review and editing. XC: Writing–review and editing. DS: Writing–review and editing. PA: Writing–review and editing. AB-P: Writing–review and editing. EF: Writing–review and editing. EK: Writing–review and editing, Writing–original draft. AS: Writing–review and editing. RB: Writing–review and editing. MK: Writing–review and editing. OT: Writing–review and editing. MW: Investigation, Methodology, Visualization, Writing–review and editing, Data curation, Software. DK: Writing–review and editing. KA: Conceptualization, Investigation, Supervision, Writing–original draft, Writing–review and editing.

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Conflict of interest

Author AB-P was employed by Joint Research Centre and Author EF was employed by DNTOX GmbH.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/ftox.2024.1285768/full#supplementary-material>

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New approach methodologies to enhance human health risk assessment of immunotoxic properties of chemicals — a PARC (Partnership for the Assessment of Risk from Chemicals) project

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As a complex system governing and interconnecting numerous functions within the human body, the immune system is unsurprisingly susceptible to the impact of toxic chemicals. Toxicants can influence the immune system through a multitude of mechanisms, resulting in immunosuppression, hypersensitivity, increased risk of autoimmune diseases and cancer development. At present, the regulatory assessment of the immunotoxicity of chemicals relies heavily on rodent models and a limited number of Organisation for Economic Co-operation

and Development (OECD) test guidelines, which only capture a fraction of potential toxic properties. Due to this limitation, various authorities, including the World Health Organization and the European Food Safety Authority have highlighted the need for the development of novel approaches without the use of animals for immunotoxicity testing of chemicals. In this paper, we present a concise overview of ongoing efforts dedicated to developing and standardizing methodologies for a comprehensive characterization of the immunotoxic effects of chemicals, which are performed under the EU-funded Partnership for the Assessment of Risk from Chemicals (PARC).

KEYWORDS

PARC, new approach methodologies, NAMs, immunotoxicology, immunosuppression, regulatory toxicology, chemical toxicology

1 Introduction

The immune system is an immensely complex hierarchical network of cells, tissues, and organs, governed by a multitude of intricately regulated mechanisms. Beyond its primary role in protecting the body against pathogens and in responding to host-originated danger signals, the immune system plays a crucial role in a large number of biological processes including metabolism (Chapman and Hangbo, 2022), neurodevelopment and central nervous system functioning (Morimoto and Nakajima, 2019; Matejuk, Vandenbark, and Offner, 2021), pregnancy (Abu-Raya et al., 2020), as well as digestion and nutrient absorption (Zhou et al., 2020; Wiertsema et al., 2021).

Given these multifaceted properties, it is not surprising that the wide range of chemicals encountered in human life can interact with the immune system and have the potential to modify its functions. Substances capable of inducing immune dysregulation are referred to as immunotoxicants. Immunotoxicants can exert their effects through various mechanisms, including immunosuppression, immunostimulation, sensitization, the development of autoimmunity, and direct toxic effects on the cells and components of the immune system (Rooney et al., 2012). In 2022, a committee of eighteen experts identified ten key characteristics of immunotoxicants, aiming to improve the understanding of their various modes of action (Germolec et al., 2022).

While estimating the exact number of potential immunotoxic substances is challenging, a variety of immunotoxicants have been identified, including chemicals, drugs, infectious agents, pollutants, and more. At present, the list of chemicals with documented immunotoxic properties consists of per- and polyfluoroalkyl substances (PFASs), polycyclic aromatic hydrocarbons (PAHs), chlorinated solvents, pesticides, and others (Veraldi et al., 2006; Naidenko et al., 2021).

Historically, the evaluation of substances for immunotoxicity has been primarily performed using animal models, with established assays such as the T-cell-dependent antibody response (TDAR) and local lymph node assays (LLNA) (Anderson, Siegel, and Meade, 2011; Lebrec et al., 2014). However, the paradigm shift towards animal-free toxicity testing, along with the emergence of novel human-relevant *in vitro* and *in silico* testing and prediction methods, provide a tremendous opportunity to establish and standardize robust, reliable, and comprehensive new approach

methodologies (NAMs) for assessing the immunotoxicological properties of various substances. Despite the highlighted demand for such novel approaches by the World Health Organization (WHO)/International Programme on Chemical Safety in 2012 (<https://apps.who.int/iris/handle/10665/330098>), a scientific consensus and widely accepted standards for immunotoxicity testing of chemicals in the post-animal-testing era are yet to be established. Consequently, only a limited number of *in vitro* immunotoxicity assessment methods have achieved the status of OECD test guidelines. These include TG 444A (IL-2 Luc assay evaluating the immunotoxic effects of chemicals on T-cells), TG 442D (ARE-Nrf2 luciferase test method assessing keratinocytes activation), and TG 442E (a series of tests to determine dendritic cell activation in the context of skin sensitization) (“OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects, 2023”).

Hence, within the European Union (EU)-funded Partnership for the Assessment of Risk from Chemicals (PARC) (<https://www.eu-parc.eu/>) (Marx-Stoelting et al., 2023), the present project aims to develop and harmonize a set of methods for immunotoxicity testing, with the ultimate goal of integrating them into risk assessment and regulatory frameworks.

In this publication, we intend to provide a description of the wide array of methodologies used by consortium partners within the PARC project. The work has been grouped and described according to the respective focus areas: immunosuppression, respiratory sensitization, and various immunotoxicity pathways, as well as the characterization and identification of novel immunotoxic substances.

2 Immunosuppression

Immunosuppression is characterized by a reduction in the functioning of the immune system or a decrease in the quantity of its components (Hussain and Khan, 2022). Immunosuppression can be induced by either an immediate impairment of immune cells or by the stimulation of immunosuppressive mediators production, as well as the alteration of regulatory cell populations (Biologic Markers in Immunotoxicology, 1992; Huaux, 2018). Multiple chemical agents, including pesticides and other organic compounds, metals, and specific immunosuppressive medicines, have been demonstrated to exhibit immunosuppressive properties

(Bou Zerdan et al., 2021). However, there exist no widely accepted chemical-agnostic *in vitro* tests specifically designed for assessing the immunosuppressive properties of chemicals (Wang et al., 2023). Therefore, there is a growing demand for developing NAMs for evaluating immunosuppression.

2.1 Institut national de la santé et de la recherche médicale (INSERM, France)—Immunosuppression under the antigenic challenge conditions

INSERM will focus on the investigation of immunosuppression using experimental models of vaccination response. The initial approach involves using a mouse model to monitor the response to an influenza vaccine in the presence or absence of various chemicals, including toxins and PFASs. To be able to see the effects in mice, concentrations around the lowest observed adverse effect level (LOAEL) will be used (Peden-Adams et al., 2008). Comprehensive cellular (histopathological), molecular (cytokine release) and omics (transcriptomics and targeted proteomics) analyses of animal material (mainly spleen and primary peripheral blood mononuclear cells (PBMCs)) will be conducted. The results of this study will provide a foundation for the development of *in vitro* tests. In parallel, the impact of these chemicals on primary PBMCs, whether activated or not by antigens (e.g., recall antigens) will be monitored using similar methods. Point of departure (POD) doses will be determined by PBBK modeling using publicly available biomonitoring values. Additionally, extracellular vesicles will be characterized. Ultimately, the effects on THP-1 and Jurkat cell lines, representing monocytes and T cells will be analysed and compared to the results from the previous models in order to consider these cell lines for a NAM. A systems toxicology integration of the data will be conducted with other tasks from PARC to identify common gene expression modules across the different experimental settings.

2.2 Norwegian Institute of Public Health (NIPH, Norway)—Assessing immunosuppression using single-cell immune profiling

NIPH will perform a comprehensive analysis of the immunosuppressive properties of PFAS and PFASs mixtures using human primary PBMCs. Initially, a systematic assessment of the cytotoxicity of the test substances will be performed to determine suitable working concentrations. Subsequently, in-depth immune profiling utilizing mass cytometry in combination with cutting-edge unsupervised data analysis methods will enable the characterization of potential immunotoxic effects at the single-cell level. The approach taken will be similar to Nygaard, et al., 2021 to explore expression of functional markers and changes to cell subsets (Nygaard et al., 2021). Additionally, the changes in cytokine levels upon exposure will be quantified using a multiplex Luminex assay. The combined data will allow to identify novel immune-related outcomes associated with PFASs exposure and will serve as valuable information for the further development of NAMs.

2.3 Wageningen Food Safety Research (WFSR, the Netherlands)—Transcriptomic characterization of immunosuppression

WFSR will study the effects of various test substances on existing models for immunotoxicity, such as primary immune cells and cell lines using transcriptomics, flow cytometry, and functional endpoints, covering likely key events for many immunotoxicity pathways. The work will focus on the development of a generic approach for mechanistic research on immunotoxicity. Available animal-free methodologies will be evaluated, assessing which immunological pathways the existing methods address, and selected for testing. Primary immune cells (PBMCs and/or subsets), as well as cell lines, will be treated with test substances (model immunotoxicants, mycotoxins, and priority chemicals such as PFASs) and analyzed using transcriptomics (RNA sequencing) and flow cytometry. Functional endpoints to be assessed include cytokine and antibody production.

2.4 Luxembourg Institute of Health (LIH, Luxembourg)—NAMs for natural killer immunotoxicity

Natural killer cells (NK) are the founding members of the innate lymphoid cell (ILC) family that are both cytotoxic and have antibody-dependent cellular cytotoxicity. LIH will study the consequences of early-life exposure to persistent organic pollutants, especially bisphenol A (BPA) analogs, on NK function, and the long-term health consequences. Our project will address the role of NK in immunosuppression based on previous human studies highlighting that these cells are durably altered in adulthood in individuals who have been exposed to environmental pollutants early in life compared with respective free living control subjects.

In vitro, NK cells derived from Human CD34⁺ Hematopoietic Stem Cells will be used to investigate how BPA analogs trigger immunosuppression or induce immunotoxicity, through an unbiased screening tool for flow cytometry data visualization, viSNE. LIH will specifically address the NK immune cell population immunophenotyping and its functionality (cytotoxicity and degranulation response assessment). An *in vivo* study, based on in-house established rat models of immuno-developmental toxicity will be performed on a selection of BPA analogs inducing NK immunosuppression to (i) validate the suitability of the *in vitro* model at modelling an early life adversity and (ii) to investigate by which mechanisms the immune system becomes impaired after early life exposure to these BPA analogs. The shift in the senescent state of NK cells will also be targeted.

2.5 University of Ljubljana (ULFFA, Slovenia)—*In vitro* substitution for whole blood cytokine release assay and investigation of glucocorticoid receptor role in lymphotoxicity

The Whole Blood Cytokine Release Assay (WBCRA) is used to assess immunosuppression by treating whole blood from a donor

with lipopolysaccharide (LPS) or Staphylococcal enterotoxin B (SEB) and measuring the cytokines released (Langezaal et al., 2002; Corsini and Roggen, 2017). However, this method requires the collection of *ex vivo* blood samples and is therefore subject to inter-individual variation. Furthermore, a lack of standardization of this method has been reported in the literature (e.g., LPS concentration, timing of activation, cytokines measured, reporting of results), making it difficult to draw conclusions and compare results between laboratories (Fullerton et al., 2016). ULFFA will develop an *in vitro* model for WBCRA using cell lines that avoids the need for *ex vivo* material, is standardized in execution and provides reliable results.

The coculture will be prepared using THP-1 and Jurkat cell lines, representing monocytes and T cells. So far, the studies using these cell lines have shown them to be a promising tool for evaluation of immunosuppression. However, the existing studies have rather been focused on cytokine gene expression in each of these lines rather than the cytokine release in their coculture (Kimura et al., 2014; Zhao et al., 2019; Kimura et al., 2021). A range of activators will be investigated to assess their effects on cytokine release from each cell type and the co-culture. Following the application of known immunosuppressants belonging to different mechanisms of action, the changes in secreted cytokines will be investigated. The immunosuppressants will be applied in nanomolar and micromolar concentrations, therefore resembling *in vivo* concentrations. A panel of cytokines that change independently of the immunosuppressive mechanism will be selected as markers for immunosuppression. In the more advanced stages of the project, the lymphoblastoid cell line LCL (Markovič et al., 2015) will be added to the co-culture to bring the model closer to whole blood. Once the model is in place, we will test whether endocrine disruptors such as bisphenols and PFAS act as immunosuppressants.

Lymphotoxicity represents the second tier of the immunosuppression evaluation. The activation of T lymphocytes is mediated via glucocorticoid receptor (GR) and results in IL-2 secretion (Northrop, Crabtree, and Mattila, 1992). However, commonly used *in vitro* models for T lymphocytes are Jurkat T cells that have a mutation in GR and in this respect do not reflect the full function of T lymphocytes (Vacca et al., 1990; Riml et al., 2004). Therefore, we will also use a GR-GAL4 Luciferase Reporter Jurkat Cell Line to investigate whether T-cell inhibition is mediated via the glucocorticoid receptor or not. Other mechanisms underlying immunosuppression will be examined on THP-1 and Jurkat cell lines to exploit the effects on signaling pathways with the aim of novel biomarker identification.

3 Respiratory sensitization

It is widely accepted that certain low-molecular-weight (LMW) chemicals are able to create allergic sensitization of the respiratory tract resulting in hypersensitivity reactions with symptoms such as asthma and rhinitis (Cochrane et al., 2015). Occupational asthma represents about 15% of all adult asthma cases (Vincent et al., 2017). The contribution of LMW chemical exposure to disease development is likely to be underestimated due to diagnostic

challenges. Inhalation is the most common exposure route for the induction of respiratory sensitization, however, skin exposure may also be an effective route (Pauluhn, Woolhiser, and Bloemen, 2005). An induction phase of immunological priming is followed by an elicitation phase triggered by the second exposure to the chemical allergen, resulting in inflammation and symptoms of a hypersensitivity reaction (Cochrane et al., 2015).

Due to the need for accurate and reliable test methods for the assessment of respiratory sensitization potential of chemicals, various testing methods are being explored. However, there are currently neither *in vivo* nor *in silico* or *in vitro* assays available that are universally accepted and validated for this purpose. It is a great challenge to develop NAMs that mimic the crucial steps known so far of the highly complex *in vivo* pathogenesis of respiratory sensitization. A single assay may not be sufficient to complete this task, but a combination of suitable assays could be used in an integrated testing strategy (Jowsey et al., 2006; Üzmezoğlu, 2021).

3.1 Medical University of Innsbruck (MUI, Austria)—NAMs for respiratory sensitization using 3D epithelial and immune cell co-cultures

MUI has developed a new method to assess the effect of long-term volatile chemical exposure on human lung epithelial cell models grown as air-liquid interface (ALI) cultures in a dose-dependent manner. The exposure technology is based on a previously developed incubator platform that generates a stable atmosphere containing defined concentrations of volatile compounds (Gostner et al., 2016). Exposure of ALI cultured epithelial and immune cells in co-cultures allows the evaluation of the effects of the test substances on the interactions of these cell types, as relevant biological processes involved in respiratory immunotoxicity and sensitization can be mimicked. Cytotoxicity and pathway focused readouts will be combined with expression data to identify relevant targets.

3.2 National Centre for Public Health and Pharmacy (NPHC, Hungary)—NAMs for the identification of respiratory sensitizers focusing on lung epithelial and dendritic cells

NPHC will explore the effects of low-molecular-weight chemical respiratory sensitizers (such as chloramine-T and piperazine) on human airway epithelial cells (BEAS-2B) and dendritic cells (DC) using a variety of cellular, biochemical and omics methods. Chemical concentrations will be determined based on cell viability assay results (da Silva et al., 2023).

Extracellular vesicles (EVs) are membrane-coated nanovesicles, actively secreted by cells (Szatmári et al., 2019). EVs contain DNA, mRNA, microRNA, lipids and proteins that are potential biomarkers of various diseases. EVs are taken up by target cells, transferring information from one cell to another, thereby influencing their behavior. It has been described that EVs in the

lung play an important role in eliciting airway inflammation and allergic immune responses (Fujita et al., 2014; Zhang et al., 2021), and analyzing the EV cargo of airway epithelial cells may be used to identify substances with respiratory sensitizing potential.

NPHC aims to develop a NAM involving dendritic cells and EVs for chemical risk assessment. EVs will be isolated from the supernatant of airway epithelial cells treated with relevant chemicals. The DC activating capacity of EVs will be investigated and compared to DC activation induced by sensitized airway epithelial cells. We hypothesize that EVs and their content might be used to predict respiratory sensitization, therefore, they might represent possible NAMs. We will also conduct *in vivo* experiments to validate our *in vitro* NAM model by using EVs isolated from the bronchoalveolar lavage fluid (BALF) of sensitized mice.

3.3 National Institute for Public Health and the Environment (RIVM, the Netherlands)—NAMs for the identification of respiratory sensitizers using air-liquid interface models

RIVM will study the effects of exposure of the Calu-3 human bronchial epithelial cell ALI model (Braakhuis et al., 2020) to a dose range of the respiratory sensitizers: chloramine-T and piperazine. Parameters are viability, barrier function, and cytokine/chemokine production. For one of the substances, the analysis will be complemented with a primary bronchial epithelial cell model.

3.4 Luxembourg Institute of Science and Technology (LIST, Luxembourg)—NAMs for the identification of respiratory sensitizers

LIST will use the 3D *in vitro* model ALIsens® representing the alveolar barrier (Chary et al., 2019) originally developed in-house to identify respiratory sensitizers. ALIsens® is based on an older model developed to identify respiratory irritants (S. G. Klein et al., 2013; 2017; Fizeşan et al., 2018; Fizeşan et al., 2019) and consists of 4 cell lines (A549, alveolar epithelial cells; THP-1 derived macrophages (PMA differentiated); THP-1 native, dendritic cells; EA.hy 926, endothelial cells) grown at the ALI in hanging inserts (Lacroix et al., 2018; Burla et al., 2023). The two chosen substances chloramine-T and piperazine will be assessed. Parameters measured will include cell viability, surface marker expression (CD54, CD86, TSLPr), cytokine/chemokine production (including IL-6, MIP-3α, MCP-1, GM-CSF, TSLP, IL-7) and potentially also the expression of a set of defined genes (CD86, CD80, CD8a, G-CSF and others).

4 Inflammatory effects

As chronic inflammation is recognized as an integral part of immunotoxicity (Kanterman, Sade-Feldman, and Baniyash, 2012), the PARC project aims to enhance methods to evaluate the proinflammatory properties of chemicals.

4.1 Institute for Risk Assessment Sciences (IRAS, the Netherlands)—*In vitro* models for lung and liver inflammation induced by chemicals

IRAS will study the effects of BPA analogs and toxins using established NAMs which reflect hub key events (KEs) that have been linked to several adverse outcome pathways (AOPs) of inflammatory diseases such as lung and liver fibrosis (Villeneuve et al., 2018).

The three hub KE are designated “Tissue resident cell activation”, “Increased proinflammatory mediators” and “Leukocyte recruitment and activation”. These KE have also been linked to AOP for skin sensitization and food allergy and focus mainly on initiating inflammation, realizing that inflammation is heavily controlled by a range of regulatory mechanisms. IRAS will build on an inflamed intestinal model that uses co-cultures of adipocytes, intestinal epithelial and immune cells (DC and T cells). This model relates to the metabolic effects of endocrine disrupting chemicals (EDCs) on immune responses initiated by tissue-resident cells. The focus of read-out parameters will be on cell stress (KE1), formation of mucus and other proinflammatory mediators (e.g., cytokines/chemokines) (KE2), and macrophage/DC/T cell activation (KE3). This research will assess whether these models can be useful as NAMs in IATA development.

5 Hazard identification and hazard characterization

In the PARC project, beside the development of NAMs for immunotoxicity which are not targeting specific substances, other partners have focused on particular data-poor substances such as BPA alternatives and mycotoxins. These PARC priority substances have been extensively reviewed by PARC partners in separate publications (Kodila, Franko, and Sollner Dolenc, 2023; Louro et al., 2024).

5.1 Mycotoxins (*Alternaria* toxins and enniatins)

5.1.1 National Institute of Occupational Health (STAMI, Norway)—Immunomodulating effects of *Alternaria* toxins

STAMI will test the immunomodulating potential of *Alternaria* mycotoxins (alternariol, alternariol monomethyl ether, altenuene, tenuazonic acid, tentoxin, altertoxin-I).

The innate immune response is crucial for early defense against infections. Certain mycotoxins inhibit the lipopolysaccharide (LPS) triggered induction of the Nuclear Factor-κB (NF-κB) pathway (Del Favero et al., 2020). Dysregulation in the NF-κB pathway, especially unappropriated activation can lead to host susceptibility to infections and disease-causing organisms (Bąska and Norbury, 2022). We will explore the potential immunomodulating effect of the *Alternaria* mycotoxins (alternariol, alternariol monomethyl ether, altenuene, tenuazonic acid, tentoxin, altertoxin-I) on NF-κB activation via innate immune receptors: toll-like receptors (TLR)

and dectin receptor signalling, using HEK-Blue™ reporter cell assays for hTLR2, hTLR4, hDectin-1a and hDectin-1b (Invivogen). The reporter cells will be exposed to mycotoxins in combination with natural activators of these receptors (LPS or LTA). This system has been suggested to be a useful tool for studying the potential of microbial components in organic dust to engage the immune system (Afanou et al., 2023; Schmeisser et al., 2023).

Previous studies indicate that physiological relevance increases in line with the complexity of models (Lacroix et al., 2018), and that advanced 3D models, which are closer to tissue and organ structure, can be more reliable for *in vitro* toxicity testing in human hazard assessment (Camassa et al., 2022). To mimic occupational inhalational exposure the biologically relevant *in vitro* human 3D mucociliary lung tissue model (EpiAirway™, Mattek) (Silva et al., 2023) will be used for pulmonary toxicity assessment of the *Alternaria* mycotoxins. The model will be complemented with the seeding of differentiated THP-1 cells on the apical side of the epithelium. EpiAirway™ trans-well inserts will be exposed to mycotoxins in the ALI system, which allows for exposures to more realistic concentrations of airborne particles compared to submerged cultures (Braakhuis et al., 2020). The co-cultures will be exposed to single and mixtures of toxins (with or without co-exposure to LPS). After exposure cells will be collected for transcriptomics and pathway analysis with a particular focus on pulmonary immunotoxicity will be carried out. The results may add to the development of a battery of NAMs needed for carrying out NGRA of *Alternaria* toxins.

5.1.2 Norwegian Veterinary Institute (NVI, Norway)—Studying immune effects of mycotoxins in gastrointestinal models

NVI will study the effects of enniatins and *Alternaria* mycotoxins on a commercially available human gastrointestinal model based on primary cells (EpiIntestinal™, MATTEK). The selected mycotoxins are widely distributed in food and feed and the gastrointestinal tract is one of the first potential targets in the body. The cell model allows studies of effects on cytokines production and barrier function as well as absorption and biotransformation and intestinal morphology.

The cells will be exposed to the toxins alone or in combination with IL-1b or LPS. This will allow studies of immune inhibition as well as immunostimulation. The effects on the production of multiple cytokines will be measured using multiplex methods and the effects on the barrier will be measured by trans-epithelial electric resistance. In addition, the model allows studies of absorption and biotransformation in the gut epithelium. All data will be compared with available information from animal experiments and more traditional human cell line models to validate the relevance of the model for human risk assessment.

5.1.3 Norwegian Institute of Public Health (NIPH, Norway)—Immunosuppressive actions of mycotoxins on PBMCs

NIPH aims to study the potential immunomodulatory properties of emerging mycotoxins. This research focuses on six *Alternaria* toxins and five enniatins. The project's experimental setup involves isolating PBMCs from both healthy male and female donors, to account for any sex effects (Sankaran-Walters

et al., 2013; S. L.; Klein and Flanagan, 2016; Abdullah et al., 2012). These PBMCs will be exposed to *Alternaria* toxins and enniatins for 24 and 48 h.

Firstly, cell viability assays will be used to determine the appropriate dose range, with a particular emphasis on avoiding cytotoxic effects. Additionally, a Luminex multiplex assay will be used to quantify cytokine levels, providing insights into immune cell functional responses when exposed to these mycotoxins. Lastly, to provide deeper insight into immune modulation by the mycotoxins, changes in various immune cell populations and their activation status as well as functional markers will be investigated using mass cytometry, described in section 2.2 by NIPH. Together, these steps will contribute to filling data gaps on the immunotoxic potential of mycotoxins.

5.1.4 German Federal Institute for Risk Assessment (BfR, Germany)—*In vivo* repeated-dose study of enniatin B1 toxicity

In addition to the various *in vitro*- and cell-based approaches delineated above, an OECD TG 408-compliant 90-day repeated-dose oral toxicity study with enniatin B1 in rats will be performed, with the aim to close data gaps regarding this substance, and to facilitate the risk assessment of enniatins. The study will first be using classic endpoints related to histopathology and clinical chemistry. In addition, tissues from the study will be utilized for NAM-based analyses. Samples from different organs will be subjected to whole transcriptome characterization, to obtain mechanistic insight into the toxicological consequences of enniatin B1 exposure, including effects on immune-related functions. More specifically directed towards immune parameters, PBMCs and spleen homogenates will be used for immunoprofiling with flow cytometry or mass cytometry. Investigation of *in vivo* material will increase our mechanistic knowledge on enniatin toxicity and comparison to *in vitro* NAM-based findings will increase confidence in the validity of *in vitro* approaches and NAMS in the field of immunotoxicity.

5.2 Bisphenol A analogs

5.2.1 University of Milan (UMIL, Italy)—Effects of BPA analogs on primary T- and B-cells

UMIL will address the effects of BPA analogs on T cell differentiation and immunoglobulin production. Literature evidence shows the ability of BPA to interact with T cells, and mainly with T helper 17. We will use PBMCs purified from buffy coats obtained from healthy donors of both sexes. PBMC will be exposed to BA for 24 h and subsequently stimulated with anti-CD3/anti-CD28 for 4 days to induce T cell activation, differentiation and expansion. After 5 days in total, cells will be harvested, and T helper cell differentiation will be assessed through the detection of intracellular cytokines or cell surface markers (by flow cytometry) and evaluation of the cytokine release (by ELISA). CD4⁺ cells will be analyzed for the expression of IFN- γ , IL-4, IL-17A to assess T helper 1, 2, and 17 populations, respectively. Regulatory T cells, CD45⁺CD4⁺CD127⁻ cells, will be analyzed based on CD25 and FoxP3 expression and for the absence of CD127. In parallel, the release of IFN- γ , IL-4, IL-17A, and TGF- β will be assessed in cell-

free supernatants. Another important class of lymphocytes is represented by B cells, whose main role is antibody production, which can be targeted by BPA analogs. We will investigate the effect of PBMCs exposed to BPA analogs for 24 h *in vitro* with subsequent stimulation with ODN2006 and rh-IL-2 for 6 days on B cells. At the end of the treatment, total IgG and IgM production will be measured in cell-free supernatants using ELISA. In addition, as positive reference control of immunosuppression, cyclosporin A and rapamycin will be used for T cell and B cell assays, respectively.

5.2.2 National Institute of Health Dr. Ricardo Jorge (INSA, Portugal)—Using co-cultures of epithelial cells with fibroblasts and macrophages to investigate the immunomodulatory effects of BPA analogs

INSA will study the effects of BPA analogs (and their mixtures) using cellular and molecular approaches. Co-cultures of polarized epithelial cells from the intestine or lung with fibroblasts and undifferentiated macrophages M0 (THP-1, a widely used model of macrophages) will be used to explore the impact of chemicals on the release of cytokines and other immune modulators (e.g., ROS, NO and PGE2), as well as changes in macrophage differentiation (M1 and M2 markers).

5.2.3 Helmholtz Centre for Environmental Research (UFZ, Germany)—Creating flow cytometry test panels to identify immune cell populations affected by chemicals

Researchers at the UFZ have established a flow cytometry-based test battery for chemicals testing on T cell subsets, MAIT cells, basophils, NK and B cells by selecting unique cell type-specific stimulus and surface and intracellular activation markers (Krause et al., 2023; Maddalon et al., 2023; Pierzchalski et al., 2023; Pierzchalski et al., 2024). These tests are designed to evaluate the immunosuppressive and immunostimulatory effects in a short time. UFZ will further develop and standardize their assays by determining which immune cell types are affected the most upon incubation of human PBMCs with BPA analogs and PFASs. In a later phase of the project, the interaction between treated immune cells/placenta 3D cultures will be studied *in vitro*. This will help to assess immune cells as mediators of toxicity towards placenta and therefore contribute to the refinement of reproductive toxicology test systems.

6 Mechanistic constructs

6.1 Adverse Outcome Pathway (AOP) development (coordinated by NIPH, Norway and MUI, Austria)

As demonstrated from the large variety of projects by numerous PARC partners above, toxicologists are generating an increased volume in data by both pathway focused and high throughput omics techniques, thus expanding the existing information volume on relevant toxic effects exerted by chemicals as well as on underlying mechanisms.

This increasing amount of data and publications pose a challenge for their timely use in chemical risk assessment.

Therefore, the concept of AOPs which integrate information along a series of events finally leading to an adverse outcome, considering the different levels of biological organization, is an important tool for the assessment of causality and human relevance. Importantly, AOPs support the use of NAMs to predict adverse health outcomes and hence AOPs are being developed in the same priority areas as the NAMs described above.

There is a recognised need for AOPs in the area of immunotoxicity, as these are underrepresented in the AOP wiki and needed to support regulatory decision making. During the first years of the PARC project there is a concerted effort to focus on the (further) development of AOPs in the areas of immunosuppression, respiratory sensitization and inappropriate immune enhancement (possibly leading to inflammatory diseases). Focus areas for immunosuppression are the development of existing AOP 14 (<https://aopwiki.org/aops/14>), related to glucocorticoid receptor activation and the inclusion of NK cell inhibition as a KE to include these important components of the innate immune system. In addition, there is work underway to further develop AOP 39, related to respiratory sensitization and to expand on the existing Molecular Initiating Events (MIEs) and KEs in the AOP wiki for sensitizer identification.

Chronic, low-grade inflammation may increase the risk of several non-communicable diseases. However, similar inflammatory processes operate in different adverse outcomes and these need to be better represented as hub KEs in several AOP framework (Villeneuve et al., 2018). Models representing these hub KEs may serve as NAMs to screen potential proinflammatory properties of chemicals. In PARC, we will incorporate inflammatory events into the targeted AOPs where appropriate, e.g., in the AOPs for fibrosis, sensitization and tumour development and non-genotoxic carcinogenesis in which inflammation is a driver of disease.

7 Concluding remarks

Establishing *in vitro* and *in silico* tests assessing immunotoxicity is among the most challenging objectives of NAMs development and animal-free science. The complexity of the immune system and the kinetic and dynamic interactions between its different components are not readily represented in simple assays. However, there are a number of recent advances that will help us make significant progress toward this objective. 1) The proposal for ten key characteristics of immunotoxicants provides the relevant biological targets that NAMs should address (Germolec et al., 2022). 2) Building AOPs in immunotoxicology will help in identifying the most relevant pathways and key events that NAMs should evaluate. This has been well illustrated in the case of skin sensitization (Clouet et al., 2019; Wang et al., 2023) and food allergy (Bilsen et al., 2017). Developing new AOPs in immunotoxicology, as described above, is also being carried out in PARC and will feed into NAMs development (De Castelbajac et al., 2023). 3) The growing knowledge on the signaling pathways triggered by drugs such as immunosuppressants is extremely useful for identifying critical signaling steps, such as in the case of calcineurin signaling (Ulengin-Talkish and Cyert, 2023). 4) The remarkable increase in understanding of pathways activated by

specific receptors, like the Arylhydrocarbon Receptor (Larigot et al., 2022) will also contribute to the further identification of the most relevant NAMs.

Since PARC is founded on science and policy interactions, we prioritize our projects accordingly. In order to support regulatory needs, the projects described here focus on respiratory sensitization and immunosuppression, which are two areas where robust assays are urgently required. However, other outcomes including inflammation promotion, developmental immunotoxicity and autoimmune diseases are also relevant. It is worth noting that the immune system is also involved in multiple diseases (e.g., cancer, gastrointestinal diseases, etc.) and, therefore, the NAMs developed here can have a wide range of applications for a spectrum of health conditions.

As the potential immunotoxicity is a significant concern for several chemicals prioritized in PARC Work Package 5, including BPA, its analogs (Kodila, Franko, and Sollner Dolenc, 2023) and certain mycotoxins (Schmutz, Cenik, and Marko, 2019; Kraft, Buchenauer, and Polte, 2021; De Felice, Spicer, and Caloni, 2023), these substances will be used in addressing data gaps and advancing the development of NAMs for immunotoxicity. Additionally, PFASs serve as model chemicals for several groups in PARC, given their ability to target the immune system (Holst et al., 2021; Ehrlich et al., 2023).

We expect this project to significantly contribute to the development of NAMs in immunotoxicology and to support the regulatory and research communities in this field. While the translation of immune tests into NAMs will be a gradual process, the effort is undoubtedly worthwhile.

Author contributions

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editing, Writing-original draft. AJ: Methodology, Writing-review and editing, Writing-original draft. SK: Methodology, Writing-review and editing, Writing-original draft. BL: Methodology, Writing-review and editing, Writing-original draft. KL: Methodology, Writing-review and editing, Writing-original draft. AM: Methodology, Writing-review and editing, Writing-original draft. SM: Methodology, Writing-review and editing, Writing-original draft. LP: Writing-review and editing, Writing-original draft, Methodology. AP: Writing-review and editing, Writing-original draft, Methodology. RP: Writing-review and editing, Writing-original draft, Methodology. MS: Writing-review and editing, Writing-original draft, Methodology. ASO: Writing-review and editing, Writing-original draft, Methodology. YS: Writing-review and editing, Writing-original draft, Methodology. AS: Writing-review and editing, Writing-original draft, Methodology. TS: Writing-review and editing, Writing-original draft, Methodology. JT: Writing-review and editing, Writing-original draft, Methodology. RV: Writing-review and editing, Writing-original draft, Methodology. AZ: Writing-review and editing, Writing-original draft, Methodology. RB: Writing-review and editing, Writing-original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

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New approach methods to assess developmental and adult neurotoxicity for regulatory use: a PARC work package 5 project

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In the European regulatory context, rodent *in vivo* studies are the predominant source of neurotoxicity information. Although they form a cornerstone of neurotoxicological assessments, they are costly and the topic of ethical debate. While the public expects chemicals and products to be safe for the developing and mature nervous systems, considerable numbers of chemicals in commerce have not, or only to a limited extent, been assessed for their potential to cause neurotoxicity. As such, there is a societal push toward the replacement of animal models with *in vitro* or alternative methods. New approach methods (NAMs) can contribute to the regulatory knowledge base, increase chemical safety, and modernize chemical hazard and risk assessment. Provided they reach an acceptable level of regulatory relevance and reliability, NAMs may be

considered as replacements for specific *in vivo* studies. The European Partnership for the Assessment of Risks from Chemicals (PARC) addresses challenges to the development and implementation of NAMs in chemical risk assessment. In collaboration with regulatory agencies, Project 5.2.1e (Neurotoxicity) aims to develop and evaluate NAMs for developmental neurotoxicity (DNT) and adult neurotoxicity (ANT) and to understand the applicability domain of specific NAMs for the detection of endocrine disruption and epigenetic perturbation. To speed up assay time and reduce costs, we identify early indicators of later-onset effects. Ultimately, we will assemble second-generation developmental neurotoxicity and first-generation adult neurotoxicity test batteries, both of which aim to provide regulatory hazard and risk assessors and industry stakeholders with robust, speedy, lower-cost, and informative next-generation hazard and risk assessment tools.

KEYWORDS

new approach method (NAM), developmental neurotoxicity (DNT), adult neurotoxicity (ANT), DNT-IVB, zebrafish, applicability domain

1 The European Partnership for the Assessment of Risks from chemicals (PARC)

The European Partnership for the Assessment of Risks from Chemicals (PARC) aims to develop next-generation chemical hazard and risk assessment tools to better protect human health and the environment (Marx-Stoelting et al., 2023). A major ambition of the project is to develop new approach methods (NAMs) for human health hazard assessment that covers developmental neurotoxicity (DNT), adult neurotoxicity (ANT), thyroid hormone disruption, immunotoxicity, and non-genotoxic carcinogens. Work package 5.2.1e aims to refine existing NAMs, develop new ones, and generate first-generation ANT and second-generation DNT test batteries. The NAMs that will be developed will be based on Key Events (KE) as identified in the Adverse Outcome Pathway (AOP) framework (Ankley et al., 2010; Leist et al., 2017; Spinu et al., 2021). The work is carried out by a consortium of over 25 experts from 10 EU research institutions and two partner institutions in non-EU countries.

2 Exposure to chemicals may pose a risk to the developing and mature nervous systems

Exposure to chemicals can adversely impact nervous system development and function across all stages of life (Costa et al., 2008; Giordano and Costa, 2012; P. Grandjean and Landrigan, 2006). Adverse chemical-dependent effects stemming from exposure of the developing offspring (including *in utero* and postnatal) to the time of sexual maturation may affect the developing nervous system (Costa et al., 2004). Such “developmental neurotoxicity” (DNT) can be long-lasting, extending far beyond the exposure period, and can vary across lifespan (Eriksson et al., 1998; Spalding et al., 2013). Note that any type of neurotoxic effect during development is of regulatory concern and relevant for developmental hazard identification. In contrast, when the mature nervous system is exposed to neurotoxic chemicals, adult neurotoxicity (ANT) effects can be immediate or they may be gradually developing and long-lasting. Depending on the type of

ANT effect, it may also be reversible (Spencer and Lein, 2014). Significant and/or severe neurotoxicity, being reversible or irreversible, immediate or delayed, is of regulatory concern.

Due to the sensitivity of the developing nervous system, exposure to low concentrations of certain chemicals may lead to structural and functional disruptions (Rice and Barone, 2000; Grandjean and Landrigan, 2014; Bennett et al., 2016). Neurodevelopmental disorders including autism spectrum disorder, intellectual disability, attention deficit/hyperactivity disorder (ADHD), neurodevelopmental motor disorders (including tic disorders), and specific learning disorders can have lifelong socioeconomic consequences, including diminished economic productivity or an increased need for learning support in schools (P. Grandjean and Landrigan, 2006). Whilst estimates were acknowledged to be uncertain, in the EU, ~30,000 disability adjusted life years (DALYs) related to neurodevelopmental disease may be the result of chemical exposure (and irrespective of a person’s genetic predisposition/sensitivity), with ~250,000 DALYs when chemical exposure was combined with underlying genetic predisposition (EC 2019). This estimate was based on a ‘top down’ assessment of impacts of pervasive neurodevelopmental disorders from the World Health Organization (WHO) and an estimate that 3% is due to environmental exposure to legacy compounds such as lead and other environmental pollutants (EC 2019). Notable socioeconomic benefits are therefore predicted via the identification of substances which are known or presumed to cause DNT and subsequent prevention of exposure (Bellanger et al., 2013; Bellanger et al., 2015).

After the developmental period, acute and/or chronic exposure to environmental chemicals may elicit toxic responses in the peripheral and/or central nervous systems and it has been suggested that exposure to specific chemical agents may increase the probability of developing neurodegenerative disorders such as Parkinson’s and Alzheimer’s Diseases, or dementia (Landrigan et al., 2005; Tanner et al., 2014; Ockleford et al.). Moreover, exposure to certain chemicals has been suspected to be linked to adolescent and adult depression, anxiety, and other psychiatric disorders in a number of academic publications (Dickerson et al., 2020; Hollander et al., 2020; Jacobson et al., 2022; Rokoff et al., 2022; Aung et al., 2023). In a study of 22 chemical inventories from 19 countries and regions, over 350,000 chemicals and mixtures of

chemicals were identified as registered for production and potentially in use (Wang et al., 2020). Despite knowledge concerning the potentially harmful impacts of environmental chemicals on the developing and mature nervous systems (P. Grandjean and Landrigan, 2006), it is understood that only a limited number of unique substances has been tested for DNT using OECD Test Guideline (TG) studies. (OECD, 2008a; Makris et al., 2009; Sachana et al., 2019; Crofton and Mundy, 2021).

3 Policy and regulatory landscapes

The EU Green Deal describes health impacts in the Zero-Pollution Action Plan, and the European Commission recently highlighted their interest in increased efforts to protect against the most harmful chemicals, by further exploring the risk management possibilities of neurotoxic and endocrine disrupting (which has been linked to DNT) substances (European Commission, 2020). In the EU, several relevant regulations are in force. For example, before entering the market or gaining approval as a biocidal or pesticidal active substance, the minimum data requirements described in the relevant EU Regulation must be fulfilled (among other conditions). EU regulations on plant protection products (Reg EC 1107; European Parliament and Council, 2009) and biocides (Reg EC 528; European Parliament and Council, 2012) can require DNT/ANT testing as part of the data requirements. Under the EU Biocides Product Regulation (Reg EC 528; European Parliament and Council, 2012), specific DNT testing, for example, OECD TG 426, recently became a mandatory information requirement for the approval process of active biocidal substances. Under REACH (European Parliament and Council, 2006), the European Regulation created to protect human health and the environment from harmful chemicals, the level of information required to identify potential neurotoxic (DNT/ANT) properties currently depends on the tonnage and identification of specific concerns that may trigger DNT or ANT tests. The available information is used to apply appropriate hazard classifications, as per the criteria specified in the CLP regulation (Reg EC 1272; European Parliament and Council, 2008), to inform on the hazardous properties of chemicals. Classification in accordance with CLP then serves to trigger or inform remedial actions in other legislation to control the hazard. The CLP regulation (Articles seven and 8) does not require DNT or ANT TGs directly but rather makes use of all available data generated in the context of relevant legislation and/or otherwise available in the public domain. In cases where such data is not available to inform on a given hazard, testing may be conducted under certain conditions including the condition that tests on animals are to be carried out only where no other alternatives, which provide adequate reliability and quality of data, are possible. This implies support and presents an opportunity for the development, validation, and implementation of NAMs.

Within the CLP Regulation, substances with DNT are addressed under the reproductive (developmental) toxicity hazard class and ANT effects are addressed under Specific Target Organ Toxicity (STOT), either single exposure (SE) or repeated exposure (RE), depending on whether the effects are caused by single or repeated exposures, respectively. The recent revision of the CLP regulation

includes a new hazard class for endocrine disruption that includes endocrine activity mediated adverse effects on the developing (and mature) nervous system (Reg EC 1272; European Parliament and Council, 2023). According to the new criteria, classification as ED Category one shall be largely based on evidence from at least one of the following: human data; animal data; non-animal data providing an equivalent predictive capacity as human data or animal data (Reg EC 1272; European Parliament and Council, 2023). Thus, the new hazard class allows for NAMs to be directly used for the purpose of this specific classification when the criteria are met.

3.1 DNT/ANT in current chemical regulations

The information needed to fulfill the data requirements under REACH and BPR is typically provided by the *in vivo* OECD TG studies defined in the relevant section of the applicable regulation, but there are also specific possibilities for adaptation (more specifically data waiving). Such adaptation possibilities can include non-animal approaches and/or the use of existing information stemming from similar substances via a read-across approach (European Parliament and Council, 2006). However, where data on human health and environmental properties are derived via adaptations to data requirements, certain conditions apply. The conditions for adaptations using *in vitro* data under REACH are specified in Annex XI, section 1.4. In the context of a read across adaptation (REACH Annex XI, section 1.5), again certain restrictive conditions apply with regard to the data that directly informs on the hazard. However, for the extrapolation of such data to other substances, there is a clear opportunity to utilize NAMs as supportive information to demonstrate similarity in the properties of the substances concerned.

It should also be noted that, depending on the applicable regulation, new tests may not be necessary if the available data is already sufficient for the regulatory purpose as given in the specific regulation. For example, the DNT study shall not be conducted under the BPR if the available data already indicate that the substance causes developmental toxicity and meets the criteria to be classified as toxic for reproduction category 1A or 1B: May damage the unborn child (H360D), and these available data are adequate to support a robust risk assessment (European Parliament and Council, 2012).

A range of OECD TG studies, including single-dose studies (e.g., OECD TG 402, 403, 420, 423, 425) and/or repeated dose toxicity studies (e.g., OECD TG 407, 408, 421, 422, 414, 443 in the absence of DNT cohorts) may inform on ANT or DNT based on clinical signs such as paralysis, convulsions, lack of coordination, or ataxia or neurohistopathology and/or alterations in brain weight (Table 1). DNT can be evaluated more comprehensively using dedicated tests such as OECD TG 426 or in the DNT cohort (cohorts 2A and 2B) of the Extended One-Generation Reproduction Toxicity Study (EOGRTS, OECD TG 443). OECD TGs dedicated to studying ANT include OECD TG 424, 418 and 419. Under REACH, ANT or specific mechanisms/modes of action with an association to (developmental) neurotoxicity can be used to trigger specific DNT studies. Substances in food intended for infants can also

TABLE 1 Description of existing OECD guideline studies that include neurotoxicity as an endpoint.

Test guideline	Primary endpoint	Neurotox endpoint	Preferred Species	Administration period	Non-behavioral endpoints related to neurotoxicity	Behavioral endpoints	Reference
OECD TG 402	Dermal Toxicity	ANT (acute)	Rat	Adults (<24 h)	No (just gross necropsy)	Autonomic and central nervous system and somatomotor activity and behavior pattern	OECD (2017)
OECD TG 403	Inhalation Toxicity	ANT (acute)	Rat	Adults (4 h)	No (just gross necropsy)	Autonomic and central nervous system and somatomotor activity and behavior pattern	OECD (2009)
OECD TG 407	Oral Toxicity/Endocrine Disruption	ANT (chronic)	Rat	Adults (daily - 28d)	Brain weight, histopathology of brain, spinal cord, and sciatic nerve	Sensory reactivity to stimuli, limb grip strength, motor activity	OECD (2008b)
OECD TG 408	Oral Toxicity/Endocrine Disruption	ANT (chronic)	Rat	Adults (daily - 90d)	Brain weight, histopathology of brain, spinal cord, and sciatic nerve	Sensory reactivity to stimuli, limb grip strength, motor activity, autonomic activity	OECD (2018a)
OECD TG 418	Neurotoxicity (OP substances)	ANT (acute)	Hen	Adults (single dose)	Neuropathology of central and peripheral nervous system, NTE and AchE activities	Behavioral abnormalities, ataxia, and paralysis	OECD (1995a)
OECD TG 419	Neurotoxicity (OP substances)	ANT (chronic)	Hen	Adults (≥28 days)	Neuropathology of central and peripheral nervous system, NTE and AchE activities	Behavioral abnormalities, ataxia, and paralysis	OECD (1995b)
OECD TG 420	Oral Toxicity	ANT (acute)	Rat	Adults (single dose)	No (just gross necropsy)	Somatomotor activity and behavior patterns	OECD (2002a)
OECD TG 423	Oral Toxicity	ANT (acute)	Rat	Adults (single dose)	No (just gross necropsy)	Somatomotor activity and behavior patterns	OECD (2002b)
OECD TG 424	Neurotoxicity	ANT (chronic)	Rat	Adults (≥28 days)	Neuropathology of central and peripheral nervous system	Sensory reactivity to stimuli, limb grip strength, motor activity	OECD (1997)
OECD TG 425	Oral Toxicity	ANT (acute)	Rat	Adults (single dose)	No (just gross necropsy)	Somatomotor activity and behavior patterns	OECD (2022)
OECD TG 426	Neurotoxicity	DNT (chronic)	Rat	Gestation & Lactation	Developmental abnormalities, Brain weights, Neuropathology	Motor activity, Motor and sensory function, Learning and memory	OECD (2007)
OECD TG 443	Reproductive Toxicity	DNT (chronic)	Rat	Premating - Pups	Neurohistopathology, Brain weight and morphometry	Auditory startle, Functional observational battery (open field, manipulative, and physiologic), motor activity	OECD (2018b)

NTE: neuropathy target esterase; AchE: acetylcholinesterase; M: male; F: female.

prompt investigations to assess potential DNT ([EFSA Scientific Committee et al., 2017](#)).

As the development of the nervous system starts prenatally and continues to develop through adolescence, reaching adult levels of neurotransmitters, synaptic plasticity, myelination and grey matter at around age of 20 in humans and around PND60 in rats ([Semple et al., 2013](#)), it is key to implement exposure throughout the whole developmental period for improving the chances to identify

developmental neurotoxicants. In an OECD TG 426, the offspring are exposed as a minimum from the time of implantation (starting on gestation day (GD) 6) throughout lactation (until postnatal day (PND) 21). In cohort 2A of an EOGRTS, the offspring are exposed via the mother *in utero*, through lactation and directly at least after weaning until termination on ~ PND 66–77. The assessed DNT parameters in specific DNT studies include as a minimum (depending on the OECD TG) motor activity, motor and sensory function, associative learning

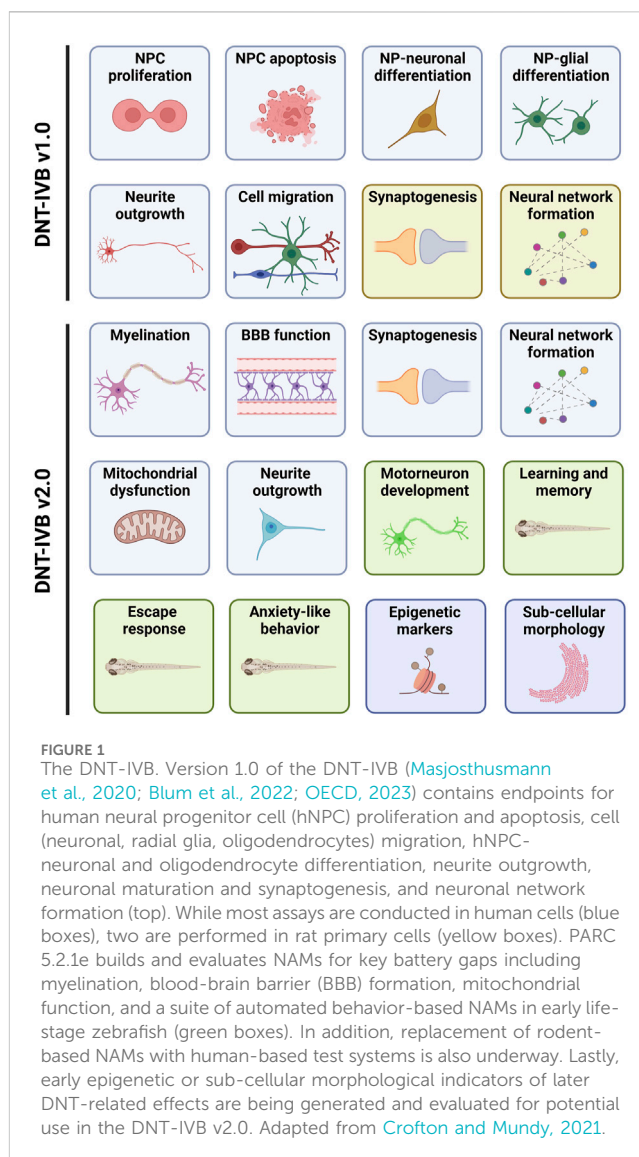
and memory (only in OECD TG 426 as standard testing), brain weight, and central and peripheral nervous system histopathology (Tsuij and Crofton, 2012). As dedicated DNT studies are often complex studies using rodents, they are resource-intensive regarding time, costs, and number of animals (Crofton et al., 2012; Smirnova et al., 2014) and only a limited number of chemicals have been tested for DNT using OECD TG DNT studies (OECD, 2008a; Makris et al., 2009; Sachana et al., 2019; Crofton and Mundy, 2021). In addition, variability in rodent neurotoxicity tests has been documented (Tsuij and Crofton, 2012; Terron and Bennekou, 2018; Sachana et al., 2019; Paparella et al., 2020; Harry et al., 2022) which indicates a need to specify, optimize, and harmonize the individual test methods used as part of the OECD TG. It also underscores the need for new tests that lack the excess variability associated with *in vivo* guideline studies for the assessment of DNT.

The risk posed by unidentified (developmental) neurotoxic agents and the limited number of timely and cost-efficient test systems (i.e., NAMs) serve as the basis for this PARC project where the ambition is the generation of an improved *in vitro* and alternative DNT test battery and a first-generation *in vitro* ANT test battery. The need to develop NAM-based next-generation hazard and risk assessment for DNT and ANT has found international support from academic scientists, industry, certain regulatory authorities, and other interest groups (Smirnova et al., 2014; Ockleford et al., 2017; Fritsche et al., 2018; Kavlock et al., 2018; Craig et al., 2019; Paparella et al., 2020; Vinken et al., 2021; Pallocca et al., 2022; Stucki et al., 2022). ECHA recently published *Key Areas of Regulatory Challenge* (ECHA, 2023), which highlights several of the known scientific and regulatory challenges that NAMs face. It further underlines the need for additional research in the field of ANT and DNT NAMs (ECHA, 2023).

NAMs have been advocated to be implemented into the regulatory hazard assessment stage of chemicals risk assessment (Stucki et al., 2022; Schmeisser et al., 2023). Currently, *in vitro* data may be used in Weight of Evidence assessment for classification and labelling (e.g., for developmental toxicity), or to trigger further DNT tests at REACH Annex IX and X, or to support grouping and read across from similar substances. High-throughput *in vitro* assays have also great potential as screening tools to prioritize chemicals and specific modes of action (MoA) for further testing (Escher et al., 2023). While such high throughput screening (HTS) tools have not yet been implemented for DNT and ANT in large scale regulatory practice, the introduction of more sophisticated *in vitro* tests and the validation of all HTS assays for DNT and ANT appear vital to improve their regulatory usefulness. It has been suggested that new *in vitro* methods should be mechanistically associated with adverse (developmental) neurotoxic outcomes (Pitzer, Shafer, and Herr, 2023). This is important to establish the toxicological relevance of endpoints measured in NAMs and/or to allow for the selection of the most informative follow-up studies to produce new information to elicit regulatory action (Smirnova et al., 2014; Ockleford et al., 2017; Fritsche et al., 2018; Kavlock et al., 2018; Craig et al., 2019; Paparella et al., 2020; Vinken et al., 2021; Pallocca et al., 2022; Stucki et al., 2022).

4 Building the DNT-IVB v2.0

One of the purposes of this PARC project is to deliver a guidance document containing a framework to facilitate the regulatory use of



data derived from a DNT *in vitro* NAM-based test battery. A basic DNT *in vitro* test battery (IVB) has already been developed (i.e., DNT-IVB v1.0). It covers several cellular neurodevelopmental processes vital for normal brain development (Bal-Price et al., 2018; Masjosthusmann et al., 2020; Crofton and Mundy, 2021; Blum et al., 2022; Koch et al., 2022; OECD, 2023). The DNT-IVB v1.0 (Figure 1) measures effects of chemicals on human neural progenitor cell (hNPC) proliferation (Baumann et al., 2014; Baumann et al., 2015; Harrill et al., 2018; Nimtz et al., 2019; Masjosthusmann et al., 2020; Koch et al., 2022) and apoptosis (Druwe et al., 2015; Harrill et al., 2018), cell migration (Baumann et al., 2015; Baumann et al., 2016; Nyffeler et al., 2017; Schmuck et al., 2017; Masjosthusmann et al., 2020; Koch et al., 2022), hNPC-neuronal differentiation (Baumann et al., 2015; Schmuck et al., 2017; Masjosthusmann et al., 2020; Koch et al., 2022), oligodendrocyte differentiation (Dach et al., 2017; Schmuck et al., 2017; Masjosthusmann et al., 2020; Klose et al., 2021; Koch et al., 2022), neurite outgrowth (human: Harrill et al., 2010; Harrill et al., 2018; Krug et al., 2013; Hoelting et al., 2016; Masjosthusmann et al., 2020; Koch et al., 2022; rat: Harrill et al., 2013; Harrill et al.,

2018), and synaptogenesis and neuronal network formation (rat: Harrill et al., 2011; Harrill et al., 2018; Brown et al., 2016; Frank et al., 2017; Shafer, 2019).

Gap analysis of the DNT-IVB v1.0 revealed a requisite for supplementary cell assays (e.g., microglia) and functions (e.g., human neuronal network formation, astrocyte function, behavior, learning, and memory) to augment coverage and increase the ability to detect potential (developmental) neurotoxicants (Crofton and Mundy, 2021). Coverage of additional targets for neurotoxicants (e.g., signaling pathways and processes) is necessary, as exemplified by nicotine, a compound identified as a false negative in the DNT-IVB v1.0 (Masjosthusmann et al., 2020; Crofton and Mundy, 2021; Blum et al., 2022). This indicates the inability to detect (developmental) neurotoxic compounds that target nicotinic receptors in these test systems (e.g., neonicotinoid insecticides) (Sheets et al., 2016; Loser et al., 2021; Blum et al., 2022). To address some of the identified gaps, there are four key areas that this PARC project aims to improve during the development of the DNT-IVB v2.0 (Figure 1). This includes refinement of existing assays, generation of new NAMs to cover essential gaps, determination of the applicability domain for relevant available NAMs, and increased cost efficiency.

4.1 Refine existing assays

The current synaptogenesis and neural network formation assays were based in primary rat cortical cells differentiated in 2D on multielectrode arrays (MEA; Brown et al., 2016; Frank et al., 2017) (Figure 1). While there is also a recently established human neural network formation (hNMF) assay (Bartmann et al., 2023), it requires commercially available human-induced pluripotent stem cells (hiPSCs), which are used to derive excitatory and inhibitory neurons and primary human astrocytes that can be plated on MEAs for a functional assessment of network formation. This assay has recently been used to evaluate the effects of pesticides on human neural network formation (Bartmann et al., 2023). To decrease the costs of the hNMF assay, the PARC consortium will re-establish and refine the protocol using non-commercially generated hiPSC-derived excitatory and inhibitory neurons, together with human astrocytes derived from hNPCs (Koch et al., 2022). Synapse assembly is a critical feature of neurodevelopment. The DNT-IVB v1.0 assay for synaptogenesis is currently based on primary rat cortical cells (Harrill et al., 2011; Harrill et al., 2018). Human iPSC-derived NPCs can be differentiated into different types of postmitotic neurons and astrocytes (Davidsen et al., 2021; Lauvås et al., 2022). Therefore, a test system, comprised of a 2D mixed culture of neurons and astrocytes undergoing differentiation, will be developed and refined using high-content imaging. To enable comparison to data generated in the rat synaptogenesis assay, a chemical test set will be evaluated (described below).

Another identified gap within the DNT-IVB v1.0 is a lack of assays that describe mitochondrial toxicity events in susceptible cell types. AOP3 (“inhibition of the mitochondrial complex I of nigro-striatal neurons leads to parkinsonian motor deficits”) describes a link between inhibition of complex I of the mitochondrial respiratory chain and motor deficits associated with parkinsonian

disorders (<https://aopwiki.org/aops/3>). The current DNT-IVB v1.0 assays are not particularly sensitive or fail to detect known mitochondrial toxicants (Masjosthusmann et al., 2020; Crofton and Mundy, 2021). To increase the sensitivity of battery assays to this class of neurotoxicants, several DNT-IVB v1.0 assays will be modified to allow for increased detection of mitochondrial toxicants. This step includes the assessment of neurite area and cell viability in human dopaminergic neurons and human immature peripheral neurons. While these endpoints are covered in the DNT-IVB v1.0, where the NAMs are performed in glucose-containing medium, in DNT-IVB v2.0, the assay will be performed in glucose-free, galactose-containing medium, which makes cells more reliant on their mitochondria and increases their sensitivity to mitochondrial toxicants (Hoelting et al., 2016; Delp et al., 2019; Delp et al., 2021).

4.2 Build new NAMs to cover essential gaps

4.2.1 Cellular gaps

In a key analysis, 29 neurotoxicity MoAs were characterized for 248 individual compounds representing 23 compound classes and 212 natural neurotoxins (Masjosthusmann et al., 2018). More comprehensive assessment of the potential for chemicals to harm the developing nervous system likely requires NAMs that cover the identified MoAs. One MoA not covered in the DNT-IVB v1.0 is the formation of a functional blood-brain barrier (BBB). The BBB determines the ability of some environmental chemicals to reach the central nervous system (Banks, 2009). Chemical exposure can affect the BBB integrity to cause DNT effects (Saili et al., 2017). Here, we will develop and use an hiPSC-based BBB NAM to test whether chemical exposure increases permeation of chemicals across the barrier, resulting in higher concentration reaching the central nervous system. According to established differentiation protocols (Appelt-Menzel et al., 2017), chemicals will be applied during cellular differentiation and transendothelial electrical resistance will be used as a readout of barrier function. One MoA considered relevant for DNT and not yet covered by the DNT-IVB v1.0 is the contribution of inflammatory reactions of glial cells. The key cell populations producing inflammatory mediators in the brain are astrocytes and microglia (Carson et al., 2006). These cells can be generated from human stem cells (Brüll et al., 2020; Spreng et al., 2022) and then either tested as pure populations, as mixed glial populations or together with various neuronal cultures (Gutbier et al., 2018; Klima et al., 2021).

4.2.2 Functional gaps

OECD TG 426 (OECD, 2007) assesses neurobehavioral endpoints which include measures of cognition (including associative learning and memory) in rodents exposed to chemicals during the developmental period. Cellular NAMs may provide information on cellular events that may eventually cause adverse effects on cognitive functions or other neurobehavioral functions but fail to provide equivalent information to neurobehavioral tests. In addition, when considering the complex integration of intracellular, intercellular, interregional, and systemic interactions that occur in development-stage and regional specific manners, *in vitro* NAMs do not cover all relevant cell types and processes, inherent within whole organisms, that are necessary to

develop and maintain a functional nervous system. In this project, the alternative (i.e., relative to rodent models) early life zebrafish model will be used to generate a range of behavior-based assays that complement the *in vitro* approaches described above.

Zebrafish (*Danio rerio*) are a 3Rs-compliant (Hooijmans et al., 2010), non-protected vertebrate model up to 5 days post fertilization (dpf) (Strähle et al., 2012; Kalueff et al., 2013). The zebrafish embryo model may represent a powerful translational system for human hazard and risk assessment as zebrafish possess orthologs for 70% of human genes (Howe et al., 2013), 80% of human disease-related genes (Howe et al., 2013), and 86% of general human drug targets (Gunnarsson et al., 2008). Zebrafish are increasingly being utilized as a model system to investigate the function of the growing list of risk genes associated with neurodevelopment disorders (Sakai et al., 2018), including motor neuron diseases (Babin et al., 2014). Zebrafish neurodevelopment starts at 24 h post-fertilization and primary neurogenesis is complete by roughly 72 h post-fertilization (depending on rearing temperature). Resulting neuroanatomy (Gupta et al., 2018), nervous system transcriptomic lineages (Raj et al., 2018), and brain asymmetry (Duboc et al., 2015) are suggested to be comparable to humans. In addition, neurotransmitter systems, including glutaminergic, cholinergic, serotonergic, dopaminergic, adrenergic, GABAergic, and histaminergic (Babin et al., 2014; Faria et al., 2015; Horzmann and Freeman, 2016) are similar to those found in humans and associated with sensory-motor outcomes. This rapid establishment of neural structures during neurodevelopment and their link to quantifiable behavioral parameters is a major asset for PARC WP5.2.1e.

Relative to *in vitro* systems, metabolically competent zebrafish embryos may address potential toxicokinetics that can affect toxicity outcomes (Chu and Sadler, 2009). Regarding neurotoxicity, the assessment of neurobehavioral effects caused by xenobiotic exposure is advantageous because these perturbations are sensitive (i.e., they occur at sub-morphological concentrations) (Noyes et al., 2015; Bruni et al., 2016; Gaballah et al., 2020; Jarema et al., 2022). Locomotor activity can function as an automated and generalized readout of neurodevelopment. A major advantage of the early life zebrafish system as compared to *in vitro* systems is that it represents an alternative whole organism animal system that is amendable to genome-wide differential expression data collection throughout early neurodevelopment (Kettleborough et al., 2013) and are expected to address comparatively more MIEs and KEs related to DNT in a single assay (e.g., neurotransmitter signaling pathways, functional BBB, myelinated axons, functional synapses, neuronal networks, and neural circuits), as compared to individual *in vitro* test systems performed in single cell types or limited co-culture systems. Moreover, zebrafish development has been mapped at the resolution of single-cell transcriptomics, allowing the detection of cell-type specific changes associated with chemical induced adversity affecting neural and non-neural components of the developing brain (Farrell et al., 2018).

In compliance with EU directive (2010/63/EU; European Parliament and Council, 2010), the majority of work will be conducted in embryos up to 5 dpf. Another key advantage of early life stage zebrafish NAMs is that they can be screened in DNT and acute modes by varying the length and timing of chemical

exposure. The DNT mode captures structural and functional deficits that alters locomotor activity in response to various stimuli. The acute mode identifies rapid, receptor-mediated changes in neuroactivity that can potentially be used as a complement to cellular ANT assays which aim to identify perturbations in signaling pathways (e.g., dopaminergic signaling) linked to ANT AOPs.

All DNT NAMs performed in early life-stage zebrafish described will be used following developmental exposure to PARC test chemicals and removed prior to behavior testing. This increases the likelihood of detecting functional or structural effects that arise from developmental perturbations in underlying behavior circuits after chemical exposure has ceased. Later, in the development of the ANT-IVB 1.0, the same assays will be applied post-neurogenesis (after three dpf) to detect the acute neuroactivity potential of test chemicals with a focus on the detection of perturbations in receptor-based neurotransmitter systems that are associated with DNT and/or ANT (e.g., dopaminergic, gabaergic, glutamatergic perturbations).

Another important functional topic is the impact of chemical exposure on associative learning and memory, which is assessed as a standard part of rodent-based OECD TG 426 for DNT (OECD, 2007), and may be included as an add on to TG 424 (OECD, 1997) for ANT and in TG 443 for DNT (OECD, 2018b). *In vitro* test systems are unable to account for these complex behavioral and cognitive aspects. Members of this consortium are developing a NAM that detects chemical-dependent disruption of non-associative learning and memory retention in early life stage zebrafish (Figure 2). An escape response NAM that identifies chemicals that specifically disrupt the motor system via the activation of reticulospinal neurons and independently of sensory processing (Dubrana et al., 2021; Knoll-Gellida et al., 2021) will be further developed and applied to screen a common set of chemicals described below. A NAM for chemical-induced developmental motor neuron toxicity will also be developed using the transgenic line Tg (nrp1a:gfp)js12 with labeled motor neurons (Sato-Maeda et al., 2006). A NAM that detects anxiety-like behavior via the detection of thigmotaxis, or the time spent along the outer edge of a well, is also under development. Finally, for a subset of test chemicals, the persistence of behavioral effects, post-exposure, will be evaluated to substantiate the detection of DNT effects in 14 dpf old larvae.

4.2.3 Determine applicability domain

The applicability domain describes the physicochemical or other properties of the chemicals for which a NAM is applicable for use (OECD, 2005). The applicability domain is generally determined using a range of reference chemicals linked to an adverse effect (OECD, 2005). Within this project, a test set of 96 reference chemicals, based on previously published work (Masjosthusmann et al., 2020; Blum et al., 2022), will be evaluated. With the exception of the zebrafish NAMs, all other DNT NAMs will be performed in human cellular assays. As these models contain a limited number of cell types and other gaps, efforts will be made to assess the applicability domain to determine whether, and to what extent, the NAMs cover established DNT AOPs, including endocrine disruption (ED) or proposed DNT AOPs such as epigenetic perturbation. Specifically, in the 2D synaptogenesis model,

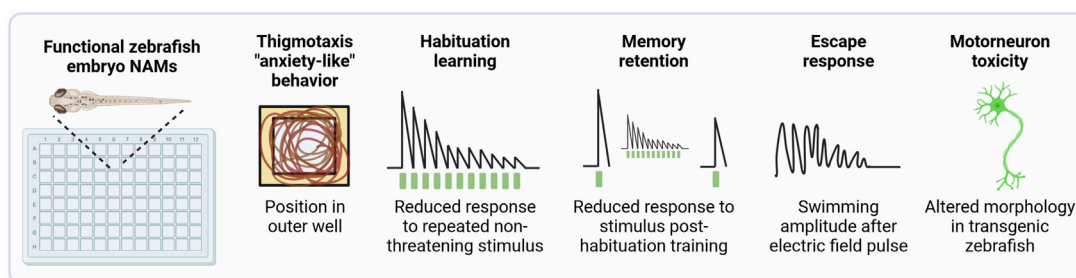


FIGURE 2

Functional zebrafish embryo NAMs performed in early life stage zebrafish. A suite of automated zebrafish-embryo behavior-based NAMs are under development for potential inclusion in the DNT-IVB v2.0. All NAMs are performed using automated tracking. Most assays will be performed in 96 square well plates for a comparable throughput to *in vitro* assays. Based on the exposure paradigm used, these assays can be performed with protocols that predict DNT and/or ANT. For the detection of DNT, chemical exposure occurs during development and is removed prior to behavior assessments. In contrast, if exposure takes place after neurogenesis is complete, any adverse effect related to the specific signalling pathways that have reached maturation may be indicative of acute ANT. However, as the organism's nervous system is still developing, it cannot be excluded that the nature of the effect may be considered within a DNT assessment framework.

characterization of differentiation up to 28 days will be performed to understand the abundance and distribution of ED-relevant receptors including retinoic acid receptor, estrogen receptor, androgen receptor, thyroid hormone receptor, glucocorticoid receptor and liver X receptor. Coverage of ED and epigenetic modes of action will also be carried out for certain cellular ANT NAMs (see below).

4.2.4 Increase molecular and cellular coverage, reliability, and cost efficiency

Mammalian TG studies for DNT testing are costly and time-consuming (Crofton et al., 2012; Smirnova et al., 2014). At the same time, mammals contain a complete nervous system with all functional components throughout the whole developmental period, and their communication with other relevant organs and systems, (e.g., gut, liver, endocrine, and immune systems) that can collectively influence neurotoxicity outcomes. Next-generation DNT and ANT testing seeks to eventually replace mammalian tests with a battery of *in vitro* and alternative test systems. Some of these next-generation test systems are lengthy and can take up to 35 days, which increases the overall cost of the potential test battery. To complement cellular and alternative assays potentially included in the DNT-IVB v2.0 and to provide a low-cost screening strategy, four approaches will be explored.

The first strategy seeks to identify early markers of later DNT-associated KEs with a focus on epigenomic or sub-cellular morphological alterations. Epigenomic processes drive cell differentiation, and chemically induced alteration in epigenetic patterns can lead to long-term changes in gene function (Baccarelli and Bollati, 2009). In this context, we will assess whether epigenetic re-arrangements precede morphological changes observed in differentiation-related assays, with the potential to shorten and/or strengthen such assays. This step will be addressed by performing and comparing different genome-wide epigenetic (i.e., DNA methylation) analyses at specific time points in cellular differentiation assays where shortening could be of interest. Cell painting is a high-throughput microscopy technique that allows researchers to simultaneously label and visualize multiple organelles

in a cell (Bray et al., 2016). It uses a cocktail of fluorescent stains and high-content imaging to obtain parallel morphological measurements on a single cell level. This project will establish and evaluate an automated cell profiling methodology for DNT assays that is cheap, fully automated, data-rich, and can operate on 2D cell monolayers, and work is ongoing to expand to 3D spheroids. Images from Cell Painting are data-rich and highly applicable for analysis with artificial intelligence methods, for example, for MoA prediction (Tian et al., 2023), and assessment of combination effects of environmental compounds (Rietdijk et al., 2022). Morphological changes will be assessed as potential early indicators of DNT or ANT outcomes in short- and longer-term cellular assays.

The second strategy employs transcriptomics to refine the search for early markers of later DNT-associated KEs with a focus on genome-wide expression patterns observed at a single cell resolution of intact developing embryo nervous systems. This single-cell transcriptome data is complemented by building transcriptomics and metabolomics technologies into an existing OECD TG for embryotoxicity (TG 236) to measure (neuro)developmental toxicity endpoints. While it was developed as one of the potential alternatives to the acute test on fish (OECD TG 203) for ecotoxicological hazard assessment, it has been reported that compared to OECD TG 203, OECD TG 236 may underestimate acute toxicity for certain types of chemicals, in particular neurotoxicants (Klüver et al., 2016; Glaberman et al., 2017; Sobanska et al., 2018). Nevertheless, OECD TG 236 has recently attracted considerable attention for its potential expansion to human health endpoints, particularly developmental (neuro) toxicity (Braunbeck et al., 2014; Krzykwa et al., 2019).

The third strategy uses a rapid, low-cost cellular neurite outgrowth assay to screen a much larger chemical test set, including human-relevant mixtures (J. Lee et al., 2022a; J. Lee et al., 2022b). For this assay, SH-SY5Y cells, sub-cloned from a neuroblastoma cell line, are used in 384 well plate format (J. Lee et al., 2022b; J. Lee et al., 2022b). The specificity and sensitivity of this assay will be compared to non-transformed, hiPSC-derived DNT models.

The fourth strategy will build *in silico* models to predict the probability of inducing DNT effects using Quantitative Structure-

Activity Relationships (QSAR) models to link chemical structural properties with measured neurotoxicity effects (Khelfaoui et al., 2021; Grillberger et al., 2023). Molecular docking combined with observed molecular dynamics will be employed to reflect interactions of organophosphates with cellular targets (e.g., membranes, proteins) identified as MIEs according to DNT-relevant AOPs (Gadaleta et al., 2022). Serine esterases and calcium transporters are currently under consideration (Knoll-Gellida et al., 2021). Method evaluation will be performed by comparing simulations with experimentally generated data. Binding affinity for the set of targets will then be predicted by using machine learning-based models, and structural alerts for pathway perturbation will be identified.

5 Demonstration of added value and identification of a minimum assay battery for DNT-IVB v2.0

A fundamental requirement for the regulatory acceptance of NAMs involves the development of test methods characterized by a high degree of robustness, performance, and readiness (Bal-Price et al., 2018), including acceptable levels of variability (Harry et al., 2022). If they are intended for future use in the context of hazard assessment, NAMs should ideally meet or exceed the sensitivity, specificity, accuracy, and reliability of the respective OECD TG, to ensure a continued level of acceptable chemical safety (Bal-Price et al., 2018). This approach ensures the use of data with a significant level of confidence. In the case of the DNT-IVB v1.0, elevated standards of readiness and robustness have already been demonstrated (Bal-Price et al., 2018; Krebs et al., 2019; Blum et al., 2022). To assess DNT-IVBv1.0 performance, a set of 45 reference (i.e., performance) compounds, consisting of 28 substances that were considered DNT positive and 17 substances that were considered DNT negative by the assay developers were used. Using these substances, an assay sensitivity of 68%, specificity of 100%, and accuracy of 80% was observed (Blum et al., 2022).

To substantiate the added value of new and refined assays within the DNT-IVB v2.0, a 96-member reference set will be used, which encompasses 45 performance compounds from the DNT-IVB v1.0 (Blum et al., 2022) and will be augmented with known modifiers targeting pathways specific to DNT (Fritsche, 2017) (e.g., mTOR (Lee, 2015), PDGFR-PLCγ1 (Kang et al., 2016), Notch (Imayoshi et al., 2013), and thyroid hormones (TH; López-Espíndola et al., 2014; Bernal et al., 2015)). Centralized chemical procurement and distribution will occur via a collaborative effort involving PARC 5.2.1e scientists and experts from the EU Joint Research Centre (EU-JRC). This structured approach facilitates standardized and comparable assessment of substances across partner laboratories, effectively minimizing uncertainties associated with substance purity, solubilization, and concentration. By testing the DNT-IVB v1.0 performance compounds at reasonable concentrations in each newly developed test method, we will gain insight into the chemical applicability domain of each assay to understand the potential added value of DNT-IVB v2.0 NAMs. If DNT-IVB v2.0 NAMs can appropriately identify DNT-IVB v1.0 false negatives as positives, this will increase the sensitivity of the resulting v2.0 test battery. In

addition, the evaluation of specific pathway agonists and antagonists will reveal the applicability domain of each assay and the whole DNT-IVB v2.0.

6 Build ANT-IVB v1.0

In contrast to DNT, there is no comparable set of NAMs with a high readiness for ANT testing that covers important MoA. ANT can be elicited through a variety of mechanisms involving neurotransmitter receptors and ion transporters which influence the transmission and processing of signals in the human brain and other parts of the nervous system (Fritsche and Hogberg, 2020; Masjosthusmann et al., 2018). Recently, a neurotoxicity MoA analysis was performed for 248 individual compounds, representing 23 compound classes and 212 natural neurotoxins (Masjosthusmann et al., 2018). The identified MoA were grouped according to ANT common key events including cholinergic, GABAergic, glycinergic, glutamatergic, adrenergic, serotonergic, and dopaminergic neurotransmission, ion channels/receptors (e.g., sodium channels, potassium channels, calcium channels, chloride channels), and a range of cellular endpoints such as mitochondrial dysfunction, oxidative stress, apoptosis, redox cycling, altered calcium signaling, cytoskeletal alterations, neuroinflammation, axonopathies, myelin toxicity, delayed neuropathy, and enzyme inhibition (Masjosthusmann et al., 2018). To enable a thorough assessment of chemicals' ANT potential, it is necessary to compile the ANT-IVB v1.0 that includes the entirety of the identified MoA, a challenge that will be addressed in our project.

To date, several assays have been established and published that cover critical KEs for neurotoxicity (Schmidt et al., 2017) but they still need refinement to meet certain criteria for regulatory acceptance of alternative methods including assessment of the domain of applicability, assay robustness and relevance, and demonstrated predictivity for adult neurotoxicity (Bal-Price et al., 2018). For the assessment of direct activation of ion channels and receptors and altered function of channels and receptors of (nociceptive) sensory neurons, several NAMs used in the DNT-IVB v2.0 can be repurposed for acute testing in mature 2D or 3D culture systems or in early life-stage zebrafish post neurogenesis. Specifically, DNT-IVB v2.0 test methods, including the myelin and BBB NAMs, cell painting in human BrainSpheres (Hartmann et al., 2023), and zebrafish learning and memory, motor system toxicity, and anxiety-like NAMs will be performed in mature cellular cultures or zebrafish embryos at time points which occur after primary neurogenesis and differentiation has occurred (R. Schmidt et al., 2013).

In addition to repurposed DNT-IVB v2.0 NAMs, several novel NAMs are being developed and applied to the ANT-IVB v1.0. One is a recently developed NAM based on hiPSC-derived nociceptor-enriched, mature sensory neurons (Holzer et al., 2022). Using this NAM after 23 days of differentiation, acute exposure to ANT reference chemicals will be carried out to evaluate the biological applicability domain of the assay. Another is the human multi-neurotransmitter assay (hMNR), which is based on hiPSC-derived mixed neuron-glia 3D BrainSpheres. The hMNR NAM assesses neuronal subtype-specific acute neurotoxicity using micro-electrode

arrays (MEA) for the recording of spontaneous electrical activity (Hartmann et al., 2023). By sorting detected signals (spikes) based on their waveform, this assay allows the distinction between glutamatergic, GABAergic, dopaminergic, serotonergic, and cholinergic responses of the mixed-neuronal co-culture, allowing for an *in vitro* MoA-based assessment of ANT (Hartmann et al., 2023). In a third NAM, Ca²⁺ signaling will be assessed at the single cell level in mature central dopaminergic neurons (LUHMES cells), which extends the coverage of the KEs to another cell type and a set of functional receptors (e.g., P2X3 receptors (Apicella and Fabbretti, 2012)). A fourth NAM under development, the Peripheral Myelin Toxicity Assay (PeriMyelinTox), assesses myelin toxicity impacting peripheral sensory and motor function, and therefore addresses the key battery gap “myelination” for peripheral ANT in human cells. In this NAM, sensory or motor neurons, along with Schwann cells, will be differentiated from hiPSCs and cultivated in co-culture (Muller et al., 2018; Schenke et al., 2020; Louit et al., 2023). A novel 3D sphere format will be developed and compared to a conventional 2D format. Myelin formation will be evaluated by quantifying myelin basic protein (MBP) or myelin protein zero (MPZ) against the pan-neuronal marker β 3-tubulin (TUJ1) through immunofluorescence staining and RT-qPCR (Chesnut et al., 2021). The assay will be optimized for automated high-throughput quantification of myelin post-exposure to a training set of potential myelin toxicants. The added value of both assays in assessing sensory and motor neuron myelin toxicity will be further evaluated.

To clearly define the applicability domains and assay-specific limitations, this project aims to develop a set of ANT reference chemicals that are known to affect the human brain, as well as negative compounds. This approach will ensure the unified characterization of the applicability domain of each assay and coverage of important human-relevant ANT MoA.

7 Outcomes and future perspectives

PARC was designed to address challenges inherent in moving from animal-based test methods to (batteries of) *in vitro* and alternative NAMs to speed up and modernize hazard identification and chemical risk assessment. Project 5.2.1e aims to improve the hazard prediction paradigm via the establishment and refinement of NAMs for DNT and ANT testing and the assembling of high performing, reproducible NAMs that provide added value into DNT and ANT test batteries. Our strategy encompasses the refinement of existing assays, generation of innovative NAMs to address identified gaps, determination of the applicability domain, and increased cost-effectiveness of lengthier assays via the demonstration of early indicators of later effects. Importantly, the development of new assays and the refinement of existing ones includes a strong focus on assay and data reliability. One critical ambition is to introduce quality control measures as described in Good Cell Culture Practices 2.0 (Pamies et al., 2022) or the GIVIMP document of the OECD (OECD, 2018). The PARC 5.2.1e consortium therefore contributes to an improved readiness, sensitivity, and overall performance of DNT NAMs to promote an increased acceptance of DNT *in vitro* and alternative assays for wider regulatory use. By the end of this project, a guidance document will be delivered that will introduce a novel framework

aiming to facilitate the regulatory use of data derived from the DNT-IVB v2.0. Such work will include considerations on how DNT-IVB data may be used in the context of an IATA or weight of evidence for hazard and risk characterization. This links seamlessly to other PARC work packages that provide information on physiologically-based kinetic (PBK) modelling to convert IVB concentrations to predicted *in vivo* doses, and to risk assessment specialists that need to consider how the predicted doses can be used to set safe exposure thresholds by, for example, considering modulatory factors in AOP or by considering variabilities and specific sensitivities in exposed populations (Schmeisser et al., 2023; Suciu et al., 2023).

In contrast to DNT, there is currently no comparable battery of NAMs for ANT testing. Therefore, the consortium will create a first-generation ANT-IVB v1.0, covering major MoAs involved in human brain functioning. Looking ahead, and to respond to PARC regulatory colleagues' requests for data on specific compound classes, 5.2.1e NAMs will be used to evaluate the potential toxicity of natural toxins, bisphenols, and per- and polyfluoroalkyl substances (PFAS). Overall, this consortium aims to offer an unprecedented opportunity to fill a longstanding gap for the commonplace assessment of neurotoxicity potential of commercial chemicals via the generation of MoA-based, robust, reproducible, fast, and inexpensive consolidated DNT and ANT testing strategies. As all of this work is conducted under the guidance of colleagues from a regulatory field and potential end users, our ambition is to revolutionize the hazard and risk assessment of DNT and ANT in Europe.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

TT: Conceptualization, Funding acquisition, Project administration, Writing-original draft, Writing-review and editing. OM: Writing-review and editing, Funding acquisition, Project administration. EF: Writing-review and editing, Funding acquisition. JR: Writing-review and editing, Funding acquisition. KC: Writing-review and editing. KAH: Writing-review and editing. CA: Writing-review and editing. PB: Writing-review and editing, Funding acquisition. BE: Writing-review and editing, Funding acquisition. HD: Writing-review and editing, Funding acquisition. KH: Writing-review and editing. KD: Writing-review and editing. YH: Writing-review and editing. SH: Writing-review and editing, Funding acquisition. KJ: Writing-review and editing. BJ: Writing-review and editing. NK: Writing-review and editing. AK-G: Writing-review and editing. BK: Writing-review and editing. MLe: Writing-review and editing, Funding acquisition. MLi: Writing-review and editing. JL: Writing-review and editing, Funding acquisition. FM: Writing-review and editing, Funding acquisition. JC: Writing-review and editing, Funding acquisition. WN: Writing-review and editing, Funding acquisition. GP: Writing-review and editing. BS: Writing-review and editing, Funding acquisition. IS: Writing-review and editing. SS: Writing-review

and editing, Funding acquisition. OL: Writing-review and editing, Funding acquisition. MT-R: Writing-review and editing, Funding acquisition. KB: Conceptualization, Project administration, Writing-original draft, Writing-review and editing.

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Conflict of interest

EF and KB are shareholders of the DNTOX GmbH offering neurotoxicity testing services.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chemical respiratory sensitization—Current status of mechanistic understanding, knowledge gaps and possible identification methods of sensitizers

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Respiratory sensitization is a complex immunological process eventually leading to hypersensitivity following re-exposure to the chemical. A frequent consequence is occupational asthma, which may occur after long latency periods. Although chemical-induced respiratory hypersensitivity has been known for decades, there are currently no comprehensive and validated approaches available for the prospective identification of chemicals that induce respiratory sensitization, while the expectations of new approach methodologies (NAMs) are high. A great hope is that due to a better understanding of the molecular key events, new methods can be developed now. However, this is a big challenge due to the different chemical classes to which respiratory sensitizers belong, as well as because of the complexity of the response and the late manifestation of symptoms. In this review article, the current information on respiratory sensitization related processes is summarized by introducing it in the available adverse outcome pathway (AOP) concept. Potentially useful models for prediction are discussed. Knowledge gaps and gaps of regulatory concern are identified.

KEYWORDS

adverse outcome pathway (AOP), chemical respiratory allergy, chemical sensitizer, chemical-induced hypersensitivity, key event, new approach methodology (NAM), occupational asthma, respiratory sensitization

1 Introduction

Chemical respiratory sensitization manifests clinically mostly as occupational asthma (OA), but also as rhinitis, conjunctivitis, and rarely as alveolitis (Cochrane et al., 2015; Seed et al., 2015; Golden et al., 2021). It is meanwhile recognized that occupational and environmental exposures can play a role in the development of almost all serious respiratory diseases in the general population (Feary et al., 2023). Even chronic low-level exposure may lead to long-term health effects. However, the link between asthma and chemical exposure is based on the patients medical history, and it is often difficult to trace the history of exposure and to identify the responsible chemicals (Vincent et al., 2017). Likewise, understanding the effects of respiratory sensitizer exposure on human health is limited due to difficulties in evaluating (occupational) exposure (Weisel, 2002; Batterman et al., 2014).

Asthma was officially recognized as the most prevalent occupational disease of the lungs in developed countries (Torén and Blanc, 2009) and is still the second-most common occupational lung disease after pneumoconiosis in developing countries (Üzmezoglu, 2021). OA represents about 15% of all adult asthma cases (Vincent et al., 2017), and sensitizer-induced OA is the most common type of OA, being induced by either high-molecular-weight (HMW) or low-molecular-weight (LMW) agents (Feary et al., 2023). An estimated 10%–25% of adult asthma cases are associated with exposure to sensitizing or irritant agents with severe consequence for those affected, as for respiratory (immune-mediated) asthma mostly cessation of exposure is required and even then, complete recovery is not guaranteed (Kuruvilla et al., 2019a). As most part of the literature does not usually make a clear distinction between respiratory reactions triggered by sensitization of the respiratory tract and those triggered by other factors, it is not possible to provide precise information on the frequency. Concurrent exposure to particles that induce inflammation or irritation can aggravate the symptoms (Staal et al., 2014). Inflammation is a key factor, and patients with severe asthma, encompassing up to 10% of all asthma patients (Kuruvilla et al., 2019b), require anti-inflammatory corticosteroids as treatment.

The definition of work-related asthma includes immunologic OA, characterized by a latency period before the onset of symptoms, non-immunologic OA, which occurs after single or multiple exposures to high concentrations of irritant materials, work-aggravated asthma, which is preexisting, or concurrent asthma exacerbated by workplace exposures, as well as variant syndromes (Mapp et al., 2005). Chemicals can induce asthma via both immunological and non-immunological (irritant-induced) mechanisms, but this is usually less discussed and distinguishing the mechanisms is sometimes problematic (Pemberton and Kimber, 2021). According to the ECHA guidance on interpretation of the classification, labelling and packaging of substances and mixtures (CLP) criteria there is no need for the demonstration of an immunological mechanism to classify a chemical as respiratory sensitizer.

As mentioned above, occupational settings are an important source of exposure, but also in the general environment we are continuously exposed to inhalable substances, e.g., from paint and coatings, but also from medicines, electronics, cosmetics and consumer care products, etc., though it is not clear to which extent this increases the number of affected people due to diagnostic difficulties. For proteins (food and feed, pollen, animal

dander, skin and sputum, invertebrates such as house dust mites), the pathophysiological mechanism of the allergic reaction that is based on IgE-induced mast cell degranulation upon inhalation is relatively well understood. However, the underlying mechanisms differ in several aspects from chemical-induced allergy for which the formation of haptens by binding to cellular macromolecules is a prerequisite to become immunogenic.

Inhalation exposure to chemicals represents the most common exposure route of concern for the induction of sensitization, but under some circumstances skin exposure may be effective for the acquisition of respiratory sensitization (Botham et al., 1988; Blaikie et al., 1995; Pauluhn et al., 2005; Tsui et al., 2020). The nature of the adaptive response is controlled by different T cell subpopulations. Overall, respiratory sensitizers preferentially elicit a T helper cell type 2 (Th2) response, while skin sensitizers promote Th1 type reactions. However, the effects of sensitizers are not limited to the airways. For example, haptenized self-proteins can contribute to the development of autoimmune diseases (Erkes and Selvan et al., 2014; Sakamoto et al., 2023).

Having entered into force in 2007, the regulation on the registration, evaluation, authorization and restriction of chemicals (REACH) is the main regulation for chemical risk assessment in the European Union that enforces the better identification of the intrinsic properties of chemicals (EC 1907/2006). Article 57 of the EU REACH regulation states that respiratory sensitizers are considered as substances of very high concern (SVHC). The current OECD testing methods for inhalable substances (no. 403: Acute Inhalation Toxicity, no. 412: Subacute Inhalation Toxicity: 28-Day Study, no. 413: Subchronic Inhalation Toxicity: 90-Day Study, no. 436: Acute Inhalation Toxicity – Acute Toxic Class Method and no. 433: Acute Inhalation Toxicity: Fixed Concentration Procedure) are still based on animal experiments, mainly utilizing rodents¹.

The causal linking of events at different levels of biological organization in adverse outcome pathway (AOP) concepts has evolved as important approach in chemical hazard and risk assessment and is also applied in multiple OECD guidelines. Yet, it is still valid what more than 10 years ago was concluded: although several attempts have been made to understand and assess the effect of respiratory sensitizers in a similar way to skin sensitizers, for which today an AOP (no. 40) and new approach methodologies (NAM) are available (Ezendam et al., 2016), no AOP is established for the identification of respiratory sensitizers and methods need to be developed further, whereby the application of the volatile chemicals and aerosols to the test system is a major issue (Rovida et al., 2013). AOP 39, which is still under development, builds on AOP 40 and addresses a mechanism of action for respiratory sensitizers that is initiated by covalent binding to protein leading to the activation of danger and inflammatory signaling and finally resulting in an allergic respiratory hypersensitivity response (Sullivan et al., 2017; Kimber et al., 2018; AOP-Wiki²). The interconnections of key events (KEs) involved in the AOP for respiratory and skin sensitization, and the connections to other AOPs are shown in Figure 1.

1 <https://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm>

2 <https://aopwiki.org>

AOP 39 provides a summary of the current knowledge regarding the mechanisms underlying respiratory sensitization and aims to identify knowledge gaps that can be addressed by targeted research. In brief, AOP 39 begins with the first key event (KE 1) covalent binding of a LMW chemical to proteins (molecular initiating event, MIE) that leads to the activation of stress response pathways and pro-inflammatory mediators in the form of danger signals, such as oxidative stress, cytokines, and chemokines released by epithelial cells (KE 2), followed by dendritic cell (DC) maturation and migration to lymph nodes (KE 3). Haptens can also directly activate DCs. Migration to the draining lymph nodes of Th2-skewed DCs elicits activation and proliferation of T cells (KE 4), marking the sensitization phase and resulting in chemical respiratory allergy. The increased allergic hypersensitivity reaction is considered the adverse outcome (AO) (Sullivan et al., 2017).

Considerable effort was made to identify respiratory sensitizers in the past, based on patients exposure histories, workplace observations, animal and cell-based studies as well as with *in silico* prediction tools. However, regulatory accepted methods to identify respiratory sensitizers are not available yet (Ponder et al., 2022). Based on structural analysis, the electrophilic nature of sensitizing chemicals is considered to be most relevant for its immunogenicity, whereby specific amino acid residues (cysteine, lysine) are suggested to be the predominant target (Enoch et al., 2009; Enoch et al., 2010). However, different mechanisms may be relevant as well, as, e.g., metals act via complexation (Thierse et al., 2005). Moreover, efforts are made to distinguish between skin and respiratory sensitizers, which is challenging because exposure via skin may also lead to respiratory sensitization and *vice versa* for some chemicals (van Och et al., 2002; De Jong et al., 2009).

We conducted a literature review using artificial intelligence to record to which extent selected molecular events and biological processes, pathological mechanisms, symptoms and adverse reactions are found to be associated with a set of respiratory sensitizers, on the one hand to emphasize the topicality of the issue and, on the other, to obtain information on potential further key events.

Herein, our objective is to elucidate comprehensively the existing knowledge pertaining to respiratory sensitization processes within the context of the AOP framework. Furthermore, we will summarize *in vitro* and *in silico* methods that are potentially applicable for the assessment of respiratory sensitization, though adaptation steps may be required. Additionally, we will identify areas where current understanding is lacking and highlight regulatory concerns that warrant further investigation.

2 Methods

2.1 Inventory of respiratory sensitizers

Literature-identified respiratory sensitizers (Enoch et al., 2009; Enoch et al., 2010; Rovida et al., 2013; Cochrane et al., 2015; Sadekar et al., 2021; Ponder et al., 2022, ECETOC TR 77³) were compared

with the official harmonized classification according to Annex VI of the Classification, Labelling and Packaging (CLP) Regulation ((EC) No 1272/2008)⁴ by using the latest consolidated version of Table 3 to Annex VI of CLP⁵ and with the classification of the Classification and Labeling (C&L) Inventory database⁶.

To estimate the overall number of respiratory sensitizers in the C&L inventory, data were download as Excel file on 15 May 2024. To specifically capture data related to respiratory sensitizers, the search query included “health hazards” with classifications “Resp. Sens. 1”, “Resp. Sens. 1A”, and “Resp. Sens. 1B” combined with an “or” operator. The filter was set to include all relevant records without any discrimination. Compounds with no CAS number and records that did not have any classification data, as well all duplicated CAS numbers were removed. Please note that the C&L inventory derived classification categories include harmonized, REACH registration and notified C&L. While the REACH registration C&L is based on self-classification by the registrant(s) and may become an official harmonized classification as soon as a classification dossier is submitted and evaluated, the notified C&L entries were not updated since the submission deadline in 2010 and thus refer to the state of knowledge at that time. In addition, companies and importers may have submitted different proposals indicating inconsistent classification categories for a certain toxicity. The database is continuously updated.

2.2 Literature search

To retrieve relevant scientific papers from PubMed, search terms related to respiratory sensitization-relevant biological targets and events that were extracted from AOP 39 and from related AOPs (no. 40 as well as other lung related AOPs), as well as related to adverse outcomes and clinical symptoms were defined. In addition, a small subset of chemical compounds was selected from Supplementary Table 1, and all PubChem listed synonyms for these compounds were used as search term. All search terms can be found in Supplementary Table 2 and are named <query_terms_events>, <query_terms_umbrella> and <query_compounds>. Subsequently, a custom R script, using the “easyPubMed” library (Fantini, 2019), was employed to query for all possible PubMed IDs (PMID) corresponding to each individual compound, event or umbrella term. These queries were constructed in the “[All Fields]” format. To maintain data integrity and eliminate redundancy, any duplicate PMIDs were systematically removed from the retrieved results. Additionally, AOP-helpFinder 2.0⁷ (Jaylet et al., 2023) was used to find relevant publications that connects stressors (the list of compounds) with a merged list of events and umbrella terms. Furthermore, we used the results from

³ <https://www.ecetoc.org/wp-content/uploads/2014/08/ECETOC-TR-077.pdf>

⁴ Classification, Labelling and Packaging (CLP) Regulation ((EC) No 1272/2008): EUR-Lex - 02008R1272-20231201 - EN - EUR-Lex (europa.eu).

⁵ Annex VI to CLP-ATP20: <https://echa.europa.eu/information-on-chemicals/annex-vi-to-clp>

⁶ <https://echa.europa.eu/information-on-chemicals/cl-inventory-database>

⁷ <https://aop-helpfinder.u-paris-sciences.fr/>

TABLE 1 List of respiratory sensitizers identified either by literature search, the official harmonized classification (Annex VI of CLP)¹¹ and/or the C&L inventory database¹².

Chemical	CAS	EC/ List no.	Skin Sens. Category (C&L inventory)	Resp. Sens. Category (C&L inventory)	Entry in annex VI of CLP	Ponder et al. (2022)	Sadekar et al. (2021)	Cochrane et al. (2015)	Enoch et al. (2010)	Rovida et al. (2013)	Enoch et al. (2009)	ECETOC TR 77
2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate	125700-67-6	423-040-4			No	A						
2-hydroxyethyl methacrylate	868-77-9	212-782-2	1		No		I					
2-methyl-4-isothiazolin-3-one	2682-20-4	220-239-6	1A		No		I					
2,4-Dichloro-5-(chlorosulphonyl) benzoic acid	3740-18-9	223-127-5			No	A						
2,4-Toluene diisocyanate	584-84-9	209-544-5	1	1	Yes				L		S	N
2,6-Toluene diisocyanate	91-08-7	202-039-0	1	1	Yes				L			
4-diazoniobenzenesulfonate	305-80-6	206-168-3			No	A						
5-chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	247-500-7	1		No		I					
6-Amino penicillanic acid	551-16-6	208-993-4			No				L			
7-Amino cephalosporanic acid	957-68-6	213-485-0	1	1	No	A			L			
Abietic acid	514-10-3	208-178-3			No						S	
Ammonium hexachloroplatinate (HCP)	16919-58-7	240-973-0	1	1	Yes	B				P		H
Ammonium Persulfate	7727-54-0	231-786-5	1	1	Yes	A						
Ampicillin	69-53-4	200-709-7	1	1	No	A			L			
Azodicarbonamide	123-77-3	204-650-8		1	Yes		I		L			
Benzyl dimethyl dodecyl ammonium chloride	139-07-1	205-351-5			No		I					

(Continued on following page)

TABLE 1 (Continued) List of respiratory sensitizers identified either by literature search, the official harmonized classification (Annex VI of CLP)¹¹ and/or the C&L inventory database¹².

Chemical	CAS	EC/ List no.	Skin Sens. Category (C&L inventory)	Resp. Sens. Category (C&L inventory)	Entry in annex VI of CLP	Ponder et al. (2022)	Sadekar et al. (2021)	Cochrane et al. (2015)	Enoch et al. (2010)	Rovida et al. (2013)	Enoch et al. (2009)	ECETOC TR 77
Benzyl n-butyl phthalate	85-68-7	201- 622-7			No		I					
C.I. 71105	4424- 06-0	224- 597-4			No				L			
Carmine	1328- 60-5	215- 527-3			No	A						
Carminic acid	1260- 17-9	215- 023-3			No				L			
Cefadroxil	50370- 12-2	256- 555-6	1	1	No	A						
Cefteram pivoxil	82547- 81-7	No results found			No	A						
Cephalexin	15686- 71-2	239- 773-6	1	1	No				L			
Chloramine T	127- 65-1	204- 854-7		1	Yes	A	C					
Chlorhexidine	55-56-1	200- 238-7			No		U		L			
Chromium	7440- 47-3	231- 157-5			No			E				
Cobalt	7440- 48-4	231- 158-0	1	1	Yes			E				
Cyanuric chloride	108- 77-0	203- 614-9	1		No		I					
Di(2-ethylhexyl) phthalate (DEHP)	117- 81-7	204- 211-0			No		I					
Dibutyl phthalate	84-74-2	201- 557-4			No		I					
Dichlorvos	62-73-7	200- 547-7	1		No				L			
Diethyl phthalate	84-66-2	201- 550-6			No		I					

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TABLE 1 (Continued) List of respiratory sensitizers identified either by literature search, the official harmonized classification (Annex VI of CLP)¹¹ and/or the C&L inventory database¹².

Chemical	CAS	EC/ List no.	Skin Sens. Category (C&L inventory)	Resp. Sens. Category (C&L inventory)	Entry in annex VI of CLP	Ponder et al. (2022)	Sadekar et al. (2021)	Cochrane et al. (2015)	Enoch et al. (2010)	Rovida et al. (2013)	Enoch et al. (2009)	ECETOC TR 77
Dimethyl ethanolamine	108-01-0	203-542-8			No				L			
Diphenylmethane diisocyanate	101-68-8	202-966-0	1	1	Yes	A	C	E	L	P		N
Epigallocatechin gallate*	989-51-5	619-381-5 and 479-560-7	1		No				L			
Ethanolamine	141-43-5	205-483-3			No				L			
Ethyl acrylate	140-88-5	205-438-8	1		No		Q					
Ethyl cyanoacrylate	7085-85-0	230-391-5			No				L			
Ethylene diamine	107-15-3	203-468-6	1	1	Yes		R	E	L		S	
Ethylene oxide	75-21-8	200-849-9			No		I					
Ethyleneimine	151-56-4	205-793-9			No		I					
Fenthion	55-38-9	200-231-9			No				L			
Formaldehyde	50-00-0	200-001-8	1		No	A	Q				S	
Glutaraldehyde	111-30-8	203-856-5	1A	1	Yes	B	R			P	S	
Glycyl compound	77430-27-4	No results found			No				L			
HBTU*	94790-37-1	619-076-7 and 423-020-5			No	A						

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TABLE 1 (Continued) List of respiratory sensitizers identified either by literature search, the official harmonized classification (Annex VI of CLP)¹¹ and/or the C&L inventory database¹².

Chemical	CAS	EC/ List no.	Skin Sens. Category (C&L inventory)	Resp. Sens. Category (C&L inventory)	Entry in annex VI of CLP	Ponder et al. (2022)	Sadekar et al. (2021)	Cochrane et al. (2015)	Enoch et al. (2010)	Rovida et al. (2013)	Enoch et al. (2009)	ECETOC TR 77
Hexachlorophene	70-30-4	200-733-8			No		I					
Hexahydro phthalic anhydride	85-42-7	201-604-9	1	1	Yes	B	C					
Hexamethylene diisocyanate	822-06-0	212-485-8	1	1	Yes	A	C	E	L	P		O
Himic anhydride	826-62-0	212-557-9	1	1	Yes		R					
Hydroxylamine	7803-49-8	232-259-2	1		No		R					
Isononanoyl oxybenzene sulfonate	123354-92-7	No results found			No		I		L			
Isophorone diamine	2855-13-2	220-666-8	1A		No		I					
Isophorone diisocyanate	4098-71-9	223-861-6	1	1	Yes		I		L			
Maleic anhydride	108-31-6	203-571-6	1A	1	Yes		R		L	P		
Menthol	1490-04-6	216-074-4			No	A						
Methyl cyanoacrylates	137-05-3	205-275-2			No		R		L			
Methyl methacrylate	80-62-6	201-297-1	1		No		I					
Methyldopa	555-30-6	209-089-2			No				L			
Methyltetrahydrophthalic anhydride	11070-44-3	234-290-7	1	1	Yes	B	R					
N-methyl morpholine	109-02-4	203-640-0			No		I					
N-methyl piperazine	109-01-3	203-639-5	1B		No		I					

(Continued on following page)

TABLE 1 (Continued) List of respiratory sensitizers identified either by literature search, the official harmonized classification (Annex VI of CLP)¹¹ and/or the C&L inventory database¹².

Chemical	CAS	EC/ List no.	Skin Sens. Category (C&L inventory)	Resp. Sens. Category (C&L inventory)	Entry in annex VI of CLP	Ponder et al. (2022)	Sadekar et al. (2021)	Cochrane et al. (2015)	Enoch et al. (2010)	Rovida et al. (2013)	Enoch et al. (2009)	ECETOC TR 77
Naphthalene diisocyanate	3173-72-6	221-641-4	1A	1	Yes		R		L			
Ninhydrin	485-47-2	207-618-1	1B		No		R					
P-phenylenediamine	106-50-3	203-404-7	1		No						S	
Penicillin G	61-33-6	200-506-3	1		No				L			
Persulfate salts (sodium persulfate)	7775-27-1	231-892-1	1	1	No		Q					
Phenylglycine acid chloride	39878-87-0	254-668-5	1	1	No	A						
Phenylglycine chloride	39478-47-2	No results found			No				L			
Phthalic anhydride	85-44-9	201-607-5	1	1	Yes	B	I	E	L		S	N
Piperacillin	61477-96-1	262-811-8	1	1	No	A			L			
Piperazine	110-85-0	203-808-3	1	1	Yes	B	C	E	L		S	
Piperazine dihydrochloride	142-64-3	205-551-2	1	1	Yes		I					
Platinum	7440-06-4	231-116-1			No			E				
Plicatic acid	16462-65-0	No results found			No	B			L			N
Potassium dichromate	7778-50-9	231-906-6	1	1	Yes	A						
Reactive orange 3R	20262-58-2	243-653-9	1	1B	No				L			

(Continued on following page)

TABLE 1 (Continued) List of respiratory sensitizers identified either by literature search, the official harmonized classification (Annex VI of CLP)¹¹ and/or the C&L inventory database¹².

Chemical	CAS	EC/ List no.	Skin Sens. Category (C&L inventory)	Resp. Sens. Category (C&L inventory)	Entry in annex VI of CLP	Ponder et al. (2022)	Sadekar et al. (2021)	Cochrane et al. (2015)	Enoch et al. (2010)	Rovida et al. (2013)	Enoch et al. (2009)	ECETOC TR 77
Rifazol black GR		No results found			No				L			
Tetrachloroisophthalodinitrile	1897- 45-6	217- 588-1	1		No				L		S	
Tetrachlorophthalic anhydride	117- 08-8	204- 171-4	1	1	Yes		R	E	L			
Thiamphenicol	15318- 45-3	239- 355-3			No	A						
Toluene diisocyanate	26471- 62-5	247- 722-4	1	1	Yes	B	C	E				
Triethanolamine	102- 71-6	203- 049-8			No		I					
Triethylene tetramine	112- 24-3	203- 950-6	1		No		I		L			
Triglycidyl isocyanurate	2451- 62-9	219- 514-3	1		No		I					
Trimellitic anhydride	552- 30-7	209- 008-0	1	1	Yes	B	C	E	L	P		N
Tungsten carbide	12070- 12-1	235- 123-0			No			E				
Tylosin	1401- 69-0	215- 754-8	1	1	No				L			
Urea formaldehyde	9011- 05-6	618- 464-3			No		I					
Vinyl benzene	100- 42-5	202- 851-5			No						S	

Compounds are listed in alphabetical order. References are listed by year of publication, starting with the most recent source. The evidence cited in the sources is abbreviated with letters (A: equal or lower than 10 cases; B: higher than 10 cases; C: compelling evidence; E: occupational asthma linked to clinical and epidemiological evidence; H: no animal evidence, human evidence; I: inadequate evidence; L: clinical studies of individuals with chemical-induced asthma-like symptoms; N: animal and human evidence; P: commented as respiratory positive controls; Q: questionable evidence; R: reasonable evidence; S: respiratory sensitization from frequent literature references; U: uncategorized). The classified respiratory sensitizers listed in the C&L inventory are highlighted in light grey, the ones listed in Annex VI of CLP in dark grey. For comparison, the C&L information for skin sensitization classification is listed, too. Please note that if the cell for the C&L inventory derived classification is empty, this can have different reasons, e.g., (i) no classification due to the lack of data, (ii) some notifiers suggest that the compound has respiratory sensitizing properties, but the majority of notifications does not include this classification, (iii) the CAS number is not available (e.g., for Rifazol black GR), and/or (iv) the compound is not listed in the ECHA C&L inventory database overview (e.g., Ceferam pivoxil). An asterisks label compounds of which the CAS numbers had a double entry in the C&L inventory, the more conservative classification was adopted. This table was updated on 15 of May. 2024.

TABLE 2 Methods that contain elements that could contribute to address the key events (KE) of AOP 39.

Method name	Test principle	Validation status for skin sensitization	Reference
MIE (KE 1) "Covalent binding, protein"			
Direct Peptide Reactivity Assay (DPRA)	Detection of sensitizers based on their reactivity with synthetic peptides containing lysine or cysteine	OECD TG 442C (2023a)	Gerberick et al. (2004)
Peroxidase Peptide Reactivity Assay (PPRA)	Builds on PPRA using dose-dependence analysis, mass spectroscopy and enzyme system to improve identification of pro-haptens		Troutman et al. (2011)
KE 2 "increased secretion of proinflammatory mediators"			
3D lung models; air-liquid interface cultures	Mimic physiological barrier, can be applied to assess barrier integrity, morphological changes, cytoprotective and cytokine responses, viability, etc.		Lacroix et al., 2018; Sullivan et al., 2017; Th�� et al., 2021
KE 3 "activation of DCs"			
Human cell line activation test (h-CLAT)	Measurement of CD86 and CD54 expression by flow cytometry in THP-1 cells	OECD TG 442E (2023b)	Sakaguchi et al. (2006)
Genomic allergen rapid detection test, adaptation for respiratory sensitizers (GARD TM air)	GARD Respiratory Prediction Signatures (GRPS) that were established based on the expression of genomic biomarker signatures in MUTZ-3 in a machine learning approach are used to classify test chemicals as respiratory sensitizers	OECD TG 442E (2023b)	Forreryd et al. (2015)
KE 4 "activation/proliferation of T cells"			
Local Lymph Node Assay (LLNA)	<i>In vivo</i> assay, measurement of proliferative responses by draining lymph node cells induced following exposure of mice to test chemicals	OECD TG 429 (2010a), OECD TG 442A (2010b), OECD TG 442B (2018)	Dearman et al. (1999)
Human T-cell priming assay (htCPA)	Measurement of IFN-�� and TNF-�� production in cocultures of na��ve T cells and chemical-modified/-pulsed monocyte-derived DC after rechallenge		Richter et al. (2013)

AOP-helpFinder to create a stressor-event network. We only included interactions predicted as moderate, high, and very-high, as well as events that occurred more than 5 times. Finally, those publications that were retrieved in both the PubMed and the AOP-helpFinder query were extracted, including information on the publication type (original article or review), and publication year. No further inclusion and exclusion criteria were applied because it is only a status assessment.

2.3 AOP 39–40 network

Data from the AOP-Wiki database² release 2.6 was downloaded on 16 June 2023. All KEs belonging to AOP 39 and AOP 40 were retained, and in cases where a KE was connected to another KE from a different AOP, it was relabeled as the associated AOP.

3 Identification/classification of respiratory sensitizers and appraisal of the topic in the scientific literature

3.1 Comparison of literature-identified sensitizers and official classification

Without prediction methods for the respiratory sensitizing potential of chemicals, it is not possible to estimate the actual

extent of the problem, which makes it difficult to take the necessary steps to increase worker and consumer safety. In the past, several initiatives were made to set up reference lists for respiratory sensitizers in order to foster the development of identification methods.

We collected a dataset of 90 compounds identified as respiratory sensitizers from scientific literature (Supplementary File S1) (ECETOC TR 77³; Enoch et al., 2009; Enoch et al., 2010; Rovida et al., 2013; Cochrane et al., 2015; Sadekar et al., 2021; Ponder et al., 2022). The assessment of the evidence for the sensitizing properties of the chemicals differs in the literature sources, the respective assessment commentaries can be compared in Table 1. More information about other types of toxicity associated with these chemicals can be found in Supplementary Table 1.

In order to be as inclusive as possible, we have used all listed chemicals for our analysis including those with low level of confidence or even questionable evidence. This decision was made because individual case reports can still suggest a potential for sensitization, even if they occur with low incidence (Sadekar et al., 2021). This is relevant to extend the AOP network to less frequently described sensitization mechanisms. Each compound was cross-referenced with its CAS number and assessed within Table 3 to Annex VI of CLP and the European Chemicals Agency’s (ECHA) C&L databases for hazard classification. Both the official harmonized C&L, the REACH registration C&L and the notified C&L entries were considered.

The results revealed that out of the 90 literature-derived compounds, 24 compounds have an official harmonized

TABLE 3 Methods used in epidemiological research and in clinical practice to address respiratory allergy. Some of these methods or variations thereof are also used with animal models in allergy research and for substance testing.

Method/Abbreviation	Description	Used in animal testing	Reference
Bronchial (methacholine) challenge test/ bronchial provocation test (BPT)/nonspecific bronchial responsiveness (NSBR)	Bronchial provocation test to measure bronchoconstriction using spirometry	Shalaby et al. (2010)	Malo et al., 2011 , Gupta et al., 2019
Airway hypersensitivity/Specific inhalation challenge test (SIC)	Controlled exposure to a suspected occupational allergen in a dose-progressive manner, as measured by spirometry, pulse oscillometry, and peak expiratory rate		Beach et al., 2007 , Gupta et al., 2019
Fractional exhaled nitric oxide test (FeNO)	Measurement of nitric oxide (NO) in exhaled air. NO is produced in airway epithelial cells, eosinophils, and macrophages by nitric oxide synthases. Higher FeNO values indicate eosinophil airway inflammation	Weicker et al. (2001)	Kuruville et al. (2019b)
Allergen-specific serum IgE level	Measurement of serum levels of allergen-specific IgE in exposed animals or human patients using immunological assays such as ELISA or the highly sensitive ImmunoCAP platform	Hilton et al. (1996)	Movérare et al., 2017 van Hage et al., 2017
Skin prick test	Intradermal allergen injection, readout is based on mast cell degranulation in the skin by quantifying skin erythema. This test correlates well with serum levels of specific IgE		Baldacci et al., 2001 , Gupta et al., 2019
Basophil activation test	<i>Ex vivo</i> test that detects allergic reactions by incubating the patient's blood with suspected allergens and measuring basophil activation, indicated by markers such as CD63 and CD203c, using flow cytometry		Vera-Berrios et al. (2019)
Patch test	An <i>in vivo</i> method in which chemicals are applied to the skin and the inflammatory eczematous reaction from delayed hypersensitivity is assessed		Popple et al., 2016 Gupta et al. (2019)

classification according to Annex VI of CLP as respiratory sensitizer (Resp. Sens.) category 1, which indicates respiratory sensitizers for which not sufficient information for sub-categorization is available. According to the C&L inventory, there are additional 8 compounds categorized as respiratory sensitizers category 1 and one compound was classified as category 1B (with a low to moderate potential to cause respiratory sensitization in humans). Furthermore, 19 literature identified compounds were either not classified for respiratory sensitization or not present in the C&L database (see [Supplementary Table 1](#)).

Interestingly, the remaining compounds have not received any CLP or C&L respiratory sensitizer classification. Thus, only approximately 27% of sensitizing compounds mentioned in literature are classified as such within the CLP regulation (37% in case of including the C&L inventory derived classified respiratory sensitizers).

Several of the compounds listed in [Table 1](#) exert also other types of toxicity ([Figure 2](#); [Supplementary Table 1](#)). As it is of interest to discriminate between respiratory and skin sensitizers, we compared the compounds in these categories. Among the 90 respiratory sensitizers mentioned in literature, 42 were recorded in the C&L inventory as skin sensitizers category 1, 5 were categorized as skin sensitizers category 1A, and 2 were labelled as skin sensitizers category 1B. When comparing the Annex VI of CLP plus C&L inventory derived classified respiratory sensitizers in our list ($n = 33$), 31 compounds were listed as skin sensitizers, too.

To gain a better understanding of the extent of the respiratory sensitization problem, the number of relevant entries in the C&L database and [Table 3](#) to CLP Annex IV was surveyed. It is important to note that we have not differentiated the type of respiratory sensitizer, thus not only LMW chemicals but all types of sensitizing compounds were included, i.e., protein allergens, particles and mixtures. Overall, currently the C&L inventory contains to date 2296 CAS numbers that are associated with a respiratory sensitization classification. [Table 3](#) to Annex VI of CLP contains 120 entries for respiratory sensitizers.

3.2 To what extent is chemical-induced respiratory sensitization reflected in the literature?

In order to better assess the knowledge gained in the last few years around the topic of respiratory sensitization we have adopted a literature search-based approach using PubMed and AOP-helpFinder 2.0, a web tool to identify and extract associations between stressor and event, and between event and event ([Jaylet et al., 2023](#)). An overview of the results of the literature search can be found in [Figure 4](#). We have set up query lists containing terms for selected molecular events and biological processes that were mentioned in the literature in the context of respiratory sensitization and insults, and umbrella terms for pathological mechanisms, symptoms and

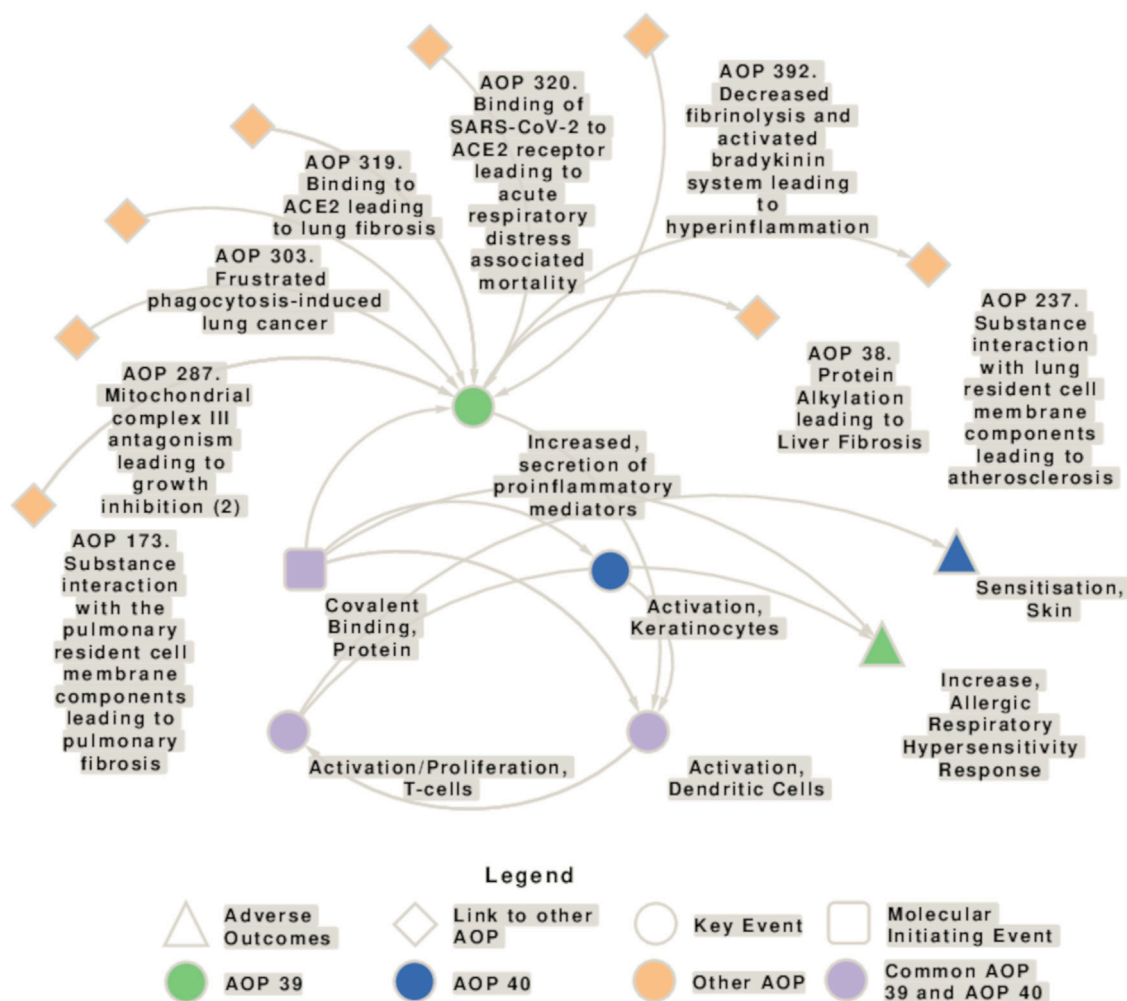


FIGURE 1

Network showing the interconnection of key events (KEs) involved in the adverse outcome pathways (AOP) for respiratory (AOP 39) and skin sensitization (AOP 40), and connections to other AOPs. AOP 39 and 40 share the molecular initiating event (MIE) "Covalent Binding, Protein," the key event (KE) "Activation, Dendritic Cells" and the KE "Activation/Proliferation, T-cells." Moreover, there are some similarities in the AOP 40 KE "activation of keratinocytes" and the AOP 39 KE "Increased secretion of proinflammatory mediators." KEs specific to AOP 39 are depicted in green, KEs specific to AOP 40 in blue, and KEs present in both AOs in purple. The squares represent the molecular initiating events (MIEs), circles the KEs, triangles the AOs, and diamonds the connection with other AOPs.

adverse reactions. Moreover, we selected a subset of 6 chemicals (Supplementary File S1), that are both literature-identified and C&L inventory derived classified respiratory sensitizers.

Chloramine T, phthalic anhydride, piperazine, toluene diisocyanate (TDI), trimellitic anhydride and ethylenediamine belong to different electrophilic mechanistic domains (Enoch et al., 2010) and are among the chemicals identified in the "in *litero*" screening as respiratory sensitizers (Sadekar et al., 2021; Ponder et al., 2022). TDI is the prototypical stressor mentioned in AOP 39 due to the availability of information on a concentration-dependent response and temporal concordance with the adverse event. The protein binding mechanism of TDI as well as of phthalic anhydride and trimellitic anhydride is acylation, whereby in particular the anhydrides show a very high electrophilic index (Enoch et al., 2009). Ethylenediamine and piperazine are metabolized to aldehydes, which undergo Schiff base formation (Enoch et al., 2010). N-chlorination is the relevant mechanism for chloramine T (Enoch et al., 2010).

For all chemicals, PubChem-listed synonyms were included as search terms. The number of publications has shown a consistent upward trend over time for all six compounds, with the most substantial increase occurring in the last 5 years, particularly notable for TDI, piperazine, and ethylenediamine (Figure 3).

However, in the PubMed search there is only a minor overlap between the compound associated publications ($n = 5263$) and the event and umbrella terms of 161 articles (Figure 4C). Yet, the network generated based on the AOP-helpFinder prediction of interaction strength of the compounds with event/umbrella terms (Figure 4D) visualizes the associations with sensitization, oxidative stress and symptoms, i.e., asthma.

4 Mechanisms described in AOP 39

As stated above, AOP 39 builds on the skin sensitization AOP 40 and is composed of the KEs: covalent binding of chemicals to

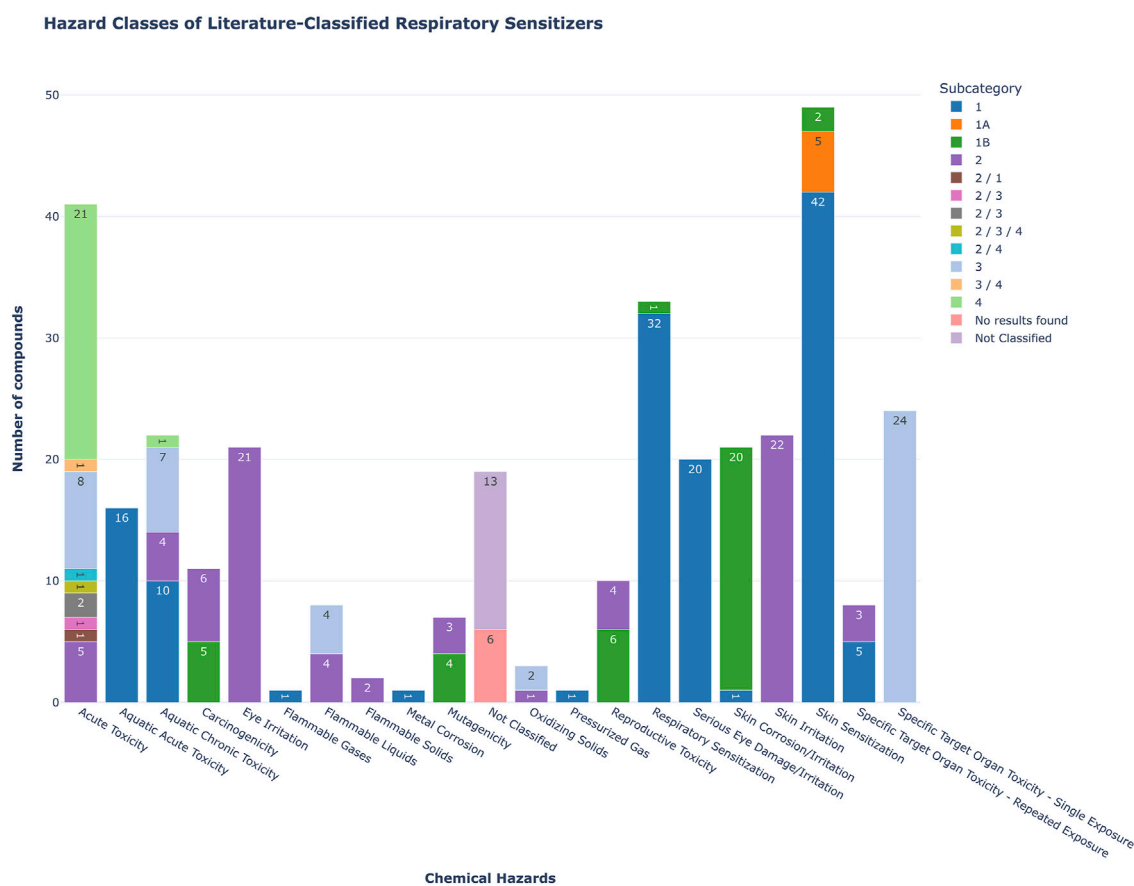


FIGURE 2

Bar plot showing the hazard classification distribution of 90 compounds previously identified as respiratory sensitizers in the literature, and queried against the ECHA C&L database. The colors indicate the subcategory, the numeric values within the bars indicate the number of compounds associated with specific hazards.

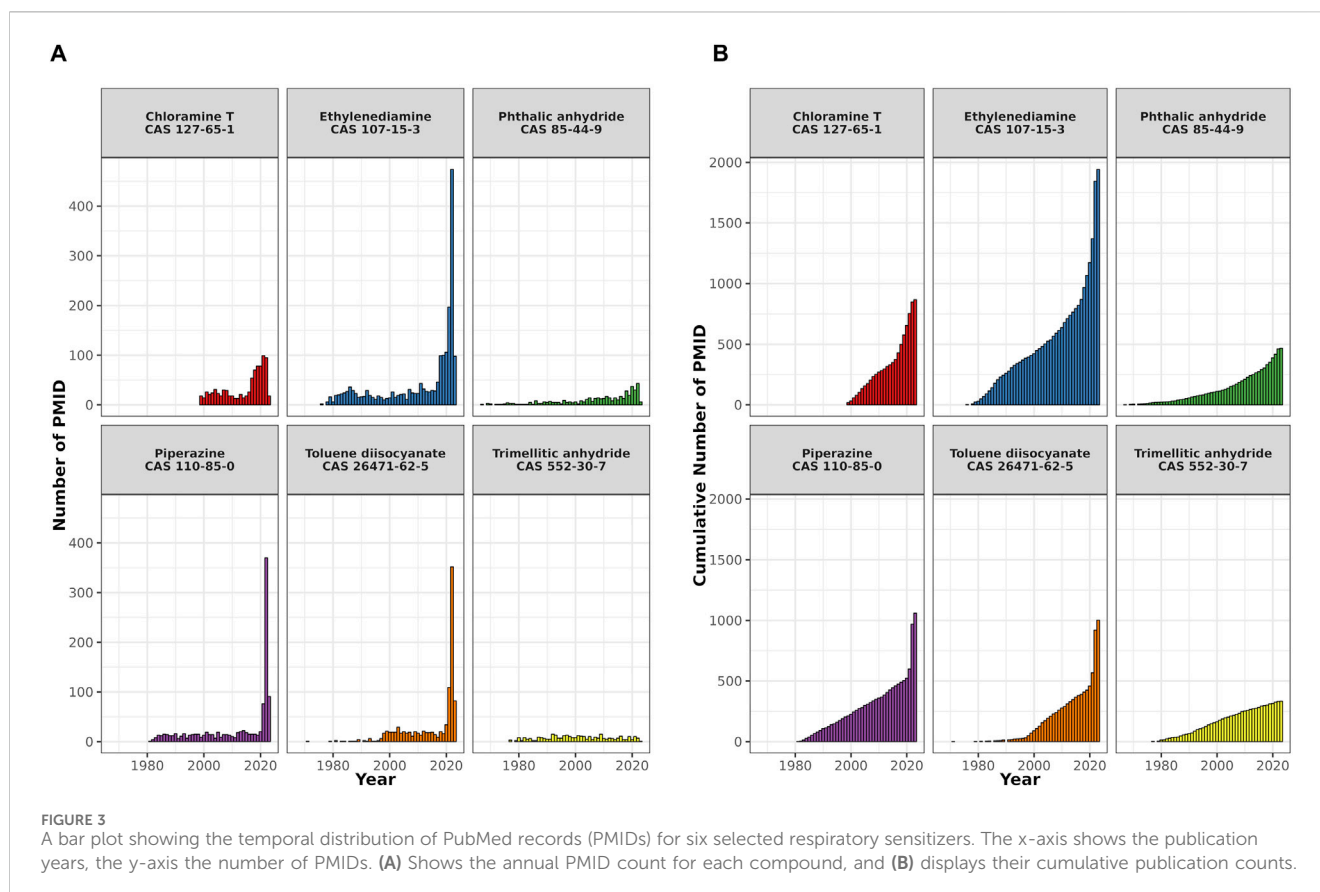
proteins (which is the MIE), increased secretion of proinflammatory mediators, dendritic cell activation, activation and proliferation of T cells, finally leading to the adverse outcome (AO) increased allergic hypersensitivity response. This AOP relies on evidence linked to low-molecular-weight chemicals and excluding other known respiratory sensitizers acting via different MIEs (<https://aopwiki.org>). The prototypical stressor is a low-molecular weight chemical, toluene diisocyanate.

4.1 MIE (KE 1): “Covalent binding to protein”, (KE 396)

The MIE represents the first key event (KE 1) in the AOP of respiratory sensitization (Sullivan et al., 2017, AOP-Wiki)¹. The covalent protein binding - or haptensation - of respiratory sensitizers was initially confirmed by those respiratory sensitizers, which tested positive in the *in vivo* local lymph node assay (LLNA) for skin sensitizers (Dearman et al., 2013) and subsequently in the *in chemico* Direct Peptide Reactivity Assay (DPRA) test (Lalko et al., 2012), which was developed and validated for the evaluation of KE 1 in the skin sensitization AOP (Gerberick et al., 2004; OECD, 2023a). The LMW chemicals, which induce respiratory sensitization

are not able to directly engage with the immune system to elicit an immune response due to their size. Small molecules, haptens, need to bind covalently to a protein and form the hapten-protein complex, which initiates the cascade of events leading to respiratory sensitization. Most respiratory sensitizers are reactive electrophilic compounds or can be converted abiotically by oxidation (pre-haptens) and/or metabolically (pro-haptens) to electrophilic species, which bind to the nucleophilic centers in carrier proteins (Enoch et al., 2009; Enoch et al., 2010). A strong correlation between the presence of multiple reactive functional groups and the ability to induce respiratory allergy has been identified. Respiratory sensitizers were categorized based on their differences in protein binding mechanisms (acylation, Michael addition, Schiff base formation, nucleophilic substitution (SN), however there are also respiratory sensitizers that do not exert an electrophilic mechanism (Enoch et al., 2010).

Different opinions exist regarding the importance of cross-linking properties of chemicals, within or between proteins and the contribution of this mechanism to respiratory sensitization (Enoch et al., 2010; Lalko et al., 2011). Lalko et al. (2012) showed in the DPRA that the majority of respiratory sensitizers favours reactivity with lysine residues, in contrast to skin sensitizers that favour cysteine binding. Lysine is considered the most important



biological nucleophile in respiratory sensitization, as cysteine is oxidized in the lung. Moreover, it has been shown that respiratory sensitizers tend to bind to serum albumin, which is rich in lysine residues (Hopkins et al., 2005). Nevertheless, preferential binding of lysine does not represent a prerequisite for respiratory sensitizers, as although the acid anhydrides display this preference (Krutz et al., 2021), other chemical classes do not bind exclusively to lysine (Dik et al., 2016).

Although skin and respiratory sensitizers share several characteristics, such as their electrophilicity, and the chemical reactivity with proteins/peptides in order to be recognized by the immune system, they ultimately lead to different forms of allergic disease. Considering that the adverse effect is differing between the two AOPs, the hypothesis that chemicals form distinct conjugates depending on their specific chemical structure and the nature of the functional groups directs the response of T cells towards preferentially either Th1 or Th2 responses, represents the first point of divergence in the pathways leading to respiratory and skin sensitization, respectively.

Importantly, non-electrophilic compounds and metals do not fall within the applicability domain of AOP 39. Transition metal complexes are non-classical haptens as their sensitization mechanism is based on the formation of coordinated complexes that are not sufficiently strong to survive antigen processing (Chipinda et al., 2011). It is suggested that these complexes either bypass the intracellular antigen processing steps or that protein is used for transport but then short-lived, high-affinity coordination sites are created within certain T cell receptor-major

histocompatibility complex (MHC) zones (Thierse et al., 2005). Moreover, some “inert” chemicals are able to bind to the MHC on the basis of their conformation rather than reactivity (Posadas and Pichler, 2007).

The role of lipophilicity and membrane penetration capacity is not widely discussed. Substances that are relatively inert but able to penetrate cell membranes can damage the lung barrier, accumulate intracellularly or are further distributed through the blood system, thereby affecting also other target organs.

4.2 KE 2: “Increased secretion of proinflammatory mediators” (KE 1496)

The second KE in AOP 39 refers to activation of inflammatory signaling by substances that promote and regulate inflammation in the respiratory tract. The exposure of the airway epithelium to respiratory sensitizers may cause loss of integrity of the epithelial barriers increasing the permeability of the epithelial cells and gaining access to dendritic cells (DC).

The mechanism of detection of inhaled environmental allergens by airway epithelial cells is realized through pattern recognition receptors (PRRs) which activate different signaling pathways. This leads to production of inflammatory cytokines and chemokines, attracting DCs. The PPRs recognize pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs). As the lung is continuously exposed to allergens (containing PAMPs and DAMPs), tolerance should occur,

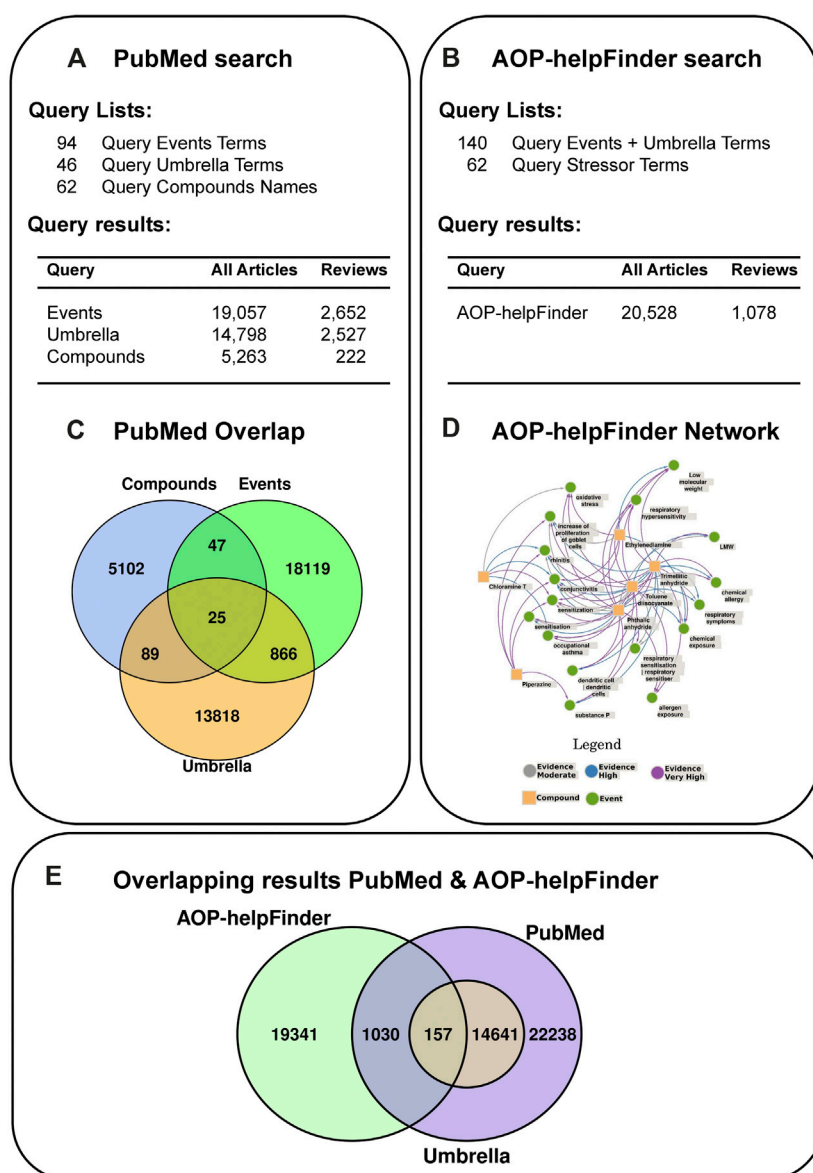


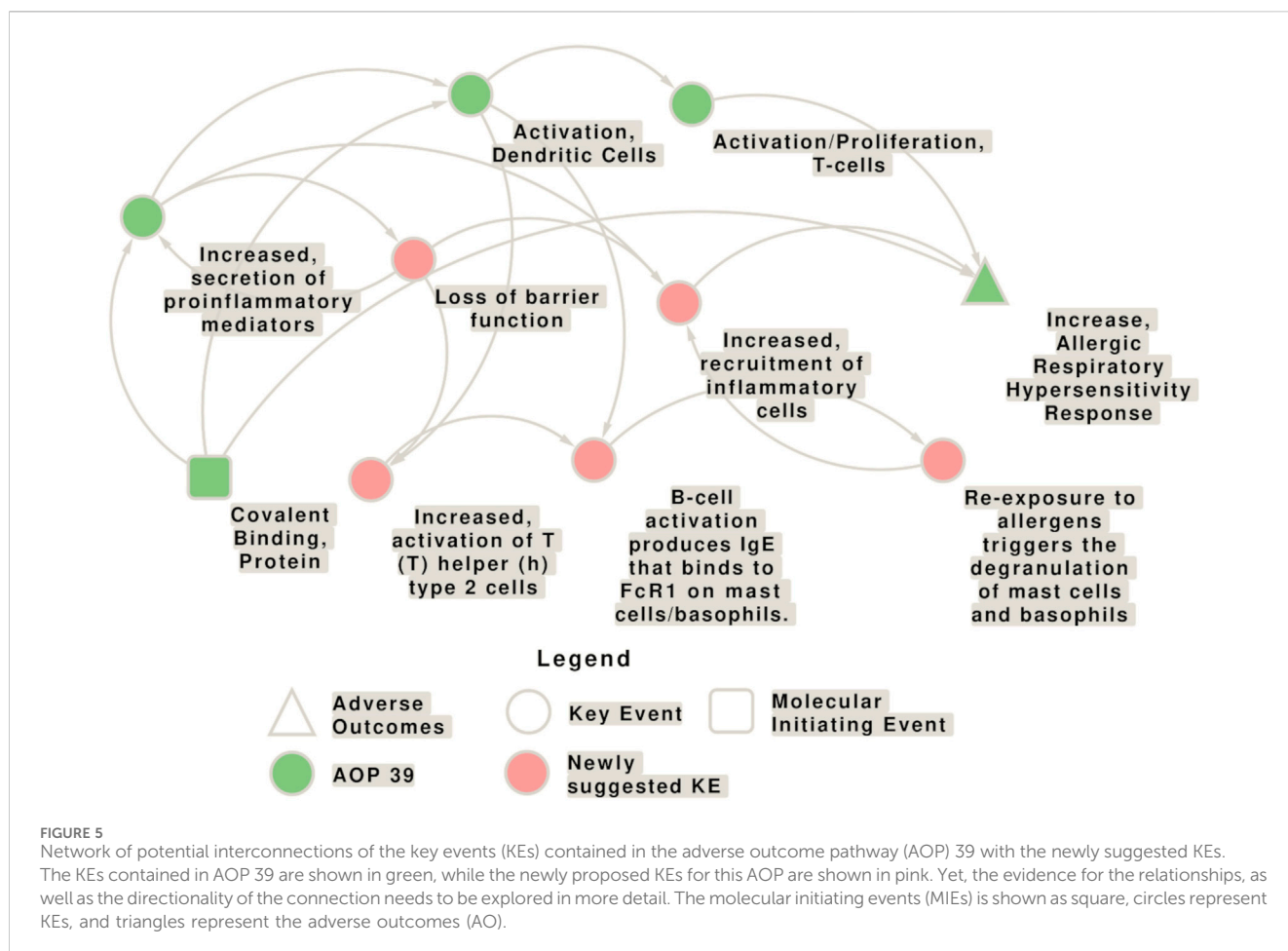
FIGURE 4

Literature search strategy using PubMed (A) and AOP-helpFinder (B). (A) The query term lists used for the PubMed search contained 1) events and targets extracted from literature, 2) clinical symptoms and/or adverse outcomes (called umbrella terms) and 3) a number of 6 selected compounds with all synonyms (chloramine T, ethylene diamine, phthalic anhydride, piperazine, toluene diisocyanate and trimellitic anhydride). The number of articles found with the individual query lists are shown. (B) For the AOP-helpFinder search, the event and umbrella terms were combined and the list of compounds served as stressors. (C) Articles that were found with more than one query list were identified. (D) A network was generated based on the predicted interactions strength from AOP-helpFinder. (E) A comparison of the results of the two different search strategies revealed 1,187 articles found using both methods.

however this is not the case in allergic diseases where inflammation is triggered. TLR4 expression on lung epithelial cells, but not on DCs, was necessary and sufficient for the induction of allergic asthma (Hammad et al., 2009). Recognition of PAMPs and DAMPs initiates host defense by triggering the release of the pro-inflammatory cytokines IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), called alarmins. Receptors of these cytokines (IL-17RA, IL1RL1 and TSLPR) are expressed on a wide variety of cells, including DCs and type 2 innate lymphoid cells (ILC2). Activation of DCs and ILCs through alarmins (KE 2) plays key roles in driving type 2 inflammatory responses in asthma by

promoting downstream production of type 2 cytokines such as IL-4, IL-5, and IL-13 from the effector cells (KE 3) (Duchesne et al., 2022).

TSLP is known for its role in initiating and maintaining Th2 responses and activating T cells (Soumelis et al., 2002; Ito et al., 2005; Rochman et al., 2007; Akamatsu et al., 2008). Its specific involvement in respiratory sensitization remains uncertain, potentially varying depending on the antigen context (Duchesne et al., 2022). TSLP-activated DCs produce chemokines that attract Th2 cells and stimulate the production of inflammatory Th2 cytokines (Soumelis et al., 2002; Pattarini et al., 2017). TSLP is highly expressed in lung and skin-derived epithelial cells, and



certain myeloid DC populations express TSLP receptors (Reche et al., 2001; Ziegler, 2012; Kitajima and Ziegler, 2013; Ochiai et al., 2014). TSLP levels in asthmatic patients correlate with Th2 cytokine expression and inversely with lung function (Ying et al., 2008; Shikotra et al., 2012). OX40L is a Th2 cell-prone costimulatory molecule. Its expression by DCs is used to identify respiratory sensitizers and to discriminate them from skin sensitizers, while for instance the expression of TSLP receptor complex components TSLPR and IL-7Ra did not provide a basis for discrimination between respiratory and skin sensitizers (Mizoguchi et al., 2023). The relevance of OX40L in T cell priming and polarization is discussed in chapter 3.1.4 (KE 4). The Th2 inducing effect of TSLP-matured DCs was further enhanced by another pro-Th2 alarmin, IL-25 (Wang et al., 2007) that is produced by epithelial cells, basophils, and eosinophils (Lambrecht and Hammad, 2009). IL-25, known for its role in parasitic infections, is also linked to asthma and allergy. Various factors such as lipopolysaccharide (LPS), ovalbumin (OVA), and epithelial cell injury trigger IL-25 release. In asthmatic patients, myeloid and plasmacytoid DCs express the IL-25 receptor IL-17RB, which is upregulated after allergen exposure. IL-25 also influences plasmacytoid DC function, impacting TLR9 expression (Tworek et al., 2016).

IL-33, part of the IL-1 cytokine family, is produced by cells in exposed tissues (skin, airways) as well as in lymphoid organs (Mitchell and O'Byrne, 2017). Mice lacking IL-33 show resistance

to respiratory allergens (Haenuki et al., 2012; Barlow et al., 2013; Nakanishi et al., 2013). IL-33 activates DCs, promoting Th2 cytokine production by inducing IL-6, IL-1 β , TNF, CCL17, and the expression of CD40, CD80, OX40L, and CCR7 *in vitro*. IL-33 recruits and activates DCs in the lungs *in vivo* (Cayrol and Girard, 2022). Another important pro-Th2 and DC attracting cytokine released by epithelial cells during allergic lung inflammation is GM-CSF. This cytokine is crucial for mouse allergic sensitization (Asquith et al., 2008; Su et al., 2008). Overexpressing GM-CSF in airways disrupts inhalation tolerance (Stämpfli et al., 1998). Neutralizing GM-CSF (or IL-33) blocks allergic sensitization to house dust mite (HDM) (Willart et al., 2012). Elevated GM-CSF levels are found in asthma patients (Saha et al., 2009). GM-CSF induces DC maturation (Bleck et al., 2006), impacting Th2/Th17 cell priming and granulocyte recruitment (Nobs et al., 2021). GM-CSF controls resident lung DCs (Greter et al., 2012).

Other cytokines and chemokines such as CCL5, CCL17, CCL11 and CCL22 were also described in different studies to trigger Th2 cell polarization upon exposure to allergens (reviewed by Morianos and Semitekolou, 2020).

Although a multitude of studies demonstrated that all these cytokines were necessary for the development of a Th2 allergic response against an antigen, mounting evidence suggests that the importance of these cytokines in the allergic response varies with the type of the allergen.

Although KE 1496 is not included in AOP 40 (Covalent Protein binding leading to Skin Sensitization; [Figure 1](#)), it is reasonable to assume that the increased secretion of pro-inflammatory mediators also occurs in the process of skin sensitization. In the description of the second key event (KE 826) of AOP 40, it is stated that the activated keratinocytes release pro-inflammatory cytokines, such as IL-18, suggesting that similar cellular responses occur after the molecular initiating event.

4.3 KE 3: “Activation of dendritic cells” (KE 398)

DCs are professional antigen presenting cells (APCs), characterized by their high surface expression of MHC molecules, co-stimulatory molecules and pattern recognition receptors. They are critical in presenting antigens to T cells, activating them and directing the immune response toward the appropriate immune reaction by migrating to the lymph nodes. DCs in lung are located in virtually all tissue compartments: throughout the conducting airways, in lung parenchyma, alveolar spaces, vasculature, pleura and bronchial lymph nodes ([GeurtsvanKessel and Lambrecht, 2008](#)). Immature DCs can exert their guarding role in the lung with direct and indirect sensing of the antigens. DCs can get into direct contact with allergens or haptens at the lung surface even in case the epithelial barrier is not compromised, due to their ability to extend dendrites into the lumen via formation of tight junctions between the epithelial cells lining the alveolar spaces with the aid of claudins and zonula-2 molecules ([Lambrecht and Hammad, 2009](#)).

The link between the MIE and activation of DCs in the AOP is well established, but the evidence for the relationship between KE 2 (activation of inflammatory signaling) and DC activation is weak due to a number of data gaps and uncertainties. Lung DCs express receptors for inflammatory mediators and DAMPs released upon tissue damage. In case of respiratory sensitization the primary site of damage is the bronchial epithelial cell layer. Hapten-protein conjugates are taken up by DCs and processed to hapten-modified peptides that can bind to major histocompatibility complex type II (MHCII) molecules ([Martin et al., 2010](#)). Antigen uptake and activation of PRRs induces the DC maturation process through which they become efficient T cell stimulators. TLR4 is expressed in abundance on conventional DCs (cDCs), and monocyte-derived DCs and are important for respiratory sensitization ([Trompette et al., 2009](#); [Blecher-Gonen et al., 2019](#)). For example, LPS, a well-known TLR4 ligand, administered as adjuvant in respiratory sensitization experiments using OVA is required to amplify Th2 responses in allergic inflammation ([Lamiabie et al., 2020](#)). In addition to TLRs, other receptors such as CD301b ([Kumamoto et al., 2013](#)), CD206 and dectins ([Parsons et al., 2014](#); [Chiffolleau, 2018](#)) were also shown to play an important role in allergen uptake by different subtypes of DCs.

Allergen-bearing DCs are stimulated to migrate to regional lymph nodes ([Lambrecht and Hammad, 2010](#)). Mature DCs migrate to the afferent lymph nodes to present their antigens to specific T cells, activate and polarize them towards Th1 or

Th2 responses. In case of skin sensitization there are extensive studies available on skin sensitizer-induced DC migration. Fibroblasts mediate migration of cytokine-matured Langerhans cells via different chemokines ([Ouwehand et al., 2008](#); [Ouwehand et al., 2012](#)), but for respiratory sensitization this indirect activation of DCs is not completely proven ([Sullivan et al., 2017](#)).

It is important to take into consideration that there are different DC subtypes, playing distinct roles in initiating and maintaining allergen-driven Th2 immune responses in the airways. Three well-defined DC populations exist in the lung: two types of conventional DCs (cDCs) and plasmacytoid DCs (pDCs). All three types of DCs are generated from progenitor cells in the bone marrow but all have different functions. Conventional DCs express high CD11c levels and can be divided in two ontogenically different subsets, cDC1s and cDC2s ([Musumeci et al., 2019](#); [Wu et al., 2020](#)). cDC1s efficiently promote cytotoxic T cell and Th1 responses, while cDC2s are the most potent in capturing and transporting allergens and inducing Th cell responses (Th1, Th2, and Th17). In addition, cDC2s are important in the allergic response. pDCs participate mostly in the antiviral defense by type I interferon (IFN) production and promote natural killer (NK) cell and T cell responses. pDCs play important roles in regulation of immune tolerance ([Lamiabie et al., 2020](#); [Xi and Upham, 2020](#)). They are reported to suppress allergic sensitization upon activation by TLR7/TLR9, by promoting the induction of regulatory T cells (Tregs) in mice ([Ouabed et al., 2008](#); [Lombardi et al., 2012](#)), thereby regulating respiratory tolerance to inoffensive inhaled antigens ([de Heer et al., 2004](#)). The presence of programmed death-ligand 1 (PD-L1) on the surface of pDCs is critical for their suppressive effect. PD-L1-deficient pDCs could not alleviate allergic airway inflammation in mice ([Kool et al., 2009](#)).

During inflammation, in addition to the above-mentioned DC subsets, monocyte-derived DCs are recruited from the blood. These inflammatory DCs are very similar to the non-activated CD11b-expressing DCs, but they express the lymphocyte antigen 6 complex C (Ly6C) protein at various levels. CD11b- DCs can directly recognize foreign particles in the airway lumen with their extended processes. In contrast, CD11b+ DCs cannot pass the epithelial barrier, they can only pick up antigens that passed the basal membrane ([GeurtsvanKessel and Lambrecht, 2008](#)).

In the steady-state, the sampling and migration of lung DCs to mediastinal lymph nodes does not occur. TLR stimulation is needed to elicit a DC response. Airway epithelial cells might be instructive in causing DC sentinel behavior and activation in the lungs, but it is not known exactly which signals from epithelial cells initiate the sampling behavior of DCs ([Hammad and Lambrecht, 2008](#)).

4.4 KE 4: “Activation/proliferation of T-cells” (KE 272)

In AOP 39, the evidence is high for the key event relationship (KER) “Activation, dendritic cells leads to activation/proliferation, T-cells” (KER 379; Society for Advancement of AOPs 2023). It is assumed that for an AO to commence, a certain number of DCs is required to be activated and to migrate to the nearest lymph node in order to stimulate the further cascade of biological events. KE 4 of AOP 39 (KE 272) is also included in AOP 40. Its taxonomic

applicability is human and mouse, and for both species the evidence is high.

Respiratory sensitization depends on the antigen-driven activation of T cells, being their proliferation and differentiation into effector and memory populations (Martin et al., 2010; Kimber et al., 2018). The mechanisms regarding T cell activation involved in respiratory sensitization caused by LMW chemicals is not fully understood.

In lymph nodes, mature DCs present the processed protein-hapten antigen to the naïve T-cell receptor (TCR) via the MHCII molecule, and if the antigen is recognized as non-self, antigen-responsive T-cells are stimulated to propagate and differentiate into effector T-helper cells (Th1, Th2, Th17) and memory T-cells (Roggen, 2014; Poppleet et al., 2016). For the activation and differentiation of naïve T-cells to Th cells TCR-MHCII interaction, as well as interaction between co-stimulatory molecules on DCs and their ligands on T-cells and specific cytokines are needed (Richter et al., 2013; Butcher and Zhu, 2021). The strength of the response depends on the degree of clonal expansion of allergen-responsive T-cells and the number of clones that are able to recognize and respond to the presented antigen (Kimber et al., 2018). If clonal expansion is of sufficient magnitude, sensitization will be acquired.

OX40-L, CD80 and CD86 costimulatory molecules were reported to be necessary in certain steps of Th2 polarization. *In vivo*, OX40L produced by DCs acted as a costimulatory signal for optimal Th2 priming and memory induction, since OX40L-deficient DCs failed to stimulate the expansion and survival of T cells (Jenkins et al., 2007). It was hypothesized that another possible way of activation of DCs towards Th2 responses was reduction of IL-12 secretion (Kimber et al., 2018). It was also reported that *in vitro*, TSLP was able to induce human DCs to express OX40L, but not IL-12. TSLP-induced DC-derived OX40L was required for triggering naïve CD4 T cells to produce IL-4, -5, and -13, but in the presence of IL-12, OX40L lost the ability to polarize Th2 cells (Ito et al., 2005). CD80/CD86 co-stimulation on DCs was shown to be only necessary during priming of naïve T cells into Th2 cells but not during restimulation of previously primed Th2 cells in the challenge phase (van Rijt et al., 2004).

The cAMP/protein kinase A (cAMP/PKA) signaling pathway is also suggested to be involved in Th2 activation. A decrease of cAMP in mouse DCs resulted in Th2 immune response with an allergic phenotype, whereas increased cAMP induced Th17 immunity (Lee et al., 2015; Kim and Kim, 2018).

Another pathway proposed to be important in Th2 differentiation is the p38 signaling pathway (Hu et al., 2012; Endo et al., 2015). Han et al. concluded that p38 signaling in DCs promoted Th2 differentiation by regulating IL-12 expression (Han et al., 2022).

4.5 AO “Increase, allergic respiratory hypersensitivity response” (AO 313)

The adverse outcome “Increase, Allergic Respiratory Hypersensitivity Response” operates at the organ (lung) level, and is the adverse outcome of AOP 39 (AOP-Wiki²). The

taxonomic applicability of AO 313 includes humans, guinea pigs (*Cavia porcellus*) and rats (*Rattus norvegicus*). High-quality evidence supports its relevance in humans, while for guinea pigs and rats, the evidence is rated as low. AO 313 is relevant during both developmental stages and adulthood, with high-quality supporting evidence (AOP-Wiki).

The most common clinical manifestations of respiratory allergy are allergic rhinitis, rhino-conjunctivitis, sinusitis and asthma. Respiratory allergy symptoms include nasal congestion, cough, sneezing, rhinorrhea, wheezing, chest tightness, shortness of breath, airflow obstruction, and bronchoconstriction (Boverhof et al., 2008; Kuruvilla et al., 2019b; Thá et al., 2021; Xie et al., 2022). Reactions can be acutely life-threatening or lead to chronic occupational asthma.

5 Gaps and suggestions for additional KEs and KERs

The AOP 39 already states that there are still unresolved aspects in respiratory sensitization (Sullivan et al., 2017), for some of which research is still needed, but in the meantime some aspects can also be covered by recent knowledge.

5.1 Epithelial barrier integrity

Importantly, effects of respiratory sensitizer exposure on lung barrier function are not considered in the current AOP, or only to a limited extent. Yet, exposure effects on barrier integrity including adherens junction proteins and the cytoskeleton have been reported. Moreover, as impaired barrier function forms a hallmark of asthma, this effect may possibly provide a link between respiratory sensitizer exposure and the OA. The role of epithelial barrier function is discussed in more detail in Section 6. The link of impaired barrier function may possibly be extended by the Th2-skewed immune response to respiratory sensitizers in humans and experimental animals, and the intricate relationship between Th2 cytokine production and reduced barrier function. Studies on effects of respiratory sensitizers on barrier function are currently largely limited to TDI, suggesting additional studies should be performed to assess whether this effect is chemical-specific or class-specific. We suggest adding the loss of epithelial barrier integrity as KE. The orphan KE “Loss of barrier function” (KE 1675) in the AOP-Wiki may be filled, accordingly.

5.2 DCs

DCs play a crucial role in shaping immune responses to chemical allergens. However, more research is necessary to understand the steps leading to a shift toward Th2 effector responses and sensitization of the respiratory tract and a wider range of chemicals than those currently known needs to be investigated. Moreover, the same DCs can elicit both Th1 and Th2 responses, raising questions about their role in determining the immune response direction and the type of sensitization acquired. It has been suggested that DCs “read” chemical

allergens or hapten-protein complexes, tailoring immune responses favoring either Th1 or Th2 types. The reading of the antigen is likely influenced by the nature of the haptenated protein and the characteristics of danger signals and the downstream immunological milieu. Through this mechanism, DCs may trigger responses resulting in sensitization of the respiratory tract (Kimber et al., 2018). In this context, a valuable approach would involve assessing the impact of different DC subsets on sensitization development. This should encompass a comparative analysis of DAMPs, cofactors, and cytokines associated with both skin and respiratory sensitization (Kimber et al., 2018). A comprehensive examination of the transcriptional signature of DCs would shed light on how early changes in DC genes contribute to the expression of maturation markers specific of cDC1 and cDC2.

5.3 T cell polarization

Th2 cells are the main drivers of eosinophilic airway inflammation (Kuruvilla et al., 2019b; Arts, 2020). In several experimental studies, chemical respiratory allergens were found to induce a Th2-type immune response (Dearman et al., 2005; Kimber et al., 2018), but other cell types may also contribute to the response (Kimber et al., 2018; Butcher and Zhu, 2021).

Th2 cells release several cytokines, such as IL-4, which stimulates the production of immunoglobulin E (IgE) by B-cells and their clonal expansion and differentiation into plasma and memory B-cells. Th2 cells also release IL-5, which plays a pivotal role in promoting the differentiation and maturation of eosinophil progenitors in the bone marrow, as well as their subsequent mobilization and survival (Menzies-Gow et al., 2007; Rosenberg et al., 2007). Furthermore, they produce IL-9, which enhances mast cell proliferation, and IL-13, which promotes goblet cell metaplasia, increased mucus secretion as well as airway hyperresponsiveness (Chary et al., 2018; Kuruvilla et al., 2019b). The major source of Th2 cytokines is Th2 cells themselves, but other cell types, such as macrophages, basophils, eosinophils and mast cells have also been shown to produce IL-4 and IL-13 upon stimulation (Liang et al., 2011). Secretion of IL-10 by Th2 cells has been suggested to downregulate the DC-derived IL-12 production and thereby leads to Th2 polarization (Aste-Amezaga et al., 1998).

Th17 cells were also shown to play an important role in the immune response through the activation of both contact hypersensitivity and airway hyperresponsiveness characterized by neutrophil inflammation (De Vooght et al., 2013; Roggen, 2014; Xie et al., 2022). IL-17A, IL-21 and IL-22 produced by Th17 cells play a critical role in the non-Th2 type asthma development (Roggen, 2014; Xie et al., 2022). It appears that both human and mouse Th17 cells show a partial Th2 phenotype, expressing IL-4, IL-5, and IL-13, too (Butcher and Zhu, 2021).

Recently, substantial evidence indicates that group 2 innate lymphoid cells (ILC2s) also play a critical role in the type 2 adaptive immune response by producing type 2 cytokines (Gold et al., 2014; Chen et al., 2017; Kuruvilla et al., 2019b). ILC2s are abundant in airway tissues and produce large quantities of IL-5 and IL-13 in response to alarmins released from epithelial cells (Yang et al., 2016; Kuruvilla et al., 2019b). Thus, it has been recognized that innate immunity also has a key role in the pathophysiology of asthma.

We suggest expanding AOP 39 with a KE on T-helper cell polarization and cytokine production. Two similar KEs are already contained in AOP-Wiki, namely, “Increased, activation of T (T) helper (h) type 2 cells” (KE 1499, included in AOP 173) and “Increase of Th2 cells producing IL-4” (KE 1712, included in AOP 314).

5.4 B cells and IgE production

The differentiation and clonal expansion of Th2 cells lead to the production of Th2 cytokines that induce immunoglobulin (Ig) class switching to production of antigen-specific IgE by B cells and clonal expansion of naïve and memory B cell populations (Dearman et al., 2003). Immature B-cells could also be directly activated by antigens through the B-cell receptor (BCR) (Dullaers et al., 2012). IgE production can occur both in the germinal centers of regional lymphoid tissues and locally in the airway mucosa, but the extent of germinal center involvement or local IgE production in respiratory sensitization is currently unknown (Chvatchko et al., 1996; Forester and Calabria, 2010; Hoddeson et al., 2010). Some human and animal studies suggest that the respiratory mucosa is the principal site of allergen-specific IgE production during allergic airway inflammation (Dullaers et al., 2012; De Schryver et al., 2015; Nelson and Wu, 2022). Plasma cells produce and release allergen-specific IgE, which has a major role in the elicitation phase. IgE binds to the high affinity IgE receptor (FcεR1), which can be found in the membrane of both basophils and mast cells (Chary et al., 2018; Kuruvilla et al., 2019b). Mast cells reside in the tissues, while basophils are recruited from the circulation to tissues, where they attain final activation (Kuruvilla et al., 2019b).

However, the role of IgE antibodies in chemical respiratory allergy is not as firmly established as in protein respiratory allergy. Many cases of occupational asthma have been reported lacking detectable IgE, which may suggest a possible IgE-independent pathway (Tee et al., 1998; Wisniewski, 2007; Thá et al., 2021). However, lack of IgE could also be caused by the short half-life of serum IgE, or by not having a “spill-over” of the mucosally-produced IgE into the circulation (Dullaers et al., 2012; De Schryver et al., 2015), or it could be due to technical challenges of measuring chemical-hapten specific IgE antibodies (Wisniewski, 2007).

A new KE could focus on B-cell activation, IgE production and binding to high affinity IgE receptors (FcεR1) on mast cells and basophils.

5.5 Degranulation of mast cells and basophils

A subsequent single or multiple exposure to the same chemical allergen leads to an accelerated and more vigorous secondary immune response. Memory T-cells are re-activated, producing cytokines and inducing granulocyte infiltration (Thá et al., 2021). Upon re-exposure to the same allergen, antigen can crosslink IgE bound to the FcεR1 on the surface of mast cells and basophils, triggering their degranulation and the release of histamine, lipid mediators (prostaglandin D2, cysteinyl leukotrienes) and pro-inflammatory factors that lead to the clinical symptoms of

asthma and rhinitis (Kimber and Dearman, 1997; Fanning and Boyce, 2013).

Nevertheless, the involvement of IgE in chemical respiratory allergy is still controversial, especially in the case of diisocyanates, and it is possible that additional pathways are also involved in the degranulation of mast cells and basophils and the development of chemical respiratory allergy (Selgrade et al., 2012; Kimber et al., 2014; Quirce, 2014).

A new KE might include the evaluation of “Re-exposure to allergens triggers the degranulation of mast cells and basophils”.

5.6 Local inflammation and recruitment of inflammatory cells

Local inflammation at the site of exposure is characterized by the influx of lymphocytes and other leukocytes, and the release of inflammatory mediators (Thá et al., 2021). Eosinophils are the principal cell types associated with a type 2 immune response, and large numbers of eosinophils are recruited via the circulation to the site of inflammation following specific pro-inflammatory mediator (cytokine and chemokine) signaling (Bentley et al., 1992; Kimber and Dearman, 1997). Upon stimulation, eosinophils release inflammatory mediators, cytokines, chemokines, granule mediators and cysteinyl leukotrienes (Kuruvilla et al., 2019b). Eosinophils stimulate bronchial fibroblasts to produce extracellular matrix proteins and collagen and thus promote the thickening of the reticular basement membrane (Durrani et al., 2011).

Airway neutrophilia has frequently been reported in patients with isocyanate-induced occupational asthma (OA), as well as in experimental murine models with sensitization through the airways (Matheson et al., 2001; Wilson et al., 2009; De Vooght et al., 2013; Choi et al., 2019; Margelidon-Cozzolino et al., 2022). In contrast, in murine models sensitization through the peritoneum is more likely to prime Th2 response and eosinophilia (Wilson et al., 2009). Neutrophilic asthma is more severe and less responsive to corticosteroids compared with the eosinophilic Th2 type asthma (Choi et al., 2019).

The KE “Local inflammation, influx of lymphocytes and granulocytes” should be included in AOP39. A similar KE is available in AOP-Wiki entitled “Increased, recruitment of inflammatory cells” (KE 1497), which is included in AOPs 173, 303, 377, 392, 409, 451, 468, and 493.

5.7 KE related to other AO

There are several other AOPs which can to a certain extent be related to immune activation in the lung: AOP 196: Volatile Organic Chemicals Activate TRPA1 Receptor to Induce Sensory Pulmonary Irritation; AOP 148: EGFR Activation Leading to Decreased Lung Function; AOP 452: Adverse outcome pathway of PM-induced respiratory toxicity; AOP 272: Deposition of energy leading to lung cancer; AP 411: Oxidative stress Leading to Decreased Lung Function. However, these AOPs have distinct AOs from respiratory sensitization. Moreover, there are several KEs included in other AOPs that could be associated with AOP 39, too, but these KEs need

further development (e.g., KE 2010 “Pulmonary inflammation”, KE 2013 “Airway remodeling”, and KE 2086 “Airway inflammation”). The network of potential interconnections of the KEs contained in AOP 39 with the newly suggested KEs is shown in Figure 5.

6 The role of the epithelial barrier in respiratory sensitization

Lung epithelium plays an essential role in the protection from environmental insults including pathogens and pollutants/chemicals, as a physical, chemical and immunological barrier. Besides mucociliary escalators and immune defense mechanisms such as secretion of antimicrobial products, the epithelial intercellular junctions are of the utmost importance for the physical separation of the environment from the subepithelial tissue as they regulate paracellular permeability (Ganesan et al., 2013; Hewitt and Lloyd, 2021). Though the cellular composition and epithelial morphology varies depending on the location in the airway, the adhesive forces that maintain physical barrier function are epithelial junctions that consist of adherens junctions (AJs), tight junctions (TJs), and hemidesmosomes (Brune et al., 2015). AJs regulate adhesion of adjacent cells through homotypic interactions between E-cadherin. Disruption of E-cadherin results in delocalisation of TJ proteins. TJs are composed of zona occludens-1 (ZO-1), occludin, claudins, and junction adhesion molecules and are the main regulators of epithelial permeability (Nawijn et al., 2011). A high trans-epithelial electrical resistance (TEER) reflects formation of a tight epithelial barrier, an important aspect of airway epithelial function. TEER measurement can be used to measure epithelial damage due to insults (Gilmour et al., 2023).

Impairment of epithelial barrier function in asthma is a key player in airway inflammation and remodelling (Heijink et al., 2020). Impaired barrier function may allow for higher penetration of allergens, microbes, microbial products, and pollutants across the epithelium, resulting in activation of the immune system and development of allergies (Xiao et al., 2011). Structural changes in the epithelium of asthmatic patients include TJ disruption, and reduced E-cadherin expression. Importantly, the asthma-related cytokine IL-13, produced by Th2 and ILC2 cells, reduced barrier function as well as junctional proteins, including claudin-18, ZO-1, occludin, and E-cadherin (Sweerus et al., 2017; Sugita et al., 2018). IL-13 and the Th2 cytokine IL-4, reduced barrier function and induced physical separation of the TJ molecules, occludin and ZO-1 (Wawrzyniak et al., 2017). Induction of E-cadherin expression reduced the expression of NF-κB, a transcription factor important for airway inflammation (Solanas et al., 2008). Gene knockdown of E-cadherin in bronchial epithelial cells resulted in a pro-inflammatory response, measured as increased expression of TARC and TSLP (Heijink et al., 2007). Airway hyperresponsiveness, another hallmark of asthma, is correlated with airway epithelial damage (Laitinen et al., 1985). Early studies have shown that respiratory sensitizers induce a Th2-skewed response (Dearman et al., 1995; van Och et al., 2002), possibly suggesting effects on epithelial barrier function.

The respiratory sensitizer TDI impaired AJ function, induced E-cadherin redistribution, and increased the permeability of

bronchial epithelial cells *in vitro* and *in vivo* (Zhao et al., 2009; Song et al., 2013). Gene profiling of 16HBE human bronchial epithelial cells revealed that exposure to 12 respiratory sensitizers affected expression of genes associated with the cytoskeleton and barrier function (Dik et al., 2015), suggesting that pulmonary barrier integrity is an important target of chemical respiratory sensitizers. Gene profiling of NCI-H292 human pulmonary cells showed that hexamethylene diisocyanate (HDI) exposure resulted in upregulation of thioredoxin reductase, aldo-keto reductase C1, stanniocalcin, and TG-interacting factor (Wisniewski et al., 2002). The first two genes are involved in cellular thiol redox homeostasis. It should be noted that the Wisniewski study may have detected HDI-specific genes and not genes that are representative of a range of respiratory sensitizers.

7 New approach methodologies (NAMs) with the potential to be used for the identification of respiratory sensitizers

Due to the urgent need for accurate and reliable test methods for the identification of respiratory sensitizers, various testing methods are being explored. However, there are currently neither *in vivo* nor *in silico* or *in vitro* assays available that are universally accepted and validated. With regard to NAMs, it is proposed that a single test will not be sufficient for a comprehensive assessment, but a combination of suitable assays could be used in an integrated testing strategy (Jowsey et al., 2006; Üzmezoğlu, 2021), similar to skin sensitization⁸. The general requirements for the tests are that they must be standardized, and the definition criteria for positive and negative results must be compared with data from animal studies and/or clinical experience. Most of the *in vitro* and *in vivo* tests described below were developed for skin sensitization, however, their relevance to correctly identify respiratory sensitizers remains to be established.

7.1 Computational methods

In recent years, there have been numerous approaches to computer-assisted prediction of the respiratory sensitization potential of chemicals, based on a wide variety of models, some of which are introduced here.

Structure-activity relationship (SAR) analysis is a powerful technique for the prediction of biological properties, including toxicity, of compounds based on their chemical structure. In 2014, Dik et al. evaluated the performance of different SAR models that aimed to predict the respiratory sensitization potential, including those developed by Graham and others using MultiCASE software (Graham et al., 1997), by Cunningham and others using cat-SAR (Cunningham et al., 2005), and by Jarvis et al. (2005) using a logistic regression model. For this, a merged airway

dataset was used, which combined data from Derek Nexus (Lhasa Ltd, 2014) and a set of alerts introduced in Enoch et al. (2012) (Dik et al., 2014). The predictivity of the available SAR models for the substances was found to be lower than their published predictive performance, indicating that no single SAR model was sufficiently reliable to draw conclusions about the potential respiratory sensitization properties of a substance. Therefore, it was concluded that the combination with additional computational, *in chimico* or *in vitro* methods is necessary to increase confidence. The profiler developed by Enoch et al. (2014) builds on the understanding of MIEs that lead to organ-level toxicity and aims to predict the respiratory toxicity of LMW chemicals as well as to compare chemical categories. The profiler was developed based on an analysis of 104 chemicals and has been accepted for inclusion in the OECD QSAR Toolbox (Dimitrov et al., 2016).

Golden et al. (2021) evaluated the structural alert model Toxtree, which was originally designed for skin sensitization, and the logistic regression model for occupational asthma from the Centre for Occupational and Environmental Health (COEH). A combined list of recognized respiratory sensitizers (Lalko et al., 2012), a screening-level dataset (Hazardous Substances Data Base; HSDB), and four highly curated chemical respiratory sensitizer datasets (Graham et al., 1997; Jarvis et al., 2005; Enoch et al., 2012; Seed et al., 2015), were included in the analysis. Toxtree had an accuracy of 71% for respiratory sensitization, while the COEH model achieved an accuracy of 76% (Golden et al., 2021).

Recent advancements in machine learning have opened up new possibilities for predicting respiratory sensitizers. Zhang et al. (2018) developed a predictive model for respiratory toxicity using a naive Bayesian classifier based on a dataset of 1241 compounds composed of the Pneumatox database and the dataset from Dik et al. (2014). This dataset was randomly divided between the external dataset (20% of the database) and the training dataset (80% of the database) (Zhang et al., 2018). To ensure the highest prediction accuracy, extended connectivity fingerprints were employed to analyze the structural characteristics of toxic and non-toxic compounds. The model achieved an accuracy of 84.3% when tested on an external dataset (Zhang et al., 2018). Wang et al. (2021) trained six different binary classifiers using various machine learning techniques on a dataset of 2529 chemicals (the Pneumatox database, the Adverse Drug Reaction Classification System (ADReCS) database and the Hazardous Chemicals Information System and compounds mentioned in relevant literature). Among the six techniques employed, support vector machine (SVM) and random forest (RF) performed the best, as indicated by the prediction results of the models.

Voutchkova-Kostal et al. (2022) developed and validated a predictive model for respiratory sensitizers based on mechanistic knowledge within the Computer-Aided Discovery and REdesign (CADRE) platform. They used a dataset of 245 compounds classified as respiratory sensitizers or non-sensitizers from peer-reviewed literature. The model employed a tiered approach, incorporating mechanistic alerts for dermal sensitization and newly developed rules for respiratory sensitizers. In the third tier, a quantum mechanics approach was utilized, considering steric factors and parameters derived from frontier molecular orbitals. The model demonstrated high accuracy, specificity, and sensitivity, with the global quadratic discriminant analysis (QDA) model achieving 93%

⁸ <https://www.ecetoc.org/wp-content/uploads/2014/08/ECETOC-TR-077.pdf>.

accuracy and the domain-specific model performing even better at 95% accuracy.

Following the detailed background on computational models for predicting respiratory sensitization, it is crucial to recognize certain limitations in the data sets used for these models. A primary concern is the relatively small number of chemicals conclusively identified as respiratory sensitizers, which poses a challenge in developing robust and accurate prediction models. To compensate, many studies have expanded their data sets to include chemicals with varying degrees of evidence of respiratory sensitization, often encompassing a wider range of respiratory toxicants. This approach, while necessary to increase the size of the dataset for model development, especially in advanced machine learning applications, can lead to ambiguities in the precise categorization of compounds as respiratory sensitizers. Therefore, interpretations of these models must take into account potential limitations arising from both the number and specific labelling of the included compounds.

7.2 (Potential) methods for the KEs of AOP 39

Among the laboratory testing methods that have been explored for their potential use in respiratory sensitization assessment (Table 2) is the DPRA, initially designed to determine the protein-binding potential of chemicals in the context of skin sensitization covering KE1 (MIE). The DPRA measures the reactivity of substances towards synthetic peptides containing lysine or cysteine, allowing for their classification into different reactivity classes and identification as sensitizers. The reactivity of a few respiratory sensitizers was tested using this method with positive responses, suggesting that the DPRA can support their identification (Gerberick et al., 2004). As a refinement of the DPRA, the peroxidase peptide reactivity assay (PPRA) has been developed to better discriminate between skin and respiratory sensitizers (Troutman et al., 2011). The PPRA incorporates dose-dependency analyses, mass spectrometry for peptide detection, and a horseradish peroxidase and hydrogen peroxide enzyme system to improve the identification of pro-haptens. However, despite these improvements which allow for better characterization of the reactivity of chemical allergens in general, the data suggest that the PPRA does not provide a significant advantage over the DPRA in distinguishing allergens as skin or respiratory sensitizers (Lalko et al., 2013; Reisinger et al., 2015).

The second key event (KE 2) for AOP 39 comprises increased production of cellular danger signals such as inflammatory cytokine, chemokine and cytoprotective gene pathways, and thus shares some similarity with the skin sensitization KE 2 “keratinocyte, activation” as these cells also secrete pro-inflammatory cytokines and induce cytoprotective cellular pathways. For skin sensitizers this can be assessed using keratinocyte activation assays such as KeratinoSens™ and LuSens (Emter et al., 2010; Natsch, 2010; Bauch et al., 2012; Reisinger et al., 2015; Sullivan et al., 2017). Both systems are based on human keratinocyte cell lines carrying a reporter gene construct composed of a luciferase gene under the control of an antioxidant/electrophile response element (ARE), thus addressing the Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor erythroid 2-related factor 2 (Nrf2)/ARE pathway (OECD, 2022). In brief and simplified, an electrophilic modification of the cysteine residues in

Keap1 leads to the release of the transcription factor Nrf-2 which shuttles in the nucleus, binds to the ARE containing promoter sequences thereby activating the transcription of cytoprotective genes. For both cell systems, the cell viability is estimated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Bauch et al., 2012; Reisinger et al., 2015). Of note, some respiratory sensitizers were shown to activate the pathway *in vitro*, but both direct induction by attacking of cysteines in Keap1 and indirect activation by changing the redox environment may be relevant (Sullivan et al., 2017). Yet, this pathway is less explored in respiratory sensitization, also due to the preferential lysine targeting of electrophilic respiratory sensitizers. However, the Nrf-2/ARE pathway is important for mediating pulmonary protection against oxidative stress and is involved in the development of lung diseases including asthma (Cho and Kleeberger, 2010).

A number of different lung cell models are available, from simple 2D submerged cultures of different lung epithelial cell lines to 3D models that are suggested to provide more robust, comprehensive responses as they better mimic the *in vivo* situation, in particular when cultivated at the air-liquid interface (ALI) (Lacroix et al., 2018). Such models were used to predict the respiratory toxicity of inhaled drugs based on measurements of viability, barrier integrity, ciliary movement and cytokine release (Mizoguchi et al., 2017; Balogh Sivars et al., 2018). 3D models of the human airway epithelium were also applied to investigate the impact of respiratory sensitizer exposure on cell viability and barrier integrity (Sullivan et al., 2017; Thá et al., 2021). Moreover, sophisticated coculture models composed of lung epithelial cells, macrophages, fibroblasts, dendritic cells are in use, and initial results in respiratory sensitizer analysis are promising (Chary et al., 2019; Mizoguchi et al., 2023).

ALIsens[®] is a 3D tetraculture model representing the alveolar barrier, which is constructed by a combination of four human cell lines (epithelial cells, macrophages, DCs and endothelial cells) grown at the ALI in hanging cell culture inserts. Following exposure to the test materials, a panel of cell surface markers, chemokines and inflammatory markers and expression of a set of relevant genes are measured allowing the differentiation between respiratory sensitizers and local irritants. The model has been shown to differentiate known sensitizers from irritants and identified correctly the proteins tested so far (Chary et al., 2019).

The ImmuLUNG™ cell model is representative of the alveolar region consisting of alveolar epithelial cells and alveolar-like macrophages (Hutter et al., 2023). The model can be utilized for the detection of irritation and sensitization markers (cell surface markers, cytokines and chemokines). An internal trial conducted with a panel of test items showed promising results for sensitization assessments.

KE 3 requires the investigation of DC activation. Several human myeloid cell lines (e.g., U937, THP-1, MUTZ-3, and KG-1) have been used to gain mechanistic insights and develop predictive assays (Sullivan et al., 2017).

The human cell line activation test (h-CLAT), which is considered one of the most advanced maturation tests for DCs, is included in OECD Guideline 442E (North et al., 2016). Using the THP-1 cell line, the h-CLAT assay measures CD86 and CD54 expression by flow cytometry (Sakaguchi et al., 2006). For

skin sensitizers, the h-CLAT showed a good concordance with the local lymph node assay (LLNA), which provides a measure for lymph node proliferation and will be described later as assay relevant for KE 4 (Ashikaga et al., 2010). It was therefore assumed that the h-CLAT is a promising screening tool to assess the potential for respiratory sensitization (North et al., 2016). Recently, a modified h-CLAT assay system was described based on a coculture of THP-1 cells with bronchial epithelial cells that aims to distinguish respiratory from skin sensitizers by measuring expressions of surface markers (CD54, CD86, OX40L) and concentrations of cytokines (IL-8, IL-33 and TSLP) (Tanabe et al., 2023).

GARDTMair, an adaptation of the genomic allergen rapid detection (GARD) test, provides binary predictions for classifying test chemicals as respiratory sensitizers or non-sensitizers. A human dendritic-like cell line was exposed to reference chemicals, genomic biomarkers were measured and expression signatures were established using pattern recognition and machine learning. Expression patterns of test chemicals are compared with those of the known chemicals. A ring trial evaluating this method demonstrated its high specificity and transferability (Forreryd et al., 2015).

An *in vivo* assay method related to KE 4 (“T-cell activation/proliferation”) is the respiratory LLNA, an adaptation of the validated test method for skin sensitization (Dearman et al., 1999; OECD, 2010). In the respiratory LLNA, mice are exposed by inhalation head/nose-only to the test material during three consecutive days, rather than applying the test material onto the skin of mice. In the respiratory LLNA both contact and respiratory allergens tested positive and could be identified by different cytokine profiles, with the exception of formaldehyde and glutaraldehyde (Arts et al., 2008; Th   et al., 2021).

Although according to the current test guideline only proliferation is measured, the LLNA itself would be in fact able to discriminate between skin and respiratory allergens by including an assessment of cytokine levels in the protocol. This is based on the fact that sensitizers induce a divergent immune response, being Th1 vs Th2 for skin and respiratory sensitizers, respectively (Dearman et al., 1995; van Och et al., 2002). Although these models were of some promise, they never gained sufficient traction to support regulatory uptake. Nevertheless, the Th1/Th2 concept for skin vs respiratory sensitizers has been a leading concept since then. To investigate the KE comprising T cell proliferation and activation for contact allergens, the human T-cell priming assay (hTCPA) was used, which measures chemical-specific T-cell frequency and antigen-specific IFN-   and TNF-   production (Richter et al., 2013). In brief, chemical-modified/-pulsed peripheral monocyte-derived DCs and na  ve T-cells derived from peripheral blood of healthy donors are co-cultured in the presence of feeder cells, co-stimulatory CD28 antibody, and cytokines. After 10 days, the secondary response by re-stimulation is measured to ensure that antigen-specific T-cells were primed. Cytokine production by T-cells is detected after a rechallenge with chemical-modified/-pulsed DCs (Richter et al., 2013). This assay is laborious and time-consuming and has high donor-to-donor variability in T-cell repertoire of blood donors, which limits reliability and reproducibility, making the standardization difficult (Martin et al., 2010; Richter et al., 2013; Ezendam et al., 2016; Mizoguchi et al., 2023). A further challenge of this assay is the low frequency of hapten-specific na  ve T-cells in the

peripheral blood, the potentially high activation threshold of those T-cells, and the delivery of chemicals to avoid toxicity but induce reactivity (Richter et al., 2013). A negative T-cell response may be caused by the failure of the chemical to react with proteins under *in vitro* conditions or because of the lack of T-cells specific for that chemical (Martin et al., 2010). It was found that the removal of regulatory T-cells can significantly improve assay sensitivity, as Treg cells may limit the extent of T-cell responses (Vocanson et al., 2008).

Other measurement methods for KE 4 may include cytokine expression and Th-cell phenotype analysis of draining lymph nodes to determine the proportions of various Th-cell subsets. In addition, cytokine profiling in the bronchoalveolar lavage fluid of lung and in serum would also be informative. Cytokine production by draining lymph node cells excised from chemical-treated mice can be measured after exposure. The preferential type 2 cytokine profile observed after exposure to respiratory sensitizers was associated exclusively with Th2 cell development (Dearman et al., 2003). A protocol variation is to measure IL-4 production following restimulation of lymph node cells with a mitogen (concanavalin A) *in vitro*, but other cytokines (IL-10, IL-13) can also be measured in the absence of re-stimulation (Dearman et al., 2003).

Furthermore, Th-cell phenotype analysis of draining lymph node cells could also be applied to determine the number of each Th-cell subset after exposure to a potential chemical allergen (Zhang et al., 2021).

Mizoguchi et al. developed an IL-4-based 3D co-culture assay consisting of peripheral na  ve T-cells and DCs stimulated previously by chemicals in a 3D co-culture system (Mizoguchi et al., 2017; Mizoguchi et al., 2023). The 3D co-culture consisted of an airway epithelial cell line (BEAS-2B), monocyte-derived DCs and a lung fibroblast cell line (MRC-5), and the chemical sensitizer was added on top of the epithelial scaffold (Mizoguchi et al., 2017; Mizoguchi et al., 2023). Shortly after the stimulation, the DC scaffold was removed and placed in a new plate, mimicking the migration of DCs to draining lymph nodes. Allogeneic na  ve T-cells were added to the co-culture system and expression of IL-4 was used as a marker of Th2 cell immune response (Mizoguchi et al., 2023). The allogeneic response is generally a polyclonal response and 10% of T-cells are allogeneic T-cells (Mizoguchi et al., 2023). An allogeneic response is stronger than a syngeneic T-cell response, and therefore in this case multiple re-stimulation is not necessary as compared to hTCPA (Mizoguchi et al., 2023). In the DC/T-cell system, respiratory sensitizers, but not skin sensitizers, enhanced expression of IL-4 and the Th2 transcription factor, GATA3 in T-cells (Mizoguchi et al., 2023). The *in vitro* models described by Chary et al. and by Mizoguchi et al. are both promising *in vitro* models to identify respiratory sensitizers, replicating several KEs.

The diagnosis of occupational respiratory allergy in patients as well as the methods used in respiratory epidemiological studies on general population samples are largely based on skin test reactivities (Baldacci et al., 1996) (Table 3). Indeed, atopy is a risk factor for asthma and bronchial hyperresponsiveness. Moreover, several respiratory sensitizers are skin sensitizers as well, which could be an explanation for the usability of the test. However, the extent to which the tests accurately predict allergy against respiratory sensitizers has not yet been systematically recorded.

An example is the formation of IgE and also IgG in chemical induced respiratory allergy. For example, Bernstein et al.

investigated the ability of trimellitic anhydride (TMA) skin testing to identify sensitized workers and found that skin prick testing was positive in 8 out of 11 workers with serum-specific IgE and intradermal testing in a further two (Bernstein et al., 2011). However, it is not possible to detect allergen-specific IgE in all patients with confirmed chemical respiratory allergy (particularly for diisocyanates) (Tee et al., 1998; Wisniewski, 2007; Thá et al., 2021). This is likely one of the reasons why some chemicals are not classified accordingly (Ponder et al., 2022). Allergen-specific IgE is detectable in 3%–39% of isocyanate-induced asthma patients, the lowest for toluene diisocyanate (TDI) (Tee et al., 1998). In the case of OA, a few weeks away from the workplace or exposure to the chemical may result in decreased serum IgE levels to an extent that it may drop below the detection limit. This situation differs from common environmental aeroallergens (e.g., pollens, dust mite), in which exposure is not restricted to a single place, but allergens are present everywhere at the same time. Therefore, a negative isocyanate-specific IgE assay without accurate exposure information may lead to misdiagnosis (Wisniewski, 2007). Interestingly, IgE can be highly elevated in the airway mucosa independently of IgE serum levels, so analysing local IgE elevation in the absence of systemic IgE could be another approach for diagnosis (De Schryver et al., 2015).

Other *ex vivo* cellular/biochemical methods for the detection of an allergic hypersensitivity response (not described in AOP 39) include (1) the measurement of total IgE in serum with ELISA (Baldacci et al., 2001; Rodriguez del Rio et al., 2022; Xie et al., 2022), (2) absolute eosinophil count (AEC) measured in whole blood, nasal secretion and sputum (Baldacci et al., 2001; Kuruvilla et al., 2019b; Rodriguez del Rio et al., 2022), (3) periostin level measured in serum as a biomarker of persistent eosinophilic airway inflammation (Kuruvilla et al., 2019b; Rodriguez del Rio et al., 2022), and (4) the level of various eosinophil-derived cationic cytotoxic proteins (eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) in serum, saliva, bronchoalveolar lavage fluid (BALF), and nasal secretion (Baldacci et al., 2001; Rodriguez del Rio et al., 2022). As an *ex vivo* cell-based assay, the lymphocyte transformation test (LTT) measured in PBMCs or T-cells isolated from blood, could indicate the presence of allergen-reactive memory T-cells (Popple et al., 2016). Moreover, transcriptomics from the bronchial tissue, sputum and blood (Kuruvilla et al., 2019b), and metabolomics measured in exhaled air (Kuruvilla et al., 2019b) could also provide a promising biomarker(s) of asthma.

Diagnosing OA can be challenging, therefore, innovative tests and a combination of multiple readouts are required to improve diagnostic accuracy (Tarlo et al., 2008). Some of the *in vivo* methods mentioned in Table 3 or variations of them have been applied in animal models, too, to assess outcomes relevant for respiratory allergy. Even though the main focus is to avoid animal experimentation as far as possible, these methods need to be discussed, since on the one hand animal experiments can also be carried out in reduced settings, and on the other hand the pathophysiological rationale behind the test methods could provide indications for the establishment of new *in vitro* methods.

The assessment of infiltrating cells, cytokines and chemokines in sputum and BALF may provide further

mechanistic information. For example, recruitment of pro-inflammatory cells can be measured using BALF cellularity (Siddiqui et al., 2008; Yu and Chen, 2018) and deliver quantitative information on the types of infiltrating cells, and cytokine and chemokine levels. Eosinophil count in induced sputum was shown to increase after exposure during the Specific Inhalation Challenge (SIC) test in which patients are exposed to a suspected occupational allergen in a dose-progressive manner in a controlled setting (Üzmezoğlu, 2021). The SIC test is widely used and considered the “gold standard” in the diagnosis of OA (Tee et al., 1998; Beach et al., 2007; Gupta et al., 2019; Pemberton and Kimber, 2021; Üzmezoğlu, 2021). In the case of TDI, some studies reported increased neutrophil count in induced sputum, but the predictive value of the changes in the neutrophil count has not been established yet (Park et al., 1999).

7.3 Methods to assess the newly suggested KE

Methods for the assessment of the newly suggested KEs are available, or could be developed based on tests used currently for research purposes.

Measuring trans-epithelial electrical resistance (TEER) is a method for quantifying the integrity of tight junctions in cell culture models of epithelial and endothelial monolayers (Srinivasan et al., 2015). A high TEER reflects the formation of a tight epithelial barrier, an important aspect of airway epithelial function and can be used to measure epithelial damage due to insults.

For the suggested KE on T-cell polarization and cytokine production the hTCPA could be applied. Another model could be the IL-4 based 3D co-culture assay, which measures Th2 response following chemical stimulation in a co-culture system with airway epithelial cells, monocyte-derived DCs, lung fibroblast cells and naive T cells (Mizoguchi et al., 2023).

The suggested KE on B cells and IgE production could be addressed by measuring allergen-specific serum IgE levels in exposed animals, for which a variety of immunological assays such as ELISA or the highly sensitive ImmunoCAP platform are available (Movérare et al., 2017; van Hage et al., 2017).

Information on the degranulation of mast cells and basophils may be derived from skin prick tests (Baldacci et al., 2001; Gupta et al., 2019) as well as from basophil activation tests, an *ex vivo* test that detects allergic reactions by incubating the patient's blood with suspected allergens and measuring basophil activation, indicated by markers such as CD63 and CD203c, measured using flow cytometry (Vera-Berrios et al., 2019).

The KE on local inflammation and recruitment of inflammatory cells can be addressed by animal experimentation with the patch test (Popple et al., 2016; Gupta et al., 2019), as well as assessment of infiltrating cells, levels and types of cytokines and chemokines in the BALF (Dearman et al., 2003; Siddiqui et al., 2008; Yu and Chen, 2018; Kim et al., 2019) and by using lung histopathology with, e.g., precision cut lung slices (PCLS) that allow the assessment of local inflammation and recruitment of inflammatory cells following exposure to chemicals (Siddiqui et al., 2008; Kim et al., 2019).

8 Conclusion

Respiratory sensitization is a highly topical issue with an urgent need for action, especially in the regulatory field due to its relevance as immune status that triggers occupational disease. In order to achieve this, it is necessary to identify and classify respiratory sensitizing chemicals with the best possible certainty, however this requires a thorough understanding of the underlying chemical and molecular mechanisms, and the existence of valid test systems.

For immune system-mediated skin sensitization and skin irritation there is a clear separation in classification, though skin sensitizers may also induce skin irritations at higher percentages. Different *in vivo* and *in vitro* validated test protocols are available. This is not the case for immune system-mediated respiratory sensitization and for respiratory irritants because of the lack of validated tests. For the CLP classification there is no need to differentiate between these two groups as ‘an immunological mechanism does not need to be demonstrated’.

Protecting workers and the general population is paramount, warranting precautionary measures even for compounds with unknown mechanisms of action. This approach may lead to an over-classification for safety’s sake, prioritizing worker protection over strict mode of action-based classification. This can be understood as a commitment to safety despite incomplete mechanistic understanding, advocating for further investigation into compounds identified as potential risks.

Thus, there are dual aspects at play: regulatory considerations and the necessity for enhanced mechanistic understanding to foster improved protection measures. Ultimately, the aim should be to advance the understanding of mechanisms to refine both classification and regulation.

For this, understanding the physicochemical properties of respiratory sensitizers is essential. In addition to lipophilicity, which determines where most of a substance is deposited in the respiratory tract, electrophilicity is an important property of many, but not all, chemicals that are suspected of having a sensitizing effect on the respiratory tract. Electrophilicity is relevant for the formation of immunogenic haptens, but is not a sufficient distinguishing feature from skin sensitizers. In addition to classical hapten-forming electrophiles, there are non-classical haptens like transition metal complexes that do not fall within the applicability domain of AOP 39. The immune-mediated mechanism of “inert” chemicals that are able to bind to the MHC on basis of their conformation rather than reactivity has also not yet been well explored. Moreover, it is not known to what extent the potential of a substance to penetrate the epithelial layer can contribute to the sensitization process, as compounds can have an indirect effect on immunological reactions by affecting cellular signalling cascades and metabolism.

One major uncertainty related to the respiratory sensitization pathway is the route of exposure through which sensitization of the respiratory tract can be achieved. As mentioned in the introduction, it was demonstrated that beside inhalation exposure, also skin exposure to relevant chemical allergens can effectively sensitize the respiratory tract, supported by animal studies and clinical cases (Tsui et al., 2020). Thus, the type of sensitization and

resulting allergic reactions induced by chemical allergens do not solely depend on the exposure route.

Consequently, theoretically proposed predictive test methods for identifying chemical respiratory sensitizers would not need to be limited to inhalation exposure or interaction with respiratory tract cells. It needs to be investigated if methods based on skin or even epithelial exposure in general could be valid for identifying chemical respiratory allergens. The use of skin reactive methods to identify respiratory allergies in clinical practice would support this conclusion.

Some KE are common for both respiratory and skin sensitization, which may explain that typical respiratory sensitizers such as diisocyanates and acid anhydrides also tested positive in skin sensitization tests. Otherwise, certain contact allergens do not typically lead to respiratory sensitization, and *vice versa*. Some chemical respiratory allergens are rarely or never linked to skin sensitization. The question is to which extent such characteristics can be modelled by NAMs. To answer this, a comparison of the performance of known skin and respiratory sensitizers in the different methods addressing different KEs would be required.

For example, in an ALI model the respiratory sensitizers OPA, TMA, and HDI all induced OX-40L expression, differentiating them from skin sensitizers, but also showed sensitizer-specific upregulation of ST2 (OPA) vs TSLPR and IL-7R (TMA and HDI) (Mizoguchi et al., 2023). Conversely, some chemicals can trigger both types of reactions. As stated in 7.2, the inclusion of cytokine measurements could enable a better discrimination, which is currently not taken into account in the guidelines, as the LLNA, for example, is used exclusively for proliferation.

To distinguish between contact and respiratory allergens, it is crucial to investigate potential chemical distinctions. Conducting a thorough comparison of the structural attributes of contact and respiratory sensitizers is essential for this purpose. Different classes of chemicals will generate different types of haptens, probably inducing also distinct mechanisms. Moreover, the direct penetration of the epithelial membrane by compounds and their potential to induce intracellular changes of the redox environment may be relevant for the activation of immunological and danger signals in a hapten-independent manner. Among the herein newly suggested KEs for AOP 39 is the assessment of barrier integrity as reduced barrier function is a hallmark of asthma, and has been convincingly shown for some respiratory sensitizers such as TDI. However, whether this is a common effect of respiratory sensitizer exposure awaits further study.

The next question that needs to be answered is whether the methods mentioned have the potential to cover the newly suggested KEs. As many of these methods currently require animal experiments, innovative approaches will need to be developed that further reduce or replace animal testing. Furthermore, although AOP 39 states that there is high evidence for the taxonomic applicability in mouse and human, this cannot be extrapolated to the new KEs without proof, as there are considerable differences in the immune system of the lung. The question is whether sufficient mechanistic understanding is already available to cover key mechanisms with *in vitro* or *in silico* methods. A particular challenge is that for immunological responses, intercellular communication is of great importance, whereby not

only different immune cells interact but also the surrounding tissue. Further research is needed to better understand the basic aspects of intercellular communication in the response to respiratory sensitizers.

A major constraint in the development of further methods is the uncertainty concerning the immunological mechanisms. For example, there is no consensus whether respiratory hypersensitivity depends on IgE-mediated mechanisms, since in some cases of chemical-induced asthma (e.g., diisocyanates) only a minority of patients display detectable IgE (Tee et al., 1998; Wisniewski, 2007). Moreover, the identification of respiratory sensitization as cause of disease in clinical practice is a major challenge. Less frequently occurring clinical symptoms are currently not taken into account in the regulatory classification of respiratory sensitizers. This could also be one of the reasons why there is only a partial overlap in our comparison of the respiratory sensitizers mentioned in the literature with the current official classification. Thus, there may be more substances that do not meet all criteria to be classified according current legislation or for which data is lacking, or for which there is inconsistent data, or the classification process is still ongoing. Moreover, there is no measure for the potency of a respiratory sensitizer, as exposure is often based on statistical coincidence only. It is unknown to what extent individual susceptibility plays a role. This not only points to the difficulty of identifying the substances themselves, but also makes it difficult to estimate the real problem size of chemical respiratory sensitization.

Knowledge of the effects of exposure to respiratory sensitizers on human health is still limited, both in terms of occupational exposure and, to an even greater extent exposure in daily life. It is even more difficult to determine causalities, especially in the case of mixed toxicities. For instance, chemical and building-related intolerance are associated with chemical exposure and inflammatory airway disorders including asthma (Anderson and Anderson, 1999; Claeson et al., 2018), as well as multiple chemical sensitivity (MCS), an adverse multisystem response to common chemicals at low doses considered non-toxic for the general population. Due to the difficulty in diagnosis such disorders are often approached from the psychiatry-psychosomatic side and not from toxicology (Hill et al., 2010; Rossi and Pitidis, 2018; Hempel et al., 2023). MCS patients may represent a group of individuals being particularly vulnerable to exposures. The role of respiratory sensitizers in the etiology of such diseases remains to be investigated.

The factors that affect the susceptibility to development and/or the clinical presentation of respiratory hypersensitivity are largely unknown but it can be assumed that factors relevant for asthma in general are important, such as genetic polymorphisms, the individual immune status and microbiome, respiratory virus infections, age (immunosenescence), air pollution and smoking, presence of indoor allergens, co-medication e.g., betablockers that may cause bronchoconstriction, and co-morbidities such as obesity (Kuruvilla et al., 2019b). Sex differences need to be considered too, as, e.g., it is known for severe asthma that there is a shift from male to female predominance after adolescence (Zein and Erzurum, 2015). In addition, psychosocial factors such as stress can affect lung development, neuroendocrine, autonomic and immune responses and thus increase reactivity to allergens (Rosenberg et al., 2014).

To summarize, we suggest to integrate the newly suggested KES in AOP 39 to get a more complete picture of the impact of test chemicals on the different steps involved in respiratory sensitization. Yet, this AOP alone may not be sufficient for all respiratory

sensitizers as it is limited by the MIE to electrophilic chemicals that form haptens, excluding, e.g., transition metals. In addition, a strategy is needed to differentiate between lung and skin sensitizers. Thus, further research is needed to decipher the relevant mechanisms for respiratory sensitization in more detail. The concentration-dependency of responses needs to be considered as for some chemicals lower concentrations lead to sensitization while at higher concentrations irritation prevails. Thus, also the exposure time and metabolic properties of the cell systems is of importance. Finally, the awareness of respiratory sensitization as a cause for disorders also needs to increase. Further translational and clinical research is needed as diagnosis of respiratory sensitizer-triggered diseases such as OA is still a major challenge.

Author contributions

RH: Conceptualization, Methodology, Writing–original draft, Writing–review and editing. LP: Conceptualization, Methodology, Writing–original draft, Writing–review and editing. TS: Conceptualization, Methodology, Writing–original draft, Writing–review and editing. PM-L: Methodology, Visualization, Writing–original draft, Writing–review and editing. VG: Writing–review and editing. KA: Methodology, Writing–review and editing. FJ: Methodology, Writing–review and editing. YS: Writing–review and editing. SB: Writing–original draft, Writing–review and editing. AC: Writing–original draft, Writing–review and editing. AG: Writing–original draft, Writing–review and editing. KL: Conceptualization, Writing–review and editing. RV: Conceptualization, Methodology, Writing–original draft, Writing–review and editing. JG: Conceptualization, Methodology, Visualization, Writing–original draft, Writing–review and editing.

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Conflict of interest

JG, RV, AG, and AC are National Experts contributing to the OECD Detailed Review Paper for respiratory sensitization. JG and RV were guest editors of *Frontiers in Toxicology*, at the time of submission. JG is co-inventor of EP3527653 and received funding

from Egger GmbH, at the time the manuscript was composed. This had no impact on the content of the manuscript. AG and AC are co-inventors of WO 2018/122219 A1. AG is the founder of INVITROLIZE and SB is an employee of the same company.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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