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Advancing pesticide risk assessment: the role of adverse outcome pathways and new approach methodologies – what's next?

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Abstract

Introduction. In recent years, the scientific community, together with regulatory authorities, institutions and stakeholders, joined in the common objective of changing the regulatory toxicology paradigm, moving from classical toxicological animal testing towards an exposure-driven risk assessment, based on mechanistic information derived from alternative methods, globally known as new approach methodologies (NAMs). The transition to the next generation risk assessment (NGRA) requires the collective effort of all involved actors. One approach addressing the needs of the NGRA is the adverse outcome pathway (AOP) concept. This construct describes a sequence of temporally and causally linked events at distinct levels of biological organization leading to an adverse health effect. The development, validation and implementation of AOPs at the regulatory level have become key objectives of several authorities.

Methods. In this paper, the AOPs and AOP networks as applied by the European Food Safety Agency are analyzed, alongside the proposed batteries of NAMs.

Results and discussion. Two case studies, (1) Parkinson's disease and pesticide exposure, and (2) exposure to substances with endocrine-disrupting properties and the development of uterine adenocarcinoma, are described. The analysis of the entire AOP construct for each case and the assembly of the specific NAMs allowed us to identify the strengths and limitations of the AOP strategy, as well as of each proposed test. Their integration and status are assessed within the current regulatory framework.

Key words

- next generation risk assessment (NGRA)
- new approach methodologies (NAMs)
- adverse outcome pathways (AOPs)
- pesticides
- regulatory toxicology
- chemical risk assessment

INTRODUCTION

Risk assessment (RA) is a rigorous, structured, and iterative process designed to address key questions regarding exposure to one or more chemical, physical or biological agents, and the associated potential risk for human, animal and environmental health. As a specialized discipline of applied science, RA involves the analysis and review of scientific information, whenever available and aligned with the regulatory framework, and/or new or existing data to evaluate the likelihood of adverse events occurring in organisms, following the exposure to these agents. The process considers the hazardous properties of the chemicals and quantifies risk, considering both hazard and exposure. If necessary, mitigation measures should be suggested and then implemented to reduce the potential risks, as part of risk management actions [1, 2].

Both scientific and technological progress, coupled with increased awareness among governments, regulatory bodies, academia and industries, have represented a great driving force for the knowledge and clarification of toxicological profiles, or "fingerprinting", of substances. This applies both to substances requiring approval before entering the market and to those already present in the environment (pollutants and contaminants) [3]. The "One Health" approach, a holistic perspective integrating the health of humans, animals, and the environment as a *unicum*, has also contributed to directing the RA towards a shared, multi-sectoral and multidisciplinary collaborative vision [4]. Similarly, the EC initiative "One Substance, One Assessment" was proposed to strengthen the chemicals strategy for sustainability (CSS), aiming to ensure coherent and transparent safety assessments of chemicals with diverse uses across various regulatory frameworks, and to build a comprehensive body of knowledge on chemicals [5]. It is im-

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portant to note, however, that the "One Substance, One Assessment" approach is most readily applicable to hazard characterization. In contrast, the broader RA process must also account for exposure, which can vary significantly, depending on how a substance is used across different sectors. For example, if the same chemical is used as a flavoring agent in candy or as an ingredient in a bath gel, the exposure scenarios, such as frequency, duration, route of exposure, and consequently the kinetics and internal dose, can differ substantially.

Historically, the classical RA process, particularly in the hazard identification and characterization phases, had extensively relied on animal testing to derive dose descriptors and, subsequently, reference values. However, the growing demand for more sustainable, ethical and species-relevant methods (i.e., those tailored for humans) has driven the scientific community to investigate, develop and implement "alternative" approaches, internationally known as new approach methodologies (NAMs). They include a range of innovative strategies, including *in vitro, in silico, in chemico, ex vivo* and *non-testing methods* or their combinations, designed to improve accuracy and relevance for human health assessments while reducing or replacing animal use (*Table 1*) [6, 7].

In addition to ethical issues, limitations of animal models, including interspecies physiological differences, varying toxicokinetic profiles and lack of interindividual susceptibility, often hinder the extrapolation of data to humans [8]. NAMs aim to address these challenges, increasingly designating animal testing as a "last resort", considering the principle of equivalent protection, meaning that the same risk assessment conclusions and risk management decisions would be reached by using NAMs instead of animals. Initially, they were developed to accelerate the screening and evaluation of the thousands of substances currently in use or under development. At the same time, their predictability, accuracy and reliability have been widely recognized [9]. The effort to develop, validate and make internationally accepted NAMs has been shared globally and reflected in initiatives starting from the Directive 2010/63/EU on animal experimentation, to EU CSS [5] and some specific EU regulations adopted for regulated chemicals and/or products before their marketing: examples include Regulation (EU) n. 283/2013, which requires that active substances used in plant protection products undergo a comparative *in vitro* metabolism study on cells derived from various species, including humans. These studies are pivotal for determining the relevance of toxicological *in vivo* data and for guiding the interpretation of findings or defining the testing strategy. Similarly, Regulation (EU) n. 1223/2009 prohibits the marketing of cosmetic products if either the ingredients or the final formulation has been subjected to animal testing.

In parallel, the European Chemicals Agency (ECHA) published on the use of NAMs within REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) and CLP (Classification, Labelling and Packaging) regulations almost 10 years ago, then set guidance updates aimed at improving the use of *in vitro*, *in silico* and read-across approaches [10], while the European Food Safety Authority (EFSA) has developed a Roadmap for NAM integration in RA [8, 11], reinforcing the convergence toward a mechanism-based, exposure-driven assessment framework. Alongside EU initiatives, similar trends toward NAM-based regulatory toxicology are underway internationally: the US-EPA is advancing the transition toward non-animal, mechanistic RA through programs such as ToxCast/Tox21 and its NAMs Work Plan [12], while the US-FDA Modernization Act 2.0 [13] formally recognizes NAMs as acceptable alternatives to animal testing in regulatory submissions.

For simple endpoints (e.g., skin and eye irritation), some stand-alone methods have already been internationally adopted, such as those included in the Organization for Economic Co-operation and Development (OECD) Test Guidelines (TGs). These methods are accepted within regulatory frameworks and widely applied, for example, in the assessment of genotoxic potential or skin sensitization. However, for more complex toxicological endpoints (e.g., systemic effects after short or long-term repeated exposure), a single test might not be sufficient on its own to provide the necessary information. For this reason, the modern approach to applying NAMs is to use integrated testing strategies rather than a single alternative test. The integrated strategies combine various methodologies, including evidence-based and non-testing approaches, to create a comprehensive path from exposure to outcome [14, 15]. In this context, the new challenge of toxicology and in particular, regulatory toxicology is "to assemble individual NAMs into a comprehensive next-generation risk assessment (NGRA) strategy" [16], as a human-relevant, exposure-led, hypothesis-driven risk assessment designed to prevent harm. Furthermore, by integrating NAMs with advanced technologies and big data, RA becomes more precise, informative, and problem-oriented, capable of addressing gaps and uncertainties [11, 17-19]. A good example is the general NGRA workflow described by the EU Scientific Committee on Consumer Safety (SCCS) for the evaluation of cosmetic ingredients [20].

To this aim, the construct of the adverse outcome pathway (AOP) has been recognized as one of the fundamental tools to this transition. An AOP maps and describes a sequence of toxicodynamic events temporally and causally linked to different levels of biological organization (i.e., molecular, cellular, tissue, organ and organism), resulting from the exposure to a stressor (e.g., chemicals, physical stress, etc.) and causes a final adverse event in human health and/or the environment [21, 22]. Several biological, genetic, physical and chemical insults contributed to the onset of the disease; however, a deeper understanding of disease mechanisms and early and key events is an essential goal shared among toxicological and regulatory areas.

The sequence of events defined by the AOP begins with a molecular initiating event (MIE), which describes the interaction between a stressor and its molecular target (e.g., receptors, proteins, enzymes, DNA). This interaction triggers a sequential chain of intermediate events, called key events (KEs), which define certain measurable biological/physiological changes that can

Table 1
Classification and characteristics of new approach methodologies (NAMs)

Models	Model/Method	Main characteristics
In vitro	Continuous cell lines	Cultured cells from a single clone, derived from tumors, pluripotent cells, or cells taken from an organism and immortalized, grown under controlled conditions
	Microorganisms	Microbial cultures grown in predetermined culture medium under controlled laboratory conditions
	Primary cells	Cells taken directly from living tissue (e.g., biopsy material) and established for growth *in vitro*
	Spheroids	Spherical cellular units that are generally cultured as free-floating aggregates
	Organoids	Self-organized cells grown in 3D using scaffolds to mimic the extracellular matrix, typically derived from stem cells (pluripotent, fetal or adult). They form structural units that resemble the organ in structure, biology and function
	Reconstructed human tissues (e.g., epidermis)	Tissue model differentiates and stratifies similar to native human tissue (3D)
	Co-cultures	Different types of cells (2D and/or 3D) grown together under controlled conditions
	Organ-on-a-chip/micro-physiological systems	Multi-channel 3D microfluidic cell culture, integrated circuit (chip) that simulates the activities, mechanics and physiological response of an entire organ or an organ system
Ex vivo	Tissues or organs	"Outside of a living body", the living tissues or entire organs are directly taken from a living organism and studied in a limited time with no or minimal alterations
In chemico	Purified targets and biochemical assays	Enzymes, receptors, proteins for evaluation of reactivity, binding, changing in conformation after exposure to an agent; they are used to detect, quantify and/or study the binding or activity of biological molecules
	Isothermal titration calorimetry	A label-free quantification method used for the evaluation of a wide variety of biomolecular interactions
In silico and non-testing methods	Extensive literature search, systematic review and metanalysis	Useful tools for evaluating the status of research on a given topic and identifying data gaps and research needs
	Read across	Relevant information from one or more compound(s) is used to predict the properties of a target compound with similar chemical/ physical / toxicological properties (e.g., genotoxicity)
	Grouping	Evaluating more than one substance at the same time such as structural analogues and/or entire classes of chemicals
	(Q)SAR	Mathematical models providing a correlation between compounds chemical structure and a property or activity (qualitative or quantitative)
	TTCs	The establishment of a generic exposure level for a class of chemicals below which there would be no appreciable risk to human health.
	PBK models	Mathematical modelling technique for predicting the ADME of synthetic or natural chemical substances
	Weight of evidence	A process in which evidence is integrated to determine the relative support for possible answers to a scientific question.
	(Q)IVIVE	*In vitro* to *in vivo* extrapolation: the qualitative or quantitative transposition of experimental results or observations made *in vitro* to predict phenomena *in vivo*
	Data waiving	Avoiding performing test if: - not scientifically necessary - not technically feasible - relevant data already exist
	Data sharing	Existing studies involving or not vertebrate animals conducted can be shared for use to avoid unnecessary animal testing or reduce costs
High-content methods, others	HTS	The use of automated equipment to rapidly test thousands to millions of samples for biological activity at organism, cellular, pathway, or molecular level
	Advanced bioimaging and bioscanning	Methods that non-invasively visualize biological processes in real time
	Omics (genomics, transcriptomics, proteomics, or metabolomics, etc.)	Genomics can facilitate analysis of entire or component genome sequences of an organism. Transcriptomics and proteomics provide significant bodies of information on temporal and spatial expression of genes and gene products, respectively, whilst metabolomics captures data for a large pool of metabolites

ADME: absorption, distribution, metabolism and excretion; HTS: high-throughput screening; PBK: physiologically based kinetic; (Q)IVIVE: quantitative *in vitro* to *in vivo* extrapolation; (Q)SAR: quantitative structure-activity relationship; TTCs: thresholds of toxicological concern.

occur if the perturbation is sufficiently relevant in terms of power, duration, and frequency to exceed the threshold of adversity. These KEs are linked by a temporal, causal, empirical relationship and biological plausibility (key event relationship – KER). The resulting AOP framework provides a clear, evidence-based progression from the MIE to the observed apical event or adverse outcome (AO) (*Figure 1*) [23, 24].

While the primary goal of developing an AOP is to systematically identify and organize existing knowledge in a structured manner, this tool may also allow for identifying current knowledge gaps that, when addressed, may further enhance its predictivity. AOPs are also considered "knowledge bridge", reflecting their role in connecting mechanistic data with AOs. The following key characteristics underscore the stepwise development of AOPs and their utility in RA:

- stepwise organization of events: sequentially link alterations at the molecular level to broader biological changes at the organelle, tissue, and organism level;
- mechanistic integration: incorporate mechanistic information into a defined, causal and plausible construct of KEs leading to the AO, allowing the appropriate use of specific NAMs;
- relevance assessment: support the evaluation of the relevance of experimental toxicological and/or epidemiological data;
- uncertainty definition: define and identify the uncertainties related to the available data;
- data gap identification: identify any missing data points easily and immediately, aiding the refinement of pathways and methodologies;
- research and regulatory prioritization: address research needs and establish criteria for prioritizing regulatory/decision-making actions [25-27].

The implementation at the regulatory level of the AOPs is among the main objectives of various European and international bodies (i.e., ECHA, EMA, EEA, JRC-EURL ECVAM, OECD, WHO, FAO, US-EPA, FDA). Specific initiatives developed within these institutions have been launched to support the adoption and refinement of AOPs, among which:

- EU PARERE Network – Preliminary Assessment of Regulatory Relevance Network – a platform for assessing the regulatory relevance of new tools and approaches (https://shorturl.at/dswAR);
- PARC – Partnership for the Assessment of Risks from Chemicals – a collaborative EU initiative to improve RA methodologies and promote innovation in the chemicals RA sector (https://www.eu-parc.eu/);
- AOP-Wiki a collaborative online platform for organizing, sharing, and refining AOP-related data and information (https://aopwiki.org) [2, 7, 10, 11].

The AOP framework might be used as a starting point not only to organize the available data and information, but also to identify knowledge gaps, evaluate uncertainties, shape future research lines and regulatory and decision-making prioritization criteria.

While the scientific foundations of AOPs have also been recently reviewed in literature, as well as AOPs related to specific endpoints or chemicals [28], the present work provides a systematic and original analysis on how AOPs have been implemented within the EFSA regulatory framework. In reconstructing the NAM-based event flows, this paper identifies practical limitations in regulatory acceptance, and highlights opportunities for improvement, e.g., by strengthening toxicokinetic data integration, empirical support, and cross-AOP consistency. This regulatory-focused perspective may offer a novel viewpoint in RA and regulatory decision-making.

METHODOLOGY

This study, covering the period from 2010 (when the AOP concept was introduced) to July 2024, follows a systematic approach to collect, evaluate, and analyze existing data. The principles of the PROMETHEUS

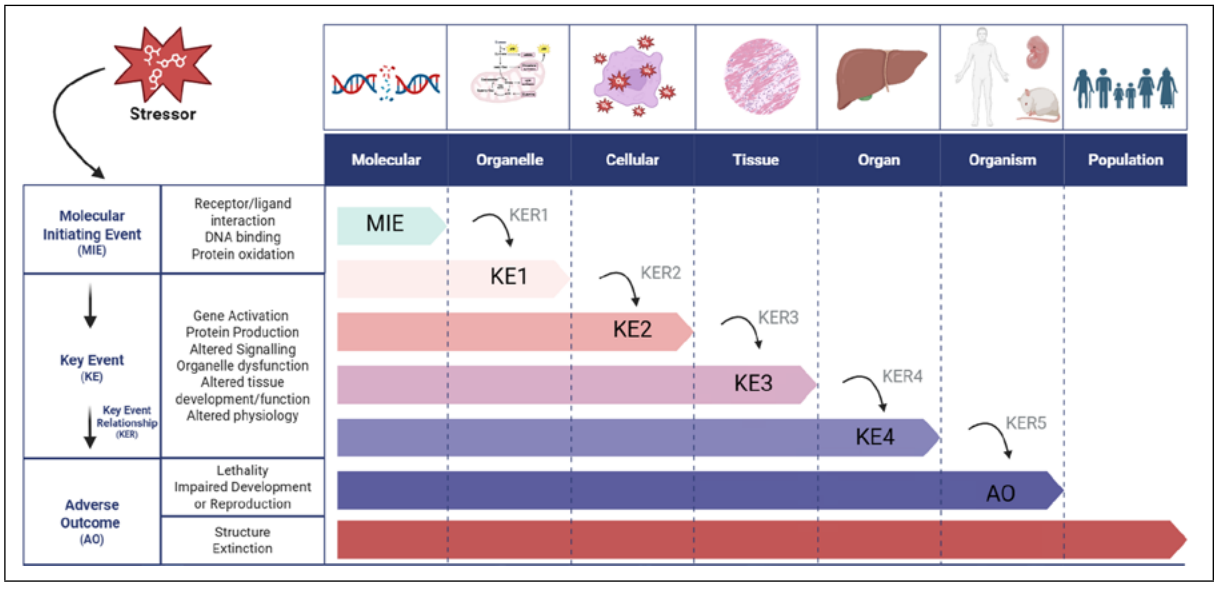

Figure 1
Summary of the adverse outcome pathway construct and levels of biological organization.

(Promoting methods for evidence use in scientific assessments) initiative, developed from EFSA, and PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) 2020 guidelines were used for this purpose [29-31]. In addition, a structured literature review using extensive literature search (ELS) methodologies was performed through three different databases, Web of Science, PubMed, and EFSA Search. The keywords were derived from the EFSA glossary and MeSH vocabulary (Medical Subject Headings). The generated search strings are reported in *Table S1 available online*.

The results were organized using EndNote Web and Rayyan software, enabling both manual and automated screening. Machine Learning (ML) tools were applied to streamline the organization of results, identify duplicates, and highlight relevant studies [32]. Studies meeting the inclusion criteria (summarized in *Table S2 available online*) underwent further analysis, while irrelevant studies were excluded.

To complement the literature review, the AOP-Wiki database was accessed to analyze and verify the status and applicability of the AOPs included in this study.

RESULTS

Figure 2 summarizes the results of the ELS, integrating systematic evidence collection with AOP evaluation to address the study objectives. Currently in the EFSA context, the use of AOPs/AOP networks is proposed for the assessment of toxicological properties linked to i) the development of Parkinson's disease (PD) and childhood leukemia (CHL) of some active substances used in plant protection products (PPPs) [33-36], ii) development of uterine adenocarcinoma by substances having endocrine disrupting properties [37, 38], iii) endocrine disrupting properties of perfluorooctanesulfonic acids (PFOS) (pollutants, excluded from this analysis) [39].

For this study, two AOPs were selected for in-depth analysis: (case 1) "mitochondrial dysfunction and neurotoxicity", relevant to PD, and (case 2) "Increased estradiol (E2) activating the ERα estrogen receptor", associated with endocrine disruption and uterine adenocarcinoma. These AOPs were chosen based on their direct relevance to PPP RA, robust scientific basis, and application within EFSA regulatory evaluations. While

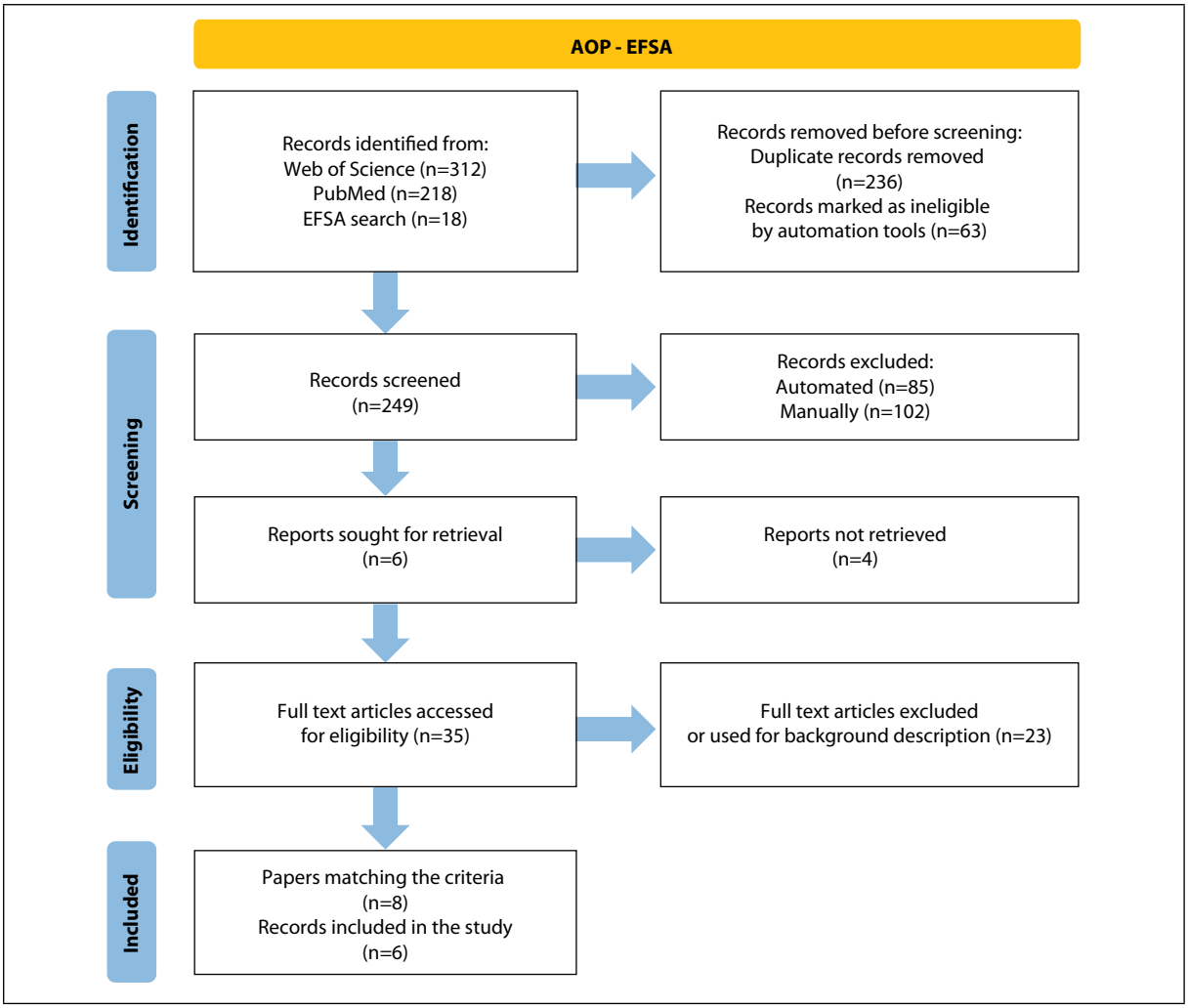

Figure 2
Preferred reporting items for systematic reviews and meta-analyses (PRISMA) flow diagram for the extensive literature search of adverse outcome pathways (AOPs) used in the European Food Safety Authority (EFSA) regulatory context.

additional AOPs were identified, including those linked to childhood leukemia and environmental pollutants (e.g., PFOS), they were excluded due to limited applicability to pesticide regulation. The selected AOPs represent distinct toxicological pathways, neurological and endocrine disruption, providing a focused yet comprehensive evaluation of the AOP framework in regulatory decision-making. The supporting publications and documents related to these AOPs were also analyzed [33-39].

In analyzing the AOPs, we considered evidence from both animal studies and NAMs, as identified through a systematic review. However, our analysis aimed to construct an AOP-based event flow using only NAMs whenever possible, critically evaluating their applicability and limitations. The resulting NAM batteries incorporate all available non-animal studies, except for the AO, where only limited alternatives currently exist.

Case 1: PPPs exposure and Parkinson's disease

In the preparatory stage, EFSA published the results of a literature review on epidemiological data linking exposure to active substances in PPPs to the occurrence of various health effects [33]. Subsequently, the EFSA Panel on Plant Protection Products and their Residues (PPR) was mandated to investigate exposure to PPPs as a risk factor for the development of PD and CHL. To define whether there was a biological plausibility between exposure to PPPs and these two diseases, the EFSA conducted a study, using the AOP construct as the most appropriate investigation tool. After an additional systematic review of the literature, the in-depth literature analysis by using the weight of evidence (WoE) approach was fundamental for the identification of the specific AOs and chemical stressors, as well as for the assessment of related MIEs, KEs and KERs [33-36].

Parkinson's disease – methodologies and AOP "mitochondrial dysfunction and neurotoxicity"

Epidemiological studies and ELS were indispensable tools for formulating the problem and the initial hypotheses. Using a top-down approach, the AO to be examined was identified. Studies on PD with genetic predisposition were excluded from the systematic review, in addition to those involving radiation and cytotoxic chemotherapeutic drugs. The studies related to mechanisms, mode of action (MoA) and KEs involved in PD pathogenesis and their organization levels were selected. Based on this groundwork, four AOPs for PD were formulated: three qualitative AOPs and a putative AOP. Two qualitative AOPs underwent structured evaluation based on OECD principles but are not fully validated, while the putative AOP remains at an early conceptual stage and requires further validation before integration into formal regulatory frameworks.

The AOP, having the inhibition of the mitochondrial complex I (Complex I – CI, NADH-ubiquinone oxidoreductase) of nigro-striatal neurons as MIE, and an AO characterized by Parkinsonian motor deficits, underwent OECD peer review, making it the most advanced among them in terms of regulatory application.

Different classes of pesticides (e.g., organochlorines, pyrethroids, carbamates, antifungals) or single active substances were analyzed in the ELS. Among these, three specific compounds were selected as prototypical neurotoxicants for assessing the MIE, KEs, and KERs: paraquat (a dipyridine insecticide), rotenone (a naturally derived insecticide/acaricide), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, a tetrahydropyridine compound). The three compounds are known to cause neurotoxicity and permanent PD symptoms.

In addition to one MIE, five KEs have been defined: three at the cellular level (Complex I inhibition, mitochondrial dysfunction and altered proteostasis) and two at the organ level (degeneration of dopaminergic neurons of the nigrostriatal pathway and neuronal inflammation). For each step, the NAMs used and/or usable for the assessment were identified, including *in vitro*, *ex vivo* tests, as well as the evaluation of existing data. The battery of tests that can be used for each event is summarized in *Table 2*.

The WoE methodological approach used in the AOP construction followed the modified Bradford-Hill criteria [28]. This approach evaluates: (1) biological plausibility, (2) empirical support in terms of linkage, (3) quantitative understanding of the relationship, (4) taxonomic applicability, life stage and gender-specific effects, (5) uncertainties and inconsistencies. Additional criteria used during this evaluation are i) analogy, i.e., a comparable association between the same outcome and a similar exposure or the same exposure and a similar outcome, and ii) coherence, in terms of concordance between the epidemiological data and the available experimental studies.

The relationships between the individual KER events were very complex and are described in detail by Terron *et al.*: eight KERs were analyzed using the WoE approach guided by the modified Bradford-Hill criteria [35].

The AOP delineated in the EFSA report (*Appendix A*) [32] was uploaded to the AOP-Wiki under the designation "mitochondrial dysfunction and neurotoxicity" (ID: 3). The evaluation process involved an initial peer review by the OECD Working Group of the National Coordinators of the Test Guidelines Programme (WNT) and the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST). Following these reviews, the AOP was endorsed. At this stage, the AOP remains open for consultation, citation, and refinement.

Case 2: AO uterine adenocarcinoma

In the second case study, the EFSA mandate to develop AOPs to be integrated into a network was examined. The AO under consideration was the development of uterine adenocarcinoma in mammals. The aim is the identification of active substances with MoA, characterizing them as endocrine-disrupting chemicals (EDCs) [37, 38].

The adopted methodology was an evidence-based approach, relying on non-testing methods with an initial mapping phase of scientific literature (e.g., ELS, literature mapping), by using a top-down approach. The

Table 2
New approach methodologies (NAMs) battery for the adverse outcome pathway (AOP) "mitochondrial dysfunction and neurotoxicity"

Event	Description	Proposed approach	Test
MIE	Binding of an inhibitor to NADH ubiquinone oxidoreductase (complex I)	*In silico* models	• Molecular docking simulations: based on mimicking the binding of chemicals to the pocket of NADH ubiquinone oxidoreductase
		In vitro/ex vivo	• Quantitative autoradiography
KE1	Inhibition of NADH ubiquinone oxidoreductase (complex I)	*In vitro* models: direct test (performed on homogenates of cells or tissues, and requires at least a partial purification of mitochondria or respiratory chain components)	• Forward electron transfer • Reverse electron transfer • Complex I activity dipstick assay
		Indirect test	• Oxygen consumption, • Intracellular ATP levels • Measurement of NADH/NAD+ ratio in mitochondria by imaging methods
KE2	Mitochondrial dysfunction	*In vitro* models: cells in culture isolated mitochondria	• Cellular oxygen consumption (ADP-to-O ratio) • Mitochondrial membrane potential • Mitochondrial PTP opening • mtDNA damage as a biomarker of mitochondrial dysfunction • ATP content assay
KE3	Impaired proteostasis	*In vitro* models: evaluation of UPS function	• General turnover assay by IHC or WB • Detection of a-synuclein aggregates by imaging, WB, MS or immuno-quantification • Proteasome reactivity assay immunocyto/histochemistry or western blotting
		Evaluation of ALP function	• Monitoring of autophagy-related molecules by mean of fluorescence-tags or quantification of lysosomes or autophagosomes
KE4	Degeneration of dopaminergic neurons of the nigrostriatal pathway	*In vitro* *	• Phenotypic histological marker measurement (TH, DAT, VMAT2) • Degenerating and/or degenerated neurons can be detected by the silver stains and the Fluoro-Jade stains • Cell counting
KE5	Neuroinflammation	*In vitro* **	• Quantification of cellular markers (e.g., pro- and anti-inflammatory cytokine expression, immunostimulatory proteins) • Quantification of released mediators
AO	Parkinsonian motor deficits	*In vitro* *In vivo* *Ex vivo*	• Determination of total contents of dopamine and its two metabolites HVA and DOPAC by HPLC-EC or HPLC-MS • Imaging by PET and SPECT • Detection of dopamine neuron terminals in the striatum • Behavioural tests • Regulatory studies (OECD 407, 408, 422, 424, 426, 443)

MIE: molecular initiating event; KE: key event; AO: adverse outcome; NADH: nicotinamide adenine dinucleotide hydrogen; ATP: adenosine triphosphate; ADP: adenosine diphosphate; PTP: permeability transition pore; UPS: ubiquitin–proteasome system; ALP: autophagy-lysosome pathway; IHC: immune-histochemistry; MS: mass spectrometry; TH: tyrosine hydroxylase; DAT: dopamine transporter; VMAT2: vesicular monoamine transporter type 2; HVA: homovanillic acid; DOPAC: dihydroxyphenylacetic; HPLC: high performance liquid chromatography; ED: electrochemical detector; PET: positron emission tomography; SPECT: single photon emission computed tomography.
*Some studies in *C. elegans*, *Drosophila*, zebrafish and *Lymnaea stagnalis* were also carried out; **some studies in zebrafish were also carried out.

WoE was used throughout the entire process, combined with expert judgment, in a structured and guided manner, i.e., Expert Knowledge Elicitation (EKE) [40]. After identifying the construct formulation questions and defining the scope of the evaluation, four AOPs were postulated with different MIEs and KEs. Through a hierarchical evaluation of the available data (human studies, *in vivo* and *in vitro*), literature mapping allowed the identification of the most plausible MIEs/KEs/KERs. Subsequently, ML techniques, including topic modelling, were employed to perform an automatic screening of the literature for the characterization and evaluation of the biological plausibility of KERs [32].

For the empirical support necessary for the WoE of KERs, two substances known to have estrogenic action, i.e., tamoxifen and estradiol, were selected. The strength of the KERs was assessed considering criteria of biological plausibility, empirical evidence, and essen-

tiality. The latter was evaluated by addressing whether blocking an upstream KE prevents downstream KEs or the AO. Finally, the developed AOPs were re-evaluated and integrated into a network.

According to a top-down approach, the common AO across the network is the development of uterine adenocarcinoma, with a key node, i.e., a meeting point between the various MIEs and/or KEs, identified as increased estradiol (E2) activating the ERα estrogen receptor. The identified MIEs were divided into uterine and extra-uterine molecular events.

Recently, the AOP 503 "Activation of uterine estrogen receptor-α leading to endometrial adenocarcinoma, via epigenetic modulation" has been formally submitted and publicly available on AOP Wiki, providing information on its applicability domain, KEs, and quantitative evidence. In AOP 503, the MIE is the activation of ERα in the uterus (KE 1065). The pathway then proceeds through four downstream KEs: (1) an epigenetic modification process (KE 2152), (2) altered expression of proliferation-related factors (KE 2153), (3) glandular epithelial hyperplasia in the endometrium (KE 772), and (4) genomic instability (KE 1896), which ultimately leads to type I endometrial (uterine) adenocarcinoma (AO 2154). A battery of tests with detailed MIE, KEs and AO was constructed and presented in *Table 3*.

Its life-stage applicability is the adult status; the taxonomic domain includes mammals, especially humans and mice. Its sex applicability is defined as female, consistent with the biology of the uterine endometrium. Empirical support remains rooted in the two agonists, which serve as prototypical stressors of ERα activation.

The evaluation of KERs is ongoing. One of the most important insights from the updated AOP is the role of epigenetic modulation (KE 2152): activation of ERα leads to changes in chromatin structure, such as histone acetylation or DNA hypomethylation, which then affect the expression of proliferation-driving genes (KE 2153). These altered expression patterns promote hyperplasia (KE 772), and over time, genomic instability (KE 1896) increases, setting the stage for malignant transformation into endometrioid adenocarcinoma.

DISCUSSION

General considerations

At a regulatory level, specific provisions govern the RA of certain substances, addressing unique regulatory needs. For complex adverse effects, such as those studied here, no single test – whether animal-based or NAM-based – is enough to fully describe toxicological properties and satisfy regulatory standards. Both the development of PD and estrogen-dependent uterine adenocarcinoma involve complex processes, with their MoA and pathogenesis not yet fully elucidated. Likewise, it remains critical to clarify and confirm whether exposure to certain substances poses a risk for the development of these pathologies. Keeping these complex questions in mind, the EFSA PPR Panel divided them into domains and considered the construction of AOPs as the most effective way for integrating information to better understand adverse events.

The goal of these constructs is to support regulatory applications by identifying those substances that share the same MIE and KE, leading to neurotoxic or endocrine-disrupting properties.

There are many approaches described in the literature for the development of an AOP: starting from the apical effect and, going backwards, building the path towards the MIE (top-down approach), or, on the contrary, starting from the MIE and building the path going towards

Table 3
NAMs battery for the AOP "ERα activation"

Event	Description	Level of biological organization	Proposed approach	Test
MIE	ERα activation	Molecular	*In vitro*	OECD 493 OECD 455
KE1	Epigenetic modulation	Cellular	*In silico* *In vitro* Omics	ToxCast Histone methylation assays DNA methylation assays miRNAs and LncRNA
KE2	Expression of factors ruling proliferation	Cellular	*in vitro* Omics	Luciferase Reporter Gene assay Protein expression (immunohistochemistry, western blot, immunoassay) transcriptome RNA sequencing
KE3	Increased proliferation (hyperplasia)	Tissue	*In vitro*	Cell proliferation assay
KE4	Genomic instability (accumulation of mutations)	Tissue	*In vitro/ex vivo*	FISH Flow cytometry OECD 476 OECD 490 OECD Guidance document 231
AO	Uterine adenocarcinoma (endometrioid adenocarcinoma Type I)	Organ	Human data *In vivo*	Human histological classification OECD 451

NAMs: new approach methodologies; AOP: adverse outcome pathway; MIE: molecular initiating event; KE: key event; AO: adverse outcome; ERα: estrogen receptor-alfa; FISH: fluorescence *in situ* hybridization.

the AO (bottom-up). Other types of approaches by analogy, case study or the middle-out approach can be considered similarly appropriate [27]. Although no single approach dominates, common steps are always involved:

1. hypothesis definition: establishing the purpose of the AOP;
2. KE and KER identification: researching and defining KEs, KERs, and tools (e.g., NAMs) for identifying and quantifying specific events;
3. biological applicability domains: defining "the boundaries" within which each KE operates. As an example, a biological event described by a KEs can be relevant only for one or a few species, gender or age (respectively, taxonomic, gender, or life stage applicability);
4. evaluation and verification: assessing assembled events and refining the AOP iteratively until sufficient confidence and predictability are achieved.

Before this assessment stage, an AOP is defined as putative, and its refinement and integration process are iterative. The confidence, predictability and regulatory fitness for an AOP must be evaluated. During all phases, ranging from development to evaluation of AOPs, what should not be underestimated is the identification and description of associated uncertainties [26].

A report is then prepared as described in the OECD guidelines and reviewed by the WNT and/or EAGMST groups to finalize the AOP endorsement (or rejection). It has been estimated that the average time to progress from the development of a single AOP to its inclusion on the AOP-Wiki, and completion of review and acceptance, exceeds three years [22, 25].

It should be emphasized that, by nature, AOPs are evidence-based constructs grounded in biological plausibility and empirical data. As such, they are living documents that evolve with technological advancements and regulatory requirements. Updates to AOPs happen as new evidence becomes available or when driven by regulatory or decision-making needs. However, as empirical evidence grows, so does the uncertainty surrounding the use of NAMs. In this context, considerable effort should be focused on assessing the relevance of both the NAMs used and the entire AOP, while also accounting for disease complexity from kinetic and dynamic viewpoints.

AOP "mitochondrial dysfunction and neurotoxicity"

In the first case study, the authors undertook the challenging task of capturing the onset of a complex disease (PD) by AOP. This AOP is included in the AOP-Wiki database and has undergone OECD evaluation and review, reaching "endorsed" status. As a result, it is open for regulatory consultation, further refinement, and application in toxicological RA.

Of the 429 AOPs currently present on the platform, only 29, approximately 7%, are in the approved state and only 10% are citable. The AOP inclusion in an internationally recognized database can encourage its use in research and chemical RA. However, the limited number of endorsed AOPs – partly due to the optional nature of the endorsement process – sheds light on the need for greater international collaboration and harmonization in chemical safety science. The applicability domains, supported by strong evidence, are adult (life stage), human and rat (taxonomy) and not sex dependent. According to all criteria, the strength of the link between each phase – MIE, KEs, KERs – and the overall AOP and between MIE and AO was evaluated as "strong" (in a scale foreseeing strong, moderate and weak). Similarly, the biological plausibility of this AOP is overall considered strong. The use of two known CI inhibitors, MPTP and rotenone, supports the WoE for the high relationship between the KEs. Experimental data with these two chemicals reveal significant concordance in dose-response relationships between MIE and AO and among KEs.

Based on the above considerations, this AOP has been considered within the overall neurotoxicity assessment, in compliance with (EC) Regulation n. 283/2013, since it is plausible that any compound that binds to the mitochondrial CI may eventually lead to Parkinsonian motor deficits.

Our analysis focuses on NAM-based tests applied within the AOP framework. This case study may represent a reference case for this aim, due to uncertainties linked to the extrapolation of data obtained with animal testing. Indeed, animal studies analyzed by EFSA PPR were carried out on knockout or mutational models. While these models may provide some information on familial PD pathogenesis, they fail to address mechanisms leading to primary idiopathic PD, which involves complex gene-environment interactions that are not fully replicated in rodents. Moreover, rodents are poorly representative of the extended human lifetime as well as of the human long-term exposure to the potential toxicants. Although rodents and humans share similar brain regions, significant differences exist in terms of size, structural organization, and complexity. Additionally, the kinetic behavior of neurotoxicants may vary significantly between species. Specifically, the prefrontal cortex, critical for higher cognitive functions in humans, is far less developed in rodents.

For these reasons, while the battery of NAMs presented in *Table 2* shows the potential for using simple and well-known tests, mainly *in vitro* or *in chemico*, several data gaps also emerge. Addressing these gaps requires targeted studies, particularly those leveraging NAMs. One example may be the establishment and implementation of mixed neurons/glial co-cultures to meet the data requirements for pesticide approval for neurotoxicity screening. These may play a key role in the development of *ad-hoc in vitro* batteries for neurotoxicity screening.

A promising scenario for advancing neurotoxicity assessment should be the use of human-derived cellular models, such as induced pluripotent stem cells (iPSCs) or brain organoids. These models allow the study of neurotoxicity in a species-specific context and support the development and validation of biomarker signatures for apical events, such as neuroinflammation, through high-content and omics methodologies. For any of the test systems selected, it should be underlined the importance of including a biokinetic study in any *in vitro* tests to understand the real cell exposure, avoiding to correlate any readout to the nominal tested

concentration(s), as also recommended by the OECD GIVIMP Document [41]. This also includes the characterization of the test system, particularly in terms of metabolic competence and transporter activity, crucial to carefully consider the biological relevance of these approaches, to enhance their translational value.

AO uterine adenocarcinoma

Recent updates to the AOP-Wiki confirm that AOP 503: activation of uterine estrogen receptor-α leading to endometrial adenocarcinoma, via epigenetic modulation is now formally deposited. Importantly, ERα activation (KE 1065) is not unique to this AOP but represents a shared MIE or early KE across several AOPs. For example, AOPs 445, 561, 565, and 609 all incorporate ERα activation as a mechanistic entry point for estrogen-mediated biological perturbations. This overlap underscores the centrality of this molecular event within endocrine-related AOP networks and highlights the necessity of evaluating uterine adenocarcinoma not through a single linear pathway, but within the broader context of interconnected AOPs converging on ERα-dependent signaling.

In AOP 503, ERα activation initiates downstream events including epigenetic modifications, altered expression of proliferation-related genes, endometrial epithelial hyperplasia, and genomic instability, ultimately leading to type I endometrial adenocarcinoma. However, because ERα activation also appears in multiple other AOPs, distinguishing tissue-specific downstream cascades (e.g., uterine *vs* mammary *vs* hypothalamic responses) remains a regulatory challenge. The presence of this shared node reinforces the need for harmonized criteria for evaluating ERα-driven mechanisms across AOPs, including standardized methodology for characterizing estrogen responsiveness, quantifying epigenetic alterations, and linking systemic and tissue-specific estradiol levels.

EFSA has identified data gaps that must be addressed before AOP 503 and its related network can be reliably used for regulatory screening of endocrine disruptors. These include the refinement of tissue-level estrogenic assays beyond receptor binding, improved quantitative correlations between circulating and uterine E2 concentrations, and NAMs specifically targeted to estrogen-dependent epigenetic mechanisms. Strengthening these areas will be essential for progressing AOP 503 toward OECD evaluation and potential regulatory adoption.

Limitations about the use of NAMs and AOP in regulatory framework

Introducing NAMs into regulatory frameworks is often hindered by specific legislative requirements. As an example, the CLP classification criteria for systemic toxicity and the corresponding information requirements under REACH refers to OECD TGs, largely based on traditional animal studies. NAM-based strategies especially for systemic toxicity end-point are not considered sufficiently valid and not validated, and are generally used for screening purposes by applicants, but not always included in the technical-scientific regulatory dossiers. It is therefore challenging to adapt NAMs to the current CLP system. Consequently, a new classification scheme is being developed, based on toxicodynamic and toxicokinetic properties obtained through NAM-based testing strategies. Under this scheme, chemicals would be ranked into three levels of concern: high, medium, and low [42].

Toxicological processes leading to an adverse outcome (AO) can be viewed as a continuum of events, starting from exposure to an external dose, progressing to the systemically available dose, and ultimately reaching the biologically effective dose at the target organ, tissue, cell, or receptor. This continuum may be described by MoA, which includes both toxicokinetic (TK) and toxicodynamic (TD), or by the AOP framework, which focuses solely on the TD dimension and is often considered "chemical agnostic". However, this distinction is important because a TK step, such as the metabolic bioactivation of a non-toxic chemical into a toxic metabolite, can itself constitute a KE. It is evident also from the case studies analyzed in this paper that among some limitations of the current AOP-based approach in pesticide RA, the TK information was not integrated into the AOP construct. The majority of KEs are anchored in a TD understanding, whereas critical determinants of internal dose are frequently absent. This limitation, together with the lack of information about *in vitro* biokinetics, restricts the extrapolation of *in vitro* NAM results (e.g., by a Quantitative *in vitro* to *in vivo* extrapolation – QIVIVE-modelling) to real exposure scenarios. Some improvements have been made, using the aggregate exposure pathway (AEP) framework or trying to quantify the KERs e.g., understanding *if* the magnitude of KE_1 is sufficient *then* to trigger KE_2 (the "if and then statement" or the biological plausibility of the relationship). Mathematical models enable the quantitative prediction of KERs using available biological data, facilitating the transition from an AOP to a qAOP [43]. While an AOP can be used to identify hazards, a qAOP can support risk evaluation, provided that an appropriate exposure scenario and complementary information on the chemical's biokinetic properties are available. Quantification is necessary for a more reliable prediction of chemical specific effects, including potency, which is a prerequisite for risk assessment [44, 45].

In addition, incorporating PBK models can help link external exposure to internal concentrations relevant to KEs [44]. Furthermore, the development of TK-TD models enables the quantification of temporal concordance and dose-response behavior along the pathway.

Furthermore, recent studies have emphasized the strong context-dependence of several neurotoxic outcomes, including Parkinsonian phenotypes, which are determined by factors such as age, developmental stage, co-exposures, and systemic inflammation [45]. It is evident that these aspects have not yet been captured by linear AOP structures. In order to enhance the regulatory applicability, specific methodological improvements can be pursued. The following four points are to be considered: (i) expanding the use of high-content and omics-based NAMs for KE quantification; (ii) de-

fining minimum performance criteria and reporting standards for NAMs included in regulatory batteries; (iii) promoting the development of qAOPs with defined quantitative KERs and exposure thresholds; and (iv) harmonizing cross-AOP analysis for shared biological nodes, such as ERα activation.

CONCLUSIONS

In conclusion, this study evaluated the application of AOPs and AOP networks by the EFSA, together with associated NAM batteries, through two case studies addressing pesticide-related Parkinson's disease and endocrine disruptor-induced uterine adenocarcinoma. The analysis of the complete AOPs and corresponding NAMs highlighted key strengths and limitations of the AOP-based approach and assessed their current level of integration within the regulatory framework. Looking to the future, the scientific and regulatory relevance of AOPs and qAOPs is expected to grow, supporting NGRA by providing reliable, human-relevant, and ethically sustainable tools for modern regulatory toxicology. This highlights the need for stronger international collaboration to streamline endorsement processes, ensure consistency in chemical safety assessments and accelerate the integration of AOPs, particularly their quantitative aspects, into both regulatory and non-regulatory frameworks.

Authors' contributions

Conceptualization and methodology, formal analysis, resources, data curation, writing – original draft preparation; writing – review and editing: TD, ET, EDC. All Authors have read and agreed to the published version of the manuscript.

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Conflict of interest statement

The Authors declare no conflict of interest.

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