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Adverse Outcome Pathways – principles and applications

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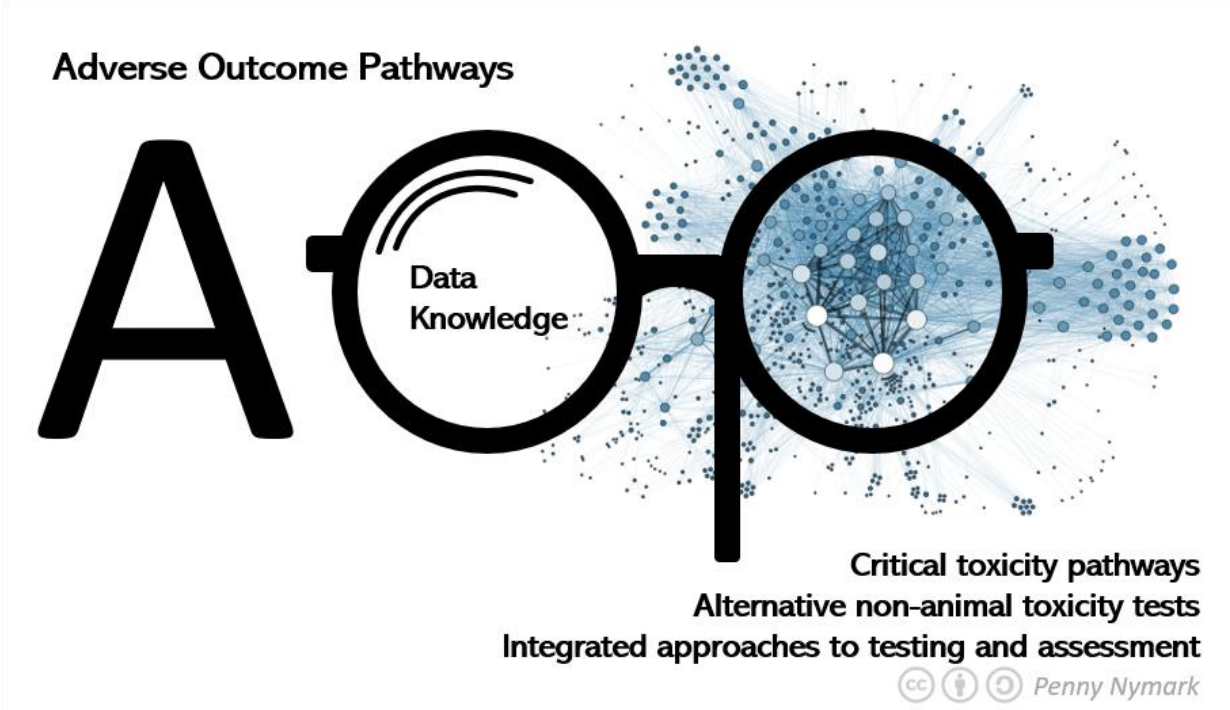

Foreword

The purpose of this report is to introduce the principles of Adverse Outcome Pathway (AOP) methodology and to describe different applications of AOPs in research, method development and risk assessment. The intended audience is primarily staff, researchers and students at the Institute of Environmental Medicine (IMM), as well as national authorities and organisations with an interest in AOP development and application.

In this report, we provide some examples to illustrate how AOPs have been developed and applied in different research and regulatory contexts. However, interest in AOPs is growing, both in academic research and at different authorities involved for example in risk assessment of chemicals, and there is an increasing body of innovative AOP-applications. A short summary in Swedish is provided.

AOPs are frameworks for systematic organization of existing mechanistic and toxicological data and knowledge and are used as tools for integrating and interpreting different types of data to draw conclusions about health risks posed by environmental factors. AOPs have been described as a platform for communication and collaboration across diverse disciplines of research and support efficient and sustainable reuse of available data and knowledge. In addition, AOPs support effective communication between research and regulation. At IMM, we have broad expertise in toxicology, epidemiology, and risk assessment, which provides an excellent basis for activities related to the development and application of AOPs. In addition, activities connected to AOPs and their use for research and risk assessment give the opportunity for cross-discipline collaborations within IMM, as well as with other institutions.

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Animation representing the AOP-driven structured organization of data and knowledge which supports identification of critical toxicity pathways, development of alternative non-animal toxicity tests, and integrated approaches to testing and assessment of chemicals and other stressors. (Data network image credits: Martin Grandjean, reproduced and built upon under CC-BY-SA 3.0 license).

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Highlights

- AOPs describe chains of key events in cells, tissues and the whole organism that link a molecular initiating event to an adverse health outcome.
- AOPs are frameworks for systematic organization of experimental and epidemiological data and knowledge.
- AOPs provide understanding of biologically plausible mechanistic pathways that underly adverse health outcomes caused by exposure to environmental stressors, such as chemicals, metals, and nanomaterials.
- AOPs can be used as frameworks for structured integration of different types of data to support risk assessment of environmental factors.
- Key events in an AOP are essential to the mechanistic pathway and are measurable biological events. Identification of such events can be used as basis for development of novel non-animal toxicity testing strategies and integrated approaches to testing and assessment (IATA).
- AOPs can be used to identify knowledge gaps and research needs.
- AOPs provide interdisciplinary collaborative platforms for researchers and risk assessors.

Svensk Sammanfattning

En Adverse Outcome Pathway (AOP) är en strukturerad sammanställning av befintlig kunskap om toxikologiska mekanismer som leder till en specifik negativ hälsoeffekt eller sjukdom. AOP:er beskriver kedjor av kausala samband som kopplar effekter på molekylär och cellulär nivå till negativa hälsoeffekter på individnivå eller populationsnivå. En AOP består av en serie kritiska steg (”key events”, KE) på olika biologiska nivåer (molekylär, cellulär, vävnad) som är sammankopplade genom så kallade ”key event relationships” (KER). Identifiering av KE och KER baseras på vetenskapliga data som utvärderas och sammanvägs enligt en strukturerad och harmoniserad metod.

AOP:er beskrevs först i början av 2010-talet av forskare vid amerikanska miljömyndigheten, US Environmental Protection Agency (EPA), som en metodik för att stödja forskning och riskbedömning inom ekotoxikologi. Sedan dess har metodiken även utvecklats inom toxikologi och riskbedömning av hälsoeffekter hos människa. Fem huvudprinciper för AOP:er är:

1. En AOP är inte bunden till en specifik stressor, till exempel en kemikalie, utan kan i teorin gälla för vilket kroppsfrämmande ämne eller faktor som helst som är kapabel att aktivera det första steget i händelseförloppet.
2. AOP:er är uppbyggda av moduler, det vill säga olika händelser (KE) och samband mellan händelserna (KER) som kan återanvändas i olika AOP:er.
3. En AOP är en pragmatisk, förenklad beskrivning av de biologiska händelser och mekanismer som leder till en särskild negativ hälsoeffekt.
4. AOP:er kan kopplas ihop till nätverk för att ge än bättre beskrivning av olika mekanistiska händelseförlopp som bidrar till en (eller fler) negativ(a) hälsoeffekt(er).
5. AOP:er är levande dokument. Allteftersom ny kunskap tillkommer kan nya data stödja eller förändra befintliga AOP:er.

Den internationella organisationen för ekonomiskt samarbete och utveckling (OECD) koordinerar idag utveckling och utvärdering av AOP:er på internationell nivå, där bland annat US EPA och Europeiska kommissionens gemensamma forskningsinstitut (Joint Research Center, JRC) är starkt drivande. Inom OECDs program utvecklas AOP:er av olika organisationer och forskargrupper världen över, enligt standardiserade metoder för att säkra strukturerad bedömning av vetenskapliga data och enhetlig beskrivning av KE, KER och AOP:er. Inom detta arbete har en AOP kunskapsbas skapats, som bland annat innefattar AOP-Wiki:n (https://aopwiki.org/). AOP-Wiki:n samlar AOP:er som beskrivits eller som är under utveckling för olika hälsoeffekter i människor och djur.

AOP:er lyfts fram som en lovande metodik för att förbättra testning och riskbedömning av kemikalier, nanomaterial och andra miljöfaktorer, och har fått ökad användning både regulatoriskt och inom forskning. Till exempel används AOP:er för att:

- Organisera befintlig kunskap om toxikologiska mekanismer och händelseförlopp som ligger bakom negativa hälsoeffekter
- Underlätta integrering och användning av olika typer av data, till exempel *in silico*, *in vitro*, djurdata (*in vivo*) och epidemiologiska data, i bedömningen av hälsoeffekter från en specifik kemikalie eller annan miljöfaktor
- Gruppera kemikalier i bedömningsgrupper baserat på toxikologiska likheter i bedömningar av effekter från kemikalieblandningar
- Stödja så kallad “mode-of-action” analys
- Identifiera kunskapsluckor och för att formulera hypoteser för forskning
- Utveckla nya djurfria metoder för toxicitetstestning

Framförallt gynnar AOP:er interdisciplinära samarbeten för att lösa specifika frågeställningar, eftersom de baseras på och kopplar ihop kunskap från olika fält, som datorbaserade modeller, toxikologi,

epidemiologi, statistik, med flera. Detta illustreras bland annat i flera pågående EU-projekt som använder AOP-metodik, till exempel EU-ToxRisk, HBM4EU, EuroMix, SmartNanoTox, PATROLS och projekten inom EURION: ATHENA, EDCMET, ENDpoiNTs, ERGO, FREIA, GOLIATH, OBERON och SCREENED. AOP:er gynnar också kopplingen mellan forskning och regulatorisk riskbedömning och beslutsfattande genom att utveckling av AOP:er samordnas internationellt av OECD och regulatoriska myndigheter med syfte att stötta riskbedömning och utveckling av testmetoder som kan användas för regulatoriskt beslutsfattande.

1 Introduction

The Adverse Outcome Pathway (AOP) concept was first described by scientists from the US Environmental Protection Agency (EPA) and in the context of providing a framework to support ecotoxicological research and risk assessment (Ankley et al. 2010). Since then, AOPs have been promoted as useful tools also in health risk assessment, in the development of Integrated Approaches to Testing and Assessment (IATA) and for developing novel animal-free test methods (e.g. Ankley and Edwards 2018; Basketter et al. 2013; Fry et al. 2019; Landesmann et al. 2013; Noyes et al. 2019; OECD 2017a,b; Perkins et al. 2019a; Vinken 2013; Wittwehr et al. 2017; Leist et al. 2014). In the recent Global Chemicals Outlook II report, the United Nations Environment Programme specifically lists accelerating development of AOPs as a measure to further advance hazard assessment of chemicals (UNEP 2019). Three key aspects of the AOP framework are believed to be central to its success in taking us further down the road of 21st century toxicity testing, as compared to other mechanism-focused toxicity concepts, such as the Mode of Action (MoA) concept; i) AOPs are systematic, structured, quality controlled and weight of evidence-based, and allow for refinement and perfection of methods, based on existing basic knowledge; ii) AOPs are supported by the OECD, which greatly facilitates their implementation into regulatory thinking; iii) AOPs allow for new ways of mechanism-driven safety assessment leading to new levels of importance, recognition and acceptance for *in vitro* and *in silico* approaches (Leist et al. 2017).

> *"An AOP describes a chain of events at different levels of biological organisation that causally connects a molecular initiating event to an adverse health outcome.*

2 What are AOPs?

Essentially, AOPs provide structured frameworks for collecting, organizing and evaluating toxicological knowledge. AOPs describe biologically plausible chains of events linking a molecular initiating event (MIE) to key events (KE) at different levels of biological organization and finally to an adverse outcome (AO), i.e. a negative health effect in an individual or on a population level (**Figure 1**). Since the AOP concept was developed within the area of regulatory (eco)toxicology, focus has been on AOs that are of regulatory relevance (Ankley et al. 2010), i.e. adverse effects which could be the focus of a regulatory risk assessment and risk mitigation. However, as AOPs gain wider application also in the field of research, the AO may not always have direct regulatory relevance.

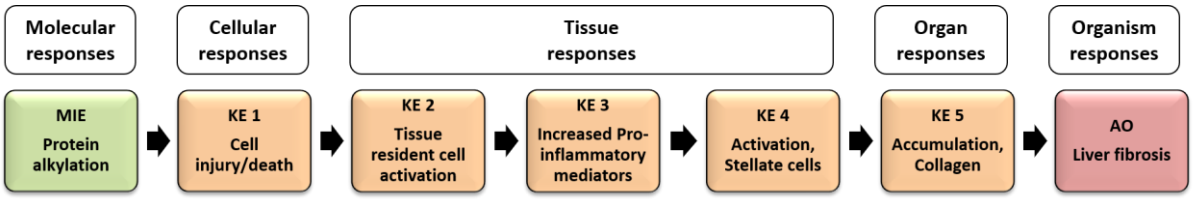

Figure 1. Example of an AOP describing a chain of events leading from a MIE (green box) via KEs (orange boxes) on different levels of biological organization to an AO (red box) on the organism level. The arrows represent key event relationships (KERs) which connect a downstream KE to an upstream KE. *(Example modified from https://aopwiki.org/aops/38.)*

AOPs support the use of a mode (and/or mechanism) of action basis for understanding and predicting adverse effects of stressors. The difference between a MoA and an AOP is that the former is described specifically for an individual stressor, while the latter is not stressor specific. Any stressor that can trigger the MIE may activate the AOP.

There are three operational defined stages of AOP development, namely putative, qualitative and quantitative AOPs. Putative AOPs provide an assembly of hypothesized KEs and Key Event Relationships (KERs) supported primarily by biological plausibility or statistical evidence. Qualitative AOPs provide more complete descriptions of KEs, including measurement possibilities, and KERs, which are supported by empirical evidence along with qualitative evaluation of the weight of evidence supporting the AOP. Finally, quantitative AOPs provide descriptions of accuracy and precision by with the KEs can be measured, as well as quantitative understanding of the magnitude and/or duration of perturbation required in an upstream KE in order to affect a downstream KE (Villeneuve et al. 2014). Quantitative information is contained within the KERs, which also allow for the capture of information on potential modulating factors such as genetic variations, pre-existing disease states, and nutritional or environmental factors (Villeneuve et al. 2014).

2.1 Five key principles of AOPs

Five key principles of AOP development were described as an outcome of a workshop on "Advancing Adverse Outcome Pathways (AOP) for Integrated Toxicology and Regulatory Applications" held in 2014 and where the US EPA, the European Commission Joint Research Centre (JRC), Environment Canada, and a number of universities were represented (Villeneuve et al. 2014). These principles are briefly described below.

Principle 1: AOPs are not stressor-specific

AOPs are not described for specific stressors. As such, they do not include considerations for kinetics, i.e. absorption, distribution, metabolism or excretion of chemicals, which are chemical-specific. Any stressor that can trigger the MIE may activate the AOP, given that the magnitude and duration of the perturbation is sufficiently severe. This reflects the ultimate goal of AOPs, i.e. that they should be useful tools to predict adverse effects of any type of stressor, including chemicals, nanomaterials, particles, radiation etc. with unknown toxic effects but for which the toxicological mechanisms (at molecular and/or cellular level) are known or can be tested.

Principle 2: AOPs are modular

AOPs are constructed from KEs and KERs. KEs are measurable events, e.g. a change in enzyme activity, and should represent steps that are essential for the AOP to progress to KEs further downstream and subsequently to the AO. In other words, if one KE is blocked, the downstream KEs and AO should be prevented. The MIE and AO are specific KEs that anchor the upstream and downstream end of the AOP, respectively. KERs connect adjacent KEs and are based on current biological knowledge and plausibility, as well as empirical evidence. The confidence in the KERs ultimately determine the overall confidence and predictive utility of the AOP.

Importantly, KEs and KERs are building blocks that can be used and re-used in many different AOPs. As such, a certain KE may provide a node where several AOPs are connected to form AOP networks (see Principle nr 4).

Principle 3: An individual AOP is a pragmatic unit of development and evaluation

For pragmatic reasons, AOPs are described as linear, unbranching pathways progressing in one direction. This is an intentional simplification of biological and toxicological processes, which commonly display branching, as well as interactions and crosstalk between pathways. This simplified approach is deemed necessary in order to create a system that makes developing, describing and curating of AOPs possible. It also enables harmonization of AOP development and assessment.

> *"AOPs are constructed from reusable key events and key event relationships and are pragmatic simplifications of real-life processes describing linear toxicity pathways.*

Principle 4: For most real-world applications, AOP networks are the functional units of prediction

AOP networks more realistically represent the complexity of biological and toxicological processes. There are probably few stressors that interact only at a single MIE (thus activating only one AOP). It is also unlikely that there are AOPs that do not branch at any point. Importantly, realistic exposure scenarios involve exposure to mixtures, potentially triggering a myriad of AOPs leading to the same AO. AOP networks can be used to investigate the interactions between different types of exposure. **Figure 2** shows an example of an AOP network describing interactions of different pathways leading to hepatic steatosis. This AOP network was used within the EU Horizon 2020 research project EuroMix (https://www.euromixproject.eu/) to develop *in vitro* methods to predict the interactions between chemicals causing steatosis via similar or dissimilar MoA.

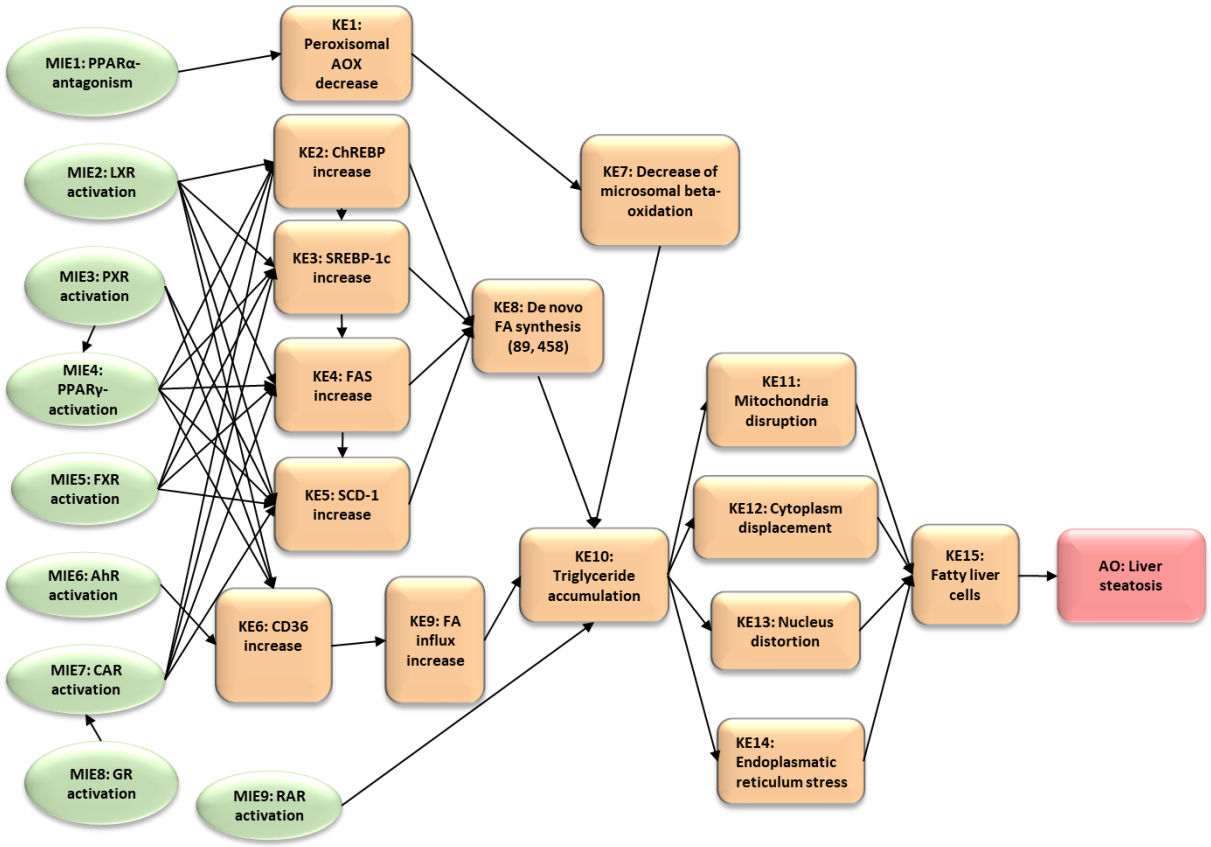

Figure 2. Example of an AOP network used for development of an *in vitro* testing strategy in the EU project EuroMix (adapted from van der Voet et al. 2019). An AOP network combines and depicts the interactions between different AOPs and represent the complexity of biological processes more realistically than individual AOPs.

Principle 5: AOPs are living documents

AOPs are never completely finalized. As our knowledge and understanding grows new KEs may be proposed; with the development of new technology, KEs may be measured with greater precision and accuracy; and new evidence may become available to support or reject KERs. AOP networks will thereby continue to grow. However, some AOPs have been endorsed by the OECD and thus can be seen as being robust and regulatory relevant. These AOPs are published in the OECD Series on Adverse Outcome Pathways (https://www.oecd-ilibrary.org/environment/oecd-series-on-adverse-outcome-pathways_2415170x).

3 The OECD AOP development programme

In 2012, the Organisation for Economic Co-operation and Development (OECD) Environmental, Health and Safety (EHS) Programme initiated a new programme on the development of AOPs[1] that is overseen by the OECD group *Extended Advisory Group for Molecular Screening and Toxicogenomics (EAGMST)*. The AOP Programme was developed as a global crowd-sourcing effort to describe existing knowledge on stressor interactions, such as chemicals, metals or other environmental factors, with a biological target leading to adverse effects. In addition to the key tasks of EAGMST to oversee and perform reviews of AOPs, EAGMST encompasses several subgroups working specifically on education, training and outreach, the AOP knowledgebase, coaching, the AOP-Wiki handbook, etc (see section 4). EAGMST members[2], nominated by their national coordinators[3], play an active role in the development of AOPs, the AOP review process, development of manuals as well as training in AOP development.

Within the AOP Development Programme, development of specific AOPs can be proposed by, for example, government representatives, academic experts, industry experts, non-governmental organisations and scientific societies in the OECD countries. Proposals are submitted to EAGMST and (if accepted) included in the AOP development workplan. The AOP proposal then undergoes an internal review by experts among EAGMST members. After revisions, a draft AOP is submitted to external expert review. Projects included in the AOP development workplan are published on the OECD website (http://www.oecd.org/chemicalsafety/testing/projects-adverse-outcome-pathways.htm), and endorsed AOPs are then published in the OECD Series on Adverse Outcome Pathways (https://www.oecd-ilibrary.org/environment/oecd-series-on-adverse-outcome-pathways_2415170x . **Figure 3** shows the current process for AOP development.

AOPs are intended to help regulatory agencies and risk assessors to utilise mechanistic data on adverse effects of stressors. To achieve this goal, EAGMST collaborates with the Working Party for Hazard Assessment (WPHA) and Working Group of National Co-ordinators of the Test Guidelines programme (WNT) to ensure increased assessment of regulatory relevance of AOP project proposals submitted to EAGMST. In line with this, the European JRC is currently performing a study on stakeholder views and needs regarding the AOP Framework to evaluate how it could be optimized to serve regulatory priorities.

[1] More information available at http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm

[2] The current SE member is Dr Emma Wincent, IMM, KI.

[3] The Swedish Chemicals Agency is National Co-ordinator for Sweden.

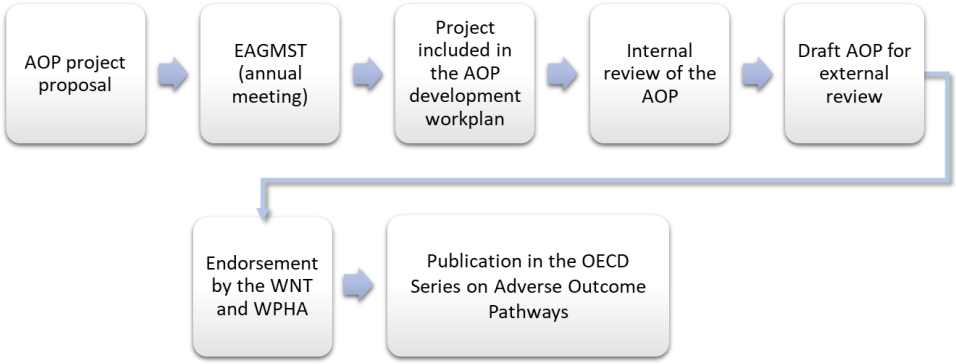

Figure 3. Overview of the AOP development process within the OECD AOP Development Programme (adjusted from http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm).

4 The AOP knowledgebase – AOP-Wiki and Effectopedia

Several tools and platforms are available to aid in harmonized AOP development. As a platform for sharing, developing and discussing AOP related knowledge, the AOP knowledge base (AOP-KB) was initiated (https://aopkb.oecd.org/index.html). The AOP-KB is maintained by the OECD in close collaboration with the European Commission JRC, the US EPA, and the US Army Engineer Research & Development Center.

The currently most prominent and well-developed resource of the AOP-KB is the AOP-Wiki, which at the time of writing contains over 300 harmonized and publicly available descriptions of AOPs at different levels of development (https://aopwiki.org/). It is the central repository for all AOPs developed within the OECD AOP Development Programme and is managed jointly by the JRC and the US EPA. In addition, the AOP-Wiki is profiled as an open-source platform for crowdsourcing and therefore also contains AOPs developed outside of OECD's program. The overall aim of the AOP-Wiki is to provide a transparent and inclusive environment for collaboration and contribution across diverse fields of research (Carusi et al. 2018).

In addition, the AOP-KB covers the Effectopedia, the AOP Explorer, and the Intermediate Effects Data Base (Carusi et al. 2018). The purpose of these tools is to provide diverse visualization and analysis opportunities. For example, the Effectopedia is a collaborative tool intended for the development and use of quantitative AOPs (https://www.effectopedia.org/). The tool is under development and can be used to visually explore the AOPs in the AOP-Wiki and to derive simple quantitative relationships between KEs. Researchers can report experimental data to be included in the Effectopedia using harmonized OECD templates.

Finally, a wide variety of data resources and life science databases have begun to provide links and analysis possibilities connected to the AOP-KB. Examples include the WikiPathways AOP portal (https://www.wikipathways.org/index.php/Portal:AOP) and the CompTox Dashboard (https://comptox.epa.gov/dashboard/chemical_lists/AOPSTRESSORS) (Martens et al. 2018; Nymark et al. 2018b; Pittman et al. 2018).

5 Applications of AOPs

AOPs are useful tools both for regulatory and research purposes. While Villeneuve et al. (2014) stated that "*the ultimate goal of AOPs is to predict adverse effects of stressors with unknown toxic effects*",

AOPs can also have other uses. For example, to identify and fill knowledge gaps, to formulate hypotheses, to develop testing strategies, to provide mechanistic understanding for how environmental factors can lead to adverse health effects, and to understand interactions between stressors with different MoA.

The OECD Guidance Document on Developing and Assessing Adverse Outcome Pathways lists different uses of AOPs (OECD 2017b). Vinken et al. (2017) also provides a comprehensive overview of the applications (as well as development and assessment) of AOPs. Here we describe some examples of uses in the regulatory setting, and in research projects.

5.1 Examples of research applications

The AOP paradigm is receiving increasing attention and use in the research community, especially in toxicology where much focus is being placed on mechanism-based approaches to investigate and predict effects of chemicals and other environmental stressors. Major objectives of applying the AOP framework in research projects include:

- development of animal-free testing strategies with relevance for human health, based on comprehensive mechanistic understanding of adverse effects induced by environmental stressors;
- optimizing planning of experimental design and research project design by summarizing prior knowledge in a systematic and biologically relevant manner, aiding in identification of knowledge gaps on links between MIEs, KEs and AOs;
- integrating novel *in silico* and *in vitro* methodologies that are relevant for regulatory purposes (Daneshian et al. 2016), e.g. to develop quantitative structure-activity relationships (QSAR) models anchored in KEs;
- integrating different types of effect data such as omics-data and targeted effect analysis;
- identifying converging pathways of toxicity and KEs that can be used in development of bioassays for mixture risk assessment and to explore potential synergies;
- establishing mechanistic links between exposures and adverse health outcomes in human populations to provide information on possible causal relationships;
- providing a structured basis for grouping chemicals or stressors into assessment groups and identifying possible up-stream KEs that can be used to calculate relative potency factors of mixture components;
- identifying biological factors influencing an AO;
- supporting development of *in silico* and *in vitro* test methods to predict different types of toxicity such as endocrine disruption, developmental toxicity, neurotoxicity, immunotoxicity, etc;
- supporting extrapolation of effects and toxicity mechanisms across vertebrate species, including humans.

The "EU-AOP strategy" has resulted in implementation of the AOP framework in several large EU Horizon 2020 projects, including e.g: EU-ToxRisk (https://www.eu-toxrisk.eu/), HBM4EU (https://www.hbm4eu.eu/), EuroMix (https://www.euromixproject.eu/, finalized in 2019), SmartNanoTox (http://www.smartnanotox.eu/), PATROLS (https://www.patrols-h2020.eu/), and the EURION cluster including the projects ATHENA, EDCMET, ENDpoiNTs, ERGO, FREIA, GOLIATH, OBERON and SCREENED receiving funding from the Horizon 2020 call "New testing and screening methods to identify endocrine disrupting chemicals" (https://eurion-cluster.eu/).

So far, these EU projects have developed or are currently developing several AOPs that can be found in the AOP-Wiki or are planned to be included in the Wiki, e.g. "Histone deacetylase inhibition leads

to neural tube defects" (https://aopwiki.org/aops/275), "Inhibition of complex I of the electron transport chain leading to chemical induced Fanconi syndrome" (https://aopwiki.org/aops/276), "α-diketone-induced bronchiolitis obliterans" (https://aopwiki.org/aops/280), "Inhibition of 17α-hydrolase/C 10,20-lyase (Cyp17A1) activity leads to birth reproductive defects (cryptorchidism) in male (mammals)" (https://aopwiki.org/aops/288), and "Substance interaction with the lung resident cell membrane components leading to lung fibrosis" (https://aopwiki.org/aops/173).

5.2 Drug development and safety assessment

Burden et al. (2015) and Hartung (2017) have addressed the potential applications of the AOP framework for safety assessment of pharmaceuticals and for drug development. The collaborative and cross-disciplinary nature of the AOPs allow for feedback of information and knowledge between diverse fields, such as disease research, toxicology and alternative methods (Nymark et al. 2018a; Nymark et al. 2021). While discussing that application of mechanistic pathways is in line with the toxicity testing in the 21st century paradigm (US NAS 2007), these articles also emphasise the need for quantitative AOPs, harmonization of development and a high level of confidence of AOPs used for regulatory purposes.

Drug-induced liver injury (steatosis, fibrosis, cholestasis) is a common reason for withdrawing pharmaceuticals from the market. The mechanisms for liver toxicity are relatively well understood and many AOPs have been developed and described in the AOP-Wiki, for example "Protein Alkylation leading to Liver Fibrosis" (https://aopwiki.org/aops/38), "Endocytic lysosomal uptake leading to liver fibrosis" (https://aopwiki.org/aops/144) and "Cholestatic Liver Injury induced by Inhibition of the Bile Salt Export Pump (ABCB11)" (https://aopwiki.org/aops/27). It has been suggested that AOPs can provide a structured basis for predicting liver injury and for developing *in silico* and *in vitro* methods for screening as well as targeted methods for pre-clinical testing to assess drug-induced liver toxicity (Vinken 2015; 2016; Kohonen et al. 2018).

AOPs have also been proposed as useful frameworks for method development and integration of *in vitro* data in personalized cancer therapy (Morgan et al. 2016). Morgan et al. present an AOP for estrogen receptor-mediated breast cancer and illustrates how it can function as a foundation for a therapeutic outcome pathway and for designing relevant *in vitro* models that reflect the targeted *in vivo* response. In line with these efforts, a novel application of the AOP framework, spurred from the ongoing coronavirus pandemic (COVID-19), has been initiated. The European Commission-funded and JRC co-ordinated project CIAO aims to model the pathogenesis of COVID-19 using the AOP framework to provide a more holistic overview of the variable disease outcomes, and to support efficient non-animal development of preventive, diagnostic, and therapeutic approaches (www.ciao-covid.net; Nymark et al. 2021).

5.3 Examples of regulatory applications

The extent to which an AOP may be used for regulatory applications depends on the overall confidence in the AOP, i.e. biological plausibility of the KERs, the essentiality of KEs and the empirical evidence supporting the KERs in the pathway (described above).

Integration of non-animal mechanistic data for regulatory purposes

The AOP paradigm was first promoted as an approach for risk assessment and predicting adverse effects of stressors based on mechanistic information and fewer animal data (Ankley et al. 2010). There is currently a rapid development of *in vitro* methods driven by stakeholder needs, academic research interests and increased regulatory focus on the 3R concept (Replacement, Reduction and Refinement of animal studies). European legislation, such as REACH and the Directive on the protection of animals used for scientific purposes (Directive 2010/63/EU), emphasize that the use of animal testing should

be kept to a minimum. Recent reports from international organisations, such as the OECD and the US National Academy of Sciences (NAS) indicate a paradigm shift and emphasize the importance of novel approaches for research and development, as well as in the regulatory context, for example for providing mechanistic understanding and potential for high-throughput screening of stressors (OECD 2017a and c; 2018b; US NAS 2007). Two important factors limiting the regulatory use of *in vitro* methods in general is i) a lack of understanding of the relationship between what is tested and the adverse effect that is being predicted, and ii) the lack of biological complexity of single *in vitro* systems, such as interactions of different cell types found in intact tissue. Here, AOPs can provide the mechanistic understanding needed to integrate data from *in vitro* and *in silico* methods to support conclusions about health effects.

Development of novel testing strategies to meet regulatory data requirements – the examples of skin sensitisation and developmental neurotoxicity

Non-animal methods are not currently able to replace animal toxicity testing of chemicals on a one-to-one basis (ECHA 2017). AOPs and AOP networks can be used for developing batteries of non-animal test methods that target certain KEs in an AOP. The OECD's activities concerning method-development to predict skin sensitization provides an example where an AOP-based approach has been used to develop a toolbox of alternative test methods for regulatory testing purposes (Delrue et al. 2016). In this case, the AOP "Covalent Protein binding leading to Skin Sensitisation" (https://aopwiki.org/aops/40), which was developed at the JRC has provided the basis for test method development (Schultz et al. 2016). A battery of different *in silico*, *in chemico*, and *in vitro* methods that target specific KEs in this AOP have been developed (Belot et al. 2017; Ezendam et al. 2016; Kleinstreuer et al. 2018; Natsch and Emter 2016; Ramirez et al. 2016). Different cases of Defined Approaches (DA) for skin sensitisation, based on these non-animal methods have been evaluated by the OECD, the JRC and the US Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM) at the National Toxicology Program (NTP) (Strickland et al. 2016). A DA describes how different *in vitro* data should be used in a defined approach to, when appropriate, replace the need for an *in vivo* experiment (OECD 2016). The ongoing OECD activities are aiming to give the DA for skin sensitisation the same regulatory recognition as the animal tests currently required under e.g. the CLP and REACH regulations. An OECD guideline for this DA is under development (latest draft in September 2019, OECD Test Guideline Programme workplan project number 4.116).

Along the same principles, initiatives have been taken at the OECD, together with the JRC, the European Food Safety Authority (EFSA), US EPA and others to develop a battery of *in vitro* test methods to screen chemicals for developmental neurotoxicity (DNT) potential, which could speed up the evaluation of thousands of chemicals that lack DNT data (Bal-Price et al. 2017; Spinu et al. 2019).

In a wider perspective, but related to the examples of skin sensitisation and DNT given above, AOPs can be used as frameworks in the development of integrated approaches to testing and assessment (IATA) of diverse stressors (e.g. Edwards et al. 2016; Fitzpatrick and Patlewicz 2017; OECD 2017a; Tollefsen et al. 2014; US EPA 2015; 2016). In this context, AOPs can be used to provide a structure for the identification and evaluation of relevant existing information on specific stressors, for identification of data gaps and which additional data are needed for assessment, as well as for development of a targeted testing strategy (OECD 2017a). Sakuratani et al. (2018) have reviewed recent OECD activities on the use of AOPs in developing IATAs.

Tiered testing frameworks for hazard characterization

Yet further related strategies supported by AOPs build on tiered testing, usually starting with broad coverage, high-throughput testing of multiple stressors and tiering towards increasingly targeted follow-up testing of selected active and/or class-representative stressors (Nel et al. 2013; Grafström

et al. 2015; Nymark et al. 2018a; Thomas et al. 2019). These approaches may be guided by or directly linked to the AOP framework supporting testing in different tiers based on the activation of biological pathways and molecules associated with MIEs, KEs, or KERs (reviewed in Nymark et al. 2020). Decisions to move to subsequent tiers may also be guided by AOPs in terms of selection of stressors to take further along tiers and regarding which model systems to employ. Initial tiers may utilize multiple cell types/model systems suitable for high-throughput assessment and cover molecular markers, pathways or cellular effects broadly linked to multiple AOPs or AOP networks, while later tiers may include more targeted testing in increasingly complex model systems representing increasingly specific AOPs. An example of a tiered testing framework proposed by and currently in implementation phase at the US EPA is shown in **Figure 4**.

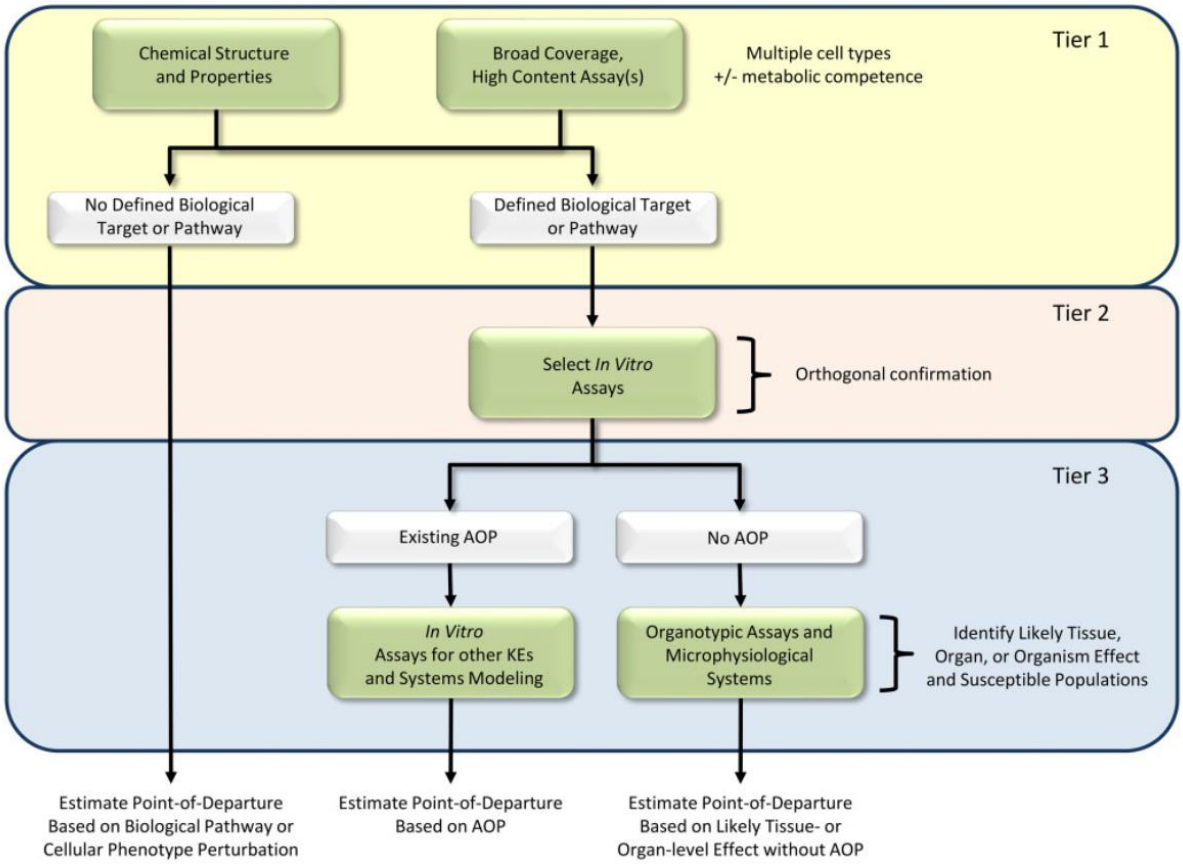

Figure 4. A next-generation tiered testing framework developed by the CompTox Initiative at the US EPA for hazard characterization (Figure reproduced with permission from Thomas et al. 2019). The approach covers grouping of stressors based on similarity in hazard potential as assessed from both structure/physicochemical properties and broad coverage high-content assays in multiple cell types (Tier 1). Next, stressors from Tier 1 which have a predicted defined biological target or pathway are tested using targeted follow-up assays (Tier 2), and finally AOP-aligned assays and/or more complex organotypic assays (Tier 3). Stressors without defined biological targets or pathways, as identified in Tier 1, will be subject to a quantitative point-of-departure estimate for the most sensitive pathway or phenotypic effect identified.

Identification and assessment of endocrine disruptors

An example of a specific regulatory application of AOPs is as support for the identification of endocrine disruptors (EDs) in the context of the EU Regulations for biocides (528/2012) and plant protection products (1107/2009). Identification of EDs in this regulatory context entails identifying endocrine activity and an adverse effect of a substance, as well as providing support for a biologically plausible link between the endocrine activity and adverse effect via MoA-analysis (ECHA/EFSA 2018). Any AOPs

already described and published either in the AOP-Wiki (see below for more information) or the open literature may be used to support the MoA-analysis and the plausibility of the link between endocrine activity and adverse effect. This includes using entire AOPs or selected KEs and KERs to describe the MoA for the specific substance under review. AOPs are also used to organize and integrate data for evaluating the endocrine activity of chemicals in the U.S. EPA's Endocrine Disruptor Screening Program (EDSP) (Browne et al. 2017).

Risk assessment of nano- and advanced materials

Risk assessment of manufactured nanomaterials and other advanced materials is challenged by among other things accelerating numbers and increasing complexity involving dynamic physicochemical characteristics that are difficult to assess, making traditional case-by-case animal-based testing and assessment virtually impossible (Ede et al. 2020). The urgent need for high-throughput alternative methods in the field has spurred significant interest in the AOP framework, and a number of putative AOPs, focused on lung and liver injuries, relevant for nanomaterials have been developed (Halappanavar et al. 2020; Gerloff et al. 2017; Nymark et al. 2018b). The OECD Working Party on Manufactured Nanomaterials (WPMN) initiated a project (NanoAOP) in 2016 for the purpose of advancing AOP application to nanomaterials, and identified six current areas of AOP application for nanomaterial risk assessment (reviewed in Ede et al. 2020), including support for hazard characterization, grouping and read across, ranking and prioritization, identification of novel biomarkers for alternative test method development, support for product development as part of safer design approaches (known as Safe by Design), and in combination with first-tier high-throughput broad coverage testing technologies, eg omics, for the purpose of guiding next-tier testing predictive of AOs (see paragraph on tiered testing frameworks above; Nymark et al. 2020; Halappanavar et al. 2021). Specific benefits of the AOP framework were described in relation to its flexibility towards assessment of the complexities of nanomaterials. For example, the MIE element in the AOP framework allows for modelling of complex physicochemical properties in relation to variable nanomaterial-related initiating events. These QSARs support identification of nanomaterial properties (including both physicochemical and biological) of concern and enable grouping and prioritization based on their ability to induce an MIE/AOP (Ede et al. 2020; Mech et al. 2019; Giusti et al. 2019; Nymark et al. 2018a). Consequently, the applicability of AOPs in regulatory risk assessment of and decision making for nanomaterials was recently reviewed and a set of nine recommendations for advancing development, use and acceptance of AOPs were provided; i) advancing nanomaterial considerations in AOP development, ii) maximizing reuse of data, iii) promoting reliable and quantitative data generation and management, iv) advancing understanding of QSARs between nanomaterial physicochemical properties and AOP elements, v) identify current applications of AOPs in nanomaterial risk assessment, vi) establish AOP-aligned test methods and protocols applicable to nanomaterials, vii) demonstrate predictive capability of AOPs and test methods, viii) provide guidance to facilitate application of AOPs in nanomaterial risk assessment, and ix) increase stakeholder communication and engagement on the use of AOPs for nanomaterials (Ede et al. 2020). Worth noting is that the recent establishment of a Nanosafety Data Interface providing Findable, Accessible, Interoperable, and Reusable (FAIR) data (Jeliazkova et al. 2021) and a FAIR implementation network, the AdvancedNanoIN, strongly supports several of the recommendations proposed (https://www.go-fair.org/implementation-networks/overview/advancednano/).

Risk assessment of combined exposures to multiple substances

There are different regulatory requirements for assessing the risks from combined exposures to multiple substances (“mixture risk assessment”) within different regulatory frameworks in the EU (reviewed in Rotter et al. 2019). EFSA, the OECD and the JRC have recently published guidance and considerations for mixture risk assessment within their different remits (Bopp et al. 2019; EFSA 2019;

OECD 2018c). These specifically describe the use of the AOP approach for grouping chemicals into relevant assessment groups. Additionally, AOPs and specifically AOP networks can be used as frameworks within which it is possible to theoretically explore the assumptions of similar and dissimilar MoA, and potential interactions between mixture components (Kienzler et al. 2016; Kortenkamp 2020; Beronius et al. 2020). Similarly, they can be used to identify effects (KEs) on which to base calculation of relative potency factors (RPFs) of mixture components (Beronius et al. 2020).

6 Principles for development and assessment of AOPs

Both regulatory and research needs and initiatives drive AOP development. The OECD has published a guidance document (OECD 2017b) as well as a User's Handbook (OECD 2018a), for the development and assessment of AOPs. While the guidance document describes the general principles for development, assessment and uses of AOPs, the User's Handbook provides practical guidance and instructions, including templates and guidance intended to improve consistency in AOPs developed by different stakeholders. The Handbook provides instructions for how AOPs, KEs and KERs should be described in the AOP-Wiki and the different sections to be included.

6.1 Main components and data used in AOP development

The main components of an AOP are the KEs (including the MIE and AO) and the KERs. AOP development entails identifying the main KEs and the MIE relevant for the AO under consideration and describing the KERs (OECD 2017b). While a MIE may initiate several different pathways, AOP development should focus on describing a single pathway, focusing on a single MIE and AO. The AO is traditionally an adverse effect of regulatory relevance (Ankley et al. 2010), such as apical endpoints investigated in regulatory test guidelines and this is the focus in the OECD AOP Development Programme. However, as AOPs are being increasingly used for different applications, such as in academic research projects, and for development of test methods and evidence integration, the AO-concept is being broadened to also include adverse effects that may not be traditionally considered as AOs in regulatory risk assessment. One example is the EFSA initiative to develop an AOP for Parkinson's disease (EFSA 2016).

KEs should be essential steps in the pathway and should be measurable. While there is no specific number of KEs that should be included in an AOP, it is preferable to describe KEs at the different levels of biological organization, i.e. on the molecular, cellular, tissue/organ and organism level (**Figure 5)**. For ecotoxicological applications, the AO is usually described at the population level, e.g. population decline.

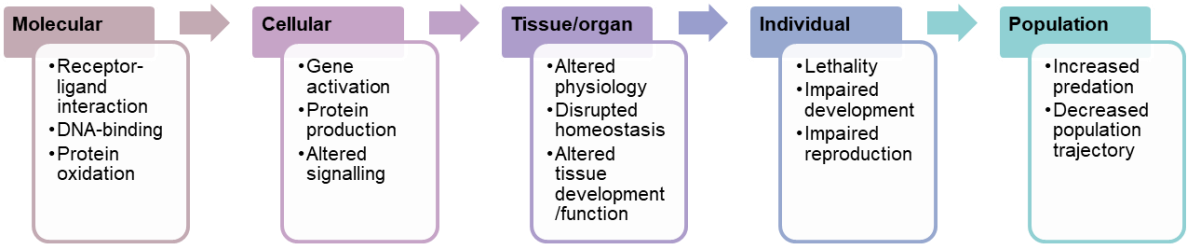

Figure 5. Examples of types of effects that can be described as KEs at different levels of biological organization in an AOP (adapted from Ankley et al. 2010).

KERs describe the association between an upstream and a downstream KE, i.e. how a change in the upstream KE influences a change in the downstream KE. A direct KER describes the association

between two adjacent KEs, while an indirect KER describes the association between an upstream KE and a downstream KE further along the pathway. KERs should be described and supported by scientific information. The plausibility, based on current biological knowledge, and the empirical evidence of the KERs are important factors that determine the overall confidence in the AOP (see AOP assessment below).

There are a wide variety of approaches applied for development of AOPs, including top-down, bottom-up and middle-out strategies that start from either the AO, the MIE or from central KEs, respectively (Villeneuve et al. 2014). In addition, so called case study approaches have been described, where one or several specific model stressors are used to describe the AOP and subsequently generalized to be applicable to other stressors; or data mining approaches, where high-throughput and high-content data, such as omics data is used to identify KEs and biomarkers (Vinken et al. 2019; Nymark et al. 2018a and b). Overall, a wide variety of data types, including *in silico*, *in chemico*, *in vitro*, *in vivo* and human data can be used to support a KER and the AOP as a whole. In addition, the stressor-agnostic aspect of the AOPs allow for modelling and use of data from a wide variety of stressors. This couples to one of the central principles of the AOP framework, which is worth repeating; i.e. that KEs and KERs are not unique to a single AOP (Villeneuve et al. 2014). The modular aspect of AOPs means that the building blocks, i.e. the KEs and KERs, including the information contained in them, are reusable in the development of new AOPs. This is important to consider in the development of AOPs in order to take full advantage of the framework aiming for efficient building on existing knowledge, support for development of AOP networks and identification of unexpected or under-appreciated biological connections between diverse AOs (Villeneuve et al. 2014).

6.2 AOP assessment

AOP assessment is a step in the AOP development in which the evidence that underpins the AOP is evaluated and described. We only describe the process very briefly here. For more detailed information the reader is referred to the OECD Revised Guidance Document on Developing and Assessing Adverse Outcome Pathways (OECD 2017b). AOP assessment includes considering the domain of biological applicability of the AOP and the level of confidence in the AOP.

The applicability domain of the AOP as a whole, in terms of sex, life-stage and taxa will depend on the most restricted KE, i.e. if most KEs in the AOP are supported by data from different species and both sexes but one KE is only supported by data from female rats, then the applicability domain for the whole AOP is, strictly speaking, limited to female rats.

The assessment of confidence of the AOP is based on modified Bradford Hill considerations (Becker et al. 2015) and the three primary considerations are the biological plausibility of the KERs, the essentiality of the KEs and the empirical support for the KERs.

> *"The confidence of an AOP depends primarily on the biological plausibility and empirical evidence for each key event relationship, as well as the essentiality of key events.*

The **biological plausibility of the KERs** is the most important consideration in an AOP assessment. Assessment of biological plausibility is based on current biological and toxicological knowledge regarding the causal relationship between two KEs. The biological plausibility is rated as high, medium or low, where high indicates that the KER is "well understood based on extensive previous documentation and has an established mechanistic basis and broad acceptance".

Assessment of the **essentiality of KEs** is based on experimental data showing that the blocking of a KE prevents or modifies downstream KEs and/or the AO.

Essentiality is rated as high, medium or low. Essentiality is considered high if the KE is supported by "direct evidence from specifically designed experimental studies illustrating prevention or corresponding impact on downstream KEs and/or the AO if upstream KEs are blocked or modified (e.g., via stop exposure/reversibility studies, antagonism, knock out models, etc.)."

The **empirical support of KERs** includes experimental data supporting the associations between KEs. Data from one or more reference chemicals can make up the evidence base. Focus is on reviewing the dose-response concordance and temporal concordance between and across KEs, i.e. whether upstream KEs are observed at lower doses and at earlier timepoints than downstream KEs, as well as whether the incidence or frequency of an upstream KE is greater than at downstream KEs at the same dose. The empirical support is also rated as high, medium or low. The empirical support is considered high when "there is dependent change in both events following exposure to a wide range of specific stressors (extensive evidence for temporal, dose-response and incidence concordance) and no or few data gaps or conflicting data".

6.3 When is an AOP accepted for regulatory purposes?

It is not straight forward to answer the question of when an AOP is considered accepted for regulatory purposes. This question depends on what is meant by "regulatory acceptance" and being aware that regulatory applications and fit-for-purpose considerations may vary (e.g. Coady et al. 2019; Perkins et al 2015). One level of acceptance is that the AOP is endorsed by the OECD and published in the OECD Series on Adverse Outcome Pathways (https://www.oecd-ilibrary.org/environment/oecd-series-on-adverse-outcome-pathways_2415170x). However, the AOP framework can still be a useful tool in different aspects of the regulatory risk/hazard assessment process, even if a specific AOP has not yet been fully developed and assessed (some examples are described above in section 5.2). For example, EFSA has used AOPs to integrate epidemiological data on pesticides in their assessments of Parkinson's disease and childhood leukaemia and conclude that "the availability of a fully elucidated AOP is not a requirement for using epidemiology studies in human health risk assessment" and "that an AOP can provide support for the definition of the biological plausibility, particularly from the mechanistic point of view" (EFSA 2018).

AOPs can be used for regulatory purposes to anchor *in vitro*, *in chemico*, or *in silico* assays for complex endpoints. The AOP for skin sensitization (https://aopwiki.org/aops/40) provides an example of an AOP that has been thoroughly evaluated and endorsed within the OECD AOP Development Programme for this purpose. It has been used as the basis for development of different *in silico* and *in vitro* methods to measure specific KEs (OECD Test Guidelines Programme project number TG442D).

Generally speaking, AOPs are useful tools for regulatory applications if they contribute to reducing the uncertainties in decision making (Perkins et al. 2015). Perkins et al. (2015) reviewed four cases of applying AOPs for different regulatory applications to explore the degree of scientific confidence and completeness required. They conclude that "AOPs at all levels of confidence can contribute to specific uses" and that one future development that can improve the use for regulatory application is the use of AOP networks to account for multiple interacting pathways.

One aspect that currently limits regulatory application and acceptance is that most AOPs are qualitative descriptions, lacking quantitative understanding. As mentioned above, development of quantitative AOPs includes quantitative descriptions of KERs, i.e. the response-to-response relationships between KEs. In other words, what magnitude of response will result at a downstream KE if there is a certain magnitude of response in an upstream KE. This requires feeding dose-response and even time-response data into the AOP, which is complex and resource intensive but improves the predictivity of AOPs and application for regulatory decision making (Conolly et al. 2017 Perkins et al.

2019a and b). Examples of such development include the use of Bayesian regression analysis to quantify KERs (Moe et al. 2021).

7 Summary and future perspectives

AOPs are frameworks for summarizing existing knowledge of toxicity pathways described according to an internationally agreed format and based on structured evaluation of evidence. Thus, they describe the mechanistic understanding of causal relationships between early molecular and cellular events, such as a stressor´s interaction with cellular receptors and changes in biochemical signalling pathways, to an adverse health outcome in an individual or a population. The AOP framework is being increasingly promoted as a useful tool for different applications in regulatory hazard and risk assessment of environmental stressors, as well as in research. Applications include:

- organisation of existing knowledge regarding toxicological pathways leading to negative health effects on the individual or population level,
- use as frameworks for integrating different types of data, e.g. epidemiological, toxicological and modelled data, to conclude about negative health effects of a specific stressor,
- use as frameworks for grouping chemicals and nanomaterials into assessment groups when assessing health risks from combined exposure to multiple substances (mixture risk assessment),
- use as basis for MoA-analysis,
- identification of knowledge gaps and generation of hypotheses,
- development of novel (animal-free) test methods and IATAs.

The AOP framework supports the progression into 21st century toxicity testing by promoting structured use of mechanistic knowledge to support conclusions about health risks from environmental stressors. It thus promotes the implementation of 3R principles and facilitates development of high-throughput test systems based on *in silico* and *in vitro* methods. The framework has for example been successfully used as basis for the development of a regulatory accepted animal-free testing strategy for skin sensitisation. It is being similarly applied in development of a battery of *in vitro* test methods for DNT-screening of chemicals, which could speed up the evaluation of thousands of chemicals that lack DNT data. International harmonization of the development and assessment of AOPs, coordinated by the OECD and involving institutions such as the European Commission JRC and the US EPA, facilitates regulatory acceptability and implementation. As such, the AOP framework also promotes a connection between science and policy, supporting increased trust among authorities in novel non-animal test methods (Carusi et al. 2018).

Notably, AOPs provide interdisciplinary collaborative platforms for researchers and risk assessors. As frameworks depicting KEs at different levels of biological organization leading to a specific adverse health effect, AOPs provide structures for integrating data from *in silico* models, *in vitro* models, *in vivo* animal studies and human epidemiological studies. This requires input from different areas of expertise but also generates opportunities and structures for collaboration. As an example, increased mechanistic understanding of toxicity pathways provided by *in silico*, *in vitro* and even *in vivo* animal data, may provide support in epidemiological studies investigating associations between exposure to environmental stressors and health effects. Such understanding can help overcome uncertainties about causality between exposure and effect caused by, for example, confounding factors. Knowledge of toxicity pathways can also form the basis for hypotheses, e.g. about interaction effects or mediation, that can be tested with epidemiological study design. The AOP framework is implemented in several large EU Horizon 2020 projects, such as EU-ToxRisk, HBM4EU, EuroMix, SmartNanoTox, PATROLS and

the EURION cluster including the projects ATHENA, EDCMET, ENDpoiNTs, ERGO, FREIA, GOLIATH, OBERON and SCREENED.

Going forward, the AOP framework is promoted by international organisations such as the OECD and the UN as an approach to improve testing and assessment of chemicals, nanomaterials and other stressors. The development of fast, reliable and animal-free testing methods, as well as structured methods for integrating and assessing different types of data is critical to meet societal needs to have safe and circular (re)use of chemicals and materials, as well as to prepare for changes in exposure to environmental stressors brought on by, for example, climate change and changes to consumer behaviours (UN, 2020).

8 Abbreviations

AO	Adverse Outcome
AOP	Adverse Outcome Pathway
AOP-KB	AOP Knowledge Base
EAGMST	The OECD Extended Advisory Group for Molecular Screening and Toxicogenomics
FAIR	Findable, Accessible, Interoperable, Reusable
IATA	Integrated Approaches to Testing and Assessment
JRC	European Commision Joint Research Center
KE	Key Event
KER	Key Event Relationship
MIE	Molecular Initiating Event
MoA	Mode of Action
OECD	The Organisation for Economic Co-operation and Development
US EPA	US Environmental Protection Agency
QSAR	Quantitative structure–activity relationship

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