

Concept Article

Beyond Chemicals: Opportunities and Challenges of Integrating Non-chemical Stressors in Adverse Outcome Pathways

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Received July 6, 2023;
Accepted October 31, 2023;
Epub November 17, 2023;
© The Authors, 2023.

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ALTEX 41(2), 233-247.
doi:10.14573/altex.2307061

Abstract

Adverse outcome pathways (AOPs) were developed to accelerate evidence-based chemical risk assessment by leveraging data from new approach methodologies. Thanks to their stressor-agnostic approach, AOPs were seen as instrumental in other fields. Here, we present AOPs that report non-chemical stressors along with the challenges encountered for their development. Challenges regarding AOPs linked to nanomaterials include non-specific molecular initiating events, limited understanding of nanomaterial biodistribution, and needs for adaptations of *in silico* modeling and testing systems. Development of AOPs for radiation faces challenges in how to incorporate ionizing events type, dose rate, energy deposition, and how to account for targeting multiple macromolecules. AOPs for COVID-19 required the inclusion of SARS-CoV-2-specific replicative steps to capture the essential events driving the disease. Developing AOPs to evaluate efficacy and toxicity of cell therapies necessitates addressing the cellular nature and the therapeutic function of the stressor. Finally, addressing toxicity of emerging biological stressors like microbial pesticides can learn from COVID-19 AOPs. We further discuss that the adaptations needed to expand AOP applicability beyond chemicals are mainly at the molecular and cellular levels, while downstream key events at tissue or organ level, such as inflammation, are shared by many AOPs initiated by various stressors. In conclusion, although it is challenging to integrate non-chemical stressors within AOPs, this expands opportunities to account for real-world scenarios, to identify vulnerable individuals, and to bridge knowledge on mechanisms of adversity.

Plain language summary

The adverse outcome pathway (AOP) framework was developed to help predict whether chemicals have toxic effects on humans. Structuring available information in an accessible database can reduce animal testing. AOPs usually capture the path from the interaction of a stressor, usually a chemical, with the human body to an adverse outcome, e.g., a disease symptom. The concept of AOPs has now been expanded to include non-chemical stressors such as nanomaterials, radiation, viruses, cells used to treat patients, and microorganisms employed as pesticides. We discuss how these stressors need to be accommodated within the framework and point out that pathways initiated by these stressors share downstream events like inflammation with chemical stressors. By integrating non-chemical stressors into the framework, real-world scenarios where people may be exposed to different stressor types can be considered, vulnerable individuals can be identified, and knowledge on toxic effects can be compounded.

1 Introduction

The adverse outcome pathway (AOP) framework emerged in the context of efforts to transform toxicity testing and risk assessment. *Toxicity Testing in the 21st Century: A Vision and a Strategy* (Andersen and Krewski, 2010; Krewski et al., 2010; NRC, 2007)

envisions “toxicity pathways” as main drivers of the paradigm shift that can enable leveraging existing knowledge and data from new approach methodologies (NAMs) to accelerate evidence-based risk assessment of the large and growing number of existing untested and newly developed chemicals. Pathway testing would require a suite of assays, ideally conducted in relevant cell lines



or tissues of human origin, that could identify the range of significant perturbations of biology resulting from chemical exposure (Krewski et al., 2010).

The AOP framework, however, aims for a wider scope compared to the “toxicity pathway”: A sequential chain of linked perturbed biological events at biological levels with increasing complexity (molecular, cellular, tissue, organ) that lead to an adverse health or ecotoxicological outcome at the individual or population level (Ankley et al., 2010). More specifically, it was initially endorsed in the field of regulatory ecotoxicology to enable a synthesis of data collected at many levels of biological organization in a way that is useful to both researchers and risk assessors (Ankley et al., 2010). Given the same challenges are pertinent to human toxicology, the approach was recognized as instrumental for informing human health risk assessment as well. In addition, such organization of existing knowledge enables identification of uncertainties and gaps to drive future relevant research. The AOP framework aims to drive the improvement of predictive approaches needed to advance regulatory (eco)toxicology to address the challenges of assessing large numbers of chemicals with minimal, effective, animal-free and species-relevant testing and assessment methodologies (Ankley et al., 2009; Volz et al., 2011; Watanabe et al., 2011; OECD, 2012a, 2020).

Early on, the AOP concept was recognized as an evolution of the mode of action approach (Boobis et al., 2006, 2008; Garcia-Reyero, 2015; Whelan and Andersen, 2013). However, with its consistent structure and terminology embedded in the online repository, named AOP-Wiki¹, AOPs provide a large platform that can embrace evidence from a wide range of chemical stressors and methodologies to facilitate identification of specific properties that trigger a particular pathway. This in a sense turns the focus from the action of particular chemical stressors to understanding the key biological perturbations and their linkages. This facilitates identification of pathways plausible and useful for toxicity prediction for stressors for which no or limited testing data is available. The AOP-aligned organization providing evidence to support that a perturbation at a lower biological level is linked to an outcome of regulatory importance has improved confidence in the development and evaluation of novel methodologies for toxicity testing and assessment. Hence AOPs are real enablers of the 21st century paradigm shift towards the reliable use of new approach methodologies (NAMs).

Following the take-off of the OECD AOP program² and the challenges highlighted by the growing number of AOPs being developed, five core principles were established. Those principles aim to guide a consistent approach for AOP development and to facilitate international involvement in building an AOP knowledge-base with high value for the regulatory and research communities. The five core principles of AOPs were described by Vilenneuve et al. (2014) as such:

1. “AOPs are not chemical specific;

2. AOPs are modular and composed of reusable elements named key events (KEs) and key event relationships (KERs);
3. an individual AOP, composed of a single sequence of KEs and KERs, is a pragmatic unit of AOP development and evaluation;
4. networks composed of multiple linear AOPs that share common KEs and are likely to be the functional units of prediction for most real-world scenarios;
5. AOPs are living documents that can evolve over time as new knowledge is generated.”

The principle that AOPs are not chemical-specific means that an AOP captures the evidence from as many stressors tested in relation to a pathway as necessary to generate a sufficient level of knowledge about the relationships from a molecular initiating event (MIE) through a series of KEs up to the adverse outcome (AO). This structured AOP then can facilitate inference about the toxicity of other chemicals with similar characteristics even in the absence of full datasets for them. Thus, this first AOP principle supports the usefulness of AOPs in supporting inference about data-poor stressors using the evidence from previously tested stressors that share common characteristics. For chemicals, the common characteristics can be represented as chemical structure fragments and patterns (e.g., functional groups, atom-centered fragments, Tanimoto index, etc.), physicochemical characteristics (e.g., molecular weight, pKow, etc.), and bioactivity (e.g., binding to a specific protein receptor or moiety). Finding the relevant common characteristics for reliable structure-function inference is not an easy task. It is not surprising then, that the QSAR (quantitative structure-activity relationship) community was one of the earliest adopters of the AOP framework and approach (OECD 2011, 2012a). For nano-sized materials, structural similarity of substances represents an additional difficulty as in general their structure is not unequivocally defined. Physicochemical parameters are chemical composition, impurities, surface area, and particle size, shape and porosity. For radiation, common attributes include the radiation type, energy, dose-rate of delivery, and spatial distribution pattern of ionization events.

Importantly, the approach can also inform the most effective testing strategies to generate the missing data and build knowledge, ideally using NAMs. This also makes AOPs instrumental for identification of environmental and human-relevant assays for development of testing strategies and for identification of needs for new testing or non-testing (such as QSAR, based on the structural characteristics of the chemicals relevant for the MIE) methods.

Because this inference based on a mechanistic understanding of the pathway can link to outcomes that might also be of scientific research relevance, a growing number of AOPs are being developed³ and used in different contexts such as read-across (OECD, 2020) or test guideline development. This increase of interest and activities outside the chemical risk assessment field generates new challenges and potential solutions that engage the AOP commu-

¹ https://aopwiki.org/info_pages/3

² <https://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm>

³ https://www.oecd-ilibrary.org/environment/oecd-series-on-adverse-outcome-pathways_2415170x

nity in revisiting, clarifying, and refining the first principle of AOP development and use.

This paper aims to outline the opportunities and challenges related to the first AOP principle, e.g., being stressor-agnostic, at the practical level considering “classical” chemical and advanced nanoscale material as well as the anticipated inclusion of other types of stressors: physical stressors such as ionizing radiation, complex biological stressors such as viruses, and cellular stressors such as therapeutics or microbial pesticides.

2 Different types of AOP stressors: why, which AOPs, and challenges

2.1 Chemicals

Why

Developed within the field of regulatory toxicology to support chemical risk assessment based on mechanistic reasoning, AOPs initially focused on chemical stressors. Specific chemicals or groups of chemicals already were considered in some early AOP-based case studies (OECD, 2012a) before the establishment of the OECD AOP program and development guidance (OECD, 2018).

Which AOPs

One of the first AOPs developed in the OECD context, even before the launch of the AOP-Wiki, is the *AOP for Skin Sensitization Initiated by Covalent Binding to Proteins*⁴ (AOP40, OECD, 2012b). AOP40 describes the perturbations of the immune system without reference to a specific chemical and includes relevant evidence for a number of chemicals. As skin sensitization is an important regulatory apical endpoint, testing over time led to relatively rich databases with evidence generated in different experimental test systems. The development of this AOP relying on evidence from many different chemical stressors endorsed the first AOP principle, e.g., the stressor-agnostic approach. As a proof of concept, this early AOP includes an Annex with evidence for one specific data-rich chemical with well-characterized skin sensitization potential *in vivo* for which data was also available at each KE of the pathway. Despite the relative richness of *in vivo*, *in vitro*, and *in chemico* data related to mechanistic aspects of skin sensitization, the AOP identifies “*lack of databases with results from assays representing KEs along the pathway*” as a significant limitation, particularly for quantitative understanding of the AOP, which is important for regulatory application. The limitation lies in the availability of relevant evidence and not in the selectivity of the approach to AOP development. AOP40 induced discussion on the potential application of AOPs to inform risk assessment of poorly tested or untested chemicals by considering structural and chemical reactivity as well as molecular and cellular bioactivity characteristics (OECD, 2012a,b). This AOP has supported the development of a series of internationally accepted test guidelines to identify chemicals with a potential to elicit skin sensitization

(OECD, 2022, 2023a,b). Furthermore, specific combinations of these testing and non-testing (e.g., QSAR) approaches have been developed as defined approaches to allow internationally aligned regulatory use for identification of skin sensitization hazard and classification (OECD, 2023c). Thus, AOP40 is a good example of the usefulness of the stressor-agnostic AOP principle.

Challenges

Following AOP40, a significant number of AOPs relevant to chemical stressors have been built within the online repository platform AOP-Wiki⁵. Some AOPs start with a MIE that is rather unspecific, such as binding to the SH(thiol)-group of proteins and/or to seleno-proteins (AOP17). For others, the MIE is an interaction in a mechanistically specific manner, such as protein alkylation (AOP38) or an interaction with a specific receptor (AOP13). This highlights specific challenges regarding what is considered the appropriate weight of evidence to support a particular pathway to be fit for a particular purpose (from hypothesis testing and gap analysis to regulatory application).

In addition, chemical-focused AOPs currently do not account for metabolic transformation nor for the dynamics of exposure, although the significance of persistent chemical accumulation has been considered for some MIEs.

2.2 Nanosized material stressors

Why

Assessing the toxicity of materials at the nanoscale is challenged by many aspects specific to physicochemical properties and behaviors of nanomaterials compared to the properties of the corresponding bulk material. The stressor-agnostic aspect of AOPs might be instrumental in developing strategies for prioritized testing of the large and constantly evolving number of nanomaterials employed in industrial innovation that pose high concern due to the uncertainties about their hazard potential (Halappanavar et al., 2020). Various toxicity pathways induced by nanomaterials share similarities with those induced by chemicals (Gerloff et al., 2017). Thus, in principle, much of the mechanistic knowledge captured in AOPs that have been developed for chemical-induced toxicity could be applicable and relevant to nanomaterials.

Which AOPs

To date, of the 15 AOPs present in the AOP-Wiki which report a nanosized material as a stressor, nine relate to lung toxicity (AOP173, AOP237, AOP303, AOP302, AOP319, AOP241, AOP409, AOP451, AOP481). In addition, in the literature, two AOPs starting from increased substance interaction and leading to lung emphysema and lung fibrosis, respectively, have been proposed (Halappanavar et al., 2020); the evidence supporting carcinogenicity of TiO₂ served for the development of one putative AOP leading to lung cancer (Braakhuis et al., 2021; Nymark et al., 2021a); and an AOP for graphene-family nanomaterial-induced lung damage was developed (Ding et al., 2023). From the

⁴ <https://aopwiki.org/aops/40>

⁵ <https://aopwiki.org/>



other AOPs in the AOP-Wiki, AOP144 (under review) describes the endocytic lysosomal uptake of NPs leading to liver fibrosis (Gerloff et al., 2017); AOP209 links perturbation of cholesterol by silica NPs to hepatotoxicity, while AOP207, AOP208, and AOP210 (currently not under OECD status) propose mechanisms leading to reproductive failure. Besides, outside the AOP-Wiki, plausible AOPs were proposed involving ingestion of TiO₂ nanoparticles, which leads to liver injury and reproductive toxicity as well as to colorectal cancer, and cardiac and kidney damage (Rolo et al., 2022). Very recently, a proposed AOP network linked nanomaterial-induced mitochondrial dysfunction to different existing AOPs for chemicals related to lung and liver outcomes but also to AOs in the cardiovascular and nervous systems (Murugadoss et al., 2024).

Challenges

Four main challenges have arisen during development of AOPs related to nanomaterials. First, the nanomaterial interactions with the biological milieu, including the formation of protein coronas around the particles, influence their potential toxicity. This depends on the nature of the milieu, changes in the milieu, and persistence and/or aging of the particles (Gerloff et al., 2017). These aspects require special considerations with regard to the domain of applicability. In addition, specifications regarding nanomaterial property-mediated deviations of the AOP and/or characterization of system-dependent properties and biophysical interactions may be needed.

Second, due to the complex behaviors and interactions of nanomaterials, their toxicity is often associated with a wide variety of molecular and cellular stress mechanisms (Nel et al., 2013). Knowledge about the initial fate/biotransformation of nanomaterials *in vivo* prior to reaching the biological target is often sparse and requires extensive material characterization both *ex* and *in situ* (Fadeel, 2022). These aspects lead to lack of clarity regarding the MIE(s), both relating to the specificity of the MIEs (in contrast to chemicals that may target specific cellular receptors or other molecules) and relating to the potential for a necessity for interactions between several MIEs in order to trigger an AOP cascade.

Third, there is still a limited understanding of nanomaterial absorption, distribution, stability, and persistence (accumulation) in the human body (Landsiedel et al., 2012), largely due to technological challenges to detect nanomaterials. There is a need for increased understanding of *in vivo* biokinetics via appropriate kinetic models to predict uptake rates, for example, as nanomaterials are extensively used in the food industry and ingestion routes have become increasingly relevant. These challenges limit the use of AOPs, as relevant exposure doses are often difficult to assess (Drasler et al., 2017).

Finally, nanomaterials require adaptation of current standards of *in silico* modelling approaches and *in vitro* testing. Test item preparation, dosing, and understanding of toxicity mechanisms are required to use the mechanistic data to build nanomaterial-relevant AOPs. As an example in that direction, a recent AOP development effort focused on an AOP for lung cancer induced by nanosized foreign matter anchored a selection of 18 standardized methods and NAMs suitable for *in silico*- and *in vitro*-based integrated as-

essment of lung carcinogenicity associated with nanomaterials (Nymark et al., 2021a). In addition, faster and more reliable methods are also needed to assess potential toxicity across multiple species and for longer-term consequences.

Thus, while chemical- and nanomaterial-induced toxicological pathways share downstream events leading to a particular AO, the major differences in the toxicodynamics of nanomaterials lie in the initial events, dose considerations, and test method adaptations.

2.3 Physical stressors such as ionizing radiation

Why

The stressor-agnostic approach has enabled the extension of the AOP framework to the field of radiation (Chauhan et al., 2019, 2021b-e, 2022b; Laurier et al., 2021). Exposure to radiation can occur in many situations ranging from occupational (e.g., nuclear worker) to environmental (e.g., radon), and medical settings (e.g., radiotherapy treatments). Health concerns include both cancer and non-cancer effects. This extends to space exploration, and the associated risk from multiple stressors on astronaut health. Most relevant data on radiation-induced diseases stems from epidemiological evidence from atomic bomb survivors, uranium miners, nuclear facility emergency workers, and medical workers. Additionally, studies in animals and cells are ongoing to better understand low-dose effects (NCRP, 2020). Many cellular constituents such as DNA, proteins, lipids, and cell membranes can be affected by ionizing radiation. Further, non-targeted effects (e.g., hormesis and adaptive responses) can increase the uncertainty in health risks for low-dose exposures. Therefore, the use of the AOP approach could help organize and consolidate data to identify relevant mechanisms best suited for understanding low-dose research needs.

Which AOPs

In 2018, the first ionizing radiation AOP to lung cancer was submitted for approval to the OECD AOP development program (Chauhan et al., 2021b). AOP272 was subsequently endorsed in 2023 by the Nuclear Energy Agency alongside the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) and Working Party on Hazard Assessment (WPHA). AOP272 was intended to illustrate the potential role AOPs could play in integrating mechanistic data with epidemiological studies. AOP272 describes the KEs leading to lung cancer predominantly supported by evidence using radiation stressors via a pathway of DNA damage response/repair. The AOP evidence base was compiled using a narrative approach, and multiple KERs in the AOP were reused from AOP296 on oxidative DNA damage leading to chromosomal aberrations and mutations, where empirical data was drawn from a chemical stressor. This strengthened the evidence for weaker relationships and demonstrated the benefits of cross-disciplinary AOPs (Chauhan et al., 2021e).

Challenges

In building AOP272, many challenges were encountered, particularly due to the stochastic nature of radiation. Typically for chemicals, a single MIE is discernible, however with radiation multiple

macromolecules can be targeted concurrently by the ionization events. AOP272 focused on DNA damage, but it was evident that follow-on work would be needed to expand the AOP to a network that included other targets at the macro-molecular level (e.g., proteins, lipids, carbohydrates). These efforts are in progress through an AOP network being built on health outcomes from space travel (Chauhan et al., 2021a). Furthermore, despite decades of research in the field of DNA damage response/repair (AOP296), a scarcity of studies met elements of the Bradford-Hill criteria, particularly dose-, time- and incidence-concordance data for adjacent KERs. Therefore, quantitative understanding of the ability to predict KERs of the AOP272 was low and its use to support risk assessment applications could not be fully recognized (Stainforth et al., 2021).

Unlike chemicals, radiation is a physical stressor, and this made it difficult to define an accurate *biological* MIE. For this reason, an all-encompassing MIE was identified, namely “deposition of energy” (DOE), which is measurable in terms of absorbed dose and essential to achieve an AO. This event leads to chemical and biological KERs and could connect all radiation AOPs within the AOP-Wiki. However, questions were raised on whether distinction was needed between an ionizing or non-ionizing injury. Although discussion continues in the radiation community, DOE is recognized as an umbrella MIE that fits all radiation types and can lead to follow-on KERs that can then possibly discern ionizing and non-ionizing radiation stressors.

Development of AOP272 also highlighted challenges in incorporating attributes of radiation exposure related to the type and dose-rate of delivery into a qualitative AOP. It is well known that a small amount of sparsely ionizing radiation will likely lead to minimal, simple damage (e.g., single DNA strand breaks); alternatively higher levels of radiation will lead to more complex damage (e.g., multiple strand breaks) that is not easily repaired by different processes such as homologous recombination and non-homologous end joining (Chatterjee and Walker, 2017). The distribution of ionization events and dose rate effects will also be important factors for progression to the AO. How best to interpret these characteristics in a qualitative AOP is an ongoing debate. An important question arises as to whether radiation AOPs should center around specific stressors in order to account for the quantity and quality of energy deposited and the period elapsed since exposure in the KER descriptions.

Another consideration is the integration of the wealth of epidemiological data in the radiation field. Although AOP272 is well-supported by epidemiological data for the KER linking MIE to AO, not many human studies have examined the early macro-molecular endpoints. A need for more experimental work using archived bio-samples from human cohort studies was identified. These could be re-examined to assess the relationship between early macromolecular endpoints (e.g., oxidative stress, DNA damage, altered signaling) and the late phenotypic effects (e.g., organ dysfunction, tissue remodeling).

A recent international survey has highlighted 25 priority questions that need to be addressed for AOPs to support radiation regu-

latory needs (Burt et al., 2022). Challenges continue to be discussed through a newly formed Radiation/Chemical Joint Topical Group (JTG) under the auspices of the Nuclear Energy Agency High Level Group on Low Dose Research to help advance the use of AOPs within the radiation field (Chauhan et al., 2022a). This includes best approaches for synthesizing the vast number of studies in the radiation field (Kozbenko et al., 2022) and integrating omics-based approaches (Azimzadeh et al., 2022; Yu et al., 2022). As the radiation community begins to build AOPs (eight currently in progress), the challenges encountered during the development and review of AOP272 will be important considerations for areas to improve (Jaylet et al., 2023; Klovov et al., 2022; Tollefsen et al., 2022). In time, AOPs relevant to the radiation field will reach a level where they will play a bigger role in the system of radiation protection.

2.4 Viral stressors such as SARS-CoV-2

Why

Expanding still further the scope of the AOPs, the international project CIAO was launched to explore the applicability of the AOP framework to support systematic organization of the diverse and fast evolving evidence related to COVID-19 pathogenesis (Wittwehr et al., 2021; Clerbaux, 2022; Nymark et al., 2021b; Kim et al., 2021). In a pandemic context, this extensive and complex evidence could be integrated via AOPs in a way that is understandable by relevant stakeholders. Exploiting the mechanistic-based approach of AOPs for a viral disease of such societal impact was believed to be instrumental in identifying knowledge gaps, relevant biomarkers, and mechanisms by which risk factors modulate outcomes but also in fostering a true interdisciplinary collaboration (Carusi et al., 2023). Over the 3 years duration of the project, CIAO⁶ brought together more than 75 scientists from more than 20 countries with distinct backgrounds and expertise.

Which AOPs

Many COVID-19-related AOPs were developed, leading to diverse AOs such as hyperinflammation (AOP392, AOP468), thromboinflammation and thrombosis (AOP412, AOP379), respiratory symptoms and lung injury (AOP320, AOP319, AOP173, AOP302), neurological syndromes including short-term anosmia (AOP394, AOP374, AOP395), intestinal disorders and gut dysbiosis (AOP422, AOP428), and liver disorders (Shahbaz et al., 2022; Hogberg et al., 2022; Clerbaux et al., 2022b,c; Vinken, 2021).

Challenges

When exploiting AOPs to model COVID-19, a viral stressor-initiated disease, the suitability of the stressor-agnostic principle was challenged. Depicting precisely the biology of the virus (i.e., the stressor) was considered essential to correctly capture the mechanisms underlying disease onset and progression. Following binding to angiotensin converting enzyme 2 (ACE2) receptor, SARS-CoV-2 enters the cell and starts its replication cycle by translating genomic RNA and producing key proteins that interact within the

⁶ <https://www.ciao-covid.net/>

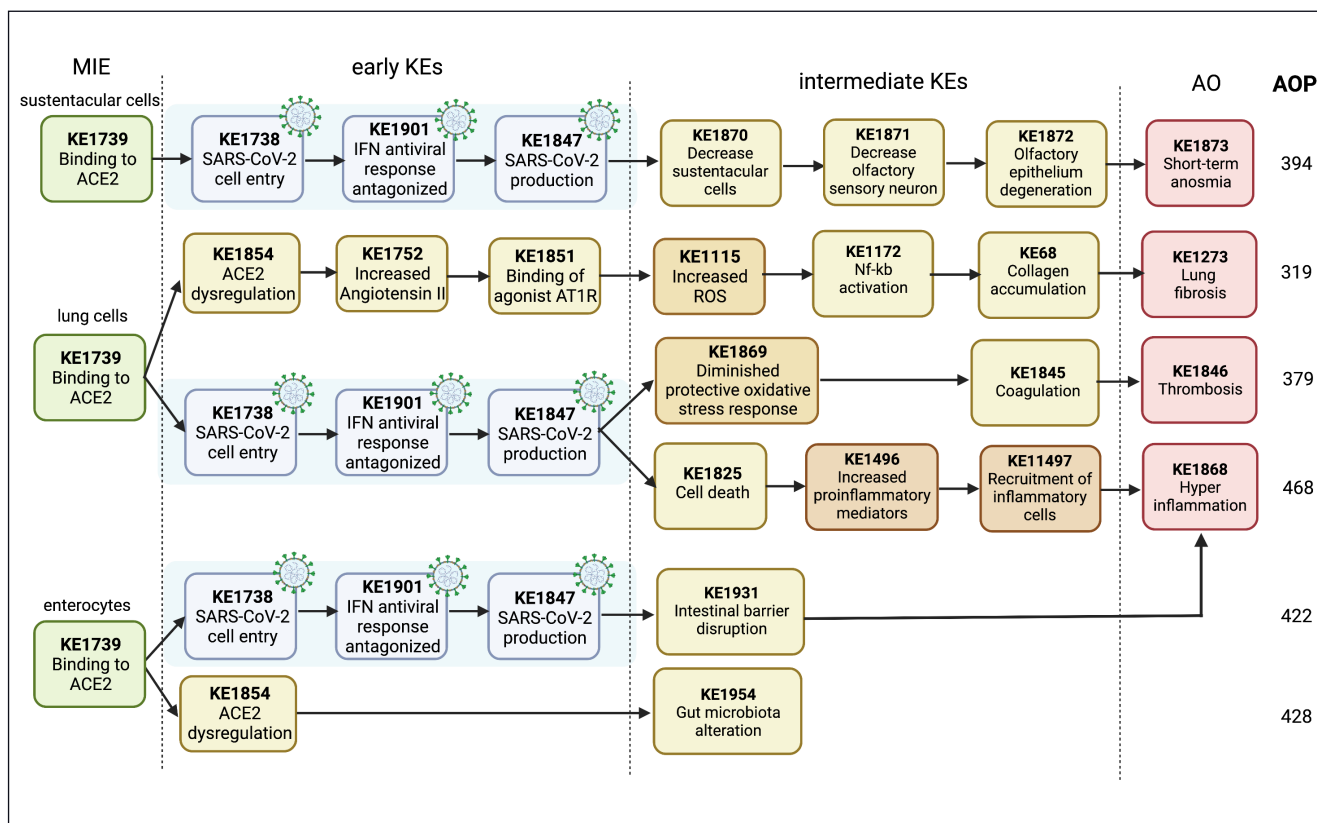


Fig. 1: Adverse outcome pathways (AOPs) of relevance for COVID-19

The six AOPs presented here are identified by their respective AOP-Wiki unique identifier. Green key events (KEs) are reported as molecular initiating events (MIE), orange ones as early or intermediate KEs, and red ones as adverse outcomes (AOs). Arrows denote adjacent key event relationships (KER). The proposed “viral hub AOP” in blue is a common series of adjacent early KEs that specifically depict SARS-CoV-2 replication (cell entry, antiviral response antagonized, and virus production) essential to induce diverse AOs in many COVID-19-related AOPs. Created with Biorender.com.

host cell to favor viral replication (Hoffmann et al., 2020; Zhou et al., 2020). Some of these proteins construct a replication factory, others are dedicated to innate immune system evasion. The latter interact with host molecular components to suppress the interferon-I antiviral response. The blocking of host innate immunity by the viral stressor components is key for viral transcription and replication to take place (Diamond and Kanneganti, 2022). Understanding this stressor-host interaction at molecular and cellular level and describing how the virus specifically accomplishes productive infection is necessary to capture the essential steps leading to the AOs. In addition, viral replication and viral load are well-established parameters correlated with bad prognosis of the disease (Brosseau et al., 2022), and most approved antivirals act on viral proteins specifically, blocking their function inside cells. Hence, in the project, a stressor-specific AOP was developed describing the replication cycle of SARS-CoV-2 from ACE2-binding up to transmission (AOP430). We proposed that this AOP430 could serve as a “hub AOP”, a re-usable unit for several AOPs that require SARS-CoV-2 replication to mediate COVID-19 AOs. As such, the proposed compromise is that only the initial three linked KEs (KE1738: SARS-CoV-2 cell entry;

KE1901: IFN antiviral response antagonized by SARS-CoV-2; KE1847: increased SARS-CoV-2 production, in blue in Fig. 1) are specific to the stressor while the downstream KEs in the different AOPs follow the stressor-agnostic principle. In addition, the MIE (KE1739: Binding to ACE2) is stressor-agnostic as not only SARS-CoV-2 can bind to this receptor, and it does not only lead to SARS-CoV-2 cell entry as exemplified here with the ACE2 dysregulation downstream event (KE1854). This also maintains the MIE open to inclusion of evidence from other stressors binding ACE2, such as SARS-Co-V (Tanonaka and Marunouchi, 2016) or ACE2 modulators.

There are other important aspects to consider when applying the AOP framework to viral infections. Viruses are not static and acquire mutations that alter interaction of specific viral proteins and consequently their interaction with host machinery, often leading to different outcomes. The CIAO COVID-19 AOPs curated the literature on viruses up to 2021, which means that the AOPs currently in the AOP-Wiki are applicable to the variants until then but are likely to fail to predict AOs for all the variants currently circulating and for future variants. Literature is already highlighting the fact that some sublineages of Omicron are more prone to

being restricted to the upper respiratory tract rather than provoking serious lower respiratory tract infections. How to address these changes in a living document such as AOPs should be evaluated carefully. One possible avenue is to have an expanded section dedicated to the stressor itself, accommodating viral evolution, mutations acquired, and changes in the disease. One may also consider that the AOP framework may be useful to identify knowledge gaps but recognize the limitations for accommodating the replication cycles of ever-evolving viruses.

Another aspect to consider with a pathogen as an AOP stressor is that an adverse outcome for one species (viral replication in human cells) may be a beneficial outcome for the pathogen (successful replication of the virus). This is a different situation than for chemicals, nanomaterials, or radiation.

Finally, this AOP-approach enabled a qualitative description, based on evidence in the literature, on how known COVID-19 risk factors alter the KERs and thus the underlying mechanisms of COVID-19, ultimately affecting disease outcomes (Clerbaux et al., 2022a). Addressing factors modulating COVID-19 is crucial since abundant clinical evidence shows that outcomes are markedly heterogeneous among patients. A majority of SARS-CoV-2 patients showed moderate symptoms or were asymptomatic, while some patients were vulnerable because of age, previous disease history, or lifestyle factors and experienced severe symptoms, often ending in death. Apart from intrinsic factors (age, sex) and pre-existing comorbidities, exposure to chemicals was investigated as a potential risk factor. Interestingly, in that context, the challenge of characterizing an exposure either as a stressor or as a modulating factor was encountered. This will be further discussed in Section 4.

2.5 Cellular stressors such as engineered cell immunotherapies

Why

Novel biologicals have also found a useful frame within the AOPs for evaluation of their effects upon interaction with the host target. Some innovative treatments for cancer and autoimmune diseases, such as advanced therapeutic medicinal products, work by altering the immune system. Amongst those, chimeric antigen receptor (CAR-T) cells are genetically modified lymphocytes of patients that express a specific receptor on their cell surface that binds to ligands on cancer cells. Following binding, activated CAR-T cells attack cancer cells and destroy them. This type of therapy has shown promising results in treating leukemia and lymphoma. CAR-T cell therapies have, however, serious side effects, with cytokine release syndrome (CRS) being the most common. Identifying potential safety issues before testing those treatments in humans still represents a major challenge notably because tests used early in drug development do not reflect the full complexity of the human immune system and tend to be based on a healthy immune system. Preclinical models failed to predict CRS elicited by CAR-T treatment. Hence, there is a need to design, develop, and deliver a range of test systems based on a comprehensive understanding of the molecular and cellular mechanisms at play. In that context, structuring the key steps along an AOP is thought to provide support to anchor relevant test systems.

Which AOPs

In a recent initiative in that direction, the AOP concept provided a scientific framework for integration of new testing paradigms by linking the mechanistic understanding of CAR-T cell induced CRS at different biological levels with appropriate targeted test systems. The AOP framework has helped to focus research, collect existing data, generate hypotheses, and identify gaps as hardly sufficient information about the underlying mechanisms was available. The CAR-T cell AOP starts with the specific recognition and binding of a CAR-T cell to antigen-expressing cancerous cells, which activate CAR-T cells, leading to the release of pro-inflammatory cytokines and chemokines, subsequently followed by recruitment and activation of leukocytes, accompanied by the release of quantitatively more inflammatory mediators, finally leading to activation of tissue-resident cells and endothelium, systemic inflammation, and CRS (Mazein et al., 2023).

Challenges

A first challenge in developing such AOPs relates to the cellular dimension of the stressor. After binding to the cancer cell, CAR-T cells are activated (activation of immune cells) and then release pro-inflammatory mediators which then recruit other immune cells that upon activation release pro-inflammatory mediators. Therefore, the first KE is not a reaction of the biological system the stressor is interacting with, it is a reaction of the cellular stressor itself. Whether those KEs should be duplicated is still a question that might need to be addressed with regards to guiding the development of test systems.

A second interesting aspect is linked to the therapeutic function of the stressor. In contrast to chemical-induced toxicity, the use of AOPs in this context is intended to evaluate the potential of new CAR-T cell therapies in balancing the efficacy and safety of the treatment. The quantitative and time dimension along with other biomarkers might be essential to assess the trade-off between protective and adverse inflammation. Such complex needs of developing an immune-related AOP along with assigned test systems to assess both efficacy and toxicity might represent a great albeit challenging opportunity to clarify within the AOP framework a physiological normal inflammatory response versus the adverse and pathological excessive inflammation relevant also to many chemical stressors.

2.6 Emerging biological stressors such as microbial pesticides

Why

Microbial pesticides are microorganisms such as bacteria, fungi, and viruses used in agriculture to control a wide range of plant diseases. Microbial pesticides emerged from a need to apply alternatives to chemical pesticides as they are more selective for targeted pests and pose lower risk to human health and the environment. However, microbial pesticide hazard testing followed the path of chemical pesticide testing and relied on existing test guidelines developed for assessing chemicals that do not consider the microbial pesticides' unique properties such as proliferation, infectivity, or secondary metabolite toxicity. As a result, either no meaningful or inconsistent data were generated that impeded the



availability of these products in the farmers' integrated pest management toolbox.

The OECD conference on Innovating Microbial Pesticide Testing⁷ was an opportunity to provide an overview of the critical innovations that are needed in hazard testing for microbial pesticides and highlight the importance of developing NAMs as alternatives to animal testing. At the conference, the SARS-CoV-2 related AOPs were recognized as inspirational and a good starting point because they clearly illustrate the applicability of the framework to model complex viral infectious diseases.

Which AOPs

A first step would be to explore the available KEs and KERs of COVID-19 AOPs and reuse them for microbial pesticides, and in particular viruses. At the moment, the regulatory community is relying on existing consensus documents (i.e., on baculoviruses) (OECD, 2002) or similar documents that are under preparation by the OECD on microbial species commonly used as biopesticides (i.e., on *Bacillus amyloliquefaciens* and *Beauveria bassiana*). These documents capture evidence on mechanistic knowledge to better guide both registrants and regulators on performing and requesting, respectively, the most appropriate and meaningful hazard testing data. For example, demonstrating lack of infectivity to mammals using AOP information could potentially prevent unnecessary animal testing. Structuring this evidence using AOPs might reduce the need to repeat information and increase the reusability of information for more microbial species if applicable.

Challenges

Another important element of the risk assessment of bacterial and fungal biopesticides includes the investigation of the risks that may result from the production of metabolites, something not applicable to viruses (OECD, 2018). Some of these metabolites are key for their normal development and proliferation and are not considered to be of concern. Other metabolites have secondary functions, including communication and/or defense against other microorganisms and nutrient sequestration, and might pose a risk to human health or the environment. Being of chemical structure makes the secondary metabolite stressors applicable to the AOP framework as the framework was first conceived to accommodate chemical safety assessment needs. Some mycotoxins such as ochratoxin A and aflatoxin B1 are already entries available in the AOP-Wiki and could form the base to broaden the mechanistic knowledge in the field, trigger development of new *in vitro*-based bioassays, and facilitate risk assessment approaches such as read-across and QSARs.

Additional opportunities for AOP development come from the research area, where resources are being invested in targeted or untargeted methodologies based on omics technologies in conjunction with the toxicity assessment of microbial pesticides. The new data would potentially facilitate the exploration of new mechanistic pathways and the development of KEs. In summary,

the regulatory community of microbial pesticides has the opportunity to evaluate mechanistic knowledge and structure it using the AOP framework to advance in the field of risk assessment for these products.

3 Practical handling of the stressor evolution in the online AOP-Wiki

Inclusion of evidence for different types of stressors triggering AOPs has evolved over time with the pace and breadth of AOP development, and this evolution has been partially reflected in the AOP-Wiki. Given that the initial context for AOPs was the field of regulatory toxicology, the AOP-Wiki stressors module⁸ and the associated guidance are still largely focused on describing chemical stressors. Currently, the identifiers available for describing each stressor in a machine-readable manner only cover chemical-specific databases (e.g., DYXID, CASRN). Other relevant databases and ontologies, including chemical-related (e.g., OECD Toolbox), are expected to follow the consideration of different stressors in the number of AOPs developed discussed above. Indeed, nanomaterials (e.g., generic term and also specific PM2.5, carbon tubes), radiation (both ionizing and non-ionizing, UVB, etc.), viral (e.g., SARS-CoV-2, influenza), and other microorganisms (bacteria, fungi) have been added to the stressor list via their inclusion in relevant pathways.

Recently the term "prototypical stressor" was introduced (version 2.5, released in July 2022). The update aligned with needs previously identified by AOP-Wiki users for a functionality that would enable to search by stressor entities associated with AOPs in the AOP-Wiki. A prototypical stressor is a stressor that is known to trigger the MIE and for which there is extensive data with respect to its impacts on the downstream KEs. Prototypical stressors often serve as a focal point for literature searches and other assembly of empirical support. The list of prototypical stressors does not aim to be exhaustive but to provide representative examples.

At present, inclusion of stressors is only possible on the AOP page. This is different from earlier versions of the AOP-Wiki where stressors used in the studies or linked to the evidence informing the description of the AOP could be identified in each KE, in addition to the overall AOP description. Moreover, the stressor-specific evidence could be assigned a semi-quantitative "score" in relation to supporting the specific KE description and the overall AOP. However, guidance on how to assess such score was not as clear as for the evaluation of the weight of evidence supporting individual KERs and the overall AOP. In addition, evaluating stressor-related evidence for individual KEs outside of the context of an AOP (e.g., essentiality) or a KER (e.g., specificity, dose concordance) was considered misleading, implying a specific level of evidence (low, medium, high) for the hazard potential of the chemical in relation to an AO based only on an individual KE perturbation. To address this uncertainty, the stressor entry has been

⁷ <https://www.oecd.org/chemicalsafety/pesticides-biocides/conferenceoninnovatingmicrobialpesticidetesting.htm>

⁸ <https://aopwiki.org/stressors>

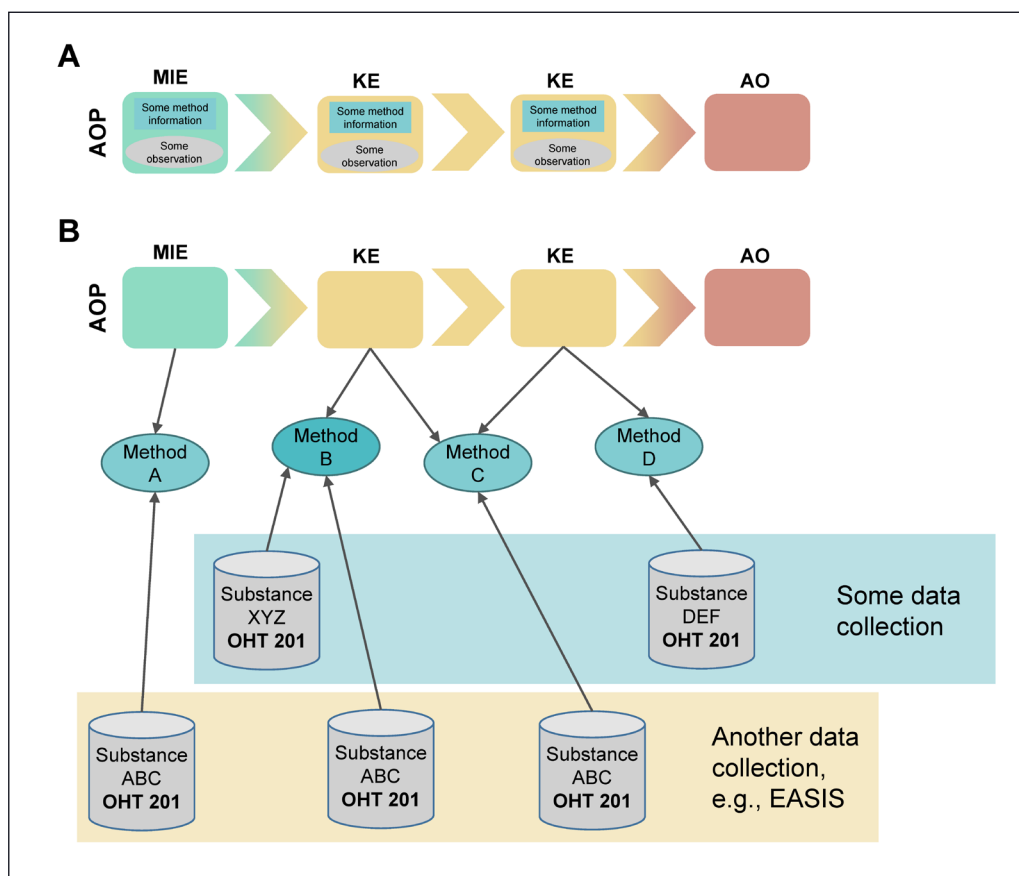


Fig. 2: Schematic current and desired situation of the integration of stressor-related evidence in the AOP-Wiki

(A) Current situation: The test method used to provide the observation as free text on the KE page is inseparably connected to one KE and cannot be re-used if the same test method was used in another KE. (B) Desired situation: The method description is separated from the KE description. One test method could be used to underpin one or several KEs, and one or several test methods could be used to produce evidence for one KE.

omitted from the KE development module in the AOP-Wiki. In this context, it may be useful to remember that application of AOP knowledge for addressing a particular regulatory or research question most often requires considering networks of interconnected AOPs (Knapen et al., 2018).

Another current challenge in the AOP-Wiki is the fact that stressor-related observation evidence is linked to the KE in an unstructured, free-text way, e.g., as a reference from a peer-reviewed paper or as a remark about an in-house experimental observation. The same applies to the other element in this connection: By describing the test method used to arrive at the observation as free text on the KE page, it is inseparably connected to this one KE and cannot be re-used if the same test method was used in another KE (Fig. 2A).

A current initiative, Methods2AOP^{2,9} suggests separating the method description from the KE description and establishing the connection between both domains by links that can depict the real situation. One test method could then be used to underpin one or several KEs, and one or several test methods could be used to produce evidence for one KE. Only then is it possible to connect stressor-related observation evidence to the KE, but – as is the

case in real life – via the test method that was used to produce it. Ideally, the stressor-related observation evidence is taken from an existing data collection, which manages and distributes data in a standardized format, e.g., the OECD Harmonised Template (OHT) format (Carnesecchi et al., 2023). Methods2AOP aims to modify the AOP-Wiki in a way that allows this more realistic depiction of the KE → test method → stressor evidence chain (Fig. 2B).

4 AOP stressors to bridge (eco)toxicology and disease biology

Considering different types of AOP stressors could be a way to bridge human toxicology, disease biology, ecotoxicology, and their communities. While those fields tend to stay in silos, in most real-world scenarios, humans and animals are exposed to not just one stressor at a time but rather to multiple stressors of different nature (chemical, physical, viral or biological). AOPs and AOP networks (Knapen et al., 2018; Villeneuve et al., 2018) can provide useful frameworks for investigating hazards and risks from combined exposures to multiple stressors. In risk as-

⁹ <https://ntp.niehs.nih.gov/sites/default/files/iccvm/meetings/sot23/sot2023-wittwehr-poster.pdf>

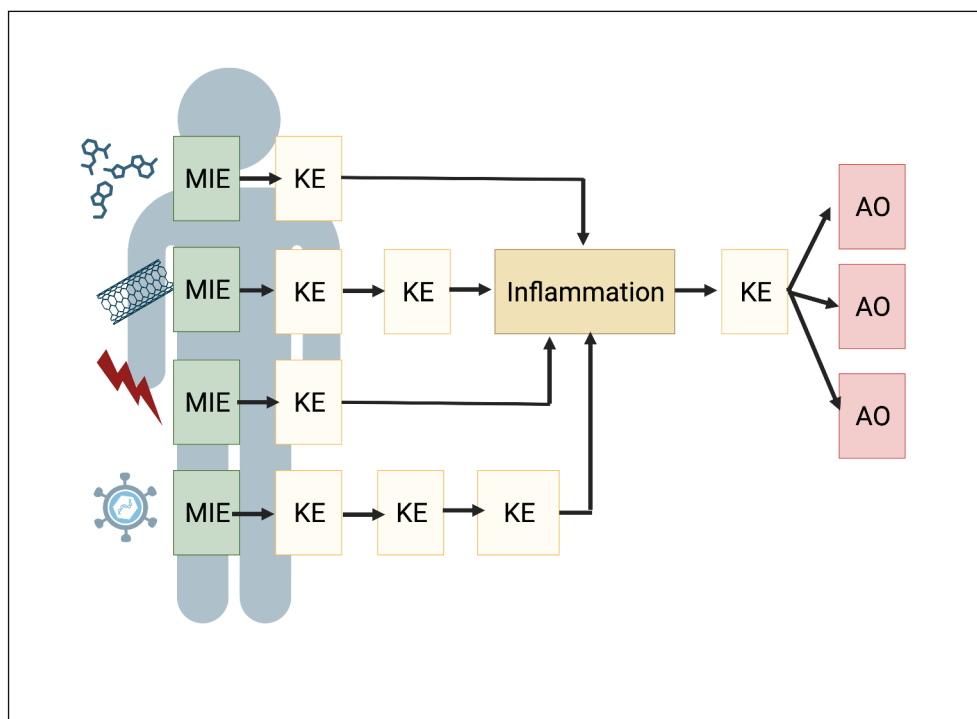


Fig. 3: Inflammatory key events (KEs) are shared by many adverse outcome pathways (AOPs) initiated by different types of stressors, chemicals, and non-chemicals in different tissues

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assessment of chemical mixtures, the AOP framework provides a structured basis for grouping substances based on common toxic effects or modes of action, identifying and collecting relevant toxicity data for the assessment, as well as identifying early/upstream KEs that can be used to calculate relative potency factors of mixture components (Beronius et al., 2020; Nymark et al., 2020). When expanding outside the chemical world, it might be challenging to know how to capture the impact of interactions between multiple types of stressors within AOP. However, it might also represent a game changer in identifying vulnerable individuals or populations.

During the development of COVID-19 related AOPs, around half of the KEs were already present in the AOP-Wiki, suggesting a broad similarity between the host response to chemical stressors and pathogens. Inflammation, for example, is shared across 30 individual AOPs describing adverse effects in diverse tissues and organisms in the AOP-Wiki. When developing AOP networks for nanomaterials (Halappanavar et al., 2020) and for SARS-CoV-2, the inflammatory response emerged as central, highlighting the cross-stressor applicability of these KEs related to inflammation (Fig. 3). The fine balance in the inflammatory response is key to determining the organisms' response strategy to stressor(s) exposure in many diseases and toxicological pathways. In the AOPs developed for excessive inflammation upon SARS-CoV-2 infection or CAR-T cell treatment, the proposed AO describes an overwhelming cytokine storm, which is not a physiological reaction. In addition, the identification of KE(s) and associated biomarkers where there is a switch from a physiologic to a pathologic reaction is currently ongoing using datasets from patients

in clinical trials. Modeling the inflammatory response within the AOP framework will facilitate insight into the critical events but also into the factors leading to overwhelmed or misdirected normal protective mechanisms. This will provide knowledge for the development of quantitative AOPs that could be instrumental to establish a threshold between physiological and pathological response. Thus, capturing the many stressors acting on the same downstream KEs can help to identify susceptibilities.

Besides inflammation, production of reactive oxygen species (ROS) is another event present in many AOPs because of its role in host defense (Pollesch et al., 2019). An ongoing international effort to harmonize the 20 ROS KEs currently existing in the AOP-Wiki will further enable improved use of the mechanistic understanding of ROS production by endogenous and exogenous stimuli and associated outcomes (Tanabe et al., 2022a,b, 2023).

Another interesting aspect when considering exposure to both chemical and viral stressors in order to identify vulnerable individuals is the distinction between a stressor and a modulating factor. According to the AOP principles, modulating factors alter quantitative aspects of the response-response function that describes the relationship between the two KEs (OECD, 2018). If stressor A and B have similar modes of action, they will act on the same MIE, activating the same AOP, leading to an additive effect on downstream KEs and AO if co-exposure to both stressors occurs. Thus, they are not considered modulating factors of each other in this scenario. In another scenario, exposure to stressor A causes induction of hepatic enzyme activity, quantitatively changing the shape of the KER and increasing sensitivity in downstream KEs in an AOP induced by stressor B. In this

case, stressor A would be considered a modulating factor. The challenge of characterizing an exposure as a stressor or a modulating factor was encountered in the CIAO⁶ project. In CIAO, exposure to environmental chemicals (specifically, perfluorinated substances, PFAS) was handled as a modulating factor that may contribute to severity of COVID-19 symptoms (Clerbaux et al., 2022a). It has been shown that exposure to PFAS during early development causes effects on immune system function (e.g., Abraham et al., 2020). It has also been hypothesized that exposure to environmental chemicals and nanoparticles can cause epigenetic regulation of critical immunologic genes and pathways affecting antiviral response and immune system function (Bulka et al., 2022; del Giudice et al., 2023). These types of effects support the role of exposure to chemicals as a modulating factor of COVID-19 pathogenesis by causing quantitative changes in immune system function. Integrity of the immune system is critical, and a dysfunctional immune system contributes to the organism response to stressor exposure (reviewed in Clerbaux et al., 2022a). However, the mechanisms of nanoparticles and PFAS immunotoxicity also share several KEs within the COVID-19 AOP-network, for example increased pro-inflammatory mediators. Hence it could be argued that environmental chemicals are also stressors of these AOPs. In general, it could be agreed that developmental exposure to chemicals that cause permanent changes in tissue and organ system function could be considered a modulating factor, distinguishing it from concurrent co-exposure to multiple stressors. However, it is likely not that simple. For example, some chemicals and nanomaterials are persistent and take years to be metabolized and excreted, if at all. Thus, theoretically they contribute to both developmental effects and concurrent exposure, i.e., “mixture effects”, later in life. Although life stage applicability is included in the AOP framework, these temporal considerations are currently difficult to capture fully.

A recent study that performed AOP enrichment with transcriptomics data suggests that an ancestral epigenetic mechanism may explain the shared immunomodulatory response to nanomaterials beyond the divergence of the individual studies (del Giudice et al., 2023). Such a conserved regulatory layer across species, anchored in new AOP-aligned tests, could bridge ecotoxicology and human toxicology, and also human toxicology (nanoparticles/chemicals could epigenetically reprogram biological systems) and disease biology (individual more susceptible to a virus exposure, for example).

Finally, developing AOPs is mainly possible though interdisciplinary collaboration bringing different expertise to the table and embracing mutual exchange. In the CIAO project, the AOP framework was effective in supporting a true interdisciplinary collaboration (Carusi et al., 2023). The framework provided a common reference point for discussion and collaboration. The AOP visuals assisted in making explicit the different perspectives brought to the knowledge about the pathways. Notably, it resulted in discovering commonalities between toxicology and virology, leading to a new understanding of how chemicals, nanoparticles, viruses, or cells used as therapeutics operate in a similar manner once the focus is on the pathways.

5 Conclusion

The AOP framework emerged in the context of regulatory toxicology to guide an effective paradigm shift towards mechanism-based chemical risk assessment. Rapidly, the framework became attractive to other fields that consider interaction of stressors with biological systems. The enthusiastic broader adoption of the AOP framework is due to its pragmatic and stressor-agnostic approach modeling pathways of biological perturbations, the common aspect of all these fields. Though inclusive in its nature and valuable in application, this principle has however been challenging within and outside of the toxicological domain of application.

Even in (eco)toxicology, new challenges arise as the community learns by doing. At present, the chemical-focused AOPs do not account for metabolic transformation or for the dynamics of the exposure, even though the significance of persistent/chronic versus acute chemical stressor exposure has been recognized in a number of AOPs and for a number of KEs, including MIEs.

Challenges related to nanomaterial AOPs include complex nanomaterial-biological system interactions where particular molecular interactions that initiate the pathway (specific MIE) cannot easily be pinpointed currently and require consideration of multiple cross-interacting MIEs. In addition, understanding of nanomaterial adsorption, distribution, stability and persistence, and adaptation needs of the current standard *in silico* modeling (Wyrzykowska et al., 2022) and *in vitro* testing systems needs further work (Gerloff et al., 2017; Halappanavar et al., 2021a,b, 2020).

Development of radiation-related AOPs highlighted twenty-five priority questions that are needed to be addressed for AOPs to support radiation research needs (Burt et al., 2022). Some challenges are related to defining KEs across different radiation types and for a varied dose rate of delivery, including accounting for the time elapsed. Furthermore, while a single MIE is discernible for chemicals, multiple macromolecules can concurrently be targeted by the ionization events, and the interpolation of these multiple KEs in quantitative risk modeling needs future consideration.

When exploiting AOPs for a viral disease, the need to describe the specific steps of SARS-CoV-2 replication was acknowledged and a viral “hub AOP” of linked stressor-specific KEs was developed. Viral production then can induce downstream stressor-agnostic KEs, such as inflammatory response shared in many AOPs triggered by diverse types of stressors (Clerbaux, 2022).

When developing an immune-related AOP for identifying potential safety issues of new cell immunotherapies, the challenges relate to the cellular nature and the therapeutic function of the stressor, as the AOP is intended to evaluate both the efficacy and toxicity of the cell-based treatment (Mazein et al., 2023).

Regarding emerging biological stressors such as microbial pesticides to control plant diseases, the human SARS-CoV-2 related AOPs were recognized as inspirational to account for the unique properties of viral and bacterial stressors such as proliferation and infectivity while the risks regarding the secondary metabolites of bacterial and fungal biopesticides, being of chemical structure, can be investigated via AOPs.



Thus, the common challenges of incorporating non-chemical stressors within the AOPs appear mainly related to the initial interactions of the stressor with the biological system. When adaptations are implemented to accommodate those considerations for MIE and early KEs, the strength of the framework to work as a mediator across disciplines is revealed in the downstream KEs. Inflammation, for example, is one of the most reported biological responses following exposure to chemical as well as non-chemical stressors. Hence inflammatory KEs are shared by many AOPs initiated by different stressors and in different tissues (Villeneuve et al., 2019).

Overall, AOP development remains a complex and challenging approach while holding great potential for better understanding the mechanisms of biological perturbations that are relevant to many fields beyond toxicology. Finding innovative ways to incorporate stressors in the AOP framework inspired by the lessons learned with the different chemical, physical, viral, and biological stressors is expected to be valuable also for the field of toxicology. In addition, by embracing diverse types of stressors, the AOP framework might represent a conceptual mediator between human toxicology, disease biology, ecotoxicology, and their communities to bridge knowledge on mechanisms of adversity and to account for real-world scenarios of combined exposures to multiple stressors.

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Conflict of interest

The authors declare they have no conflicts of interest.

Data availability

No datasets were generated or analyzed during this research.

Acknowledgements

The authors thank all the researchers involved in discussions to continuously accelerate and expand the use and development of the AOP framework.