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Research Article

A Strategy Towards the Generation of Testable Adverse Outcome Pathways for Nanomaterials

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Abstract

Manufactured nanomaterials (NMs) are increasingly used in a wide range of industrial applications leading to a constant increase in the market size of nano-enabled products. The increased production and use of NMs are constantly raising concerns among different stakeholder groups with regard to their effects on human and environmental health. Currently, nanosafety hazard assessment is still widely performed using *in vivo* (animal) models, however the development of robust and regulatory relevant strategies is required to prioritize and/or reduce animal testing. Adverse outcome pathways (AOPs) are a structured representation of biological events that start from a molecular initiating event (MIE) leading to an adverse outcome (AO) through a series of key events (KEs). The AOP framework offers great advancement to risk assessment and regulatory safety assessments. While AOPs for chemicals have been more frequently reported, AOPs collection for NMs is narrow. By using existing AOPs, we aimed to generate simple and testable strategies to predict if a given NM has the potential to induce a MIE leading to an AO through a series of KEs. Firstly, we identified potential MIEs or initial KEs reported for NMs in the literature. Then, we searched the identified MIE or initial KEs as keywords in the AOP-Wiki to find associated AOPs. Finally, using two case studies, we demonstrated here how *in vitro* strategies can be used for testing the identified MIE/KEs.

1 Introduction

Manufactured nanomaterials (NMs) are increasingly used in a wide range of industrial applications and novel NM-enabled products are routinely introduced into the market (Vance et al., 2015; Stark et al., 2015). Constant increase in production and use of NMs is raising concerns among different stakeholder groups including consumers, regulatory authorities and policy makers with regard to effects of NMs on human and environmental health. Decades of nanotoxicological research have revealed that small size and enhanced surface reactivity of NMs may induce adverse effects at both cellular and whole organism level (Shi et al., 2013; Murugadoss et al., 2017). However, analyses revealed that the time needed to complete *in vivo* toxicological evaluation of existing NMs by 2009 would take at least three to five decades (Choi et al., 2009). Consequently, there is demand for robust and regulatory relevant strategies to prioritize and/or to reduce animal testing. Global efforts are being made to implement the 3Rs (Replacement, Reduction and Refinement) concept (European Commission, 2020) that seek for alternative animal-free testing methodologies (Collins et al., 2017; Ostermann et al., 2020). However, many *in vitro* approaches for NMs toxicity evaluation are complicated due to the unique nano-specific properties that may induce different interferences with the test system and thus require either adaptation of existing or development of new methods less prone to biased results (Ostermann et al., 2020). Moreover, even small variabilities in the physico-chemical properties of NMs have been shown to influence the toxicological outcome, which further challenges the grouping and read across analysis of NMs. The development of intelligent and more efficient methodologies at lower costs are therefore urgently needed for hazard and risk assessment (RA) of NMs.

The Adverse Outcome Pathways (AOPs) framework can significantly support the advancement of RA approaches by developing predictive methods that utilize mechanistic and evidence-based data. The AOPs, as described a decade ago (Ankley et al., 2010), refer to conceptual structures portraying biological failures initiated by the interaction of a chemical with

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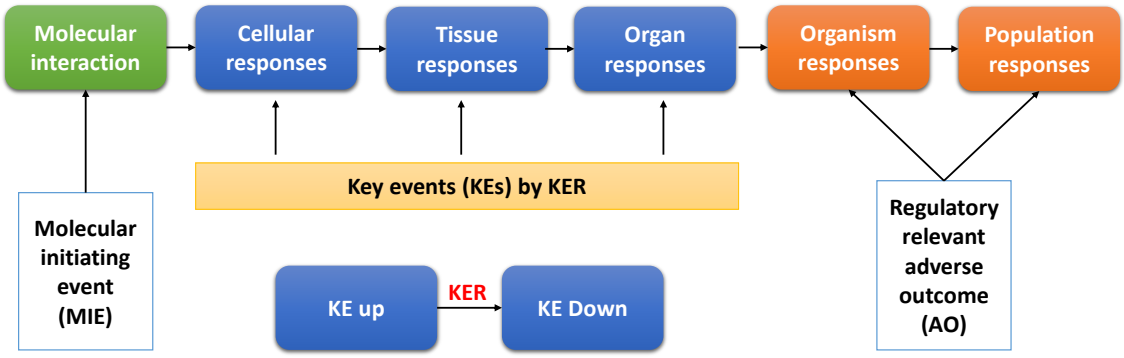

Fig. 1: A schematic representation of the Adverse Outcome Pathway (AOP) framework (inspired by Sachana et al. 2018) An AOP is triggered by a Molecular Initiating Event (MIE), an initial interaction with a biological target (Anchor 1) that leads to a sequential cascade of cellular, tissue and organ responses (Key Events), linked to each other by key event relationship (KER) to result in an adverse outcome (AO) of regulatory relevance.

a biomolecule or biosystem that can perturb normal biology, impairing critical function and leading to adverse outcome(s) (AO) at organism or population level. As shown in Figure 1, AOP comprise a series of key events (KEs) along a biological pathway from the molecular initiating event (MIE) to the AO. As such, AOPs framework provides systematic knowledge about key toxic mechanisms, thus being very effective to characterize the individual biological impact and toxicological potential of substances and significantly improving the prediction of adverse effects. Worldwide, there are many initiatives for further development and advancement of AOPs framework; at European level, the OECD has made significant efforts in this direction and initiated an AOP Development Programme in 2012. In joint collaboration with the U.S. EPA and the U.S. Army Engineer Research and Development Center, the EC's Joint Research Centre launched in 2014 the AOP Knowledge Base (AOP-KB) as a web-based tool encompassing the e.AOP.Portal, the AOP-Wiki, the Effectopedia, the AOP Xplorer and the Intermediate Effects Database (Delrue et al., 2016). In 2016, NanoAOP project (OECD, 2020) was started by the OECD Working Party on Manufactured Nanomaterials (WPMN) to support the development of future AOPs for NMs RA and categorization. The AOPs framework offers great advancement to RA and human hazard assessments of NMs that often dissent from classical dose-response relationships and exhibit particulate-specific toxicity. Indeed, even small changes in their physico-chemical properties may significantly impair the nano-bio interface, aggravating predictability of traditional RA tools and methods.

It has been discussed that existing AOPs for chemicals can be potentially used for NMs as these may share similar KEs with chemicals (Ede et al., 2020). The AOP provides the mechanistic representation of an AO initiated by a MIE, thus reflecting the molecular level and making possible the connection with NMs' physico-chemical properties via *in silico* tools, such as quantitative analyses of structure-activity relationships (QSARs). While extensive efforts have been given to the development of AOPs for chemicals, AOPs specific to NMs are still scarce. At the end of 2020, the OECD WPMN, as part of the NanoAOP project, reported a methodology to identify, analyze and evaluate existing scientific data to prioritize NMs-relevant KEs and finally to contribute to the development of a knowledge base to inform AOP development and assessment for NMs (OECD, 2020). One of the main outcomes of this project was the development of a case study on inflammation pathway and analysis of a specific KE for this pathway to establish an approach to advance future NMs-related AOP (OECD, 2020). Gerloff et al. (2017) in his study attempted to merge existing chemically induced liver fibrosis AOPs and proposed a putative AOP for metal oxide NMs by combining *in vivo* and *in vitro* literature data obtained for TiO_2 and SiO_2 NMs. However, the potential application of chemical AOPs to NMs is not comprehensively explored.

Thus, we aimed to generate simple and testable strategies for development of AOPs for NMs that are of relevance for human health. Our approach is based on using existing AOPs to predict if a given NM has the potential to induce an MIE leading to an AO through a series of KEs. Firstly, we identified potential MIEs reported for NMs in the literature. Then, we searched in the AOP-Wiki using the identified MIE (as keywords) to find associated AOPs that can be applied to NMs and can be verified using *in vitro* and *in silico* approaches, through testing the KEs involved.

2 Methodology

The first step of generating testable AOPs for NMs was a literature search using the following scientific databases: PUBMED, EMBASE, SCOPUS and WEB OF SCIENCE. The search, performed in the period until 1/12/2020 using the key words "adverse outcome pathway" OR "AOP" AND "nano*", resulted in 960 papers in total. After careful analysis and refinement on duplicates, reviews, AOPs/AOs reports and type of organisms studied therein, 32 papers were selected for further analysis that covered both *in vitro* and *in vivo* studies on mammals. Next, each of the selected papers was evaluated by the software-based tool ToxRTool (European Commission, 2013) , which assesses the reliability of *in vivo* or *in vitro* human toxicity studies. According to the criteria described by (Klimisch et al., 1997), this evaluation revealed that only 15% of selected papers provided reliable data with some restrictions, i.e. data are potentially useful, but their relevance should be checked for intended purpose. The rest of papers (85%) were evaluated as providing reliable data without restrictions. The papers were then analyzed for data extraction, which included the identification of NMs-induced AOs and MIEs relevant for NMs. In this study, we focused on the identification of initiating events relevant for NMs because chemicals may share common KEs with NMs but major differences lie in their way of interaction with biological targets. To achieve this, the AOs and the respective first event (mainly molecular/cellular level key event) reported in each study were summarized. When analyzing these data, we found that certain events were consistently reported**.** Finally, the identified initiating events were used to search the AOP-Wiki for potential AOPs

applicable for NMs and all the AOPs linked to each of used keywords were summarized. Since inhalation and ingestion are the primary routes of NM exposure, we focused on lung and liver fibrosis, respectively, to describe our strategy to generate testable AOPs.

3 Results

3.1 Identification of (molecular) initiating events relevant for NMs

To identify potential MIEs relevant for NMs, AOs reported in each of the selected research papers and their respective first event reported/identified were consolidated as presented in Table 1. The KEs can be described as a measurable change in the biological state representing essential event for further biological effect(s) and progression towards the AO, but not bridging levels of biological organization. The critical step in the AOP development is the identification of MIE that is defined as "the initial interaction between a molecule and a biomolecule or biosystem that can be causally linked to an outcome via a pathway" (Villeneuve et al., 2014). In this definition, "a molecule" can be replaced by a NM, but the chemistry of MIE should be carefully described to provide a coherent link between the physico-chemical properties of NMs and MIE that is stronger than the links to adverse endpoints (Allen et al., 2014). When analyzing the extracted data from papers (Table 1), five potential MIEs for NMs were identified: (i) Interaction of particles/fibers with cell membranes/biomolecules (ii) reactive oxygen species (ROS) formation/generation, (iii) lysosomal injury/damage/disruption (iv) DNA damage/methylation and (v) inflammation induction. All these initial KEs were obtained from both *in vitro* and *in vivo* studies. Instead of MIE, the term "initial key event (initial KE)" was used in subsequent sections because not all identified events occur at the molecular level.

Tab. 1: Summary of AO and their respective (M)IE

Reference	Types of particles used	Adverse outcomes (AO)	Models	First event reported in the study
Ndika et al., 2018	Single-walled (SWCNTs) and Multi-walled carbon nanotubes (MWCNTs)	Cell death and DNA repair impairment	*in vitro*	Interaction of fibres with cell membranes
Barosova et al., 2020	MWCNTs and silica quartz particles	Lung fibrosis	*in vitro*	Interaction of particles/fibres with cell membranes
Bezerra et al., 2020	SWCNTs, Titanium dioxide (TiO_2) nanoparticles (NPs) and fullerenes	Skin sensitization	*in vitro*	Interaction of particles with skin proteins
Zhang et al., 2018a	rare earth oxide, Zinc oxide (ZnO), Silver (Ag), TiO_2 and iron oxide NPs	Compromised phagocytosis	*in vitro*	Interaction of particles with biomolecules/membrane
Nikota et al., 2017	Multi-walled carbon nanotubes (MWCNTs)	Lung fibrosis	*in vivo*	Interaction of fibres with cell membranes
Huaux et al., 2016	MWCNTs	Mesothelioma	*in vivo*	Interaction of fibres with cell membranes
Labib et al., 2016	MWCNTs	Lung fibrosis	*in vivo*	Interaction of fibres with cell membranes
Shvedova et al., 2016	MWCNTs	Pulmonary inflammation and fibrosis	*in vivo*	Interaction of fibres with cell membranes
Nikota et al., 2016	MWCNTs	Lung fibrosis	*in vivo*	Interaction of fibres with cell membranes
Pavan and Fubini, 2017	Crystalline silica	Persistent lung inflammation	*in vitro* and *in vivo*	Interaction of particles with cell membranes and membrinolysis
Dekkers et al., 2018	Ag, ZnO and cerium oxide (CeO_2) NPs	Death and cancer progression	*in vitro*	ROS formation
Garcia-Reyero et al., 2014	PVP coated Ag NPs	Liver and brain damage	*in vitro*	ROS formation and dopamine receptor antagonism
Boyles et al., 2016	Copper oxide (CuO) NPs	Apoptosis	*in vitro*	ROS formation and accumulation of amino acid and glycerophosphocholine
Pisani et al., 2015	Fumed silica NPs	Cell death	*in vitro*	ROS formation
Duan et al., 2016	Silica, Fe_3O_4 and cobalt oxide (CoO) nanoparticles	Apoptosis	*in vitro*	ROS formation
Yang et al., 2010	Copper (Cu) NPs	Weight loss	*in vivo*	ROS formation
Lei et al., 2015	Cu NPs	Liver and kidney damage	*in vivo*	MDA formation and mitochondrial dysfunction
Hansjosten et al., 2018	CeO_2, ZnO, TiO_2, Ag and silica NPs	Cell death	*in vitro*	Lysosomal acidification
Wang et al., 2015	SWCNTS, graphene and graphene oxide	Lung fibrosis	*in vivo* and *in vitro*	Lysosome injury

Wang et al., 2015	SWCNTs	Collagen deposition	*in vitro* and *in vivo*	Lysosome injury
Bourdon et al., 2013	Carbon black NPs	Lung fibrosis	*in vitro*	DNA damage
Chen et al., 2017	Ag, Gold (Au), TiO_2, ZnO, CNTs and graphene oxide	Impaired cytoskeleton	*in vitro*	DNA methylation
Scala, 2018	10 different types of carbon NPs	Cancer	*in vitro*	DNA methylation
Gomes et al., 2017	Coated and uncoated Ag NPs	Decreased reproduction and increased mortality	*in vivo*	DNA damage, apoptosis stimulation and ROS formation
Pisani et al., 2017	Magnetic (core-FE_2O_3) mesoporous silica nanocarriers	Cholestatic liver injury	*in vitro*	Induction of IL1 and TNFα/BSEP-inhibition
Ma et al., 2017	Coated and uncoated MWCNTs	Systemic inflammation and anemia	*in vivo*	Induction of IL6
Aragon et al., 2017	MWCNTs	Systemic (neuro) inflammation	*in vivo*	Inflammation in the lung
Ma et al., 2016	Carboxylated MWCNTs	Arthritis	*in vivo* and *in vitro*	Induction of IL1β and TNFα *in vitro* or TNFα and IL6 *in vivo*
Poon et al., 2017	TiO_2, ZnO and Ag NPs	Immune system dysregulation	*in vitro*	Activation of intracellular pattern recognition receptors
Thai et al., 2019	TiO_2 and CeO_2 NPs	Liver and Lung damage	*in vitro*	Altered signaling pathways associated with cytotoxicity
Hao et al., 2017	ZnO NPs	Systemic shortage of lipid or hepatic steatosis	*in vivo*	Altered expression of lipid synthesis of liver growth factors and apoptotic genes
Zhang et al., 2018b	Gadolinium and Manganese oxide NPs	Kidney damage	*in vivo*	Interruption of calcium homeostasis

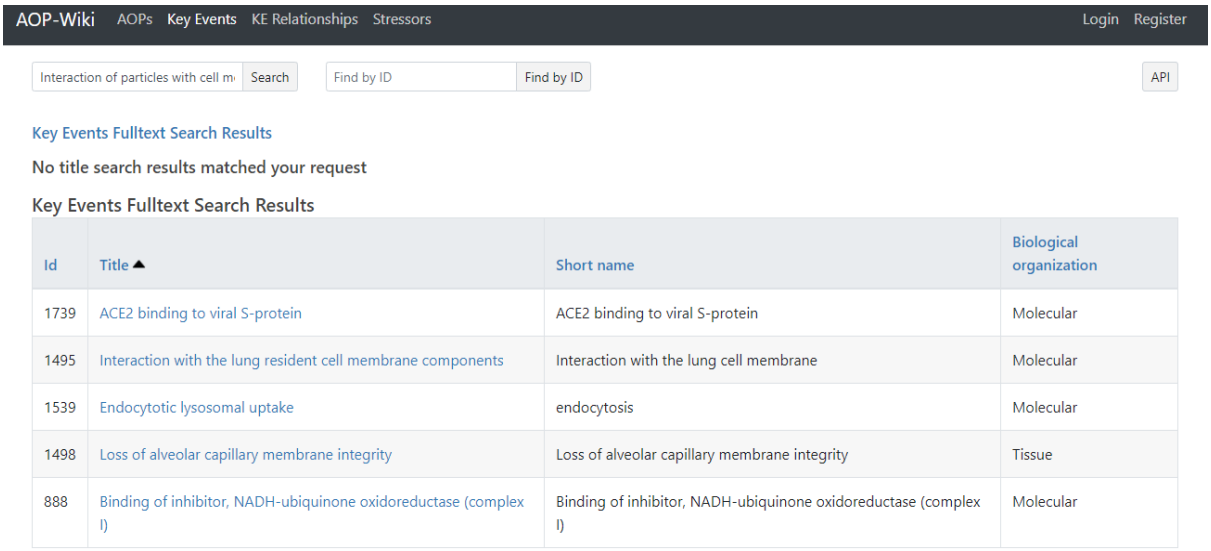

Fig. 2: Screenshot of AOP-Wiki page during the search for potential AOPs using "Interaction of particles with cell membranes" in "key event" search tab

3.2 Identification of potential AOPs in the AOP-Wiki

The AOP-Wiki "Key Events" module of the freely accessible web-based AOP-KB was used to identify AOPs applicable for NMs. Search revealed several titles linked to initial KEs identified in the first step (Table 1), e.g., the results for "Interaction of particles with cell membranes" as depicted in Figure 2. Then the AOPs linked to each of these titles were retrieved: Table 2 shows the potential AOPs found in the AOP-Wiki linked to each of these initial KEs. We did not use the term "inflammation" in the search as it was widely recognized as KE rather than initiating event (Halappanavar et al., 2019). A detailed analysis of titles linked to different keyword search and identification of associated AOPs is provided in file S1[1].

3.3 Generation of testable strategies using simple *in vitro/in silico* experiments

During this work, initial KEs are considered as one critical component that can be shared by more than one pathway. The sequence of intermediate KEs connecting the initial KE with AOs should be described including the definition of the biological state, methods used for intermediate KEs observation and measurement as well as evaluation of taxonomic applicability of a

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Tab. 2: Summary of AOPs associated with NMs-relevant initial KEs identified from the literature search
Different keywords for each initial KE were used to retrieve all AOPs from the AOP-Wiki that can be explored for NMs.

Key word search	Associated AOPs	AOP number
Interaction of particles/fibres with cell membranes, Interaction of particles/fibres with biomolecules	Substance interaction with the lung cell membrane leading to **lung fibrosis**	173
	Ionizing energy leading to **lung cancer**	272
	lysosomal uptake induced **liver fibrosis**	144
	Mitochondrial complex inhibition leading to **liver injury**	273
	Lung surfactant function inhibition leading to immediate **adverse lung effects**	302
	ACE2 binding to viral S protein, Acute **respiratory distress**	320
	Mitochondrial dysfunction and **neurotoxicity**	3
	Chemical binding to tubulin in oocytes leading to **aneuploid offspring**	106
	Complex I inhibition leads to **Fanconi syndrome**	276
	Receptor mediated endocytosis and lysosomal overload leading to **kidney toxicity**	257
	ionotropic glutamatergic receptors and **cognition**	48
Lysosomal damage, lysosomal disruption, lysosomal injury	Substance interaction with the lung cell membrane leading to **lung fibrosis**	173
	Lysosomal uptake induced **liver fibrosis**	144
	Protein alkylation to **liver fibrosis**	38
	IKK complex inhibition leading to **liver injury**	278
	Mitochondrial complex inhibition leading to **liver injury**	273
	Increased DNA damage leading to **breast cancer**	293
	RONS leading to **breast cancer**	294
	Oxidative stress and d**evelopmental impairment** in learning and memory	17
	Receptor mediated endocytosis and lysosomal overload leading to **kidney toxicity**	257
	Mitochondrial dysfunction and **neurotoxicity**	3
	ionotropic glutamatergic receptors and **cognition**	48
	Binding of antagonist to NMDARs impairs **cognition**	13
	Binding of antagonist to NMDARs can lead to **neuroinflammation and neurodegeneration**	12
	AChE inhibition leading to **neurodegeneration**	281
DNA damage, Oxidative DNA damage, DNA strand breaks, DNA methylation	Oxidative DNA damage, chromosomal aberrations and **mutations**	296
	ER activation to **breast cancer**	200
	Increased DNA damage leading to **breast cancer**	293
	RONS leading to **breast cancer**	294
	Excessive ROS leading to **mortality**	330
	Frustrated phagocytosis-induced **lung cancer**	303
	Ionizing energy leading to **lung cancer**	272
	ROS production leading to population decline via **follicular atresia**	216
	Uncoupling of OXPHOS leading to **growth inhibition**	266
	Thermal stress leading to **population decline**	325
	NADPH oxidase activation leading to **reproductive failure**	207
	Alkylation of DNA leading to **reduced sperm count**	322
	DNMT inhibition leading to **population decline** (1)	336
	DNMT inhibition leading to **population decline** (2)	337
	DNMT inhibition leading to **transgenerational effects** (1)	340
	DNMT inhibition leading to **transgenerational effects** (2)	341
	PPARG modification leading to **adipogenesis**	72
	Thermal stress leading to **population decline** (3)	326
Reactive oxygen species, ROS formation, ROS generation	Chronic ROS leading to human treatment-resistant **gastric cancer**	298
	Frustrated phagocytosis-induced **lung cancer**	303
	Mitochondrial complex inhibition leading to **liver injury**	273
	Cholestatic Liver Injury induced by Inhibition of the Bile Salt Export Pump (ABCB11)	27
	Inhibition fatty acid beta oxidation leading to **nonalcoholic steatohepatitiis** (NASH)	213
	unknown MIE **renal failure**	186
	Calcium-mediated neuronal ROS production and **energy imbalance**	26
	Excessive ROS leading to **mortality**	327
	Excessive ROS leading to **mortality**	328
	Excessive ROS leading to **mortality**	329
	Excessive ROS leading to **mortality**	330
	Uncoupling of OXPHOS leading to **growth inhibition**	266
	Uncoupling of OXPHOS leading to **growth inhibition**	267
	Uncoupling of OXPHOS leading to **growth inhibition**	268
	ROS production leading to **population decline** via mitochondrial dysfunction	311
	ROS production leading to **population decline** via follicular atresia	216
	Thermal stress leading to **population decline**	325
	Thermal stress leading to **population decline**	326
	NADPH oxidase activation leading to **reproductive failure**	207
	Reactive oxygen species generated from photoreactive chemicals leading to **phototoxic reactions**	282
	ROS production leading to population decline via **reduced FAO**	299
	ROS production leading to population **decline via LPO**	238

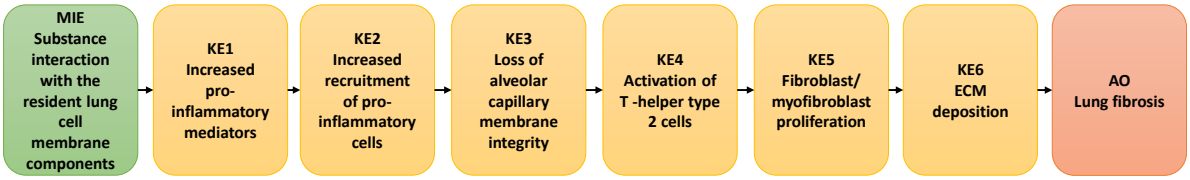

Fig. 3: Schematic representation of the AOP 173

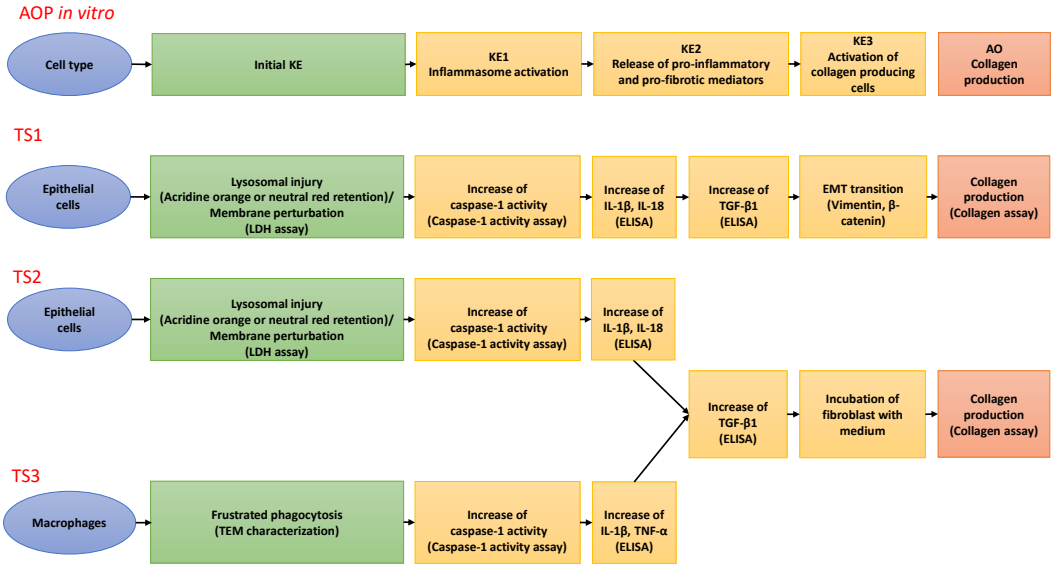

Fig. 4: Proposed in vitro strategy to test the potential of a NM to induce lung fibrosis

particular KE (Villeneuve et al., 2014). Another important AOP component is the KE relationship (KER) that is supported by empirical evidences and establishes directed and quantitative relationships between KEs. Weight of evidence for KER can be obtained by literature search (as in our case), targeted experiments, data mining, or modelling approaches. Finally, the utilization of a particular AOP within AOP-KB as basis for AOP network relies on a simple AOP description. It is important to mention here that our main objective was to extract and integrate relevant information from the literature/database to build a strategy to test the potential of an NM to induce an initiating KE leading to AO through causally linked KEs. Our final goal is to generate AOPs that can be tested by *in vitro*/*in silico* tools in compliance with the 3Rs principle.

3.3.1 Case study 1: Lung fibrosis

AOP 173 (Figure 3, substance interaction with lung epithelial and macrophage cell membrane leading to lung fibrosis) has been the most discussed AOP for its potential application for NMs. Briefly, the interaction between the substance and components of the cellular membrane (MIE) leads to the release of pro-inflammatory mediators (KE1) that promote the recruitment of pro-inflammatory cells into the lungs (KE2). Persistent inflammation leads to the loss of alveolar capillary membrane integrity (KE3) and activation of adaptive immune response (T Helper type 2 activation) (KE4), during which anti-inflammatory and pro-repair/fibrotic molecules are secreted. The repair and healing process stimulates fibroblast proliferation and myofibroblast differentiation (KE5), leading to synthesis and deposition of an extracellular matrix or collagen (KE6), and eventually lung fibrosis (AO). It appeared that some of the components of this AOP cannot be replaced with *in vitro* cellular assays (such as KE2, KE3 and KE4).

NMs, particularly carbon nanotubes (CNTs), were shown to induce lung fibrosis *in vivo* via different interactions and pathways. When mining the literature, we found a recent comprehensive pathway analysis of *in vitro* results relating to multi-walled (MW) CNTs-induced lung fibrosis (Vietti et al., 2016). Based on this information, we propose an AOP consisting of the major KEs that can be tested/verified under *in vitro* settings. Figure 4 shows the AOP aligned initial KE-KEs-AO pattern that can be measured *in vitro* to predict the lung fibrotic responses *in vivo* and different strategies to test the potential of a given NM to induce an AO related to lung fibrosis. In this *in vitro* testable AOP, we propose KE6 of AOP 173 to become AO.

Frustrated phagocytosis and lysosomal injury of MWCNTs are the key determinants of lung fibrosis initiating events (Vietti et al., 2016). When mining the literature, we have also found that high aspect ratio nanomaterials such as nanowires, nanorods and other NMs, such as fumed silica and cerium oxide also induce inflammasome activation via lysosomal injury, membrane perturbation and/or frustrated phagocytosis (Wang et al. 2017). Despite the lack of information that inflammasome activation induced by these NMs potentially can lead to lung fibrosis, the downstream biological processes of the inflammasome activation induced by different NMs could be similar to MWCNTs. Therefore, we use the existing information specific for MWCNTs and propose the following strategy to test the potential of NM to induce an AO related to lung fibrosis.

Epithelial cells are the first to encounter with NMs once they reach the deeper parts of the lung such as bronchioles and alveoli. The NM interaction with epithelial cells (bronchial or alveolar) could activate NLRP3 (NOD-like receptor family,

LRR- and pyrin domain containing 3) inflammasome activation either by lysosomal injury (can be measured by assays such as acridine orange or neutral red uptake), or membrane perturbation (assay such as lactate dehydrogenase (LDH) release), and promote pro-inflammatory and pro-fibrotic mediators release such as IL-1β and IL-18. Caspase-1 activation is an essential component of inflammasome activation and processing of IL-1β and IL-18. Therefore, caspase-1 activity can be measured as an indicator of inflammasome activation (KE 1). Subsequently IL-1β and IL-18 release can be measured by ELISA in the medium of the cell cultures to quantify pro-inflammatory and pro-fibrotic mediators release (KE 2).

As secreted cytokines may act in different pathways (see Figure 4), we propose three test-strategies (TS):

TS1: IL-1β promotes the secretion of TGF-β1, which plays a key role in the epithelial-mesenchymal transition (EMT) (KE3). EMT transition can be measured by quantifying mesenchymal cell markers such as vimentin and β catenin. These polarized epithelial cells are involved in the production of collagen (measured by collagen assay).

TS2: IL-1β promotes the secretion of TGF-β1, which plays a key role in fibroblast activation and proliferation. Activated fibroblasts (KE3) are involved in the production of collagen. IL-18 is also involved in the direct activation of fibroblasts. Collagen production in exposed epithelial cells (TS1) and lung fibroblasts (TS2) can be measured as a representative *in vitro* AO to predict lung fibrosis *in vivo.*

TS3: Macrophages, the first line of defense that engulfs NMs by phagocytosis, also play a key role in the development of lung fibrosis. Upon inflammasome activation due to frustrated phagocytosis (characterized by TEM), macrophages secrete IL-1β and TNF-α, which are involved in promoting TGF-β1, which in-turn activates fibroblasts (KE3) and promotes collagen production.

From the obtained results, the potency of a NM to trigger a MIE leading to AO (collagen production) via any of these pathways (TS1, 2 and 3) can be determined.

3.3.2 Case study 2: Liver fibrosis

The liver is known to be one of the main target organs for ingested NMs. Therefore, we explored the AOPs for liver fibrosis presented in AOP-Wiki to generate *in vitro* test strategies for NMs.

The scheme's shown in Figure 5 both lead to liver fibrosis as follows:

- AOP 144 with MIE endocytic lysosomal uptake: Endocytic lysosomal uptake (MIE) of stressor leads to lysosomal disruption (KE1), which induces subsequent KEs at the cellular level such as mitochondrial dysfunction (KE2), cell injury and apoptosis/necrosis (KE3). Cell death leads to increased production of pro-inflammatory mediators (KE4), which attract and activate leukocytes (KE5). Activated leukocytes through molecular mediators activate hepatic stellate cells (HSCs) (KE6), which increase the accumulation of collagen (KE7) leading to extracellular matrix (ECM) alteration and AO - liver fibrosis.
- AOP 38 with protein alkylation: presented with similar downstream KEs as in AOP 144 (KE3, KE6 AND KE7) except that liver tissue resident macrophages released mediators, which activate HSC (instead of mediators released by leukocytes) and with protein alkylation as MIE (Figure 5).

In the literature, it has been reported that several NMs induce lysosomal disruption and apoptosis/necrosis via lysosomal membrane permeabilization (LMP) (Stern et al., 2012). Therefore, we propose test strategies using simple *in vitro* experiments with LMP as an initial KE (Figure 6).

TS 1: Hepatocytes (epithelial cells) can be used as a cell model as ingested NMs, once entering the liver via portal vein, encounter the epithelial layer of the liver. NM-induced LMP (measured by assays such as neutral red/acridine orange) and KE1 mitochondrial dysfunction (mitochondrial membrane potential, MMP) of hepatocytes lead to KE2 cell injury (cytotoxicity assays such as WST-1, LDH). Release of damage associated molecular pattern (DAMP) mediators, and cytokines such as TNF-α and TGF-β, are involved in the direct activation of HSCs (KE3), a major source of collagen producing cells (AO) in the liver (Liu et al., 2012; Li et al., 2008). HSCs also become activated upon engulfment of DNA fragments from apoptotic hepatocytes (Li et al., 2008). To verify this, HSCs can be incubated with cell culture medium collected from NM-exposed hepatocytes cultures and collagen production in exposed HSCs can be measured as a representative *in vitro* AO to predict liver fibrosis *in vivo*.

TS2: Leukocytes, upon activation secrete TGF-β1 and TNF-α (KE3), which in turn activate HSCs (KE4) that produce collagen[2]. Therefore, leukocytes such as monocytes can be incubated with NMs and subsequently, HSCs can be incubated with the medium collected from monocytes, and collagen production in exposed HSCs can be measured as a representative *in vitro* AO to predict liver fibrosis *in vivo.*

TS3: Kupffer cells, liver resident macrophages, also play a key role in the development of liver fibrosis. Activated Kupffer cells can secrete pro-inflammatory mediators such as TNF-α and IL-6 and pro-fibrotic mediator TGF-β, which leads to HSC activation[3] (Liu et al., 2012). To verify this, Kupffer cells can be incubated with NMs and subsequently, HSCs can be incubated with the medium collected from Kupffer cells, and collagen production in exposed HSC cells can be measured.

From the obtained results, the potency of a NM to trigger an initial KE leading to AO (collagen production) via any of these pathways (TS1, 2 and 3) can be determined.

[2] AOP-Wiki. Activation, Stellate cells leads to Accumulation, Collagen, KER 295. https://aopwiki.org/relationships/295 [Accessed February 14, 2021].
[3] AOP-Wiki. Tissue resident cell activation, KE 1492. https://aopwiki.org/events/1492 [Accessed February 14, 2021].

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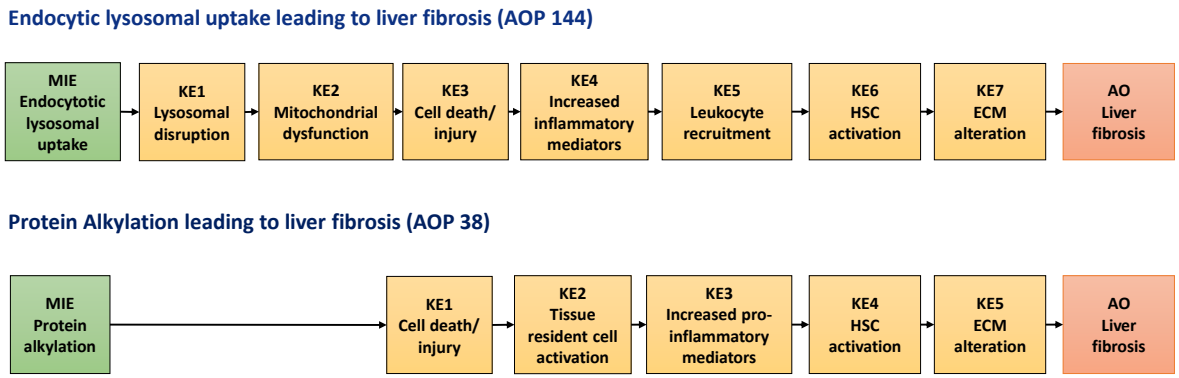

Fig. 5: Schematic representation of liver fibrosis AOPs as presented in the AOP-Wiki

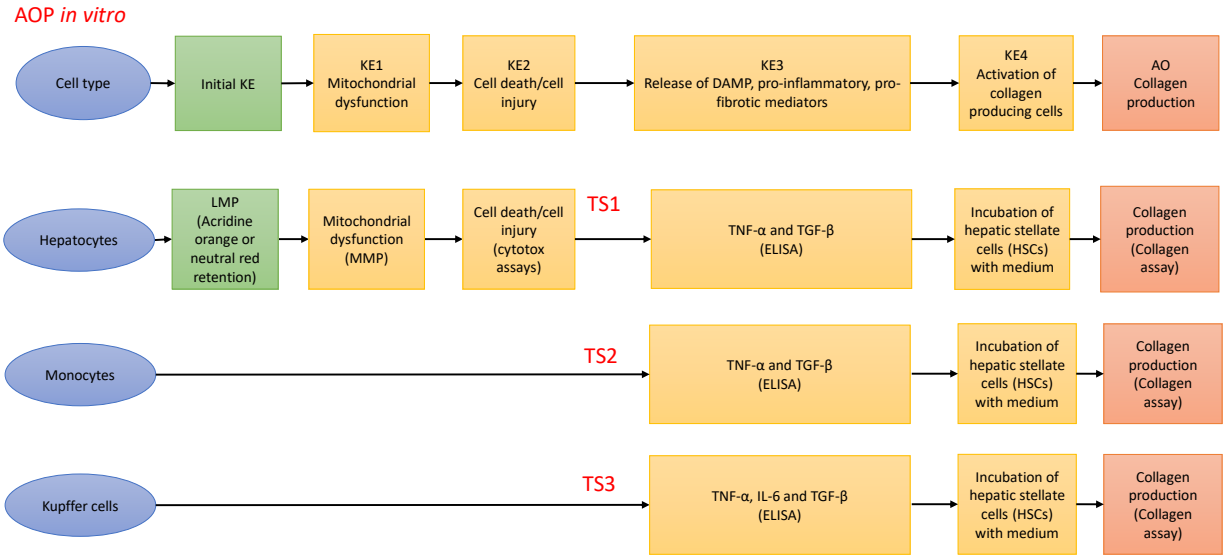

Fig. 6: Proposed in vitro strategy to test the potential of a NM to induce liver fibrosis

4 Discussion

The increasing number of applications of nanotechnology and the fast-growing market of nanoproducts creates an urgent need for the development of strategies to perform a fast and reliable safety testing and hazard assessment of NMs. The AOPs framework represents an important regulatory-relevant aid in predicting the adverse effects of NMs that may foster reduction/elimination of animal testing. In this paper, we described a simple strategy for AOP implementation in nanotoxicology to facilitate the fast screening of NMs' safety and to provide an efficient aid in regulatory decision-making and safe(r)-by-design approach to the development and use of NMs.

Chemical AOPs are not stressor specific and we assumed that they can be used to postulate the downstream effects of NMs if proper MIEs relevant for NMs were identified. Therefore, we systematically explored existing AOPs in AOP-Wiki using NMs-relevant initiating KEs identified in the literature and proposed *in vitro* strategies to test the potential of a NM to induce lung and liver fibrosis as two AO case studies. In addition to these AOPs, we realized that several AOPs identified in the AOP-Wiki related to liver injury (AOP 273), kidney toxicity (AOP 257), neurotoxicity (AOP 3), and breast cancer (AOP 293) can also be potentially explored for NMs. However, more information on NMs biodistribution in organs such as brain and breast are required in order to justify the use of these AOPs that were proposed for conventional chemicals.

A recent review by Halappanavar et al. (2021) suggest that cell death, membrane integrity, ROS/RNS and cytokines are some of the *in vitro* biological endpoints that are widely reported in nanotoxicological studies, but, their relevance in terms of predicting the AO is unknown. AOPs are relevant in the context of organizing the existing information to establish relationships between key biological endpoints for the AO prediction. Despite more than 200 AOPs currently available in the AOP-Wiki, it is important to note that only a handful of AOPs have been formally validated or endorsed by the OECD. Furthermore, *in vitro* exposure models and assays that are currently being used to measure KEs need to be validated for testing of NMs. Until reaching the stage of availability of validated in *vitro* assays and exposure models, as well as AOPs for NMs, our strategy based on existing AOPs is useful in the following contexts: (i) addressing the knowledge gaps in AOPs, (ii) early screening of NM safety assessment to prioritize animal research, and (iii) determining the influence of NM property, NM concentration and duration of exposure in observing AO. In order to test the strategies proposed for lung and liver fibrosis, it

could be worth focusing on MWCNTs because of the countless number of variants differ in length, diameter, rigidness, functional groups and impurities and the data already for some of them pointing out an effect on lung fibrosis *in vivo* (Porter et al., 2010; Mercer et al., 2011; Duke and Bonner, 2018) .

Very recently, based on *in vitro* and *in vivo* studies, AOPs related to the carcinogenicity of TiO_2 NMs (Braakhuis et al., 2021) and AOPs related to steatosis, oedema and fibrosis in the liver induced upon TiO_2 exposure (Brand et al., 2020; Gerloff et al., 2017) have been postulated. When analyzing these AOPs, we found that lysosomal injury or lysosomal membrane permeabilization, ROS formation and DNA damage have been described as early KEs. These initiating KEs have been identified also in our study indicating their potential usefulness in the characterization of NMs-related MIEs. Particularly, with the application of high throughput screening and high content assays, a large amount of NMs can be screened for their potentially hazardous nature. Application of nano-specific AOPs for human health risk assessment cannot be efficient without understanding the *in vitro*-*in vivo* correlation. Predictability of *in vitro* methods for *in vivo* AOs remains a critical issue and mainly concerns: a) NMs dose-selection and dose-metrics, b) *in vitro* assays including cell type and assay conditions, and c) the nano-relevant reference materials including both negative or positive controls (Dobrovolskaia and McNeil, 2013). In the case of conventional chemicals, OECD adopted and validated a certain number of *in vitro* assays against an *in vivo* response. The largest knowledge gap for NMs is related to existing *in vivo* data that would provide validity of an *in vitro* assay to in vivo AOs. The severity of the endpoint represents an important factor in determining the extent of validation that would be required. Thus, selection and prioritization strategy would involve targeting those KEs that are shared by number of AOPs with the most severe health outcomes and for which established *in vitro* assays are available. For example, both case studies presented here share common immunotoxic response for which the Nanotechnology Characterization Lab's (NCL's) recommended *in vitro* assays with high potential of *in vivo* predictability (Dobrovolskaia and McNeil, 2013).

The choice of the *in vitro* model is the next crucial step for the successful application of *in vitro* testing strategies. The model must have the ability to exhibit crucial KEs upon exposure to NMs. For instance, in testing lung fibrosis AOP, selected epithelial cells must have the ability to undergo EMT transition, whereas epithelial cells and macrophages must have the ability to release sufficient levels of pro-inflammatory and pro-fibrotic mediators. Agents that can induce these effects (positive agents) can be used to characterize the abilities of cell types to exhibit these KEs. As an example, TGF-β1, a strong promoter of EMT transition, can be used to check the ability of the selected cell type to undergo EMT transition. However, the most validated *in vitro* tests are based on 2D monocultures that do not reliably represent the architecture and physiology of an organ and the interactions within an organism. It has been recommended to develop and validate test systems based on a combination of cell cultures, co-cultures, tissue and tissue culture models (Halappanavar et al., 2021). The AOP development for nanosafety assessment should benefit from advanced biological models such as reconstructed epithelia, 3D cultures and microfluidic-based platforms that are continuously developed in the 3Rs spirit, particularly those that allow long-term and/or low-dose exposure to predict better chronic effects (Drasler et al., 2017) (Ruzycka et al., 2019; Barosova et al., 2020). Such models, developed to mimic human physiology and metabolism, hold great promises for RA of engineered NMs (Burden et al., 2021). As an example, advanced *in vitro* models of the human lung and liver have been used in the EU H2020 projects "Physiologically Anchored Tools for Realistic nanOmateriaL hazard aSsessment" (PATROLS[4]) and "Smart Tools for Gauging Nano Hazards" (SmartNanoTox[5]).

Secondly, one should consider the reliability, reproducibility and accessibility of the *in vitro* testing approach for RA of NMs with respect to nanospecific challenges, e.g., the heterogeneity of NMs, the interference of NMs with assays, and the lack of standardized protocols (Savolainen et al., 2010; Shah et al., 2014). An *in vitro* toolbox should include selected critical checkpoints to avoid any undesirable interactions of NMs with assay components and/or detection systems caused by NMs' properties or high exposure concentration, which may lead to erroneous results (Vinković Vrček et al., 2015; Hoet et al., 2013; Guadagnini et al., 2015; Ostermann et al., 2020; Kroll et al., 2011; Seiffert et al., 2012; Kroll et al., 2012). Despite many studies evidencing that nanospecific properties, such as high adsorption capacity, hydrophobicity, surface charge, optical and magnetic properties, or catalytic activity may induce interferences with *in vitro* methods, this issue has still not been adequately considered in nanotoxicology and nanomedicine. Any false positive and false negative results caused by NM-induced interferences would undoubtedly create errors in the interpretation of in *vitro* assessment of KEs. Moreover, it has been well documented that many NM types, especially metal-based, interact with biological structures, affecting their fate (toxicokinetics and toxicodynamics) in biological systems (Feliu et al., 2016). In biological media, aggregation, agglomeration, dissolution and degradation of NMs may occur leading to the generation and co-existence of different sizes and forms (NMs, ions and complex salts forms), illustrating the need for detailed analytical tools able to pick up this mixture of chemical forms and particle types. Some of these new species may trigger MIEs and KEs that are not any more nano-specific. Nano-bio interactions should be assessed and used to govern selection of *in vitro* tests by considering specific endpoints that derive from nano-related properties and transformations driven by the biological environment present around the material. For example, lysosomal enzyme release and quantification of metal can be used to assess lysosomal dysfunction as KE resulting from the active cellular uptake of metallic NMs and their transformation in endosomes. In any case, no single *in vitro* model is sufficient in providing a comprehensive answer about safety or hazards of NMs. While validation of assays with regard to their relevance, reliability, and specificity represents the enduring need for risk governance of nanotechnology, we are still faced with the huge lack in human exposure and effects data for NMs that would foster adaptation and development of such methods.

The choice of exposure durations (short-term or long-term) and exposure concentrations is critical for the safety assessment of NMs as most of them exhibit very low acute toxicity (Annangi et al., 2016; Xi et al., 2019; Chen et al., 2016). Indeed, most *in vitro* studies evaluated the toxic potential of NMs after short-term exposure (24 to 72 h), while long-term and repeated low-concentration exposure studies are scarce, but are extremely important as they mimic better the real-life exposure (e.g., workers in production and consumers through food). Since NM safety assessment by AOP testing is in early stages of

[4] https://www.patrols-h2020.eu/
[5] http://www.smartnanotox.eu/

development, use of exposure conditions and relevant *in vitro* models that mimic more closely the realistic exposure situation should be encouraged.

Considering that the regulatory relevant AOP networks extend and enhance the toxicity testing strategy via providing insight into the mechanism of action, they also support the development of relevant approaches for toxicity prediction including computationally-based predictive models. Hence, linking the AOP framework with *in silico* methods may facilitate the safety prognosis of NMs. *In silico* models may be used to predict biological responses of potential concern for the occurrence of the AO, instead of predicting the apical changes measured at the phenotypic level (Jagiello et al., 2021). However, the relevance of the response used for modelling of the eventual AO needs to be first justified. In effect, the AOP-anchored predictive models (including Quantitative Structure-Activity Relationship (QSAR)) would be delivered. The development of mathematical models (including QSARs) as predictive tools for early KEs is now one of the long-term actions according to the OECD report (OECD, 2015). The QSAR models have been widely used for predicting the toxicity of chemicals. These models are based on the assumption that substances with similar chemical structures will have similar toxicological effects/mechanism of actions, i.e., will follow similar pattern of interactions with possible molecular targets (e.g. protein, receptor, membrane) and/or induce similar changes at the cellular level (Kubinyi, 1997). The first reports on the use of QSAR methodology for NMs, so-called NanoQSAR, appeared approximately a decade ago (Puzyn et al., 2011). The existing NanoQSAR methods used in nanotoxicology still need adjustments due to the fact that most of the models developed so far refer to the phenotypic response, mainly cytotoxicity (Puzyn et al., 2011; Pan et al., 2016; Gajewicz et al., 2015). In fact, the relevance of modelled activity for the eventual nano-related AOs is not clear. This situation reveals that the challenge for future directions is to construct AOP-informed NanoQSAR models closely connected with AOP models. Such models will link physico-chemical properties of NMs with the AOP-relevant responses. Another computational strategy being developed to foster the validation of *in vitro* methods for *in vivo* AOs is *in vitro*-*in vivo* extrapolation (IVIVE) approach employing historical *in vivo* data sets as used by PATROLS and SmartNanoTox projects. As suggested (Halappanavar et al., 2020), analysis of heterogeneous and huge data sets on NMs toxicity by sophisticated computational models and machine learning will enable integration and description of biologically relevant information to be used for determination of causative link between KEs and KERs within AOP.

The simplest solution would be to link NMs properties to the MIEs they may induce. However, this is not possible currently due to the fact that the AOP development has not been focused on toxicological mechanisms relevant for NMs, so far (Halappanavar et al., 2019). There is no practical knowledge on whether events and outcomes of AOPs integrated so far in the AOP-Wiki may be related to nano-related structural features (e.g., shape, size, surface coating). Secondly, there is no structured data that clearly and comprehensively describe NMs-related MIEs (Halappanavar et al., 2020). Thus, additional efforts should be invested to critically review existing scientific literature and databases towards identification of all possible NMs-related MIEs. Finally, lessons on biological effects of NMs learned so far suggest that biological actions of NMs are often non-specific (Halappanavar et al., 2020). More extensively defined in relation to NMs-relevant AOPs are early KEs, which are the first identified biological responses essential for the occurrence of the AO manifested at the organism level. Responses, such as inflammation, oxidative stress or cytotoxicity, are repeatedly listed as KEs in the nanotoxicology literature. All these toxicological effects may lead to the AOs confirmed for NMs, e.g., lung fibrosis, lung emphysema and lung cancer that are already described in the AOP-Wiki as AOP 173, AOP 1.25 and AOP 303, respectively (Halappanavar et al., 2020). Recently, a novel strategy for modelling NMs' toxicity was proposed (Jagiello et al., 2021), which employed a nano-QSAR model able to predict transcriptomic response related to the early KEs regarding the AOP 173. Such an approach could be used for showing the relationship between the structural features of NMs and the AO considered by AOP (Jagiello et al., 2021). Further development of computationally assisted AOP development for NMs should be therefore based on the provision of high-quality data that support establishing full AOPs and creating predictive models. One aspect that is generally missing in biological testing is the evaluation of the uncertainty in measurements (ISO, 2008), which would facilitate the assessment of the reliability of measurements and comparison of the results obtained in different laboratories. We would therefore recommend the inclusion of measurements uncertainty as one of the important parameters for the overall data analysis.

Combined *in vitro/in silico* approaches may significantly facilitate the AOP acceptance by all relevant stakeholder groups coming in contact with NMs with regards to the evaluation of existing information, identification of data gaps, generation of new knowledge and iterative decision making (Ede et al., 2020). Finally, the AOP framework also finds its utility in the nano-product design and development as mechanistic aid, thereby having relevance beyond regulatory applications.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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