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Research Article

Linking Nanomaterial-Induced Mitochondrial Dysfunction to Existing Adverse Outcome Pathways for Chemicals

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Abstract

The Adverse Outcome Pathway (AOP) framework plays a crucial role in the paradigm shift of toxicity testing towards the development and use of new approach methodologies. AOPs developed for chemicals are in theory applicable to nanomaterials (NMs). However, only subtle efforts have been made to integrate information on NM-induced toxicity into existing AOPs. In a previous study, we identified AOPs in the AOP-Wiki associated with the molecular initiating events (MIEs) and key events (KEs) reported for NMs in scientific literature. In a next step, we analyzed these AOPs and found that mitochondrial toxicity plays a significant role in several of them at the molecular and cellular levels. In this study, we aimed to generate hypothesis-based AOPs related to NM-induced mitochondrial toxicity. This was achieved by integrating science-based information collected on NM-induced mitochondrial toxicity into all existing AOPs in the AOP-Wiki, which already includes mitochondrial toxicity as a MIE/KE. The results showed that several AOPs in the AOP-Wiki related to the lung, liver, cardiovascular and nervous system, with extensively defined KEs and key event relationships (KERs), could be utilized to develop AOPs that are relevant for NMs. Our results also indicate that the majority of the studies included in our literature review were of poor quality, particularly in reporting NM physico-chemical characteristics, and NM-relevant mitochondrial MIEs were scarcely reported. This study highlights the potential role of NM-induced mitochondrial toxicity in human-relevant adverse outcomes and identifies useful AOPs in the AOP-Wiki for the development AOPs that are relevant for NMs.

1 Introduction

The adverse Outcome Pathway (AOP) framework plays a crucial role in the paradigm shift of toxicity testing towards the development and use of New Approach Methodologies (NAMs) (Burden et al., 2015; Bajard et al., 2023; Brescia et al., 2023). AOPs allow the collection and logical organization of experimental data from different sources, and the identification of essential biological events that are both physiologically and chemically plausible. Additionally, AOPs play an important role in the development of alternative strategies to animal testing to inform various steps of the human health risk assessment (RA) process (OECD, 2020a).

The RA related to nanomaterials (NMs) is challenging due to high number and high variability in their physico-chemical properties and associated nano-specific effects. AOPs provide a framework for understanding the molecular and cellular events that lead to adverse outcomes (AOs) and can help to identify the mode of toxicity specific to critical properties of NMs (Vietti et al., 2016; Rolo et al., 2022). By utilizing AOPs, researchers and regulators can overcome these challenges, ultimately leading to more accurate and comprehensive risk assessments (Rolo et al., 2022).

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AOPs are stressor agnostic, and consequently, AOPs developed for chemicals are also applicable to NMs (Halappanavar, Ede, et al., 2020). In recent years, efforts have been made to develop nano-relevant AOPs by adapting existing AOPs for NMs (Nymark et al., 2018; Brand et al., 2020; Halappanavar, Van Den Brule, et al., 2020; Murugadoss, 2021). Based on a nanotoxicological systematic literature review, an AOP network relevant to NM was proposed by combining linear AOPs for lung fibrosis (AOP 173), lung emphysema (AOP 1.25), acute lung toxicity (AOP 302), lung cancer (AOP 303) and atherosclerotic plaque formation (AOP 237), as an outcome of the OECD Working Party for Manufactured Nano-materials (WPMN) project on advancing the development of nano-relevant AOPs (Halappanavar, Van Den Brule, et al., 2020). By leveraging AOP 144 and AOP 34, AOPs associated with steatosis, edema, and fibrosis in the liver upon based in existing information on TiO_2, including its nanoform, have been postulated (Brand et al., 2020). Gerloff et al. (2017) also employed AOP 144 and proposed a putative liver fibrosis AOP based on available information for metal oxide NMs (Gerloff et al., 2017). Furthermore, several ongoing EU and international projects, including RiskGONE, seek to further the development and utilization of AOPs for NMs (Ede et al., 2020).

Recently, we presented a simple and testable strategy to develop AOPs for NMs based on existing AOPs (Murugadoss, Vrček, et al., 2021). This work was focused on searching molecular initiating events (MIEs) and key events (KEs) reported for NMs in scientific literature and identifying AOPs associated with these MIEs and KEs in the AOP-Wiki. In a next step, we analyzed these AOPs and found that mitochondrial toxicity plays a significant role in several of them at the molecular and cellular levels. Although there is a growing body of studies on NM-induced mitochondrial toxicity (Wu et al., 2020), the extent to which it contributes to adverse effects is not yet fully understood.

Stressor-induced mitochondrial toxicity primarily results in mitochondrial dysfunction. The term "mitochondrial dysfunction" encompasses a wide variety of changes in the structure and functioning of the mitochondria. The most reported dysfunction is the disturbances in the production of adenosine triphosphate (ATP) via oxidative phosphorylation. Other dysfunctions encompass the loss of mitochondrial membrane potential (MMP) and pore permeability changes, inhibition of protein complexes in the electron transport chain (ETC), failure to produce enzymes that detoxify reactive oxygen species (ROS) (e.g. manganese superoxide dismutase), disruption in mitochondrial network formation and structure, impaired clearance of dysfunctional mitochondria through mitophagy, dysregulation of cytoplasmic and mitochondrial matrix transport of Ca^{2+} ions, induction of pro-inflammatory and apoptotic pathways, or damage to the mitochondrial DNA (mtDNA), including also the induction of mtDNA adducts impairing mitochondrial transcription or alteration of the activity of DNA polymerase gamma (Wallace, 2012; Meyer et al., 2013, 2018; Vuda and Kamath, 2016; Vyas et al., 2016; Fetterman et al., 2017; West, 2017; Massart et al., 2018; Daiber et al., 2020). Owing to their unique characteristics and functionality, mitochondria are prone to be affected by various chemical stressors. The negatively charged and alkaline mitochondrial matrix enables the accumulation of amphiphilic xenobiotics and metals such as lead, cadmium, mercury, or manganese (Cohen, 2010), while the high lipid content of mitochondrial membrane facilitates the internalization of lipophilic compounds such as polycyclic aromatic hydrocarbons (PAHs) (Backer and Weinstein, 1980). Moreover, mtDNA is more susceptible to damage compared to nuclear DNA, probably due to its vicinity to the ETC, the lack of histones, and the deficiency in certain DNA repair mechanisms (Khalifa et al., 2021).

Occupational studies have revealed that exposure to chemical stressors such as pesticides, benzene, PAHs, metal rich particulate matter (PM) and particle containing welding fumes is associated with mitochondrial dysfunction and mtDNA damage (Roubicek and de Souza-Pinto, 2017). Additionally, mitochondrial dysfunction can also be induced by a broad range of other stressors, including polychlorinated biphenyls, dioxins/furans, metals/metalloids (arsenic, lead, copper, chromium, cadmium, nickel, and vanadium), air pollutants (diesel exhaust and ambient ultrafine particles, sulfur dioxide, nitrogen oxides), tobacco smoke (Fetterman et al., 2017), and algal toxins (Jayasundara, 2017).

Clinical perspectives on chemical exposure and mitochondrial dysfunction have been excellently reviewed (Zolkipli-Cunningham and Falk, 2017; Gorini et al., 2018; Tang et al., 2022) and it can be stated that various organs and tissues (brain, heart, liver, kidney, pancreas, muscles, arteries) can be affected by mitochondrial dysfunction and damage (Hayden, 2022). Due to its high-energy demand, the heart is one of the major organs where mitochondrial disturbances have marked consequences like myocardial infarction, cardiomyopathy, and heart failure, all of which have been linked to the accumulation of aberrant mitochondria (Kirichenko et al., 2022; Li et al., 2022). Moreover, accumulation of aberrant mitochondria due to impaired mitophagy (that selectively degrades damaged mitochondria) in the myocardium has been reported for several diseases, such as obesity, impaired glucose tolerance, type 2 diabetes mellitus, insulin resistance, and metabolic syndrome (Hayden, 2022). Furthermore, impairment of mitochondrial dynamics has also been linked to vascular endothelial dysfunction (Qu et al., 2022). Mitochondria have been shown to play a crucial role not only in cardiovascular disturbances but also in several neurological disorders, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, ischemic stroke, traumatic brain injury, and epilepsy (Cabral-Costa and Kowaltowski, 2020; Norat et al., 2020; Delp et al., 2021).

In light of the potential adverse effects induced by NMs, it is crucial to identify the role of NM-induced mitochondrial toxicity. This can be achieved by utilizing the AOP framework. Therefore, the objective of this study was to generate hypothesis-based AOPs related to NM-induced mitochondrial toxicity by integrating science-based information on NM-induced mitochondrial toxicity into all existing AOPs in the AOP-Wiki, which already includes mitochondrial toxicity as a MIE/KE.

2 Methods

The approach to generate hypothesis-based AOPs related to nanomaterial (NM)-induced mitochondrial toxicity is schematically depicted in Fig. 1. Firstly, we analyzed AOPs identified in Murugadoss (2021) that are associated with MIEs and KEs reported for NMs and found that mitochondrial toxicity plays a key role in several of them at the molecular and cellular levels (Tab. 1). Furthermore, we performed a keyword search for "mitochondria" in the "Key Events" of the AOP-Wiki, which revealed that mitochondrial toxicity is involved in several other AOPs that are not included in Tab. 1. Here, we aimed to establish a plausible link between NMs-induced mitochondrial toxicity and existing AOPs in the AOP-Wiki and to propose a conceptual AOP (network) relevant to human health effects.

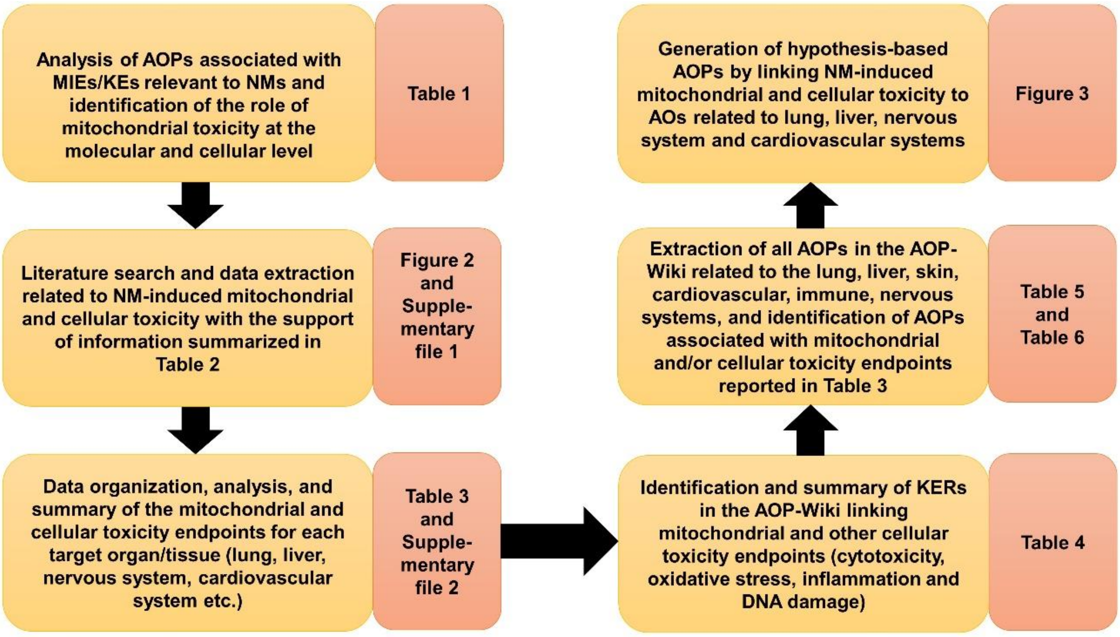

Fig.1:
Schematic workflow of the approach to generate hypothesis-based adverse outcome pathways (AOPs) related to nanomaterial (NM)-induced mitochondrial toxicity
MIEs - molecular initiating events; KEs - key events; KERs - key event relationships; AOs - adverse outcomes.

Tab.1: Adverse outcome pathways (AOPs) in the AOP-Wiki associated with mitochondrial initiating events (MIEs)/ key events (KEs) reported for nanomaterials (NMs) and mitochondrial toxicity

AOP number	AO	KE title	KE number	Status of the AOP
256	Kidney toxicity	Dysfunction, Mitochondria (Cellular)	1483	Under development
258	Kidney toxicity	Dysfunction, Mitochondria (Cellular)/Decrease, Mitochondrial ATP production (Cellular)	1483/40	Under development
276	Fanconi syndrome	Decreased mitochondrial oxidative phosphorylation (Cellular)	1477	Under development
284	Kidney disease	Disruption, Mitochondrial electron transport chain (Cellular)	178	Under development
144	Liver fibrosis	Mitochondrial dysfunction (Cellular)	177	EAGMST Under Review
273	Liver injury	Decrease in mitochondrial oxidative phosphorylation/Increased ROS (in the mitochondria) (Organelle)/ Mitochondrial Injury (Cellular)	1545/1546/1547	Under development
362	Hepatitis	Mitochondrial dysfunction and reduced ATP synthesis (Cellular)	1816	Under development
3	Neurotoxicity	Mitochondrial dysfunction (Cellular)	177	WPHA/WNT Endorsed
48	Cognition	Mitochondrial dysfunction (Cellular)	177	WPHA/WNT Endorsed
207*	Reproductive failure	Mitochondrial damage (Cellular)	176	Under development
311*	Population decline	MMP decrease (Cellular)/Decreased mitochondrial oxidative phosphorylation (Cellular)	1770/1477	Under development
325	Population decline	Mitochondrial dysfunction (Molecular) MIE	177	Under development
324	Population decline	Mitochondrial dysfunction (Molecular) MIE	177	Under development
328*	Mortality	MMP decrease (Cellular)/Decreased mitochondrial oxidative phosphorylation (Cellular)	1770/1477	Under development
200	Breast cancer	Mitochondrial dysfunction (Cellular)	177	Under development
26	Energy imbalance	Disruption, Mitochondrial electron transport chain (Cellular)/Decrease, Mitochondrial ATP production (Cellular)	178/40	Under development

AOPs indicated with (*) are currently not applicable to humans.
AO - adverse outcome; EAGMST- Extended Advisory Group on Molecular Screening and Toxicogenomics; WNT - Working Group of the National Coordinators of the Test Guidelines Programme; WPHA - Working Party on Hazard Assessment; ATP - adenosine triphosphate; MMP - mitochondrial membrane potential

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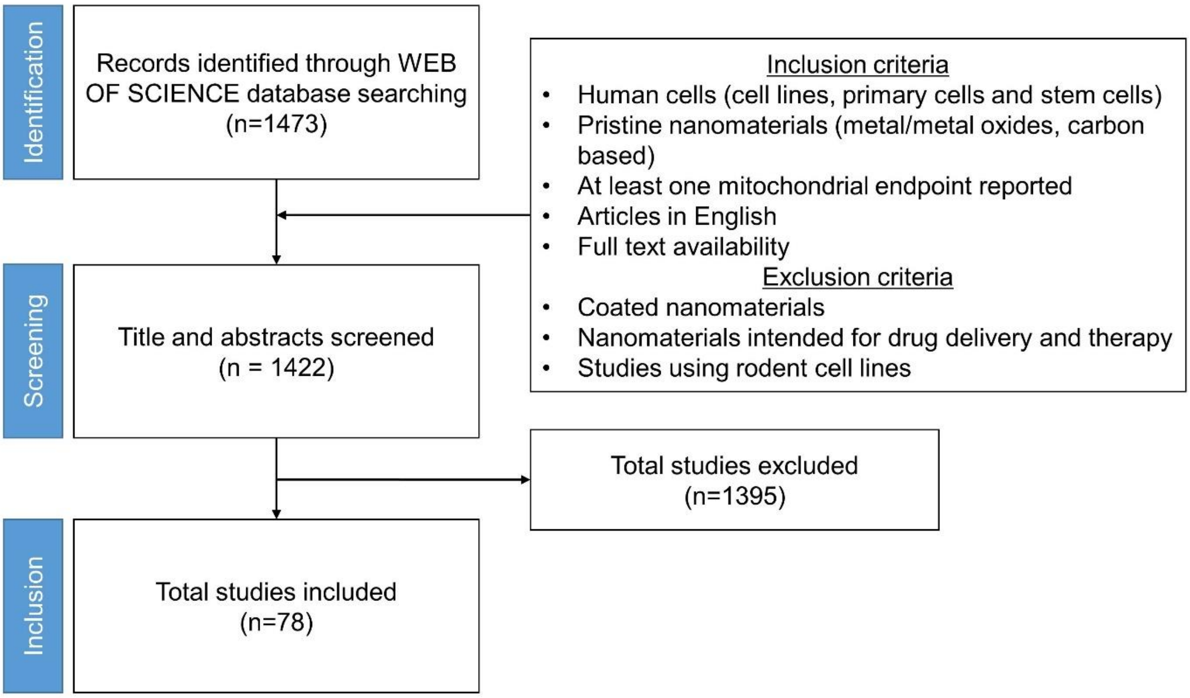

Fig. 2: Systematic selection process to identify relevant studies

Tab.2: Summary of mitochondrial endpoints related to key events (KEs) provided in Tab. 1 and associated methods/assays in AOP-Wiki

Endpoints related to KEs provided in Table 1	Methods/Assays
Mitochondrial complex inhibition	Complex inhibition assays specifically for complex (IV, V, III), MitoTox (inhibition of Complex I), Enzyme activity assays, mitochondrial membrane potential (MMP) measurement by fluorescent dyes
Decrease in oxidative phosphorylation	MMP measurement by mitochondrial dyes (JC-1, rhodamine 123, DiOC6, TMRE), Extracellular lactate reflecting an increase in glycolytic rate (colorimetric assay), Oxygen consumption measurement by the Seahorse assay
Decrease in MMP	MMP measurement by mitochondrial dyes (JC-1, rhodamine 123, DiOC6, TMRE)
Decrease in adenosine triphosphate (ATP) synthesis	ATP bioluminescent assay, ATP synthase assay, Complex V activity assay
Increase in mitochondrial reactive oxygen species (ROS)	MitoROS (targeting mitochondrial ROS)
Increase in mitochondrial injury/damage/disruption	Cellular oxygen consumption, MMP, enzymatic activity of the electron transport system, ATP content.

To identify relevant studies, we conducted a literature review on mitochondrial toxicity induced by NMs (Fig. 2). A combination of keywords used in the Web of Science (WoS) database to search for relevant studies included [*(nanoparticles or nanomaterials)* AND *(mitochondria)* AND *(in vitro)*]. The search resulted in 1473 papers in total for studies published before 25/01/2023. After screening and applying different exclusion and inclusion criteria (see Fig. 2), 78 studies covering *in vitro* experiments on human cells and pristine NMs were selected for further analysis.

These 78 studies were then evaluated by data quality scoring approach based on the GUIDEnano system (Fernández-Cruz et al., 2018). This approach follows the principles of the Klimisch score (K score), related to test design and reporting considerations, and it is complemented by the GUIDEnano S score that considers the reported physicochemical properties of the NMs, including those characterized in the exposure medium. The K score and the S score are combined to obtain an overall quality score (Q score), which is numerically classified as follows: Q=1 (very high quality), Q=0.8 (high quality), Q=0.5 (medium quality), and Q=0 (unacceptable quality).

To ensure the correct identification of mitochondrial MIEs and KEs and their reliable connection to AOPs in the AOP-Wiki, we analyzed the descriptions of mitochondrial KEs in Tab. 1 and summarized mitochondrial toxicity endpoints and related methods/assays to measure them (Tab. 2). This includes endpoints such as mitochondrial complex inhibition, decrease in oxidative phosphorylation, MMP, ATP production as well as increase in mitochondrial ROS production and mitochondrial damage/dysfunction/disruption. These endpoints were then used to design a template, which was used to extract and consolidate data from the selected studies.

We extracted data from the selected studies and analyzed it in two steps:

1) Analysis and evaluation of the evidence for NM-induced mitochondrial toxicity. This step summarized the studies reporting mitochondrial and cellular toxicity endpoints induced by NMs (such as titanium dioxide (TiO_2), amorphous silica (SiO_2), silver

(Ag)), etc) in human cell types that are representative of a potential target organ/tissue (such as lung, liver, etc) or system (such as nervous).

2) Establishment of a biologically plausible link between NM-induced mitochondrial toxicity and existing AOPs in the AOP-Wiki, and proposal of a conceptual AOP network relevant for NMs. This step focused on finding AOPs related to each target organ/tissue/system in the AOP-Wiki that had mitochondrial endpoints identified in step 1 and linking NM-induced mitochondrial toxicity to these AOPs via other cellular toxicity endpoints.

3 Results

3.1 Evidence analysis of mitochondrial toxicity induced by different NMs

Supplementary file 1[1] includes the list of selected studies, extracted raw data, and evaluation of these studies using a data quality scoring approach based on the GUIDEnano system. All studies are also marked by quality Q score. However, in order to address the gaps in the available data, we did not exclude studies based on their data quality but included all 78 studies in our data analyses. Indeed, only 25 out of the 78 studies had an acceptable Q value with very high, high or medium quality.

In Supplementary file 2[2], we organized the data from individual studies related to mitochondrial and cellular toxicity endpoints, cell types used, and their corresponding organs/tissues/system. Each study was assigned to one row in the file and information on whether a positive or negative outcome observed for each endpoint as well as the Q score associated with each study was also included. A summary of the findings with positive outcome was presented for each target organ/tissue/system in Tab. 3. The analysis revealed that multiple studies have investigated the mitochondrial and cellular toxicity induced by various types of NMs across different human cell types that are related to the lung, liver, skin, cardiovascular, immune or nervous systems. The low number of studies for other target organs/tissues, such as the kidney or eye, may be due to the scarcity of available cell models, or at least their infrequent utilization in toxicology testing. Based on these findings, AOPs related to these target organs/tissues/system in the AOP-Wiki were considered for further investigation.

Tab.3: Summary of mitochondrial and cellular toxicity endpoints induced by nanomaterials (NMs) in human cell types representing different target organs/tissues/systems
Reference to individual studies are provided in supplementary file 2[2].

Target organ/system	Cell line or type	NM	No. of studies	Type of mitochondrial toxicity endpoint observed (positive outcome)	Cellular toxicity endpoints (positive outcome)
Lung	A549, Calu-1, NCI-H358, primary lung fibroblasts, IMR-90, BEAS-2B, 16HBE14o-, H1355, BECs, HPAEpiC	ZnO nanorods, Ag, CeO_2, Carbon Black, CuO, Al_2O_3, ZnO, Fe_2O_3/Fe_3O_4, MWCNTs, Au nanoprisms, Cr_2O_3	15	Increase in mitochondrial reactive oxygen species (ROS), decrease in mitochondrial membrane potential (MMP), increase in mitochondrial protein oxidation, altered mitochondrial function, changes in mitochondrial morphology, altered mitochondrial calcium, decrease in adenosine triphosphate (ATP), expression of mitochondria-dependent apoptosis related proteins, mitophagy, altered mitochondrial abundance, effect on oxidative phosphorylation, decrease in mitochondrial oxygen consumption	Cytotoxicity (apoptosis/necrosis), pro-inflammatory responses (increase in cytokines), oxidative stress (increase in ROS), DNA damage
Liver	HL7702, HepG2,L02, Hep2,BEL-7402, HepaRG	TiO_2, Ag, SPION, ZnO, Amorphous SiO_2, CdS Quantum dots, Fe_3O_4, Fe_2O_3, Co_3O_4	14	Decrease in MMP, increase in mitochondrial ROS, altered mitochondria permeability transition, effect on mitochondria associated endoplasmic reticulum membranes, decrease in ATP changes in mitochondrial morphology, inhibition of mitochondrial fission	Cytotoxicity (apoptosis/necrosis), pro-inflammatory responses (increase in cytokines), oxidative stress (increase in ROS, lipid peroxidation, decrease in glutathione), DNA damage
Nervous system	LUHMES cells and brain spheres (Neural progenitor cells differentiated from iPSC), SHSY-5Y, U373, LN229, U87 MG, U251 MG, U87	Au, Fe_3O_4, Ag, SiO_2, TiO_2, Gd_2O_3, Mn_3O_4, Graphene, Graphene oxide	11	Decrease in MMP, altered mitochondrial function, mitochondria depolarization, altered oxidative phosphorylation, decrease in electron transport chain (ETC) enzyme activities (complex I, III and IV), decrease in ATP, decrease in mitochondrial membrane permeability, increase in mitochondrial ROS, changes in mitochondrial morphology	Cytotoxicity (apoptosis/necrosis), pro-inflammatory responses, oxidative stress (increase in ROS, lipid peroxidation), DNA damage, cell cycle arrest

[1] doi:10.14573/altex.2305011s1
[2] doi:10.14573/altex.2305011s2

Target organ/system	Cell line or type	NM	No. of studies	Type of mitochondrial toxicity endpoint observed (positive outcome)	Cellular toxicity endpoints (positive outcome)
Cardiovascular system	HUVECs, EA.hy926, HCASMC, , HPAEC and aortic VSMC	Amorphous SiO_2, SWCNTs, MWCNTs, ZnO, NiO	8	Increase in mitochondrial ROS, inhibition of mitochondrial fission, decrease in MMP, decrease in mitochondrial number, decrease in mitochondrial mass, internalization in mitochondria, changes in mitochondrial morphology	Oxidative stress (increase in ROS), decrease in cell viability, pro-inflammatory responses (increase in interleukin-6)
Immune system	PBMCs, MM cells, THP1, human leukemia Jurkat, HL-60 cells, HS-5, human lymphocytes and erythrocytes	TiO_2, Pd, ZnO, Pt, MWCNT, Graphene	7	Changes in mitochondrial morphology, decrease in ATP, decrease in MMP increase in adenosine diphosphate (ADP)/ATP ratio	Cytotoxicity (apoptosis), oxidative stress (increase in ROS), DNA damage, cell cycle arrest
Skin	melanoma cells, HaCaT, human epidermal keratinocytes, human skin fibroblasts	ZnO nanorods, Y_2O_3/ZrO_2, Bismuth oxychloride, TiO_2, ZnO, CuO, MoS_2	7	Decrease in MMP, effect on mitochondrial tricarboxylic acid (TCA) cycle, increase in mitochondrial ROS, decrease in ETC enzyme activities (complex I and III), decrease in ATP	Oxidative stress (increase in ROS), lysosomal integrity, cell cycle arrest
Intestine	HCT 116, Caco2, HT29	Ag	4	Decrease in MMP, changes in mitochondrial morphology, altered mitochondrial function, decrease in ATP	Cytotoxicity (apoptosis) oxidative stress (increase in ROS), cell cycle arrest, DNA damage
Kidney	HEK cells	ZnO, CdTe, Quantum dots,	2	Decrease in MMP, mitochondrial membrane permeability transition	Oxidative stress (increase in ROS)
Breast	MCF-7, HBL100	Ag, Au	3	Changes in mitochondrial morphology, decrease in MMP, altered oxidative phosphorylation	Oxidative stress (increase in ROS)
Development (Embryo)	HTR-8/SVneo, FECH15 and NAF1nor	Cu NMs, Mn_3O_4	2	Decrease in MMP, altered oxidative phosphorylation	N/A
Reproductive system	HeLa	Co_3O_4, Au, ZrO_2	3	Decrease in MMP, decrease in ATP, decrease in mitochondrial oxygen consumption	Cytotoxicity (apoptosis), oxidative stress (increase in ROS)
Eye	hCECs, ARPE-19 and HCjECs	SiO_2, Carbon black, ZnO	3	Decrease in MMP	Cytotoxicity, oxidative stress (increase in ROS)
Gums	CRL-2014	Ag	1	Expression of mitochondria-dependent apoptosis related proteins	DNA damage
Placenta	BeWo	CuO	1	Decrease in MMP	Cytotoxicity (Apoptosis), oxidative stress (increase in ROS, decrease in total GSH and in activity of antioxidant enzymes)

BECs - bronchial epithelial primary cells; HPAEpiC - Human Pulmonary Alveolar Epithelial Cells; HUVECS-human umbilical vein endothelial cells; LUHMES - Lund human mesencephalic; HCASMC - primary human coronary artery smooth muscle cells; HPAEC - primary human pulmonary artery endothelial cells; VSMC - vascular smooth muscle cells; PBMCs - peripheral blood mononuclear cells; HEK - human embryonic kidney; hCECs - human corneal epithelial cells; hCjECs- human conjunctival epithelial cells; MM- Multiple myeloma

3.2 Plausible linking of NM-induced mitochondrial toxicity to existing AOPs in the AOP-Wiki

The summary of cellular toxicity endpoints in the studies reporting mitochondrial MIEs and KEs (Tab. 3), suggests that mitochondrial toxicity induced by NMs (upstream KEs) can be potentially linked to cellular toxicity endpoints such as cytotoxicity, oxidative stress, pro-inflammation, and DNA damage (downstream KEs). The biological plausibility of mitochondrial toxicity leading to oxidative stress and/or cytotoxicity, and their subsequent cellular toxic responses, such as pro-inflammatory responses and DNA damage, are well established in the literature as well as through the KERs described in the AOP-Wiki (Tab. 4). We have also indicated the status of the AOPs in which these KERs are included.

To make a biologically plausible and causal link of NM-induced mitochondrial and cellular toxicity to existing AOPs, we searched for AOPs in the AOP-Wiki related to lung, liver, skin, cardiovascular, immune, and nervous systems using keywords indicated in Tab. 5. Then, it was assessed whether a given AOP contains KEs related to mitochondrial and/or cellular toxicity endpoints (cytotoxicity, oxidative stress, pro-inflammatory responses, and DNA damage). This resulted in 28 AOPs that include

Tab.4: Key event relationships (KERs) describing a causal link between mitochondrial and/or cellular toxicity endpoints and the related adverse outcome pathways (AOPs)

KER	KER number	Included in OECD endorsed AOP	Included in AOPs under review	Included in AOPs under development but well described
Mitochondrial dysfunction leads to cell injury/death	363	AOP 48	AOP 144	
Inhibition of ETC complexes of the respiratory chain leads to oxidative stress	2565			AOP 437
Cell injury/death leads to increased pro-inflammatory mediators	1776		AOP 144	
Oxidative stress leads to cell injury/death	1690	AOP 17		
Oxidative stress leads to increased pro-inflammatory mediators	2772			AOP 470
Oxidative stress leads to increased DNA strand breaks	2811			AOP 478, AOP 483

OECD - Organization for Economic Co-operation and Development; ETC - electron transport chain

Tab.5: Adverse outcome pathways (AOPs) in the AOP-Wiki related to the target organs/tissues/systems corresponding to the cellular systems for which nanomaterial (NM)-induced mitochondrial toxicity was observed

Target Organ/tissue system	Keywords	Total number of relevant AOPs found	AOPs that can be potentially linked to mitochondrial toxicity
Lung	Lung	19	272, 411, 424, 425, 171, 451, 414, 206 and 173
Liver	Liver	17	34, 38, 144, 220, 273, 278 and 362
Nervous system	Nervous system	19	3,17, 12, 48,260, 374
Cardiovascular system	Cardiovascular, heart, cardiotoxicity	17	264,265, 268, 438, 479, 480
Immune system	Immunotoxicity	3	N/A
Skin	Skin, dermal	3	N/A

N/A - not available

Tab.6: Summary of adverse outcome pathways (AOPs) with mitochondrial and cellular key events (KEs) indicated in Table 4

Organ	AO	AOP number	Status of the AOP	Pathway	Mitochondrial KE	cellular toxicity KE
Lung	Decreased lung function	411	Under development	Via direct effect on ciliary beat frequency	N/A	Oxidative stress
		424	Under development	Via decrease in CTFR function	N/A	Oxidative stress
		425	Under development	Via decrease in FOXJ1	N/A	Oxidative stress
		414	Under development	Via AhR pathway	N/A	Oxidative stress, cytotoxicity
	Lung cancer	451	Under development	Via interaction with lung resident cell membrane components	N/A	Cytotoxicity, Oxidative stress
		272	EAGMST Approved	Via DNA damage	N/A	DNA strand breaks
		414	Under development	Via IL-6 or AhR pathway	N/A	Oxidative stress, DNA damage/ mutation
	Lung fibrosis	173	EAGMST Under Review	Via interaction with lung resident cell membrane components	N/A	Pro-inflammatory response, cell death
	Mesothelioma	171	Under development	Via chronic cytotoxicity of the serous membrane	N/A	Oxidative stress, pro-inflammation
		409	Under development	Frustrated phagocytosis leads to malignant mesothelioma	N/A	Oxidative stress, DNA damage
Liver	Liver Injury	273	Under development	Via mitochondrial complex inhibition	Mitochondrial dysfunction	Cytotoxicity

		278	Under development	Via IKK complex inhibition	N/A	Cytotoxicity
	Liver fibrosis	38	WPHA/WNT Endorsed	Via protein alkylation	N/A	Cytotoxicity, pro-inflammatory response
		144	EAGMST Under Review	Via endocytic lysosomal uptake	Mitochondrial dysfunction	Cytotoxicity, pro-inflammatory response
		383	Under development	Via inhibition of angiotensin-converting enzyme 2	N/A	Oxidative stress
	Immune mediated hepatitis	362	Under development	Via reactive metabolites	Mitochondrial dysfunction	Cytotoxicity, Pro-inflammatory response
	Cholestasis	27	Under development	Via bile acid accumulation		Pro-inflammation, Oxidative stress, cytotoxicity
	Liver steatosis	34	Under development	Via LXR activation	Mitochondrial damage	
	Liver cancer	220	WPHA/WNT Endorsed	Via Cyp2E1 activation	N/A	Oxidative stress, cytotoxicity
Nervous system	Impairment of learning and memory	12	WPHA/WNT Endorsed	Via NMDARs inhibition	N/A	Cytotoxicity, neuro-inflammation
		48	WPHA/WNT Endorsed	Via over activation of NMDARs	Mitochondrial dysfunction	Cytotoxicity, neuro-inflammation
		17	WPHA/WNT Endorsed	Via binding to SH/seleno proteins	N/A	Oxidative stress, cytotoxicity
	Neurodegeneration	260	Under development	Via CYP2E1 activation	N/A	Oxidative stress, Cytotoxicity
		374	Under development	Via neuroinflammation		Neuro-inflammation
	Parkinsonian motor deficits	3	WPHA/WNT Endorsed	Via Inhibition of the mitochondrial complex I	Mitochondrial dysfunction	Neuro-inflammation, neuro-degeneration
Cardiovascular system	Decreased growth	264	Under development	Via ATP depletion	Mitochondrial dysfunction	Cytotoxicity
		265	Under development	Via increased cytosolic calcium	Mitochondrial dysfunction	Cytotoxicity
		268	Under development	Via increased protein oxidation	Mitochondrial dysfunction	Cytotoxicity
	Cardiovascular diseases	438	Under development	Via oxidative stress	Mitochondrial dysfunction	Oxidative stress
	Heart failure	479	Under development	Via oxidative stress	Mitochondrial dysfunction	Oxidative stress, cytotoxicity
	Heart failure	480	Under development	Via decrease in ATP production	Mitochondrial dysfunction	N/A

CTFR - cystic fibrosis transmembrane regulator; FOXJ1 - forkhead box J1; AhR - Aryl hydrocarbon receptor; IL - interleukin; IKK - IkappaB kinase; LXR - liver X receptor; CYP2E1 - Cytochrome P450 2E1; NMDAR - N-methyl-D-aspartate receptor; ATP – adenosine triphosphate; EAGMST- Extended Advisory Group on Molecular Screening and Toxicogenomics; WNT - Working Group of the National Coordinators of the Test Guidelines Programme WPHA - Working Party on Hazard Assessment; N/A - not available

the selected target organs/tissues/systems. Among them, several AOPs were identified for cardiovascular (n=9), lung, (n=7), liver (n=6) and nervous systems (n=6), that can be potentially linked to mitochondrial toxicity induced by NMs. No potential AOPs related to the mitochondrial toxicity for the immune system and skin were found in the AOP-Wiki (Tab. 5).

The summary of AOPs with mitochondrial and/or cellular KEs corresponding to the KERs (indicated in Tab. 4) is summarized in Tab. 6. The following AOs were identified for each target organ/tissue/system:

Lung: decreased lung function, lung cancer, lung fibrosis, and mesothelioma; Liver: liver steatosis, liver injury, liver fibrosis, immune mediated hepatitis, cholestasis, and liver cancer; Nervous system: neurodegeneration, parkinsonian motor deficits, and impairment of learning and memory; Cardiovascular system: heart failure, decreased cardiovascular growth, increased cardiovascular morbidity and mortality of cardiovascular diseases.

Inhibition of mitochondrial complexes, uncoupling of oxidative phosphorylation, and mtDNA damage are identified as potential MIEs leading to mitochondrial dysfunction (Dreier et al., 2019) and these were also observed in some studies concerning NMs (in Tab. 3). Thus, we propose these endpoints as MIEs for the conceptual AOP network on NM-induced mitochondrial

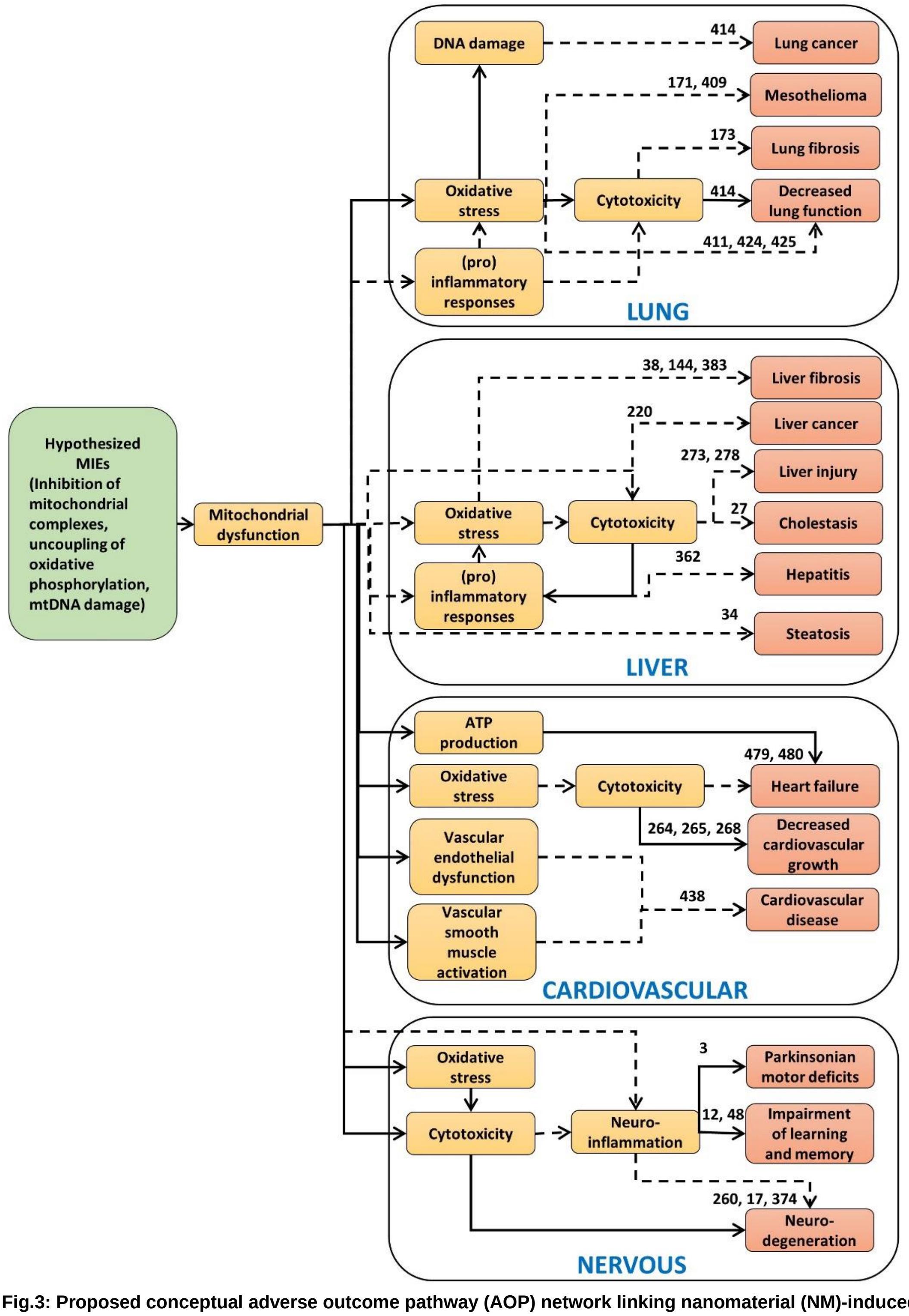

Fig.3: Proposed conceptual adverse outcome pathway (AOP) network linking nanomaterial (NM)-induced mitochondrial and cellular toxicity to different AOPs related to different organ/tissues/systems
The dotted lines indicate that additional key events (KEs) are included in the referenced AOP but they are not included in this figure.

toxicity. From Tab. 3, we also noticed that other endpoints, such as the decrease in mitochondrial MMP, ATP production, and oxygen consumption, as well as an increase in mitochondrial ROS production and physical damage to the mitochondria are widely reported as a sign of mitochondrial dysfunction at the organelle or cellular level. Thus, we centralized mitochondrial dysfunction as the subsequent KE hub and linked it to oxidative stress, cytotoxicity, inflammatory responses, and DNA damage, including interconnections. Fig. 3 presents this conceptual AOP network connecting different AOPs related to different target organs/tissues/systems.

4 Discussion

In this study, we applied a systematic approach and established a biologically plausible link between mitochondrial toxicity induced by NMs and already existing AOPs in the AOP-Wiki. This strategy can not only inform on the potential role of NMs-induced mitochondrial toxicity in several human-relevant AOs but also identify potential AOs and AOPs in the AOP-Wiki that can be prioritized in the further development of nano-relevant AOPs. AOPs are, in principle, designed to be modular with re-usable elements and stressor-agnostic. Our results demonstrate how several components from the AOP-Wiki that were extensively defined (such as KEs and KERs) can be utilized to develop NM-relevant AOPs, rather than investing considerable resources in developing new nano-related AOPs.

In order to identify and address gaps in the available data, the quality of the studies was analyzed using the GUIDEnano approach (Fernández-Cruz et al., 2018). The results of this analysis revealed that only 25 out of 78 studies had an acceptable Q score, which is a result of a different combination of K and S scores (Supplementary file 1[1]). Further analysis of the K and S scores showed that the S score, which is based on the reported physicochemical properties of the NMs, had a greater impact on the overall Q score. A closer examination of these physico-chemical data revealed that most studies failed to report relevant information such as endotoxin content, impurities, and NM concentration and stability in the exposure medium. In addition, the surface area and hydrodynamic diameter of the NM at the beginning and/or end of the exposure period were also scarcely reported. To improve the overall quality, reusability and reliability of the data for specific purposes, such as the development of nano-relevant AOPs with less overall risk of bias and/or meta-analyses, it is important to address these characteristics in future *in vitro* nanotoxicity studies.

Linking physicochemical characteristics of the test material with the toxicological responses is crucial in NM hazard assessment and for Safer-by-design (SbD) approaches to develop and promote the use of safer NMs. However, such linking is not yet possible in our study due to the lack of experimental data related to the characterization of NMs before and after dispersing in the exposure medium. There is some consensus on the minimal set of material-specific properties (e.g., size, shape, surface area, chemical composition, surface charge, surface reactivity, agglomeration/aggregation, and solubility) that are essential to be evaluated in NM toxicological assessment (ISO, 2012). When assessing the studies in our review, we found that the majority of the studies did not report this minimal set of characteristics, which makes it difficult to even predict the toxicological behaviour of the NMs with the same composition.

Material-specific properties can be largely influenced by experimental conditions such as the use of serum. The reactive surfaces of NMs can interact with their environment and may lead to the formation of a corona (e.g., of proteins) on the particle surface, which can further modify their chemical behavior (Barbir et al., 2021). NMs also tend to agglomerate in cell culture media, which can affect their cellular uptake behavior. On the other hand, sonication, a widely used technique to disperse NMs, can introduce changes to size, shape and destroy the surface properties of the NMs under consideration. Murugadoss et al (Murugadoss, Das, et al., 2021) demonstrated that the agglomerate size in exposure medium is an important nanodescriptor to assess the toxicological effects of TiO_2 NMs in *in vitro* submerged conditions and emphasized that the agglomeration state of NMs can be potentially influenced by *in vitro* exposure conditions. Upon examining the supplementary file 1[1], it becomes clear that the amount of serum used and applied sonication protocols varied significantly across different studies. Moreover, as indicated previously, in addition to other characteristics, often the agglomeration of the NMs at the beginning and/or at the end of the exposure period was also scarcely reported in studies reviewed and analyzed here. Determination of the influence of exposure conditions on physico-chemical characteristics is crucial because such influences could be just a confounder in broader contexts such as SbD approaches, which require linking of material-specific properties to the toxicological outcome.

As previously indicated (Barbir et al., 2021; Murugadoss, Das, et al., 2021; Cheimarios et al., 2022), to reliably link physico-chemical properties to toxicological effects, one should start with the standardization of NM dispersion protocols and experimental conditions. In this way, one can utilize the data from the literature to perform a meta-analysis with minimal bias introduced by experimental conditions and establish reliable linking. Several dispersion protocols have been established, such as NANOGENOTOX and ENPRA (Hartmann et al., 2015), Deloid et al. (2017) or Nanodefine (Mech et al., 2020) that can be applied for many types of NMs, but ideally, there is also a regulatory ambition to move towards one substance-one assessment. Secondly, systematic and case studies should be designed to establish an in-depth understanding of the influence of material-specific properties, such as primary size, shape, dissolution, surface properties, surface reactivity, crystal phase, surface functionalization, and exposure medium specific properties such as agglomeration in different experimental conditions. Alternatively, the same NM should be tested under different experimental conditions such as with and without serum, and NM characteristics such as catalytic property, the release of ions, etc., should be thoroughly characterized in each condition. In this way, we can establish the scientific understanding of the link between material-specific properties and agglomeration and therefore, a better understanding of the association of material-specific properties to the observed toxicity.

Establishing a dose-effect relationship for NMs is vital for hazard characterization, hazard potency ranking, and hazard testing according to the SbD principles. However, establishing the dose-effect dependency for NMs based on existing literature is quite challenging for several reasons. One of the main difficulties is the absence of a common dosimetry approach in reporting such data, which may cause unreliable and biased conclusions about NMs hazard properties. At the scientific and regulatory level, there is still no consensus on what is the best approach and which units should be used for reporting dose-response results following exposure and treatments with NMs (mg/L or number of particles/L or specific surface area/L). Furthermore, the administered dose can differ significantly from the delivered dose reaching the cells. Under *in vitro* submerged conditions, NMs are introduced alongside the cell culture medium and the subsequent settling of NMs depends on their density, size, and the properties of the cell-culture medium (Pyrgiotakis et al., 2013). Moreover, in a submerged environment, NMs often experience dynamic agglomeration in the exposure medium, which significantly impacts the dose reaching the cells. Approaches are now available to model delivered dose including the distorted grid (DG) (Deloid et al., 2017) or ISD3 model (Thomas et al., 2018). These models require the effective density of NMs as input, among others. Effective density is the density of NM in the dispersion medium, and in the case of agglomerates, this includes the density of medium trapped inside the agglomerates. The effective density can be measured using the volume centrifugation method (VCM) or analytical ultracentrifugation (AUC).

Underlining this significance, Murugadoss et al., utilizing the VCM and DG model, showed that about 56-58% of the applied doses of nano-TiO_2 were delivered in a 24-hour exposure, whereas only 7-9% of nano-SiO_2 was delivered at the same time under identical experimental conditions (Murugadoss, Brassinne, et al., 2020; Murugadoss, Van Den Brule, et al., 2020). This difference might be attributed to the density and effective density of the nano-SiO_2 being similar to that of the density of the cell culture medium. Pal et al. (2015) demonstrated that using a variety of NMs, the delivered dose can differ significantly from the administered dose (Pal et al., 2015). Correcting for the delivered dose led to a substantial shift in the hazard ranking of several NMs, aligning more closely with *in vivo* inflammation data. In our review, NM-induced *in vitro* effects were typically observed at unrealistically high doses. However, most studies in our review, conducted under *in vitro* submerged conditions, presented results in terms of administered doses/concentrations without evaluating the delivered doses/concentrations. This oversight currently obstructs meaningful hazard potency evaluations at this moment, emphasizing that the estimation of delivered dose under *in vitro* submerged conditions is an absolute requirement in future studies. An alternative solution involves using an air-liquid interface (ALI) for inhalation or ingestion exposures. Here, NMs can be directly administered to the cells, and a quartz crystal microbalance (QCM) might offer precise measurements of the deposited dose. It has also been shown that NMs can cause toxic effects at deposited doses significantly lower than those administered in submerged conditions (Diabaté et al., 2020; Bessa et al., 2021), indicating that toxicity observed in submerged condition could lead to underestimation of NM induced effects due to discrepancies between administered and delivered doses. Another effort to tackle this issue was made by Cheimarios et al. (2022) who provided a web application for cellular dosimetry based on the DG model, which enables the prediction of the NM concentration reaching the cell surface (Cheimarios et al., 2022). The use of this open access web tool allows correlation of the real exposure concentration with the observed toxicity, which may significantly increase the reliability of toxicity data.

The identification NM-relevant MIEs is also crucial in the development of predictive models for MIE activation, including Quantitative Structure-Activity Relationship (QSAR) models, which assume that different compounds showing similar structural features may have similar mechanisms of action and induce similar toxicological effects (Singh and Gupta, 2014). These tools, such as QSARs, could be used to predict whether a given NM with certain characteristics would trigger these MIEs and thus could be useful for initial screening or prioritization of NMs for hazard assessment. The development of predictive models defining early KEs as well as MIE is now one of the long-term perspectives in the next generation risk assessment (NGRA), according to the OECD (OECD, 2020b). Our analysis showed that most studies investigated mitochondrial toxicity as a KE but not as MIE. This means that no attention has been paid so far to the direct interaction of NMs with mitochondria, or potential mitochondria-related MIEs, such as mtDNA damage, uncoupling, redox cycling, or inhibition of particular protein complexes (Dreier et al., 2019). Future nanotoxicity studies should pay more attention to characterize nano-relevant mitochondrial MIEs as well as subsequent KEs.

AOPs can serve multiple purposes in the RA framework, particularly as informing Integrated Approaches to Testing and Assessment (IATA) and as an integral part of next generation risk assessment workflows (Bajard et al., 2023). AOP-based IATAs are science-based approaches that integrate NAMs and mechanistic knowledge for hazard characterization within a specific regulatory context, potentially eliminating the need for animal testing and fully supporting the 3R's concept (Russell and Burch, 1959). The AOP framework can be particularly useful in identifying the most suitable assays for measuring MIE or KEs that can assess the likelihood of an AO (van der Zalm et al., 2022). Several IATA OECD case studies are already based on AOPs describing pathways for non-genotoxic carcinogens, skin sensitization, chemical-induced liver steatosis, and neural development to assess the applicability of *in vitro* testing batteries for hazard identification and characterization (OECD, 2017; Jacobs et al., 2020; Bajard et al., 2023; Kubickova and Jacobs, 2023). These case studies illustrate that AOPs can help increase confidence in the predictive capabilities of NAMs and further promote their regulatory acceptance. For example, the endorsed AOP 3, which includes mitochondrial dysfunction as a KE, has informed an OECD IATA case study on the identification and characterization of the Parkinsonian hazard liability of rotenone and deguelin, two structurally similar mitochondrial complex I inhibitors (Alimohammadi et al., 2023).

As shown here, NM-induced mitochondrial dysfunction has also been associated with cardiovascular disease (Tab. 6). The heart is particularly vulnerable to changes in energy production, as it is the organ with the highest energy demand per kilogram (Wang et al., 2010) and proper cardiac contractile function requires a constant supply of ATP (Werbner et al., 2023). Therefore, mitochondrial dysfunction can cause cardiomyocyte cell death, resulting in increased cardiac remodeling and, consequently an elevated risk for heart failure (Werbner et al., 2023). However, the current RA of chemicals, including NMs, does not adequately cover cardiotoxicity (Schaffert et al., 2023). Developing AOPs that specifically address cardiovascular AOs through mitochondrial dysfunction may be particularly useful to improve regulatory safety assessment of cardiotoxicity. There are ongoing efforts to address this need, such as the EU 2020 Horizon project ALTERNATIVE[3], which aims to develop AOPs based on mitochondrial dysfunction leading to heart failure via oxidative stress or ATP production decrease (AOP-Wiki AOPs 479 and 480, respectively). The mechanistic knowledge of these AOPs will be used for drafting of an IATA in addressing cardiotoxicity assessment.

The implementation of the AOP framework in RA can bridge the gap between mechanistic toxicological data and regulatory safety assessment for NMs. Ideally, AOPs suitable for RA should undergo a thorough weight-of-evidence evaluation process and be reviewed and endorsed/approved by experts. However, the number of such AOPs is still limited. Among the AOPs identified here that are NM-relevant and cover mitochondrial KEs, the majority of approved AOPs are related to the nervous system, whereas those for the lung, liver, and cardiovascular system are either not included in the OECD work plan or still under development (Tab. 6). These AOPs should be further developed toward OECD approval to increase their regulatory acceptance. The endorsement of NM-relevant AOPs could be facilitated by the discovery of substantial evidence for KERs between MIEs induced by NMs and the KEs in an endorsed AOP. This can not only significantly contribute to the regulatory safety assessment of NMs, but also save substantial resources that would be needed to develop new nano-related AOPs.

[3] https://alternative-project.eu/

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Conflict of interest

The authors declare no conflict of interest

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Data availability

All data of this publication are made publicly available.

Electronic supplementary material

Supplementary file 1[1] includes the list of selected studies, extracted raw data, and evaluation of these studies using a data quality scoring approach based on the GUIDEnano system.

Supplementary file 2[2] presents the data extracted from individual studies (provided in supplementary file 1[1]) on mitochondrial and cellular toxicity endpoints, along with the corresponding cell types and organs/tissues. Each study is represented in a row in the file, which also indicates whether a positive or negative outcome was observed for each endpoint as well as the Q score associated with each study.