SCIENCE-BASED RISK GOVERNANCE
OF NANO-TECHNOLOGY

RISK GONE

REPORT ON THE EXPERT MEETING AOP DRAFT REVIEW

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Abstract

Background, Motivation and Objective

Adverse Outcome Pathways (AOPs) refer to conceptual structures portraying biologic failures initiated by the interaction of a chemical with some biomolecule in the body perturbing normal biology, impairing critical function of the organism and leading finally to the adverse outcome (AO). AOPs depict a series of key events (KEs) along a biological pathway from the molecular initiating event (MIE) to the AO. AOPs are promising tools in risk assessment (RA) and regulatory safety assessments. While extensive efforts have been given to the development of AOPs for chemicals, AOPs for engineered nanomaterials (ENMs) are still scarce. One of the goals of the Work package (WP) on human hazard assessment (WP5) in the RiskGone H2020 project is to evaluate existing AOPs for ENMs in the literature and to generate testable AOPs for ENMs using the existing knowledge. Here, we present our strategy to generate testable AOPs for human hazard assessment of ENMs.

Methodology

In our systematic literature search, pre-defined key words “adverse outcome pathway” OR “AOP” AND “nano*” were used in databases such as PUBMED, EMBASE, SCOPUS and WEB OF SCIENCE. In the first hit, 790 peer-reviewed papers were gathered in total. After removing duplicates, 679 papers were retrieved and screened for title and abstract. Papers not reporting AOPs/AOs and papers reporting on non-mammalian organisms were excluded. Finally, 43 papers were selected for further analysis. An excel template to capture key information such as ENMs characteristics, exposure conditions and biological endpoints (MIEs, KEs and AOs) was created. The ToxR and GuideNano tools were also used for scoring of the data quality of the papers. Then the papers were distributed among the partners and data were extracted. Our first strategy was to verify the cross applicability of existing AOPs in AOP Wiki and to generate testable AOPs for ENMs. In order to achieve this, we identified potential MIEs/KEs reported for ENMs. Using these MIEs/KEs as keywords, potential AOPs in the AOP Wiki were searched, identified and inspected further for the cross applicability for ENMs.

Analysis/Discussion

We identified several AOPs in the AOP Wiki relevant to ENMs and ones that were constructed with MIE/KEs reported also for ENMs. When reviewing these AOPs closer (such as lung and liver fibrosis), we realized that these AOPs could also be applied for ENMs by adapting the MIE based on existing knowledge in the literature. Given the variants of ENMs in the market and prioritizing 3R’s (reduction, refinement and replacement) principle to reduce animal experiments, we propose testable AOPs using *in vitro* experiments with biological plausibility and measurability. These simple and (linear) AOPs can be used to characterize the hazardous potential of a given ENM for induction of a specific MIE leading to an AO via series of measurable KEs. The proposed AOPs provide a rational, biologically based arguments to address uncertainty in risk assessment of ENMs.

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1. Introduction

Engineered nanomaterials (ENMs), because of their appealing physico-chemical properties, are increasingly used in industrial and medical applications, and nano-products are routinely introduced into the consumer market[1][2][3]. Carbon based materials, titanium dioxide (TiO_2), silver (Ag) and silica are the most abundantly used ENMs in these products among others[2]. Such increased production and use of ENMs raised concern among the consumers, regulatory authorities and policy makers regarding their potential human health effects.

Decades of nanotoxicological research revealed that because of their small size and enhanced surface reactivity, ENMs may induce adverse effects in humans as observed for some ENMs in animals (*in vivo*) and in cell cultures (*in vitro*)[3,1]. While animal testing is widely used in risk/hazard assessments because it can rather mimic real-life human exposure, it is considered as not feasible for the hazard evaluation of the tremendous number of ENMs as it will be too expensive, time-consuming and it is not ethical/legal in many countries[5][6]. It has recently been estimated that the time taken to complete the toxicological evaluation of existing ENMs by animal testing would take at least three to five decades[7]. Moreover, small variability in physico-chemical properties of same ENMs are shown to influence the toxicological outcome, making individual hazard assessment of each variant of ENMs by animal testing impossible. Therefore, there is an urgent need to develop simple strategies, but with regulatory relevance, to prioritize and/or to reduce animal testing.

Adverse Outcome Pathways (AOPs) refer to conceptual structures portraying biologic failures initiated by the interaction of a chemical with some biomolecule(s) in the body perturbing normal biology, impairing critical function of the organism and leading finally to the adverse outcome (AO). As shown in Figure 1, AOPs depict a series of key events (KEs) along a biological pathway from the molecular initiating event (MIE) to the AO. Such framework provides systematic knowledge about key toxic mechanisms, very effective in characterizing the individual biological and toxicological potential of substances, and is an important aid in predicting adverse effects of regulatory relevance. AOPs are promising tools in risk assessment (RA) and regulatory safety assessments. While extensive efforts have been given to the development of AOPs for chemicals, AOPs for ENMs are still scarce.

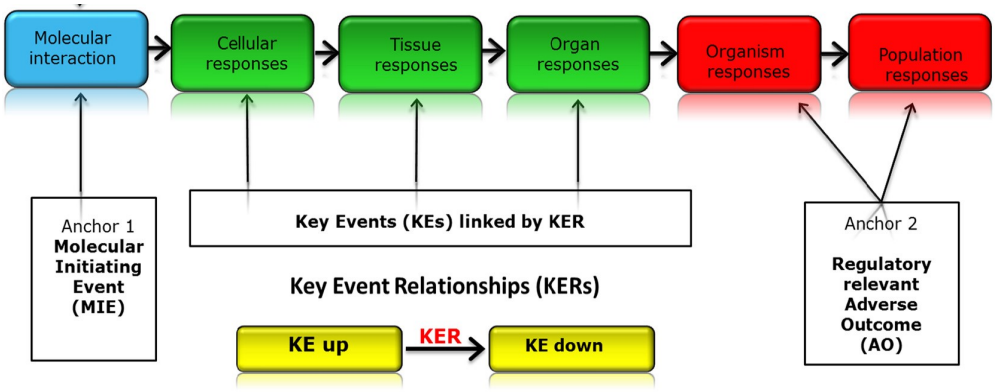

Figure 1: *A schematic representation of the Adverse Outcome Pathway (AOP) framework as depicted in* [8]. *An AOP is triggered by an Molecular Initiating Event (MIE), an initial interaction with a biological*

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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) resulting in an adverse outcome (AO) of regulatory relevance.

Therefore, in this report, we demonstrate how we performed a systematic literature search to identify and evaluate existing AOPs for ENMs. It has also been discussed that the existing AOPs in the AOP wiki can be potentially applied for ENMs as chemicals and ENMs that induce the same AO can share similar KEs/biological endpoints. Therefore, we have also shown how we can generate simple and testable *in vitro* strategies using existing AOPs to predict a given ENM has the potential to induce a MIE leading to an AO through a series of KE.

2. Methodology

Systematic literature search

Five partners (KUL, IMI, UiB, ANSES and BIGCAT) from WP5 were collaborated to perform this task. A schematic representation of our literature search to identify AOPs for ENMs is depicted in Figure 2. In our systematic literature search, pre-defined key words "adverse outcome pathway" OR "AOP" AND nano*" were used in databases such as PubMed, Embase, Scopus (performed by KUL) and Web of Science (performed by IMI). In the first hit, 790 peer-reviewed papers published in English were gathered in total. After removing duplicates, 679 papers were retrieved and screened for title and abstract. Papers not reporting AOPs/AOs and papers reporting on non-mammalian organisms were excluded. Finally, 43 papers covering both *in vitro* and *in vivo* studies were selected for further analysis. An excel template to capture key information such as ENMs characteristics, exposure conditions and biological endpoints (MIEs, KEs and AOs) was created (Table 1). The ToxR and GuideNano tools were also used for scoring of the data quality of the papers. Then the papers were distributed among the partners (KUL, IMI, UiB, ANSES and BIGCAT) and data were extracted.

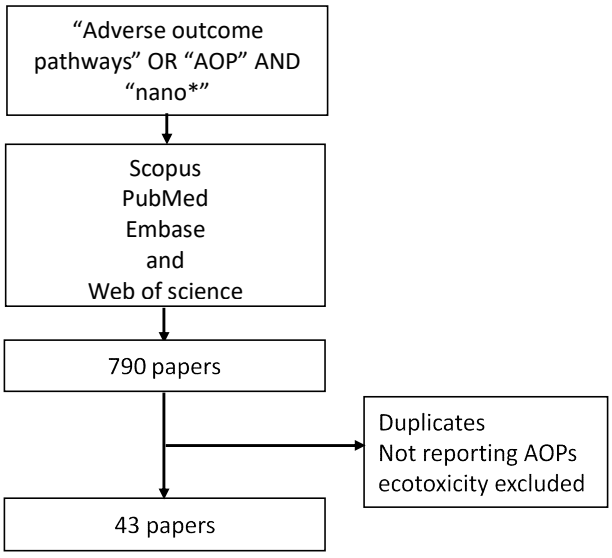

Figure 2: *Scheme of systematic literature search to find peer-reviewed papers reporting nano-relevant AOP*

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Table 1: *AOP template used for data extraction. A-empty template; B-template with data extracted from Nikota et al 2017*

(A)

No.	Nanomaterials (stressor)	Cell type *in vitro*	Animal *in vivo*	Important phy-chem characteristics	Exposure condition		Molecular initiating event	key event 1	key event 2	key event 3	key event 4	Adverse outcome	
					conc	duration		organelle response	Cellular response	Tissue response	organ response	Organism level	population level

(B)

Nanomaterials (stressor)	Animal *in vivo*	Important phy-chem characteristics	Exposure condition		Molecular initiating event	key event 1	key event 2	key event 3	key event 4	Adverse outcome	
			conc	duration		organelle response	Cellular response	Tissue response	organ response	Organism level	population level
MWCNTS	C57BL/6 mice	L-3.86 µm and D ± 13.4 nm	162 µg in a 50 µl	1 and days 28 exposure.	Cellular sensing	induction of cytokines CXCL1, IL-6, and IL-12	Persistent inflammation neutrophils persistent increase	increase of pro-fibrotic genes CCL2, OPN (osteopontin) and TGF-β	Excessive ECM increased collagen deposition Fibroblast proliferation increased vimentin signal	Lung Fibrosis	

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3. Analysis (scientific progress)

Part 1-Identify and evaluate existing AOPs for ENMs

Table 2 depicts a bird's eye view of data reported in papers identified from the literature search. Briefly, carbon-based ENMs (CNTs in particular) are widely investigated in these studies. In terms of AOs, lung (such as lung fibrosis, lung tumors, and mesothelioma) and liver-based AOs (such as liver steatosis and cholestatic liver injury) are majorly reported.

***Table 2**: Summary of ENMs and AOs reported in papers identified from the literature search*

Particle type	*AOs*	*Identified as*	*Link to the AOP wiki source*
CNTs, graphenes and CB	Lung fibrosis/collagen deposition	AO	https://aopwiki.org/aops/206
CNTs and multiNPs	Cell death/apoptosis	AO	https://aopwiki.org/aops/205
C,Ag,ZnO and CeO and TiO2	Cancer/Lung tumors	AO	https://aopwiki.org/aops/139
CNTs	Mesothelioma	AO	https://aopwiki.org/aops/171
TiO2,CeO2 and Ag	Death	AO	https://aopwiki.org/aops/96
CuO	Decreased body weight	AO	https://aopwiki.org/aops/6
Ag	Decreased reproduction and increased mortality	AO	https://aopwiki.org/aops/290
Fullerene,CNTs TiO2 and PM2.5	Effect on offspring	AO	https://aopwiki.org/aops/42
Mesoporous SiO2	Cholestatic liver injury	AO	https://aopwiki.org/aops/27
ZnO	Liver steatosis	AO	https://aopwiki.org/aops/34
SiO2, Fe2O3,CoO,REO,Ag,ZnO and crystalline silica	Inflammation/dysregulation	KE	https://aopwiki.org/aops/303
CNTs	Neuro inflammation	KE	https://aopwiki.org/aops/17
Ag and GO	Impaired cytoskeleton	KE	https://aopwiki.org/aops/70
GdO, MnO and CuO	Liver and kidney damage	N/A	
CNTs	Systemic arthritis	N/A	

We have also performed a search in AOP Wiki page to identify how these AOs are reported in AOP Wiki (Table 2). Most AOs reported in these papers are presented as AOs in AOP Wiki except inflammation and impaired cytoskeleton, which are presented as KEs. In future work, we will perform in-depth analyses of the data to realize how far we are in implementing AOPs in the risk assessment of ENMs and provide future directions for implementing AOPs (i.e., main steps to be undertaken will be summarized).

Part 2 -Apply AOPs for chemicals to ENMs

In this part, we present how we generated simple and testable *in vitro* strategies using existing AOPs to predict a given ENM has the potential to induce a MIE leading to an AO through a series of KE. A systematic review on AOPs for ENMs was presented in part 1. Firstly, we consolidated MIEs and AO of papers reported in our review to identify potential MIE reported for ENMs. Then we explored the AOP wiki for all AOPs relevant for ENMs and used the previously identified MIEs as keywords to search for AOPs that can be potentially explored for ENMs. Finally, simple and linear AOPs using *in vitro* experiments, but with biological plausibility, were proposed to test the potential of ENMs to induce an AOP.

Identification of (molecular) initiating events relevant to ENMs:

To identify potential (molecular) initiating events relevant for ENMs, AOs reported in each of these studies and their respective (M)IEs or first event reported/identified were consolidated (Table 3).

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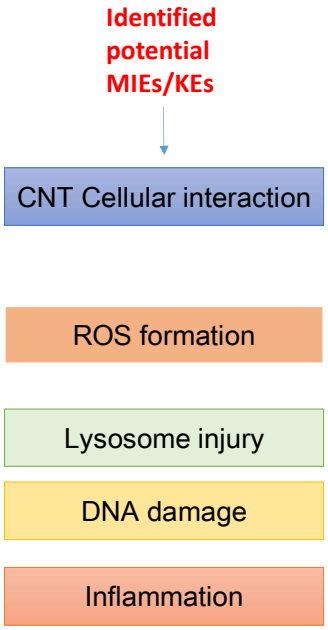
Table 3: *Summary of AO and their respective (M)IE*

Adverse outcomes (AO)	Models	Molecular Initiating event (or first event reported in the study)
Lung fibrosis	in vivo	CNT cellular interaction
Mesothelioma	in vivo	CNT cellular/tissue interaction
Lung fibrosis	in vivo	CNT cellular/tissue interaction
Cardiac dysfunction in fetuses/offspring	in vivo	CNT cellular/tissue interaction
Cell death and DNA repair impairment	in vitro	CNT cellular interaction
Pulmonary inflammation and fibrosis	in vivo	CNT cellular/tissue interaction
Mesothelioma	in vivo	CNT cellular/tissue interaction
Lung fibrosis	in vivo	CNT cellular/tissue interaction
Antioxidant defense, Inflammation, impaired men	in vitro	NP direct interaction with biomolecules/membranes
Persistent lung inflammation (proposed)	in vitro and in vivo	surface silanol disorganization and Membrinolysis
Death and cancer progression	in vitro	ROS formation
weight loss	in vivo	Free radical (ROS) formation
Liver and brain damage	in vitro	ROS formation and dopamine receptor antagonist
Apoptosis	in vitro	ROS formation/amino acid and Glycerophosphocholine accumulation
Cell death	in vitro	ROS formation?
Apoptosis	in vitro	ROS formation
Liver and kidney damage	in vivo	MDA fomation and mitochondrial dysfunction
Lung fibrosis	in vivo and in vitro	Lysosome injury
Cell death	in vitro	Lysosomal acidification
Collagen deposition	in vitro and in vivo	Lysosome injury
Lung fibrosis	in vitro	Genotoxicity
Decreased reproduction and increased mortality	in vivo	Apoptotic stimuli/ROS formation/DNA damage
Impaired cytoskeleton	in vitro	DNA methylation?
Cancer	in vitro	DNA methylation?
Arthritis	in vivo and in vitro	Induction of IL1β and TNFα (TNFα and IL6 in invivo)
Cholestatic Liver injury	in vitro	induction of IL1 and TNFα/BSEP- inhibition
Systemic inflammation and anemia	in vivo	Induction of IL6
Systemic (neuro) inflammation	in vivo	inflammation in the lung?
Kidney damage	in vivo	interuption of calcium homeostatis
Liver and Lung damage	in vitro	altered signalling pathways associated with cyotoxicity ?
Systemic shortage of lipid or hepatic steatosis	in vivo	altered expression of lipid systhesis liver growth factors and apoptotic genes?
Immune system dysregulation	in vitro	activation of intracellular pattern recognition receptors
Lung tumors	in vivo	lung overload?



It is clear from the table that CNT cellular interaction, ROS formation, Lysosome injury, DNA damage and inflammation are the most reported first (initiating) events reported for ENMs.

Identification of potential AOPs in AOP Wiki relevant to ENMs:

In order to identify AOPs in AOP-Wiki applicable to ENMs, we developed a Jupyter notebook (https://github.com/h2020-riskgone/workflows) to explore the AOP-Wiki (https://aopwiki.org/ . It uses the AOP-Wiki RDF for automated extraction of data from the AOP-Wiki and uses the ChEBI ontology to identify ENMs among the chemicals that are present in the AOP-Wiki. Whereas none of the 275 unique chemicals with CAS-ID are considered a ENM, textual mapping indicated that at least 8 stressors do describe ENMs, which are linked to 8 AOPs in the AOP-Wiki (Figure 3A). Furthermore, combining these with textual mapping for 'nano' in AOPs and KEs has led to a list of 14 unique AOPs potentially linked to ENMs in the AOP-Wiki (Figure 3B).

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Stressor	StressorTitle	Part of
Stressor 224	nanoparticles	http://identifiers.org/aop.events/1539
Stressor 224	nanoparticles	http://identifiers.org/aop/144
Stressor 252	Silver nanoparticles	http://identifiers.org/aop/207
Stressor 253	UV-activated Titanium dioxide nanoparticles	http://identifiers.org/aop/208
Stressor 254	Silica nanoparticles	http://identifiers.org/aop/209
Stressor 255	Graphene oxide nanoparticles	http://identifiers.org/aop/210
Stressor 255	Graphene oxide nanoparticles	http://identifiers.org/aop/237
Stressor 318	Carbon nanotubes	http://identifiers.org/aop/237
Stressor 318	Carbon nanotubes	http://identifiers.org/aop/241
Stressor 338	Carbon nanotubes, Multi-walled carbon nanotubes, single-walled carbon nanotubes, carbon nanofibres	http://identifiers.org/aop.events/1458
Stressor 338	Carbon nanotubes, Multi-walled carbon nanotubes, single-walled carbon nanotubes, carbon nanofibres	http://identifiers.org/aop/173
Stressor 338	Carbon nanotubes, Multi-walled carbon nanotubes, single-walled carbon nanotubes, carbon nanofibres	http://identifiers.org/aop/241
Stressor 377	Insoluble nano-sized particles	http://identifiers.org/aop/237

B

AOPID	shortAOPTitle	AdverseOutcome	AOP
AOP 106	Tubulin binding and aneuploidy	Increase, Aneuploid offspring	http://identifiers.org/aop/106
AOP 144	lysosomal uptake induced liver fibrosis	N/A, Liver fibrosis	http://identifiers.org/aop/144
AOP 152	Transthyretin interference	Cognitive Function, Decreased	http://identifiers.org/aop/152
AOP 173	Substance interaction with the lung cell membrane leading to lung fibrosis	Pulmonary fibrosis	http://identifiers.org/aop/173
AOP 207	NADPH oxidase activation leading to reproductive failure	Reproductive failure	http://identifiers.org/aop/207
AOP 208	JAK/STAT and TGF-beta pathways activation leading to reproductive failure	Reproductive failure	http://identifiers.org/aop/208
AOP 209	Cholesterol and glutathione leading to hepatotoxicity: Multi-OMICS approach	Hepatotoxicity	http://identifiers.org/aop/209
AOP 210	JNK, FOXO and WNT alteration leading to reproductive failure: Multi-OMICS approach	Reproductive failure	http://identifiers.org/aop/210
AOP 237	Secretion of inflammatory cytokines leading to plaque progression	Plaque progression in arteries	http://identifiers.org/aop/237
AOP 241	Latent TGFbeta1 activation leads to pulmonary fibrosis	Pulmonary fibrosis	http://identifiers.org/aop/241
AOP 293	Increased DNA damage leading to breast cancer	Increased, Ductal Hyperplasia	http://identifiers.org/aop/293
AOP 293	Increased DNA damage leading to breast cancer	N/A, Breast Cancer	http://identifiers.org/aop/293
AOP 293	Increased DNA damage leading to breast cancer	Increase, Mutations	http://identifiers.org/aop/293
AOP 294	RONS leading to breast cancer	Increase, Mutations	http://identifiers.org/aop/294
AOP 294	RONS leading to breast cancer	N/A, Breast Cancer	http://identifiers.org/aop/294
AOP 294	RONS leading to breast cancer	Increase, DNA damage	http://identifiers.org/aop/294
AOP 294	RONS leading to breast cancer	Increased, Ductal Hyperplasia	http://identifiers.org/aop/294
AOP 296	Oxidative DNA damage, chromosomal aberrations and mutations	Increase, Chromosomal aberrations	http://identifiers.org/aop/296
AOP 296	Oxidative DNA damage, chromosomal aberrations and mutations	Increase, Mutations	http://identifiers.org/aop/296
AOP 303	Frustrated phagocytosis-induced lung cancer	Lung cancer	http://identifiers.org/aop/303

Figure 3: *Screenshots of the Jupyter notebook to extract AOPs applicable for ENMs based on Stressors (A) and AOP/KE textual mapping (B).*

To explore the AOP in more detail, we used the identified MIEs, to search for potential AOPs applicable for ENMs (AOP Wiki/key events/keyword search/MIE). In the first hit, we found several titles linked to each of these keywords (see Figure 4 for lysosomal injury). Therefore, we explored each title and consolidated all the AOPs linked to lysosomal injury. (Figure 5A).

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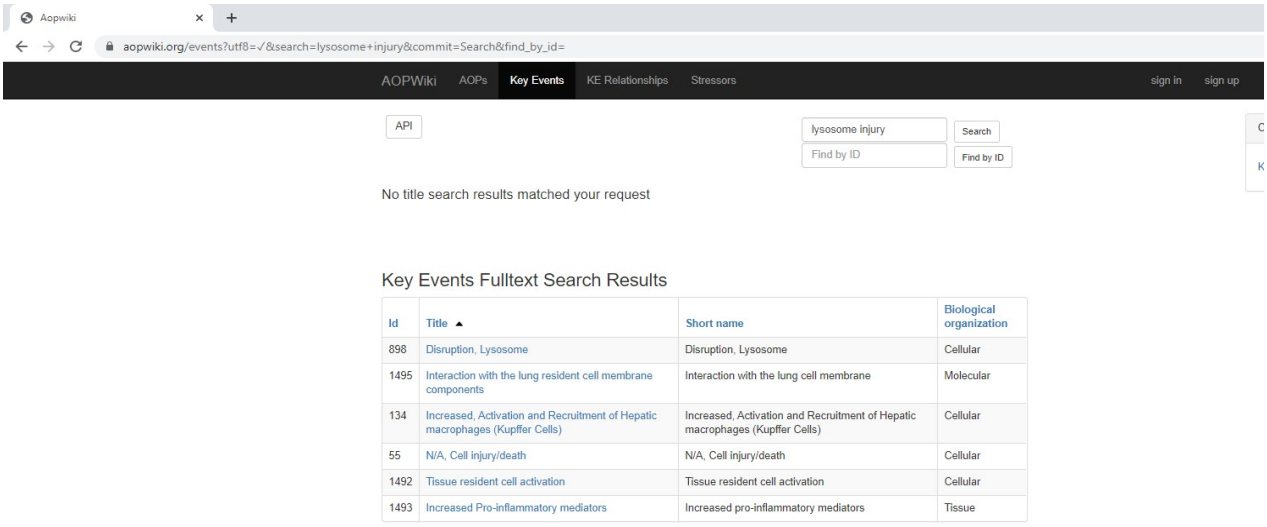
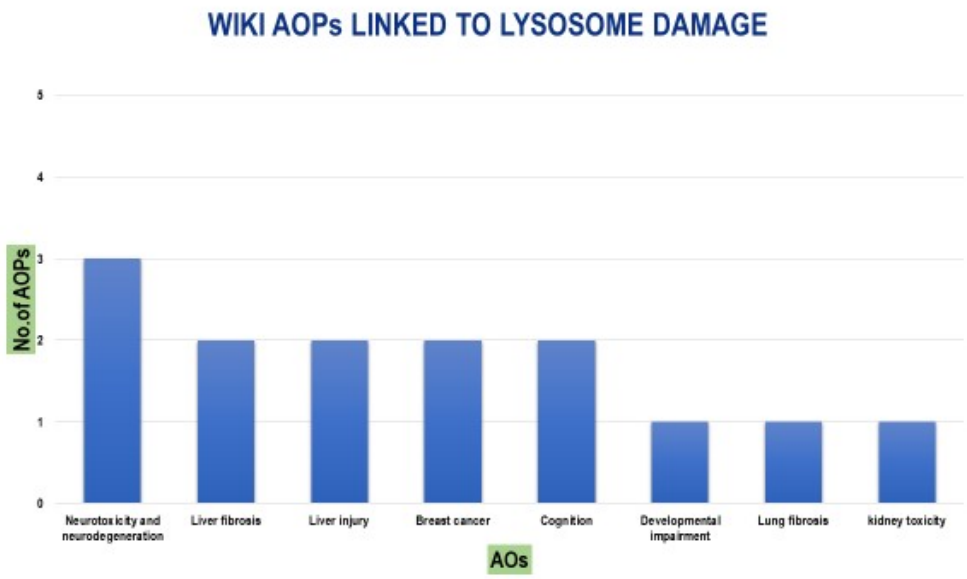
Similar analyses were performed with other MIEs such as CNT cellular interaction, ROS formation (Figure 5B) and DNA damage (Figure 5C). Table 4 shows the total number of AOPs (after removing duplicates). In total, 37 AOPs including lung and liver fibrosis were identified in AOP wiki that can be potentially explored for the cross applicability for ENMs.



Id	Title ▲	Short name	Biological organization
898	Disruption, Lysosome	Disruption, Lysosome	Cellular
1495	Interaction with the lung resident cell membrane components	Interaction with the lung cell membrane	Molecular
134	Increased, Activation and Recruitment of Hepatic macrophages (Kupffer Cells)	Increased, Activation and Recruitment of Hepatic macrophages (Kupffer Cells)	Cellular
55	N/A, Cell injury/death	N/A, Cell injury/death	Cellular
1492	Tissue resident cell activation	Tissue resident cell activation	Cellular
1493	Increased Pro-inflammatory mediators	Increased pro-inflammatory mediators	Tissue

Figure 4: *Screenshot of AOP Wiki page during the search for potential AOPs using lysosomal damage in key event search tab*

A



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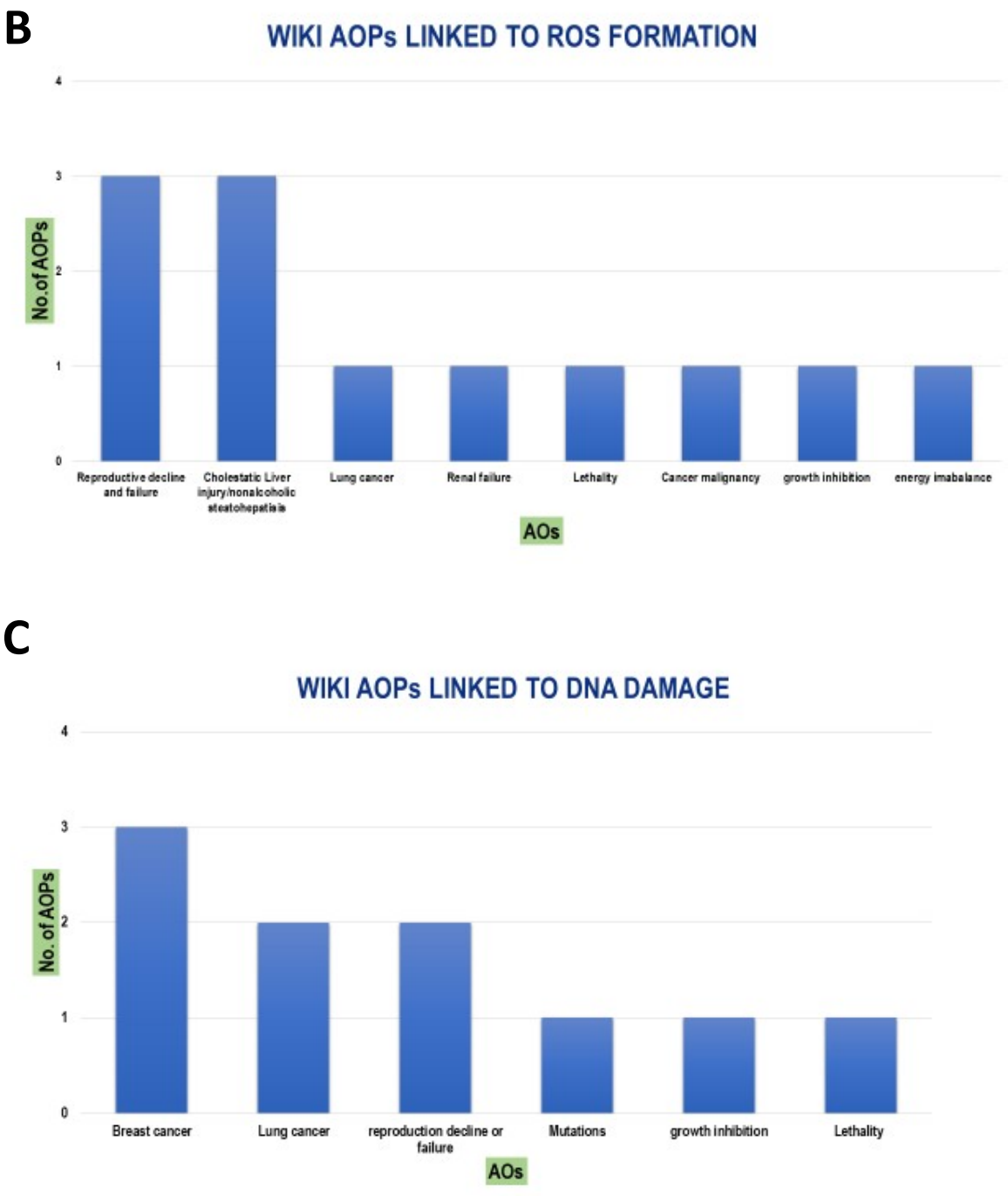

Figure 5: *Summary of AOPs linked to MIEs reported for ENMs. Lysosomal injury (A), ROS formation (B) and DNA damage (C)*

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Table 4: *AOPs identified to be explored for ENMs*

MIE/KE	No.of AOPs
CNT cellular interaction	1
Lysosome injury	14
ROS formation	12
DNA damage	10

Generation of testable strategies using simple in vitro experiments

Since inhalation and ingestion are the primary routes of ENM exposure, we chose to focus on lung and liver fibrosis (AOPs linked to lysosomal injury), respectively, to describe our strategy to generate testable AOPs.

Case study 1: Lung fibrosis

AOP 173 (substance interaction with lung epithelial and macrophage cell membrane leading to lung fibrosis) has been the most discussed AOP for its potential application for ENMs (Figure 6). Briefly, the interaction between the substance and components of the cellular membrane 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 extracellular matrix or collagen (KE6), and eventually lung fibrosis (AO).

It appears that some of the components of this AOP cannot be replaced with *in vitro* cellular assays (such as KE3 and KE4). It is important to mention here that our main objective is to extract relevant information from the literature and integrate to build a strategy to test a potential of a ENM to induce a MIE leading to AO through causally linked KEs.

Multi-walled carbon nanotubes (MWCNTs) are 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 MWCNTs induced lung fibrosis [9]. Based on this information, we propose an AOP of which the major KEs can be tested/verified in *in vitro* testing. Figure 7 shows the AOP aligned **MIE-KEs-AO** that can be measured *in vitro* to predict the lung fibrotic responses *in vivo* and different strategies to test the potential of a given ENM to induce an AO.

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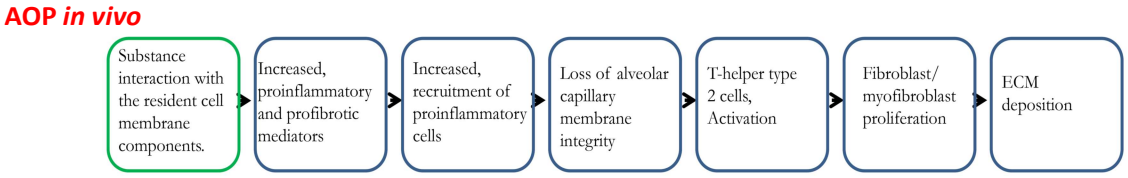

Figure 6: *Schematic representation of AOP 173*

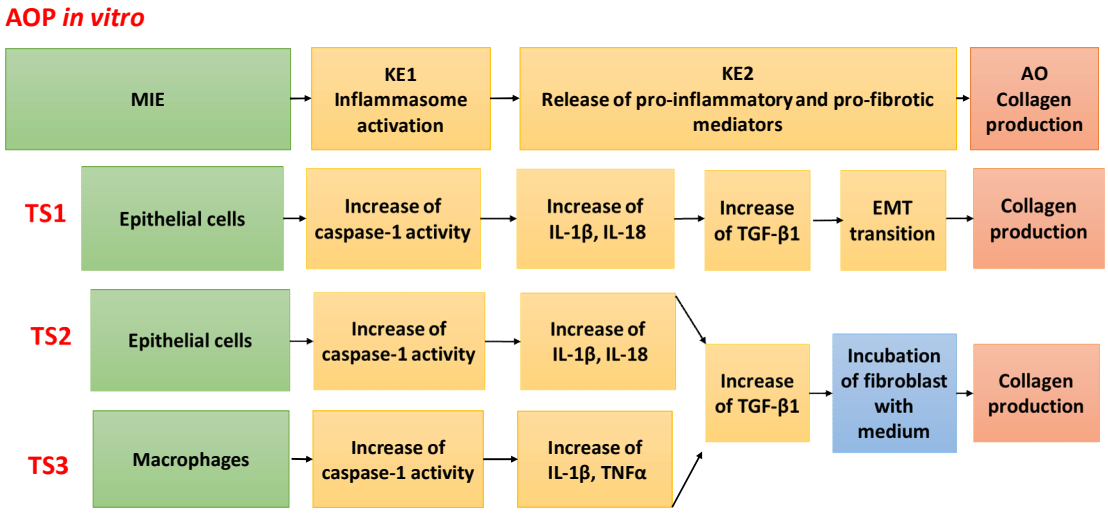

Figure 7: *Proposed in vitro strategy to test the potential of a ENM to induce lung fibrosis*

Frustrated phagocytosis and inflammasome activation induced by MWCNTs are determined as initiating mechanisms of lung fibrosis [9] . When mining the literature, we have also found that high aspect ratio ENMs such as nanowires, nanorods, as well as other ENMs such as fumed silica and cerium oxide also induce inflammasome activation via lysosomal injury, membrane perturbation and/ or frustrated phagocytosis[10]. Despite we lack of information that the inflammasome activation induced by these ENMs led to lung fibrosis, the downstream biological processes of inflammasome activation induced by different ENMs could be similar to MWCNTs. Therefore, we use the existing information specific for MWCNTs and propose the following strategy to test the potential of ENM to induce an AO.

ENM (MWCNTs) interaction with epithelial cells could activate NLRP3 (NOD-like receptor family, pyrin domain containing 3) inflammasome activation and, promote pro-inflammatory and profibrotic mediators release such as IL-1β and IL-18. Caspase-1 activation is an

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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 increase can be measured in the supernatant of the cell cultures to quantify pro-inflammatory and profibrotic mediators release (KE 2).

Here, secreted cytokines act in different pathways (see Figure 7);

Test-strategy (TS) 1: IL-1β promotes the secretion of TGF-β1, which plays a key role in epithelial-mesenchymal transition (EMT). These polarized cells act as an alternative source of fibroblasts and are involved in the production of collagen.

TS2: IL-1β promotes the secretion of TGF-β1, which plays a key role in fibroblast activation, proliferation and collagen production. 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 representing *in vitro* AO to predict lung fibrosis *in vivo.*

TS3: Macrophages also play a key role in the development of lung fibrosis. Upon inflammasome activation, macrophages secrete IL-1β and TNF-α, which are involved in promoting TGF-β1, which in-turn activate fibroblasts and promote collagen production.

Case study 2: Liver fibrosis

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

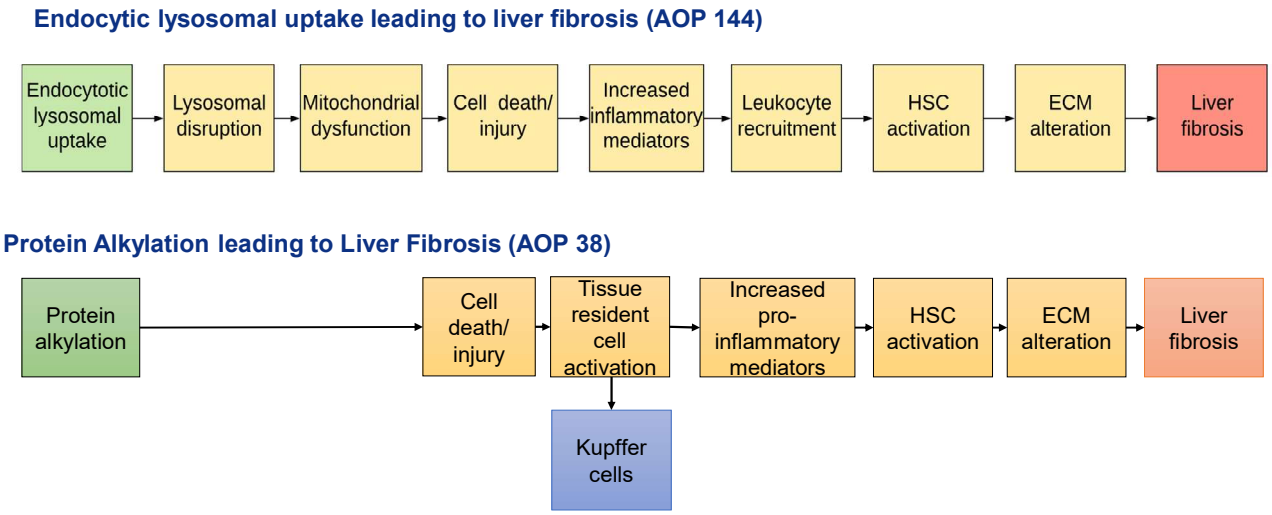

Figure 8: *Schematic representation of liver fibrosis AOPs presented in AOP Wiki*

The scheme of endocytic lysosomal uptake leading to liver fibrosis (AOP 144) is shown in Figure 8. Endocytic lysosomal uptake (MIE) of stressor leads to lysosomal disruption (KE1), which induces subsequent KEs at the cellular level such as mitochondrial dysfunction, cell injury and apoptosis/necrosis. Cell death leads to increased production of pro-inflammatory mediators, which attract and activate leukocytes. Activated leukocytes through molecular mediators activate hepatic stellate cells (HSC), which increases the accumulation of collagen and leading to AO - liver fibrosis. AOP 38 (protein alkylation leading to liver fibrosis) also

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presented with similar KEs except that liver tissue resident cells released mediators activate HSC (instead of mediators released by leukocytes) and with protein alkylation as MIE (Figure 8).

In the literature, it has been reported that several ENMs induce lysosomal disruption and apoptosis/necrosis via lysosomal membrane permeabilization (LMP)[11]. Therefore, we propose test strategies using simple *in vitro* experiments with LMP as a (M)IE (Figure 9).

Test-strategy (TS) 1: Hepatocytes (epithelial cells) can be used as a cell model as ingested ENMs, once entering the blood circulation, encounter the epithelial layer of the liver. ENM-induced LMP (measured by assays such as neutral red/acridine orange) and mitochondrial dysfunction (mitochondrial membrane potential, MMP) of hepatocytes lead to cell injury (cytotoxicity assays such as WST-1, LDH). TGF-β1 secreted by leukocytes play a key role in transforming injured epithelial cells into mesenchymal cells (EMT transition), which produce collagen. Therefore, TGF-β1 can be provided externally to the epithelial cell cultures and collagen production in exposed hepatocytes can be measured as a representative *in vitro* AO to predict lung fibrosis *in vivo.*

TS2: Mediators secreted by injured hepatocytes play a key role in the activation of hepatic stellate cells (HSCs) and collagen production. To verify this, HSCs can be incubated with cell culture medium collected from ENM exposed cell cultures (hepatocytes) and collagen production in exposed HSCs can be measured as a representative of AO.

TS3: Kupffer cells, liver resident macrophages, also play a key role in the development of liver fibrosis. Kupffer cells encounter with ENM and secretion of TGF-β also play a key role in HSC activation. To verify this, HSCs can be incubated with cell culture medium collected from ENM exposed cell cultures (Kupffer cells) and collagen production in exposed HSC cells can be measured.

From the obtained results, it can be determined the potency of a ENM to trigger a MIE leading to AO (collagen production) via any of these pathways (TS1, 2 and 3) or not (no collagen production).

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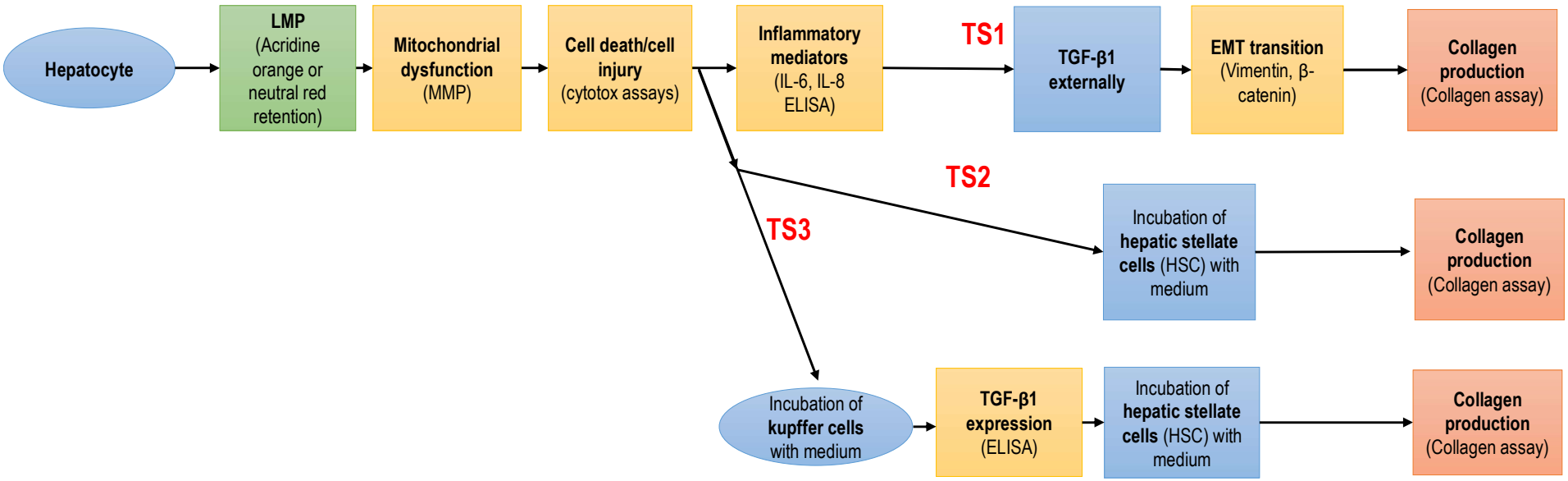

Figure 9: *Proposed in vitro strategy to test the potential of a ENM to induce liver fibrosis*

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4. Webinar Summary

To discuss our findings with the experts and formulate the way forward, a webinar topic entitled “Adverse Outcome Pathways for Nanomaterials” was organized by RiskGONE WP5 partners and presented on 5th June 2020. Nearly 40 participants (experts/members) attended the meeting and few important issues were discussed. The total duration of the webinar was 90 minutes.

The webinar was presented in three sessions. The first session of the webinar was launched by Dr. Ivana Vinković Vrček, Institute for Medical Research and Occupational Health, Zagreb, Croatia. In this first session, the concept of AOP was introduced. A brief description of available AOP knowledge bases (such as AOP Wiki) and an overview on how to use these tools was presented. Finally, approaches required to contribute to the upgrading of these knowledge bases was proposed and discussed. Questions regarding the accessibility (such as publicly available or not) of AOP knowledge bases were raised during the discussion session of the webinar. Ivana informed the knowledge bases that are freely available to the public (Such as AOP Wiki).

Prof. Peter Hoet and Mr.Sivakumar Murugadoss from KU Leuven presented the session 2. In this session, an overview of a systematic literature search summarizing existing AOPs for ENMs (performed in riskGONE project) was presented. A summary of existing chemical AOPs with MIE and/or KE specific for ENMs was shown. Finally, a few examples were demonstrated on how to generate a testable hypothesis for AO of ENMs from chemical AOPs. At the end of the webinar, Prof. Peter Hoet and Sivakumar addressed the question from participants regarding the development of AOPs and discussed the potential application of mRNA, extracellular vesicles, proteins and genes in AOP construction. Sivakumar involved in discussion with the experts/participants about inflammation, a major KE in ENM induced AOPs. In addition, Prof. Peter Hoet discussed the factors such as class of ENMs, dose and concentrations, and their influences in the construction of AOPs.

The final session was presented by Dr. Karolina Jagiello QSAR lab, Gdańsk, Poland. In this session, the concept of quantitative structure activity relationship (QSAR) was introduced and the importance of QSAR-AOP integration was discussed. An example on how to integrate OMICs data with ENMs structure was shown. Finally, potential information needed in the future, such as nano-specific MIEs/KEs for further development of QSAR-AOP, was discussed.

Mainly, experts/participants questioned how AOPs are useful in establishing QSAR and in the risk assessment of ENMs. Dr. Jagiello demonstrated the potential application of transcriptomic-based and AOP-anchored QSAR approach in the risk assessment of ENMs. Altogether, the discussion with experts shed more light on the technical and experimental aspects of AOP construction, and its scope in silico hazard/risk assessment of ENMs.

5. Conclusion

From the initial analysis, it appears that there is a lot of potential to generate testable AOPs for ENMs by combining existing AOPs in AOP Wiki and existing knowledge from the literature. Such a strategy is useful to reduce animal testing in the long term; however,

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animal studies are required to obtain toxicokinetics information. Further, validation of these *in vitro* AOPs by animal testing is needed. In addition, these strategies are useful to generate mechanistic information to represent the knowledge gaps in hazard assessment and to reduce the complexity of the experimental approach.

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