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Research paper

A systematic process for identifying key events for advancing the development of nanomaterial relevant adverse outcome pathways

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ABSTRACT

Adverse outcome pathways (AOPs) represent a sequence of key events (KEs) between a molecular initiating event (MIE) and an adverse outcome (AO) and span many levels of biological organization including molecular, cellular, tissue, organ, organism, and population. AOPs link specific biological observations with AOs. AOPs are not specific to individual chemicals; however, they were traditionally developed for chemicals. The objective of the present study is to advance the identification of KEs using existing nanotoxicology literature as part of an Organisation for Economic Cooperation and Development (OECD) Working Party on Manufactured Nanomaterials (WPMN) project aiming to support the future development of AOPs relevant for Manufactured Nanomaterials (MNs). A database of 11,000 nanotoxicology studies published between 2000 and 2013 was assessed for the types of MNs investigated and for the assays, endpoints and toxicity effects presented. Since tissue inflammation is one of the consistently observed and reported effects following MN exposure, the large database was processed to select those studies reporting specifically on inflammation to identify inflammation-associated KEs. This exercise resulted in 191 publications describing ~60 different endpoints for 45 different MNs, which were used in identification of MN-induced KEs and selection of single or multiple KEs that are relevant to AOs of regulatory interest. This report summarises the key findings of the study describing the various KEs identified, and the reported assays and specific measurements used to assess the KEs. The report also describes a single KE 'Tissue Injury', selected by the process for further development in a case study as part of the OECD WPMN project to show its relevance to MN-induced AOs, and in turn, to future MN risk assessment. Finally, the challenges and limitations of the existing nanotoxicology literature for the development of MN-relevant AOPs are highlighted.

1. Introduction

The diversity and number of manufactured nanomaterials (MNs) emerging in the market requires novel approaches to assess their risks. Adverse Outcome Pathways (AOPs) are conceptual frameworks that link key biological events occurring post-substance exposure to

Abbreviations: MN, manufactured nanomaterial; AOP, adverse outcome pathway; KE, key event; MIE, molecular initiating event; AO, adverse outcome; KER, key event relationship; OECD, Organisation for Economic Co-operation and Development; WPMN, Working Party on Manufactured Nanomaterials; SmartNanoTox, Smart Tools for Gauging Nano Hazards; EU, European Union; H2020, Horizon 2020; PATROLS, Physiologically Anchored Tools for Realistic nanomaterial hazard aSsessment; NanoAOP project, Advancing Adverse Outcome Pathway Development for Nanomaterial Risk Assessment and Categorisation; FOPH, Swiss Federal Office of Public Health; EAGMST, Extended Advisory Group on Molecular Screening and Toxicogenomics; NanoTox 2018, 9th International Conference on Nanotoxicology; VCI, German Chemicals Industry Association; BALF, bronchoalveolar lavage fluid; NOEL, no-observable-effects-level; LOEL, lowest-observable-effects-level; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BALF, bronchoalveolar lavage fluid; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LDH, lactate dehydrogenase; MCP, monocyte chemoattractant protein; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; PCR, polymerase chain reaction; PMN, polymorphic mononuclear cell; RT, real time; SOD, superoxide dismutase; SWCNT, single walled carbon nanotube; TARC, thymus and activation regulated chemokine; TEM, transmission electron microscopy; TGFβ, transforming growth factor beta; TLR, toll-like receptor; TNF-α, tumour necrosis factor α; ATP, adenosine triphosphate; IFN, interferon; TEER, transepithelial electrical resistance

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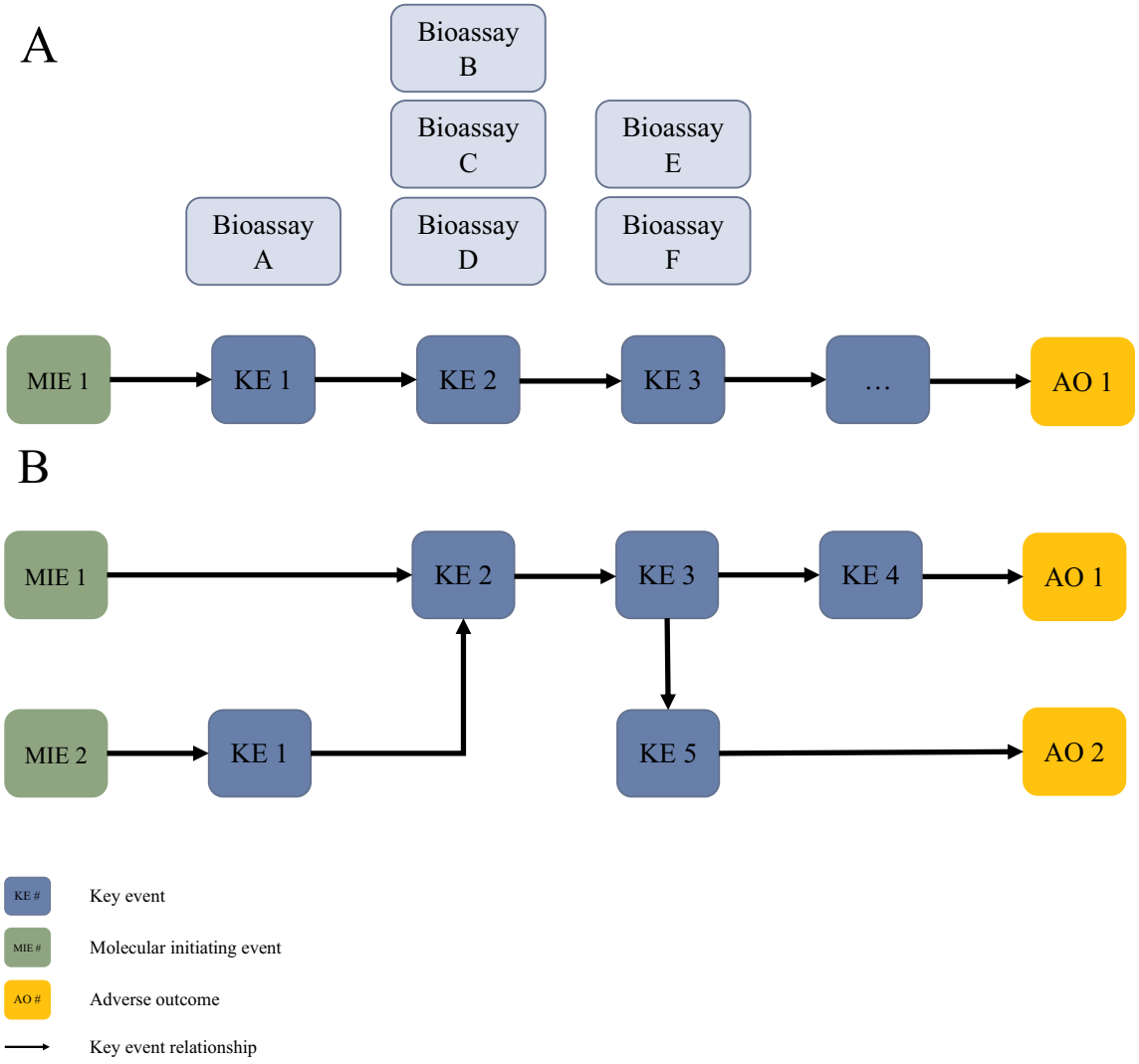

Fig. 1. (A) Generalised AOP showing how KEs can be used to design and develop targeted bioassays predictive of an AO. (B) Demonstration of how multiple AOPs form networks of MIEs, KEs and AOs, representing the complex biology underlying disease processes.
(Adapted from Villeneuve et al. (2014a) and Carusi et al. (2018).)

eventual health impacts of regulatory relevance. AOPs are explicitly designed to organize toxicological information in support of regulatory decision-making (Villeneuve et al., 2014a) and are highlighted as important tools for several applications in risk assessment. AOPs have the potential to advance grouping, categorisation and read-across efforts; offer mechanistic insights and weight of evidence in developing integrated approaches to testing and assessment; improve or refine *in vivo* testing strategies; develop targeted mechanism-based *in vitro* or alternative testing strategies; and provide mechanistic support for models such as quantitative structure-activity relationships to enhance their use (OECD, 2016; *Validation of Alternative Methods for Toxicity Testing*, 2016).

AOPs represent a sequence of key events (KEs) that link a molecular initiating event (MIE), which is a specialised KE, to an adverse outcome (AO) (Villeneuve et al., 2014a). AOs can span different levels of biological organization and include organ, organism, and population responses that result in an adverse environmental or human health effect. The causal/predictive relationships linking adjacent KEs and ultimately to AOs are known as Key Event Relationships (KERs). Fig. 1 depicts a generalised AOP (Panel A) and demonstrates in a simplified example how AOPs form networks of MIEs, KEs and AOs (Panel B), representing the complex biology underlying disease processes. Bioassays and endpoints are characterized or developed to measure a single or multiple KEs expected to be predictive of the ultimate AO, using single or multiple *in vivo* and/or *in vitro* methods. As AOPs represent mechanistic biological processes leading to an AO, they are by definition not substance specific (Villeneuve et al., 2014a). However, AOPs to date have mainly focused on known toxicological mechanisms of chemicals. Although AOPs that capture MN-induced AOs are emerging, there is a need to support future development of AOPs that contain KEs relevant for MNs in support of MN risk assessment.

In its report on "*Alternative Testing Strategies in Risk Assessment of Manufactured Nanomaterials: Current State of Knowledge and Research Needs to Advance Their Use*" the Organisation for Economic Co-operation and Development (OECD) Working Party on Manufactured Nanomaterials (WPMN) recommended developing and validating AOP frameworks for MN risk assessment (OECD, 2016). The need for MN-related AOPs was further emphasized by a European Union (EU) report, due to MN-specific issues such as deeper tissue infiltration and accumulation potential, ability to bind biomolecules and alter their functions, and other mechanisms not typically observed for chemical substances (Gerloff et al., 2017). In essence, the size-associated aspects of MNs require further investigation into the mechanisms of toxicity and how these mechanisms may lead to an AO. Although in their infancy, several efforts are currently underway to develop AOPs with direct relevance for MNs (Labib et al., 2016). In addition, SmartNanoTox

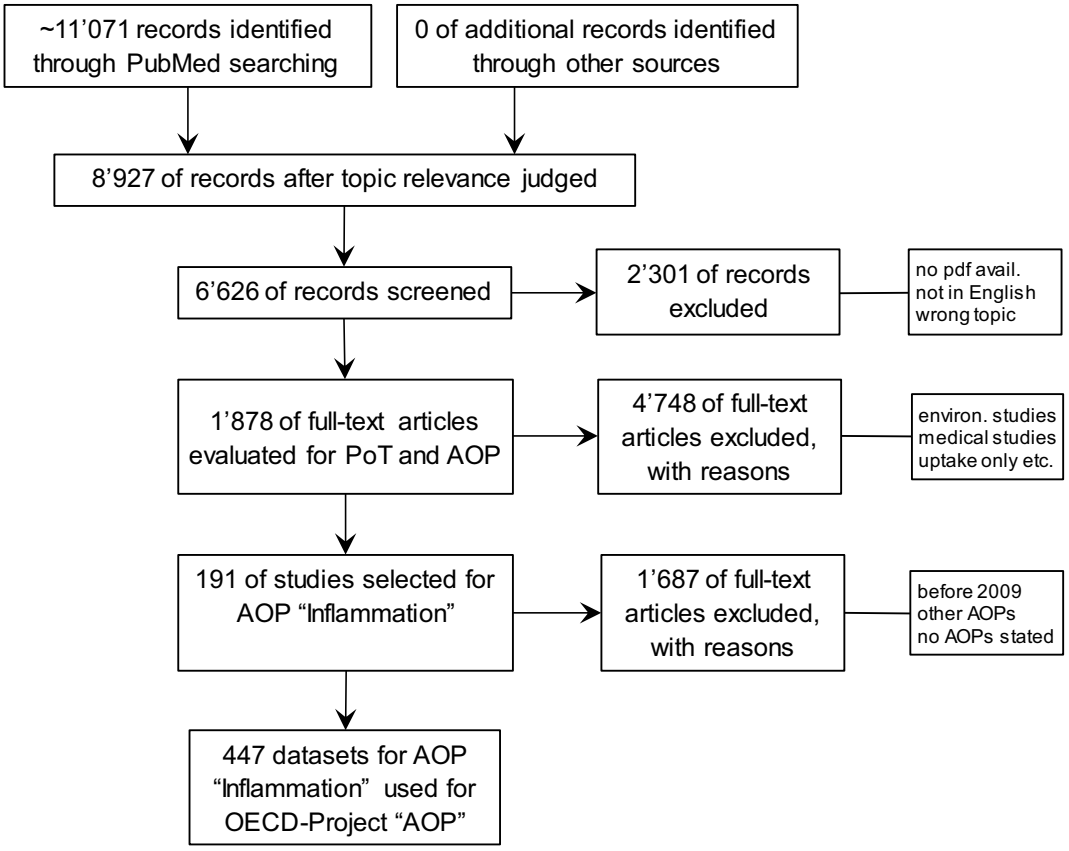

Fig. 2. Scheme of evaluation of the literature between 2000 and May 2013 and the selection process of the 191 studies taken for the actual AOP-project. From more than 11,000 publications the number was reduced by several exclusion processes. Excluded have been all studies without any toxicological content or no biological systems investigated. Moreover, papers in a different language or when no pdf file was downloadable were omitted as well. Only papers referring to "inflammation" were selected for the present study. As each study may consist of more than one dataset (*e.g.*, more than one material, more than one cell type or different sizes of a certain material have been tested) the 191 selected studies resulted in 447 datasets.

(Smart Tools for Gauging Nano Hazards), a EU Horizon 2020 (H2020)-funded project, is aiming to identify potential KEs in the respiratory pathways activated by MNs using *in vivo*, *in vitro* and *in silico* research to develop MN-relevant AOPs. Similarly, another EU H2020-funded project, PATROLS (Physiologically Anchored Tools for Realistic nanOmateriaL hazard aSsessment), is developing mechanisms-based, non-animal (*in vitro*) models and computational tools for MN hazard assessment, targeting the KEs in established AOPs (*e.g.* AOP 173 in the OECD AOPWiki), and other putative AOPs that are being developed as part of the ongoing EU H2020 projects.

Although complete quantitative or qualitative AOPs with various KEs identified and KERs established are highly desired, in the context of MNs, the existing toxicological information is not sufficient to support the full development of AOPs. Thus, an OECD WPMN project "Advancing Adverse Outcome Pathway Development for Nanomaterial Risk Assessment and Categorisation" (NanoAOP project) is investigating, through a case study approach, how incorporation of data on KEs from the existing nanotoxicology literature potentially linked to AOs can advance the future development of AOPs in support of the MN risk assessment process. The NanoAOP project is led by the Canadian delegation (Health Canada & Environment and Climate Change Canada) of the OECD WPMN, with support from Alberta Innovates. Participants include the National Institute for Public Health and the Environment in the Netherlands, NanoCASE and the Swiss Federal Office of Public Health (FOPH) in Switzerland, the National Institute for Occupational Safety and Health in the United States, the National Institute for Occupational Health in South Africa and the University of Birmingham in the United Kingdom. Vireo Advisors in the United States provides coordination support for the project.

The specific and the overarching objective of the project is to establish an approach to advance future AOP development relevant for MNs using the available nanotoxicity literature. The immediate term sub-objectives include (1) identification of KEs induced by MNs, (2) development of a methodology, through a case study approach, for evaluating the usefulness or relevance of a selected KE for informing the process of future MN risk assessment and (3) convening of expert workshops to gain feedback on the current status, use and future needs for AOPs relevant to MNs in support of risk assessment. It is important to note that the aim of the project was not to develop a full AOP for a specific AO induced by MNs, but to develop a case study that describes an approach and provides a methodology for identifying and developing specific KEs and their KERs using the existing nanotoxicology literature in support of development of potential AOPs relevant to MNs, in accordance with the OECD Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST) committee guidelines. The project focuses specifically on KEs in the inflammation pathway as (1) there is a substantial literature base examining the inflammation processes for MNs to build the proposed method and case study and (2) inflammation occurs in many AOPs and is a precursor to several AOs. This article summarises the approach developed to identify MN-relevant KEs from the literature and describes the selected KE that will be used in a case study. In addition, it presents key outcomes of a workshop convened during the 9th International Conference on Nanotoxicology (NanoTox 2018).

2. Material and methods

2.1. Approach for identifying potential KEs using nanotoxicity literature

2.1.1. Swiss-VCI database

Switzerland contributed a robust database of nanotoxicology literature on which to base the first phase of the project which identified MN-induced KEs from the literature. The database was originally developed as part of a project to evaluate the potential health, exposure and environmental effects of MNs (Krug, 2014). Co-financed by the Swiss FOPH and the German Chemicals Industry Association (VCI, Verband der Chemischen Industrie e.V.), the Swiss-VCI database contains information from studies published between 2000 and May 2013 and was co-opted to serve as the foundation for this work.

At the time of this writing, approximately 11,000 studies published from 2000 to 2013 have been assessed for their toxicological content (Fig. 2) following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses evaluation scheme (Liberati et al., 2009). A selection process was implemented to screen for papers from the database of 11,000 investigating inflammation in biological systems, and excluding papers not written in English or that were inaccessible (Fig. 2). This resulted in a database that included 191 studies, representing 447 records reporting on inflammation, spanning ~60 endpoints (*e.g.*, inflammation, cytokine secretion, cell death), for 45 different types of MNs. The term record is used to capture individual datasets within the study (*e.g.* if a study reported on five individual nanomaterials, it would count as 5 records). Most studies contributed multiple records since they investigated different forms of nanomaterials and/or different endpoints. A complete list of the 191 studies captured in the database is provided as Supplemental Table 1.

Until this step, no selection criteria for study quality had been applied. Although a quality scheme (DaNa methodology) was developed, applying it strictly would result in a very small number of studies and would make it impossible to generate a list of KEs induced by MNs (Krug et al., 2018). For example, quality assessment of the 447 identified records following the DaNa quality criteria catalogue resulted in the following outcome:

109 records: from poor studies (missing material characterization and/or usage of ultra-high concentrations or doses and/or no controls for biological endpoints have been included *etc.*)

322 records: from acceptable studies (most quality criteria fulfilled but not strictly all)

16 records: from good studies (quality criteria fulfilled)

Thus, a quality screening was not applied to the 191 studies (447 records) included in the KE analysis.

2.1.2. Database analysis to identify potential KEs

Inflammation is an important and consistently observed response following exposure to a variety of MNs. While inflammation occurring acutely following exposure to a toxic substance is protective and part of an organism's defense mechanism against foreign stimuli, sustained inflammation in response to recurring exposure or tissue persistence of a toxic substance can result in chronic or persistent inflammation and tissue injury, which is associated with a number of AOs. Thus, the selected 191 studies were analysed individually to identify the various biological events elicited by MNs, including those that may be associated with inflammation but not necessarily connected to an AO. The analysis documented the type of MN, the experimental model and mode of MN exposure, the post-exposure sampling timepoint, the specific assays used and the various measurements (endpoints) reported. Where possible, endpoints were assigned to a potential KE and an AO using expert judgment. It is noteworthy that all biological events may not be considered KEs. The KEs assigned to measurements such as cytokine expression and differential cell counts in bronchoalveolar lavage fluid (BALF), which are directly related to inflammation, were framed according to the guidance published in Villeneuve et al., 2018 for representing inflammation as a KE in an AOP.

In addition, the database of 191 studies was analysed to explore the possibility of deriving potential dose-response and property-response matrices for MNs. The reported physical and chemical properties (*e.g.*, size, shape, crystallinity) and toxicity values (*e.g.*, no-observed-effect-level [NOEL], lowest-observed-effect-level [LOEL]) for two MNs—titanium dioxide and silicon dioxide—were captured. These materials were evaluated for establishing (1) potential relationships or correlations between their physical and chemical properties and reported biological events, and (2) potential dose-response relationships. However, the available data were too disparate in terms of physical and chemical property information reported, types of assays used, and endpoints reported to allow such analysis.

2.2. Selecting a single KE for case study development

A series of criteria were developed to prioritize the potential KEs identified in the database analysis to select one to serve as a case study. These criteria were centered around three main objectives, ensuring that the selected KE is: (1) plausible, (2) measurable, and (3) relevant for regulatory considerations. The generated list of potential KEs was reviewed and assessed against these criteria.

2.3. Expert workshop

An expert workshop was held during NanoTox 2018, titled "Advancing Adverse Outcome Pathway (AOP) Development for Nanomaterial Risk Assessment and Categorisation." The workshop was designed as an interactive forum intended to gain expert input on: (1) perspectives on inflammation and the associated challenges for AOPs; (2) the technical, scientific and research questions surrounding AOPs, including feedback on the selection of a KE for the case study and the specific approach and (3) use of the AOP framework for the risk assessment of MNs. A list of expert panelists who participated in the workshop is provided in Supplemental Table 2.

The workshop format included an opening discussion on the dichotomous role of inflammation as an adverse *versus* normal response and associated implications for AOPs, followed by two interactive panels. The first panel discussed the technical, scientific and research questions surrounding AOPs with a focus on the project approach and the KE selected for a case study, while the second panel addressed the application and use of the AOP framework specifically for MN risk assessment purposes.

3. Results

3.1. Potential KEs identified using nanotoxicity literature

The database provided robust datasets for the analysis that included a diverse set of both *in vivo* and *in vitro* studies and model systems. *In vivo* studies involved routinely used mammalian models such as mice and rats, as well as non-mammalian models, such as drosophila, medaka, nematodes and zebrafish. Similarly, the *in vitro* dataset consisted of over 45 different cell types derived from various species (data not shown). Together, these studies identified biological events occurring after exposure to ~45 different types of MNs.

Studies on the inhalation exposure route were especially well represented in the database. In inhalation studies, MNs were deposited using whole body or nose-only inhalation, intratracheal instillation, oropharyngeal aspiration and other modes. Many biological events following inhalation exposure were observed in the lung, though effects were also reported in other organs such as the brain, heart, liver and spleen, suggesting systemic responses. The oral route of exposure administered *via* oral gavage or through food/water and dermal exposure routes were also well represented in the database, and biological events resulting from MN exposure *via* these routes were reported in several

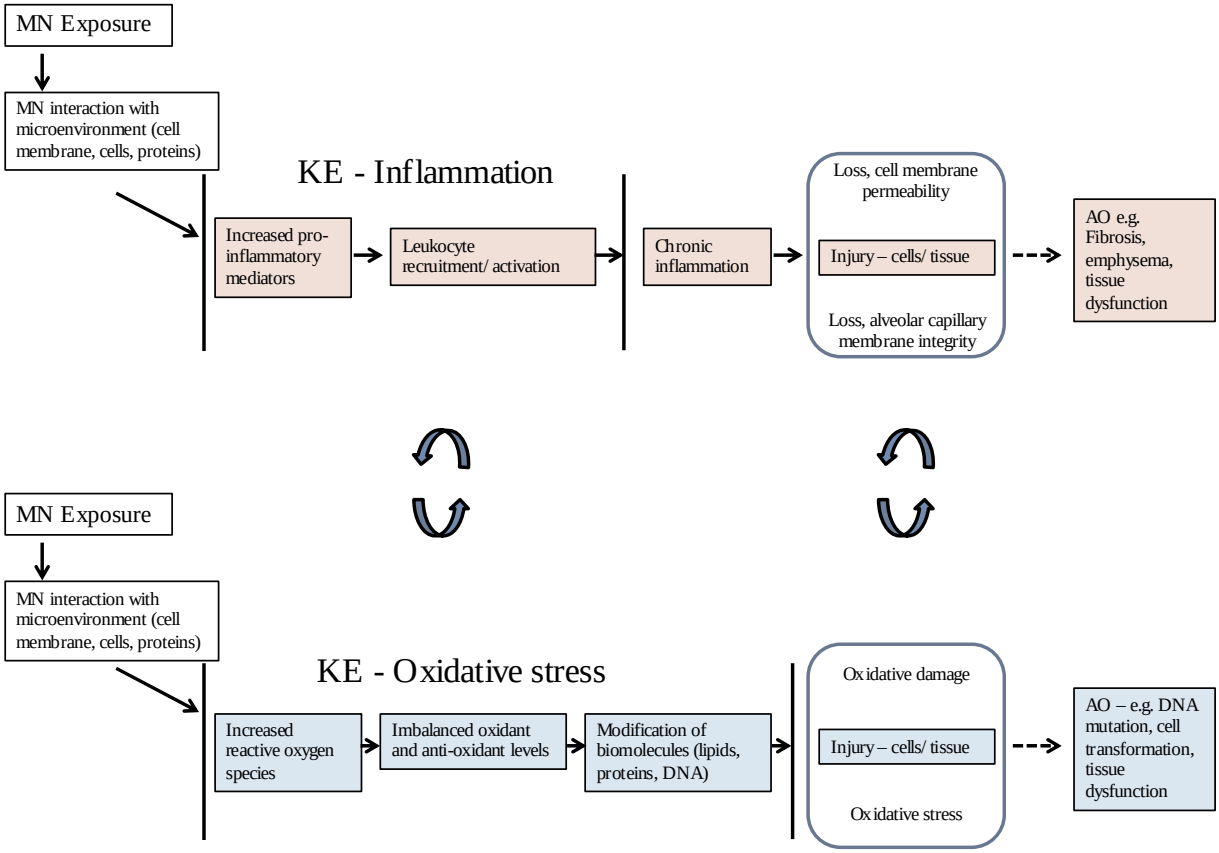

Fig. 3. Potential MN-induced KEs from Table 1 and Supplemental Table 3 organized in relationship to one another and known processes for inflammation (top panel) and oxidative stress (bottom panel). The KE 'chronic inflammation' is not listed; however, it is used to reflect the temporal aspects of the inflammation KE, which may be used to distinguish between the benign inflammation from an adverse one. The cycling arrows depict the feedback loop between the oxidative stress and the pro-inflammatory process. The colored boxes depict the KEs assigned to the measurements listed in Table 1 and Supplemental Table 3. The dashed arrow suggests that the exact relationship shared between the tissue injury KE and the hypothetical AOs could be both direct and indirect. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

organs. In contrast, direct and deliberate exposures, such as intraperitoneal and intravenous exposures, were less well represented in the literature examined.

To isolate potential KEs from the literature in the Swiss-VCI database, biological events reported following MN exposure were captured and were assigned to potential KEs and AOs. Table 1 lists 7 randomly picked studies from the 191, demonstrating how the data analysis was performed and the key biological events induced following MN exposure were identified. Supplemental Table 3 is an expanded analysis of 25 studies, and Supplemental Table 4 summarises the results for all 191 studies, grouped by exposure route, organ where the effect was observed, and observation type (*e.g.*, clinical symptoms, histopathology). Expert judgment was used to apply consistent terminology across studies when summarising the results in Supplemental Table 4. Biological events reported in non-mammalian models such as drosophila or zebrafish, and *in vitro* studies are not captured in Table 1 or Supplemental Table 3.

The biological events reported in the literature spanned various levels of biological organization including observations at the molecular through the tissue or organ level (Supplemental Tables 3, 4). Some of these biological events fell upstream of inflammation (regulatory events such as inflammasome activation), while others were associated directly with the inflammatory process (*e.g.*, increased pro-inflammatory mediators such as cytokines, or leukocyte recruitment), and still others occurred downstream of inflammation (*e.g.*, increased alveolar thickness, granuloma formation). There were some that were viewed to function parallel to the process of inflammation (*e.g.*, oxidative stress), assumed to form positive feedback loops propagating and advancing the process of inflammation. In the case of events downstream of inflammation, the events were typically the consequences from an adverse inflammatory response (un-resolved or persistent/ chronic inflammation), such as tissue injury. Of the reported biological events following MN exposure, those associated directly with the inflammatory process were reported most frequently, though tissue injury, which occurs downstream of inflammation, was also a frequently reported biological event. The list of measurements or potential KEs identified in Table 1 and Supplemental Table 3 is organized in a schematic (Fig. 3) to visualise their relationship with each other, to inflammation, and to a hypothetical AO. KEs identified were mainly inflammatory in association or downstream of inflammation (top panel, Fig. 3) and were reflective of the pro-inflammatory process, cell or tissue injury/damage (*e.g.*, measurements such as total protein, LDH release, and histopathology). The other widely investigated response was oxidative stress, which was frequently reported through measurements such as synthesis of reactive oxygen species, altered expression of antioxidant genes or proteins, lipid peroxidation, and protein modification. These measurements were grouped under an overarching KE 'oxidative stress' and were assigned more generalised KE titles. Oxidative stress was considered as an associative KE to an inflammation-induced AO, and thus was presented in a pathway parallel (lower panel, Fig. 3) to inflammation. The majority of these studies investigated oxidative stress as indicative of cell or tissue damage. As suggested earlier, oxidative stress may play an important role in the pro-inflammatory pathway leading to inflammation-associated cell or tissue injury, which is depicted by the cycling arrows in Fig. 3.

Table 1
Snapshot of analysis performed to identify potential KEs from the Swiss-VCI database.

Reference	Experimental model and mode of exposure	Materials studied	Post-exposure sampling time[a]	Biological/toxicological assays	Actual measurements	Potential key events associated with the measurements[b]	Potential adverse outcomes associated with the key event[c]
Teeguarden et al., 2011	Mouse, aspiration	SWCNTs, ultrafine carbon black, crocidolite asbestos	3 weeks	Differential cell counts - BALF	Total cells, alveolar macrophages, PMNs	Increased leukocyte recruitment/ activation	Tissue inflammation, granuloma
				ELISA for cytokine analysis - BALF	Several cytokines (*e.g.*, TARC, IL-12, Macrophage derived cytokine, and others)	Increased pro-inflammatory mediators	Tissue inflammation
				Histopathology - lung	Morphological, structural and pathological changes (material uptake, distribution, histiocytosis, distribution of inflammatory cells), collagen accumulation	Increased leukocyte recruitment/ activation; Increase deposition of extracellular matrix	Tissue inflammation, lung fibrosis
				Sircol collagen assay - lung	Total collagen	Increased deposition of extracellular matrix	Lung fibrosis
				Proteomics - lung	Global protein profiles	Not identified - subjective to the protein signatures identified	Not identified - subjective to the protein signatures identified
Gosens et al., 2010	Rat, intratracheal instillation	Gold particles (spherical), quartz	3 h, 24 h	Differential cell counts - BALF	Total cells, alveolar macrophages, PMNs	Increased leukocyte recruitment/ activation	Tissue inflammation
				ELISA for cytokine analysis - BALF	Several cytokines (*e.g.*, MCP-1, TNF-α, IL-6)	Increased pro-inflammatory mediators	Tissue inflammation
				Protein assay (assay not specified) -BALF	Total protein - BALF	Loss of alveolar capillary membrane integrity (epithelial cell damage/ tissue injury)	Tissue dysfunction
				Albumin assay (assay not specified) - BALF	Total albumin - BALF	Loss of alveolar capillary membrane integrity (epithelial cell damage/ tissue injury)	Tissue dysfunction
				Alkaline phosphatase assay - BALF	Cytotoxicity - BALF	Increased cell death (cell/tissue injury)	Tissue dysfunction
				LDH assay - BALF	Cell membrane permeability - BALF	Increased cytotoxicity/cell death (cell/tissue injury)	Tissue dysfunction
				Biochemical analysis - blood	Blood total cell number, differential cell count, fibrinogen, C-reactive protein	Increased systemic inflammation and immune response	Systemic inflammation
				TEM - lung	Particle uptake	Increased substance interaction with components of the cell membrane	Not identified
Cui et al., 2011	Mouse - intragastric administration	Nano titanium dioxide	60d	Histopathology, TEM - liver	Morphological and structural changes - fatty degenerations, large vacuoles, congestion in cells, recruitment of inflammatory cells	Increased hepatocyte apoptotic cell death/necrosis; increased tissue injury; increased, leukocyte recruitment/activation	Liver tissue dysfunction, inflammation
				Body weight, liver weight	Coefficients of liver	Increased tissue injury	Liver dysfunction
				Biochemical analysis - blood	Liver enzymes (ALT, ALP, AST, LDH, and others)	Increased tissue injury	Liver dysfunction
				RT PCR for pro-inflammatory and signalling molecules	Cytokines (*e.g.*,TLR-2, TLR-4, IL-2, TNF-α, and others)	Increased pro-inflammatory mediators	Tissue inflammation
				ELISA for pro-inflammatory and signalling molecules	Cytokines (*e.g.*, TLR-2, TLR-4, IL-2, TNF-α, NF-kB and others)	Increased pro-inflammatory mediators	Tissue inflammation
Y. Wang et al., 2011	Mouse, intranasal instillation	Nano ferric oxide particles	3 h (after 40d intranasal exposure)	Neuropathology, Nissl staining - brain	Morphological and pathological changes in brain	Cellular swelling, vacuolar degeneration, nuclear chromatin condensation and fragmentation (brain tissue injury)	Neuropathology
				Neuropathology, Nissl staining - brain	Nuclear chromatin condensation and fragmentation	Increased cell death/cytotoxicity (tissue injury)	Neuronal cell death, tissue dysfunction
				Immunohistochemistry (Cd11b antibody) - brain	Microglial activation	Increased microglial activation and proliferation	Neurotoxicity

(*continued on next page*)

Table 1 (*continued*)

Reference	Experimental model and mode of exposure	Materials studied	Post-exposure sampling time[a]	Biological/toxicological assays	Actual measurements	Potential key events associated with the measurements[b]	Potential adverse outcomes associated with the key event[c]
Srinivas et al., 2011	Rat, head and nose inhalation	Nano cerium oxide particles	1d, 2d, 14d	Hematology analysis	Total erythrocytes, platelet count, leukocytes and leukocytes differential counts, levels of hemoglobin, hematocrit, mean cell volume, mean corpuscular hemoglobin	Increased pro-inflammatory markers	Systemic inflammation
				Biochemical analysis - blood	Total urea, creatinine, total protein, albumin, aspartate transaminase, *etc.*	Increased systemic injury/damage	Systemic diseases
				Differential cell counts - BALF	Total cells, macrophages, neutrophils, eosinophils, lymphocytes	Increased pro-inflammatory cells	Tissue inflammation
				LDH assay - BALF	Cell membrane permeability - BALF	Increased cytotoxicity/cell death (cell/tissue injury)	Tissue dysfunction
				Protein assay (assay not specified) - BALF	Total protein - BALF	Loss of alveolar capillary membrane integrity (epithelial cell damage/ tissue injury)	Tissue dysfunction
				ALP assay - BALF	ALP activity	Altered Type II alveolar epithelial cell secretory activity (cell/tissue injury)	Tissue dysfunction
	Rat, head and nose inhalation	Nano cerium oxide particles	1d, 2d, 14d	Trypan blue assay - BALF	Cell viability	Increased cytotoxicity/cell death; (cell/tissue injury) Increased cell proliferation	Tissue dysfunction
				ELISA assay	Cytokines (IL-1b, TNF-α and IL-6)	Increased pro-inflammatory mediators	Tissue inflammation
				Malondialdehyde assay - BALF	Lipid peroxidation/modification	Increased oxidative stress, oxidative cell/tissue injury	Oxidative damage, DNA damage
				Antioxidant assay	Glutathione levels	Increased oxidative stress, oxidative cell/tissue injury	Oxidative damage, DNA damage
				Glutathione peroxidase enzyme activity assay	Glutathione activity	Increased oxidative stress, oxidative cell/tissue injury	Oxidative damage, DNA damage
				Histopathology	Morphological changes, distribution of pro-inflammatory cells	Increased, leukocyte recruitment/ activation	Tissue inflammation, granuloma, alveolitis
Nemmar et al., 2011	Rats, intratracheal instillation	Nano titanium dioxide rods	1d	Differential cell counts - BALF	Increase in macrophages, neutrophils	Increased recruitment of leukocytes/ activation	Tissue inflammation
				LDH assay - BALF	Cell membrane permeability- BALF	Increased cytotoxicity/cell death (cell/tissue injury)	Tissue dysfunction
	Rats, intratracheal instillation	Nano titanium dioxide rods	1d	ALT - BALF and/or blood	ALT levels - BALF	Increased cytotoxicity/cell death (cell/tissue injury)	Tissue dysfunction
				AST - BALF and/or blood	AST levels - BALF	Increased cytotoxicity/cell death (cell/tissue injury)	Tissue dysfunction
				ELISA assay	Cytokines (IL-6, TNF-α)	Increased pro-inflammatory mediators	Tissue inflammation
				Platelet aggregation - blood	Platelet numbers	Increased platelet aggregation	Tissue inflammation
				Histopathology	Morphological changes, distribution of pro-inflammatory cells, inflammatory lesions	Increased, leukocyte recruitment/ activation	Tissue inflammation
				Functional tests	Systolic blood pressure	Altered vasomotor balance	Cardiovascular diseases
				Functional tests	Heart rate	Altered vasomotor balance	Cardiovascular diseases
				Levels of antioxidants (assay not specified) - BALF, blood	SOD	Increased oxidative stress/oxidant burden; Imbalanced oxidant/ antioxidant levels, oxidative cell/ tissue injury	Oxidative damage, DNA damage
				Glutathione assay, (assay not specified) - BALF blood	Reduced form of glutathione	Increased oxidative stress/oxidant burden; imbalanced oxidant/ antioxidant levels, oxidative cell/ tissue injury	Oxidative damage, DNA damage

(*continued on next page*)

Table 1 (*continued*)

Reference	Experimental model and mode of exposure	Materials studied	Post-exposure sampling time[a]	Biological/toxicological assays	Actual measurements	Potential key events associated with the measurements[b]	Potential adverse outcomes associated with the key event[c]
Gui et al., 2011	Mouse, Intragastric administration	Nano titanium dioxide particles	90d	Coefficient of kidney	Ratio of kidney (wet) to body weight	Increased coefficient of kidney (tissue injury)	Nephritic dysfunction
				Biochemical analysis - blood	Total uric acid, blood urea nitrogen, creatinine and others.	Impaired nephritic function (tissue injury)	Tissue dysfunction
				ELISA assay - kidney	Cytokines (IL-2, TNF-α, IL-4, IL-6, IL-10, TGFβ1, and others)	Increased pro-inflammatory mediators	Kidney injury
				Quantitative RT PCR - kidney	Targeted gene expression (IL-2, TNF-α, IL-4, IL-6, IL-10, TGFβ1, and others)	Increased pro-inflammatory mediators	Kidney injury
				Histopathology - kidney	Morphological and pathological changes	Cell death (cell/tissue injury)	Inflammation, kidney injury

The details of the study design including the material properties, results of the other endpoints assessed and more importantly, temporal aspects should be considered in categorising tissue inflammation as adverse. Thus, tissue inflammation could potentially be an AO associated with inflammation KEs.

Abbreviations: ALP (alkaline phosphatase), ALT (alanine aminotransferase), AST (aspartate aminotransferase), BALF (bronchoalveolar lavage fluid), DNA (deoxyribonucleic acid), ELISA (enzyme-linked immunosorbent assay), IL (interleukin), LDH (lactate dehydrogenase), MCP (monocyte chemoattractant protein), NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells), PCR (polymerase chain reaction), PMN (polymorphic mononuclear cell), RT (real time), SOD (superoxide dismutase), SWCNT (single walled carbon nanotube), TARC (thymus and activation regulated chemokine), TEM (transmission electron microscopy), TGFβ (transforming growth factor beta), TLR (toll-like receptor), TNF-α (tumour necrosis factor α).

[a] This does not indicate the exact dose regime.

[b] Where possible, KEs are stated following the guidelines of the OECD EAGMST.

[c] Tissue inflammation is a process that involves multiple KEs and when not resolved in a timely manner, persistent or chronic inflammation can be considered as an AO.

3.2. KE prioritization and selection for case study

The database analysis identified numerous biological events that are reported to occur following MN exposure (Table 1, Supplemental Table 3). These biological events represent potential KEs in an AOP and were therefore considered candidates for the KE case study. To narrow this list to a single case study subject, in addition to expert judgment, the various KEs were assessed for three main criteria: plausibility, measurability, and regulatory relevance. Table 2 presents these criteria, along with the rationale for their inclusion. Furthermore, the KEs were also assessed for cross-AOP application.

KEs that occurred acutely after exposure and were transitory or reversible were avoided for a case study. KEs that occurred post-inflammation were preferred as they were more closely linked to a specific AO. Accounting for these considerations and the criteria described in Table 2, tissue injury, defined as damage to tissues involving structural and/or functional changes, was selected as the KE to serve as a case study.

3.2.1. Tissue injury

In general, tissue (a complex assembly of cells and associated extracellular matrix of the same origin that work together to carry out a specific function) injury or tissue damage can be described as the stress or toxicity that a tissue suffers due to (1) external stimuli such as physical, chemical, infectious and others; or, (2) internal stimuli arising secondary to substance exposure or due to internal biological/physiological processes. Tissue injury or damage results in the disruption or loss of the ability for tissue to maintain structural integrity, function and homeostasis. Depending on the type and extent of exposure (exposure dose or substance properties), the damage can be repaired, and function restored, or, in the case of repeated or persistent exposure, severe damage to tissues can result in complete dysfunction and impairment leading to a disease or an AO. Additional factors such as susceptibility (genetic make-up, prior history of disease) play a role in the ultimate outcome of the tissue injury. At the cellular level, the loss of energy (adenosine triphosphate depletion, not routinely measured for MNs *in vivo*), mitochondrial damage (*in vitro* biological event reported for MNs), loss of calcium homeostasis (*in vitro* biological event reported for MNs), defects in plasma membrane permeability (measured by cytotoxicity assays such as LDH assay both *in vivo* and *in vitro*; *in vivo* total protein content; and histopathology), and generation of reactive oxygen species (oxidative stress, a well reported biological event both *in vitro* and *in vivo* for MN) are some of the biochemical mechanisms that serve as warning signs of impending tissue injury. At the organ level, tissue injury precedes tissue dysfunction.

Tissue inflammation is proposed to be both the trigger and the corollary of tissue injury. Initial interaction of pathological or injurious substances with the cellular components of tissue leads to receptor-dependent or independent cell signalling resulting in the initiation of host-mediated tissue inflammatory response (*i.e.*, acute inflammation), the purpose of which is to contain and clear the invading stimulus. However, repeated or persistent stimuli will lead to uncontrolled or chronic inflammation, which can result in tissue injury. The components, cell types and molecules involved in the inflammatory process, in turn, contribute to the advancement of tissue injury. For example, macrophages and neutrophils recruited during an inflammatory process synthesize and secrete cytokines and enzymes that are destructive of connective tissue elements and tissue vasculature. The cytokines secreted by inflammatory cells can trigger cell death, which can augment tissue injury (Wallach et al., 2014). Unrepaired tissue injury results in tissue dysfunction and can be considered a tipping point from which inflammation will lead to pathological outcomes. Tissue inflammation and tissue injury are the commonly observed and reported responses following MN exposure. Diseases such as fibrosis, granuloma, mesothelioma and emphysema are among the commonly observed MN-induced AOs that are associated with lung tissue injury. Critically,

Table 2
Criteria for prioritizing KEs.

Objective	Criterion	Rationale
Plausibility	Frequency the KE is reported to be elicited by MN exposure	To ensure that the selected KE is routinely measured for MNs.
	Number and variety of MN that elicit the KE	To ensure the KE is applicable to a broad set of MNs of varying classes and physical chemical properties.
	Available publications in the literature, including *in vivo* and *in vitro* data	To ensure a robust dataset from which to develop and evaluate the KE.
Measurability	Endpoints, methods and assays available to measure the KE	To ensure the KE is a measurable and quantifiable biological event, both *in vitro* and *in vivo*.
Regulatory relevance	The potential significance of the KE and relevance to AO in risk assessment	To ensure the KE is associated with an AO of interest to regulatory decision making.
		To ensure that the KE is not representative of an acute or adaptive response that is transitory.
	Cross species application	To ensure that the KE is observed across species and relevant to human disease.

tissue injury meets the definition of a KE, which must be a measurable or observable biological event that is essential for toxicity (Villeneuve et al., 2014a). Tissue injury can be measured using a number of biomarkers, which can be characterized with various *in vivo* and *in vitro* methods (Table 3), discussed in greater detail in Section 3.3.

3.3. Methods and assays used to measure the tissue injury KE

Following the selection of the tissue injury KE, the database was revisited to focus on reported assays that allow for quantifiable measurement of tissue injury both *in vivo* and *in vitro*. As discussed, this analysis was essential to ensure tissue injury represents a measurable change in biological state, fulfilling the requirement for KEs (Villeneuve et al., 2014b). This included an analysis of reported endpoints that can be measured for tissue injury (tissue injury biomarkers), and the associated methods and assays used to assess them. A summary of the results of this analysis is in Table 3.

There are several reported biomarkers for tissue injury *in vivo* that include evidence of chronic inflammation (temporal), such as sustained increases in pro-inflammatory cells and mediators (*e.g.*, cytokines in BALF). Tissue injury can also be measured *in vivo* by evidence of chronic oxidative stress, such as depletion of antioxidants in tissue or increased lipid peroxidation. Both inflammation and oxidative stress are triggered acutely following substance exposure as a response to organism's defense mechanisms. However, persistent or sustained inflammation and/or oxidative stress can inflict tissue injury. Thus, it is important to differentiate between the acute and adaptive responses from the toxicity related to pro-inflammatory and oxidative stress responses. This can be achieved by including time series experiments. Finally, the presence of biopersistent substances is normally associated with histopathological changes that are also indicative of tissue injury. Such indicators include cytotoxicity, granuloma formation, thickened alveolar lining and extracellular matrix degradation.

There are a number of *in vitro* biomarkers of the tissue injury KE. This includes cytotoxicity which can be measured with cell viability assays, such as the LDH assay, measurements of cell death, and cell survival assays. The presence of chronic oxidative stress is also a marker of tissue injury *in vitro*; however, temporal evidence of chronic oxidative stress can be harder to assess *in vitro*, requiring a different experimental set up that can measure changes over time. Disruptions in

Table 3
Tissue injury measurements *in vivo* and *in vitro*.

Tissue injury biomarkers		Method or assay[a]
In vivo	Chronic inflammation (temporal)	BALF differential cell counts (measuring sustained or persistent increase in recruitment of leukocytes in BALF)
		Chronically elevated expression of specific pro-inflammatory mediators in BALF (altered levels of cytokine genes; *e.g.,* RT PCR/custom PCR arrays or microarrays) or protein expression (*e.g.,* ELISA, bead array, targeted Western blots)
	Proteinosis	Increased protein content, increased albumin in BALF (*e.g.,* Bradford assay)
	Oxidative stress (temporal)	Sustained depletion of antioxidants (altered expression levels of genes (*e.g.*, RT PCR) or proteins (*e.g.,* ELISA, Western blot))
		Increase of pro-oxidants (*e.g.,* increased synthesis of reactive oxygen species)
		Lipid peroxidation (*e.g.,* proteomics, targeted assays)
		Protein modification (*e.g.,* protein carbonylation)
		DNA damage (*e.g.,* COMET assay)
	Histopathology	Cytotoxicity (*e.g.*, apoptotic bodies, DNA fragmentation, apoptosis, necrosis)
		Granuloma formation
		Thickened alveolar lining, biopersistent substance
		Excessive extracellular matrix deposition or degradation
In vitro	Cytotoxicity	Cell viability assays, including membrane integrity and mitochondrial function (*e.g.*, LDH assay, ATP depletion)
		Cell death assays (*e.g.*, Caspase activation, LDH assay)
	Oxidative stress	Fluorescence assays measuring increased reactive oxygen species synthesis, depletion of glutathione (a variety of assays available)
	Cellular barrier/membrane permeability	Loss of gap junctions
		Loss of tight junctions
		TEER (multi-cell-type cultures)
	Lysosomal uptake autophagy	Immunohistochemistry, microscopic observations for lysosomal destabilization, lysosomal rupture
	Imbalanced levels of proteases/antiproteases	Altered expression of protein and/or mRNA of proteases and antiproteases
	Inflammation	Expression of pro-inflammatory and pro-fibrotic mediators *e.g.*, TNF-α, IL-1β, IFNγ (*e.g.*, Protein ELISA and/or mRNA analysis by RT-PCR)

Abbreviations: Adenosine triphosphate (ATP), BALF (bronchoalveolar lavage fluid), DNA (deoxyribonucleic acid), ELISA (enzyme-linked immunosorbent assay), IL (interleukin), IFN (interferon), LDH (lactate dehydrogenase), mRNA (messenger RNA), PCR (polymerase chain reaction), RT (real time), TEER (transepithelial electrical resistance), TNF-α (tumour necrosis factor α).

[a] List of assays found in the database of 191 studies potentially used to assess tissue injury, demonstrating the heterogeneous ways of assessing this KE. This is not a recommended assay list.

cellular communication, such as loss of gap junctions, disruptions in the barrier integrity of cultured monolayers of cells, measured through a loss of tight junctions or a loss of transepithelial electrical resistance are reflective of injured cells. Intracellular events such as lysosomal destabilization or rupture and the associated autophagy and vacuolization are additional measures of the tissue injury KE *in vitro*, along with secretion of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β) and interferon gamma (IFNγ). The long list of measurements informs the available techniques and tools to measure tissue injury; however, a careful review of these methodologies will be needed in the future to select the most appropriate methods to use *in vitro* techniques to measure tissue injury. It remains to be decided how many of the *in vitro* measurements for tissue injury as a KE are needed to attain high *in vivo* predictive capacity.

3.4. Outcomes from an expert workshop

An expert workshop was convened at NanoTox 2018 in order to get a generalised consensus on inflammation-associated tissue injury as a relevant KE to analyze further in a case study. The case study will aim to understand the feasibility of measuring tissue injury following MN exposure *in vitro* and to gain guidance on how it should be measured or the techniques to be used. Experts in the areas of inhalation toxicology, inflammation, development and application of AOPs, risk assessment and regulation of MNs from academia, industry and government participated in panel discussions and provided feedback. A general discussion was also held on the need for developing AOPs and their current status and future use toward improved risk assessment of MNs.

In general, the panelists agreed that tissue injury is a relevant and a valid KE that has significance to MN-induced AO; however, concerns were raised regarding the demonstrated scientific evidence linking it to an AO and its ability to predict a potential AO. Concerns were also raised regarding challenges associated with inflammation-mediated AOPs including: (1) the need to distinguish between inflammation as an adverse *versus* normal tissue response; (2) the need to differentiate among different types of inflammation; (3) the complexity of measuring and quantifying inflammation *in vitro*; and (4) the current limitations of the nanotoxicity literature for AOP development due to insufficient consideration of interspecies differences in inflammatory responses. Discussions identified areas where additional technical work is needed, including: an investigation of appropriate *in vitro* measures of tissue injury and inflammation; the mechanisms and role of tissue injury in the inflammation pathway; and clear links to an AO.

During the workshop, discussion also focussed on whether AOPs are necessary for MN risk assessment. The general consensus was that AOPs are not a 'must have' for MN risk assessment, but they will most certainly improve risk assessment strategies, including furthering our understanding of toxicity mechanisms and potency, and allowing grouping and categorisation. Panelists discussed how AOPs can fit into current decision-making frameworks, including their use in hazard identification, development of alternative testing strategies relevant for MNs, for screening, for improving testing efficiency and to improve the basis for grouping and categorisation efforts. Some of these points were controversial, but most participants agreed that the value of AOPs toward MN risk assessment is the improved mechanistic insights they offer, which is integral to the design and development of mechanism-based *in vitro* assays, supporting the vision and the strategy outlined in NRC (2007).

4. Discussion

AOP frameworks are a tool that can help link KEs elicited from MN exposure to potential AOs at the organ, organism, or even population level without the burden of extensive animal testing. The process of constructing AOPs using the existing toxicology literature is tedious. Disease pathways are seldom linear as depicted by the simplified AOP frameworks and often involve multiple toxicity pathways and intricate processes. AOPs are a series of KEs that link the exposure to an eventual AO. Thus, an additional challenge is to identify the most representative key biological events that are sequential and essential for toxicity from the multitude of events triggered and processes that are altered following a disease stimulus. In the NanoAOP project, an approach has been demonstrated using the existing nanotoxicology literature to identify potential KEs induced by MN exposure and prioritize them for AOP development based on their likelihood to contribute to an AO of relevance to MN risk assessment.

Tissue inflammation is not always considered adverse and is not an endpoint of regulatory relevance; however, it is one of the most consistently observed and reported tissue responses following MN exposure. Chemical-induced inflammation is a precursor for many target organ toxicities and human diseases. It is a complex biological process involving a cascade of causal events at the molecular, cellular and tissue level, which can be sequentially organized into an AOP framework of its own. The inflammation process as a KE is regulated by a range of upstream signalling events and is linked to several downstream effects. Although complex, the process of inflammation has several common features that can be generalised across different tissues and its pathways are conserved across different species including humans (Villeneuve et al., 2018). Thus, the initial analysis of the Swiss-VCI database consisting of more than 11,000 published studies was limited to a total of 191 studies that reported on inflammation associated observations. These studies described a wide variety of exposure models and systems including, *in vivo*, *in vitro*, multiple routes and modes of exposure, target organs and systemic effects, all measured through several different types of assays and methods. The emphasis was to select a single KE associated with inflammation to serve as a case study. In addition to the snap shot of KEs presented in the Table 1 and Supplemental Table 3, the results of the entire database analysis of 191 studies (Supplemental Table 4) revealed several MN-induced potential KEs including recruitment of leukocytes/activation, lysosomal destabilization, DNA damage, inflammasome activation, and oxidative stress that also partially meet the criteria defined in Table 2 for prioritization for further development in a case study. For example, DNA damage is an event involved in the process of carcinogenic transformation and is of high relevance to regulatory decision making. It is suggested that MN-induced DNA damage may be the indirect consequence of processes such as inflammation and oxidative stress. However, evidence to support the occurrence of DNA damage following MN exposure is scarce and inconsistent. Several studies have shown inflammasome activation in cells and tissues following MN exposure; however, how activated inflammasomes induce a known AO relevant to MN exposure is not clear. Thus, tissue injury, a KE that is most frequently reported and is associated with the inflammation pathway and to a known AO induced by MN, was selected for further development in a case study.

Although the process of MN risk assessment can greatly benefit from completely developed quantitative AOPs, owing to their complex and versatile properties, the underlying mechanisms of MN toxicity are not completely deduced. Moreover, conventionally, experimental study designs did not incorporate AOP thinking; a typical *in vivo* inhalation study focussed on histopathological endpoints rather than sequential KEs underlying such endpoints. Thus, with the available toxicological data, construction of quantitative AOPs for MNs is not feasible and it may not be an option to wait until the complete AOPs are developed to conduct MN risk assessments. In the interim, a single or multiple KEs that constitute a part of the known MN-induced toxicity pathways may be identified and used to generate much needed data in support prioritization, grouping and categorisation for risk assessment. Tissue injury, the KE identified for the case study, satisfies the critical criteria in that it is measurable, it is essential for downstream toxicity and has been investigated following exposure to many MNs, and can be used to generate data in support of MN risk assessment. However, there is significant inconsistency in how tissue injury is measured *in vivo* and *in*

vitro, and how it is reported. Chronic inflammation and cytotoxicity are some of the routinely measured tissue events following MN exposure but are not always reported as tissue injury. Also, chronic inflammation is a time-dependent event. It is not clear for how long studies should be carried out to characterize a response as chronic inflammation and it is also not clear what the characteristics of chronic inflammation are that are reflective of tissue injury or an adverse outcome. Similar to inflammation, tissue injury can also be a reversible event; identifying the threshold beyond which it becomes adverse would be critical for its successful implementation in MN risk assessment. Moreover, there is no harmonised guidance on how to measure this KE in different experimental models. Additional work will be required to validate the KE 'Tissue Injury' in the context of how exposure dose and MN properties will influence its occurrence or the magnitude of injury.

5. Conclusions

Despite the perceived value of AOPs for the purpose of risk assessment in general and for MNs, some outstanding technical, scientific and research questions surrounding AOPs remain. This proof-of-principle study highlighted various issues related to the available toxicological literature for developing MN relevant AOPs. For example, of the 447 datasets reporting on inflammation, less than 20 were deemed high quality publications with appropriate level of details on material characterisations, use of physiologically relevant exposure doses and justification of the models and endpoints assessed, highlighting the need for streamlining the experimental design and reporting standards for MN toxicity studies. The robust database was not sufficient to produce a dose-response or property-response relationship for any MN, suggesting the need for careful selection of MNs and their variants for inclusion in studies. Lastly, the study also highlighted the need for mechanistic understanding of the processes elicited by MNs in *in vitro* models and their relevance to *in vivo* responses. In the context of MN-induced tissue injury, the need for differentiating between the adaptive *versus* adverse consequences of tissue injury, understanding of the temporal aspects associated with it and more importantly, careful selection of the *in vivo* predictive *in vitro* measurements for tissue injury that is relevant to humans was highlighted.

Albeit the limitations, the study narrowed down a single KE - tissue injury - as both important and relevant for MN assessment. Next, the Swiss-VCI database will be updated with studies through 2017 and will be used for validating the selected KE. Further assessment will include: quantitative analysis of how many MNs induce tissue injury; the dose and property-response relationships; evaluation of the existing *in vitro* methodologies for measuring tissue injury and selection of the most sensitive and predictive *in vitro* assay/s for further consideration; development of guidance on an experimental design to assess tissue injury; and developing recommendations on how toxicological studies can be designed to support AOP development in general.

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References

Carusi, A., Davies, M.R., Grandis, G.D., Escher, B.I., Hodges, G., Leung, K.M.Y., Whelan, M., Willett, C., Ankley, G.T., 2018. Harvesting the promise of AOPs: an assessment and recommendations. Sci. Total Environ. 628, 1542–1556.

Cui, Y., Liu, H., Zhou, M., Duan, Y., Li, N., Gong, X., Hong, F., 2011. Signaling pathway of inflammatory responses in the mouse liver caused by TiO_2 nanoparticles. J. Biomed. Mater. Res. A 96 (1), 221–229. https://doi.org/10.1002/jbm.a.32976.

Gerloff, K., Landesmann, B., Worth, A., Munn, S., Palosaari, T., Whelan, M., 2017. The adverse outcome pathway approach in nanotoxicology. Comput. Toxicol. 1, 3–11. https://doi.org/10.1016/j.comtox.2016.07.001.

Gosens, I., Post, J.A., de la Fonteyne, L.J., Jansen, E.H., Geus, J.W., Cassee, F.R., de Jong, W.H., 2010. Impact of agglomeration state of nano- and submicron sized gold particles on pulmonary inflammation. Part. Fibre Toxicol. 7 (1), 37. https://doi.org/10.1186/1743-8977-7-37.

Gui, S., Zhang, Z., Zheng, L., Cui, Y., Liu, X., Li, N., Hong, F., 2011. Molecular mechanism of kidney injury of mice caused by exposure to titanium dioxide nanoparticles. J. Hazard. Mater. 195, 365–370. https://doi.org/10.1016/j.jhazmat.2011.08.055.

Krug, H.F., 2014. Nanosafety research—are we on the right track? Angew. Chem. Int. Ed. Engl. 53 (46), 12304–12319. https://doi.org/10.1002/anie.201403367.

Krug, H.F., Bohmer, N., Kühnel, D., Marquardt, C., Nau, K., Steinbach, C., 2018. The DaNa (2.0) knowledge base nanomaterials-an important measure accompanying nanomaterials development. Nanomaterials (Basel, Switzerland) 8 (4), 204. https://doi.org/10.3390/nano8040204.

Labib, S., Williams, A., Yauk, C.L., Nikota, J.K., Wallin, H., Vogel, U., Halappanavar, S., 2016. Nano-risk Science: application of toxicogenomics in an adverse outcome pathway framework for risk assessment of multi-walled carbon nanotubes. Part. Fibre Toxicol. 13 (15). https://doi.org/10.1186/s12989-016-0125-9.

Liberati, A., Altman, D.G., Tetzlaff, J., Mulrow, C., Gøtzsche, P.C., Ioannidis, J.P.A., Moher, D., 2009. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: explanation and elaboration. BMJ 339, b2700. https://doi.org/10.1136/bmj.b2700.

National Research Council, 2007. Toxicity Testing in the 21st Century: A Vision and a Strategy. The National Academies Press, Washington, DC.

Nemmar, A., Melghit, K., Al-Salam, S., Zia, S., Dhanasekaran, S., Attoub, S., Ali, B.H., 2011. Acute respiratory and systemic toxicity of pulmonary exposure to rutile Fe-doped TiO(2) nanorods. Toxicology 279 (1–3), 167–175. https://doi.org/10.1016/j.tox.2010.10.007.

OECD, 2016. Alternative Testing Strategies in Risk Assessment of Manufactured Nanomaterials: Current State of Knowledge and Research Needs to Advance their Use Retrieved from. http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2016)63&docLanguage=En.

Srinivas, A., Rao, P.J., Selvam, G., Murthy, P.B., Reddy, P.N., 2011. Acute inhalation toxicity of cerium oxide nanoparticles in rats. Toxicol. Lett. 205 (2), 105–115. https://doi.org/10.1016/j.toxlet.2011.05.1027.

Teeguarden, J.G., Webb-Robertson, B.J., Waters, K.M., Murray, A.R., Kisin, E.R., Varnum, S.M., Shvedova, A.A., 2011. Comparative proteomics and pulmonary toxicity of instilled single-walled carbon nanotubes, crocidolite asbestos, and ultrafine carbon black in mice. Toxicol. Sci. 120 (1), 123–135. https://doi.org/10.1093/toxsci/kfq363.

Validation of Alternative Methods for Toxicity Testing. (2016). (C. Eskes & M. Whelan Eds.). Switzerland: Springer, Cham.

Villeneuve, D.L., Crump, D., Garcia-Reyero, N., Hecker, M., Hutchinson, T.H., LaLone, C.A., Whelan, M., 2014a. Adverse outcome pathway (AOP) development I: strategies and principles. Toxicol. Sci. 142 (2), 312–320. https://doi.org/10.1093/toxsci/kfu199.

Villeneuve, D.L., Crump, D., Garcia-Reyero, N., Hecker, M., Hutchinson, T.H., LaLone, C.A., Whelan, M., 2014b. Adverse outcome pathway development II: best practices. Toxicol. Sci. 142 (2), 321–330. https://doi.org/10.1093/toxsci/kfu200.

Villeneuve, D.L., Landesmann, B., Allavena, P., Ashley, N., Bal-Price, A., Corsini, E., Tschudi-Monnet, F., 2018. Representing the process of inflammation as key events in adverse outcome pathways. Toxicol. Sci. 163 (2), 346–352. https://doi.org/10.1093/toxsci/kfy047.

Wallach, D., Kang, T.B., Kovalenko, A., 2014. Concepts of tissue injury and cell death in inflammation: a historical perspective. Nat. Rev. Immunol. 14 (1), 51–59. https://doi.org/10.1038/nri3561.

Wang, Y., Wang, B., Zhu, M.T., Li, M., Wang, H.J., Wang, M., Zhao, Y.L., 2011. Microglial activation, recruitment and phagocytosis as linked phenomena in ferric oxide nanoparticle exposure. Toxicol. Lett. 205 (1), 26–37. https://doi.org/10.1016/j.toxlet.2011.05.001.