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Advancing Adverse Outcome Pathway (AOP) Development for Nanomaterial Risk Assessment and Categorisation Part 2: Case Study on Tissue Injury

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Advancing Adverse Outcome Pathway (AOP) Development for Nanomaterial Risk Assessment and Categorisation Part 2: Case Study on Tissue Injury

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Table of contents

Foreword

1. The OECD has a key role in standardising methodologies for hazard testing and assessment and promoting best practices for the safe use of chemicals and the protection of human health and the environment. The OECD has established a number of programmes addressing different aspects of chemical safety enabling a sound harmonised approach for industrial chemical management. The Working Party on Manufactured Nanomaterials (WPMN) was established to ensure that the approaches for hazard, exposure and risk assessment for manufactured nanomaterials are properly integrated in the assessment of chemicals and aligned with the high quality, science-based and internationally harmonized tools developed by the OECD Chemicals Programme.

2. With this in mind, the WPMN launched the project *Advancing Adverse Outcome Pathway (AOP) Development for Nanomaterial Risk Assessment and Categorisation*. The objective is to contribute to the future development and application of AOPs for MN regulatory decision making, by following the principles established by the OECD Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST). The outcomes of the project are presented in three complementary documents addressing:

- The scope of the project, its development and summary of the main conclusions. The document includes a methodology to identify, analyse and evaluate existing nanotoxicology literature with the objective to prioritize Key Events (KEs) relevant for MNs;
- A case study focused on a specific Key Event (KE) in the inflammation pathway to analyse the empirical evidence and contribute to the development of a knowledge base to inform AOP development and assessment for MNs; and
- The report from the OECD workshop *Advancing Adverse Outcome Pathway (AOP) Development for Nanomaterial Risk Assessment and Categorisation*, which was organised in collaboration with the European Union (EU) Horizon 2020 projects *SmartNanoTox* and *Physiologically Anchored Tools for Realistic nanOmateriaL hazard aSsessment* (PATROLS). At this workshop, stakeholders had an opportunity to provide feedback on the methodology proposed, as well as on the case study, and to reach consensus on areas that could be further explored in the short, medium and long term.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology of the OECD.

Executive Summary

3. The Organisation for Economic Cooperation and Development (OECD) Working Party on Manufactured Nanomaterials (WPMN) project, "Advancing Adverse Outcome Pathway Development for Nanomaterial Risk Assessment and Categorisation (NanoAOP project)" contributes to both the future development and the potential application of AOPs for manufactured nanomaterial (MN) regulatory decision making. This document was developed as part of the OECD project *Advancing Adverse Outcome Pathway (AOP) Development for Nanomaterial Risk Assessment and Categorisation*. It outlines the methodology, through a case study approach, for identifying key events (KEs) that could support development of AOPs relevant for MNs. This included the development of the NanoAOP database, the quality evaluation of the published literature, and a process to support the identification of a KE, which were used to identify literature reporting on inflammation and associated endpoints following exposure *in vitro* or *in vivo* to MN. Finally, the results were discussed at the OECD workshop on "Advancing Adverse Outcome Pathway (NanoAOP) Development for Nanomaterials Risk Assessment and Categorisation" (September 2019) for review and feedback on (i) the methodology developed and (ii) the current status, use and future needs of AOPs relevant to MNs in support of their use in human health risk assessment and decision making.

4. Based on the development and quality evaluation of the NanoAOP Database[1], the case study focused on the identification of 'tissue injury' as a KE relevant for MNs. A review of the literature included in the NanoAOP database found that events related to inflammation, oxidative stress and cytotoxicity were the three most commonly assessed and reported biological events following MN exposure with a direct inference to tissue injury. These frequently reported biological events were thus believed to represent the upstream KEs of 'tissue injury'. As such, unresolved inflammation, oxidative stress and cytotoxicity induce injury to tissues. Since tissue injury precedes tissue dysfunction and plays a role in several adverse outcomes (e.g. fibrosis, granuloma, mesothelioma, and emphysema in the lung) of regulatory relevance to MNs, it was selected for further development in the case study. As evidenced by the NanoAOP database, each of these upstream KEs is measurable both *in vivo* and *in vitro*. The three KEs can be measured in all types of potential target cells. Moreover, a number of *in vivo* and *in vitro* endpoints, methods and assays have been used to measure the KEs for tissue injury and are readily available. Tissue injury is observed following exposure to a variety of MNs of diverse properties. Thus, the case study showed that *in vitro* cellular level assays can be used to predict the occurrence of tissue injury *in vivo*.

5. The KEs identified at cellular level reflect a change in the biological state that is critical for occurrence of the 'tissue injury' KE at organ level. However, much work needs to be completed before one can derive recommendations or provide specific guidance on experimental design for assessing tissue injury as indicative of an MN-induced adverse event.

[1] See document, Part1: Final Project Report and Recommendations with Methodology to Prioritize Key Events (KEs) Relevant for MNs [ENV/JM/MONO(2020)33] and Halappanavar et al., 2019.

1 Introduction

6. MNs are engineered substances with a diverse number of applications and potential benefits to society. However, adoption is hindered by the lack of accepted and efficient methods to evaluate the safety[2] of the growing number, diversity, and complexity of MNs entering the market. The current approach requires each MN to be individually assessed for health and environmental risks using traditional animal testing methods, an approach that is too time- and cost-intensive to be practical and is undesirable for ethical reasons. Adverse Outcome Pathways (AOPs) are conceptual frameworks that link key biological events (key events; KEs) resulting from chemical (material) exposure to adverse health or environmental impacts important for evaluating safety (OECD, 2016a). AOPs are designed as frameworks to organise toxicological information and offer a systematic, mechanistic approach to develop, assess, use, and interpret alternative testing strategies as part of an integrated approach to testing and assessment (IATA), ultimately expected to reduce reliance on animal testing conditional to the availability of validated methods (OECD, 2016b).

Problem statement

7. Significant advances have been made in AOP development over the last decade. However, a number of challenges remain to operationalise this framework, and to translate these advances into established mechanistic pathways for use in MN safety assessment and decision making. As AOPs represent mechanistic frameworks outlining biological processes that lead to an adverse outcome (AO), they are by definition not substance-specific. However, AOPs to date have mainly focused on known toxicological mechanisms of chemicals. Thus, there is a need to: (i) support the future development of AOPs that capture toxicological mechanisms consisting of KEs relevant for MNs; (ii) evaluate the use of the AOP framework as part of an IATA for MNs; and (iii) identify how it can be adopted for real-world applications in MN safety assessment and decision making.

[2] The OECD has a dedicated programme to ensure the development of standard methods for safety testing of chemicals, which includes nanomaterials. This programme ensure the methods meet regulatory needs; reflect scientific progress; address animal welfare aspects, and improve cost-effectiveness of test methods. See: https://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm

2 Background

Project overview

8. The Organisation for Economic Cooperation and Development (OECD) WPMN project "Advancing Adverse Outcome Pathway Development for Nanomaterial Risk Assessment and Categorisation (NanoAOP project)" is contributing to both the future development and potential application of AOPs for MN regulatory decision making. The overall goal of the project is to establish an approach to advance future AOP development relevant for MNs using existing and available nanotoxicity literature. This is accomplished through two main objectives. First, there was a systematic process for searching and mining the nanotoxicity literature to identify KEs relevant for MNs (Halappanavar et al., 2019). Secondly, the project developed a methodology, through a case study approach, for identifying KEs that could support the development of KEs relevant for MNs, from evidence gathered from the literature. The results of the latter are presented in this document. Lastly, as part of its outcomes, the project involved convening a workshop (held in September 2019) to gather expert feedback on (i) the methodology developed under this project and (ii) the current status, use and future needs of AOPs relevant to MNs in support of their use in risk assessment and decision making.

9. In order to move forward, the NanoAOP project developed a case study and agreed on a specific KE in the inflammation pathway as: (i) there is a substantial literature base examining the inflammation processes for MNs to build the proposed method and case study; and (ii) inflammation is an identified KE in many AOPs and precursor to several AOs relevant to MNs (e.g. fibrosis).

10. It is important to note that the focus was to propose a methodology for identifying and developing specific KEs using the existing nanotoxicology literature to inform future MN risk assessment, following the principles used by the OECD Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST). Therefore, the purpose was not to develop a full AOP for a specific AO induced by MNs.

Criteria and methods to identify and prioritise potential Key Events (KEs)

11. The analysis was completed with a significant database of nanotoxicity literature from a project developed by the Swiss Small-Medium Enterprise (SME) NanoCASE Gesellschaft mit beschränkter Haftung (GMBH) with the financial support of the Swiss Federal Office of Public Health and the German Chemicals Industry Association (VCI). The Swiss-VCI 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. The large database was processed to select those studies reporting specifically on inflammation to identify potential inflammation associated KEs and resulted in 191 publications spanning ~60 different endpoints for 45 different MNs. Analysis of the database identified numerous biological events that are reported to occur following MN exposure and represent potential KEs in an AOP relevant for MNs. Halappanavar et al., 2019 presents an overview of the developed strategy for identifying KEs from the MN literature and results of this analysis. This work was presented to experts for feedback during a workshop hosted at the 9th International Conference on Nanotoxicology (NanoTox, 2018) and was recently published in the journal NanoImpact (Halappanavar et al., 2019).

12. The Swiss-VCI database was further expanded with additional literature and an amended database called NanoAOP database was established in January 2018 with the main goal of evaluating the biological plausibility of the prioritised KE from Halappanavar et al., 2019.

Selection of Tissue Injury as a Key Event (KE)

13. To prioritise the identified potential KEs for selection and to select a KE to serve as a case study, KEs were assessed with three main criteria to ensure the selected KE is: (i) plausible; (ii) measurable; and (iii) relevant for regulatory considerations. Potential KEs that occurred acutely after exposure and were transitory or reversible were avoided for case study selection. Potential KEs that occurred post inflammation were preferred as they were more closely linked to a specific AO. Accounting for these considerations, tissue injury, defined as damage to tissues involving structural and/or functional changes, was selected as the potential KE to serve as a case study.

Weight-of-Evidence toward Tissue Injury as a Key Event (KE)

14. Tissue injury was a strong candidate for selection in the case study as it meets the definition of a KE, which must be a measurable or observable biological event that is essential for toxicity (Villeneuve et al., 2014a). It was highly plausible, given that it was one of the most frequently reported biological events in the Swiss-VCI database following exposure to a variety of MNs, it is measurable with numerous endpoints, methods and assays reported both *in vivo* and *in vitro*, and it has high regulatory relevance, being associated with significant adverse human health outcomes, including fibrosis, granuloma, mesothelioma, and emphysema in the lung.

Assays and Endpoints

15. Following the selection of the tissue injury KE, the Swiss-VCI database was revisited to focus on reported endpoints and assays that allow for quantifiable measurement of tissue injury both *in vivo* and *in vitro*. As noted above, this analysis was essential to ensure that 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. Several endpoints of tissue injury were identified *in vivo* (e.g. inflammation, oxidative stress, proteinosis) and *in vitro* (e.g. inflammation, oxidative stress, cytotoxicity) with numerous reported methods or assays to assess them. These are reported in Table 3 of Halappanavar et al., 2019.

3 Case Study: Overview

16. The inflammatory response is complex and is characterised by the activation, proliferation, and recruitment of different types of immune cells, with a common goal of combating an invading pathogen or harmful stimulant and repairing the damage caused by them. However, failure to resolve inflammation or persistent inflammation, can cause cell/tissue injury and damage.

17. Similar to inflammation, tissue injury, which is defined as damage to tissues involving structural and/or functional changes, is a very common consequence/result observed and measured following exposure to MNs. While tissue injury incited acutely after exposure to stressors results in the release of signalling molecules that activate tissue repair and regeneration process, which includes an early inflammatory response, tissue injury that follows unresolved inflammation or failed tissue repair attempts can be detrimental to the organism. 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 external stimuli, such as physical, chemical, infectious and others, or 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 of the 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 are impairment leading to a disease or an AO. Tissue injury precedes tissue dysfunction. Tissue injury, among other processes, can lead to long term effect such as cancer via promoting clonal expansion.

18. Below, the step-wise approach taken to evaluate biological plausibility of tissue injury KE following MN exposure using the NanoAOP database is presented.

3.1. Identification of Upstream Key Events (KEs) for Tissue Injury and Endpoints, Methods, and Assays to Measure Them

(a) Methods

19. Having chosen ‘tissue injury’ as a KE for the case study, the first task under Objective 2 was to evaluate how this KE is assessed and reported in the scientific literature.

Updated database and search strategy

20. The Swiss-VCI database contained studies reporting on inflammation from MN exposures that were published between 2009 to May 2013, and evaluated for quality following the DaNa scheme (see Halappanavar et al., 2019 for details). A subset of the Swiss-VCI database evaluating tissue injury (119 studies total) was updated with relevant studies published between January 2014 and December 2017 (NanoAOP database) by project partner University of Birmingham funded via the European Union (EU) Horizon 2020 (H2020) NanoCommons project. After an initial assessment of the current state of the literature, seven MNs were selected to update the database: Silver, Cerium Oxide, Copper Oxide, Multi-

walled carbon nanotubes (MWCNT), Single-walled carbon nanotubes (SWCNT), Titanium Dioxide, and Zinc Oxide.

21. A Boolean-based search model with related words was used to identify relevant publications for NanoAOP database development for the seven prioritised types of MNs. Appendix 1 outlines the details of the literature search strategy and the inclusion/exclusion criteria for AOP database development. During the update, 15 additional parameters, specific to inflammation and/or tissue injury, were added to the database to aid in the Objective 2 analysis.

Quality evaluation

22. A quality assessment was conducted for the additional literature update (research articles published between 2013 and 2017). For papers meeting the inclusion criteria (see Appendix 1), a two-step quality evaluation was completed (with slight modification) to ensure papers populating the database were of a high overall quality. Studies were evaluated along two primary axes.

23. The first axis evaluated how well studies characterised and reported key physical and chemical properties of the studied MNs. Those studies which did not meet minimum characterisation requirements were excluded from the NanoAOP database. The second analysis axis evaluated the experimental design and reporting with a set of parameters (e.g. reporting of applied concentrations, dispersion procedures employed, inclusion of appropriate negative and positive controls; see Appendix 1 for the full list). Studies were scored as 0, 1 or 2 for each of the criteria. If no information was provided, a criterion was scored as 0. 'Not applicable' was used instead of 0, where warranted. A score of 1 was given if the study provided minimum information on the criteria. A score of 2 was given if the information or data provided on a particular criterion was considered to be appropriate and detailed. The individual scorings were summed up and the published studies were 'binned' into three groups:

1. Discarded studies from the first MN characterisation step – Group 1, and not included in the database.
2. Studies with <24 points – Group 2
3. Studies with ≥ 24 points – Group 3

24. Studies in Group 2 and 3 were judged to be of adequate quality and were used for updating the NanoAOP database.

(b) Results

25. The original Swiss-VCI database consisted of data from 119 peer-reviewed papers published in the period 2009-2013 addressing inflammation. The additional literature search and quality evaluation completed under Objective 2 identified a total of 126 new peer-reviewed papers, covering seven MNs, and a total of 485 unique parameters. The resulting NanoAOP database contained 245 publications, with many materials having numerous variants (*e.g.* uncoated or coated, positively or negatively charged, different shapes etc.). If an individual publication evaluated more than one type of MN, data were separated by MN type and each was considered as a separate 'study'. The final Nano-AOP database includes 294 studies. Appendix 1 has a full description of the methodology and results of the literature review, quality evaluation, and database updating. The developed NanoAOP database formed the basis for the work conducted under Objective 2.

Identification of upstream KEs for tissue injury

26. Inflammatory events (increased recruitment of pro-inflammatory cells and increased secretion of pro-inflammatory mediators), events related to oxidative stress (increased synthesis of reactive oxygen or nitrogen species (ROS/RNS), differential expression of pro- or anti-oxidant genes, oxidative modification

of biomolecules) and cytotoxicity events were the three most commonly assessed and reported endpoints in the NanoAOP database with a direct inference to tissue damage and injury. As such, the three key biological events – inflammation, oxidative stress and cytotoxicity - are interconnected and play a prominent role in tissue injury in general and also in inflammation-mediated injury.

27. At the cellular level, injury inflicted by the acute interaction of irritants, pathogens and toxic materials with cells serves to induce signalling pathways that in turn, lead to activation of host defence mechanisms, including immune and inflammatory responses. This initial injury is not intrinsically detrimental. Once inflammation is signalled in a tissue in an organism, the ensuing cytokine storm involving secretion of complement proteins, enzymes and cytokines that exhibit destructive capabilities, and together with the activity of infiltrated or resident leukocytes, leads to the synthesis of ROS and results in unintended exacerbation of cell injury and cell death. At the tissue level, uncontrolled cell injury results in extracellular matrix degradation, vascular damage and eventually in tissue dysfunction. Thus, it was inferred that the three frequently assessed and reported KEs can be used as upstream KEs to the 'tissue injury' KE, which would also allow identification of its downstream effector KEs, as well as the various methods and assays for measuring them using *in vivo* and *in vitro* models. Furthermore, each upstream KE (i.e. inflammation, oxidative stress and cytotoxicity) was represented by distinct associative events. For example, the upstream KE inflammation was represented by 'leukocyte recruitment/activation' and 'increased pro-inflammatory mediators', which are referred to here as hub-KEs of inflammation. Similarly, the oxidative stress KE was represented in the database by hub-KEs 'increased reactive oxygen species (ROS) synthesis', 'imbalanced oxidant and anti-oxidant levels' and 'modification of biomolecules' (Villeneuve et al., 2018). Cytotoxicity KE was represented by the hub-KEs 'altered membrane integrity' and 'cytotoxicity'. The upstream and associated hub-KEs were arranged as such that they showed the causal sequence (solid line) but also in parallel (dotted line), functioning in a feedback loop (Figure 1).

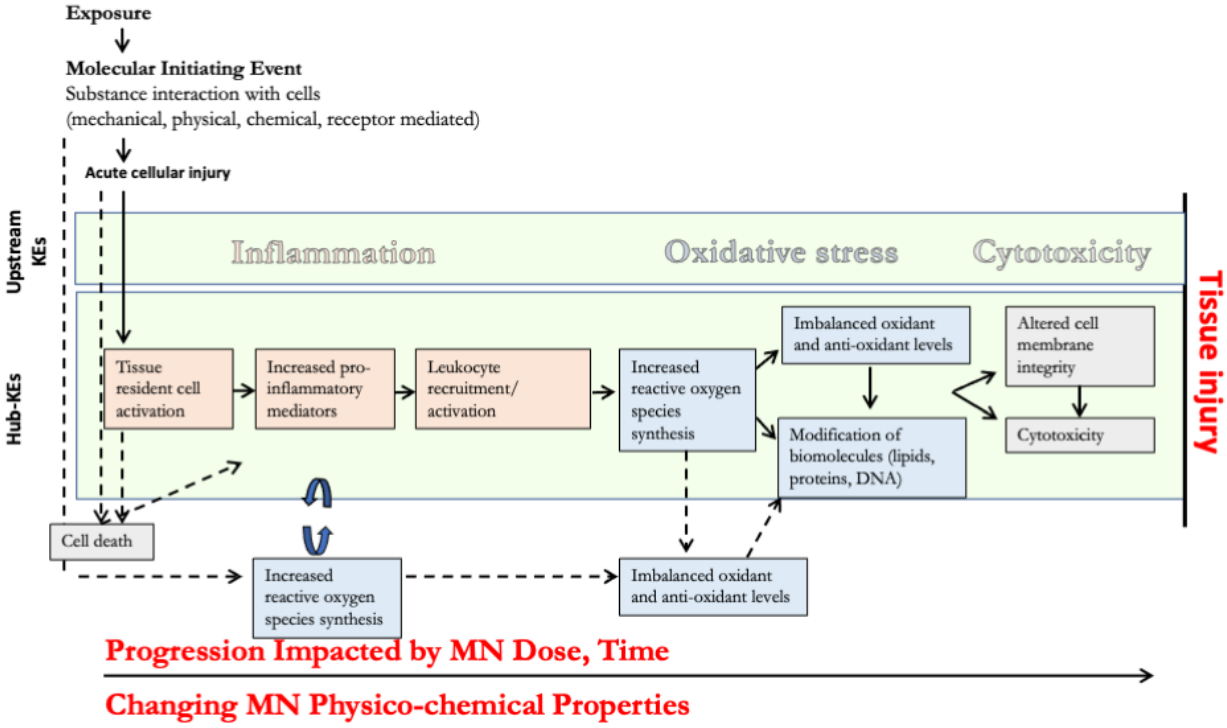


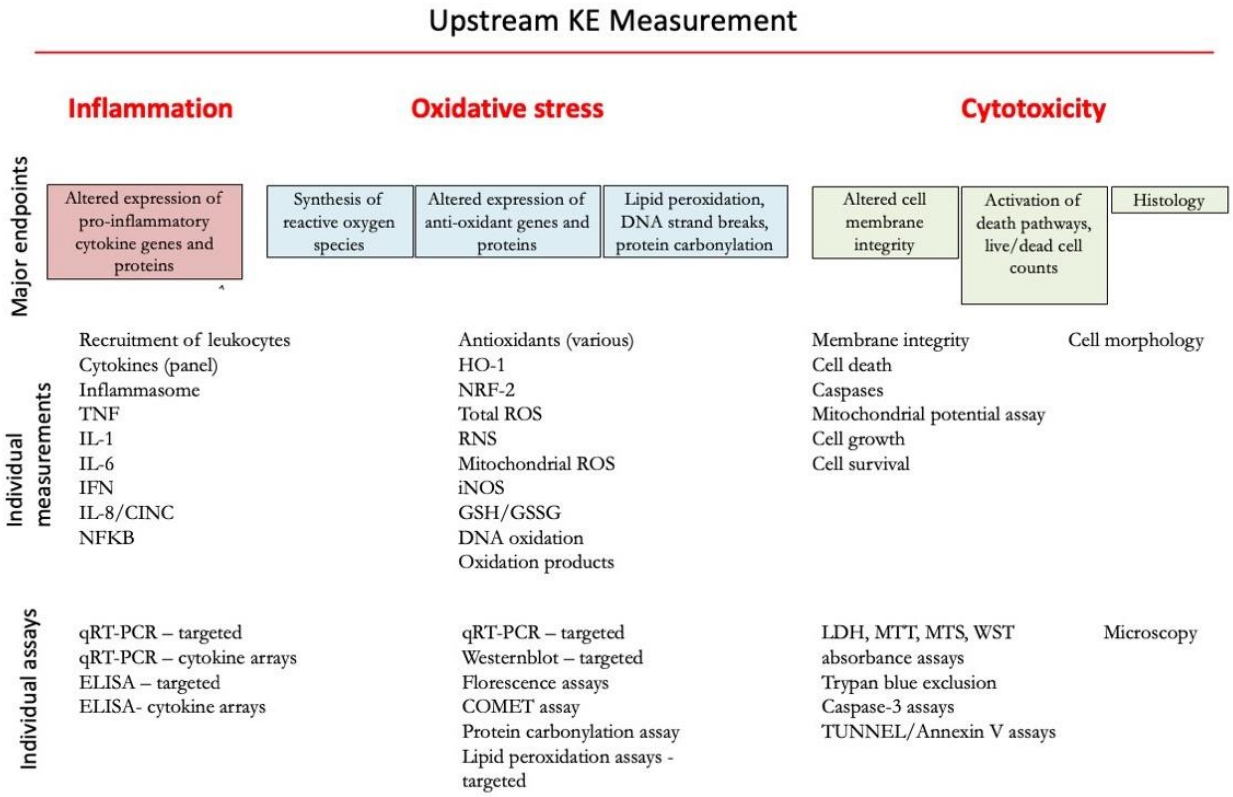
Figure 1. Substance-induced tissue injury is an interplay between inflammation, oxidative stress and cytotoxicity events, and respective hub-KEs.

28. The identified upstream KEs reflect a change in the biological homeostasis that is critical for occurrence of the 'tissue injury' KE; however, they may not be sufficient on their own to cause an adverse effect. As evidenced by the NanoAOP database, each of the upstream KEs is measurable and the same three KEs are measured irrespective of the tissue type in both *in vivo* and *in vitro* models. However, due to a long list of cytokines and chemokines (inflammation) or antioxidant genes and proteins (oxidative stress) that can be effectively measured, and the current lack of guidance or clear understanding of the relative importance of any of these specific biological entities in the actual event, details with respect to how many entities or which specific ones to be assessed in an assay, remains to be determined. For now, it is not clear if all upstream KEs require assessment in a single experiment as indicative of 'tissue injury' as quantitative understanding has not been established yet.

Identification of Endpoints, Methods and Assays to Assess KEs of Tissue Injury

29. Next, the NanoAOP database was reviewed extensively to identify the various measurements (endpoints) and assays used to measure the upstream KEs. Since one of the primary goals of AOP development is to design effective *in vitro* assays to measure the KEs identified in an AOP, the focus was placed on *in vitro* studies. *In vitro* studies generally reported on at least one of the upstream KEs of 'tissue injury'; however, the specific types of assays and endpoints varied widely between studies. For example, most *in vitro* studies assessed inflammation by measuring the altered expression of pro-inflammatory mediators; however, the number and type of mediators varied across the studies. Three studies assessed synthesis of nitric oxide as indicative of initiation of inflammation. Cytotoxicity was measured using a wide variety of assays targeting different biological phases of cytotoxicity; the majority of these used assays that are indicative of altered cell membrane permeability, one of the first signs of cytotoxicity. Other studies measured alteration in the levels of total adenosine triphosphate (ATP); decreased ATP levels are associated with programmed cell death and necrotic death. Still others directly measured activation of death pathways or used assays that measure the percentage of dead versus live cells. In addition, some studies documented histological changes (altered morphology) to support the occurrence of cytotoxicity. Similarly, different assays were used to assess the synthesis of ROS and altered expression of anti-oxidant genes in support of oxidative stress. Furthermore, it was observed that several different cell types were used across the studies representing different organ systems. Thus, a large heterogeneity was observed across the studies with regards to the cell types, specific endpoints and assays used. Figure 2 shows different endpoints and individual measurements used to evaluate the three upstream KEs, and the specific assays utilised.

Figure 2. Individual measurements and assays used for measurement of upstream KEs



3.2. Evidence to Support Upstream Key Events (KEs) for Tissue Injury

30. Next, the NanoAOP database was reorganised to reflect the KE 'tissue injury", arranged by the three upstream KEs: inflammation, oxidative stress and cytotoxicity. Database columns reporting the individual measurements were sorted and assigned to specific upstream KEs. Although histology is one of the endpoints measured in support of the upstream KE cytotoxicity, it was separately assessed as it can be used as direct evidence for clinical manifestation of the 'tissue injury'.

(a) Methods

Analysis by MN & evidence-based assessment of available literature

31. The updated NanoAOP database was organised to align the three upstream KEs proposed for measuring tissue injury (*i.e.* inflammation, oxidative stress, cytotoxicity) and histology with biological endpoints commonly reported to measure them, both *in vivo* and *in vitro* (Table 1). The NanoAOP database was sorted by MN type, with the analysis focusing on seven materials: silver, cerium oxide, copper oxide, MWCNT, SWCNT, titanium dioxide and zinc oxide.

Table 1. Endpoints commonly reported and captured in the NanoAOP database for measuring the 3 upstream KEs of tissue injury, plus histology

Inflammation	Oxidative Stress	Cytotoxicity	Histology
Increased recruitment of leukocytes	***Altered expression of antioxidant genes/proteins***	Membrane integrity	Microscopy
Increased pro-inflammatory mediators	Anti-Ox.	Cell death	Histology
TNF	HO-1	Caspases	
IL-1	NRF-2	Mitochondrial membrane potential	
IL-6	***Increased synthesis of ROS***	Cell growth/colony forming	
IFN	Total ROS	Survival	
IL8/CINC	iNOS		
NFkB	RNS		
Cytokines (other)	Mitochondrial ROS		
	Intracellular glutathione levels		
	GSH/GSSH		
	Modification of biomolecules		
	DNA oxidation		
	Oxidation products		

32. The updated NanoAOP database contained a total of 245 publications; however, some studies examined several different types of MNs. For the purpose of this analysis, those papers were broken down by MN type (i.e. a single study is counted more than once if it evaluated more than one type of MN) and each MN is considered a study. This resulted in a total of 294 studies.

33. The analysis examined the number of studies that: (i) reported (i.e. measured) each of the three upstream KEs and histology endpoint; and (ii) found significant induction of each upstream KE following MN exposure at any of the concentrations tested in the study. An upstream KE (e.g. cytotoxicity) was considered to be reported if at least one endpoint (e.g. membrane integrity, cell death, caspases, mitochondrial membrane potential, cell growth/colony formation, survival) was included in the experimental design. An upstream KE was considered to be induced following MN exposure if at least one measured endpoint showed a statistically significant difference (as determined in the experiment) in the exposed groups in comparison to un-exposed control groups in at least one of the concentrations tested in the study. Based on these criteria, all 294 studies were reviewed and assigned a value of 1 for each upstream KE if it was measured, in order to quantify the number of studies reporting each upstream KE. Similarly, in a separate analysis, all studies were reviewed and each KE assigned a value of 1 if the study found significant induction after MN exposure. This analysis was used to subsequently determine the number of studies that (i) measured/assessed and (ii) found significant induction with MN exposure of 0, 1, 2, or 3 upstream KEs as well as histology within a single experimental design.

34. A similar approach was adopted to quantify the number of studies that (i) measured/assessed and (ii) found significant induction of each endpoint used to determine each upstream KE (e.g. membrane integrity for the cytotoxicity KE) in response to MN exposure. Due to differences in study numbers between

materials (*e.g*. 71 studies evaluating titanium dioxide versus 16 studies evaluating copper oxide), these values are expressed as percentages.

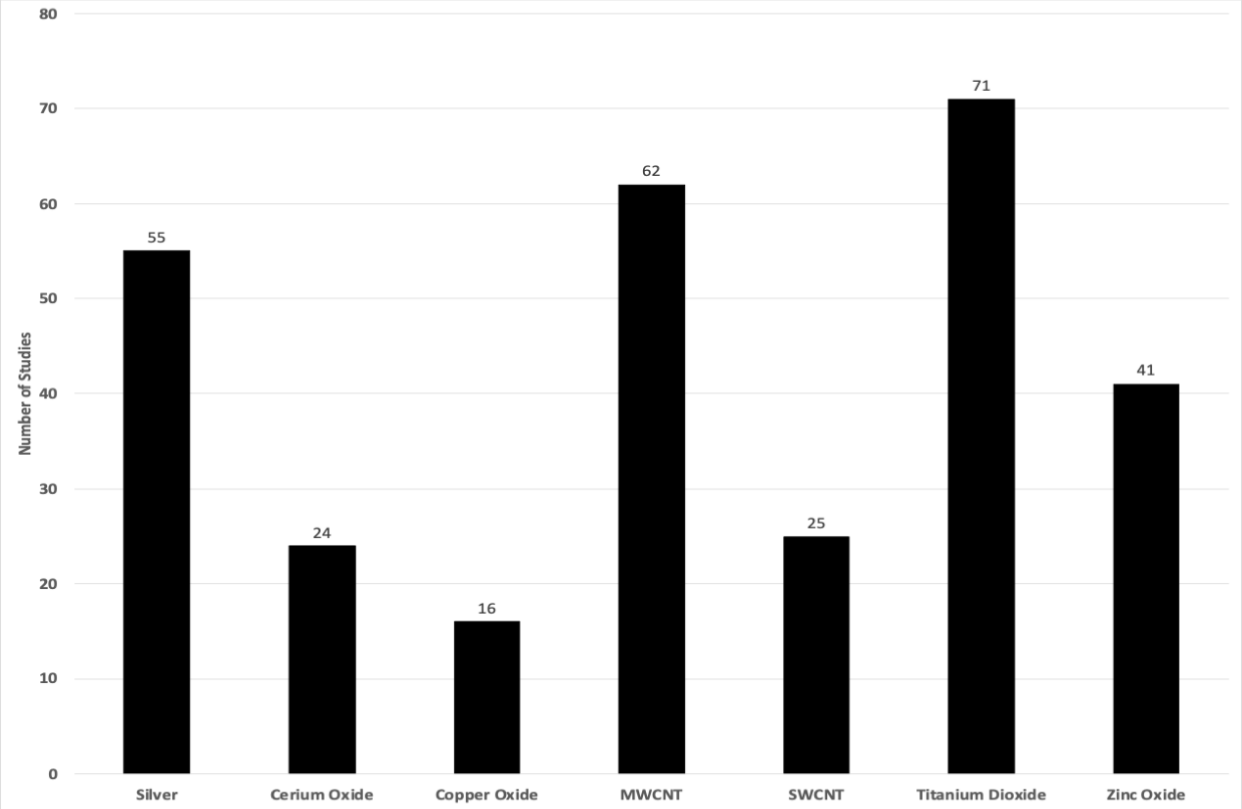
(b) Results

Evidence to support upstream KEs

35. The analysis included the number of studies that have assessed: (i) the individual upstream KEs for each MN; (ii) the number of studies that assessed specific endpoints under each upstream KE; and (iii) the number of the studies that found significant induction of the upstream KE with MN exposure and the measurements used to assess them. In addition, for each MN, a summary of how many upstream KEs were assessed per study was recorded.

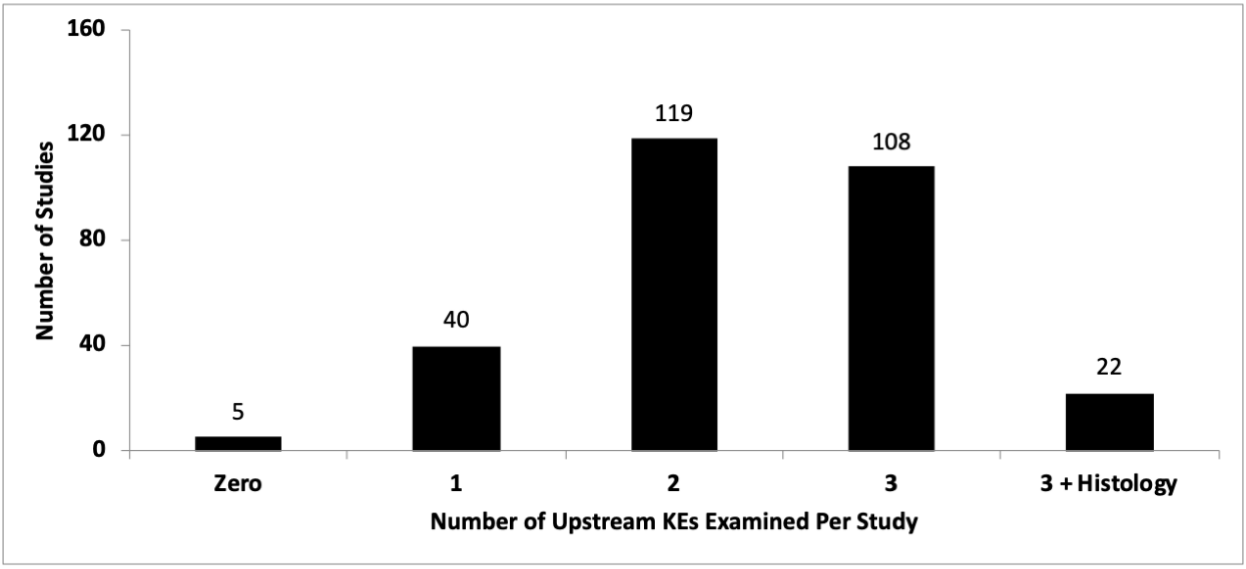
36. In total, the NanoAOP database consists of 294 studies, highly enriched with studies reporting on titanium dioxide (71), MWCNTs (62), silver (55) and zinc oxide (41). The other studies involved the investigation of SWCNTs (25), cerium oxide (24), and copper oxide (16) (Figure 3).

Figure 3. Number of studies analysed for each MN in the NanoAOP Database



37. Further analysis of the number of upstream KEs that individual studies investigated revealed that 40, 119 and 108 of them investigated one, two, or three upstream KEs respectively. Five studies did not investigate any of the upstream KEs identified for tissue injury in the case study. Only 22 studies investigated all three upstream KEs as well as histology (three upstream KEs + histology; Figure 4). Although it is currently not clear if it is necessary to measure all three upstream KEs as indicative of tissue injury, the 108 studies that report on all three upstream KEs provide a reasonable basis for building the Key Event Relationships (KERs) that connect them.

Figure 4. Number of studies in the NanoAOP database measuring 0, 1, 2 or 3 upstream KEs of tissue injury, or 3 upstream KEs plus histopathology

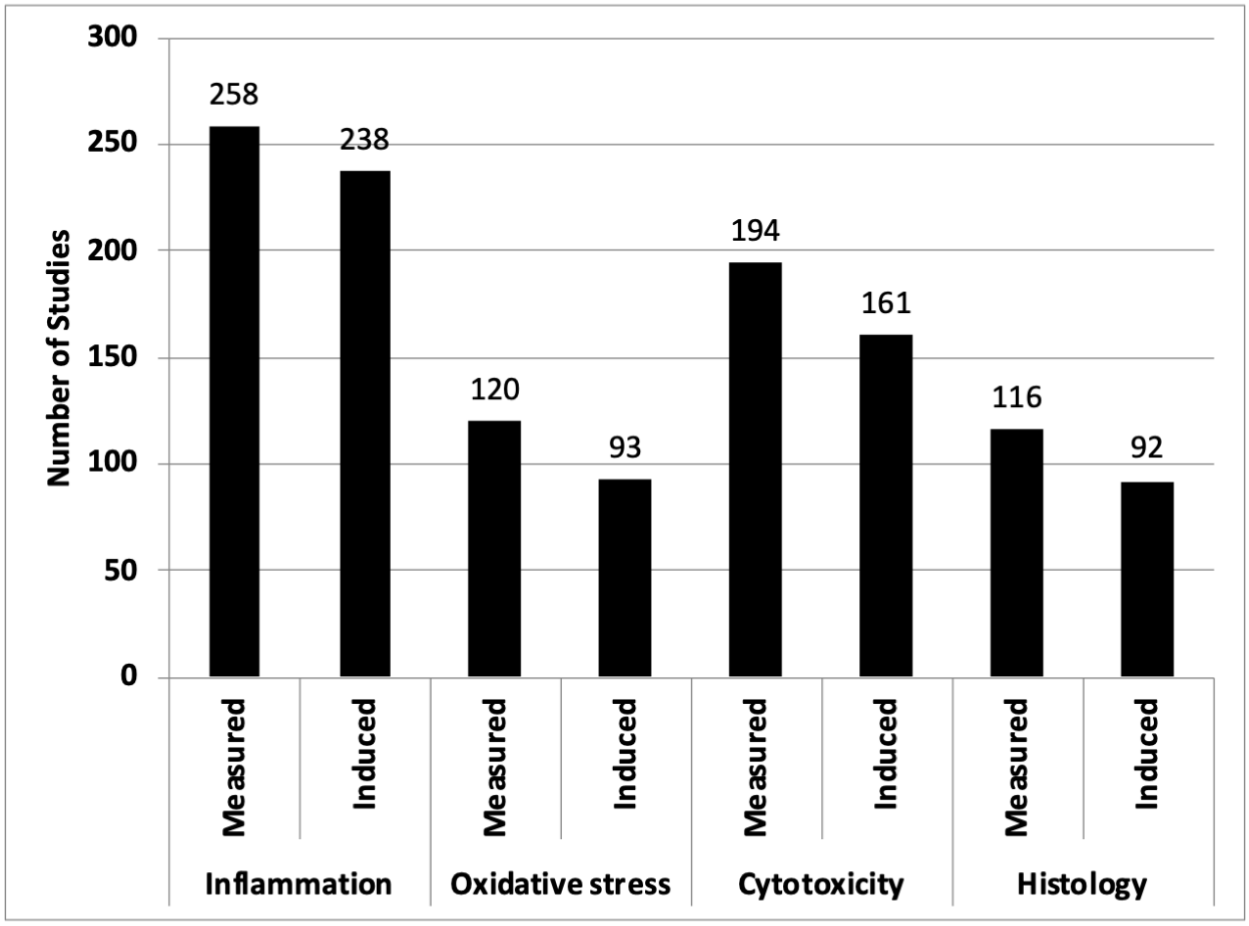


Overall Findings

Tissue Injury upstream KEs most commonly reported/elicited by MN exposure

38. Inflammation was the most commonly reported and induced upstream KE for tissue injury. Almost all the studies that assessed inflammation (KE1) reported induction, where induction was defined as any significant change in at least one endpoint (e.g. increased recruitment of leukocytes; others in Table 1) following MN exposure, relative to the unexposed control. As summarised in Figure 5, there were a total of 258 studies that assessed inflammation and 238 of these studies found significant induction with MN exposure. The second most commonly reported and induced upstream KE for tissue injury was cytotoxicity (KE3); a total of 194 papers assessed at least one cytotoxicity endpoint, of which, 161 found significant induction following MN exposure compared to controls. A total of 93 of 120 studies that examined oxidative stress reported this as being induced. Oxidative stress (KE2) can be measured by multiple assays including synthesis of ROS, depletion or imbalanced oxidant/antioxidant levels and oxidative modification of biomolecules. However, the analysis did not discriminate between the number and types of assays employed in making the 'induced' call. Finally, 116 studies reported on the histology endpoint with 92 studies finding significant changes with MN exposure compared to untreated controls. It is important to note that most of the 116 studies were conducted *in vivo*.

Figure 5. Results for total measured versus induced upstream KEs.



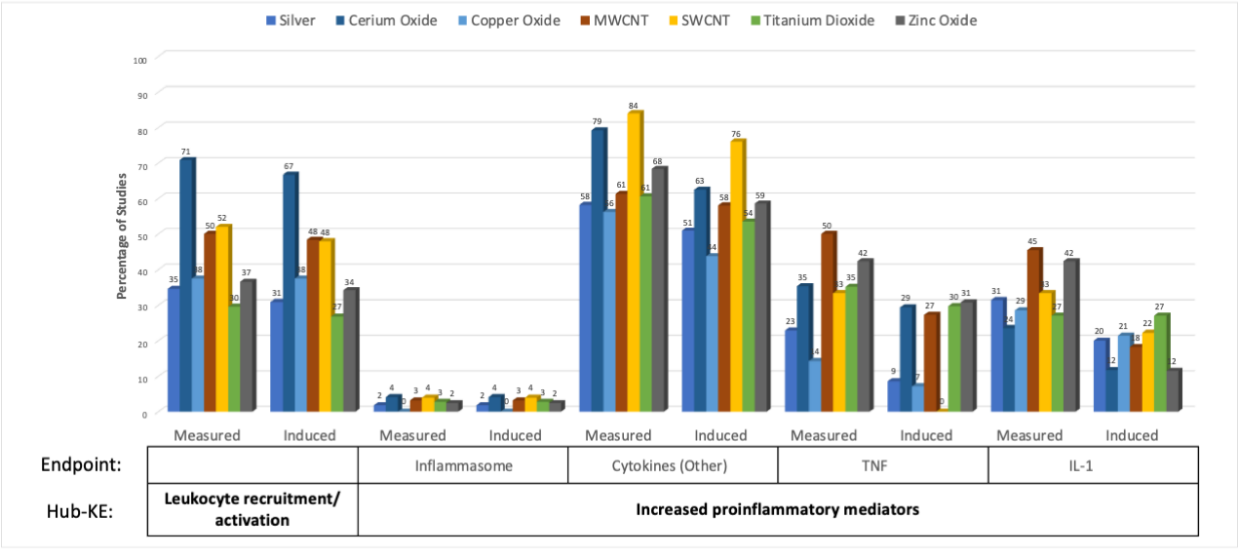
Endpoints most commonly reported to measure upstream KEs

39. Analysis by the type of endpoints used for each individual upstream KE revealed that for upstream KE inflammation, altered expression of pro-inflammatory mediators was the most assessed endpoint. The NanoAOP database recorded the number of studies examining TNF, IL-1, IL-6, IFN, IL-8/CINC and NFkB directly, all other pro-inflammatory mediators were captured under the category 'Cytokines (Other)'.

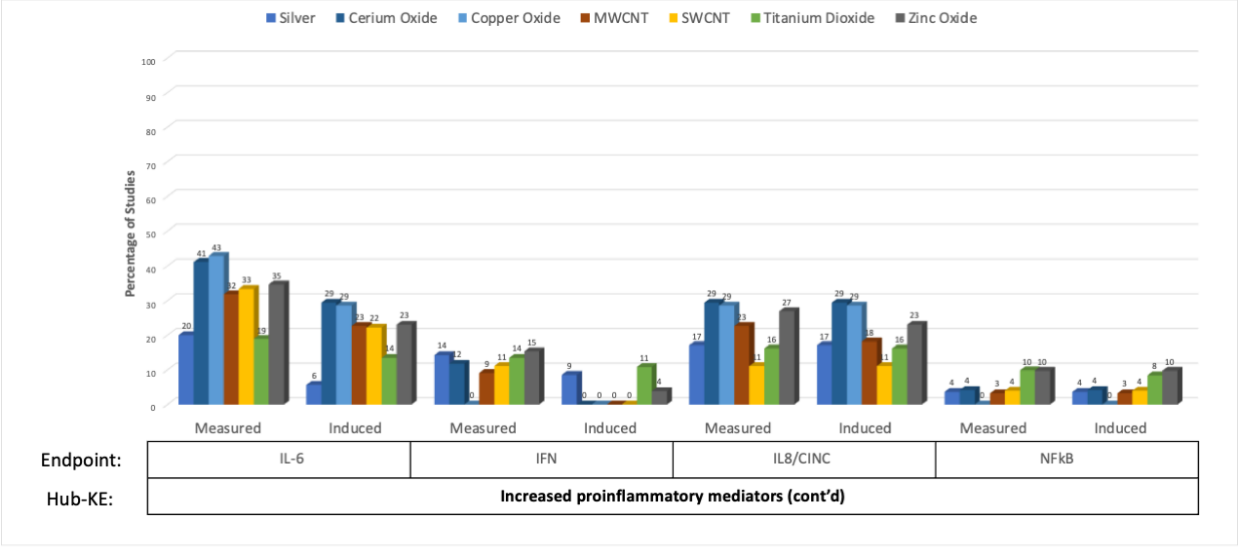
40. Recruitment of leukocytes was also a commonly assessed endpoint, with 41% studies measuring it, and of those, 39% finding significant recruitment with MN exposure. Inflammasome activation was far less reported in the literature as an indicator of inflammation, with 3% of studies examining its induction following MN exposure. From the analysis, it was not possible to deduce any MN or MN property-specific trends (Figure 6).

Figure 6. Measured versus induced endpoints for the inflammation KE.

A.



B.



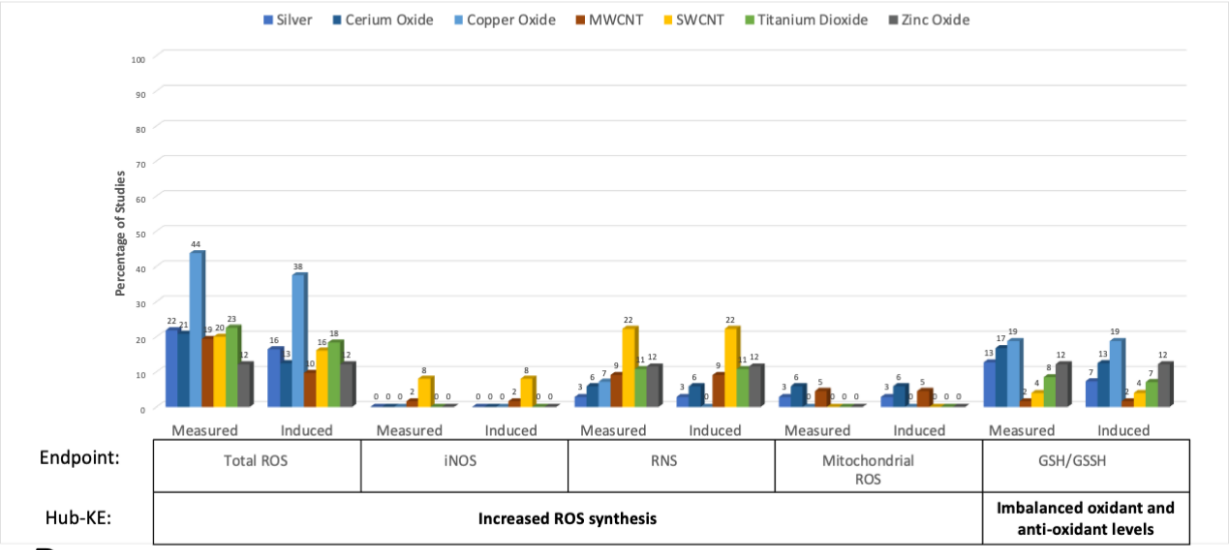
41. For the upstream KE oxidative stress, the analysis did not differentiate between *in vivo* and *in vitro* measurements. The most assessed oxidative stress endpoint in the MN literature was Total ROS, with 21% of studies examining it, and 16% finding induction with MN exposure. Other highly examined endpoints include: Oxidation products (11% of studies measured, 8% found induction), Reactive Nitrogen Species (RNS; 9% measured; 8% found induction), ratio of reduced glutathione/oxidised glutathione (GSH/GSSH; 9% measured, 7% found induction) and Anti-Ox (4% measured, 3% found induction).Less commonly reported endpoints were activation of HO-1 and NRF2 signalling pathways, altered expression of iNOS; synthesis of mitochondrial ROS, and DNA oxidation (Figure 7).

42. For the upstream KE cytotoxicity, cell death was the most predominantly assessed endpoint for *in vivo* and *in vitro* studies, with 44% of studies examining it, and 35% finding significant cell death with MN exposure. Membrane integrity was also frequently assessed, with 35% studies examining it, and 29% finding MN exposure leading to compromised membrane integrity compared to controls (Figure 8). The endpoints caspases, mitochondrial membrane potential, cell growth/colony formation and survival were less commonly assessed, with a total of 10%, 6%, 2%, and 0% of studies examining them, respectively.

43. For histology, cell morphology was the most commonly reported endpoint *in vitro*, with 19% studies examining it, and 13% finding significant changes in morphology after MN exposure. *In vivo*, tissue histology was the most commonly reported endpoint, and a direct measure of tissue injury was recording significant changes in tissue structure following MN exposure. Of the 37% of studies examining this endpoint, 28% reported a significant change in tissue structure following MN exposure.

Figure 7. Measured and induced endpoints for oxidative stress KE.

A.



B.

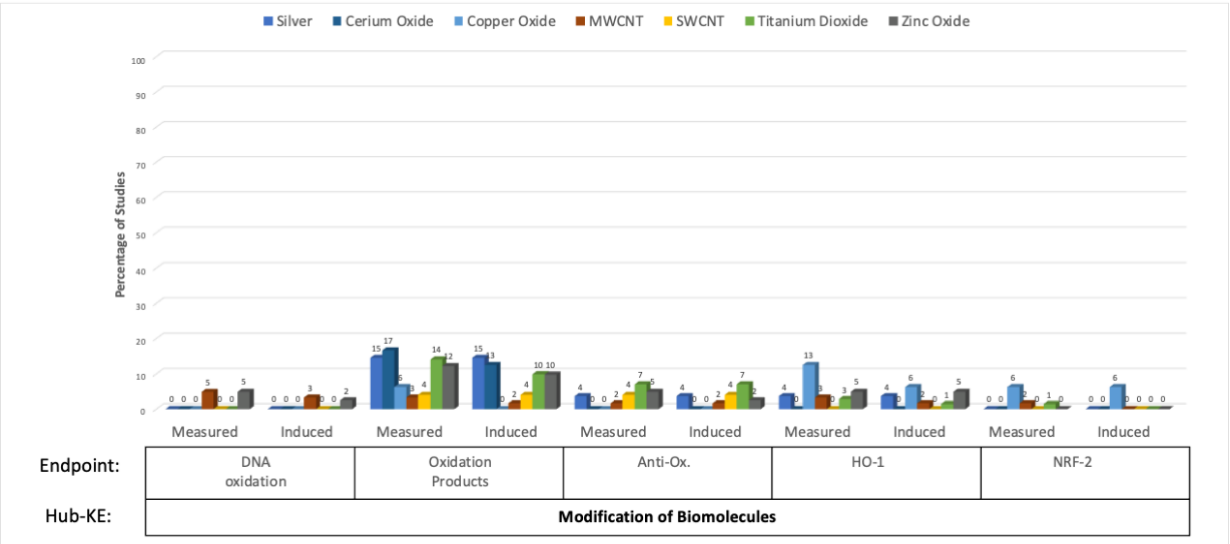
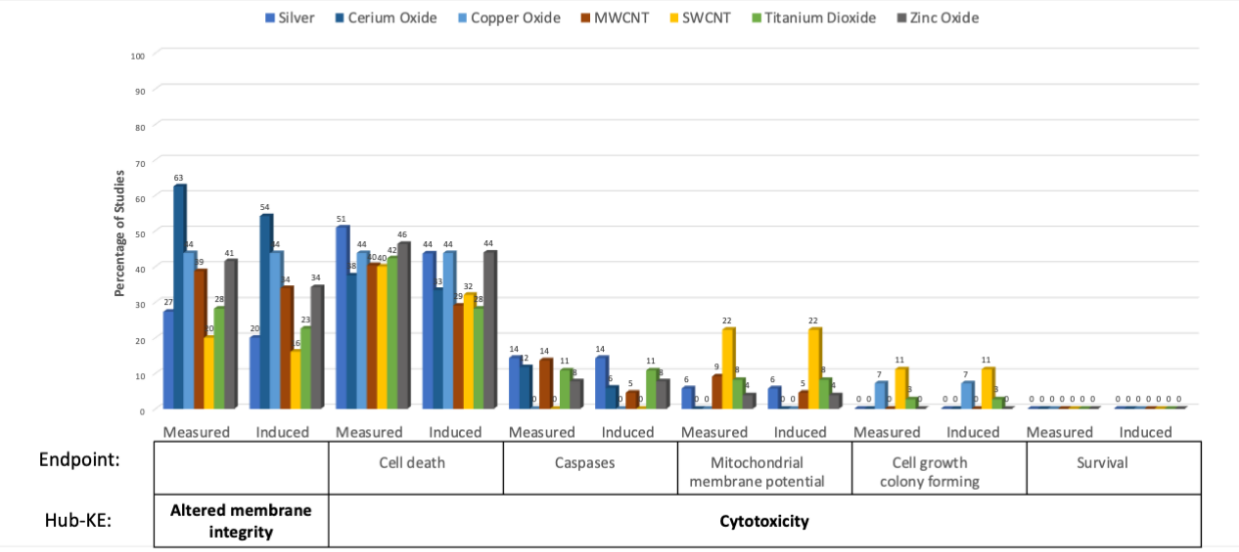


Figure 8. Measured and induced endpoints for the cytotoxicity KE.



4 Limitations

44. Although the Swiss-VCI database provided an excellent starting point, the database was not specifically designed to assess tissue injury. Without this goal in mind, the parameters collected in the database and the criteria for capturing this information from the peer reviewed literature were not ideal for development of the qualitative or quantitative tissue injury KE. For example, it would have been useful within the NanoAOP database to collect the full range of concentrations tested and the post-exposure sampling time points assessed within each experiment, which would enable building of dose-response and temporal relationships. Tissue injury evolves over time and physico-chemical properties of MNs, duration of exposure and the specific exposure concentrations are important factors that determine its manifestation. It is important to appreciate the fact that injury leading to permanent damage or functional dysfunction ensues only when endogenous defence mechanisms are outdone.

45. Biological processes and functions exhibit redundancy. This redundancy makes it very difficult to recommend a panel of endpoints (e.g. genes/proteins/metabolites/etc.) as being sufficient for measuring a specific upstream KE. For example, altered expression of pro-inflammatory mediators, an inflammatory KE, is assessed by measuring the change in expression of a single or multiple cytokines/chemokines. The number of mediators assessed and the specific types depend on the experience of the individual researcher/laboratory and the resources available. Similarly, many studies opt to measure cytotoxicity using more than one assay. How many pro-inflammatory mediators or cytotoxicity assays should be included in the assessment as sufficient evidence or whether an assay requires validation by another assay measuring the same endpoint is not yet known. At present, the evidentiary basis of *in vitro* toxicological science is insufficient, particularly for MNs, to develop recommendations addressing these questions.

46. The other major limitations with respect to assessing the weight-of-evidence for an upstream KE is the heterogeneity in experimental design, including differences in the *in vivo* species, route of exposure, and post-exposure duration; variability in the *in vitro* cell type; and the specific assays employed in both *in vivo* and *in vitro* studies. For example, for the cytotoxicity KE, cell death is a commonly reported endpoint; however, it has been assessed with a variety of methods and assays (e.g. metabolic assays, live/dead cell count). Different assays for a given endpoint cannot be directly compared to evaluate trends between MNs, or their properties. Moreover, cytotoxicity assays are reported as cell viability, cell survival, cell proliferation and cell death; however, the assays employed to measure the listed endpoints are often the same. For example, the LDH assay is employed for measuring both cell viability and cell death. Designing the NanoAOP database using consistent ontology (e.g. capturing and grouping all the assays that measure membrane permeability under a 'loss of membrane permeability' endpoint) would permit (i) consistent analysis of the types of assays/methods used to assess a particular endpoint and a KE; and (ii) would allow the data to be organised by assay type, to allow for better comparisons between MNs and their properties.

47. Finally, due to the structure of the NanoAOP database, manual assessment of the studies was required to record the (i) number of studies examining the upstream KEs and (ii) number of studies examining each of the endpoints to assess those KEs. This required significant time to review all of these variables, for all 294 studies. Streamlining the ontology used for recording data and automated tallying would make the analysis and subsequent KE development more efficient.

48. Lastly, it is acknowledged that the suggested upstream KEs pose challenges for use as indirect measurements of one single KE tissue injury. Moreover, tissue injury in some cases could be regarded as an AO. Thus, guidance on how many upstream KEs need assessment as predictive of tissue injury occurrence is important. While it is tempting to suggest that one endpoint measuring each of the upstream KEs showing dose and temporal progression in a cell type that is relevant to the tissue type or route of exposure investigated should be sufficient, further discussions have to be held in the community to agree on a set of recommendations.

5 Conclusions

49. The case study has outlined a methodology for developing KEs relevant for MNs from evidence gathered from the literature. The methodology includes an approach to develop an expert-curated database; complete a literature search and quality evaluation; and the subsequent use of that database to gather evidence (i) for the occurrence of a KE following MN exposure and (ii) to support its development as a KE in an AOP.

50. The case study focused on development of tissue injury as a KE relevant for MNs. A review of the literature in the developed NanoAOP database found that events related to inflammation, oxidative stress and cytotoxicity were the three most commonly assessed and reported biological events following MN exposure with a direct inference to tissue injury. Thus, it was interpreted that these frequently reported biological events can be used as upstream KEs to the 'tissue injury' KE. As evidenced by the NanoAOP database, each of the upstream KEs is measurable and the same three upstream KEs can be measured irrespective of the tissue type in both *in vivo* and *in vitro* models. For some tissues such as brain or liver, additional parameters reflective of tissue function are measured. A review of the NanoAOP database identified the various *in vitro* endpoints, methods and assays used to measure the upstream KEs for tissue injury.

51. The analysis outlined the use of the NanoAOP database as an evidence-based assessment of tissue injury as a KE. The upstream KEs reported were found to be induced by almost all types of MNs examined. The analysis also examined the endpoints most commonly reported to measure each upstream KE for MNs. For the inflammation KE, altered expression of pro-inflammatory mediators was the most assessed endpoint and was reported following exposure to almost all MNs examined. For the oxidative stress KE, production of RNS and total ROS were the most commonly measured and reported mixed with endpoint and endpoint following MN exposure, while for the cytotoxicity KE, cell death was the most predominantly assessed and reported endpoint that occurs following MN exposure. The analysis separately evaluated histology as one of the endpoints in support of the KE cytotoxicity, as it can be used as direct evidence for clinical manifestation of 'tissue injury'. Histology was found to be measured and reported in the literature following exposure to almost all types of MNs examined.

52. Together, these data have developed tissue injury as a KE relevant for MNs as it is both a measurable and observed biological event following MN exposure that is essential for toxicity. It was one of the most frequently observed biological events in the NanoAOP database following exposure to a variety of MNs; it is measurable with numerous endpoints, methods and assays reported both *in vivo* and *in vitro*; and it has high regulatory relevance, being associated with significant adverse human health outcomes in a variety of tissues (e.g. fibrosis, granuloma, mesothelioma, and emphysema in the lung).

53. The KEs identified reflect a change in the biological state that is critical for occurrence of the 'tissue injury' KE, although it is not yet clear whether all upstream KEs require assessment in a single experiment as indicative of 'tissue injury'. However, much additional work needs to be completed before one can derive recommendations or provide specific guidance on experimental design for assessing tissue injury as indicative of an MN-induced adverse event.

6 Next steps

54. From this work, a series of next steps are proposed to further develop and advance the use of this proposed methodology for MN-relevant KE development.

- Outline how the proposed methodology can be used to build KERs. The analysis identified 108 studies examining three upstream KEs for tissue injury and 22 studies examining three upstream KEs and histology. Since these studies examine three upstream KEs for tissue injury in a single experiment, they are prime candidates for: (i) developing KERs that exist between these KEs; (ii) for linking *in vitro* methods to assess tissue injury to *in vivo* methods to assess tissue injury; and (iii) for linking tissue injury to eventual adverse health outcomes;
- Refine the NanoAOP database template to use consistent ontology and terminology being developed in related projects, such as ongoing projects across the EU H2020 consortia;
- Refine the NanoAOP database template to capture the various methods reported for endpoints, and consider how this can be used to enable better comparisons between data sets;
- Identify the studies in the NanoAOP database with sufficient quantitative dose-response information for deriving comparative potency across MNs for specific biological outcomes, and whether sufficient information is available to investigate structure-activity relationships between MN properties and biological outcomes;
- Identify the relevant or suitable cell types to consider, and the relevant exposure models to use. Significant efforts are being made in some of the partner labs and as part of EU H2020 research consortia, to design and validate appropriate *in vitro* models to assess the safety of MNs. Potential collaboration between these efforts can help streamline a path forward for assessing tissue injury, enabling expansion of the domain of applicability (*i.e.* range of NMs assessed, species specificity, developmental stage applicability, age and sex specificity), utilising weight-of-evidence methods, and assessing the relationship between tissue injury endpoints and dose (by various metrics including total particle mass, volume, and/or surface area). Such information would facilitate building KEs as well as KERs for upstream KEs identified in a pathway of interest such as tissue injury and eventually inform AOP development.

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Acknowledgments

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Appendix 1: Literature Reviews, Quality Evaluation and AOP Database

Updating of the Existing Database

1. The data entries related to AOP/PoT (Pathways of Toxicity) 'inflammation' in the Swiss-VCI database were the basis for developing the case study for the KE tissue injury. This database, which covered literature spanning the period 2009 to May 2013 and consisted of 119 publications, had 101 columns to record the various parameters or variables tested/reported in the peer-review published literature and 447 rows of unique data entries (with 1 row per MN type, cell or animal type). The details of the Swiss-VCI database, its search strategy and quality evaluation, have been presented in Halappanavar et al., 2019.

2. University of Birmingham was responsible for updating the Swiss-VCI database and relevant studies published between January 2014 and December 2017 were added to the database. Also, 15 additional parameters or variables, very specific to inflammation or tissue injury, were added as columns in the database to aid in further, more detailed analysis to support the development of the tissue injury KE. Seven MNs were selected to update the database: titanium dioxide, silver, copper oxide, cerium oxide, zinc oxide, SWCNT and MWCNT.

3. The basic search query tab in Web of Science (WoS) included the 'topic' field, with a customised time range of 2014-2017, and the terms "nano*" AND "inflamma*" combined with suitable truncated terms to include the MN type. The Boolean operator NOT was used in the same search query to reject publications reporting intravenous mode of exposure. Inclusion (*e.g.* inhalation exposure) and exclusion criteria (*e.g.* experiments with environmental organisms, such as *Daphnia magna*, zebrafish) were developed and applied for selecting/identifying the studies to include in the database (Box 1). By this search mechanism, ~ 1294 new publications were identified for the seven MNs, of which only 136 publications satisfied the inclusion/exclusion criteria. Since one of the objectives of the present study was to assess whether existing data are supportive of the development of KEs and AOPs of relevance to MNs, the inclusion/exclusion criteria in some cases were not stringently applied to enable inclusion of a maximum number of studies (some of which were later removed after deliberation during the analysis step). After a scan of the full paper these publications were then assessed for quality as described below.

Quality Evaluation

4. A two-step evaluation (originally proposed by RIVM) was adopted with some modifications. As a first step, a quality control (QC) assessment was done in which it was checked if the ENM used in the study has been characterised. If the authors had characterised the data or had given references to characterisation data, or if manufacturer provided data was reported and it was easily located, the study was included for scoring. If the selected published study lacked characterisation, it was rejected and not included in the database. As a second step, studies were scored as 0, 1, 2 for each of the criterion outlined (details in Annex). If no information was provided for a particular criterion it was scored as 0. Not applicable

or not relevant was used instead of 0, where warranted. A score of 1 was given if the study provided minimum information on the criteria. A score of 2 was given if the information or data provided on a particular criterion was considered to be appropriate and detailed. Then, the individual scorings were summed up and the published studies were divided into three groups.

1. Discarded studies from the first QC step – group 1, and not included in the database.

2. Studies with <24 points – group 2

3. Studies with ≥ 24 points – group 3

4. Group 3 studies, i.e., studies with ≥ 24 points, can be seen as high quality studies, as sufficient information has then been given and/or additional pieces of useful information provided as per the quality criteria developed for the study. Of 136 studies identified in the literature search, 126 papers were binned as Group 2 or 3 and included in the NanoAOP database.

5. There is no doubt that there will be subjectivity in the quality assessment, however, this evaluation was meant to identify quickly the various aspects in which the papers published on nanotoxicology were limited and to suggest a way forward in terms of design of studies to enable subsequent users of the data to make relevant interpretations or for future meta-analysis to support decision making in MNs risk categorisation. Studies in the second and third groups were used for building the NanoAOP database. This quality assessment was done only for the updated database (original research articles published between 2013 and 2017 especially evaluated for KE and AOP development).

Results

6. The original Swiss-VCI database consisted of data from 119 studies published in the period 2009-2013. The updated database (papers from 2014-2017) has an additional 126 peer-reviewed papers, covering the 7 MNs listed in Table 2, and consisting of 485 rows with unique entries. Therefore, the database used for MN AOP development has 245 publications which resulted in 932 rows with unique entries, covering 30 distinct families of NMs, with many MNs having numerous variants (*e.g.* uncoated or coated, positively or negatively charged, different shapes etc.).

Box 1. Criteria to identify relevant papers that could support development of the KE tissue injury

Criteria for inclusion of papers

- Studies published online between 1 January 2014 and 31 December 2017
- Inhalation exposure, intratracheal instillation, and orpharyngeal aspiration, ingestion for *in vivo* studies
- Contains data on one or more of: Reactive oxygen species (ROS), injury (kidney, liver, etc.), membrane permeability (LDH), membrane potential changes, cytotoxicity (LDH), tight junction changes, inflammatory mediator releases - cytokine (ILα,ILβ TNFα, IFNγ, lymphocyte, monocyte chemoattractant protein) and chemokine release (especially IL8), nitric oxide, leucocyte recruitment, proteases (metalloproteinases/tissue inhibitor metalloproteinase), necrosis, fibrosis, lysosome stability
- English Language publications
- Papers focused on Toxicity (for *in vitro* studies) and that do not have 'inflammation' as a word in the abstract but do have results for: 1) ROS generation, 2) membrane permeability, 3) membrane potential change, 4) caspases or cytokine release, 5) pro-inflammatory gene activation, 6) oxidative DNA damage, 7) Colony formation / proliferation assay. At least four (out of these 7 end-points) should be addressed in a paper for its inclusion.

Criteria for exclusion of papers:

- *In vivo* or *in vitro* data related to environmental organisms (*e.g.* zebrafish, *C. elegans*, daphnia, bivalves)
- Papers with intravenous injection as the route of exposure
- Publications on nanomedical applications
- Papers on cytotoxicity or cell death (*in vitro*) not clearly linked to inflammation or ROS or tissue injury
- If the paper only reports results of increase in transcription factors of genes related to inflammation.

Annex

7. The criteria identified for quality assessment are:

A. MNs that are intended to be tested in studies must be characterised sufficiently beforehand. Information on the following characteristics must be provided:

a. Chemical composition

b. Particle size

c. Shape

d. Aspect ratio (where relevant)

e. Crystal phase in the case of TiO_2 and SiO_2

f. Redox state in the case of CeO_2

g. Surface chemistry (*i.e.* composition at the surface, chemical composition of any coating), chemical composition of any functionalising groups, surface charge (negative, neutral or positive)).

B. Sufficient information must be provided to enable evaluation of the validity and suitability of the selected test methods.

a. *In vivo* studies:

i. Applied quantities (concentration/dose)
ii. Dispersion procedure
iii. Route of exposure
iv. Appropriate negative and positive controls
v. The stability of daily applied MN dispersion during the test
vi. The frequency and duration of exposure as well as the time points of observations
vii. Species
viii. Number of animals per group
ix. (Meta) data related to uncertainty, *e.g.* a standard deviation and the number of replicates.

b. *In vitro studies:*

x. Applied quantities (concentration)
xi. Dispersion procedure
xii. Frequency and duration of exposure as well as time points of observations
xiii. Assessment of potential interference of the MN with the test system
xiv. Appropriate proteins in the medium
xv. Appropriate negative and positive controls are applied
xvi. The stability of the MN dispersion during the test
xvii. The origin of the cells in terms of cell line
xviii. An appropriate light regime is applied when studying TiO2 MNs
xix. (Meta) data related to uncertainty e.g. a standard deviation and the number of replicates.

Table A-1. Search terms and results after scan of abstracts (only for articles)

Nanomaterials	Search terms (in topic field of web of science)	Results of search	Number of papers in the first round of download into Endnote X8
Titanium Dioxide	titan* nano* inflamma* NOT intravenous	105	66
Silver Nanoparticle	silver nano* inflamma* NOT intravenous	503	33
CuO or Copper nanoparticle	coppe* nano* inflamma*	76	23
Zinc or Zinc oxide nanoparticle	zinc nano* inflamma*	244	62
Ceria or cerium oxide nanoparticle	ceri* nano* inflamma*	112	27
Carbon Nanotube (CNT)	nano* CNT inflamma*	89	26
Carbon Nanotube (CNT)	nano* MWCNT inflamma*	128	42
Carbon Nanotube (CNT)	nano* SWCNT inflamma*	37	8

Appendix 2: Glossary of terms related to Adverse Outcome Pathways in this document

Adverse Outcome (AO)

An AO is a specialised type of KE that is generally accepted as being of regulatory significance on the basis of correspondence to an established protection goal or equivalence to an apical endpoint in an accepted regulatory guideline toxicity test (OECD, 2016a).

Note: Depending on whether the protection goal is for human health or ecological health, the endpoints considered may differ.

Adverse Outcome Pathway (AOP)

Conceptually, an AOP can be viewed as a sequence of events commencing with initial interactions of a stressor with a biomolecule in a target cell or tissue (i.e., molecular initiating event), progressing through a dependent series of intermediate events and culminating with an AO. AOPs are typically represented sequentially, moving from one KE to another, as compensatory mechanisms and feedback loops are overcome (OECD, 2016a).

Endpoint

The recorded observation coming from an in chemico method, or an *in vitro* or *in vivo* assay.

Integrated Approaches to Testing and Assessment (IATA)

An IATA is an approach based on multiple information sources used for hazard identification, hazard characterisation and/or safety assessment of chemicals. An IATA integrates and weights all relevant existing evidence and guides the targeted generation of new data, where required, to inform regulatory decision-making regarding potential hazard and/or risk. Within an IATA, data from various information sources (i.e. physicochemical properties, in silico models, grouping and read-across approaches, *in vitro* methods, *in vivo* tests and human data) are evaluated and integrated to draw conclusions on the hazard and/or risk of chemicals. Within this process, the incorporation of data generated with non-animal testing and non-testing methods is expected to contribute considerably to a reduction of testing in animals (OECD,

2016b). The output of an IATA is a conclusion that, along with other considerations, informs regulatory decision-making (OECD, 2016b).

Key Event (KE)

A KE is a change in biological state that is both measurable and essential to the progression of a defined biological perturbation leading to a specific adverse outcome (OECD, 2016a).

Key Event Relationship (KER)

A KER is a scientifically-based relationship that connects one KE to another, defines a directed relationship between the two (i.e. identifies one as upstream and the other as downstream), and facilitates inference or extrapolation of the state of the downstream KE from the known, measured, or predicted state of the upstream KE (OECD, 2016a).

Weight of Evidence (WoE) [3]

WoE is a comprehensive, integrated, often qualitative judgment of the extent and quality of information supporting an hypothesis for which the approaches and tools vary, depending on the context (Weed, 2005; WHO-UNEP, 2012). For AOPs, the WoE is addressed based on a specified subset of considerations modified from those proposed by Bradford Hill (B/H) for assessment of causality in epidemiological studies (Hill, 1965), drawing on previous experience in mode of action analysis. Defining questions and the nature of supporting data for each of the relevant considerations is included in the document of the Users' Handbook to the OECD Guidance on AOPs (OECD, 2016a).

[3] "Guiding Principles and Key Elements for Establishing a Weight of Evidence for Chemical Assessment", Series on Testing and Assessment No. 311 (OECD,2019)

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