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A methodology for developing key events to advance nanomaterial-relevant adverse outcome pathways to inform risk assessment

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Abstract

Significant advances have been made in the development of Adverse Outcome Pathways (AOPs) over the last decade, mainly focused on the toxicity mechanisms of chemicals. These AOPs, although relevant to manufactured nanomaterials (MNs), do not currently capture the reported roles of size-associated properties of MNs on toxicity. Moreover, some AOs of relevance to airborne exposures to MNs such as lung inflammation and fibrosis shown in animal studies may not be targeted in routine regulatory decision making. The primary objective of the present study was to establish an approach to advance the development of AOPs of relevance to MNs using existing, publicly available, nanotoxicology literature. A systematic methodology was created for curating, organizing and applying the available literature for identifying key events (KEs). Using a case study approach, the study applied the available literature to build the biological plausibility for 'tissue injury', a KE of regulatory relevance to MNs. The results of the analysis reveal the various endpoints, assays and specific biological markers used for assessing and reporting tissue

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Supplemental data for this article can be accessed here

injury. The study elaborates on the limitations and opportunities of the current nanotoxicology literature and provides recommendations for the future reporting of nanotoxicology results that will expedite not only the development of AOPs for MNs but also aid in application of existing data for decision making.

Keywords

Adverse outcome pathway; key event; nanomaterial; risk assessment

1. Introduction

Manufactured Nanomaterials (MN/MNs) are a diverse class of materials, defined as ‘material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm’ (European Commission 2011). Because of their nanoscale dimensions, MNs exhibit unique structural, chemical, physical, optical, electrical, and thermal properties, making them desirable for a wide range of applications from consumer products to medical diagnostics (Maynard, Warheit, and Philbert 2011). In the context of biology, because of their small size, MNs (1) can be easily taken up by cells; (2) accumulate in resident macrophages and translocate to tissues, including reaching deeper, highly vascular areas such as lung tissues; and (3) exhibit larger surface area per unit mass compared to their bulk counterparts and thus, exhibit higher reactivity with biomolecules and potentially higher toxicity (Nel et al. 2006) for a given mass. In addition, MNs are often application-tailored which involves modifications to their surfaces with chemical groups and/or charges that can impact their toxicity. More recently, novel generations of hybrid MNs (MN core is coated with a shell of another composition) are penetrating the consumer market (Hafner et al. 2014), leading to advanced materials (i.e. materials with engineered properties) such as composites, ceramics, biomaterials, polymers, and others. Because of their numerous applications, the production of MNs and other advanced materials has increased and the availability of nano-enabled consumer products on the market is also on the rise (Boyadzhiev et al. 2020). Consequently, exposure to these substances in occupational settings and throughout the lifecycle of the MNs is expected to increase.

Two decades of toxicological research has revealed that (1) unique MN properties influence their behavior in biological and environmental matrices and are reciprocally influenced by the surrounding biological or environmental milieu (Wagner et al. 2014; Garner and Keller 2014; Geitner et al. 2020); (2) physicochemical properties (such as dissolution, size, shape, density, dustiness, fiber rigidity, specific surface area, surface reactivity, band gap energy and surface bond strain) have been associated with differences in toxicity (Nel et al. 2006; Warheit et al. 2007; Cho et al. 2010; Braakhuis et al. 2014; Halappanavar et al. 2015, Halappanavar, Ede, et al. 2019; Poulsen et al. 2016; Schmid and Stoeger 2016; Rahman et al. 2017) although validated models are not yet available to predict toxicity based on these properties, which may necessitate case-by-case investigation of individual MNs (Danielsen et al. 2020; Knudsen et al. 2019; Nel et al. 2013; Poulsen et al. 2017; Wohlleben et al.

2019); (3) some of the existing methods for material characterization and toxicity testing approaches for assessing risks to human health and the environment require modifications or adjustments for MNs and, in some cases, new methodology is needed (Hirsch et al. 2011; Krug 2014); and (4) some MNs can be toxic to the environment and/or humans, and, the toxicity mechanisms or the eventual adverse effects may be similar to ones known for chemicals (Labib et al. 2016; Nikota et al. 2016; Laux et al. 2018) or to microscale particles of similar chemical composition, although at lower mass concentrations (Saber et al. 2019). Conventional toxicity testing methods have been used to characterize MN toxicity; however, given their variability and number, methods that heavily rely on animal experimentation are not operationally feasible (Choi et al. 2009). Alternatives to animal testing exist, including increasing use of toxicogenomics and *in silico* methods, but will require justification and validation before their application in nanotoxicology and regulatory decision making for MNs (Afantitis et al. 2020; Kinaret et al. 2020).

In the last 10 years, advances have been made in the field of regulatory toxicology to move away from decision making relying on testing for apical effects in animals to a mechanistic approach that is based on a holistic understanding of the biological pathway perturbations at the molecular, cellular, tissue and whole organism levels (Shatkin et al. 2016; Ede et al. 2020). The resultant mechanisms-based testing tools and assays are expected to reduce overall animal use and help predict the human and environmental health impacts from exposure to chemicals in a cost, time and resource-effective manner (NRC 2007). However, the effectiveness or success of such a scheme depends on a detailed understanding of the mechanisms of toxicity and the development of targeted, sensitive, and reliable assays that enable accurate measurements of essential components of the mechanisms, allowing predictions of the outcomes in an organism. All of this requires: (1) careful curation of the large body of scientific information that describes how chemicals perturb the normal biological pathways and functions to exert their toxic effects; (2) organization of the complex mechanistic information in a meaningful and simplified format that is open and accessible; and (3) development of guidance for application of this information to decision-making.

In order to facilitate a systematic curation, organization and application of mechanistic information, the Adverse Outcome Pathways (AOPs) framework was developed (OECD 2016). AOPs capture the complex mechanistic basis of toxicity in linear modules of causally linked biological events spanning multiple levels of biological organization, from molecular, cellular, tissue and organ levels to individuals and whole populations (Ankley et al. 2010; Vinken 2013). AOPs connect an initial trigger of toxicity at a molecular level (molecular interactions with stressors, termed molecular initiating events; MIEs) to an apparent toxicity or adverse outcome (AO) associated with chemical exposure. AOPs are ‘chemical agnostic’ (i.e. AOPs can be triggered by various stressors) and describe dynamic processes of toxicity. AOs are measured at higher levels of biological organization such as human health (organ or organism) or environmental endpoints (organisms, populations or ecosystems) that are important for regulatory decision-making (Carusi et al. 2018). The individual components of the AOP include a MIE, a series of key events (KEs), which are measurable biological changes that occur between a MIE and its eventual AO, linked by the key event relationships (KERs; Halappanavar, Ede, et al. 2019).

Several efforts worldwide have been promoting the development of AOPs as next-generation tools for knowledge transfer across the research community, guiding risk assessment strategies, and regulatory decision-making (Pollesch, Villeneuve, and O'Brien 2019; Knapen et al. 2018; Villeneuve et al. 2018a). From a risk assessment and regulatory perspective (*e.g.* grouping, categorization and readacross), AOPs provide a structured framework to develop, assess and use data generated from alternative testing strategies (e.g. *in chemico*, *in silico*, *in vitro*, *ex vivo* and systems biology) as part of an integrated approach to testing and assessment (IATA) (Delrue et al. 2016).

The underlying mechanisms of toxicity induced by MNs may be similar to those for larger particulate materials. However, MN-induced toxicity could exhibit differences in severity compared to their microscale particle counterparts due to size-associated changes in the physicochemical and structural properties of MNs. Evaluating the evidence available for utilizing or further developing AOPs, the focus of this paper, is relevant for application of AOPs in occupational and environmental health risk assessment. Particle size is also associated with particle dissolution rate or their distribution kinetics, which in turn both play a role in distribution of particulate and chemical substances in the biological system and the potential toxicity. Detection and quantification of MNs in biological tissues has been challenging, which hampers assessment of particle doses associated with toxicity (Johnston et al. 2013). It is important to note that conventional toxicity testing is mostly based on uniform distribution of a test substance in the exposure vehicle, and in cells and tissues post-exposure, because of which conventional toxicity testing may not be applicable to assessing MN-induced toxicity (Drasler et al. 2017). In addition, the unique surface properties of MNs govern their interaction with biomolecules and cells and can result in cellular uptake and internalization, a critical biological event or MIE for MN-induced tissue responses, but which is not captured in AOPs for chemicals. The application of AOPs in the field of nanotoxicology was evaluated by Gerloff et al. (2017), focusing specifically on MN-induced liver toxicity. The authors found that mechanistic knowledge from chemically induced toxicity and captured in AOPs is generally relevant for MNs; however, key differences between chemicals and MNs include consideration of toxicokinetics and the nature of the initial interaction of MNs with biological systems (i.e. the MIE). These differences between chemicals and MNs need to be accounted for and evaluated to ensure the applicability of AOPs for MN risk assessment.

In a recent review by Halappanavar et al. (2020), the authors stated that for most MN-induced toxicity, MIEs constitute MN interaction with cells and biomolecules in the surrounding microenvironment (e.g. lung fluid or serum *in vivo*, cell culture medium *in vitro*) and thus, may not always represent molecular level interactions. For now, MN-related MIEs are nonspecific and insufficiently understood. Moreover, how these interactions are governed by the individual properties of MNs is not completely known. Thus, AOPs developed for chemicals may require further modification, or new AOPs of relevance to MNs that incorporate MN property-specific deviations in toxicity, may be needed (Ede et al. 2020; Halappanavar et al. 2020). Many groups are working to develop and validate AOPs for MN risk assessment and a roadmap for development of AOPs for MN risk assessment has recently been published (Ede et al. 2020).

The work presented here builds on the previous efforts of Halappanavar, Ede, et al. (2019) and summarizes the outcomes of a recently completed multi-stakeholder project, the primary objective of which was to develop a methodology to extract, curate and apply the existing nanotoxicology literature in support of advancing the future development of MN-relevant AOPs. Halappanavar, Ede, et al. (2019) outlined (i) a systematic process for mining the nanotoxicology literature to identify potential KEs relevant for MNs and (ii) a strategy to prioritize potential KEs for development. The study also selected 'tissue injury' as an appropriate KE of relevance to MN-induced AOs, for further development. In the present follow-up study, using a case study approach, we examine the available evidence in the literature for assessing the (i) biological plausibility, (ii) measurability, and (iii) regulatory relevance of the KE 'tissue injury' following exposure to MNs and its applicability for future development of AOPs. These criteria align with the evolved Bradford Hill criteria described by Becker et al. (2017). The various challenges concerning the utility of the available nanotoxicology literature are discussed and preliminary insights and guidance are provided on standard reporting of nanotoxicity study results of relevance to AOP development. In addition, a second case study is presented that demonstrates the utility of the Nano-AOP database, the primary outcome of the project, to provide additional weight of evidence for identified KEs and MN-induced AOs and to build KERs in support of future development of AOPs.

2. Material and methods

2.1. Strategy for assessing tissue injury

The results of the Swiss-VCI database (*in vivo* and *in vitro* studies) evaluation conducted in Halappanavar, Ede, et al. (2019), which mainly targeted tissue inflammation and associated events, revealed that events of inflammation, oxidative stress and cytotoxicity were the most commonly assessed and reported in the database, that had a direct inference to tissue damage and injury. The evaluation of the relationships shared between the three main reported events —inflammation, oxidative stress and cytotoxicity—showed that they are interconnected and play a prominent role in tissue injury in general and also in inflammation-mediated injury. Thus, it was inferred that these three frequently assessed and reported biological events can be used as upstream KEs to the 'tissue injury' KE (Figure 1), which would also allow identification of its downstream effector KEs, as well as the various methods and assays for their measurement using *in vivo* and *in vitro* models. Furthermore, each upstream KE (i.e. inflammation, oxidative stress and cytotoxicity) is represented by distinct associative events. For example, the upstream KE inflammation is represented by 'tissue resident cell activation', 'leukocyte recruitment/activation' and 'increased pro-inflammatory mediators', which are referred to here as hub-KEs of inflammation. Similarly, the oxidative stress KE is represented in the database by the hub-KEs of 'increased reactive oxygen (or nitrogen) species (ROS) synthesis', 'imbalanced oxidant and anti-oxidant levels' and 'modification of biomolecules'. Cytotoxicity KE is represented by the hub-KEs 'altered membrane integrity' and 'cytotoxicity'. Positioning the upstream and associated hub-KEs in an AOP framework shows that they occur in a causal sequence from exposure to an adverse event, but also in parallel, including functioning in a feedback loop (Figure 1).

2.2. Literature review and Nano-AOP database development

The review of literature and establishment of the database was conducted in two phases, as shown schematically in Supplemental Figure S1. The details of the phase-1 literature review strategies and resulting Swiss-VCI database are described in Halappanavar, Ede, et al. 2019. The Swiss-VCI database (Krug 2014) was used for identifying MN-relevant KEs and prioritizing a tissue injury KE for the case study. The preliminary Swiss-VCI database consisted of publications from 2000 to 2013 (a total of ~11 000 studies), which were evaluated following the 'Preferred Reporting Items for Systematic Reviews and Meta-Analyses' (PRISMA) scheme (Liberati et al. 2009). The Swiss-VCI database was filtered to identify studies specifically reporting on 'inflammation', which were then evaluated for quality with the scheme developed within the DaNa project [www.nanoobjects.info]; however, the quality criteria proposed in the DaNa project were not applied stringently as is it was found that it would result in an insufficient number of records for any type of downstream analysis (Halappanavar, Ede, et al. 2019).

In phase-2, the original Swiss-VCI database was updated within the context of the NanoCommons research infrastructure project with studies published from 2014 to 2017 and reorganized to suit the needs of the tissue injury case study presented here. Specifically, literature was extracted from the Swiss-VCI database and updated for a select set of seven MNs (Silver, Cerium Oxide, Copper Oxide, Multi-walled Carbon Nanotubes, Single-walled Carbon Nanotubes, Titanium Dioxide, Zinc Oxide) and a modified database called the 'Nano-AOP database' was created. These seven MNs were chosen as they are widely researched and represent a range of MN properties including differences in chemical composition, solubility and shape. The literature search was limited to the key term 'inflammation' and one of the seven MNs. Addition of the search term 'tissue injury' resulted in many irrelevant results and thus was not used in the review strategy. Only the Web of Science (WoS) portal was used for the systematic review, as Scopus and Pubmed resulted in fewer results with the search query used.

The WoS's basic search query tab included the 'topic' field, with a customized 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 (Supplemental Information; Appendix 1; Box S1). By this search mechanism, ~1294 new publications were identified for the seven MNs, of which only 136 publications satisfied the inclusion/exclusion criteria (Supplemental Information; Appendix 1; Box S1). 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 (details in Supplemental Information; Appendix 1).

A two-step quality assessment process (Supplemental Information; Appendix 1) was applied to the 136 publications. The first step specifically addressed whether a particular publication sufficiently completed and reported a set of minimum MN characterization data. Publications that did not provide sufficient material characterization data were always rejected. The second step addressed the experimental design (e.g. animals, cell lines, dose/concentrations, route of exposure, inclusion of appropriate controls, etc.), as per the criteria provided in the Supplemental Information (Supplemental Appendix 1). Finally, a total of 124 publications were included in the 'Nano-AOP database' (Supplemental Figure S1). If an individual publication evaluated more than one type of MN, data was separated by MN type and each was considered as a separate 'study'. Thus, the final 'Nano-AOP database', reporting specifically on the seven selected MNs, consisted of publications from the original Swiss-VCI database (119 publications) and new publications from the phase-2 literature review (124 publications), leading to a grand total of 243 publications and 294 studies.

The developed NanoAOP database is aligned with the FAIR (Findable, Accessible, Interoperable, and Reusable) guiding principles. A manuscript detailing its development and curation is currently in development and the database will be accessible as part of the KnowledgeBase (https://ssl.biomax.de/nanocommons/) being developed under the NanoCommons project via which it will be provided with a unique Digital Object Identifier. The ontologies developed under the NanoCommons project are applied consistently in the NanoAOP database to make it interoperable with the additional datasets developed within that project. Recently, the FAIRness of datasets included in the NanoCommons database has been evaluated, and the majority of the criteria defined by the FAIR maturity indicators were met; use of standard schema for metadata was suggested to further increase the FAIRness of the data (Ammar et al. 2020).

2.3. Database organization for gathering evidence in support of the KE tissue injury

The Nano-AOP database was organized by grouping the reported biological endpoints from individual studies under the three main upstream KEs identified for tissue injury in Halappanavar, Ede, et al. (2019): inflammation, oxidative stress, and cytotoxicity (Figure 1). The KE inflammation is represented in this evaluation by two of the three individual hub-KEs (Figure 1) of inflammation: 'increased pro-inflammatory mediators' and 'leukocyte recruitment/activation' in the database, as recommended by EAGMST (Villeneuve et al. 2018b), and individual endpoints assessing the hub-KEs were sorted in the database accordingly (Table 1). This breakdown of the upstream KEs provided clarity on the specific endpoints assessed, the actual measurements reported in the literature, and the relevance of the measurements to the final KE of interest, i.e. tissue injury. Since histology is one of the *in vivo* endpoints of relevance to health risk and regulatory assessments, and since several studies in the database reported histological findings (i.e. histopathology, fibrosis, granuloma formation) *in vivo* and related endpoints in *in vitro* models (e.g. cell morphology), histology was added as a separate endpoint for consideration in the analysis. Histology is one of the measures of pulmonary fibrosis, which can be an AO of tissue injury.

2.4. Analyses of the Nano-AOP database

The Nano-AOP database, consisting of both *in vivo* and *in vitro* studies, was analyzed to gather experimental support for the KE plausibility, measurability, and regulatory relevance; the same three criteria applied to select the tissue injury KE as a case study (Halappanavar, Ede, et al. 2019). For the analysis of 'regulatory relevance', both *in vivo* and *in vitro* studies were used to evaluate no-observed effect levels (NOELs) or or lowest-observed effect levels (LOEL); however, only *in vivo* studies were employed to examine benchmark dose (BMD) estimates. Further details of these analyses are provided below. A full list of literature available in the NanoAOP database is provided in Supplemental Information, Appendix 3.

2.4.1. KE plausibility—For each MN, the analyses examined the number of studies that: (i) reported (*i.e.* measured) each of the three upstream KEs and the histology endpoint; and (ii) found significant induction of each upstream KE following MN exposure at any of the concentrations tested in the study. For example, the upstream KE cytotoxicity was considered to be reported if at least one endpoint (*e.g.* altered membrane integrity, cell death, caspases, mitochondrial membrane potential, cell growth/colony formation, survival; Table 1) was included in the experimental design for assessment as reflective of one or both hub-KEs of cytotoxicity. An upstream KE was considered to be induced following MN exposure if at least one measured endpoint assessing any one hub-KE showed a statistically significant difference (as determined in the experiment) in exposed groups in comparison to the 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 measured and the number of studies reporting each upstream KE was noted. Similarly, in a separate analysis, all studies were reviewed and each upstream KE assigned a value of 1 if the study found significant induction of that KE after MN exposure. This analysis was used to determine the number of studies that (i) reported and (ii) found significant induction with MN exposure of 0, 1, 2, or 3 upstream KEs and/or histology within a single experimental design.

2.4.2. KE measurability—Following a similar approach as described above, the analysis quantified the number of studies that (i) reported and (ii) found significant induction with MN exposure of each individual endpoint (e.g. TNF, IL-1, IL-6, IFN, IL8/CINC, NF-kB, Cytokines (other), Inflammasome), under each upstream KE (*e.g.* inflammation) (Table 1). The quantified number of studies was then converted to a percentage of studies measuring or finding induction, to enable comparisons between each type of MN. Individual assays used to measure an endpoint and thus an upstream KE were identified.

2.4.3. Regulatory relevance of the KE—The relevance of the tissue injury KE for regulatory decision-making was assessed in part by evaluating the availability of dose-response data for endpoints within the upstream KEs. Two types of quantitative dose estimates were evaluated using the Nano-AOP database: (1) BMD estimates, and (2) NOEL or LOEL estimates. These point estimates of dose are typically used as points of departure in quantitative risk assessments (depending on the response endpoints) and could also be used as estimates of hazard potency in grouping or ranking of MNs. Studies reporting AOs of relevance to humans and conducted according to the OECD study guidelines (e.g.

subchronic inhalation studies) are more typically used in quantitative risk assessment for exposure limit derivation, although establishment of KERs and AOPs could be useful in the development of predictive models based on earlier (upstream) biological responses.

2.4.3.1 BMD estimates.: Sufficient data reported in the publications were required for dose-response modeling. This information includes at least two dose groups in addition to controls, quantitative response endpoints of interest, and sufficient summary statistics (mean or proportion, error, group size).

Endpoints selected as being relevant to occupational health risk assessment and used here for BMD estimation include *in vivo* pulmonary inflammation and pulmonary fibrosis. Pulmonary neutrophilic inflammation is measured as an increase in the percentage of polymorphonuclear leukocytes (PMNs) in the bronchioloalveolar lavage fluid (BALF) in rodents at 1–3 days post-exposure. The percentage of PMNs (of the total cells recovered in BALF) was either reported in the paper or calculated from the cell differential data reported. The benchmark response (BMR) for modeling was a 4% increase in PMN above the control mean response, which has been considered to be biologically relevant in rodents and humans and used in previous analyses (e.g. Drew et al. 2017, NIOSH 2011).

In addition, pulmonary fibrosis is a recognized downstream consequence of tissue injury and an adverse health effect in workers exposed to airborne respirable particles (e.g. coal dust, silica). It has been used as a response endpoint in quantitative risk assessments of MNs, e.g. CNTs (NIOSH 2013). Pulmonary fibrosis was reported quantitatively as the amount of collagen or hydroxyproline in lung tissue or as histopathology severity scores (e.g. proportion of animals with fibrosis per dose group). A biologically-based adverse level of collagen or hydroxyproline was not identified, so the BMR was defined statistically as the level at 1.1 standard deviation above the control mean response (Crump 1995).

2.4.3.2. NOELs and LOELs.: NOEL and LOEL estimates were recorded during database development for the various biological response endpoints within the upstream KEs (Section 2.3). NOELs and LOELs were either reported in the journal articles or determined from the statistical significance of the responses associated with the doses. If a NOEL was not identified for a particular endpoint, the highest dose in the study was estimated as the NOEL for that endpoint. LOELs in this database were recorded as being less than the lowest dose, indicating that LOELs were not reported in those studies.

Within the upstream KEs, ‘Inflammation’ and ‘Cytotoxicity’ were selected as the first KE and last KE preceding the adverse outcome of tissue injury (Figure 1). The following measurements and associated NOELs or LOELs were selected for investigation: *Inflammation*—cytokines (Altered levels of pro-inflammatory mediators) or cell number (Leukocyte recruitment/activation); and *Cytotoxicity*—cell death and altered membrane integrity. The measurement selected for the tissue injury outcome was Histology—fibrosis. Specific cytokine information (IL-1, IL-6, IL-8, TNFα, IFNγ, etc.) was recorded only in the updated database (i.e. papers published between 2014 and 2017). In the original database, all types of cytokines were combined and reported as NOELs or LOELs (with additional information included in comments). Cell number represented either increased leukocyte

number or total cell number in BALF, in the updated or original database, respectively. Fibrosis was reported based on findings from histopathology examination or biochemical measures (collagen or hydroxyproline) in lung tissue.

Of the studies reporting the selected endpoints, the data were divided into *in vivo* and *in vitro* experiments, which were further stratified by: (1) rat or mouse model if *in vivo*; and (2) rat, mouse, or human model if *in vitro*. The rat and mouse *in vivo* datasets were further stratified based on route of exposure: (1) inhalation; or (2) administered dose (intratracheal instillation or pharyngeal aspiration).

2.5. Application of Nano-AOP database to support development of other KEs of relevance to different routes of exposure and other AOs induced by MNs

A second case study was conducted to evaluate the use of the Nano-AOP database to provide additional weight of evidence to the already identified KEs, MN-induced AOs or to build KERs for known KEs connecting particle exposure to an AO. The details of the case study are provided in Supplemental Information, Appendix 2. In brief, the database was searched for cellular events such as endoplasmic reticulum (ER) stress, ROS synthesis, NLRP3 activation/caspase increase, and changes in IL-1/TNF levels. These events represent a series of KEs putatively linking chronic exposures to titanium dioxide (TiO_2) MNs with type-2 diabetes (T2D). TiO_2 MN is one of the most widely assessed of the 7 MNs in the Nano-AOP database (Figure 2). The presence of TiO_2 MNs was observed in the pancreas of eight individual type-2 diabetic (T2D) patients (4 with and 4 without pancreatitis), while TiO_2 NPs were not detected in the non-T2D controls (Heller, Jarvis, and Coffman 2018). Several studies (for details, refer to Supplemental Appendix 2) have shown that the potential mechanisms of T2D following exposure to particles involve the KEs mentioned above. It should be noted that the study aimed to gather weight of evidence for these KEs, not for TiO_2 NP-induced T2D which is still a hypothesized pathway.

The Nano-AOP database was searched using the strategy described in Supplemental Appendix 2. The individual studies in the database were analyzed after filtering for 'TiO_2' MN, and individual entries in the database were checked for the cellular events/KEs 'ER stress', 'ROS', 'inflammasome', 'caspase', 'IL-1/TNF'. Out of 180 experiments with TiO_2 MN, 77 were found for 'Titanium dioxide' (any size/shape/coating, *in vivo* and *in vitro* – various cell lines and doses) that measured 'ROS' (53) or 'inflammasome' (20) or 'caspase-1' (4), and were used for further analysis (it was found that the KE of ER stress was not measured in any of the studies in the database). Eight experiments (3 publications) measured 'ROS', 'IL-1' and 'TNF-α'. Sixteen experiments (4 publications) measured 'ROS' and 'TNF-α'. Nineteen experiments (6 publications) measured 'inflammasome' and 'IL-1'. No experiment measured 'inflammasome', IL-1' and 'NF-κB'. In all, the search resulted in 46 experiments belonging to 11 publications that were evaluated separately for weight of evidence.

3. Results

3.1. The updated Nano-AOP database

The original Swiss-VCI database consisted of data from 191 peer-reviewed journal articles published in the period 2009–2013 addressing inflammation, which were reduced to 119 following additional analysis to determine their suitability for tissue injury assessment. From the additional literature search (2014–2017) and further iterations regarding inclusion criteria, a total of 124 new peer-reviewed publications were used for the analysis of plausibility and measurability of KEs covering seven selected MN types. The Nano-AOP database is highly enriched with studies reporting on nano TiO_2 (71 studies), MWCNT (62 studies), silver (55 studies), zinc oxide (41 studies), SWCNT (25 studies), cerium oxide (24 studies), and copper oxide (16 studies) (Figure 2).

3.2. Tissue injury KE plausibility

A total of 41, 119 and 107 studies assessed 1, 2 or 3 of the upstream KEs, respectively. Five studies did not assess any of the upstream KEs for tissue injury. Only 22 studies assessed all three upstream KEs in addition to the histology endpoint (Figure 3), although in total 116 papers reported some histology.

Inflammation was the most commonly measured upstream KE and the most commonly induced. Almost all the studies that assessed inflammation (Figure 4) reported induction, where induction was defined as any statistically significant change in at least one endpoint (*e.g.* leukocytes recruitment) following MN exposure for at least one tested concentration, relative to the unexposed control. As summarized in Figure 4, there were a total of 259 studies that assessed inflammation and 240 of these found significant induction with MN exposure. The second most commonly reported and induced upstream KE for tissue injury was cytotoxicity (Figure 4); a total of 192 studies assessed at least one cytotoxicity endpoint (see Table 1 for list of endpoints), of which 159 found significant induction following MN exposure compared to controls. Ninety three of the 120 studies that examined the upstream KE oxidative stress reported it as being induced. It is important to note that the analysis did not discriminate between the number and types of endpoints used in making the ‘induced’ call. Finally, of the 116 studies that reported on histology 92 found significant changes with MN exposure compared to untreated controls.

3.3. Tissue injury KE measurability

The measurability of the tissue injury KE was assessed by analyzing the number of studies that evaluated specific endpoints under each upstream KE and the biomarkers/assays used to assess and measure them.

Review of the Nano-AOP database identified the various endpoints and biomarkers/assays used to measure the upstream KEs. Studies generally reported on at least one of the upstream KEs; however, the specific endpoint and biomarker/assays varied widely between studies. The upstream KE inflammation was measured *in vitro* mainly by assessing the hub-KE altered levels of pro-inflammatory mediators; however, the number and type of mediators assessed varied across the studies.

The upstream KE cytotoxicity was measured using a wide variety of assays targeting different hub-KEs (Table 1); the majority of these used assays that are indicative of altered membrane integrity. Other studies assessed cytotoxicity through assays measuring cell death, caspases, mitochondrial membrane potential, cell growth/colony formation, or survival. In addition, some studies documented histological changes (e.g. altered morphology) to support the occurrence of cytotoxicity.

Different endpoints were used to assess the upstream KE oxidative stress. Oxidative stress was measured by multiple endpoints under its three hub-KEs and 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. Table 1 shows the different endpoints used to evaluate the three upstream KEs and histology endpoint, broken out by their respective hub-KEs.

3.3.1. Types of endpoints assessed for each upstream KE

3.3.1.1. Inflammation Upstream KE.: Analysis by the type of endpoints used for each individual upstream KE revealed that for inflammation, from the individually reported cytokines, TNF-α, IL-1, and IL-6 were the most frequently measured (34, 33 and 29% of studies, respectively) and induced (21, 19 and 18% of studies, respectively) following MN exposure. Figure 5 presents results of this analysis, demonstrating the percentage of studies measuring and finding induction for each endpoint under the upstream KE inflammation, for each of the seven classes of MNs.

Leukocyte recruitment/activation was also a commonly assessed endpoint, with 41% of examined studies measuring it, and 39% finding significant recruitment following MN exposure. Inflammasome activation was far less reported in the selected literature as an indicator of inflammation, with just 7% of studies examining it. From this overview analysis, which did not take into account dose, MN physicochemical properties, or experimental factors, it was not possible to deduce any MN or MN property-specific trends (Figure 5). Rather, the purpose of this analysis was to determine the amount of information available in the literature—and as represented in the Nano AOP database—for further evaluation of a tissue injury KE for MNs.

3.3.1.2. Oxidative stress Upstream KE.: The most assessed oxidative stress endpoint in the MN literature was total ROS, with 21% of studies examining it and 16% finding induction. Other examined endpoints include: RNS (9% of studies measured; 8% found induction), oxidation products (11% measured, 8% found induction), GSH/GSSH (9% measured, 7% found induction) and antioxidant gene expression (Anti-Ox; 4% measured, 3% found induction). Less commonly reported endpoints were activation of HO-1 and NRF2 signaling pathways, altered expression of iNOS; synthesis of mitochondrial ROS, and DNA oxidation (Figure 6).

3.3.1.3. Cytotoxicity Upstream KE.: For the upstream KE cytotoxicity, cell death was the most predominantly assessed endpoint, with 44% of studies examining it, and 35% reporting significant cell death following MN exposure. Altered membrane integrity was also frequently assessed, with 35% of studies examining it, and 29% reporting

compromised membrane integrity following MN treatment, compared to controls (Figure 7). The endpoints caspases, mitochondrial membrane potential, cell growth/colony formation and survival were less commonly assessed, with a total of 9%, 6%, 2%, and 0% of studies examining them, respectively.

3.3.1.4. Histology endpoint.: For histology, cell morphology was the most commonly reported endpoint *in vitro*, with 19% of studies examining it, and 13% reporting significant changes in morphology after MN exposure. Tissue histology was the most commonly reported endpoint, and provided a direct measure of tissue injury. Damage to tissue structure was frequently reported; 37% of studies examined this endpoint, with 28% reporting significant change in tissue morphology following MN exposure.

3.4. Regulatory relevance and application of database to quantitative analysis

The regulatory relevance and application were assessed, in part, by evaluating whether data could be used to derive potency estimates for grouping/ranking or points of departure for quantitative risk assessment.

3.4.1. BMD estimates—In an evaluation of the Nano-AOP database, 39 *in vivo* studies were selected as reporting sufficient dose-response data to perform BMD modeling for the endpoint 'recruitment of leukocytes,' a hub-KE of inflammation (measured as %PMNs in BALF). BMDs could be estimated for 45 experiments from 20 of those studies. In addition, 19 studies were selected as reporting quantitative data for the AO of pulmonary fibrosis (measured as amount of collagen or hydroxyproline or as histopathology severity score); these studies are being evaluated further. An experiment refers here to any treatment group that differs from another treatment group in any aspect (e.g. material/modification, species/strain/sex, exposure time/post-exposure time). In addition, an experiment consists of the unique dose-response data for a given MN under specific experimental conditions; i.e. one BMD estimate is obtained per experiment.

For most studies, BMDs could not be estimated because of incomplete or insufficient data for dose-response modeling (including only one exposure group; no standard deviation reported; or data reported as fold of control). Also, some data did not show a dose-response trend and a model could not be fit to the data, or the BMD estimate required extrapolation beyond the highest dose in the experiment. Fitting models to these data required several adjustments/assumptions, including relaxing the goodness of fit criteria from $p > 0.05$ to $p > 0.1$, removing the highest dose group from the model (if more than two dose groups in addition to control), or assuming a lognormal distribution (exponential model).

3.4.2. NOELs—Study-reported NOELs were ranked within strata by experiment (*in vitro* vs. *in vivo*); endpoint (cell number, cytokines, cell death, LDH, or fibrosis); and species (rat or mouse *in vivo*, and human, rat, or mouse cells *in vitro*). The NOELs from the *in vivo* studies were also stratified by route of exposure based on dose units (e.g. mg/kg body weight vs. mg/m^3). The number of NOELs per stratum ranged from 0 to 138 for *in vitro* experiments, and from 1 to 40 for *in vivo* experiments. LDH and cytokines were commonly reported endpoints in both *in vivo* and *in vitro* studies in the Nano-AOP database. Cell

death was typically reported only in the *in vitro* studies. Cell number (in BALF) and fibrosis are endpoints specific to *in vivo* studies. Further analyses of these results are underway, including normalizing dose across species and route/duration of exposure, which would allow pooling across some strata and increasing the number of NOELs for comparisons, and comparisons of results *in vitro* and *in vivo*.

Other differences among these experiments within each stratum include exposure duration and post-exposure duration differences. Within the Nano-AOP database, there are a few differences in the information provided in the phase-1 and phase-2 versions of the database (e.g. post-exposure duration was added in phase-2, and cell number variable was revised from neutrophils to total cells), which would be expected to contribute to heterogeneity among NOELs within strata. Additional stratification of experiments by organ (*in vivo*) and cell type (*in vitro*) may further reduce variability and provide a clearer picture of any patterns in the data for selected KE endpoints.

3.5. Application of Nano-AOP database for identifying other KEs of relevance to different routes of exposure and other AOs induced by MN

The second case study search resulted in a total of 46 individual experiments belonging to 11 publications (details in Supplemental information) which were evaluated separately for weight of evidence. The analysis provided evidence for activation of KEs NLRP3- and caspase-1 activation, ROS, IL1-β activation and TNF-α release and the data supported the causal relationship between the KEs. The effects were also found to be TiO_2 NP size dependent (for more details, refer to Supplemental Appendix 2). This analysis demonstrates that the Nano-AOP database can be used to gather preliminary evidence in support of KEs or AOs of particular interest.

4. Discussion

4.1. Limitations of the nanotoxicology literature

Publicly available nanotoxicology literature is rich with more than 50 000 peer-reviewed publications to date covering a wide variety of MN classes and properties, which are easily accessible on PubMed (Krug, 2018). However, there are only a few that would actually pass a rigorous quality assessment for use in risk assessment. For example, in the original Swiss-VCI database, less than 20% of the 11 000 publications passed the stringent quality criteria applied (Halappanavar, Ede, et al. 2019; Krug et al. 2018; Kühnel et al. 2014). In another recent study, a targeted literature search for studies reporting on silica nanoparticle induced toxicity (publications between 2013 and 2019), resulted in less than 25% of the selected publications fulfilling the applied quality check criteria (Krug, personal communication). Thus, a major impediment for researchers attempting to develop AOPs or identify KEs of relevance to MN toxicity, is the lack of quality scientific information and the heterogeneity of endpoints reported. However, of the 136 papers from 2014 to 2017 that met the inclusion/exclusion criteria outlined in Supplemental Appendix 1, 126 publications (89%) passed the two-step quality assessment that ensures minimum physical-chemical and study design criteria are included and reported, suggesting that the quality of nanosafety literature/reporting is improving.

A majority of the studies in the Nano-AOP database did not include information relevant for dose-response assessment. As a result, in the present work, the results could only be reported as ‘assessed’ and ‘induced’ and does not include a dose component. A subset of studies was identified with adequate dose-response data for BMD modeling, and those data were obtained directly from the publications (Section 2.4.3.1). Furthermore, the current literature mainly captures the results from papers showing some degree of altered host response following MN exposure, largely excluding studies that show no response (negative or noeffect studies), which are important in establishing appropriate experimental controls and benchmark response levels but which are traditionally harder to publish. These limitations will make future quantitative AOP development difficult with currently available literature. Although quantitative AOP development and modeling is still in its infancy, the field is gaining momentum and several qAOPs have been proposed (Spinu et al. 2020). As future efforts aim to validate, harmonize and gain regulatory acceptance of quantitative AOP models, guidance should be provided on the information that should be reported in the nanotoxicity literature to support their development; some initial recommendations are included in Section 4.4, and a proposal for community driven metadata standards for nanosafety has been proposed recently (Papadiamantis et al. 2020).

Regardless of the deficiencies, the Nano-AOP and Swiss-VCI databases provide a preliminary but rich platform to build upon. Although the present work focuses on seven specific MN types, inflammation and associated events, and mainly the pulmonary system as the exposure target, which is the most commonly reported in this database, the Nano-AOP and Swiss-VCI databases can be used to support preliminary investigations of KEs of relevance to other routes of exposure, MN type, or tissue type responses (Supplemental Appendix 2), and different types of KEs such as genotoxicity (for studies up to 2013 in the Swiss-VCI dataset) which can then be supplemented with more recent studies as demonstrated here.

4.2. Biological plausibility, measurability, and regulatory relevance of tissue injury as a KE

Tissue injury, which is defined as damage to tissues involving structural and/or functional changes, is a very common response observed and measured following exposure to MN. While tissue injury incited acutely after exposure to stressors results in the release of signaling molecules that activate tissue repair and regeneration processes, which includes an early inflammatory response, tissue injury that follows unresolved inflammation or failed tissue repair attempts can be detrimental to the organism. Tissue is a complex assembly of cells and associated extracellular matrix of the same origin that work together to carry out a specific function. Tissue injury or 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 and impairment leading to a

disease or an AO. Tissue injury precedes tissue dysfunction and plays a role in several adverse outcomes of regulatory relevance to MNs (*e.g.* fibrosis (AOP 173 2019), granuloma, mesothelioma, and emphysema in the lung (Nagai et al. 2011; Morimoto, Izumi, and Kuroda 2014; Halappanavar et al. 2020). Tissue injury, among other processes, plays an important role in diseases such as cancer by promoting clonal expansion.

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. Thus, the frequently reported biological events were believed to represent the upstream KEs of 'tissue injury'. As such, unresolved inflammation, oxidative stress and cytotoxicity induce injury to cells and tissues. Since each of these upstream events involves complex processes and multiple biological events, potentially at different levels of biological organization, they were broken down into hub-KEs. The identified upstream and associated hub-KEs reflect a change in the biological state 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 and hub-KEs are measurable and the same three upstream KEs are measured irrespective of the tissue or cell type in both *in vivo* and *in vitro* models. The three KEs can be measured in all tissue/cell types. 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 (Table 1). Tissue injury is observed following exposure to a variety of MNs of diverse properties. Thus, from the case study, it can be construed that *in vitro* cellular level assays could be used in developing predictive models of the occurrence of tissue injury, a tissue level effect *in vivo*.

4.3. Limitations of the case study

One of the important challenges for assessing the evidence in support of the tissue injury KE was the relatively limited number of specific endpoints that were assessed. For example, altered levels of pro-inflammatory mediators, an inflammatory hub-KE, is assessed by measuring the change in expression of single or multiple cytokines/chemokines. The number of mediators assessed and the specific types may depend on the experience of the individual researcher/laboratory and the resources available. The relative importance of any of these specific biological entities in the actual event, and the granularity with respect to how many entities, or which specific ones to be assessed in an assay, remains to be determined. 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. Lastly, although 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, it is acknowledged that the suggested upstream KEs and the associated hub-KEs may be too many 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 or provide specific guidance on experimental design for assessing tissue injury *in vitro* as indicative of an MN-induced adverse event *in vivo*.

This study also focused on seven MNs; as a result, MN properties such as sizes, surface coatings, even if part of the same study, were not evaluated separately with respect to the measurement and induction of an upstream KE. If a study used a MN doped with elements or a combination of two MNs (*e.g.* diesel exhaust particles and cerium oxide MNs), the data points specific to these property deviants and MNs other than the seven prioritized in the study were not included in the analyses. This impeded the ability to evaluate the influence of MN property on induction of tissue injury, an important piece of information for consideration in regulatory applications such as read across and grouping.

The other major limitations with respect to assessing the weight-of-evidence for an upstream KE was the heterogeneity in experimental designs, 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, 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 or different types of death (e.g. apoptosis and necrosis). 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 organized by assay type, to allow for better comparisons between MNs and their properties.

Lastly, the Swiss-VCI database was not specifically designed to assess tissue injury, the parameters collected in the database and the criteria for capturing this were not ideal for development of the qualitative or quantitative tissue injury KE. The NOEL and LOEL estimates, which were available from the information reported in this NanoAOP database, were limited in their utility for quantitative comparison of hazard potency across MN. In addition to the inherent uncertainty in NOEL or LOEL estimates compared to BMD estimates (Crump 2002; US EPA 2012), the experiments in the database without a reported NOEL or LOEL resulted in additional uncertainty about the values that were estimated here. For example, for the unreported LOELs, estimating the LOELs as the lowest experimental dose (which was statistically significant) may overestimate that value since the true LOEL may be lower and may represent a possible bias in a nonprotective direction. In

contrast, estimating the unreported NOELs as the highest experimental dose (which was not statistically significant) may underestimate the NOEL and represent a possible bias in an overly protective direction. These uncertainties would need to be taken into account in using the estimated NOEL and LOEL values in this database. In addition, the heterogeneity in experimental design resulted in relatively few studies within each stratum for comparison of NOELs or LOELs, and likely contributed to obscuring any patterns in the potency rankings across MNs. The same challenge of experimental heterogeneity hampers comparison of BMD estimates across these studies, as it would for other measures such as LD_{50} or EC_{50} from *in vivo* or *in vitro* experiments. Normalizing these values to better account for differences in experimental factors, or obtaining a larger set of comparable studies, would be needed for more reliable comparative potency analyses. A challenge of using the NOEL, LOEL, or BMD estimates to assess upstream KEs is that these point estimates may vary by assay or endpoint. Evidence-based criteria would be needed for selecting a given upstream KE as a point of departure for risk assessment or determining how these findings could be integrated into an overall assessment.

4.4. Recommendations

- Although the present case study largely employed manual curation, a combination of human intelligence with machine learning is required in the future to extract and organize relevant data for AOP building. The high-quality human-annotated datasets derived from past publications can aid in training machine learning algorithms for accurate identification of KEs and their relation to a specific AOP. This requires consultation and engagement of experts in the community from various disciplines including biochemistry, toxicology, medicine, materials science, physics, biology, *etc.*, to establish the KE ontologies.
- Study reporting templates (e.g. excel sheets) should be developed that allow for capturing the study details, which should include modules such as toxicology module (doses, duration of exposure, post-exposure time points), exposure module (mode and route of exposure, exposure system [submerged, air liquid interface, preparation of exposure material, etc.]), endpoints and assays module (specific endpoint, assay and included in the publication as Supplementary information, etc.). Reporting standards and guidelines must be developed for each of these modules (e.g. the ISA-TAB-Nano specifications: https://wiki.nci.nih.gov/display/ICR/ISA-TAB-Nano). Best practices for toxicity testing should be developed. Such detailed reporting would allow application of machine learning and text mining approaches to extract the data from the disparate literature into the agreed database structure, in a time effective manner.
- Most current approaches are based on text-mining algorithms, although, some software for digitizing plots in pdfs is available but would require thorough validation against the raw data for regulatory use. For a specific set of KEs that are already identified, the international research community can be called upon to report the data in a usable format (e.g. Supplemental Tables). Therefore, building on the lessons obtained here, once ontologies and *in vitro* assays have

been formalized and their reporting guidelines have been established, machine learning and text mining approaches will be able to scale-up the extraction of data from the disparate literature.

- Quantitative analyses are important to AOP development because of the inherent relationship between dose and response that is critical to the validation of an AOP. Evaluation of responses across materials depends on the dose as well as experimental factors. Thus, study reporting should enable collection of 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. This is important as 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 (Figure 1). It is also important to appreciate the fact that injury leading to permanent damage or functional dysfunction ensues only when endogenous defence mechanisms are compromised or are insufficient to repair the injury; although the repair process itself, e.g. if it results in scarring of lung tissue, can contribute to the adverse outcome of tissue injury.

Conclusions

One of the most critical caveats of the present day nanotoxicology is that the best practices and reporting standards for most measurements are not yet established. Despite the many limitations, this study demonstrated a systematic approach to reviewing the available nanotoxicology literature for identifying KEs of relevance to MNs, a process for establishing the KE ontologies, and a process for evaluating the weight of evidence using the available literature for a given KE leading to an AO. The study established a KE ‘tissue injury’ and showed how available literature with its limitations can be used to assess its biological plausibility. The exercise allowed identification of the gaps in the literature that pose impediments to developing KEs and potential AOPs in the future and opportunities for improvements. The Swiss-VCI and Nano-AOP databases are rich sources of information that are open to amendments and expansion to allow future KE or AOP development of relevance to specific route of exposure, tissue, or MN type.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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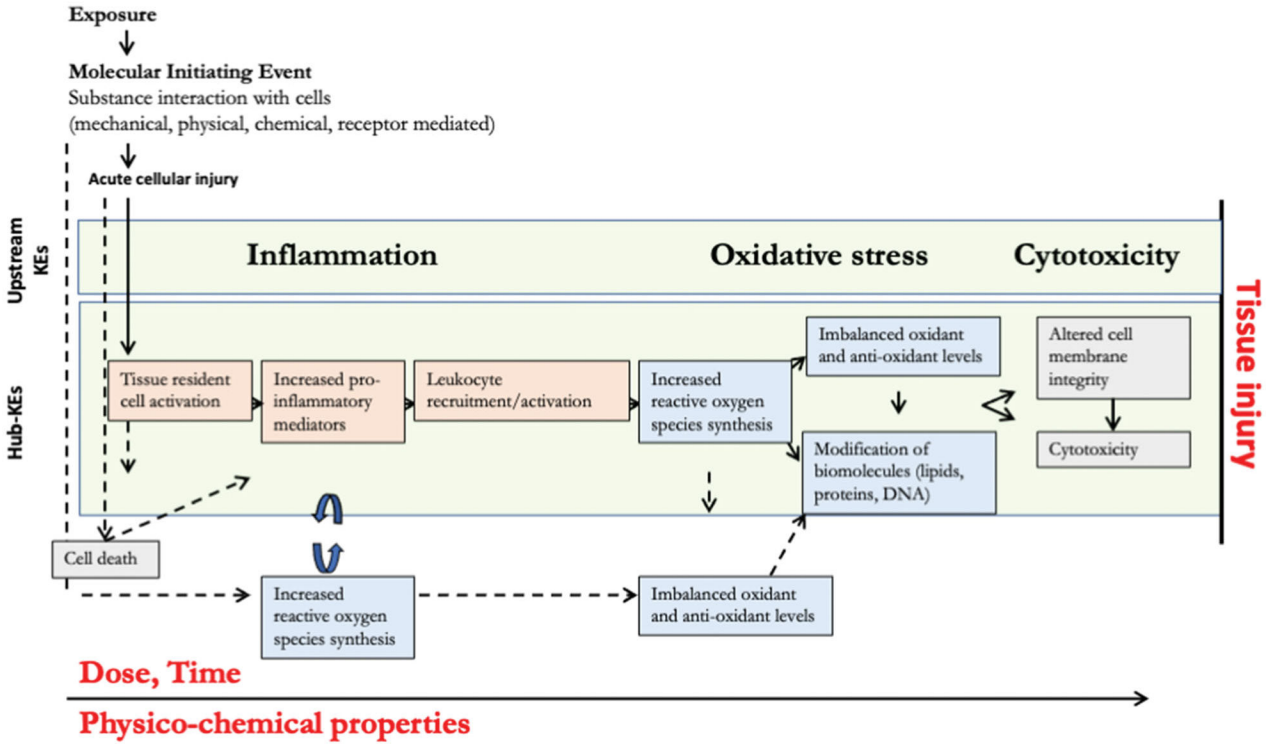

Figure 1.

Schematic depiction of the mechanism underlying tissue injury KE. Substance-induced tissue injury is an interplay between inflammation, oxidative stress and cytotoxicity events which are thus considered as upstream KEs. The individual upstream KEs presented here reflect both cellular and tissue level events and are represented by additional associative events in the database, which are referred to as hub-KEs. The dashed arrows describe parallel events and the solid arrows describe the main pathway to tissue injury. Cyclic arrows describe feedback loops. At the cellular level, injury inflicted by the acute interaction of irritants, pathogens and toxic materials with cells (Molecular Initiating Event) serves to induce signaling pathways that in turn, lead to activation of host defence mechanisms, including immune and pro-inflammatory responses. This initial injury is not intrinsically detrimental. The activated inflammatory process involves secretion of complement proteins, enzymes and cytokines and recruitment of pro-inflammatory cells to the injury site. The metabolic activity of pro-inflammatory cells results in ROS synthesis leading to exacerbation of cell injury and cell death. At the tissue level, uncontrolled cell injury results in extracellular matrix degradation, vascular damage and eventual tissue dysfunction. The surface reactivity (oxidative potential) of MNs can directly induce ROS synthesis, which in turn, can activate pro-inflammatory process leading to cell injury and cell death. Negative or inhibitory processes are not shown.

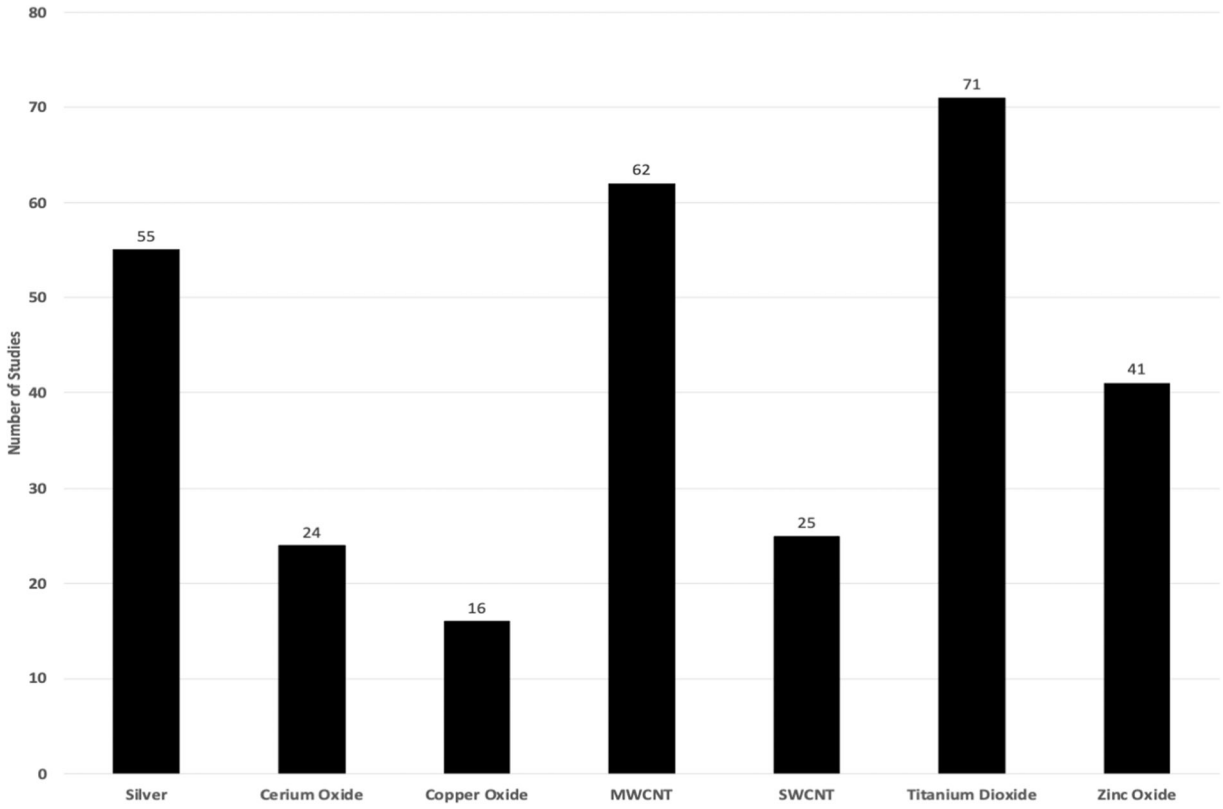

Figure 2.
Number of studies (*in vivo* and *in vitro*) analyzed for each MN in the Nano-AOP database.

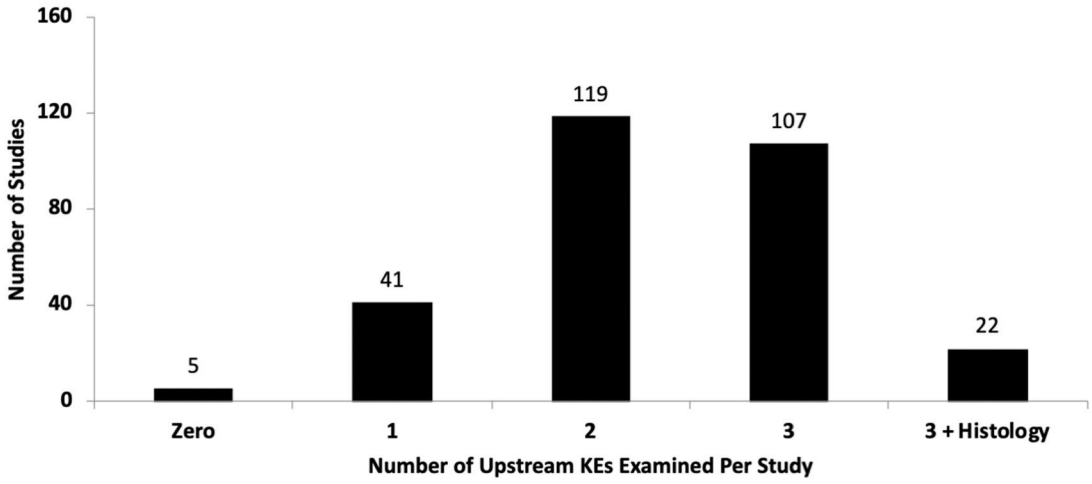

Figure 3.

Number of studies (*in vivo* and *in vitro*) in the database measuring 0, 1, 2 or 3 upstream KEs of tissue injury, or 3 upstream KEs plus histology.

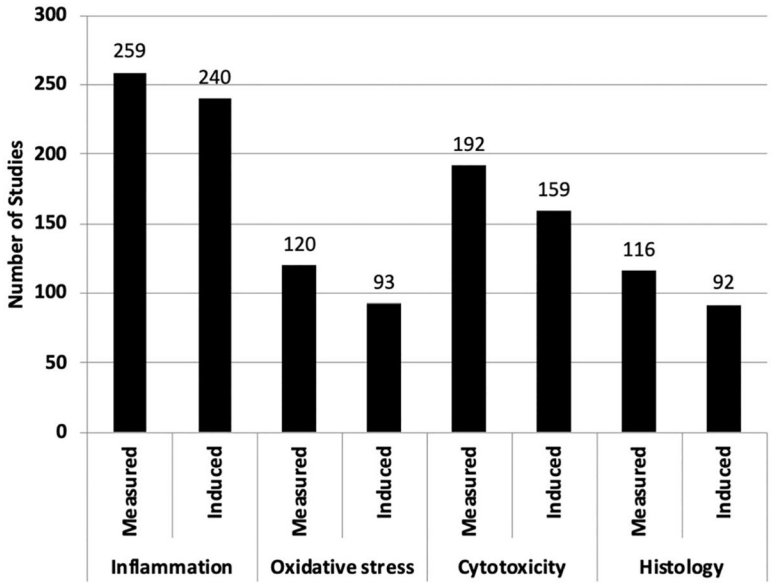

Figure 4.

Number of studies (*in vivo* and *in vitro*) that measured each individual upstream KE and number of studies that reported induction of a KE.

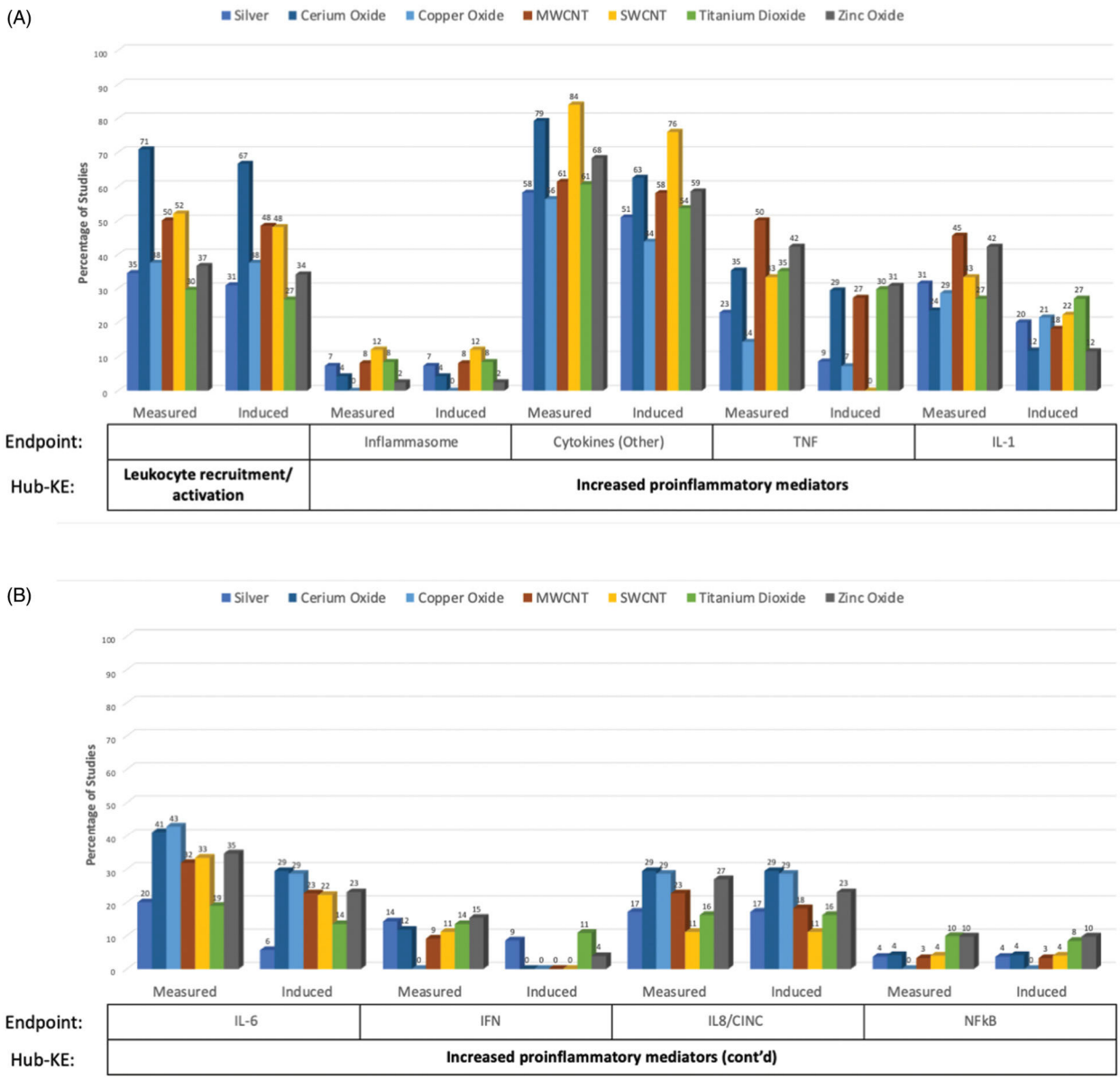

Figure 5.

Percentage of studies (*in vivo* and *in vitro*) in the Nano-AOP database that (i) measured and (ii) found significant induction of endpoints assessing the upstream KE inflammation for seven types of MNs. Endpoints for the inflammation hub-KE 'Leukocyte recruitment/ activation' are shown in A, while endpoints for the inflammation hub-KE 'increased proinflammatory mediators' are shown in A, B.

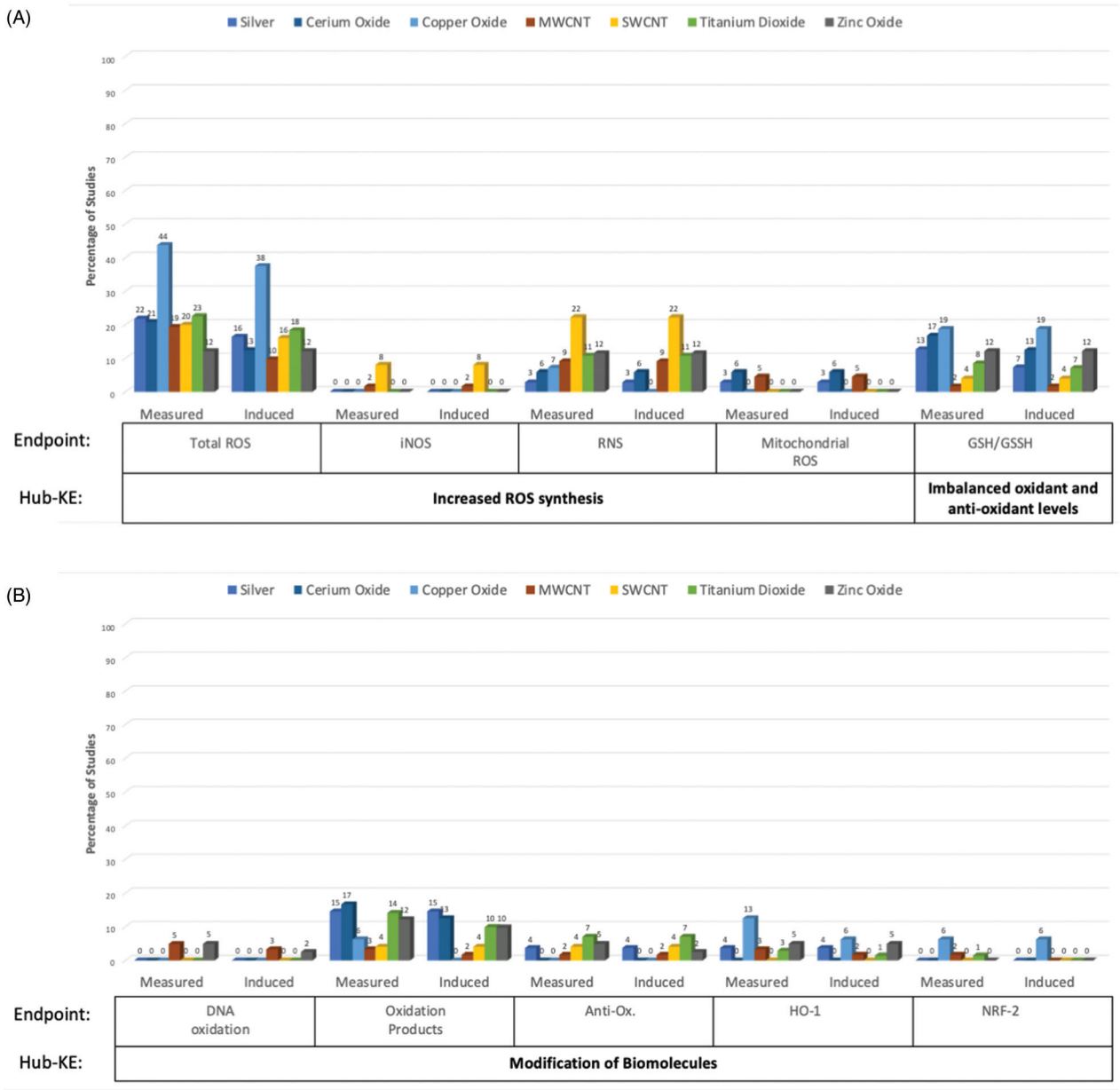

Figure 6.

Percentage of studies (*in vivo* and *in vitro*) in the Nano-AOP database that (i) measured and (ii) found significant induction of endpoints assessing the upstream KE oxidative stress for the seven types of MNs. Endpoints for the oxidative stress hub-KE 'increased ROS synthesis' and 'Imbalanced oxidant and anti-oxidant levels' are shown in A, while endpoints for the oxidative stress hub-KE 'Modification of biomolecules' are shown in B.

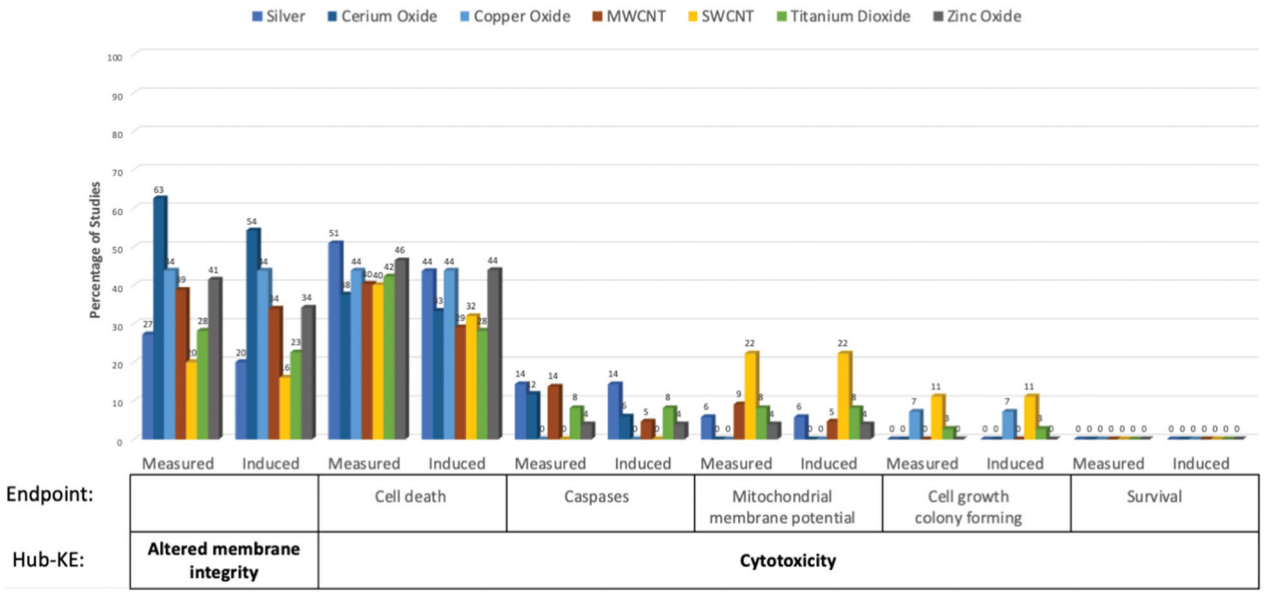

Figure 7.

Percentage of studies (*in vivo* and *in vitro*) in the Nano-AOP database that (i) measured and (ii) found significant induction of endpoints assessing the upstream KE cytotoxicity for the seven types of MNs. Endpoints for the cytotoxicity hub-KE 'Altered membrane integrity' and 'Cytotoxicity' are shown.

Table 1.

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

Inflammation[a]	**Oxidative Stress**[a]	**Cytotoxicity**[a]	**Histology**
Increased pro-inflammatory mediators [b]	*Modification of biomolecules (Lipids, Proteins, DNA)* [b]	*Altered membrane integrity* [b]	Cell morphology
TNF	HO-1	*Cytotoxicity* [b]	Tissue histology
IL-1	NRF-2	Cell death	
IL-6	DNA oxidation	Caspases	
IFN	Oxidation products	Mitochondrial membrane potential	
IL8/CINC	*Increased ROS Synthesis* [b]	Cell growth/colony forming	
NF-κB	Total ROS	Survival	
Cytokines (Other)	iNOS		
Inflammasome	RNS		
Leukocyte recruitment/ activation [b]	Mitochondrial ROS		
	Imbalanced oxidant and anti-oxidant levels [b] GSH/GSSH		

[a] Upstream KE

[b] Hub-KEs for each Upstream KE.