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Advancing Adverse Outcome Pathway (AOP) Development for Nanomaterial Risk Assessment and Categorization Part3: Workshop Report and Recommendations

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Advancing Adverse Outcome Pathway (AOP) Development for Nanomaterial Risk Assessment and Categorization Part3: Workshop Report and Recommendations

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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.

1 Introduction

3. Project partners of the Organisation for Economic Co-Operation and Development (OECD) Working Party on Manufactured Nanomaterials (WPMN) project, "Advancing Adverse Outcome Pathway Development for Nanomaterial Assessment and Categorisation (NanoAOP project)", hosted a workshop in collaboration with the European Union (EU) Horizon 2020 projects SmartNanoTox and Physiologically Anchored Tools for Realistic nanOmateriaL hazard aSsessment (PATROLS). This event took place on 11 September 2019 at the OECD Conference Centre in Boulogne-Billancourt, France. The main objective of the NanoAOP project is to advance the knowledge of the benefits and requirements of Adverse Outcome Pathways (AOPs) for manufactured nanomaterials (MNs) in the context of a risk assessment. The project outlines an approach to advance future development of AOPs using existing nanotoxicity literature, that includes the establishment of criteria for a quality evaluation of published literature, literature database development, and the identification of key events (KEs) reported to be induced following exposure to MNs that are of potential relevance to MN-induced adverse outcomes (AOs). Using a single KE case study on tissue injury, the project demonstrated how the developed literature database can be used to analyse biological plausibility, measurability and regulatory relevance of a KE and provide empirical evidence available in the literature to build a KE for future AOP development and use for MN regulatory decision making.

4. The workshop gathered OECD experts, including those from the Working Party on Manufactured Nanomaterials (WPMN), and from the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST), as well as experts from the SmartNanoTox and PATROLS projects, providing a unique set of expertise gathering policy makers, regulators, academia, industry and animal welfare organisations. Invited experts provided input on (1) the completed objectives and outcomes of the NanoAOP project to date, and (2) the status, use, and future needs for use of AOPs in the risk assessment of MNs. This document summarises the discussions, recommendations and outcomes derived from the workshop held on 11 September 2019.

2 Setting the Scene: Plenary Presentations

5. Plenary presentations provided workshop attendees with an overview of efforts to date by EAGMST, and the NanoAOP, SmartNanoTox and PATROLS projects. Dr. Magdalini Sachana (OECD Secretariat) presented an overview of efforts being led by EAGMST on the OECD AOP Development Programme, including approaches, guidance and tools currently available or in development. Drs. Sabina Halappanavar (Health Canada) and Jo Anne Shatkin (Vireo Advisors) provided an overview of the NanoAOP project, its objectives and outcomes. The presentations included results and conclusions from the literature evaluation, database development, identification and prioritisation of potential KEs, and the tissue injury case study. Limitations from the approach, and potential next steps for the NanoAOP project were discussed.

6. Attendees were given a background document prepared in advanced (See Annex, p.13) produced in collaboration with SmartNanoTox, outlining recommended actions to advance the development and adoption of MN-relevant AOPs. Feedback received from expert participants was used to update the general recommendations so they reflect the views of a diversity of stakeholders/attendees including experts, researchers, policymakers, regulators, risk assessors and industry representatives. A major outcome of this workshop was a number of recommendations to advance the development, application, and acceptance of the AOP framework and related testing tools for use in MN risk assessment and risk management decision making (see Annex). Each recommendation is broken out into in short-, medium- and long-term actions to achieve them. The summary is made available in Table 1.

Table.1. Recommendations to advance the development, application, and acceptance of the AOP framework and related testing tools for use in MN risk assessment and risk management decision making

Recommendation	Description
Advance MN-relevant and Advanced Material Considerations in AOP Development:	Success of the AOP framework requires continued development of the pathways that incorporate consideration of MN toxicity.
Utilise Existing Data from Literature and Previous Projects	Existing data already generated for MNs should be used to advance knowledge and identify opportunities for AOP development. This requires extensive expert-driven curation efforts.
Promote Reliable and Quantitative MN Data Development	Data development needs are essential to ensure MN-relevant AOPs can be developed and used in decision making. Guidance is needed on the types of data and reporting standards to enable AOP use in regulatory decision making. Efforts should be coordinated among stakeholders to ensure efficiency and limit additional testing.

Advance Knowledge of Quantitative Relationships Between MN Physical and Chemical Characteristics and AOP Elements	A better understanding of the quantitative relationships between MN physical and chemical characteristics and toxicological outcomes is required.
Identify Current Applications of the AOP Framework for MN Decision Making	Current applications of the AOP framework (e.g. prioritisation, grouping and read-across) can be adopted into decision making.
Establish Test Methods and Protocols Useful for MN Decision Making	Test methods to accurately measure MN-relevant molecular initiating events (MIEs) and KEs are required to advance use of AOPs as part of an IATA for MN decision making.
Demonstrate Predictive Capability of AOPs and In Vitro Test Methods	A coordinated effort is needed to ensure alternative testing strategies are predictive of AOs of regulatory relevance.
Guidance to Facilitate Adoption of MN-relevant AOPs for MN Decision Making	Efforts are needed to translate and incorporate advances in AOPs and related alternative testing strategies into regulatory decision making.
Stakeholder Communication & Engagement on Use of AOPs for MN Decision Making	To facilitate the development, adoption and use of the AOP framework for MN decision making, engagement of multiple stakeholders with a broad range of expertise is essential and coordination and cooperation are needed.

7. The second session presented outcomes from the SmartNanoTox project. Drs. Carole Seidel (French National Research and Safety Institute), Jorid Sorli (National Research Centre for the Working Environment; NRCWE) and Ulla Vogel (NRCWE) presented the AOPs for Frustrated phagocytosis-induced lung cancer (AOP 303), Lung surfactant function disruption leading to acute inhalation toxicity (AOP 302), and Secretion of inflammatory cytokines after cellular sensing of the stressor leading to plaque progression (AOP 237), developed under the SmartNanoTox project with direct relevance to MNs. Dr. Sabina Halappanavar (Health Canada) presented AOP 173 - Substance interaction with lung resident cell membrane components leading to lung fibrosis.

8. The final plenary session updated workshop participants on current efforts being led by PATROLS to develop alternative testing methods to support AOPs. Dr. Shareen Doak (Swansea University) gave an overview of the PATROLS project and the *in vitro* methods currently under development for human health hazard assessment including advanced lung, liver and gastrointestinal models. Dr. Tomasz Puzyn (QSAR Lab) gave an overview of his efforts to integrate knowledge from quantitative structure-activity relationships (QSARs) for MNs and AOPs to model and predict the roles of MN physical and chemical properties in inducing molecular initiating events (MIEs).

9. Three breakout sessions each addressed different charge questions to gather expert input on the results of the three projects to date and how they can be used to advance future AOP development and decision making for MN risk assessment. . The first discussion group was chaired by Sabina Halappanavar (Health Canada) and the rapporteur was James Ede (Vireo Advisors). The second break out group was chaired by Jo Anne Shatkin (Vireo Advisors) and the rapporteur was Claire Skentelbery (Nanotechnology Industries Association). The third group was chaired by Ulla Vogel (NRCWE) and the rapporteur was Deborah Ashby (Health Canada). The questions addressed by participants together with the main discussion points and recommendations are summarised below.

3 Discussions and Recommendations

Breakout Group 1. Facilitator: Sabina Halappanavar (Health Canada) Rapporteur: James Ede (Vireo Advisors)

- ***What is needed for future development of MN-relevant AOPs and supporting data?***

10. Experts discussed the limitations toward the use of available MN literature for AOP development including: uncertainty from different exposure conditions used between studies; different models used in each study (*e.g.* assays, cell lines, etc.); consideration of MN dispersion; the general lack of physical and chemical characterisation of MNs (especially with earlier studies); and others. Guidance is needed to use these data for risk assessment purposes, including future AOP development. The field would also benefit from guidance outlining the types of data that would be relevant for regulatory decision making to guide future testing and reporting, particularly for advanced materials which were not represented in the database.

11. Recommendation that the database developed in the NanoAOP project be used to revisit these limitations and investigate if it can advise ways to address the identified shortcomings of the literature.

12. Recommendation to extend and reorganise the NanoAOP database, including the addition of information pertinent to addressing the limitations above.

- ***How can the nanotoxicology literature data mining demonstrated in the OECD WPMN NanoAOP project be more efficient to facilitate its use for future AOP development?***

13. To make the database most useful, the database needs to be designed and populated with consideration of the specific questions being addressed. For example, in the context of future AOP development, the database structure should be updated to better capture potential MIEs. A formalised template should be developed and shared across various projects working on similar objectives (*e.g.* a template developed by the NanoCommons consortia[1] can be used to begin this effort).

14. The NanoAOP case study analysis would be improved by denoting *in vitro* versus *in vivo* data, and how those data factor into a weight-of-evidence analysis for supporting KE or AOP development.

- ***What other data are available that can be added to the NanoAOP database? Should the database be harmonised with developed reporting standards so that global data can be collected in one place as a future resource?***

15. Several additional resources were identified that could be added to the NanoAOP project database including the Registration, Evaluation, Authorisation, Restriction and Chemicals (REACH) dossiers, data from the NanoCommons project, as well as traditional chemical databases such as the U.S. EPA's ToxCast in the United States.

[1] See; https://www.epa.gov/chemical-research/exploring-toxcast-data-downloadable-data

16. It was recommended that ecotoxicological endpoints should also be included in the database. Some ecotoxicology models can be used to inform human health KEs (*e.g.* zebrafish models; Saleem *et al.*, 2018).

17. Harmonising the format for reporting data within the database is extremely complex and several projects are currently working to gain consensus. In the interim, making raw toxicology data publicly available was seen by experts as important for helping to move the entire field forward. For example, many journals have a mandatory requirement for making high content data such as "omics" data publicly available by depositing them in a public repository. Similar requirements for other apical endpoint data would help advance the development of a nanotoxicology database that can be used for several purposes including building AOPs.

▪ *What can be done to improve the adequacy of available data to better support quantitative AOP development? Can a harmonised experimental design involving the upstream KE measurements help?*

18. Experts agreed that a key first step was addressing the limitations of the current nanotoxicology literature, identified above.

19. Developing guidance on the assays/methods that should be used to assess different classes of MNs, how these studies should be conducted as well as reported was suggested. Participants emphasised the importance of dispersion and adequate physical and chemical characterisation as part of MN study design. Experts emphasised the need to ensure developed methods and assays are predictive of *in vivo* outcomes for MNs.

20. It was agreed that considering what an *in vitro* assay measures in the context of an *in vivo* response is an important factor when designing an *in vitro* experiment or reporting the results.

▪ *Are the existing data sufficient for MIE identification and development? If not, what data are needed?*

21. An issue facing MN-relevant AOP development is the lack of identified MIEs. To address this, experts suggested updating the NanoAOP database to better capture MIEs from the literature and the associated biology such as membrane interactions and subsequent endocytosis of MNs.

22. It was recommended to review the MIEs currently in the Collaborative Adverse Outcome Pathway Wiki (AOP Wiki)[2] for their relevance to MNs and prioritise them for future MN-relevant AOP development according to EAGMST principles. For example, cationic nanoparticles have been shown to bind angiotensin-converting enzyme 2 (ACE2)[3] which plays a role in induced acute lung injury[4].

Breakout Group 2. Facilitator: Jo Anne Shatkin (Vireo Advisors) Rapporteur: Claire Skentelbery (Nanotechnology Industries Association)

▪ *How can the use of the AOP framework be advanced for decision making about the safety of MNs? What are the current applications?*

23. Experts agreed that currently the AOP framework can be used for: (i) ranking and prioritising MNs; (ii) identifying critical KEs to develop alternative testing strategies for; (iii) product development as

[2] See; https://aopwiki.org/

[3] Cationic nanoparticles directly bind angiotensin-converting enzyme 2 and induce acute lung injury in mice. Part Fibre Toxicol. 2015 Mar 7;12:4

[4] AOP 319. ACE2 inhibition leading to lung fibrosis. Available at: https://aopwiki.org/aops/319

part of a safer manufacturing approach; and (iv) together with 'omics' strategies, AOPs can be used to propose testing that could be predictive of AOs.

24. In the future, the goal is to be able to facilitate the use AOPs as the basis for regulatory decision making for MNs as part of an Integrated Approach to Testing and Assessment (IATA).

▪ *From these projects, what lessons are learnt about applying AOPs in decision making for MNs?*

25. To use AOPs for decision making about MNs, there is a need to identify which KEs are critical for testing as part of an IATA. Critical KEs could be prioritised by examining which events are shared across multiple AOPs. For each critical KE identified, methods/assays that are appropriate and predictive for characterising them can be identified and developed.

26. Experts recommended developing guidance on what the 'gold standard' would be for testing MNs to develop data useful for an AOP framework, in a risk assessment context. This could include picking a critical KE (either one that occurs in many AOPs, or that represents a 'point of no return') or a suite of KEs and developing key tests/methods necessary to demonstrate these KEs in order to help guide regulatory decisions based on AOPs. This could be done by adopting a weight-of-evidence approach to narrow and prioritise the KEs and methods/assays for development.

27. Guidance should be established on the types of data the research community needs to develop and report for their work to be useful for regulators to address regulatory issues.

▪ *What are the key issues impeding the adoption of AOPs for decision making about MNs?*

28. Currently the AOP framework does not account for considerations of exposure, a key determinant of risk and a necessity for applying AOPs in MN regulatory decision making.

29. Many key biological events, although triggered with exposure, can resolve over time and do not result in an AO. Such repair mechanisms may not be accounted for in the framework and identifying 'points of no return' towards an AO is important for using the AOP framework for decision making. Consideration of dose-response (*i.e*. exposure) within the framework would help account for repair mechanisms.

30. For MNs, many MIEs are physical rather than molecular (*e.g.* frustrated phagocytosis); future research should focus on these particle-specific mechanisms.

31. To ensure relevance for regulatory applications, experts suggested that for AOP development, it is important to start with an AO of relevance and work backwards.

32. Better data curation is required to advance the application of the AOP framework, including reporting of negative data. NanoCommons was suggested as a platform to make negative data publicly available.

Breakout Group 3. Facilitator: Ulla Vogel (NRCWE) Rapporteur: Deborah Ashby (Health Canada)

• How can the development and adoption of in vitro assays be targeted to KEs to enable use of AOPs as part of an IATA for MN decision making? What is the current state of in vitro assay development to measure the KEs in AOPs?

33. AOPs can help focus efforts on development of *in vitro* assays with greatest relevance to KEs. A suggestion was made to prioritise KEs according to how close they are to an AO to ensure relevance for decision making. In some cases, several assays/methods may need to be developed to adequately characterise a given KE.

34. Additional work needed to move forward includes: (i) prioritising/ranking both KEs and the assays/methods to characterise them for development; (ii) establishing standard methods to enable comparisons between tests; (iii) developing standard nomenclature and definitions to ensure proper interpretation of test results; (iv) understanding how the assays/methods perform under different conditions and with different types of MNs.

• How do you determine the suitability of an in vitro assay for measuring a KE?

35. In evaluating readiness of an assay/method, validation will be an important component. It would be useful to have guidance on the minimum level of validation required to use a given assay/method for decision making. *In vitro* assay validation may require a different approach compared to traditional validation procedures followed for *in vivo* assays. Moreover, it is important to state what the assay will be validated against. Traditionally, for the existing *in vitro* assays that are OECD adopted, validation was conducted against an *in vivo* response. In the context of MNs, there are few *in vivo* data available. To validate these assays, MNs that score high on predictive *in vitro* assays should be prioritised to complete side-by-side animal experiments to confirm the predictiveness of an *in vitro* assay to *in vivo* outcomes. The severity of the endpoint will be an important factor in determining the extent of validation required (*e.g.* skin irritation versus carcinogenicity).

36. Assays/methods will have to be evaluated for their relevance to a given AOP; some assays may be relevant to more than one AO. In such cases, multiple assays may be required to evaluate KEs and ensure specificity to a given AOP (*e.g.* assays looking at cytotoxicity via different mechanisms such as apoptosis versus necrosis).

37. Reproducibility and accessibility (*i.e.* testing that can be completed with readily available and affordable equipment) need to be considered when developing *in vitro* assays.

• Can we agree on one or two assays that effectively measure the three broader sub-KEs identified for tissue injury?

38. As the project characterised tissue injury into three upstream KEs it is likely that three or more assays will be needed to predict the 'tissue injury' KE proposed. There are assays available for each upstream KE, but they are mostly not validated (see Halappanavar *et al.*, 2019).

• Are we at a place where we can develop guidance for measuring the upstream KEs identified in the NanoAOP project?

39. However, there is no standard for measuring of the upstream KEs.

40. Guidance could be developed both for the assessor (*e.g.* how to judge and evaluate available data for decision making) and for the study director (*e.g.* on the types of methods/assays and how to conduct the tests taking MN considerations into account and how to report the data for maximal utility).

41. In developing guidance, 'nano-specificity' will be especially important for MIEs. Many other KEs have established guidance for measurement based on our experience with chemicals (*e.g.* cytotoxicity); however, they need to be tailored for MNs addressing issues such as physical and chemical characterisation, dispersion, and dosing including differentiation between cell-attachment versus cellular internalisation, for example.

- ***Do new MNs need to be tested with a battery of in vitro tests spanning all KEs in an AOP to predict an AO or can one or a few be used?***

42. The weight-of-evidence would depend on the severity of the AO, and whether results suggested a positive or negative response. A higher burden of proof, requiring a battery of tests spanning several KEs, would be required for more severe outcomes (*e.g.* fibrosis) than for less severe AOs and to confirm negative results truly mean that no AO manifests.

Annex. Translating Scientific Advances in the AOP Framework to Decision Making for Nanomaterials

1 Introduction

1. This annex was first developed by Vireo Advisors in collaboration with the Horizon2020 SmartNanoTox Project. The purpose was to serve as a discussion document by providing an overview on the current status, needs and requirements to facilitate the future use of AOP in the risk assessment of manufactured nanomaterials.

2. As such, the document provides:

1) the state of the art and potential next steps for the development and use of the adverse outcome pathway (AOP) framework in decision making about the safety of manufactured nanomaterials (MNs);

2) Opportunities and challenges toward the advancement and adoption of AOPs as part of an integrated approach to testing and assessment (IATA) of MNs;

3) and specific actions proposed to advance the development, use and acceptance of the AOP framework and associated testing strategies for MN risk assessment and decision making.

3. As mentioned above the document was shared with participants to the two workshops “Advancing Adverse Outcome Pathway Development for Nanomaterial Assessment and Categorisation (NanoAOP project)” and “Stakeholder Workshop” and was later amended based on their feedback. This document compiles the main findings of the discussions based on the feedback from three projects on AOP for MNs: 1) OECD project Advancing Adverse Outcome Pathway (AOP) Development for Nanomaterial Risk Assessment and Categorisation; 2) H2020 Project SmartNanoTox; and 3) H2020 project PATROLS.

2 Translating Scientific Advances in the AOP Framework to Decision Making for Nanomaterials

4. Manufactured nanomaterials (MNs) as well as other advanced and emerging materials offer significant benefits to consumers including technological innovation, and safer and more sustainable alternatives to current technologies. However, safety evaluations of these materials are lagging due to safety testing challenges and the number, diversity and complexity of MNs coming to market. Currently, the toxicological assessment of MNs requires individual assessments of health and environmental endpoints using traditional animal testing methods, an approach that is too time- and cost-intensive to be practical and is undesirable ethically.

5. There has been significant international investment to uncover the scientific relationships between the physical, chemical and biological aspects of MNs, and recent efforts to link these in the Adverse Outcome Pathway (AOP) framework to advance mechanistic understanding of toxicological pathways. The next steps relate to how these efforts can be adopted for use by decision makers and include filling critical data and knowledge gaps.

6. The AOP framework is a promising and evidence-based approach toward solving several twenty-first century challenges of chemical and nano-specific safety assessments. Significant progress has been made in AOP development, application and use over the last decade including methods, resources and tools to adopt AOPs as part of an integrated approach to testing and assessment (IATA). The AOP framework offers a systematic, mechanistic approach to develop, assess, use and interpret alternative testing strategies for risk assessment and decision making, thereby reducing reliance on animal testing. Although the science behind AOPs has advanced considerably, a number of barriers remain toward adoption of alternative testing strategies approaches, an obstacle toward use of AOPs for risk assessment of MNs. An international, coordinated effort is needed to address the technical and translational issues in order to realise the potential of AOPs for evidence-based decision making about the safety of MNs.

7. Challenges regarding the adoption and use of AOPs for MN decision making include specific issues for MNs as well as ongoing research, technical needs and barriers that exist for chemicals more generally. Projects worldwide are focusing on meeting these challenges and advancing the adoption of AOPs for use by decision makers.

3 Recommendations in moving forward

8. Following the approach of the ProSafe White Paper (ProSafe 2017), recommendations are made to advance the use of concepts in the AOP framework for risk assessment and decision making for MNs. Significant advancement in understanding the relationships between MN properties and their modes of action is being made by the European Union (EU) NanoSafety Cluster projects from investments by the European Horizon2020 programme. Because MNs are advanced materials and enabling technologies they feature in a diversity of areas and applications. Several of the priority clusters for research and innovation proposed under the EU Horizon Strategic Plan overlap with, or would benefit from, advancement in MN decision making, including Health (Cluster 1); Digital, Industry and Space (Cluster 4); Climate, Energy and Mobility (Cluster 5); and Food, Bioeconomy, Natural Resources, Agriculture, and Environment (Cluster 6).[5] The following focused efforts are proposed to advance the development, application, and acceptance of the AOP framework and related testing tools for use in MN risk assessment and risk management decision making. The recommendations are organised into short-, medium- and long-term actions.

SUMMARY RECOMMENDATIONS: SHORT,MEDIUM, LONG TERM	
1. Advance MN-relevant and Advanced Material Considerations in AOP Development The AOP framework requires continued development of predictive pathways, building on the efforts of the AOP Wiki. Needs include updates to toxicological mechanisms within AOPs that identify processes and considerations relevant to MN toxicity. Further, the path for emerging advanced and hybrid materials to use the AOP Framework for early stage safety decisions can be outlined from these efforts. Recommendations include:	
	Short-term Actions a) Establish the types of data required to develop AOPs for MNs and identify existing NM-relevant AOPs; b) Compare molecular initiating events (MIEs), key events (KEs), key event relationships (KERs) and adverse outcomes (AOs) identified for MNs to AOPs in the AOP Wiki; c) Identify MN-relevant MIEs, KEs, and KERs. Research should include identifying MN-relevant mechanisms and MIEs, which are often due to physical interactions with MNs instead of molecular ones (e.g. frustrated phagocytosis and particle-surface induced reactive oxygen species).
	Medium-term Actions d) Identify similarities and differences in MIEs, KEs, and KERs between other emerging advanced materials and MNs; e) Conduct targeted research on MNs to elucidate the effect of interspecies variability on AOPs and related testing strategy development (Saber, Poulsen et al. 2019; Vogel and Cassee 2018). This can include side-by-side testing of in vitro cell lines and 3D models from a number of species exposed to a suite of MNs to examine conserved mechanisms and potencies; f) Use 'omics' approaches to identify interspecies variability in gene, protein and metabolite responses to MN exposure (e.g. using heatmaps) and their implications for AOP development (Labib, Williams et al. 2016).

[5] Orientations towards the first Strategic Plan implementing the research and innovation framework programme Horizon Europe. Available here: https://clepa.eu/wp-content/uploads/2019/07/Horizon-Europe-Strategic-Planning-Summer-2019.pdf

Long-term Actions

g) Develop data sets for quantitative AOPs (qAOPs) that include consideration of exposure conditions necessary for MN risk assessment. This includes adopting formal definitions and structure for qAOPs and developing case studies outlining the development and use of qAOPs for MNs;
h) Develop a testing strategy for advanced and hybrid materials (smart and responsive materials) to identify and quantify MIEs, KEs, KERs and AOs;
i) Convene experts to discuss how AOPs can account for individual (e.g. sex or life-stage) and inter-species variation that exists and can thus be used to reduce the associated uncertainty for decision making.

2. Utilise Existing Data from Literature and Previous Projects

A diverse set of data has been developed that may be useful for furthering the development, application and use of AOPs for MN risk assessment. In so far as possible, these data should be taken advantage of to advance knowledge and identify opportunities for additional AOP development. Recommendations include:

Short-term Actions

a) Evaluate data quality from the peer review literature and current suite of *in vitro* assays based on identified KEs;
b) Encourage researchers (and publishers) to make their raw toxicology data from peer reviewed literature available publicly;
c) Harmonise formats for reporting toxicology data (including negative results) to facilitate database development;
d) Build searchable databases for priority MNs that includes funded research and literature (*e.g.* available data developed under NanoCommons, NanoReg2, GRACIOUS and DaNa projects) as well as traditional chemical databases (*e.g.* TOXCAST). Efforts should include collecting negative data.

Medium-term Actions

a) Develop guidance on how the existing nanotoxicity literature, despite documented limitations (e.g. minimal reporting of physical and chemical characteristics), can be used for AOP development and decision making;
b) Broaden access and use of existing data sources (e.g. Nanomaterial-Biological Interactions Knowledgebase, Nanomaterials Knowledge Informatics Commons (NIKC); eNanoMapper, NanoCommons KnowledgeBase) and other resources;
c) Evaluate publicly available REACH data for MNs in terms of use in AO and predictive modelling;
d) Identify novel biomarkers for hazard evaluation;
e) Develop research projects to fill data gaps for identified endpoints.

Long-term Actions

f) Create processes to continually update publicly available databases as new data is developed.

3. Promote Reliable and Quantitative MN Data Development

Data development needs require coordination to ensure MN-relevant AOPs can be developed and used in regulatory decision making. Guidance is needed on the types of data and reporting standards toward use of AOPs in regulatory decision making for MN. Recommendations include

Short-term Actions

a) Identify priority AOs observed with MNs and initiate research into AOP development for these AOs;
b) Standardise the endpoints and reporting elements of assays evaluating MIEs, KEs and KERs to ensure high quality, comparable data is generated, published and added to databases, including reporting of negative data;
c) Develop guidance on the types of data that need to be developed and reported by the research community for their work to be useful in regulatory decision making.

Medium-term Actions

d) Develop data to allow for grouping – data collection/mining to determine the mode of action using MNs that can represent groups of MNs/ functionalisations;
e) Develop MN specific resources (for the AOP Wiki) to encourage coordination and cooperation among stakeholders needed for efficient, high-quality AOP development.

Long-term Actions

f) Advance modelling and QSAR databases and link to physical and chemical attributes of MNs;
g) Adopt iterative decision making, including increased confidence in non-traditional methods and use of non-traditional data and methods to improve weight-of-evidence in decision making.

4. Advance Knowledge of Quantitative Relationships Between MN Physical and Chemical Characteristics and AOP Elements

A better understanding of the quantitative relationships between MN physical and chemical characteristics and toxicological

outcomes is required. It is recommended to:

Short-term Actions

a) Review findings of existing data and research on the relationships between physical and chemical properties and MN KEs, including MIEs, AOs and KERs;
b) Develop hypotheses of predictive relationships between MN physical and chemical properties and biological outcomes.

Medium-term Actions

c) Test predictive physical and chemical relationships of MNs to biological outcomes using carefully controlled changes within and across materials (furthering the work of projects that have begun this effort such as SmartNanoTox and NanoMILE);
d) Assess the importance of using alternative dose metrics to mass (*e.g.* surface area, particle number) in predicting toxicological outcomes for MNs;
e) Where appropriate, incorporate alternative dose metrics into developed benchmark levels for MNs for screening and risk assessment.

Long-term Actions

f) Develop Quantitative Structure-Activity Relationships (QSAR) as predictive tools for KEs.

5. Identify Current Applications of the AOP Framework for MN Decision Making

Selected applications of the AOP framework have been proposed for current use in MN safety decision making. It is recommended to:

Short-term Actions

a) Identify screening-level MN safety decisions that are fit-for-purpose/can rely on AOPs;
b) Incorporate AOP elements into grouping and read-across decision trees for MNs;

Medium-term Actions

c) Adopt a testing scheme/decision tree for MN grouping and read-across;
d) Develop guidance and case studies for use of AOPs in regulatory decision making (*e.g.* MN prioritisation; grouping, categorisation and read-across; and hazard identification and ranking).

6. Establish Test Methods and Protocols Useful for MN Decision Making

Test methods to accurately measure MN-relevant MIEs and KEs are necessary to advance use of AOPs in MN risk assessment. Development (and verification) of harmonised, standardised MN-relevant test methods is needed:

Short-term Actions

a) Evaluate, advance or develop physical and chemical characterisation protocols for MNs and how they can be used to identify MIE, KE and AO portions of the AOP framework;
b) Prioritise KEs and the assays/methods to characterise them for development, with KEs closer to an AO being prioritised to ensure relevance for regulatory decision making as part of an IATA;
c) Evaluate, advance or develop *in silico*, *in chemico*, *in vitro* and *ex vivo* assays for MNs and how they can be used to characterise MIE, KE and AO portions of the AOP framework;
d) Initiate Test Guideline development for assays tailored to MNs that address considerations such as physical and chemical characterisation, dispersion and dosing relevant to AOPs;
e) Develop guidance on the minimum level of validation required to use a given *in vitro* assay or method for regulatory decision making.

Medium-term Actions

f) Create voluntary standard methods for IATA;
g) Consider formal adoption of IATA for certain MN hazard or risk decisions;
h) Advance new *in vitro* test development to screen for MIEs and KEs;
i) Identify test methods (including *in silico*) for high throughput screening.

Long-term Actions

j) Develop OECD Test Guidelines for MNs that relate to MIEs, KEs and AOs;
k) Where appropriate, formally adopt IATA for MNs;
l) Adopt harmonised, standardised tests for high throughput screening.

7. Demonstrate Predictive Capability of AOPs and *In Vitro* Test Methods

To be useful for decision making, a coordinated effort is required to ensure that the alternative testing models for KEs in an AOP can be predictive of an AO of regulatory relevance. Recommendations include:

Short-term Actions

a) Assess the strength of evidence for considering dose-response relationships in AOPs as predictive tools for MN risk assessment.

	Medium-term Actions b) Compare the predictive capability of in vitro assays for MIEs and KEs with in vivo observations or epidemiological data; c) Design and conduct side-by-side in vitro and in vivo testing for representative MNs to compare toxicity mechanisms and potency across MNs and assays (furthering the work of projects that have begun this effort such as PATROLS).
	Long-term Actions d) Develop and test predictive alternative testing models; e) Validate predictive alternative testing models.
8. Guidance to Facilitate Adoption of MN-relevant AOPs for Decision Making The science required to address the technical challenges of transitioning to alternative (non-animal) toxicity testing is progressing, but efforts are needed to translate and incorporate these developments into decision making about the safety of MNs. Recommendations include:	
	Short-term Actions a) Identify MN-relevant and MN-specific AOPs, KEs and KERs, including an assessment of the AOPs which have been officially OECD-endorsed, approved, or are under review and under active development for MN-relevance.
	Medium-term Actions b) Develop and validate an IATA based on KEs, KERs and AOPs that can be used in risk assessments of new nanoscale materials. This includes identifying and prioritising which KEs are critical for testing as part of an IATA, building on the work currently ongoing in NanoSolveIT; c) Develop guidance for risk assessors on developing an IATA based on AOP frameworks to complete MN safety assessments. It should include how to pick critical KEs, or a suite of KEs, for testing.
	Long-term Actions d) Incorporate technical developments into specific regulatory guidance/policy documents.
9. Stakeholder Communication & Engagement on Use of AOPs for MN Decision Making To facilitate the development, adoption and use of the AOP framework for MN decision making, engagement of multiple stakeholders with a broad range of expertise is essential and coordination and cooperation are needed. Recommendations include:	
	Short-term Actions a) Develop communication and educational materials on the use of the AOP framework for MN decision making for non-technical stakeholders; b) Organise additional workshops targeted to encourage participation from various stakeholders with a vested interest in AOP development and application for MN decision making including academics, policy-makers, regulators, and industry.

4 Problem Formulation

9. Much of the current innovation in new chemicals is focused on advanced materials, including manufactured nanomaterials (MNs). MNs can display unique attributes and behaviors and may be biologically and physically complex, making them valuable across a wide range of applications. However, as the number, diversity and complexity of MNs coming to market continues to grow, assessing their individual health and environmental risks with traditional animal testing approaches is too time- and cost-intensive to be practical, and is undesirable for ethical reasons. New approaches are needed that meet current requirements for regulatory risk assessment while reducing reliance on animal testing. The adverse outcome pathway (AOP) framework presents a sound model for advancement of MN decision making. Yet there are currently gaps in the technical and policy aspects of AOPs that hinder adoption and use for MN risk assessment and safety decision making. This paper outlines the current situation and recommends specific efforts to advance use of the AOP framework in the context of regulatory decision making for MNs and emerging advanced materials.

5 Background

10. The applications for MNs and nano-enabled products are rapidly emerging, while regulators are still working toward hazard and risk assessment approaches for this emerging class of materials. Recognising the importance of assessing the safety of these materials for humans and the environment, regulators and risk assessors have been challenged to adapt and develop risk assessment strategies to address specific challenges posed by MNs. To help meet this need, the EU has published guidance for the safety assessment of MNs in a variety of regulated applications, including food and feed (European Food Safety Authority 2018), cosmetics (EU Scientific Committee on Consumer Safety 2012) and chemicals (Commission Regulation (EU) 2018/1881). These documents provide valuable information but also highlight requirements for more efficient testing strategies that address MN-specific challenges as well as issues that have not yet been resolved for conventional materials. The maturation and adoption of such strategies hold the potential to advance both MN-specific toxicity testing and the development of methods and approaches needed for advanced materials and other emerging substance decision making.

11. MN toxicity testing is challenging in part because of the unique physical and chemical attributes of MNs. MN dose-response relationships may differ from those of conventional materials. Dose-response relationships of conventional materials are based on dose in mass terms and do not account for the potentially enhanced toxicity caused by the particle aspects and increased specific surface area of MNs. As such, dose-response relationships for MNs cannot be predicted based on current chemical substance models, whereas air pollution toxicology and nanotoxicology share similarities (Stone, Miller et al. 2017). In addition, identifying groups for categorisation based on biological effects arising from small changes in MN physical and chemical characteristics are not easily predicted using today's tools.

12. MNs are an enabling technology with applications in a diversity of areas and sectors. However, the adoption & commercialisation of MNs is hindered by the lack of accepted methods for toxicity evaluation (ProSafe Project Office 2017) with an associated potential for lost benefits to European consumers of safer and more sustainable goods. Regulatory agencies have recognised that more efficient testing strategies are needed for MNs but require further development and verification before incorporation into regulatory testing guidance documents. Several of the priority clusters for research and innovation proposed under the EU Horizon Strategic Plan overlap with, or could benefit from, further advancement in MN decision making including Health (Cluster 1); Digital, Industry and Space (Cluster 4); Climate, Energy and Mobility (Cluster 5); and Food, Bioeconomy, Natural Resources, Agriculture, and Environment (Cluster 6) (Horizon Europe 2019).

13. Ideally, developing strategies that help address the technical challenges associated with MN toxicity testing will also help address problems with chemical testing more generally, including a strong reliance on animal studies. Traditional toxicity studies rely heavily on animal testing. The growing number of new chemicals and the associated testing required to assess their risks has led to a dramatic increase in the number of animals sacrificed each year. In the EU alone, it is estimated that over 11.5 million animals were used for experiments in 2011 (European Commission 2013) and despite the introduction of legislation to reduce animal testing in 2013, an estimated 9.39 million animals were still used in 2017 (European Commission 2020). This use of animal testing is causing ethical controversy, and several calls have been made to reduce the number of animals required for regulatory assessments of new chemicals.

For example, the Frank R. Lautenberg Chemical Safety for the 21st Century Act requires the United States (US) Environmental Protection Agency (EPA) to promote the development and implementation of alternative test methods and strategies to reduce, refine or replace animal testing and efforts are underway to develop these methodologies, and the EPA Administrator recently announced efforts to reduce, refine or replace vertebrate animal testing.

14. At the same time, today's chemical toxicity testing landscape is facing pressure to enhance transparency and incorporate up-to-date scientific understanding in risk assessment and related decision making. Regulators must accurately assess the safety of tens of thousands of new and existing chemical substances, but resources allotted for safety assessments are often static or decreasing (Carusi, Davies et al. 2018). Policy is shifting globally towards reduction and eventual elimination of animal studies, favouring alternative methods for all chemicals (e.g., in the EU (European Commission 2020) and the US (US EPA 2019).

15. The science required to address the technical challenges of transitioning to alternative (non-animal) toxicity testing is progressing, but efforts are needed to incorporate these developments into decision making and to guide the science to address data and methodological gaps hindering regulatory risk assessment. Over the past several decades, significant advances have been made in toxicological evaluations that utilise *in silico*, *in chemico*, *in vitro* and *ex vivo* approaches to predict the hazards of new chemicals, including MNs, without animal testing (Carusi, Davies et al. 2018). Improvements in computational capability have made *in silico* modeling experiments more accurate and the ability to model complex system responses more feasible. Advances in genomics and proteomics have improved understanding of how changes in gene and protein expression may lead to adverse outcomes in organisms, and bioinformatics has led to the development of methods and tools to analyse the enormous amounts of data generated through these approaches. *In vitro* models have advanced, improving the characterisation of complex biological systems. These new toxicological tools evaluate biological responses on the molecular, sub-cellular, cellular and tissue levels and offer risk assessors more mechanistic information than ever before.

16. Currently, however, few *in vitro* and *ex vivo* tests are accepted for use in regulatory decision making, largely due to lack of formal validation. The widely acknowledged challenges for alternative testing approaches apply to conventional chemicals and MNs alike, but these issues are further complicated for MNs by their particulate nature which affects dosimetry and provides surface reactivity. How to incorporate and use *in vitro* and *ex vivo* data in assessing the risks of MNs and other new chemicals often remains unclear, and the translation of these approaches from experimental to regulatory relevance remains difficult.

17. The dual challenge of advancing MN risk assessment while reducing reliance on animal testing presents an opportunity to develop smarter approaches to screening and prioritising novel nanoscale materials. Doing so will require a coordinated response that adopts science into decision making and bridges current knowledge gaps. The AOP framework links biological mechanisms of action to observed adverse effects of regulatory relevance and thereby offers a promising evidence-based and toxicologically realistic approach that ultimately reduces the resources required for testing, applies and develops relevant MN-specific data, support the advancement and validation of alternative testing methods for all chemicals and provides a more structured basis for prioritisation and risk assessment.

18. Recognising this value, a number of initiatives are working toward development of MN-relevant AOPs. Notably, the EU and the OECD are both spearheading major initiatives, including the EU H2020 SmartNanoTox program and related projects, as well as the OECD's AOP Development Programme. The promise of the AOP framework is confirmed in MN-specific guidance documents, such as the European Food Safety Authority "Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain," which states:

> *The Organisation for Economic Co-operation and Development now also explores Integrated Approach to Testing and Assessment (IATA) and promotes the use of AOPs to build risk assessment, while assessing all the existing data.*

19. At the same time, however, more work is needed. The EFSA guidance, for instance, goes on to say:

> *The developments in efficient testing strategies and AOPs for nanomaterials are highly acknowledged, though they need further development and verification before incorporation into guidance documents can be considered.*

20. Efforts continue to make critical advances in AOP development for MNs and other chemicals, but specific policy support is needed to take advantage of, and operationalise, this work. The remainder of this document describes the AOP framework and its current status in greater detail, outlines challenges facing its advancement and adoption and recommends specific actions needed to mature the concept and advance its use for decision making specifically for MNs.

6 Overview of AOP Framework and Current Status

21. AOPs clarify relationships across biological levels of organisation (including molecular, sub-cellular, cellular, tissue, organ, organism and whole populations), using cause-and-effect relationships to connect molecular initiating events (MIE) to adverse outcomes (AO). AOs are negative biological consequences resulting from chemical exposure; they are typically measured at higher levels of biological organisation important for regulatory decision making, such as human health (organ or organism) or environmental endpoints (organism or populations). The pathways connecting MIEs and AOs are defined by key events (KEs), which represent measurable biological changes, and key event relationships (KERs), the directed, predictive relationships among those KEs. AOPs are the unit of development for the AOP framework, and represent a single, non-branching sequence of KEs, linked by KERs, connecting a single MIE to a single AO.

22. Figure 1 gives an example of a generalised AOP and its components (Panel A) and shows how AOPs can form interlinked networks based on overlapping MIEs, KEs and AOs (Panel B) that represent the complex biology underlying disease processes (Halappanavar et al. 2019). Bioassays targeting the MIE and KEs in an AOP are developed, characterised and used as endpoints as part of an IATA. The AOP framework defines this entire conceptual approach that assembles and organises mechanistic knowledge to communicate causal linkages between biological perturbations and adverse health outcomes meaningful to chemical risk assessment and regulatory decision making (Ankley, Bennett et al. 2010; Carusi, Davies et al. 2018).

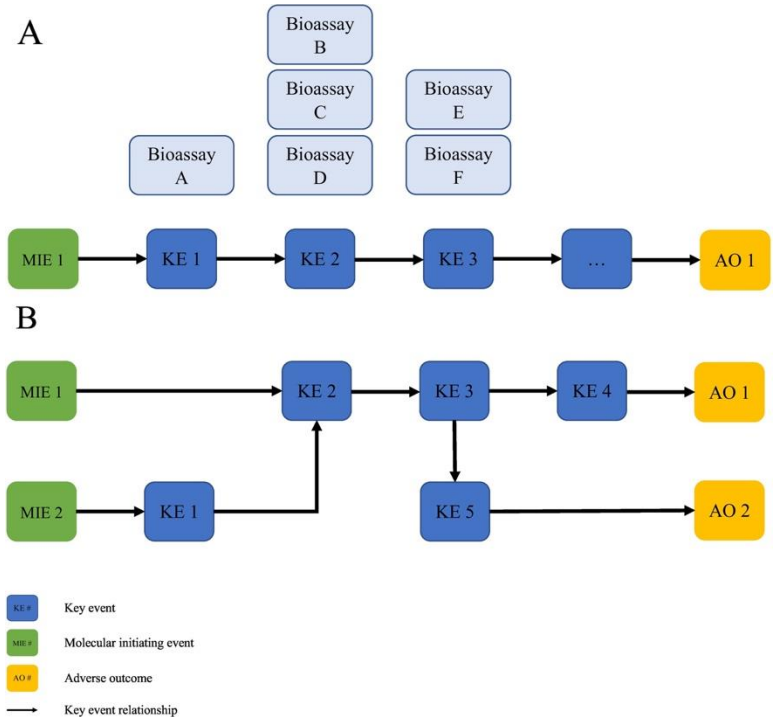

Figure 1. (A) Generalized AOP showing the relationship between MIE, KEs and AOs and the KERs that connect them. Bioassays targeting the MIE and KEs in an AOP are characterized or developed as part of an IATA. (B) AOPs can form interlinked networks based on overlapping MIEs, KEs and AOs that better capture the complex biology of disease processes. (From Halappanavar et al. 2019)

23. To help accelerate AOP development, strategies, principles and best practices have been established to help scientists, regulators and decision makers understand and contribute to, the AOP framework. In 2014, Villeneuve et al. proposed a set of five core principles to guide AOP development (Villeneuve, Crump et al. 2014); these are highlighted in Box 1.

Box 1. Core Tenets of AOPs (Villeneuve, Crump et al. 2014)

1. AOPs are not chemical-specific. Specificity limits the predictive utility of AOPs for new substances.
2. AOPs are designed with modular units. These components should be reusable to enhance flexibility, and they should be designed to accommodate differing levels of detail based on evidence.
3. AOPs are a unit of development. An individual AOP is defined as a single, non-branching sequence of KEs, linked by KERs, connecting a single MIE to a single AO. This structure reduces complexity and is a practical unit for development and evaluation.
4. AOPs form networks. Multiple AOPs, sharing one or more common KE or KER, form networks that more realistically represent the complexity of biological systems needed to make accurate biological predictions of adverse toxicological outcomes.
5. AOPs should be continuously updated. New research should be used to inform and refine existing AOPs.

Current status

24. Tremendous progress in AOP research and development has occurred since AOPs were first described in 2010 (Ankley, Bennett et al. 2010). A survey of papers published annually on the topic from 2010-2019 indicates exponential growth, with over 100 publications expected in 2019 based on a

PubMed literature search. This growth suggests the AOP concept is gaining widespread traction and acceptance in the academic community (Figure 2; Langley 2017).

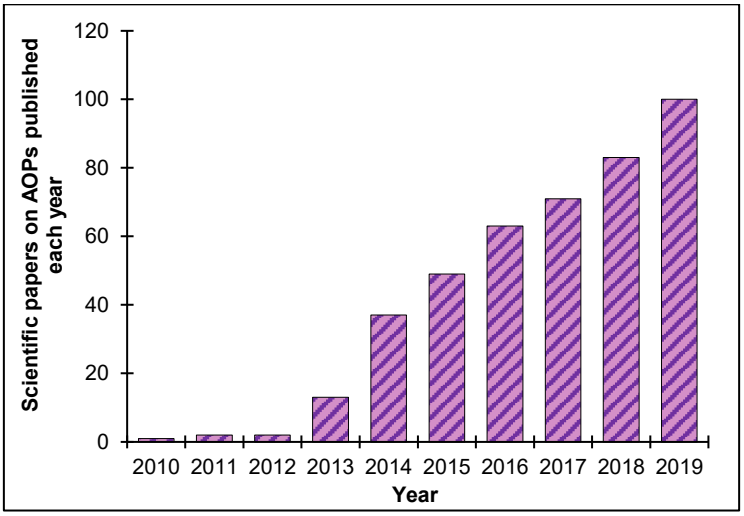

Figure 2. Scientific papers on AOPs published annually, 2010-2019. PubMed was searched for published papers containing the text words "adverse outcome pathway" on July 30, 2019. The result for 2019 is estimated given the number of publications that have been published per month.

25. A number of efforts worldwide are contributing to AOP development; of these, the OECD is spearheading one of the largest. The OECD AOP Development Programme was started in 2012 and is overseen by the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST). Its goal is to develop, review and officially endorse AOPs. AOP development is based on voluntary contributions from member countries and stakeholders. It involves an internal review within EAGMST to ensure compliance with AOP principles, as well as an external review by subject matter experts to assess the scientific merit of the proposed pathway. There are currently nine OECD-endorsed AOPs, seven that have received approved status, fourteen under review (indicating they are at a late stage of the endorsement process), and twenty-four proposals under active development.

26. The OECD, together with contributions from the US EPA, the European Commission's Joint Research Centre, and the U.S. Army Engineer Research and Development Center has also led the development of the AOP Knowledge Base (AOP-KB). Launched in 2014, this web-based tool consists of five modules to enable and promote development and application of AOPs: the e.AOP.Portal, the AOP-Wiki, the Effectopedia, the AOP Xplorer and the Intermediate Effects Database (Delrue, Sachana et al. 2016). The AOP-Wiki serves as the primary repository for qualitative AOPs developed (including those endorsed by the OECD) or under development. It is intended to foster collaboration among various stakeholders contributing to AOP development following the standard OECD principles for developing and assessing AOPs (OECD 2017). The AOP Wiki currently contains more than 200 AOPs, including more than 2,000 defined KEs (Figure 3).

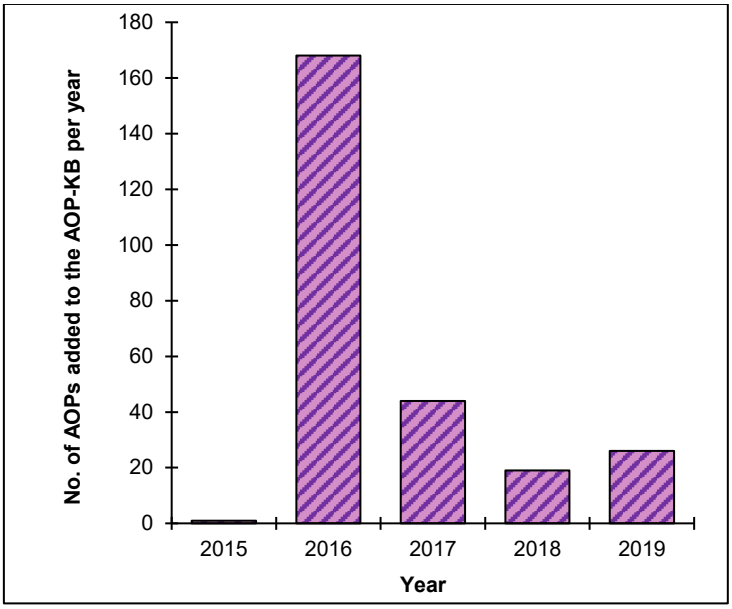

Figure 3. AOPs created in the AOP-KB each year, 2015-2019 (until July 31, 2019).

27. In addition to these efforts, various stakeholders have supported projects aimed at evaluating and promoting the development of AOPs for MNs. The EU, through its H2020 initiative, has supported several projects focused on development and application of AOPs for MNs; these include SmartNanoTox (Smart Tools for Gauging Nano Hazards) and PATROLS (Physiologically Anchored Tools for Realistic nanOmateriaL hazard aSsessment) (Villeneuve, Crump et al. 2014).

28. Since 2016, the OECD Working Party on Manufactured Nanomaterials (WPMN) has included in its programme of work the project *Advancing Adverse Outcome Pathway (AOP) Development for Nanomaterial Risk Assessment and Categorisation*. These projects and their outcomes and contributions to AOP development are discussed in Section 5.

7 Potential Applications of AOPs

29. Progress in AOP development has enabled a range of potential applications. In chemical risk assessment, the AOP framework is intended to guide and develop integrated approaches to testing and assessment (IATA). IATA is an approach to characterising the hazard of chemicals that integrates: (i) analysis of existing information with (ii) generation of new information through targeted testing strategies (Delrue, Sachana et al. 2016). From a risk assessment perspective, the AOP framework provides:

1) A structured framework to evaluate existing information available for a chemical of interest; potential sources include *in chemico*, *in silico*, *in vitro*, *ex vivo*, *in vivo* and 'omics' data;
2) A way to identify data gaps and efficiently generate missing information to increase confidence in decision making and assessment of risk;
3) A framework to apply an iterative approach until sufficient information is gathered for decision making.

30. Within this context, the AOP framework has several specific applications and benefits to improve risk assessment and decision making; these are summarised in Table 1 (Delrue, Sachana et al. 2016, Langley 2017, Fadeel et al. 2018). Of particular interest, the AOP concept offers a systematic, mechanistic framework to develop, assess, use and interpret alternative testing strategies for chemical risk assessment and decision making, thereby reducing reliance on new substance testing. Applications in chemical safety assessment are especially useful to regulatory toxicologists, risk assessors and risk managers, as well as to industry stakeholders responsible for product stewardship and compliance with regulatory requirements (Carusi, Davies et al. 2018).

Table 1. Potential applications and benefits of the AOP framework in chemical risk assessment

1. Evaluation of existing information
• improve chemical grouping and categorisation for filling data gaps by read-across • utilise data developed from advances in alternative testing strategies such as *in chemico*, *in vitro*, *ex vivo* and 'omic' data for decision making • improve predictivity in safety assessment (*e.g.,* quantitative structure-activity relationships)
2. Identification of data gaps and generation of new data
• improve high-throughput screening for chemical prioritisation • identify data gaps to inform relevant research • identify novel biomarkers for hazard evaluation • develop novel, non-animal approaches for hazard characterisation • reduce reliance on animal testing
3. Iterative decision making
• increase confidence in non-traditional methods • use non-traditional data and methods to improve weight-of-evidence in decision making

31. One area of proposed application of AOPs is for MN risk assessment and decision making. Because of their particulate aspects, MNs do not necessarily display classical dose-response relationships, and their toxicity is not always predictable from chemical substance models. Moreover, the biological effects of small changes in their physical and chemical makeup are not easily predicted with

today's risk assessment toolbox and models. The benefits of a framework better suited to assessing the impacts of such MN modifications can accrue to regulators, researchers and product developers.

32. While not the focus of this discussion, the AOP framework has other potential applications that extend beyond risk assessment (Carusi, Davies et al. 2018). AOPs provide utility in product discovery and development, especially in the pharmaceutical and agrochemical industries; here, they can support preclinical safety assessments to identify compounds potentially harmful to human, animal or environmental health early in development. In medicine and health, clinicians and researchers can use AOP knowledge to understand disease pathways across multiple biological levels, informing prevention, diagnosis and treatment efforts. Other stakeholders that may benefit from AOP applications include academics (who may benefit from a unifying framework to increase the real-world impact of their work) and non-governmental organisations (NGOs) including animal welfare and environmental NGOs. As mentioned above, several focus areas in the proposed Horizon Europe Strategic Plan can benefit from advancement and adoption of AOPs in decision making.

8 AOPs Development and Application for MNs

33. Projects worldwide are focusing on meeting the challenges of AOP development and promoting the use of AOPs for decision making. Below, projects contributing to the development, use, and adoption of AOPs for MNs are highlighted.

Progress in AOP Development for Manufactured Nanomaterials

34. Inhalation exposure to powdered forms of MNs, especially during manufacture and handling, is a highly relevant risk scenario. Some studies have documented significant AOs such as fibrosis and cancer following exposure to certain forms of MNs (*e.g.* carbon nanotubes); however, considerable uncertainty about the physical and chemical properties influencing these outcomes remain. *In vivo* inhalation experiments to characterise the hazard of MNs are challenging and resource-intensive. The AOP framework, as part of an IATA, offers a more time- and cost-efficient alternative approach to assessing the potential risks from inhaled MNs, however significant development and verification of the pathway is necessary before such an approach could be used for decision making.

35. The SmartNanoTox project is using results from *in vivo*, *in vitro* and *in silico* research to develop AOPs for adverse pulmonary effects following MN exposure. The effort is using representative sets of MNs to identify critical KEs and KERs to construct AOPs and relate them to interactions at the bio-nano interface. Using the data generated from various KEs and KERs along the AOP, SmartNanoTox aims to develop quantitative structure-activity relationships (QSARs) to enable predictions of whether a MN can trigger an AOP and lead to an AO and to enable grouping, categorisation and read-across of MNs for these endpoints.

36. A number of efforts such as SmartNanoTox are developing AOPs directly. Other related efforts are developing data, methods and tools useful for AOP development. For example, the EU's NanoSolveIT project is developing: (i) innovative modelling techniques and tools for nanoinformatics; (ii) an IATA to identify the specific characteristics of MNs that are responsible for adverse effects on human health or the environment; and (iii) *in silico* methods, models and tools useful for AOP development. The grouping strategies, data and methods in the H2020 projects GRACIOUS and NanoReg 2 are being incorporated into IATA (Mech et al. 2019). Similarly, NanoCommons aims to deliver a nanoinformatics research infrastructure including a database to facilitate reuse of existing nanosafety data, and *in silico* tools for analysis and prediction of MN impacts. The Data and Knowledge on Nanomaterial (DaNa) project has compiled data on the applications of MNs and the current state of knowledge. The EU Cluster of Systems of Metadata for Official Statistics (COSMOS) is identifying common sets of metadata objects with standard definitions and methods to build better metadata repositories. All of these efforts can make significant contributions to AOP development.

Progress in Application and Use of AOPs

37. One of the first AOPs officially endorsed by the OECD was the skin sensitisation AOP (AOP No. 40 in the AOP-Wiki) (Langley 2017). Since its endorsement, the mechanistic knowledge gained on skin sensitisation has been used to develop and validate three standardised *in vitro* tests targeting the KEs in the AOP. These test guidelines have been published and are now accepted for regulatory use as a viable alternative to traditional animal testing for skin sensitisation. The skin sensitisation AOP is a success story, demonstrating how AOP development can help identify, promote development of, and validate alternative testing strategies for chemical risk assessment without the use of animals.

38. While significant progress has been made in AOP development, application and use over the last decade, for endpoints beyond skin sensitisation, there is still a significant distance from current reliance on, to reduction of, and replacement of animal testing with non-animal approaches for risk assessment and regulatory decision making. The OECD WPMN NanoAOP project is contributing to both the development and application of AOPs for MN risk assessment. The goal of the project was to develop a methodology and approach to use existing nanotoxicology literature to support MN-relevant AOP development. While not developing an AOP, a case study outlines how the literature can be mined to identify and develop specific KEs to support AOP development. As part of its outcomes, the project convened two workshops to gain expert feedback on the current status, use and future needs for AOPs relevant to MNs in support of risk assessment. The consensus from experts is that currently, the primary applications of AOPs are to support hazard identification; grouping, categorisation and read-across; ranking and prioritising MNs; identification of novel biomarkers for alternative test method development; for product development as part of a safer manufacturing approach; and together with 'omics' strategies, AOPs can be used to propose testing that could be predictive of AOs. The ultimate goal is to use AOPs as the basis for regulatory decision making for MNs; however, this goal has several challenges identified by experts that need to be overcome to advance future use of AOPs for MN risk assessment (discussed in Section 6). Experts suggest that to ensure relevance of AOP frameworks for regulatory decision making, development should proceed by first choosing an AO relevant for regulators, and developing the pathway working backwards towards an MIE.

39. Several projects are contributing directly to the application and use of AOPs for MN risk assessment. SmartNanoTox and PATROLS are developing *in vitro* models, methods and computational tools for MN hazard assessment targeting the KEs in developed AOPs. This is an important step to ensure that identified KEs in an AOP can be assessed with validated methods and tests and thus to support their inclusion in an IATA for MN risk assessment. SmartNanoTox is constructing simplified *in vitro* or *in silico* tests for the AOPs developed in their project, targeting identified MIEs and KEs for adverse respiratory outcomes from MN inhalation. PATROLS is focused on developing mechanism-based, non-animal methods, models and computational tools for MN hazard characterisation, targeting the KEs in established AOPs. This includes *in silico* hazard testing systems, *in vitro* human tissue models, ecotoxicology models and methods for MN characterisation in biological systems.

9 Challenges of AOPs for MNs

40. Substantial progress has been made toward the development and application of AOPs, but a number of challenges remain before their full potential can be realised. A global horizon scanning exercise to identify current challenges toward regulatory adoption of the AOP framework is one of the largest efforts to advance AOP development and application (LaLone, Ankley et al. 2017). The key findings from that effort are summarised below, as are expert insights from the workshops held as part of the OECD NanoAOP project. Limitations identified through these efforts include challenges specific to MNs as well as outstanding research and technical needs hindering AOP development more generally.

Nanomaterial-specific Challenges

41. The development and application of AOPs for MN decision making poses a specific set of challenges:

- ***Limitations of Current Literature***. Although there have been significant advancements in nanosafety and nanotoxicology research over the last two decades (Fadeel et al. 2018), there are several limitations of this literature for AOP development and use. Limitations include uncertainty from the different exposure conditions used between studies; different models used in each study (*e.g.* assays, cell lines, etc.); consideration of MN dispersion and dosimetry; general lack of physical and chemical characterisation of MNs (see *Influence of Physical and Chemical Properties,* below), and fragmentation of the data. Better data creation and management processes for MNs are required to advance the development and use of the AOP framework and future data reporting needs to include a set of minimum information requirements (Faria et al. 2018) and negative results.
- ***Assays and Methods to Assess MIEs and KEs***. To use AOPs for decision making about MNs, there is a need to identify which KEs are critical for testing as part of an IATA. To apply AOPs to chemical risk assessment, strategies to evaluate specific MIEs and KEs must be developed and verified. Many of these assays and testing strategies exist for conventional chemicals, but toxicity testing of MNs often require modification (Stone, Johnston et al. 2009). Development, including verification of MN-appropriate methods, is needed and remains an on-going challenge. Highlighted needs specific for MNs arising from the expert discussions include incorporation of accurate dosimetry into toxicity testing, which includes consideration of the most relevant dose metrics (*e.g.* mass, surface area, or particle number) and exposure conditions (*e.g.* stability of suspensions and characterisation of agglomerates)(DeLoid et al. 2015). In developing assays and methods to assess MIEs and KEs, reproducibility and accessibility to testing methods needs to be considered. Further, assays and methods will have to be evaluated for their relevance to a given AOP; some assays may be relevant to more than one AO. In such cases, multiple assays may be required to evaluate KEs and ensure specificity to a given AOP (*e.g.* assays looking at cytotoxicity via different mechanisms such as apoptosis versus necrosis).
- ***Influence of MN Physical and Chemical Properties***. Traditional chemical properties such as solubility and chemical composition are known to influence the toxicity of MNs. However, MN toxicity can also be influenced by distinct physical-chemical properties, such as dispersibility and

aggregation in solution, large surface area to volume ratios or increased surface reactivity (Labib, Williams et al. 2016). AOPs are generally developed with data from conventional, bulk chemicals; their application to MN risk assessment requires evaluation to ensure they are capturing MN-relevant mechanisms, MIEs and KEs (Halappanavar et al. 2019). In particular, experts identified that an issue facing MN-relevant AOP development is a lack of identified MIEs. For MNs, MIEs may be physical rather than molecular in nature (*e.g.* frustrated phagocytosis); research and AOP development will need to account for these particle-specific mechanisms. Currently, there are nine OECD-endorsed AOPs, seven that have received approved status, fourteen under review, and twenty-four proposals under active development which could be assessed for their relevance to MNs.

42. Despite these challenges, experts concluded that the AOP framework would improve risk assessment strategies for MNs as well as further the community's understanding of toxicity mechanisms and potency. Realising this potential requires addressing outstanding technical challenges and barriers to adoption, both those specific to MNs (described above) and those that apply to AOPs more generally.

Technical Challenges

- ***AOP Networks***. Individual AOPs are constructed as linear sequences of biological events connecting a MIE to an AO. However, exposure to chemicals, including MNs and other emerging substances, may affect more than one MIE or KE and result in one or many AOs. Individual AOP units are intended to form networks of inter-connected KEs to reflect this complexity, but there is little guidance currently available to develop, analyse, and evaluate these networks.
- ***Exposure and Dose***. Risk assessment requires information on the exposure conditions (*e.g.*, route, dose, duration and frequency) needed to cause an AO. Quantitative AOPs (qAOPs), which use quantitative data to predict risk of an AO given specified exposure conditions, are proposed as a solution to address these needs, but few examples of qAOPs currently exist.
- ***Individual and Inter-species Differences.*** Additional challenges include how to develop AOPs that can account for individual variation, such as life stage, immune status or sex, and how to reduce uncertainty that arises from inter-species differences, including sensitivity, potency and metabolic diversity.
- ***Repair Mechanisms.*** Many key biological events, although triggered with exposure, can resolve over time and do not result in an AO. Such repair mechanisms may not be accounted for in the AOP framework and identifying 'points of no return' towards an AO is important for using the AOP framework for decision making. Consideration of dose-response (*i.e*. exposure) within the framework would help account for repair mechanisms.

Barriers to Adoption

- ***Lack of Guidance for Risk Assessors.*** Guidance is needed for risk assessors outlining the use and applications of AOPs for decision making. Such guidance should help risk assessors determine whether the level of development for an AOP and weight of evidence provided when using an AOP as part of an IATA is adequate for decision making. The emerging consensus is that the level of understanding and degree of confidence needed will depend on the intended application and severity of the AO, *i.e.*, whether an AOP is 'fit-for-purpose' must be evaluated on a case-by-case basis. For example, for AOPs with more severe outcomes (*e.g.* fibrosis) an IATA would likely require a battery of validated tests spanning several KEs in the pathway to establish

the weight-of-evidence required for decision making. Further, negative results would require a higher burden of proof. Establishing guidance on this topic would help accelerate AOP adoption.

- ***Engagement of Multiple Stakeholders.*** Development of AOPs requires a significant investment of resources, time and expertise. Engagement of multiple stakeholders with a broad range of expertise is essential; coordination and cooperation are needed for efficient, high-quality AOP development. Multi-stakeholder participation is critical to increase confidence in the framework and to support its transition toward use in policy, decision making and regulatory applications, but challenges exist in engaging participants. These include, among others, lack of adequate incentives, problems with information control and ownership, reputational and liability risks associated with developing AOPs for decision making purposes, resource demands and limitations, cross-discipline communication challenges and the need for oversight (Carusi, Davies et al. 2018).
- ***Communication*.** Even among experts, misconceptions about the AOP framework exist. In the horizon scanning effort, community collaboration and communication were identified as critical components of AOP development and acceptance.

10 Summary

43. Adoption of AOPs for advanced materials and MNs is hindered by the lack of accepted methods for toxicity evaluation and over-reliance on resource-intensive animal testing. There is a need and an opportunity to develop smarter approaches for screening and prioritising of novel nanoscale materials for decision making. The AOP framework is a promising and toxicologically realistic approach to help address several current challenges in nano-specific safety assessments. Significant progress has been made in AOP development, application and use over the last decade including methods, tools and assessment approaches. However, we are still a long way from reliance on and replacement of animal testing with non-animal approaches, where MNs bring additional challenges. Several challenges remain and must be addressed to realise the full potential of AOPs and their acceptance and adoption in decision making about nanoscale and emerging substances. Projects worldwide are focusing on meeting these challenges and advancing the use of AOPs for decision making. International cross-disciplinary analysis and deliberation will improve adoption and acceptance of the AOP framework including for MNs.

11 Potential Next Steps

44. To advance the development, use and acceptance of the AOP framework for MN risk assessment and decision making, and to help overcome the challenges identified above, several actions are suggested aimed at promoting nine central recommendations (Table 2). The 'central recommendations' (Table 2) are meant to be high-level goals, with the 'actions' the means of achieving them, broken out by timing. Although these recommendations and corresponding actions are tailored to promote the development, use and acceptance of AOPs, they share overlapping goals with the nanosafety research community at large.

Table 2. Central Recommendations for Promoting the Development, Use and Acceptance of the AOP Framework for MN Decision Making.

1. *Advance MN-relevant and Advanced Material Considerations in AOP Development*
Success of the AOP framework requires continued development of the pathways that incorporate consideration of MN toxicity.
2. Utilise Existing Data from Literature and Previous Projects
Existing data already generated for MNs should be used to advance knowledge and identify opportunities for AOP development. This requires extensive expert-driven curation efforts.
3. *Promote Reliable and Quantitative MN Data Development*
Data development needs are essential to ensure MN-relevant AOPs can be developed and used in decision making. Guidance is needed on the types of data and reporting standards to enable AOP use in regulatory decision making. Efforts should be coordinated among stakeholders to ensure efficiency and limit additional testing.
4. *Advance Knowledge of Quantitative Relationships Between MN Physical and Chemical Characteristics and AOP Elements*
A better understanding of the quantitative relationships between MN physical and chemical characteristics and toxicological outcomes is required.
5. *Identify Current Applications of the AOP Framework for MN Decision Making*
Current applications of the AOP framework (*e.g.* prioritisation, grouping and read-across) can be adopted into decision making.
6. *Establish Test Methods and Protocols Useful for MN Decision Making*
Test methods to accurately measure MN-relevant MIEs and KEs are required to advance use of AOPs as part of an IATA for MN decision making.
7. *Demonstrate Predictive Capability of AOPs and* In Vitro *Test Methods*
A coordinated effort is needed to ensure alternative testing strategies are predictive of adverse outcomes of regulatory relevance.
8. *Guidance to Facilitate Adoption of MN-relevant AOPs for MN Decision Making*
Efforts are needed to translate and incorporate advances in AOPs and related alternative testing strategies into regulatory decision making.
9. *Stakeholder Communication & Engagement on Use of AOPs for MN Decision Making*
To facilitate the development, adoption and use of the AOP framework for MN decision making, engagement of multiple stakeholders with a broad range of expertise is essential and coordination and cooperation are needed.

45. Actions addressing these central recommendations are organised by timelines expected for completion: short-, medium- and long-term.

Short-term Actions	Medium-term Actions	Long-term Actions
- Identify MN-relevant and MN-specific AOPs, KEs and KERs, including an assessment of the AOPs which have been officially OECD-endorsed, approved, under review and under active development for MN-relevance; - Compare MIEs, KEs, KERs and AOs identified for MNs to AOPs in the AOP Wiki; - Identify MN-relevant MIEs, KEs, and KERs. Research should include identifying MN-relevant mechanisms and MIEs, which are often due to physical interactions with MNs instead of molecular ones (e.g. frustrated phagocytosis and particle-surface induced reactive oxygen species); - Identify screening-level MN safety decisions that are fit-for-purpose/can rely on AOPs; - Incorporate AOP elements into grouping and read-across decision trees for MNs; - Establish the types of data required to develop AOPs for MNs and identify existing NM-relevant AOPs; - Identify priority AOs observed with MNs and initiate research into AOP development for these AOs; - Evaluate, advance or develop physical and chemical characterisation protocols for MNs and how they can be used to identify MIE, KE and AO portions of the AOP framework; - Evaluate, advance or develop in silico, in chemico, in vitro and ex vivo assays for MNs and how they can be used to characterise MIE, KE and AO portions of the AOP framework;	- Develop and validate an IATA based on KEs, KERs and AOPs that can be used in risk assessments of new nanoscale materials. This includes identifying and prioritising which KEs are critical for testing as part of an IATA; building on the work currently ongoing in NanoSolveIT; - Create voluntary standard methods for IATA; - Consider formal adoption of IATA for certain MN hazard or risk decisions; - Identify test methods (including in silico) for high throughput screening; - Advance new in vitro test development to screen for MIEs and KEs; - Compare the predictive capability of in vitro assays for MIEs and KEs with in vivo observations or epidemiological data; - Identify similarities and differences in MIEs, KEs, KERs between other emerging advanced materials and MNs; - Identify novel biomarkers for hazard evaluation; - Design and conduct side-by-side in vitro and in vivo testing for representative MNs to compare toxicity mechanisms and potency across MNs and assays (furthering the work of projects that have begun this effort such as PATROLS); - Test predictive physical and chemical relationships of MNs to biological outcomes using carefully controlled changes within and across materials (furthering the work of projects that have begun this effort such as SmartNanoTox and nanoMILE);	- Advance modeling and QSAR databases and link to physical and chemical attributes of MNs; - Create processes to continually update publicly available databases as new data is developed; - Develop QSARs as predictive tools for KEs; - Develop and test predictive alternative testing models; - Validate predictive alternative testing models; - Develop a testing strategy for advanced and hybrid materials (smart and responsive materials) to identify and quantify MIEs, KEs, KERs and AOs; - Develop OECD Test Guidelines for MNs that relate to MIEs, KEs and AOs; - Adopt harmonised, standardised tests for high throughput screening; - Where appropriate, formally adopt IATA for MNs; - Develop data sets for qAOPs that include consideration of exposure conditions necessary for MN risk assessment. This includes adopting formal definitions and structure for qAOPs and developing case studies outlining the development and use of qAOPs for MNs; - Convene experts to discuss how AOPs can account for individual (*e.g.* sex or life-stage) and inter-species variation that exists and can thus be used to reduce the associated uncertainty for decision making; - Incorporate technical developments into specific regulatory guidance/policy documents;

- Prioritise KEs and the assays/methods to characterise them for development, with KEs closer to an AO prioritised to ensure relevance for regulatory decision making as part of an IATA; - Evaluate data quality from the peer review literature and current suite of in vitro assays based on identified KEs; - Encourage researchers (and publishers) to make raw toxicology data from peer reviewed literature available publicly; - Harmonise formats for reporting toxicology data (including negative results) to facilitate database development; - Standardise the endpoints and reporting elements of assays evaluating MIEs, KEs and KERs to ensure high quality, comparable data is generated, published and added to databases, including reporting of negative data; - Build searchable databases for priority MNs that includes funded research and literature (e.g. available data developed under NanoCommons, NanoReg2, GRACIOUS and DaNa projects) as well as traditional chemical databases (e.g. TOXCAST). Efforts should include collecting negative data; - Develop guidance on the types of data that need to be developed and reported by the research community for their work to be useful in regulatory decision making; - Review findings of existing data and research on the relationships between physical and chemical properties and MN KEs, including MIEs, AOs and KERs; - Develop hypotheses of predictive relationships between MN physical and chemical properties and biological outcomes; - Initiate Test Guideline development for assays tailored to MNs that address considerations such as physical and chemical characterisation, dispersion and dosing relevant to AOPs;	- Assess the importance of using alternative dose metrics to mass (e.g. surface area, particle number) in predicting toxicological outcomes for MNs; - Where appropriate, incorporate alternative dose metrics into developed benchmark levels for MNs for screening and risk assessment; - Conduct targeted research on MNs to elucidate the effect of interspecies variability on AOPs and related testing strategy development (Saber, Poulsen et al. 2019; Vogel and Cassee 2018). This can include side-by-side testing of in vitro cell lines and 3D models from a number of species exposed to a suite of MNs to examine conserved mechanisms and potencies; - Use 'omics' approaches to identify interspecies variability in gene, protein and metabolite responses to MN exposure (e.g. using heatmaps) and their implications for AOP development (Labib, Williams et al. 2016); - Broaden access and use of existing data sources (e.g. Nanomaterial-Biological Interactions Knowledgebase, Nanomaterials Knowledge Informatics Commons (NIKC); eNanoMapper, NanoCommons KnowledgeBase) and other resources; - Develop guidance on how the existing nanotoxicity literature, despite documented limitations (e.g. minimal reporting of physical and chemical characteristics), can be used for AOP development and decision making; - Develop research projects to fill data gaps for identified endpoints. - Evaluate publicly available REACH data for MNs in terms of use in AO and predictive modeling; - Develop data to allow for grouping – data collection/mining to determine the mode of action using MNs that can represent groups of MNs/ functionalisations;	- Adopt iterative decision making, including increased confidence in non-traditional methods and use non-traditional data and methods to improve weight-of-evidence in decision making.

- Develop guidance on the minimum level of validation required to use a given in vitro assay or method for regulatory decision making; - Assess the strength of evidence for considering dose-response relationships in AOPs as predictive tools for MN risk assessment; - Develop communication and educational materials on the use of the AOP framework for MN decision making for non-technical stakeholders; - Organise additional workshops targeted to encourage participation from various stakeholders with a vested interest in AOP development and application for MN decision making including academics, policy-makers, regulators, and industry.	- Adopt a testing scheme/decision tree for MN grouping and read-across; - Develop guidance and case studies for use of AOPs in regulatory decision making (e.g. MN prioritisation; grouping, categorisation and read-across; and hazard identification and ranking); - Develop guidance for risk assessors on developing an IATA based on AOP frameworks to complete MN safety assessments. It should include how to pick critical KEs, or a suite of KEs, for testing; - Develop MN specific resources (for the AOP Wiki) to encourage coordination and cooperation among stakeholders needed for efficient, high-quality AOP development.	

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