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Adverse Outcome Pathway External Review Report

AOP 173: Substance interaction with the lung resident cell membrane components leading to lung fibrosis

Short name: Substance interaction with the lung cell membrane leading to lung fibrosis

This document has been prepared by the review manager of AOP 173 scientific review.
It compiles the views and comments of the reviewers and explains how the authors of the AOP plan to address these comments.
It provides the basis to EAGMST for determining if AOP 173 has been adequately revised by their authors following the review and if it can be released to the Working group of the National Coordinators of the Test Guidelines Programme and to the Working Party on Hazard Assessment for endorsement.

Enter contact names here.
Nathalie Delrue: Nathalie.delrue@oecd.org

External review of AOP 173: Substance interaction with the lung resident cell membrane components leading to lung fibrosis

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1. Introduction and background to AOP 173

Background

The project for development of the AOP173: *Substance interaction with the lung resident cell membrane components leading to lung fibrosis* was submitted to the AOPs Development Programme in 2015 (project 1.32) by Canada.

AOP173 has undergone an internal review and modifications in early 2018 (Internal review AOP 173). Based on these, the Extended Advisory Group for Molecular Screening and Toxicogenomics (EAGMST) agreed at its June 2018 meeting, that the draft AOP173 was a candidate for external review. Scientific review started in November 2019 on the AOP173 snapshot from 13-12-2019 [PDF].

A scientific review panel (Annex1) was selected by an independent review manager based on the positive response to the call for experts by the OECD secretariat.

The review panel was charged with reviewing the scientific content of the draft AOP based on the charge questions (CQ) previously agreed by the EAGMST:

CQ1 Scientific quality:

- Does the AOP incorporate the appropriate scientific literature?
- Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?

CQ2 Weight of evidence:

- In your opinion, is the rationale for the weight of evidence judgement/scoring well described and justified based on the evidence presented? If not, please explain?
- Please consider for each KER and the AOP as a whole

CQ3 Additional observations:

- Do you have any additional observations or comments for the authors (e.g., what do you consider to be critical data gaps and how might they be filled)?

The review was conducted during December 2019 and March 2020. Based on the initial responses to the charge questions (Annex 2) main issues (Section 2) were discussed at a teleconference on 10 February 2020 (Section 3). Based on the discussion at the teleconference, further written discussion and agreed actions (Section 5), authors outlined a summary of planned revisions (Section 5) to include in the AOP before its submission to the EAGMST.

Introduction

AOP173: *Substance interaction with the lung resident cell membrane components leading to lung fibrosis* (short title: Substance interaction with the lung cell membrane leading to lung fibrosis) includes the description and assessment of the critical elements of the pathway initiated by the interaction of a range of different substances with the different membrane components of resident lung cells leading to activation of the endogenous inflammatory processes which if unresolved results in lung fibrosis.

The Molecular Initiating Event (MIE) of AOP173 (Figure 1) in the review draft is described as a set of interactions of different types of respiratory stressors (bleomycin, carbon nanotubes[1], carbon nanofibres, specified in the review draft) with different cell membrane components. Interactions covered by the MIE include nonspecific physico-chemical interactions of the fibrogenic stressors with the lipid and protein components, but also more specific, receptor interactions mediated e.g. via the Toll Like Receptors (TLR) and Scavenger Receptors, by the results of the non-specific interactions: frustrated phagocytosis and /or release of DAMP[2]s (alarmins) from dying or injured cells. Such molecular interactions with the lung resident cells (airway epithelial cells, alveolar, interstitial macrophages and dendritic cells) initiate activation of intracellular signalling and gene expression pathways that lead to synthesis of pro-inflammatory mediators (e.g. IL1α) characteristic for the innate immune response and normal process of tissue repair.

The pro-inflammatory mediators signal further recruitment and proliferation of bone-marrow originating pro-inflammatory cells (macrophages, neutrophils) to the lungs. Under conditions of continuous stimulus or persistent stressor, the non-resolving inflammation leads to further tissue injury including loss of the integrity of the alveolar capillary membrane and activation of T-helper type 2 (Th2) cells at the site of injury. The Th2 adaptive immune response is marked by release of anti-inflammatory and pro-repair/fibrotic mediators stimulating proliferation and differentiation of fibroblasts and myofibroblasts which deposit excess of extracellular matrix components (ECMs) in the alveolar space, thus causing alveolar septa thickening, decrease in total lung volume and lung fibrosis (Adverse Outcome). These histological changes at the organ level interfere with the critical function of gas exchange in the lungs ultimately leading to increasing mortality at organism level.

[1] Single-welled and multi-walled

[2] Damage-associated molecular patterns

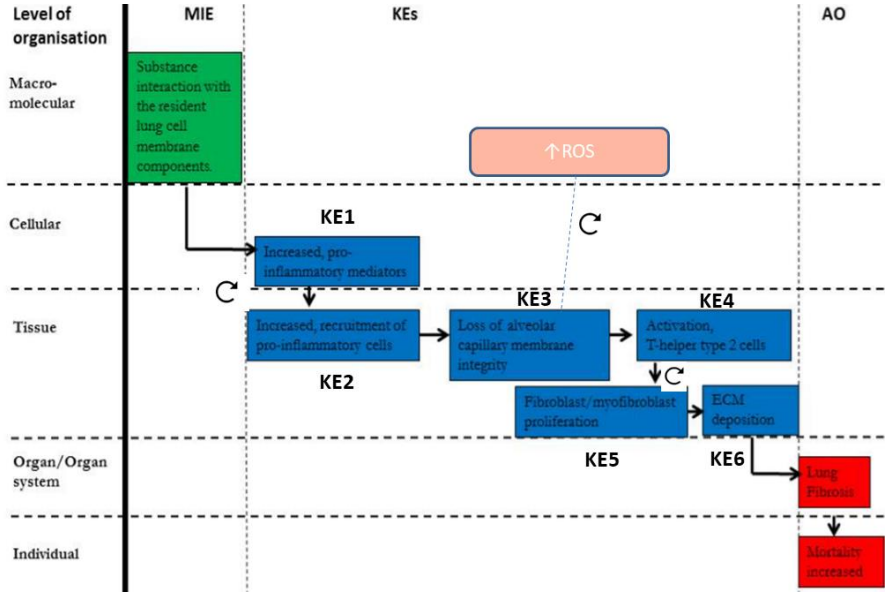

Figure1: Graphical representation of the components of AOP173. Feed forward loops are included in this graph (they are not on the AOP wiki) based on the discussion in the AOP draft text

Pro-fibrotic stressors linked to the perturbation of the inflammatory pathway and the adversity in the draft AOP173, include substances with varied physico-chemical properties, including insoluble particulate substances such as silica dust, asbestos, carbon nanotubes, multi-walled carbon nanotubes (MWCN), single-walled carbon nanotubes (SWCN), carbon nanofibres but also soluble substances such as Bleomycin. However, the AOP was specifically assembled keeping in mind a novel class of engineered materials (nanomaterials) exhibiting sophisticated properties that have been shown to induce lung fibrosis via this mechanism.

Overall weight of the evidence (WoE) for the draft AOP173 was assessed as high, based on the evidence for each KER being scored as high. Authors present AOP173 as qualitative. However, the evidence for most KERs was assessed as providing high understanding for the quantitative dose response, except MIE-KE1 (unspecified) and KE3-KE4 (moderate).

Evidence from a number of studies with mammals was used to support the wide taxonomic applicability of the AOP, from rodents to humans. Notably, sex and age differences have been observed in mice and humans indicating that aged males are more susceptible to chemically induced as well as idiopathic pulmonary fibrosis.

Essentiality of the particular KE was not assessed individually but as part of the overall weight of evidence for the KERs. Individual KERs are also not described in individual KER Wiki pages. Authors argue that animal or cell culture experiments are generally not designed to measure parameters that inform these KEs separately and as a result, there is not enough empirical support to build individual KERs.

Certainly, the process of development of AOPs, including the complex

inflammatory hub, has been challenging (Villeneuve et al., 2018[3]). Inconsistencies in the evidence for AOP173 with some stressors is discussed as possibly due to the multifaceted and interlinked signalling pathways associated with the innate and adoptive immunity. Indeed, evidence discussed in AOP 173 indicates that KE1 and KE2 act in a positive feedback loop mechanism and propagate the pro-inflammatory environment while KE4 and KE5 can function in parallel in a positive feedback loop perpetuating and magnifying the response at each stage. Furthermore, the infiltrating inflammatory cells (neutrophils and macrophages) generate reactive oxygen species (ROS) in KE3, leading to increased airspace epithelial permeability, increased cell death and increased expression of pro-inflammatory genes, all of which lead to secretion of inflammatory cytokines/chemokines generating another self-perpetuating loop that results in prolonged and chronic inflammation.

Some knowledge gaps are identified to be targeted in the future, including additional studies to support the essentiality of the KEs and to build KERs as well as better elucidation of the mode or type of interactions between the resident cell membrane and a substance.

[3] Villeneuve et al., 2018. Representing the Process of Inflammation as Key Events in Adverse Outcome Pathways. Toxicological Sciences, 163(2) 346-352.

2. Synthesis of main issues of the review

Individual review comments are available in Annex 2 of this report.

Summary of responses to CQ 1 - Scientific Quality

Overall initial reviewers' comments acknowledged the extensive coverage of the scientific literature and evidence covering the inflammatory process leading to lung fibrosis in AOP173. However, additional references were suggested (some listed in Annex 2) to beater cover some aspects, such as:

- Lung fibrosis in general, including cellular pathophysiology
- Evidence related to other relevant (nano-)material characteristics leading to lung inflammation and fibrosis (e.g. ROS generation by metallic contaminants of CNTs), and other cellular consequences of interaction and uptake (e.g. apoptosis, frustrated phagocytosis)
- Literature addressing the limitations and advantages characteristic for the specific models used in the studies

Review comments acknowledged that the scientific content of AOP173 generally reflects the current scientific knowledge relating to lung fibrosis mediated by inflammation. However, the following uncertainties were highlighted:

- Lack of clarity about the specific molecular interaction(s) covered by the MIE
- Chemical applicability domain: link to other potential stressors and associated evidence/literature (e.g. nanostructured metal oxides)
- WoE for the essentiality assessment of the early KEs
- Limited evidence for any particular (or combination of) inflammatory mediators specific for the AOP173
- Limited discussion for the role of particular cell types at different points of the AOP
- Need for better representation, graphical and also in discussing the rationale, for the persistency/reversibility of the inflammatory process, and for the positive feedback (feed-forward) already discussed in the AOP
- Limited evidence supporting the quantitative linkages between events in the AOP

Summary of responses to CQ 2 - Weight of Evidence

Acknowledging the complexity of the inflammatory process in the lungs and the available evidence, reviewers identified few aspects of the justification description

and scoring for the weight of evidence calls that may need additional considerations and/or revisions:

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- Representing the rationale and the related evidence in tabular format consistent with the AOP User's handbook, both for KEs and KERs.
- Further supporting the rationale by including additional evidence from occupational and other relevant human epidemiological studies, (e.g. evidence with inhalation of asbestos / silica and for cigarette smoking)
- Downgrading the High WoE call for the linkages between KE1-KE2-KE3 to moderate.

Summary of responses to CQ3 - Additional observations

Most of the additional considerations emphasised the uncertainties already identified by CQ 1 and CQ2, including: (i) uncertainties with the evidence to support the essentiality of the early KE, (ii) challenges with availability of appropriate test models to address specific elements of AOP173; (iii) the uncertainty about the specificity of particular mediators and (iv) the role of different cell types in the pro-fibrotic inflammatory process and, (vi) the lack of clarity about a defined and measurable molecular mechanisms covered by the MIE. It was suggested that these uncertainties could be addressed by inclusion of relevant evidence from studies with other stressors. Alternatively, one reviewer suggested that omitting the MIE from AOP173 could result in better representation of the inflammatory process leading to inflammation mediated lung fibrosis with the current evidence.

Better/more detailed graphical presentation of the AOP elements was brought up in the additional considerations again.

In addition, two reviewers suggested that oxidative stress and the role of ROS should be considered as an early KE of AOP173 in addition to the ROS association with KE3.

Also, a suggestion was given for consideration of additional methods for measurement of KE2: Increased, recruitment of inflammatory cells, including co-culture transwell chemotaxis assays and differential expression of integrins/selectins (literature reference provided by the reviewer in Annex 2).

It was also noted that in the assessment of the evidence from the in vivo studies, little consideration is given to the time-course of the occurrence of different KEs. This is specific aspect of the uncertainties already raised for the WoE evidence and rationale under CQ2, but as additional consideration emphasises the need for time course considerations to better inform new experimental model designs.

The need for considerations and better understanding of the dose-response relationship in defining the delivered dose/concentration relevant to the adverse outcome was raised in relation to AOP173 and the AOP framework in general.

The complexity of the inflammatory process in the lung and its linkages to the other related processes, including carcinogenesis, emphysema, asthma but also resolution of the process, was emphasised as a point for consideration in the future.

Finally, one reviewer suggested that in would be useful to farther clarify the potential applications related to AOP173.

3. Summary record of the teleconference

End-of-review teleconference (TC) was attended by all reviewers, two authors and the review manager (Annex 1).

Before the TC authors provided some general and specific responses (Annex 3) that were a starting point for the discussion.

For the end-of review teleconference participants agreed that comments focused roughly around nine, to a large extent interlinked, aspects (items a. to i. Section 3.1),.

3.1. TC agenda

Agenda for AOP 173 end-of-review teleconference discussion

10 February 2020, 2-5pm Paris time

1. Introduction of participants
2. Short introduction by RM
3. CQ1 and elated additional observation

a. <u>Incorporation of additional literature re:</u>

Some references provided by reviewers, discuss path forward for potentially expanding the coverage of specific aspects (identify critical aspects needing additional coverage) – **Comments No: 1,2,3,11,12**

- fibrosis AOP and cell pathophysiology
- relevant (nano-)material characteristics
- cellular consequences of interaction or uptake
- limitations related to specific models used in the studies (in general limitations and advantages of the different methods described as used for measurements?) – also Com: 32

b. <u>Reflecting the current scientific knowledge relevant to the AOP:</u>

(How) can the content be structured/clarified to better reflect the relevant knowledge and address the points raised in relation to: **Comments no: 5, 6, 7, 8**

- link between significantly different stressors and MIE/KEs
- description of the MIE and early KEs (also Q3 points No: 23, 29 oxidative stress?)

- essentiality assessment for KE
- inflammatory mediators specific for the AOP
- applicability domain (cell types?)
- Feedback loops/reversibility or progression

c. Evidence supporting Quantitative Understanding: (comments No: 9)

- Rationale for High/Moderate Quantitative Understanding (QU) calls in the KER table sufficient?
- Can QU description for AOP173 be improved at present point, and if so how?

4. CQ2 – rationale for WoE calls for KERs and for AOP173 overall

d. Evidence supporting essentiality of KE

- General: Formatting/representation of evidence (Comment No: 14)
- Specific: additional evidence (toxicogenomic & from human ocup. exposure) related to different stressors (Comment No: 15, 19)
- Evidence for stressors specifically driving lung fibrosis and not cancer: is it possible to emphasize/clarify? (Comment No: 17)

e. WoE calls

- KER1703: KE1 (Increased, secretion of proinflammatory and profibrotic mediators) directly leads to KE2 (Increased, recruitment of inflammatory cells) – Comment No: 13
- KER1704 KE2 (Increased recruitment of pro-inflammatory cells) directly leads to KE3 (loss of alveolar capillary membrane integrity) - Comment No: 18

f. Inconsistencies in the evidence linking inflammation with pulmonary fibrosis (Comment No: 16)

Inconsistencies in the evidence are discussed in the draft AOP overall assessment page. Discuss how to improve or reflect within the WoE calls.

5. CQ3 – Additional observations (not discussed under CQ1)

g. Additional assays for measurement of KE 2 (1497): Increased, recruitment of inflammatory cells (Comment No: 24), e.g.

- co-culture transwell chemotaxis assays
- differential expression of integrins/selectins

h. **Additional considerations for time-response relationship(s) Comment No: 31)**

i. **Increase usefulness/clarity; Reduce complexity: (Comment No: 23, 26, 29)**

- Limit the AOP evidence to particular type of stressor (CNT proposed)
- Reconsider oxidative stress in this context
- Could this be addressed by potential revisions streaming from CQ1 and CQ2?

3.2. Main issues and responses during the call

The discussion loosely followed the agenda as issues were interlinked, particularly literature coverage for the evidence and WoE and additional considerations streaming from that.

Coverage of the evidence

General
At the start, reviewers reiterated their comments to include additional literature regarding the biology of lung fibrosis earlier in the AOP Background to clarify also some aspects of cell types involved. In particular it was noted that the role of the different cell types (epithelial type 1 and II and macrophages) was missing.

In fact the roles of the different cell types in the different events along the AOP173 was a major topic of discussion during the TC, it come up at different points of the agenda and this was recognised as very important issue to cover better in the AOP, even as a knowledge gap based on the evidence specific for AOP173.

Authors pointed out that one of the issues raised during the internal review was that there is too much text book biology in the AOP and so this was taken out. However, they recognised that further discussion regarding what is known about the roles of the different cell types in the lung fibrosis could increase clarity and they agreed to consider the suggested references, and look for newer references to include. They also agreed to consider the suggested references and revisit the background to include some of them earlier in the AOP, even before discussion on specific KE.

In addition, the author informed the group that there is ‘omics’ evidence including from their lab, that addresses lung fibrosis “signature” using in vivo and ex-vivo models and single cell genomic analysis. This evidence (under review) is expected to shed more light on the type of lung cells and also genes (therefore potentially mechanisms) affected by pro-fibrotic stressors (CNTs and bleomycin tested).

Reviewers encouraged the authors to consider including the ‘omics’ evidence in the revised AOP173, if possible, as it may address a lot of the uncertainties identified in the current draft, including quantitative understanding (see below, under the WoE assessment).

Evidence for range of stressors

Another recurring and intensely debated topic in the discussion was the coverage of the evidence for lung fibrosis from studies with different stressors. One reviewer argued for inclusion of evidence from human epidemiological studies with silica, asbestos and cigarette smoke exposure, and another for inclusion of human and animal studies with exposure to persisting metallic oxide dust. The request for inclusion of this evidence was justified by: (i) the MIE is unspecific and allows for inclusion of stressors instigating different initiating mechanisms/events that result in pro-inflammatory and pro-fibrotic response, (ii) the WoE for inflammation mediated lung fibrosis would be strengthened by adding the evidence in the context of the current MIE.

Author argued that the evidence for some of the stressors, particularly related to metals and metal oxides was not considered as their major effect is not lung fibrosis but emphysema and there is another AOP under development dealing with the latter AO. Some evidence for silica, asbestos and cigarette smoke is already included in AOP173, but it was agreed to consider where it can be used to strengthen the WoE for particular aspects of the AOP.

Molecular Initiating Event
There were divergent opinions on the value of having such mechanistically unspecific MIE, encompassing different events, some measured directly (e.g. frustrated phagocytosis), some indirectly (e.g. activation of TLR responsive genes), and others by the markers also linked to the common consequence KE1, i.e. release of pro-inflammatory mediators. It appears that during its long history within the AOP programme, the AOP has gone through different stages, including a stage where the increase of pro-inflammatory mediators was the first event. The draft for internal review had the same MIE and although the reviewers had similar discussion it was finally decided not to revise the MIE. Therefore the author was reluctant to revise the AOP with regard to AOP173 and since the group could not reach a final agreement it is considered appropriate that the wider EAGMSTG discusses the issue and decides on the best way forward for AOP173 in regard to the issues raised by the reviewers for the MIE.

Early KEs – persistency of inflammation
The discussion regarding the coverage of the evidence for relevant stressors also touched on the importance of the exposure aspects relevant to the outline of the AOP presented for review. One reviewer questioned using the evidence from bleomycin studies particularly given the unspecific description of the MIE and also the main intended applicability of the AOP in the context of the WPMNM.

Considering that AOPs should be stressor agnostic, it was agreed by all that bleomycin represents a well-studied typical stressor for lung fibrosis mediated via inflammation and that the evidence is valuable even though the primary site of exposure, and therefore the cells and interactions at different early KE stages, may not be the same. The author however agreed that it could be helpful to include additional information to clarify: (i) that the AOP including the particular MIE, is relevant to persistent stress(ors) associated with inhalation exposure and, (ii) how the evidence included in the AOP for other known types of exposure (bleomycin) and even unknown (e.g. environmental risks and ILF) links to the critical KE hub of the AOP i.e. the inflammation as a common and underlying principle. It was left for the author to find the best place to include these considerations but early in the background and the summary was suggested.

ROS and oxidative stress

Given the central role of the inflammatory hub in AOP173, the role of the oxidative stress and the reactive oxygen species (ROS) generated in the process was discussed extensively and at different points of the agenda. Reviewers argued that:

(i) persistent/not-resolving oxidative stress could be better covered in terms of outlining the feed forward loops between KE in the inflammatory hub leading to fibrosis,

(ii) there is a place and evidence for additional consideration of the role of ROS earlier in the pathway, namely ROS generated by the initial stress (by some stressors) as well as the ROS generated during the later events during the inflammatory pathway.

Author maintained that the major inflammation persisting/potentiating role of ROS is related to KE3 as currently outlined and that the 'acute' or early generation of ROS is indicative but not sufficiently predictive of the AO. However, it was recognised that additional consideration could be included to address role of ROS released later in the inflammatory process on the earlier KEs and potentiating the early KER linkages, thus contributing to persistence of the process which is critical in driving the pathway to the final AO as opposed to resolution.

WoE of evidence assessment:

All participants noted the inconsistency of the 'High' WoE calls in the AOP tables throughout the draft document, with the statements about the limited availability of evidence to address particular early KE linkages, in the draft text as well as in the initial responses by the author (Annex 3). In relation to this, some reviewers suggested downgrading the 'High' to 'Moderate' for the KERs involving the early KEs, MIE-KE1-KE2-KE3.

The author indicated that the current 'High' evidence calls may be an unchanged default option due to technical issue and that in fact 'Moderate' would be more appropriate for these KERs. However, considering the discussion about expanding the coverage of the evidence (above) it was agreed that the author would revisit the WoE calls also providing the rationale more specifically considering the handbook guidance. It was agreed that it would be very helpful to better communicate the rationale for the calls, if at least some relevant text essential to the KER WoE analysis from the external overall AOP WoE assessment table is transferred to the KER specific pages, most of which are unpopulated in the current draft.

The quantitative understanding calls of the KERs (mostly 'High') were also discussed. Author explained that the rationale for these calls was that there is evidence showing dose response even though not to the extent that would allow bench mark dose identification. While participants agreed that most AOPs are at a similar point of understanding, if rationale is better specified to align the assessment criteria for the call with the available evidence, the calls would be clearer and more consistent.

It was again agreed that the omics data, if possible to include, could strengthen the rationale for the quantitative understanding calls. It was recognised however, that the AOPs are living documents and this info may be included at a later stage. The AOP provides a scaffold for inclusion of such data to further support the usefulness of the AOP.

Time response relationship (item h) was not specifically discussed at the TC due to time constraints together with the complexity of the issue. Following the TC the reviewer indicated that no further discussion is necessary as the time aspect is difficult always, and particularly with inflammation as

a process that loops, and it would be difficult to expand more than it already is in the draft AOP, namely there is short reference to this issue in the overall assessment under Concordance of dose and time-response relationships.

Additional considerations:

Additional Assays for measurements of KE or combinations
With regard to the assays described to measure particular KEs along AOP173 and considering the limited evidence for the role of specific types of cells and mediators (discussed above), it was suggested by reviewers to consider including some specific co-culture methods (some references provided with the initial comments Annex 3).

Authors agree to consider them even though for simplicity they prefer to keep the assays to the most commonly used at present. It was again pointed out that more evidence would be available from the 'omics' data and that these methods could be included in the future. However it was agreed that the proposed references would be considered.

Usefulness and gaps
Recognising that identification of knowledge gaps is one of the important aspects of the AOP development and usefulness, it was suggested that emphasising the gaps in the evidence and methods addressing specific relevant cell types and mediators could represent a particular advantage of the current AOP, that would help drive appropriate assay development to address existing evidence gaps.

Finally the usability/usefulness of the AOP173 was discussed, i.e. how it can be used/applied with the present stage of development taking into account revisions based on this review. Reviewers agreed that AOP173 represents a good starting platform to develop new testing methods to further elucidate the details of the critical KEs in the pathway (specific cell types, modulators) and also guide testing approaches for more materials in the future. Reviewers urged authors to peruse the revisions as agreed to add value to this important pathway that will represent the scaffold for the future development of other important converging and diverging AOPs.

3.3. Action list

For Authors

1. Revise early section of the AOP (Background and early in overall AOP assessment) to discuss and cite literature on general lung fibrosis, role of epithelial type I versus type II cells, macrophages and fibroblasts in the response to injury and also in promoting injury and inflammation. Consider specific references provided by reviewers in Annex 2, but also cite already included literature that is considered later in the AOP.
    - Also expand the AO event with literature general references and discussion on human biology relevant to fibrosis
2. Consider using the 'omics' data including from the authors lab (if published before submission to the EAGMSTG to support the KERs and the quantitative understanding.

3. Include additional evidence from human and also animal studies with persisting metal oxide dusts, asbestos, silica and cigarette smoke, that support particular KERs, including quantitative understanding in the inflammatory hub **and/or** the final AO, as appropriate. Add references particularly on metallic oxide NMs provided by reviewers following the TC (see section 5 on further discussion).
4. Clarify the role of ROS in the AOP173. How it connects to earlier events and its role in propagating the inflammation through positive feedback (feed forward) loop. Provide a schematic to clarify the point.
5. Clarify that the AOP is relevant to persistent stress(ors) associated with inhalation exposure and improve/clarify the discussion of how the evidence included in the AOP for other known types of exposure (bleomycn) and even unknown (e.g. environmental risks and ILF) links to the critical KE hub, the inflammation as a common and underlying principle.
6. Revise the WoE calls for KERs between early KEs, MIE-KE1-KE2-KE3, based on the discussion and the initial responses which point out limited evidence in relation to these early KERs. Suggestion was made to revise to Moderate.
    - Re-visit the supporting evidence and provide the appropriate (wording) for the rationale based on the Users's handbook.
    - Transfer the relevant content from the external Table 1 to the relevant KER pages as much as possible.
    - For quantitative understanding specify the rationale using the guidance from the Handbook. Not necessarily downgrade needed but the rationale should be clearly stated in the KER pages.
7. Revisit the assessment/rationale of the essentiality of KEs according to the scoring suggested in the handbook. Even if evidence is, scarce, indirect or points out to inconsistences, use it as a rationale to provide the score rather than omit it.
8. Include and reference to newer cellular (co-culture) models in the relevant KEs (how it is measured).
    - reference better the section(s) dealing with limitations of certain models used in the study of lung fibrosis
9. Expand the *Uncertainties, inconsistencies and data gap* section with the discussion about the knowledge gap with regard to the specific involvement/relevance of particular cell types and mediators for lung fibrosis and the on-going attempts to address them in terms of assay availability and development.

For EAGMSTG

1. The wider EAGMSTG should consider the different opinions expressed described in Section 3.2, in relation to the evidence for the range of stressors covered, the mechanistic coverage of the MIE and the complexity of the pathway, particularly the early elements of inflammation, and provide advice on the way forward for AOP 173:
    - can the MIE be considered sufficiently consistent with the overall AOP framework, considering the specificities of the stressors/interactions discussed?

- if not, is AOP173 a special case that will help the development of AOPs within the WPMN community?
- if approved following the revisions recommended by the review but with the current mechanistic coverage of the MIE, what would be the steps needed to encourage and monitor the development of this living document to support its relevance and usefulness in the wider community, if applicable?

4. Summary of (planned) revisions

1. Revise early section of the AOP (Background and early in overall AOP assessment) to discuss and cite literature on general lung fibrosis, role of epithelial type I versus type II cells and fibroblasts in the response to injury and also in promoting injury and inflammation. Consider specific references provided by reviewers in Appendix X, but also cite already included literature that is considered later in the AOP.
    - Also expand the AO event with literature general references and discussion on human biology relevant to fibrosis

Planned revision (feedback from authors): The reviewers have agreed that the AOP173 document is already comprehensive and has covered the literature as extensively as possible. Thus, the suggestion to include additional references will be reviewed and where possible or necessary an attempt will be made to include them. The description of AO will be expanded to include human physiology and the cell types potentially known to be involved in the human lung fibrosis adding relevant references. However, we do not agree that the AOP should capture literature covering historical perspectives to current state-of-the-art. AOPs should not be viewed as review documents and it is not necessary to cite all published literature. Moreover, background section in the AOP document is optional.

2. Consider using the 'omics' data including from the authors lab (if published before submission to the EAGMSTG to support the KERs and the quantitative understanding.

Planned revision: One of the reviewers suggested to include one of the omics papers from our lab in support of weight of evidence for KEs. We will review the suggested reference and add it to the weight of evidence table.

3. Include additional evidence from human and also animal studies with persisting metal oxide dusts, asbestos, silica and cigarette smoke, that support particular KERs, including quantitative understanding in the inflammatory hub **and/or** the final AO, as appropriate. Additional references particularly on metallic oxide NMs provided by reviewers following the TC (See section 5 on further discussion).

Planned revision: As agreed on the call, if a suggested study supports weight of evidence and if it is missing from the present document, we will add that study to the list; however, we do not agree to conduct systematic literature review to build an exhaustive weight of evidence table. We do not agree that lung fibrosis is a primary adverse outcome for several of the metal oxides. One of the reviewer was in agreement with our argument that the primary adverse outcome for metal oxides is emphysema and not lung fibrosis. Thus, additional literature pertinent to metal oxides will only be included if supportive of weight of evidence in this AOP. Again, systematic review will not be conducted to update the reference list. We also do not believe that it is necessary to name all stressors.

4. Clarify the role of ROS in the AOP173. How it connects to earlier events and its role in propagating the inflammation through positive feedback (feed forward) loop. Provide a schematic to clarify the point.

Planned revision: Many xenobiotics including the stressors named in this AOP are capable of inducing ROS acutely after exposure, which serves as signalling mechanism to alert the organism

of the impending invasion and signal the inflammatory response. However, ROS is not an absolute requirement at this stage for initiating the inflammatory cascade but if present, can help build the acute inflammatory response. Thus, authors are of the opinion that ROS should only be described as a detrimental associative event in the presence of continuing inflammation, injury and exposure. However, considering the reviewers comments, a brief description of this will be added in the beginning of the document and a separate schematic will be prepared to show how initial ROS synthesis works in propagating inflammation.

5. Clarify that the AOP is relevant to persistent stress(ors) associated with inhalation exposure and improve/clarify the discussion of how the evidence included in the AOP for other known types of exposure (bleomycin) and even unknown (e.g. environmental risks and ILF) links to the critical KE hub, the inflammation as a common and undelaying principle.

Planned revisions: A brief section will be added to explain how this AOP will is applicable to a wide variety of stressors of different properties. Bleomycin is included in the AOP as it is used as prototypic model for lung fibrosis and is one of the most commonly and extensively studied models. This will be clarified.

6. Revise the WoE calls for KERs between early KEs, MIE-KE1-KE2-KE3, based on the discussion and the initial responses which point out limited evidence in relation to these early KERs. Suggestion was made to revise to Moderate.
    - Revisit the supporting evidence and provide the appropriate (wording) for the rationale based on the Users's handbook.
    - Transfer the relevant content from the external Table 1 to the relevant KER pages as much as possible.
    - For quantitative understanding specify the rationale using the guidance from the Handbook. Not necessarily downgrade needed but the rationale should be clearly stated in the KER pages.

Planned revisions: WOE calls will be revised as appropriate. However, what is not clear is how many studies should be cited for WOE to be qualified as high.

The appropriate efforts will be made to present the KER information using the guidance provide in the handbook. Rationale will be clarified using the guidance provided.

7. Revisit the assessment/rationale of the essentiality of KEs according to the scoring suggested in the handbook. Even if evidence is scarce, indirect or points out to inconsistences, use it as a rationale to provide the score rather than omit it.

Planned revisions: Scoring suggested in the handbook will be considered in revising the essentiality assessment.

8. Include and reference to newer cellular (co-culture) models in the relevant KE (how it is measured).
    a. reference better the section(s) dealing with limitations of certain models used in the study of lung fibrosis

Planed revisions: New cell culture models will be added as appropriate and referenced under KE measurement. We are not sure if the AOP should discuss the limitations of certain models. Again,

this is not a review article and we can only list the assays that are most commonly used and are readily available for the assessment of KEs.

9. Expand the *Uncertainties, inconsistencies and data gap* section with the discussion about the knowledge gap with regard to the specific involvement/relevance of particular cell types and mediators for lung fibrosis and the on-going attempts to address them in terms of assay availability and development.

Planned revisions: We will add brief paragraphs categorically addressing some of the inconsistencies and data gaps discussed during the TC.

5. Further discussion

Following the discussion at the end of review teleconference and circulation of the draft report with planed revisions, one reviewer emphasised few points and provided additional references for consideration (added in Annex 2).

Discussion under *Evidence for range of stressors (Section 3.2) w*ith regard to the author's argument that the evidence particularly related to metals and metal oxides was not considered in the AOP173 as their major effect is not lung fibrosis buy emphysema:

Reviewer 1: This is also a matter of dose and retention/accumulation of the stressor (in compliance with the AOP hypothesis). One could also argue that lung cancers are the primary outcomes of stressors such as silica, CNT, asbestos and silica. In fact, "inert" metallic particles also induce inflammatory responses comparable to those mentioned for the above stressors.
Perhaps a differentiation is necessary to differentiate between focal and diffuse interstitial fibrosis.
This is not a trivial issue as, for example, it is currently not quite clear, if the fibrotic lesion of CNT is (also) due to the inherent cobalt contaminant (cf. hard metal lung disease).

Authors response
We disagree that we are required to cover every single chemical or a substance that has been shown to induce lung fibrosis. We also disagree that we have to distinguish the effects of metal contaminants of CNTs. For every reference that supports weight of evidence, there may be a study providing a counter argument. We have used best evidence available to support the mechanism presented duly noting the inconsistencies. As stated above in many places, if there is compelling evidence that clearly supports KERs, we will make a sincere effort to cite that study and make use of that information.
It is correct that anything that is inhaled has a potential to induce inflammation and this includes metals and metal oxides. But inflammation in each case has a different signature. For example, inflammation in emphysematic lung and fibrotic lung is different. Characterising the nature of inflammation and defining the threshold above which the disease is induced and below which the response is reversible, is what is needed. This is one of the challenges and AOP173 will initiate work in that direction.

The AOP presents a mechanism that involves early inflammatory component leading to fibrosis in lungs. Examples of different types of stressors that induce the disease via this mechanism is provided. I believe that substantial amount of evidence is presented to support the validity of the mechanism. We do not agree addition of more stressors would enhance the validity of the AOP.

Discussion under *ROS and oxidative stress (Section 3.2) w*ith regard to the author's argument that the major inflammation persisting/potentiating role of ROS is related to KE3 as currently outlined and that the 'acute' or early generation of

ROS is indicative but not sufficiently predictive of the AO:

Reviewer 1; This might be true. However, one might wonder, if "membrane interaction" is more predictive? ROS has the additional advantage of being measureable, and indicative of both stressor-inherent (acellular) and host defence (cellular) induced oxidative stress.

Generally reviewers urge the authors to carefully address action point 4 (section 4) and highlight (i) the role of ROS in the initiation of the inflammatory process in the lung leading to fibrosis; (ii) the interconnectedness of inflammatory and immunological processes at the organ (ling) and organism level.

Authors response
As already stated, the consequence of the interaction is measured and not the interaction itself. It is not about what is easy to measure but rather what is more predictive. As reviewer agrees that potentiating role of ROS is related to KE3, we believe measuring it as a MIE does not make it right. However, we are working on this specific point and how to best describe it and where does it serve best to measure it. Hopefully we will find a way to include it in a way it helps all.

Inflammatory process induced by the stressors described in this AOP can be independent of ROS and this is one of the reasons why we don't consider it as a MIE. There have been several efforts made by researchers with commercial incentives to design assays to measure ROS as predictive of a toxicity potential of nanomaterials and so far there has been no evidence to prove that it is predictive.

Please see responses drafted by us (Annex below) for more detailed response to this comment.

Planned revisions response under point 3 (Section 4) with regard to not conducting a systematic review for evidence related to additional types of stressors:

Reviewer 1: As it is a major objective of the AOP to provide alternative testing strategies for the large number of nanomaterials, available (in vivo) information on those related to lung fibrosis induction. A considerable number of commercialised nanomaterials are metals and metal oxides.

Review manager's note: In addition to the above, it is noted that the current draft AOP173 aims to be applicable to wide range of stresses as stated in the Abstract: Lung fibrosis is frequently observed in miners and welders exposed to metal dusts, making this AOP relevant to occupational exposures"; and in the Domain of applicability: "This AOP is applicable to occupational exposures as lung fibrosis is frequently observed in miners and welders exposed to metal dusts.". Therefore, the consideration of the additional evidence recommended by the reviewers, appears highly relevant for the AOP173.

Authors response
We will consider reviewer's comments and we will revise the document as necessary and we will submit the revised version at the earliest possible for further consideration.

6. Outcome of the external review

AOP173 tackles an important and complex toxicity pathway in the lungs leading to lung fibrosis that can be initiated by exposure to different toxicants, including the novel materials such as carbon nanotubes and other nanomaterials with varied physico-chemical properties.

In considerable detail, AOP173 describes the main components of the complex inflammatory process from activation of resident cells, release of pro-inflammatory mediators and recruitment/activation of leucocytes to the site of stress/injury to lung fibrosis. Therefore, the description of the KEs and KERs in AOP173 represents an important key element for the future building of the complex network of processes involved in the inflammatory response, its drivers and modulators in the lung and at the organism level.

While reviewers recognise that the issue of networking AOPs cannot be resolved at this stage, they encourage mindful revisions that would facilitate future developments to help unravel if we can predict whether a substance/particle will induce a specific AO or whether it can induce multiple AOs depending on the dose, deposited region, time etc. In addition, future network of AOP173 as its initial building block could guide the development of appropriate assays to measure the key events predictive of inflammation-mediated chronic health impacts, and aid in screening a large array of inhalation toxicants that are inflammogenic, for their potential to induce lung fibrosis and potentially other adverse effects related to inflammation in the lungs. Ultimately, it should facilitate developing of Integrated Approaches for Testing and Assessment that could inform regulatory assessment of nanomaterials.

Therefore reviewers urge the authors to consider the review discussion and revise AOP173 accordingly before submission to the EAGMSTG for consideration for endorsement.

Annex 1: Table of review participants

Reviewers	Affiliation
Barbara Rothen-Rutishauser	Adolphe Merkle Institute Université de Fribourg,CH
Frank Herzberg	The German Federal Institute for Risk Assessment (BfR), Germany
Hedwig Braakhuis	Netherlands National Institute for Public Health and the Environment, Netherlands

Authors	Affiliation
Sabina Halappanavar	Environmental Health Science and Research Bureau, Health Canada, Ottawa
Monita Sharma	PETA International Science Consortium Ltd., London, United Kingdom

Review Manager	Affiliation
Julija Filipovska	Independent Consultant

Annex 2: Individual reviewers' comments with initial responses from the authors

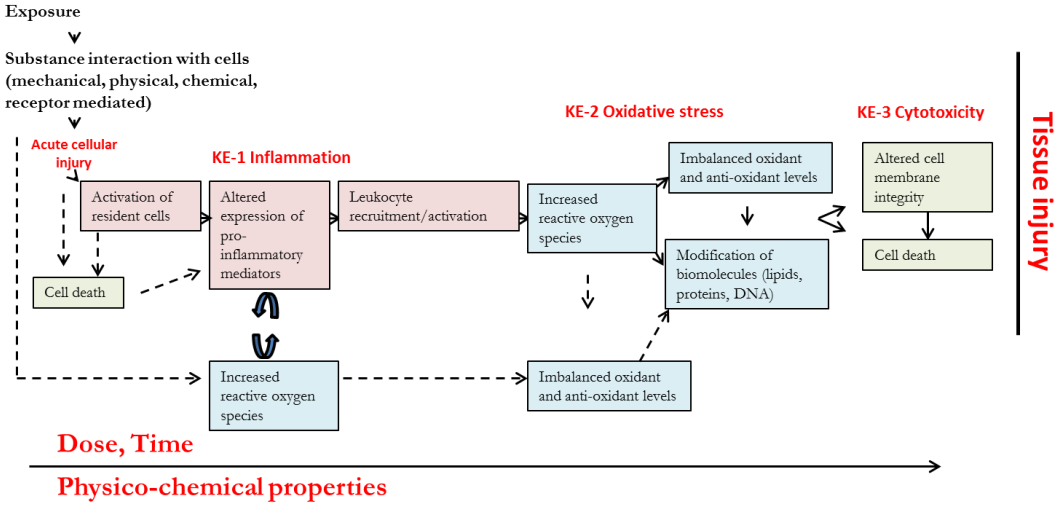
General responses to overall comments:

Issue group 1: Vague MIE, suggested interventions, stressor specificity, and is MIE required?
Response: We accept and agree that MIE description is vague and not specific. Also, we agree that MIE itself is not measured, rather the consequence of triggering the MIE. The MIE in AOP173 has evolved over last four years from 'secretion of cytokines', 'sensing of danger', 'binding of IL1R receptor', 'resident cell activation, to 'substance interaction', the latter being the most accepted MIE for lung fibrosis induced by nanomaterials. We have published multiple AOP173 roadmaps using the various MIE titles. Some did not describe the molecular level event and were rejected. The MIE specifying receptor binding was experimentally proven insufficient in our hands and after several rounds of literature reviews and experiments, we then settled down to a non-specific description of the MIE as is now.
For nanomaterials, one of the target groups of stressors for this AOP, material interaction with lung cells is a must. Without interaction, there is no AOP for certain. Thus, the suggestion to leave out the MIE altogether from the AOP is not supported.
Consideration of splitting specific interaction types, all leading to KE1 is also not possible. There isn't sufficient information to consider each individual interaction type separately. One of the suggestion is to limit this AOP to CNTs only with frustrated phagocytosis as a MIE. However, we don't agree with that. There is no evidence to prove that all CNTs inducing frustrated phagocytosis will lead to fibrosis and similarly, it is not proven that CNTs that do not induce frustrated phagocytosis do not induce fibrosis. Moreover, CNTs are suggested to engage receptors such as selectins, TLRs directly or PRRs through DAMPs. There is no evidence to suggest that one material will initiate one type of interaction. In most cases multiple interactions are assumed, all of which can activate robust inflammatory cascade, which then becomes detrimental. Even in the case of bleomycin, studies have shown that at low doses belomycin interacts with cellular receptors and inflammation, and at higher doses, DNA damage. In a recent study (Putzyn et al, in peer review), we have made an attempt to use toxicgenomics data to develop a QSAR model that predicts structural properties of CNTs responsible for triggering inflammation. We show that some CNTs known to induce frustrated phagocytosis could be engaging selectin-like receptors that consequently mediate the downstream events of inflammation. Thus, it is premature at this stage to conclusively side with one or the other MIE.

Consideration of ROS as a MIE – ROS is as non-specific as anything else. Acute ROS is a part of organisms' defense mechanism. There is no evidence to show that acute ROS synthesis will result in lung fibrosis. Moreover, acute ROS is reversible and in fact functions as a signaling molecule relaying the danger signal. Thus, acutely, ROS synthesis can be used to measure the MIE but not as a MIE.
However, chronic ROS can cause oxidative modification of biomolecules including DNA, cause DNA damage injury, which can be causal to genotoxicity, as described in the AOP for lung cancer (alluded to in the comment document). In the lung cancer AOP, the oxidative stress is a KE. There, it merits the place of a KE as the AOP describes genotoxicity and oxidative stress plays a causative role. In that case, oxidative injury should lead to DNA damage, proliferation of damaged cells with mutated DNA and then cancer. Thus, this oxidative injury is different from what is discussed in AOP173 where oxidative stress may induce cell death, which initiates and or promote inflammatory reaction leading to further injury and activation of healing process.

An important point to note here is that the same material that induces frustrated phagocytosis can induce both cancer and fibrosis. In some cases, fibrosis may precede cancer!? Can this be differentiated at the stage of MIE – not possible for now.

Lastly, there is an ongoing discussion among EAGMST members concerning how best to frame oxidative stress in an AOP framework? When does oxidative stress become a KE in an AOP? Is increased ROS synthesis enough to indicate adverse outcome? Etc.
As noted by one of the reviewer, especially for nanomaterials, oxidative stress should be considered at the early KEs. Although we agree that nanomaterials can induce oxidative stress because of their surface properties, we are yet to link acute ROS induced by nanomaterials with eventual pathological outcome. We (Halappanavar's lab) have shown that even inert nanomaterials induce ROS synthesis and the extent of ROS is not associated with cell death (Decan et al 2016). In another recent study involving air pollutants, we show that ambient urban air samples of different chemical composition induce robust ROS but not cytotoxicity (Halapanavar et al., in preparation). Thus, at best, oxidative stress can cause cell death acutely, initiate injury, fuel pro-inflammatory KEs and mid-way into the disease process, ROS can aggravate the injury. For that reason, it is included later in the AOP as an associative event, where its involvement in cell injury may play a detrimental role.
Below, the outcome of a recently completed OECD WPMN project on identifying KEs for furthering the development of AOPs, is shown. The schematic demonstrates the most commonly reported inflammation associated KEs in nanotoxicology literature and linkages between the reported KEs. The schematic breaks down the oxidative stress event into hub events describing specific aspects of it and what may be required for the injury to ensue. It also shows how oxidative stress by itself or in collaboration with inflammatory events, can help propel the injury axis (Halappanavar et al 2019, NANOIMPACT). Thus, authors of AOP173 have considered the various aspects or complexity of the fibrosis disease process. We fully accept that there are some questions that cannot be answered at this point in time. More importantly, we firmly believe that the MIE presented in AOP173 is an exception to the EAGMST MIE convention and should be accommodated as is because of the nature of the biology involved.



Lastly, authors agree that as new information becomes available, we can add or separate the non-specific all-inclusive MIE into concrete bits and provide evidence to show how they link to the eventual AO. In our opinion, the expectation that the MIE should be modified extensively at this stage is not supported.

Networks of AOPs

We agree that there is value in connecting different linear AOPs to get a comprehensive view of the biology perturbed. The corresponding author of this AOP is privileged to be part of several of the nano-relevant AOPs that are under development and included on the work plan of EAGMST

(AOPwiki). A manuscript (Halappanavar and Vogel et al., under peer review) has been put together briefly summarizing all nano-relevant AOPs. The manuscript specifically demonstrates how individual linear AOPs can be connected in a network and how networks aid in understanding the overall biology. The manuscript also describes a simple strategy for using AOP173 in decision making. The take home message of this exercise is that all these AOPs share MIEs that describe various nano-bio interaction. This could mean that a single nanomaterial can initiate multiple AOs or multiple interactions can lead to the same AO. Several KEs overlap and these can be used in the tier-1 assessment. Thus, authors are completely aware of what is coming and how incoming new information can be used to validate or modify AOP173. However, none of these AOPs are fully developed and it remains to be seen if MIEs and KEs identified will stay. Should the manuscript be accepted before submission of the revised AOP draft, we will summarize the results under alternate mechanisms section.

Stressor specificity

Stressor specific information is not included in MIE description. However, we have added a section summarizing specific stressors that induce the MIE and AO. But this is not normal as AOPs (MIE, KEs) are stressor agnostic.

Insufficient stressors discussed

I (Halappanavar) have served as an internal reviewer of several AOPs and presently serving as an AOP coach. I can confidently state that AOP173 discusses more stressors than many out there. Most are developed based on one stressor. Having said that, if available, we will add more information on stressors.

Issue group 2: Inflammatory KEs, target cell specificity, biomarker specificity (assays), reversibility (essentiality), feedback loops

Response: Inflammation is in itself a complicated process. Involves multiple cell types, multiple biomolecules, and the changing microenvironment. The microenvironment plays an important role here as it decides the fate of the activated inflammatory process – that is to resolve, progress to chronicity/adaptation and commitment to disease. Each cell type and the mediator play multiple roles and the entire process is temporal. Because it is an important defence mechanism of the organism, it is equipped with compensating mechanisms to help should one mechanism fail or one mediator go missing. Not all pathways and the specific actors involved are entirely understood. For these reasons, proving essentiality is difficult. For example, because of the compensatory pathways, knocking down one mediator or one pathway will not stop the disease process. In some cases, inhibition of an important inflammatory pathway can lead to exacerbation of disease. Failure to remove the exogenous material due to the missing signalling can result in persistence of the material and cell/tissue injury, which is an essential event for fibrosis. From our study, we have shown that abrogation of both acute and adaptive phase of the inflammation reduces the disease process, however, does not completely stop the disease from manifesting. Complete inhibition of inflammation may have grave consequences to the organism. Thus, a special consideration has to be given for how essentiality of these early KEs can be proven.

Routine cell types used to study inflammation include macrophages, neutrophils and epithelial cells. Most studies are conducted using monocultures and in the recent times, co-culture system is gaining popularity. Again, there is no consensus on what is good or better or reliable. Similarly,

TNF-a, IL1b, and IL-6 are some of favourites and are used routinely to assess inflammation process. Apart from the fact that these are the most commonly assessed pro-inflammatory mediators, there is no real evidence to suggest that they are the most suitable or if all of them should be assessed to imply activation of inflammation or assessing one of them is sufficient. In addition, guidance on specific cell types for assessing them is also not available. As alluded to by one of the reviewers, recent advances in omics techniques should help with it. Rightfully so, we (Halappanavar's lab) have used omics and investigated more than 75 different nanomaterials to date in a single species in addition to lung responses to cigarette smoke, air pollutants among others. The results identified SAA3 as one of the most consistently induced acute phase reactant following all of these exposures. Specifically, for nanomaterials, we (Vogel et al) showed that the magnitude of SAA3 expression directly correlates with extent of neutrophil influx, directly linking altered expression to leukocyte influx. More importantly, these results suggested that extent of SAA3 expression can be used to assess toxic potency of materials. However, SAA3 is like CRP and is non-specific.

In addition, the vast amount of omics data (publicly available and in-house generated) was used by us (Halappanavar et al) to identify a gene signature of 17 genes that is predictive of lung fibrosis (Manuscript in peer review). This 17-gene signature spans multiple KEs in AOP173 and has been pre-validated to assess in vivo fibrotic responses induced by CNTs and ex vivo pro-fibrotic responses induced by bleomycin. Furthermore, using publicly available single cell omics data in bleomycin model, we have identified the cell types that express these 17 genes. There are more than a few types involved in the fibrotic response. Since at present there is no consensus on the cell culture models to use for assessing pro-inflammatory and pro-fibrotic responses, we (Halappanavar lab) have optimised an ex vivo lung slice technique as an interim alternative. Conditional to further validation, we propose that combined 17-gene signature and the ex vivo lung slice model as a promising alternative to screen pro-fibrotic stressors. Should the manuscript be accepted before the submission of the revised AOP draft, they both will be included under the assay sections. We have ongoing studies to expand this signature using additional bioinformatics analysis, which should be published soon. Thus, a serious thought is given to the various issues that are correctly identified by the reviewers. However, it is important to note that there are no ready solutions to these questions for now.

<u>Reversibility of the process, addition of feedback loops</u>

It is true that the inflammatory process is reversible and providing clarity on key features of the inflammation process that signifies adversity is important. This will require additional experiments involving dose and time series and identifying a threshold response below which inflammation is inert and above which it is harmful. This is work in progress. However, we do not agree that we have to include feedback loops and inhibitory pathways to the schematic or discuss them in the main text. It is granted that the AOP depicts the path forward to an AO despite of all inhibitory loops. Adding feedback loops will simply complicate it further.

Issue group 3: Weight of evidence need to be strengthened, essentiality table may be needed

for all KEs
Response: Where possible, we will add information

Issue group 4: Missing or incorrect references
Response: We agree that some references may be left out and there may be errors. The text will be carefully reviewed to update the reference list. Where possible we will add additional references. But as reviewers point out, the document is already comprehensive and exhaustive and thus, it may not be necessary to add all available references.

In conclusion, authors would like to thank the reviewers for their time and efforts in reviewing the AOP. We appreciate the valuable comments provided by them and we will make full efforts to address them where possible. But we would like to request that due considerations be given to the important questions that reviewers have raised, for which there are no ready answers at present as reviewers may acknowledge.

	Charge Question 1: Scientific quality: *Does the AOP incorporate the critical scientific literature and evidence?* *Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?*		**Authors Initial Response**
1	Reviewer 1	The cited scientific literature is comprehensive, adequate and useful to inform on the background and the events for the development of AOP 173. It is acknowledged that the literature cannot be exhaustively covered due to the complexity of the matter and processes involved.	
2	Reviewer 1	Regarding CNTs as exemplary stressors, there are a number of publications/reviews addressing fibrosis and AOP development which should also be cited already on p.4 (e.g. Dong 2019, 2018, 2016, Duke 2017, Vietti 2016, 2016a).	We agree that there is much more available out there and could have been referenced. It is a possibility that some references may have been left out due to the exhaustive number of references already added to the text. We will go through the text and add additional references where necessary.
3	Reviewer 1	Further literature may be included which discusses relevant (nano-)material characteristics for cellular interaction (MIE) **and** inflammatory response induction (e.g. ROS generation by metallic contaminants of CNTs) and cellular consequences of interaction or uptake, respectively (e.g. apoptosis, immobilisation/chemoattraction, autophagy/ER-stress).	Addition of literature – where possible and necessary, we will add additional citations. We agree that ROS generation can be viewed as a consequence of triggered MIE and can be used to measure the MIE. This information can be used under the measurement section as an endpoint of consideration. However, the suggested literature related to metallic contaminants of nanomaterials may not be

			necessary. As such, in our experience, even the most inert nanomaterials induce ROS and it is not detrimental. However, it is important to note that AOPs are stressor agnostic and MIE/KEs have to be described independent of stressors inducing them. Thus, the AOP describes the most commonly accepted mechanism of fibrosis. Some evidence is provided to support the occurrence of MIE/KEs but specific details are avoided. Some other points to consider: the target stressors for this AOP are not limited to nanomaterials and the AOP should not be viewed as a review of nanomaterial literature or to specify nanomaterial property mediated differences. Where necessary, this point is already emphasised in the text. Moreover, at this point in time, there is incongruence in the literature available in the context of specific properties or characteristics-dependent responses. There is no consensus in the literature. AOP 173 focuses on the most commonly accepted mechanism of fibrosis and is applicable to a wide variety of stressors but not necessarily to every single nanomaterial present out there.
4	Reviewer 1	Some lack of congruency has been found upon cursory checking citations in the text and the corresponding reference lists. For instance, KE Description of event 1497 (p. 19) cites Zuo, 2002 and Beamer, 2013 (2012?), both missing in the reference list on the same page. It is recommended to carefully check for missing or incorrect citations in all reference lists.	Thank you for pointing to this. We will check the text for such omissions and revise accordingly.
5	Reviewer 1	The topic is well covered considering the current scientific knowledge with regard to providing evidence for KER, also addressing critical issues and knowledge gaps. Altogether, the scientific quality is high and up to date. However, the somewhat indiscriminative treatment of soluble and particulate (fibrous) stressors CNTs (exemplified by bleomycin and CNTs, respectively) would need more critical appraisal, e.g. in terms of target cells, types of cell injury and mediators involved in inflammation and fibrosis.	We agree that the target cells, type of cell injury and mediators involved could all differ. The consensus from the literature is that the acute injury mediated by pro-fibrotic stressors mainly involves alveolar macrophages and epithelial cells. Chronic or adaptive phase of the disease mainly involves macrophages and fibroblasts. In allergen induced fibrosis, eosinophils are involved in addition to the others. Similarly, types of pro-inflammatory mediators secreted

			acutely vary. In some cases, same mediators may be secreted but to a different magnitude. Also, there is no consensus on how many mediators are sufficient to make a 'positive' call. This was one of the topics for discussion at the 2017 expert workshop on inflammation. It was agreed that in most cases, what is measured for inflammation depends on individual experiences/expertise and available resources in a given laboratory. We agree that more thought has to be given to this aspect.; however, this is something we are working on in our own laboratory. We are using meta-analysis approaches to identify clusters of genes that can be used for predicting occurrence of inflammation and even the AO. By the time this AOP is revised, the manuscript describing one of the gene clusters potentially predictive of the AO will be published and we will accordingly revise the text.
6	Reviewer 2	**Overall, the scientific quality of the AOP is strong.** There is evidence for each of the KEs in the pathway that these can be related to the AO pulmonary fibrosis. In addition, the authors provide substantial evidence from literature, including data interpretation and discussion of uncertainties and inconsistencies. Especially the evidence for the later KEs and the AO is strong. A limitation of the AOP is that there is some incongruence in the essentiality of the MIE and some KE for the eventual AO. The authors do describe this in the document, but they could be more clear. MIE substance interaction, KE1 increased pro-inflammatory mediators and KE2 increased recruitment of pro-inflammatory cells are all part of an inflammation response. Inflammation does not necessarily lead to pulmonary fibrosis. Therefore, the authors need to be more clear on which type of substance **interactions** with membrane components can be linked to pulmonary fibrosis, **which** pro-inflammatory **mediators** can be linked to fibrosis and **which** pro-inflammatory **cells** can be linked to fibrosis. Now, it is not specific enough to understand the mechanism as inflammation could also lead to pulmonary emphysema, cancer or decreased lung function.	We agree with the comment and have discussed extensively the specified incongruence. The AOP 173 applies to those stressors that mediate their effects via immune and inflammatory reactions. The argument that inflammation is not necessary for eventual AO comes from the transgenic studies that lack a single pro-inflammatory mediator and inactivation of a specific pro-inflammatory pathway. However, as noted in many places in the AOP 173 text, these early responses to stressor exposure serve as defence mechanisms. As a result, they exhibit high level of redundancy and pleiotropy, which is absolutely needed for the organism's survival. As pointed out in one of our studies, unless we are able to completely abrogate the acute as well as adaptive immune responses, one cannot stop the fibrotic disease. Fibrosis is a progressive disease and involves signalling and cross talk between several cell types, mediators and molecules. Its occurrence is highly dependent on the material properties (persistence), level of injury

			(repeated exposure) and the temporal microenvironment. At each stage, there is a feedback signalling that either propagates the AO response or inhibits it from further progress. AOP173 describes a path forward towards the AO that has overcome the inhibitory loops. Again, there are 'classical' pro-fibrotic markers that have been routinely used in the literature; however, in our experience, they don't work always. The only way to specify a set of genes/proteins with confidence is to derive a set by meta-data analysis. In our own laboratory, we have conducted meta-analysis of high content data and have come up with a 17-gene signature that is promising. Although further validation of the 17-gene set is necessary, for now we can confidently say that these genes are induced both by bleomycin and CNTs, and the results can be correlated to histopathological findings. In fibrosis, the acute inflammatory phase will also secrete pro-fibrotic markers. Please note that we are not referring to diagnostic markers of fibrosis that are present in the fibrotic lesion. We are referring to markers that promote the process of fibrosis and can be used to predict its occurrence. Similarly, in emphysema, we see several metalloproteinase secreted during the acute phase that we don't see following pro-fibrotic stressors. Halappanavar's group is developing a parallel AOP for lung emphysema (on EAGMST workplan) and it is clear that acute inflammatory phase can be discriminatory. In cancer, it is a different story. Fibrosis can precede cancer growth if material properties and exposure scenarios are conducive. There is a lot of debate about this and in our opinion, we are no way closer to solving this debate. Finally harmonising the

			inflammatory key events – while the higher order title 'Altered expression of pro-inflammatory mediators' can be common to all AOPs, specifications in the context of the AO described will have to be included. For example, in AOP 173, we define this KE as 'Altered expression of pro-inflammatory and pro-fibrotic mediators'. Similarly, in emphysema AOP it is defined as ''Altered expression of pro-inflammatory and metallo proteinases'. There is no way around this. At present, there are about 5-6 AOPs of relevance to nanomaterials. The MIE in each of these is the same – nano-bio interaction. However, each AOP describes this interaction with subtle differences. For example, frustrated phagocytosis is the MIE in the AOP for lung cancer. Interaction with surfactants is the MIE n the AOP for acute lung toxicity. However, no one is really measuring the MIE itself, rather the consequence of such interaction. At the same time, frustrated phagocytosis is one type of interaction that can lead to lung fibrosis. The same material that induces frustrated phagocytosis can also interact with surfactants and inhibit their activity, thus inducing acute inhalation toxicity. So the point is that the MIE in this AOP does not follow the prescribed norms and it is essential for it to be that way. Especially for nanomaterials, this interaction can vary from material to material and more than one type of interaction can occur at the same time. Even for bleomycin, higher doses of bleomycin can interact with DNA and induce DNA damage but the low doses can bind to specific receptors and initiate fibrosis via inflammation.
7	Reviewer 2	More specifically, the MIE is still a bit vague: interaction of substances (physical, chemical or receptor-mediated) with membrane components (receptors, lipids) leading to danger signals. The MIE itself is usually not measured and thus not specified. The danger signals are identified as	As described above, MIE is non-specific intentionally. We would argue that MIEs that lead to defence mechanisms such as inflammation or healing process will be redundant in most cases. There is another AOP

		alarmins that initiate an immune response. Alarmins can induce a cascade leading to fibrosis, however, alarmins can also induce other diseases such as cancer. Therefore, the MIE seems not specific for the AO. In addition, it is not clear which cell types are involved here (macrophages? Or epithelial cells? Both are mentioned) and whether there should be a specific pro-inflammatory response to start the cascade leading to pulmonary fibrosis.	for lung fibrosis that describes specific binding and inhibition of PPAR receptor leading to fibrosis. In this AOP, inflammation plays a role ofassociative event and not a KE. From our own work, we have seen that carbon nanotubes that induce frustrated phagocytosis also potentially interact with selectins, receptors required for agranulocyte diapedesis. The binding is assumed to result in inhibition of selectin-mediated signalling and impede diapedesis. ROS increase is as vague as the MIE described in this AOP. ROS increase may be used for metal oxides but not for other nanomaterials. In our own work we have observed that even most inert nanomaterials induce increase in ROS. Metal oxides induce emphysema or metal fever more than fibrosis. TLR4 activation is an example. Our collaborator and a co-author of this AOP are investigating this interaction in their laboratory. For now, the evidence is scarce and inconsistent. New content – In my opinion, ROS increase is vague and is not prescriptive of eventual AO. It is surely an associative event as described in the AOP173; however not a detrimental KE. As part of the ongoing collaborative work with various European nano consortia, Dr. Halappanavar is aware of all AOPs submitted or on the work plan of EAGMST committee. The proposal for these AOPs are on AOPwiki. In fact, a review article (Halappanavar and Vogel et al) is under peer review at the moment that summarises all of these AOPs and demonstrates how these AOPs can be connected in a network to identify the overlapping KEs that can be prioritised for testing.
8	Revi	The inflammation response plays a critical role in	When KE essentiality (How much of

	ewer 2	the AOP. Inflammation can be reversible, as is also explained in the section on biological plausibility, coherence and consistency. This leads to inconsistent results. The AOP could reflect the feedback loops.	KE1 is required to trigger KE2) is built, this has to be taken into consideration. At the moment, there is not enough information in the literature to recommend a threshold below which inflammation is reversible and above which inflammation progresses to AO. For this, we will need to agree on the set of inflammatory markers that will be assessed commonly and then define thresholds. We are working on it. This is work in progress. For now, we know that if exposure persists, inflammation persists and will lead to AO. However, we would like to bring up the example of expression of SAA3 following exposure to all types of nanomaterials and other pro-fibrotic stressors that involve inflammation. SAA3 expression in mouse lungs is directly proportional to the extent of neutrophil influx and potential potency of stressors (in this example, nanomaterials) to induce pathology. Further work is needed to validate if this is an appropriate marker. A lot of work is being taken up by Dr. Vogel's group on this. For humans, several SAA genes exist. Regarding reversibility - The AOP is supposed to describe a mechanism that leads to AO and not inhibitory loops that inhibit the disease process. Granted that all disease processes have two trajectories but the AOP describes the trajectory that surpasses the inhibitory loop to the final AO. All disease processes are complex and not just lung fibrosis. Aren't AOPs supposed to depict complex biology in a linear modular format? Feedback loops need not be added to the graphical representation, If we start adding all feedback loops as suggested, it will not be anymore an AOP, it will be a MOA.
9	Revi ewer 2	Finally, there is an overall lack of quantitative information on the KERs. The authors explain that many studies only tested a single dose and that therefore quantitative information is missing. This	Yes, absolutely agreed. Quantitative information is lacking and should be one of the objectives for a future research project.

		is an important point as quantification of KERs would help to increase the understanding of the AOP. Especially for the KEs related to inflammation, quantitative information could help to understand at which doses inflammation can progress into fibrosis and at which doses the inflammation is reversible. Maybe for a future project, it would be feasible to collect available quantitative data on each of the KERs and start modelling the data to find quantitative KERs.	Where possible, we will try to expand and add text or information. However, we have already agreed that this is a qualitative AOP.
10	Reviewer 3	Lung fibrosis as an adverse outcome (AO) is a chronic and progressive disease that leads to scarred alveolar tissue. Occupational and environmental factors as well as medication can induce lung fibrosis, but rare cases also occur without a known cause, which are summarized as idiopathic pulmonary fibrosis (IPF). The sequence of effects includes: the interaction of the substance / chemical / drug / material with the outer cell membrane, i.e. molecular initiating event (MIE), pro-inflammatory mediator release (KE1), recruitment of inflammatory cells into the lung tissue (KE2), alveolar capillary membrane integrity loss (KE3), activation of adaptive immune response by T Helper type 2 cell signalling accompanied by anti-inflammatory and pro-repair/fibrotic mediator release (KE4), fibroblast proliferation and myofibroblast differentiation (KE5), which finally leads to the synthesis and deposition extracellular matrix deposition (KE6). All these events culminate in a thickening of the alveolar septa, a decrease in lung volume and lung fibrosis (AO). It is a comprehensive work on this AOP with a careful revision based on the comments of the internal reviewers. It provides clear guidance for in vitro and in vivo work to coordinate and conduct specific experiments to assess the potential of a chemical / substance / nanomaterial to induce fibrosis.	
11	Reviewer 3	As mentioned above, the information provided is comprehensive and includes the relevant scientific literature. The focus is on data from animal experiments, which of course have the advantage of providing more information about KE and KERs. I only miss some references from "old-school" lung anatomy and pathophysiological books describing lung fibrosis. For example, the targeted injury of type II alveolar epithelial cells which are unable to repair damaged type 1 pneumocyte tissue, is an important information, especially for the design of future (in vitro) studies.	We will try to include these new references that the reviewer is suggesting. Old school text book language was criticised by the EAGMST internal reviewers, which resulted in removal and rewriting of much of the text that was part of the earlier draft.
1	Revi	In the overall assessment of the AOP, the different	We will look at the suggested

2	ewer 3	animal species used for this research area are listed. It is found that the key characteristic events for fibrosis are more or less the same, with some minor differences such as inter-species variation in the respiratory system. It is recommended to add some references that summarize the limitations of the different models, e.g. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5532376 https://err.ersjournals.com/content/28/153/190029 https://onlinelibrary.wiley.com/doi/full/10.1002/path.4658	reading.
	Charge Question 2: Weight of evidence: *In your opinion, is the rationale for the weight of evidence judgement/scoring well described and justified based on the evidence presented? If not, please explain.*		
13	Revi ewer 1	Regarding the WoE for the KERs, comments are provided for each KER described, also addressing uncertainties, inconsistencies and challenges in terms of quantification study and inconsistencies are identified for several studies. The KER table on p.3 provides "high" evidence scoring for all KERs. The described KERs (MIE-K1E, KE1-KE2) emphasize the decisive role of the alarmin IL-1a/ NFk B signaling pathway in triggering KE2. However, the example of MWCNT-macrophage interaction shows that more signaling pathways may be involved (Vietti, 2016, Li, 2018), and IL-1a/inflammasome activation appears to be predominantly induced by fibre-like MWCNTs exposure resulting in macrophage necrosis (Palomäki, 2011). Likewise, Il-1R1 knock-out models which provide strong evidence for the relevance for IL-1 signaling in PMN recruitment also tested fibre-like but no other types of MWCNTs (Nikota, 2017). Because of the narrow evidence base in relation to its suggested comprehensive applicability, downgrading the WoE evidence from "high" to "moderate" for the first KER is recommended.	We will look into this.
14	Revi ewer 1	Regarding the essentiality assessment of all KE's in section 4, it would be helpful to organize a separate Excel sheet. The table provided, termed "weight of evidence", which lists key studies for different stressors and the KEs they address. It is not clear this table is intended to be used as an equivalent for Table 5 in the OECD Handbook. In any case, it is recommended to use the format of the Handbook for appraising the quality of the available evidence (direct, indirect, no or contradictory evidence), considering specificities of stressors.	We will look into this

15	Reviewer 1	Weight of evidence for involvement of specific stressors in the different KEs is provided from the scientific literature, most often for bleomycin, CNT, asbestos, silica, as well as IPF. However, the evidence is rather anecdotal and it is questionable if reported changes of the expression or secretion of cytokines (varying with type of stressor) are sufficient to predict the sequential occurrence of KEs such as inflammatory cell recruitment or loss of capillary membrane integrity. Moreover, as dose-response concordance is missing it is difficult to substantiate hypothesized KERs with respective data. In this context it is surprising that the BMDs related to specific KEs derived from toxicogenomics analysis by the AOP developers has not been considered yet (Labib, 2016) for a concordance table, as suggested in the OECD Handbook.	Please see responses to comments above. The reason we hesitate deriving conclusions based on what is available is the heterogenous nature of info on inflammation. As mentioned above, increased expression of SAA3 is directly correlative of neutrophil influx in mouse lungs. There are other cytokines and chomokines which are also highly expressed in the same study and not all correlate with neutrophil influx. Thus, it is important to define or agree on which cytokines and chemokines we consider as relevant to inflammation and then build such concordance relations. This is something we are doing at the moment.
16	Reviewer 2	There are a number of studies supporting the AOP leading to pulmonary fibrosis. From these studies, there are some inconsistencies that are pointed out in the WoE table. There are some inconsistencies that seem to indicate that the correlation between inflammation and pulmonary fibrosis is not that clear. For example, in knock-out mice that lack specific genes (for a receptor for example), the inflammation response is not changed, while the fibrosis is decreased or even absent. In addition, in mice lacking IL1R1 signalling, the inflammation response is first suppressed, while later on the fibrosis is exacerbated. This makes clear that the inflammation response is complex and that the correlation between inflammation and fibrosis is still not completely clear.	Absolutely. Please see the responses to earlier comments. The classical way of gathering weight of evidence as described in AOP guidance document is not possible for this AOP. While we know and we all agree that inflammation is important for lung fibrosis, because of its complex nature and more importantly, the redundancy involved, it is not possible to really nail this down to one or the other molecule. In fact, if we were to forcefully stop the inflammation altogether following exposure, the organism will die or will suffer from another grave disease.
17	Reviewer 2	Another observation from the WoE table is that the number of substances that lead to pulmonary fibrosis seem limited. The WoE is mainly based on Bleomycin, some carbon nanotubes (depending on the characteristics), asbestos and silica. For CNTs, asbestos and silica, it is known that these could also induce cancer. Therefore, it is important to better understand when the inflammation that is induced progresses into fibrosis or into cancer or when it is still able to resolve.	Most endorsed AOPs are built on one or two stressors. AOP173 provides evidence from several of stressors. Role of inflammation in cancer is yet to be defined. Identifying the specific markers that allow differentiation between inflammation leading to cancer vs inflammation leading to lung fibrosis is a research program in itself. Thus, we will not attempt to address this here. Please see other responses above.
18	Reviewer 3	Yes, the evidence and qualitative understanding is high for most KEs, as there is many human and animal data that clearly underline the described KEs and KERs. Only for the integrity of alveolar	We agree that it is difficult to prove.

		capillary membrane, the KER is difficult to prove with animal data, and more data must be generated by in vitro model to support KE3 with quantitative data.	
19		Weight of evidence support for dose and time-response relationship focuses on data for carbon nanotubes. But a lot of human data exists for occupational exposure, such as inhalation of asbestos / silica and for cigarette smoking. Is it possible to make a similar estimation from these data as for CNTs?	The text already references several human studies. We will look into what additional information can be added.
	Charge Question 3: Additional observations: *Do you have any additional observations or comments for the authors (e.g., what do you consider to be critical data gaps and how might they be filled)?*		
20	Reviewer 1	This AOP is quite ambitious as it spans a large distance from a stressor's portal of entry to fibrosis, thereby linking highly complex processes and mechanisms such as acute and chronic inflammation with fibrotic effects, considering respective cells, signalling pathways, and soluble mediators involved in culminating in adverse fibrotic scarring. The developers of the AOP emphasize its generic features and events, irrespective of the nature of the stressor (soluble chemical, [nano]particle, fibre, biological pathogen, unknown stressor). Converging evidence is provided for both, soluble (exemplified by bleomycin) and a particulate stressors (exemplified by CNT). The rationale for AOP project reads as if its development is primarily motivated by an alternative strategy for nanomaterial testing and assessment. Evidence for nanomaterials and solid materials predominates the database. Bleomycin and ILF data is included primarily because these models provide well established mechanistic information, thus supporting the universal character of the AOP. The later events are indeed independent of the type of stressor, as long as the stress persists. However, the type of stressor matters in terms of the MIE and upstream inflammatory events. For instance, there are profound differences in solid and soluble stressors with regard to toxic properties, compartmentalisation cellular interaction. These should be pointed out more clearly.	MIE and KEs should be described independent of stressors. We have clearly stated that this AOP is applicable to any pro-fibrotic stressor that initiates immune and inflammation response. Although MIE and the first two KEs seem generic, the magnitude of their activation is dictated by the property of materials. Thus, the injury and consequent DAMP release following interaction of 'toxic' materials with lung cells will be many fold higher compared to the response observed following exposure to inert materials. From the 75 different nanomaterials that we have studied, we can clearly see a gradient of inflammatory response that can be associated to eventual pathological potential of each material. This is all work in progress. It is also important to note that metal oxides activate proteinases, which are not observed following CNT exposure. In addition, the magnitude (how many, how much) of proteinase response again varies based on how toxic a material is. Some pro-emphysematic stressors can also induce fibrosis. Again, for the diseases that are mediated by immune and inflammation response, there is a thin line that differentiates the final outcome and it is all dictated by the

			material properties and changing microenvironment. It has to be acknowledged that stressors that induce a robust inflammatory and immune response can induce multiple AOs.
21	Reviewer 1	Measurements for KEs on the cellular and partially on the tissue level heavily rely on PCR and ELISA methods to investigate the expression and secretion of soluble mediators. Because pro-inflammatory mediators can be induced depending on stressors and because mediators are usually multifunctional, it can become difficult to agree on a set of mediators predictive for fibrogenic inflammation. To circumvent this issue, a large set of mediators may be screened, e.g. by using array and –omics techniques. However, such an approach is still facing a high degree of uncertainty, reproducibility issues and random in interpretation of results. Likewise, primary target cells differ depending on the type of stressor. Thus, agreeing on relevant cells for in vitro assays can be challenging.	Agreed. We have taken the omics and meta-analysis approach and we have come up with a 17-gene signature (paper in peer review). We have validated with the data available and we also have optimised ex vivo technique that circumvents the issue of selecting specific cells until we have an agreement on that. So there is definitely hope and we are getting closer to get some answers on that.
22	Reviewer 1	MIE 1495: "Interaction with the lung resident cell membrane components" is very vague terminology (perturbation, chemical interaction, receptor-binding, etc.?). Lack of precision is likely due to the broad range of stressors the AOP is developed for but should be avoided. In fact, depending on the sort of stressor, it is reasonable to assume that there is more than one MIE. For the time being it is recommended to do without a MIE and use the earliest known KE instead, in accordance with the OECD Handbook.	For nanomaterials, it is accepted that nano-bio interaction is a must for any response to occur, without which there is no AOP. Thus, even though vague, the MIE is important to have. As such, vagueness describes the non-specificity of such interaction. This brings us back to the same points discussed above – one stressor can initiate multiple interactions. The MIE in AOP 173 is unconventional and may be it is important that autors acknowledge that.
23	Reviewer 1	The uncertainty deteriorates the usefulness of this AOP for regulatory purposes. It is therefore recommended to adapt and limit the AOP to specific types or class of stressors. For instance, limiting it to CNTs, inflammation-promoting interaction would clarify on primary target cells (resident AM) and interactions (phagocytosis, piercing). In this case, oxidative stress (due to substance-related or cell-dependent ROS generation) would act as an initiating or upstream event in terms of inflammation compared to its current role defined as KE associative event No.3 in the context of loss of ACM integrity only. Accordingly, both cellular and acellular ROS would need to be measured.	AOPs are stressor agnostic. MIE and KEs have to be described independent of stressors involved. We do not agree that there is conclusive literature on what cells CNTs engage and CNTs inducing phagocytosis will definitely induce lung fibrosis. CNTs can induce multiple AOs and cellular interaction, inflammation, injury are the routine observations. Interventions proposed are not helpful. They will add more confusion.

24	Reviewer 1	KE 2 (1497): Increased, recruitment of inflammatory cells Authors admitted that measurement in vitro is difficult, thus referring to the detection of proinflammatory cytokines as indicators of cellular recruitment. Alternatively, the appropriateness of co-culture transwell chemotaxis assays and differential expression of integrins/selectins could be checked (e.g. see Zemans RL, Colgan SP, Downey GP. Transepithelial migration of neutrophils: mechanisms and implications for acute lung injury. Am J Respir Cell Mol Biol. 2009;40(5):519–535. doi:10.1165/rcmb.2008-0348TR).	Please see responses above. We have a 17-gene signature, which will be expanded further adding more genes representing multiple KEs including inflammation KE. It is work in progress. For now, it is not wise to recommend anything as definite. It is best to leave it with some routinely assessed markers until we are able to recommend a definite set with confidence. In fact, we have optimised an ex vivo lung slice culture method as an in between in vivo and in vitro model as an interim solution. This model allows us to see recruitment of macrophages, neutrophils to the site of injury as well as cellular piercing, cell injury and finally the collagen deposition (the paper is in review). We will add this to the assay. We will also add the 17-gene signature (in review) to the recommended assay. We intend to validate multiple cell culture models in the near future and validate the predictive signature in these models.
25	Reviewer 1	Potential applications This topic is insufficiently addressed. It should be elaborated more in detail what this AOP is aiming for and how it can be applied to inform risk assessment (including screening, grouping). Does it aim at replacing animal testing, such as repeat dose inhalation testing? If so, this would have an impact on sampling and measurement of tissue-level KEs in vivo (e.g. histopathology analysis, BALF analysis, use of primary cells, etc.).	The potential application of AOP 173 is well summarised in the following text. We have evidence to show that we have used AOP173 for each of the applications described below including QSAR modelling (paper in peer review). 'Generally it could be foreseen that AOPs can be used for: development of in vitro and ex vivo assays that detect chemical effects or responses at the different level of organisation, as well as screening assays for targets related to the MIEs; in the development of IATA or ITS strategies for any given endpoint; and developing chemical categories and further development of the OECD QSAR toolbox'.
26	Reviewer 1	Altogether, AOP 173 is certainly a project that deserves to be followed-up, provided that there is an agreement on its applicability but also on its limitations. Refinement and strengthening of the MIE/KEs and KERs appears to be most effectively achieved when focusing on a specific class of stressors (even if redundancy of stressors is deemed a common feature of AOPs). This would	We do not agree that intervention strategies suggested will help reduce the complexity. Instead, authors will add details under alternative mechanisms section to elaborate on potential deviations in the pathway proposed in AOP173.

		also help reducing the complexity of this AOP, specifying the involved cellular interactions, pathways and molecules, and fostering the integration of its linear approach into respective AOP networks that highlight further stressors and KEs.	
27	Reviewer 2	The authors put a lot of effort in describing the AOP, there is a lot of information available in the document that is very helpful for understanding the mechanism behind pulmonary fibrosis. Such an AOP can be very valuable for developing in vitro methods that can be used for screening for the potential of substances/particles to induce pulmonary fibrosis. In my opinion, the main issue of this AOP are the MIE and the earlier KEs. There is uncertainty whether these are essential for the AO and they seem not specific for the AO. The MIE and early KEs can be grouped as the inflammation response. Pulmonary inflammation can be resolved, or it can lead to many different outcomes. Recent insight show that mechanisms related to chronic inflammation, fibrosis and cancer are all interrelated. This is the case for CNTs, asbestos and silica, which are used in the WoE for the AOP. More information on the complex interplay between these mechanisms is needed to refine the AOP and to be able to use it. A suggestion forward is to start building networks of AOPs, as many share the same KEs. By gathering available data on each of the KEs and the AO, as is done in the current AOP 173 but then including cancer and chronic inflammation, one could unravel if there are specific pro-inflammatory mediators or specific inflammatory cells involved in the different AOs. In addition, one could start to model the KERs. Probably, by unravelling the quantitative KERs, the correlation between each of the KEs and the AO becomes more clear.	As mentioned above, we have just submitted a paper that shows how various AOPs involving inflammation and injury KEs can be connected to AOP173 in a network and how this information can be used to support decision making. So there is significant work that has already been taken up by the authors of the AOP to validate the AOP173. Just to stress, even if we are able to build a network of AOPs, we will still not be able to solve the issue related to selection of specific cell types, biomarkers, etc. AOP173 may not be able to solve all these problems now.
28	Reviewer 3	The AOP is applicable to a broad group of chemicals / substances / drugs / materials and can also be applied to engineered nanomaterials. For carbon nanotubes a lot of data is provided. In this area there is an ongoing and intensive discussion about dose-response relationship. This is not covered in the current AOP concept, where only the MIE is relevant, but not the delivered dose / concentration. The quantitative understanding of this class of materials should be considered as relevant information.	There may be dose-response studies but they do not assess all KEs in the AOP173. Most only look at late KEs and some report only on early KEs. If there are studies that the reviewers are aware of that can help support AOP173 (assays, target cells, dose-response etc), we would very much appreciate receiving those from them.
29		Currently, oxidative stress response is not covered as the first KE, but the oxidative stress paradigm for nanomaterials is an accepted fact. Since this endpoint, in addition with inflammatory mediators, would facilitate cross-species comparison, this should be reconsidered and assays to cover this	Oxidative stress is another higher order KE that involves multiple hub KEs. Acute oxidative stress can play a signalling role and can be reversed. Similar to feedback loops involved in inflammation, multiple

		point should be expanded.	feedback loops are involved in oxidative stress. However, there is no clarity as to when does oxidative stress becomes detrimental. Although oxidative stress is assessed for nanomaterials, a clear link between nanomaterial induced oxidative stress and a pathological outcome is yet to be demonstrated. For these reasons, we have only included it at a later stage where it will play an important role in propagating the injury and thus helping the disease process. Moreover, I (Halappanavar) have reviewed several AOPs submitted to EAGMST that involve oxidative stress as a KE. However, EAGMST feels that more discussion has to take place to define oxidative stress as a KE.
30		The description of the KEs and the graphical illustration imply that MIE and then the cell and tissue effects occur sequential. However, as I understand it - and what is partially described in the text - inflammation is chronic and other KEs may occur simultaneously. This aspect should be better discussed to also guide researchers for future investigations.	In our opinion, nothing in biology is sequential. Everything (positive and negative trajectories) is initiated in parallel. It is the exposure, material property and changing microenvironment consequential to signalling is what determines the fate. This is very important to take into consideration for the AOP development. AOP framework recognises that there are parallel processes and feedback loops. But the AOP will depict the most important set of events required for the AO to occur. If we add all those parallel processes and feedback loops, it will be a MOA and not an AOP.
31		For in vivo studies, little information is given about the time-line of the occurrence of different KEs. Could this be expanded to include recommendation on the duration of an experiment? It is obvious that in humans or animals the final KE, which proliferation / activation of fibroblasts and deposition of ECM, only occurs after months / years.	
32		An overview about the challenges and limitations of animal vs cell models to study this AOP could be given, this would also help to identify optimal experimental approaches for additional relevant data to fill some of the gaps. For instance, KE3 is more difficult to study in vivo, while the influx of immune cells (KE2) is difficult to mimic in vitro.	

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