

EAGMST endorsement phase - Comments received on AOP 173 – by 30 August 2022

Comments were received from Japan, Germany and the US

| Member country | Comments | Responses |
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| | General comments | |
| Japan | <p>I don't have any specific comment for AOP173 and 363 since I was involved in earlier stages of the AOP review verification.</p> <p>I am just wondering whether the title of AOP 173 "Substance interaction with the lung resident cell membrane componets leading to lung fibrosis" is okay with "lung" since AO (KE1458) is titled "pulmonary fibrosis". It might be discussed earlier and there might be some agreement, so this is just for confirmation to authors.</p> | <p>The Title is changed to 'pulmonary', as it is used by other AOPs as well. It is now made clear at the onset that the terms 'lung' and 'pulmonary' mean the same and therefore, are used throughout the AOP description in an interchangeable manner. We hope this is satisfactory.</p> |
| Germany | <p>Pulmonary fibrosis vs lung fibrosis</p> <p>Pulmonary fibrosis is used more often than lung fibrosis and this is also mentioned as AO. Therefore it is proposed to rename the title of the AOP to "Substance interaction with the lung cell membrane leading to lung fibrosis". It is also proposed to use the same ontology throughout the entire document.</p> | <p>The title is changed. It is now made clear at the onset that the terms 'lung' and 'pulmonary' mean the same and therefore, are used throughout the AOP description in an interchangeable manner. We hope this is satisfactory.</p> |
| US | <p>It is well known that nanomaterials' physicochemical properties can have significant effects on their interaction with cells, which may lead to a mechanistically unspecific MIE. Since these factors were not taken into consideration for nanomaterial stressors, the authors should explain how this AOP applies to a broad group of substances with diverse properties.</p> | <p>The term 'substances' is changed to 'stressors'. These stressors could be pharmacological products, fibres, chemicals, microorganisms or over expression of specific inflammatory mediators and nanomaterials. For nanomaterials, in addition to aspect ratio of fibers, their persistence in general is</p> |

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| | | <p>suggested to play a role in fibrosis. Crystallinity is the other property.</p> <p>A sentence as follows has been added to the context section.</p> <p>'Specifically, nanomaterial properties such as aspect ratio, tube/fiber rigidity, crystallinity and persistence are suggested to play a role in the induction of pulmonary fibrosis'.</p> |
| | Specific comments | |
| | Page 2/3 (background) | |
| Germany | a-SMA should be introduced as alpha-smooth muscle actin (α -SMA) here; there are also other abbreviations which were not introduced yet (e.g. MARCO, PDGF). | These terms have been expanded in the background section and there onwards, abbreviations are used. |
| | Page 1 (Graphical representation) | |
| Germany | The AOP is titled "Substance interaction with the lung resident cell membrane components leading to lung fibrosis", but the graphical representation depicts "increased mortality" as an AO. As this is not an event included in this AOP, "increased mortality" should be removed from the graphical representation to avoid misunderstanding. | <p>That is a neat observation. True, it is not discussed. However, instead of totally removing it, as it is the ultimate AO at the individual level, a new sentence has been added as follows, in the abstract and the specific KE (Mortality, increased) in the graphical representation is not coloured in red. We hope that this is acceptable.</p> <p>'At the individual level, pulmonary fibrosis will lead to death, which is the ultimate AO (Mortality, increased); however, it is not discussed in the AOP description. Thus, for this AOP, pulmonary fibrosis is the final AO (Event 1458)'.</p> |

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| | Page 4 | |
| Germany | (CNT) - NIOSH should be introduced here | The reference is added. |
| US | <p>Paragraph 3:</p> <p>Stressors</p> <p>Carbon nanotubes, multi-walled carbon nanotubes, single-walled carbon nanotubes, and carbon nanofibers were identified as stressors known to trigger MIE and scored the evidence as high. However, evidence is provided for only a single type of pristine MWCNT (Mitsui-7). Since evidence for all the listed nanomaterial stressors is scored as high, it is recommended to include relevant literature for each stressor. Also, the validity of the AOP would be enhanced if the authors could provide evidence for the listed stressors that have varying physicochemical properties. If existing evidence is confined to rigid MWCNTs with a high aspect ratio, the AOP should only be focused on MWCNTs.</p> | <p>As such, with our genomics experiments, we have identified several types of CNTs including SWCNTs to be capable of inducing fibrosis with varying potency. However, not all these studies have looked for histological evidence. We have selected some studies that have stronger evidence, for inclusion in the narrative. Evidence on MWCNT7 is stronger than for other CNTs. There are references throughout the AOP text for specific stressors.</p> <p>Plenty of evidence is presented to show how many different kinds of stressors induce fibrosis involving the same set of KEs. We do not agree that we need to add more stressor-specific references.</p> |
| | Page 5 (Domain of applicability) | |
| Germany | The authors described in this section that species other than rats and mice have been used to study fibrosis with higher order organisms (e.g. dogs, cats or horses) having a closer resemblance to human idiopathic pulmonary fibrosis. While the limitations and lack of suitability of using these higher order organisms as models for fibrosis research are acknowledged, it would be beneficial to include and address these other species under taxonomic applicability even if the evidence is low. Furthermore, data from other species (i.e. non-rodent) that support any event in this AOP should be added and discussed here. This would then support the | We have already highlighted the use of other experimental animals in fibrosis research. We disagree that further elaboration or extensive discussion and review of literature concerning other species is necessary to support the taxonomy applicability. Most of latest research involves rodents and not higher order animals. Thus, focus on rodents is justified. Also, in our opinion, the |

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| | authors' conclusion that the "cross-species applicability for this AOP is strong". This point was already brought up during the external review by Reviewer 3. | information provided is sufficient to justify 'strong' cross species applicability call. |
| | Page 5 | |
| Germany | <i>at least partial resolution following 28 days</i> 28 days post exposure? | It is corrected |
| | Page 6 | |
| Germany | <i>In addition to these model stressors, exposure to various metals including uranium, arsenic, cadmium, and soluble copper can lead to fibrotic outcomes in humans</i> Some of the examples given here are related to the nano form, are these effects restricted to the nano form of the respective metals? Please specify! Also for silica the question arises to what form/size this applies. SMAD and CXCL1 have not been introduced yet. | We haven't made size specific differentiation in responses here. Some of the examples used do not specify the size. Occupational exposures are not limited to nanoform. It is important to recognize that the exposure could consist of nano forms. For silica, crystalline silica dust is added to the text to specify the form. All abbreviations are corrected now. |
| US | Weight of Evidence Summary- Concordance of Dose-Response Relationships The authors stated that there is only limited evidence on dose-response relationships and have presented AOP 173 as qualitative. However, many key biological events can resolve over time without causing any adverse outcomes. Hence, in order to make informed regulatory decisions, it is imperative to have a quantitative understanding of the dose-response. | It is correct that for informed regulatory decision making, quantitative evidence is needed. However, this AOP, in its present state, is not quantitative. Targeted experiments are underway to validate the AOP. These results will be added over time. It is also true that some KEs may resolve overtime depending on the exposure duration, stressor properties and dose. This is true for most KEs in AOPwiki. Significant qualitative evidence is presented to support the essentiality of each KE in the AOP. |

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| | Page 8 - KE 3; Event 1498 | |
| Germany | there evidence from knock-out models is lacking -> evidence from knock-out models is lacking | Corrected. |
| US | <p>KE 3; Event 1498</p> <p>It is well known that all nanomaterial stressors listed in AOP 173 have the potential to generate reactive oxygen species (ROS) even in the absence of cells. Therefore, it is pertinent to consider oxidative stress as an initiating or upstream event rather than a downstream event.</p> | While they may induce acellular ROS, in vitro or in vivo, its synthesis is inconsistent and its role is not defined in the context of disease outcome. We have conducted several experiments in our own lab to understand the role of ROS and the results are inconclusive. At this point, we only see ROS as an associative KE and not the main KE, and thus it is presented as an associative event only. In the future, with more experimental evidence becoming available, it may be possible to move it up. |
| | Page 9 - KE 4; Event 1499 | |
| Germany | <p><i>In mice deficient in STAT6, MWCNT treatment reduced the Th2 response</i></p> <p>Don't STAT6 deficient mice have a reduced Th2 cytokine response anyway?</p> | <p>The reviewer is right. The sentence is modified as follows:</p> <p>In mice deficient in STAT6 with reduced Th2 response, MWCNT-induced fibrotic response including fibroblast proliferation, and eventual formation of fibrotic lesions, was reduced.</p> |
| | Page 9 - KE 5 - Event 1500 | |
| Germany | myofibroblast phenotype which expresse -> which expresses | Corrected. |
| | Page 9 Associative Event 1: Chronic Inflammation | |

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| Germany | <ol style="list-style-type: none"> 1. Shouldn't this be renamed to chronic lung inflammation? 2. Shouldn't this be rather a separate AO? 3. This event should be added as a distinct KE to AOP wiki as it doesn't seem to be appropriate to include an additional type of relationship | Corrected. No it is not a separate KE as it is an associative event. It is now possible to include other (e.g. indirect or associative relationships) in the AOP. |
| Page 10 - Associative Event 2: oxidative stress | | |
| Germany | A corresponding key event is already contained in AOP wiki (KE 1392) which should be used here | We have listed KE1392 and used the first two lines to define the KE as follows: 'KE1392 (AOPwiki) describes 'Oxidative stress' as an imbalance in the production of reactive oxygen species (ROS) and antioxidant defences. High levels of oxidizing free radicals can be very damaging to cells and molecules within the cell. As a result, the cell has important defense mechanisms to protect itself from ROS. For example, Nrf2 is a transcription factor and master regulator of the oxidative stress response. During periods of oxidative stress, Nrf2-dependent changes in gene expression are important in regaining cellular homeostasis (Nguyen, et al. 2009) and can be used as indicators of the presence of oxidative stress in the cell'. The rest of the text is retained as it provides some AOP specific context. |
| Page 10 - Associative Event 3: macrophage polarisation | | |
| Germany | It is suggested to include this as a separate KE. | We disagree as it is not the main KE and its essentiality is inconsistent. It is important enough to merit its inclusion as |

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| | | an associative event but it does not have enough evidence to support its inclusion as the main KE in the context of this AOP. We have addressed this comment in the previous round as part of the external review process. |
| | Page 12 - Relationship 1704 | |
| Germany | which have shown empirically shown a relationship -> which have empirically shown a relationship | Unable to locate this sentence. |
| | Page 12 - Relationship 1705 | |
| Germany | have directly assessed the direct of ACM integrity loss -> have directly assessed ACM integrity loss | Corrected. |
| | Page 12 - Relationship 1706 | |
| Germany | the Th2 cytokines IL-4 and IL-13 induces -> the Th2 cytokines IL-4 and IL-13 induce | Corrected. |
| | Page 12 - Relationship 1629 | |
| Germany | in the context of prolong inflammation -> in the context of prolonged inflammation | corrected |
| | Page 13 | |
| Germany | <i>The mode or type of interactions between the resident cell membrane and a substance is dependent on the specific physical-chemical characteristics of the substance</i> Can examples be given here? Is this mostly the particle size? How about hydrophobicity and polarity? | The following is added to the text: (e.g. aspect ratio, crystallinity, persistence, surface charge, size, etc.). |
| | Page 14 | |

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| <p>Germany</p> | <p>An AOP network is mentioned here, will this be included in AOP wiki? AOPs should not be stressor-specific, is the network relevant to non-nano stressors as well?</p> | <p>When all KEs and associated KERs in the network are developed (partially or completely), the network will be uploaded to AOPwiki. Since the time of developing the network, it has become apparent, that most components of the network are widely applicable to lung injury models in general. Thus, when it will be added, appropriate language will be used to make it stressor-independent.</p> |
| <p>Page 15</p> | | |
| <p>Germany</p> | <p><i>Thus, the AOP can be extended to represent fibrosis in other organs</i> It could be indicated here that an AOP for liver fibrosis already exists and that the event 68 collagen, accumulation is shared by several fibrosis-related AOPs.</p> | <p>Added. The KE68 has been used in this AOP.</p> |
| <p>Page 19 - (Event: 1495: Substance interaction with the lung resident cell membrane components)</p> | | |
| <p>Germany</p> | <p>While we understand the authors' rationale of keeping this MIE non-specific, we also see the concerns expressed by the reviewers during the external scientific review. A suggestion of keeping this MIE heading as is and yet having a clear scientific understanding of this event could be to clearly delineate the types of substance interactions expected in lung resident cells in its description, e.g. as numbered points like (1) activation of PRRs, (2) interaction with scavenger receptors, (3) direct damage of lung epithelial cell membrane, etc. Subsequently, it would also be good to demonstrate in the event description which methods or assays are appropriate for which types of interactions, e.g. ELISA for detection of DAMPs or HAMPs vs. phagocytosis for uptake of nanomaterials. This way, at least the readers will clearly know what types of substance interactions are being discussed here and which methods are most appropriate for which type of interaction.</p> | <p>This MIE description was changed to merge different types of interactions under one main paragraph as per the comments received during the first phase of the internal review process (previously it was sectioned under Receptor-mediated interactions, Physical or mechanical interactions, etc.) We see value in keeping them merged as each interaction may or may not occur individually. Moreover, the assays used to assess the MIE are not directly assessing the interaction, rather the outcome of such</p> |

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| | <p>Next, under the section “How it is Measured or Detected”, methods such as western blots or qRT-PCR are only described briefly under ELISA. These methods deserve separate and further elaboration in this section as they are well-known and commonly used for measuring expression of proteins or genes, respectively. In addition, the description of methods for measuring phagocytosis is extremely sparse (currently just “frustrated phagocytosis is assessed using microscopic techniques”) and should therefore be expanded. For example, phagocytosis can also be measured by uptake of fluorescent polystyrene microbeads followed by flow cytometry or fluorescence plate reader.</p> <p>Please consider restructuring the presentation of this event along with further elaboration of other methods for measuring this event.</p> | <p>interaction. For example, Under this MIE, any interaction leading to cell injury will release DAMPs. Assessment of any DAMP is sufficient and will be indicative of MIE activation. We do not have to list all possible DAMPs in the AOP.</p> <p>We are currently exploring the possibilities of developing individual interactions under the MIE as a group of MIEs. We are assessing the role of certain receptors in these interactions using knockout models. If we are successful, we will add the new information to the AOP at a later time.</p> <p>Any rigid and high aspect ratio material can induce frustrated phagocytosis and therefore, we do not have to specify the stressor in specific assays.</p> <p>Both qRT-PCR and ELISA methods are described in sufficient length and depth. We disagree that further elaboration is needed, as these methods come with manufacturer protocols and may vary according to the protein or gene they target.</p> <p>The assays mentioned are the most common ones used and do not necessarily cover all the assays available out in the literature.</p> |
| Page 21 | | |

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| Germany | <p>How it is measured or detected?</p> <p>The DAMPs typically assessed and how they can be measured should be mentioned here. In the following text only ELISA is mentioned, aren't there also luciferase-based assays available, e.g. for ATP and HMGB1?</p> | <p>We are only describing the most common ones used and not necessarily list all assays available.</p> |
| Page 22 | | |
| US | <p>Frustrated phagocytosis and cellular uptake of NMs</p> <p>The methods described to evaluate cellular uptake under the section " Frustrated phagocytosis and cellular uptake of NMs:" may not be applicable for carbon nanomaterials. In the referenced literature, fluorescently labelled polystyrene or silica particles are used to demonstrate cellular uptake. However, the stressors mentioned in AOP 173 are carbon nanomaterials. It is therefore recommended that authors clarify whether the same methods are appropriate and reliable for evaluating the cellular uptake of carbon nanomaterials. In addition, given the crucial role cell-material interactions play in triggering AOP, providing reliable methods for measuring nanomaterial interactions with cells is extremely important.</p> | <p>The sentence is revised to add more information.</p> <p>Frustrated phagocytosis is assessed using microscopic techniques such as, time-lapse microscopy, backscatter electron microscopy and others (Start et al., 2017; Donaldson et al., 2010; Donaldson et al., 2012; Murphy et al., 2012). In addition, MIE 1668 of AOP303 notes other indirect methods for measuring frustrated phagocytosis.</p> <p>The following references are added.</p> <p>Padmore T, Stark C, Turkevich LA, Champion JA. Quantitative analysis of the role of fiber length on phagocytosis and inflammatory response by alveolar macrophages. <i>Biochimica et biophysica acta</i>. 2017;1861 2:58-67;</p> <p>Schinwald A, Donaldson K. Use of back-scatter electron signals to visualise cell/nanowires interactions in vitro and in vivo; frustrated phagocytosis of long fibres in macrophages and compartmentalisation</p> |

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| | | <p>in mesothelial cells in vivo. Particle and fibre toxicology. 2012;9:34;</p> <p>Murphy FA, Schinwald A, Poland CA, Donaldson K. The mechanism of pleural inflammation by long carbon nanotubes: interaction of long fibres with macrophages stimulates them to amplify pro-inflammatory responses in mesothelial cells. Particle and fibre toxicology. 2012;9:8;</p> |
| | Page 26 | |
| Germany | <p>How it is measured or detected?</p> <p>There are also luciferase-based assays available, e.g. the IL2 LUC which already has been validated (see doi: 10.1016/j.tiv.2020.104832)</p> | There may be many assays available but those that are most commonly used are included in the text. |
| | Page 31 | |
| Germany | <p><i>Exposure to SWCNTs</i></p> <p>SWCNTs not introduced yet</p> <p>Should they be discussed already earlier on? What are the differences in reactions observed for SWCNTs to MWCNTs?</p> <p>markers of oxidative modification of lipids and proteins <u>as a result of oxidative stress</u></p> | <p>SWCNT is spelled out. The sentence is revised.</p> <p>Discussion on differences observed between SWCNT and MWCNTs is beyond the scope of this AOP.</p> |
| | Page 32 | |
| Germany | Other: The second sentence (EpiAlveolar model system) is related to TEER and should be rather moved to this paragraph | It is already stated that EpiAlveolar model can be used to assess ECM loss and TEER. |
| | Page 34 - (Event: 1499: Increased, activation of T (T) helper (h) type 2 cells) | |

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| Germany | There are other methods to detect increased activation of Th2 cells such as flow cytometry for measuring surface markers of cells. It would be good to include and describe additional methods under "How it is measured or Detected" for this event. | There are no standard or validated methods for measuring these KEs. The most common ones are included in the text but it is not necessary to include all available assays. |
| | Page 36 | |
| Germany | <i>Immunohistochemistry</i> It is suggested to delete the rather detailed protocol here. Instead a reference should be added. | In our opinion, the text is fine as is. Immunohistochemistry method can vary in details based on the type of antigen being detected. Moreover, the manufacturer provides antigen specific protocols. |
| US | Paragraph 7 Fibroblast proliferation assay Carbon nanomaterials have been demonstrated to interfere with conventional absorbance-based in vitro assays (WST-1), leading to bias in the results. Therefore, alternative reliable and reproducible methods should be provided for the assessment of fibroblast proliferation. | Vietti, et al., (<i>Part Fibre Toxicol</i> 10 , 52 (2013). https://doi.org/10.1186/1743-8977-10-52) have optimised the WST-1 assay for carbon nanotubes. Thus, it does seem like a good assay to assess fibroblast proliferation after exposure to CNTs. |
| | Page 40 | |
| Germany | References should be in alphabetical order. | References were listed in alphabetical order |
| | Page 41 | |
| Germany | <i>Bleomycin</i> References should be in alphabetical order. <i>Carbon nanotubes</i> This introductory paragraph would better fit to the section types of stressors (page 6). | We think that the intro provides context and is fine on page 41. References were listed in alphabetical order |

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| | Page 43 | |
| US | There is no space in "leukocytesinduces." Change to "leukocytes induces" | Corrected |
| | Page 44 | |
| Germany | <ul style="list-style-type: none"> - polyhexamethyleneguanidine phosphate should be introduced here, e.g.: a humidifier disinfectant, is known to cause lung toxicity, including inflammation and pulmonary fibrosis; it seems that there are some more recent studies available (e.g. https://www.nature.com/articles/s41598-021-85662-z, https://link.springer.com/article/10.1007/s13273-021-00169-y) - TRAF and IRAK were not introduced yet - Thapsigargin should be introduced, e.g. a tumor promoter in mammalian cells - <u>human granulocyte macrophage colony-stimulating factor</u> (GMC-CSF) - CXCL 8 <p>Throughout the document IL-8 is used</p> | The AOP has presented sufficient evidence and references in support of KE essentiality. It is not possible to cite each of the stressors and as such, not necessary for the AOP narrative. We have included it in the stressor section but not in the main text. |
| | Page 48 | |
| Germany | PM2.5, Mt2, Saa1, and Saa2 as well as ATII cells, CINC-3 and eotaxin are not introduced | Acronyms were introduced |
| | Page 49 | |
| Germany | coated quantum dots, <u>Cd-based nanoparticles</u> , | Corrected |
| | Page 52 | |
| Germany | <p>an increase in the levels <u>of both metalloproteinases and the corresponding tissue inhibitors of metalloproteinases</u> (MMP-2, TIMP-1, MMP-9, TIMP-2) mRNA expression</p> <p><i>For example, exposure to crystalline silica generates oxidative stress</i></p> | We haven't made size specific differentiation in responses here, which is not necessary in the context of this AOP, except where studies specifically state the use of nanomaterials in the experiment. |

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| | <p><i>Arras et al. (2001) studied the effect of IL-9 on the development of lung fibrosis after crystalline silica particle</i></p> <p>Are both referring to the nano form? This is not entirely clear. Since crystalline silica is used more often throughout the document, it might be helpful to indicate when nano forms were used.</p> | |
| | Page 53 | |
| Germany | The levels of TBARS and 8-OHdG, <u>which are biomarkers for oxidative stress,</u> increased after 3 days of instillation | “which are biomarkers for oxidative stress” was added |
| | Page 57 | |
| Germany | an increase in the expression of <u>surfactant protein D (SP-D)</u> mRNA | SP-D was defined |
| | Page 59 | |
| Germany | evaluated the role of <u>regulatory T cells</u> (Treg cells) | “regulatory T cells” as added |
| | Page 63 | |
| Germany | <p>FN1, FSP, Ki67 and PCNA expression, <u>which are markers of proliferation</u></p> <p>At week 4, the expression of α-SMA, collagen-I, and picro-sirius red increased</p> | Sentences were modified and corrected |