Organisation for Economic Co-operation and Development

DOCUMENT CODE

For Official Use **English - Or. English**

Adverse Outcome Pathway External Review Report

AOP 144: Endocytic Lysosomal Uptake Leading to Liver Fibrosis

This document has been prepared by Ms Julija Filipovska, review manager of AOP 144 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 144 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

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Table of contents

1. Introduction and background to specific AOP

Background

The project for development of AOP144: *Endocytic Lysosomal Uptake Leading to Liver Fibrosis* was submitted to the AOPs Development Programme in 2016 (project 1.47, original title *Lysosomal damage leading to liver inflammation*) by the European Commission.

AOP144 has undergone an internal review and modifications during 2019-2020 (Internal review AOP 144). Based on these, the OECD Secretariat to the Extended Advisory Group for Molecular Screening and Toxicogenomics (EAGMST) organised the scientific review of AOP144 snapshot [PDF].

A scientific review panel (Annex 1) 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 February 2021 and May 2021. Based on the initial responses to the charge questions (Annex 2) main issues (Section 2) were discussed at a teleconference on 24 April 2021 (Section 3.1). Based on the discussion at the teleconference (Section 3.2) and the agreed actions (Section 3.3), authors responded to the initial comments (Annex 2) and will revise the AOP following the agreed action list before submission to the EAGMST for approval.

Introduction

AOP144 links endocytic lysosomal uptake and the formation of liver fibrosis. The molecular initiating event (MIE) is endocytic lysosomal uptake of chemicals, leading to lysosomal disruption, the first key event (KE). Lysosomal disruption induces mitochondrial dysfunction, which leads to cell injury and both apoptosis and necrosis. Cell death results in release of damage-associated molecular patterns (DAMPs) which lead to increased production of pro-inflammatory mediators, the next KE along the path. Inflammatory mediators attract and activate leukocytes, which in turn release specific molecular mediators that activate hepatic stellate cells to synthesise increased amount of collagen I and III. Collagen accumulation at the tissue level leads to adverse outcome (AO) - liver fibrosis, which changes the normal functioning of the whole organ. Oxidative stress is also an important on-going process throughout the pathway, and is described as a modulating factor in the relevant KEs and KERs.

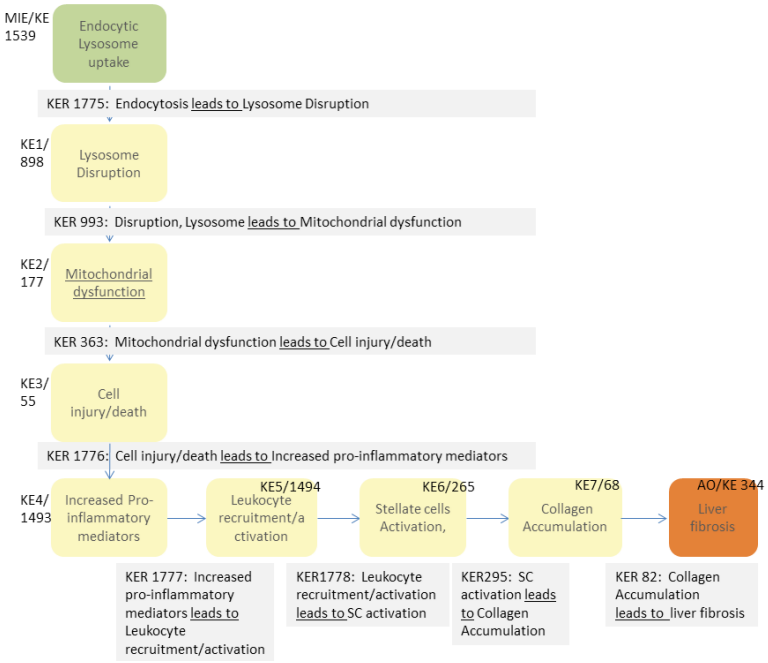


Figure1: Graphical representation of the components of AOP144.

The AOP *Endocytic lysosomal uptake to liver fibrosis* has high biological plausibility, supported with empirical evidence. However, quantitative data and temporal sequences between KEs are currently lacking and further efforts are necessary for their provision, but where temporal sequences between KEs are available, they are presented.

The essentiality of almost all of the KEs in this AOP was rated high as there is much experimental evidence that the blocking of one KE prevents the next downstream KE and therefore the whole AOP. Only the essentiality of KE2: Mitochondrial dysfunction was rated as moderate, as there are two pathways to apoptosis (the next downstream KE3), intrinsic and extrinsic, and only intrinsic pathway includes

mitochondrial dysfunction.

Based on the above and the level of empirical evidence for the KE relationships, the overall weight of the evidence (WoE) for the AOP144 is assessed as high. The gaps and uncertainties in the evidence are addressed, particularly for the linkages KE5 to KE7.

Evidence from a number of studies with mammals was used to support the wide taxonomic applicability of the AOP, from rodents to humans. There is no evidence to support sex or life stage specific differences in the pathway.

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.

A number of pro-fibrotic stressors linked to the perturbation of the inflammatory pathway and the adversity in the AOP144 are considered. They include drugs, nanomaterials and lysosomotropic detergents and are well characterised for their specific lysosomotropic properties at the subcellular level in the MIE.

The value of this AOP is that it might support chemical risk assessment by identifying upstream biomarkers for adverse outcome, even though the adequate cell model is not available. This systematic organisation of existing knowledge, but also of present uncertainties can facilitate regulatory processes, and help identify the need and opportunities for development of new testing methods.

2. Synthesis of main issues of the initial review comments

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

Summary of responses to CQ 1 - Scientific Quality

Overall initial reviewers' comments acknowledged that the authors have done a good job in collecting many relevant studies, clustering them per KE/KER and substantiating the AOP. In particular, the description of the evidence on the lysosomal disruption (KE1/898) was assessed as commendable.

However, there was concern that the bulk of the scientific literature incorporated in the AOP144 dates back before 2011 and it was recommended to revisit the more recent literature on nanomaterial (NM) hazard, especially in-depth mechanistic studies. Particular studies, specifically related to inflammasome activation were suggested by some reviewers (See Annex 2).

Summary of responses to CQ 2 - Weight of Evidence

Acknowledging the complexity of the inflammatory process, reviewers identified few aspects of the justification description and scoring for the weight of evidence calls that may need additional considerations and/or revisions. Specifically:

KER 1775: Endocytosis leads to Disruption, Lysosome

Reviewers agreed that the WoE for this KER is high and well justified.

It was noted that it may be useful to specify/clarify which types of endocytic pathways are covered by the upstream KE (MIE), i.e passive diffusion, clathrin-independent, caveolin-mediated, and the cell type (domain of applicability) to which the process is specific i.e. hepatocyte and/or Kupffer cells.

It was also suggested to elaborate/define the process of autophagy by which nanoparticle (NP) material is taken up by a cell and transported to the lysosome.

KER 993: Disruption, Lysosome leads to N/A, Mitochondrial dysfunction 1

There was no agreement on the "high" WoE for this KER in the initial review comments.

One reviewer suggested "moderate" call for the described WoE.

It was acknowledged that WoE for this KER is difficult to assess, especially since mitochondrial dysfunction is a key player in many cellular processes, including

proinflammatory status of the cell. Suggestions were made to include additional considerations for evidence on Endoplasmic Reticulum (ER) stress, inflammasome activation and apoptosis. Specific suggestions were made by some reviewers and this KER was specifically discussed at the end-of review teleconference under agenda items 5 and 6 (see section 3.1)

KER 363: Mitochondrial dysfunction 1 leads to Cell injury/death

Most reviewers agreed that the WoE for this KER is moderate and well justified. One reviewer was of the opinion that the evidence supporting this link is “high”, even if cell death can be achieved through alternatives pathways (e.g. extrinsic apoptosis or necrosis).

Another reviewer noted that the evidence described in this KER relates to neuronal tissue and suggested to add evidence relevant to liver tissue.

The suggestion on more detailed and reordered outline for the KEs in the KERs linking MIE up to KE *cell death/inflammation* (see KER993, agenda item 5) was also relevant for this KER, and was discussed under agenda item 5.

Consideration of ROS as an initiating stressor for the AOP was questioned and discussed at the TC (agenda item 4c).

KER 1776: Cell injury/death leads to Increased pro-inflammatory mediators

Reviewers agreed that the WoE for this KER is ‘high’ and well justified.

However, it was noted that the overlapping AOP38 follows a bit different KE link order, including tissue resident cell activation in-between. Potential alignment with AOP38 was discussed at the end-of-review TC integrally under agenda items 4 and 8.

KER 1777: Increased pro-inflammatory mediators leads to Leukocyte recruitment/activation

Overall reviewers agreed that the WoE for this KER is ‘high’ and well justified.

However it was noted that the current KER includes limited liver specific evidence in support of the KER for lymphocytes as infiltrating cells.

In addition, one reviewer raised an uncertainty about the downstream KE1494: *Rrecruitment of leukocytes* being the essential and sufficient KE to elicit fibrotic phenotype further downstream (subject of KER1778). Furthermore they pointed out specific evidence (see reference in Annex 2, reviewer 5) in support activation of Kupffer cells being sufficient to elicit the required level of HSC activation, although

lack of quantitative data was acknowledged (see below KER1778).

Potential future alignment of AOP144 and particularly the downstream KE 1494: *Leukocyte recruitment,* with AOP38 which contains KE 1493 (increased pro-inflammatory mediators but not KE 1494), was raised again and discussed also in the context of KER1778 (agenda item 10).

KER1778: Leukocyte recruitment/activation leads to Activation, Stellate cells

Overall reviewers agreed that the WoE for this KER is high. However suggestions were made for additional discussion to better describe and justify this evaluation:

- Potentially include description of the Stellate cell activation process as a two-step process: an initiation phase e.g. by injured hepatocytes, ROS or Kupffer cells, and a subsequent stimulation phase. Discuss the role of TGF-β1 specifically (link to AOP38 was pointed out again).
- One reviewer specifically questioned the essentiality assessment of the upstream KE1494: *Leukocyte recruitment/activation* based on the evidence currently included in this KE. Furthermore, it was recommended to include and discuss specific evidence regarding the critical role of recruited and resident macrophages, including Kupffer cells, in enhancing the fibrinogenic process by promoting the survival of activated HSCs in a NF-κB–dependent manner. Suggested evidence: Pradere et al, 2013; doi: 10.1002/hep.26429).

KER295: Activation, Stellate cells leads to Accumulation, Collagen

Reviewers agreed that the WoE for this KER is high and well justified.

KER 82: Accumulation, Collagen leads to N/A, Liver fibrosis

Reviewers agreed that the WoE for this KER is high and well justified.

WoE assessment for AOP144 as a whole (Biological plausibility, Concordance, Uncertainties)

For the overall AOP WoE assessment few points for improvements were made:

- Consider if better alignment with AOP38: Protein Alkylation leading to Liver Fibrosis. At the minimum, include in the overall WoE AOP144, a discussion of the uncertainties regarding the limited coverage of compensatory mechanisms that may counterbalance/modulate the KERs leading to adversity e.g. Arch Toxicol [2017] 91(11), 3477-350, as discussed in AOP38.
- Discuss the description of NMs dynamics in the body i.e. how the stressors access hepatic and inflammatory cells.

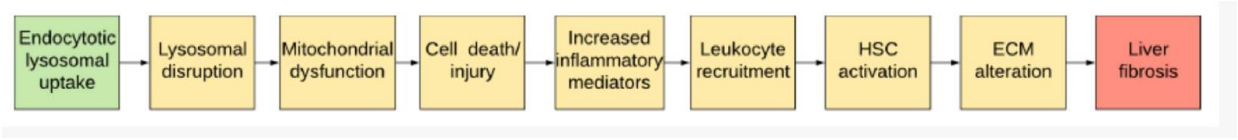
- Highlight the uncertainty regarding limitations in terms of availability of evidence on temporal and quantitative/dose-response aspects of the AOP.
- Highlight the complexity of mechanisms encompassed within KER 363: Mitochondrial dysfunction leads to Cell injury/death and discuss and clarify the involvement of ER stress.

Summary of Additional/general observations

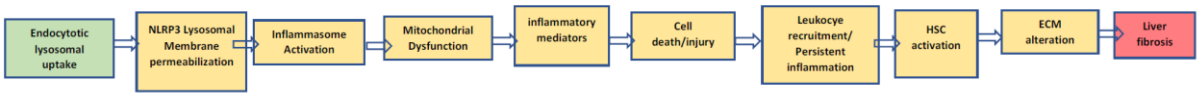
Some of the additional considerations emphasised the uncertainties already identified by CQ 1 and CQ2, including:

- Lack of evidence on quantitative aspects.
- Need for more detailed consideration of aspects of inflammation in particular: (i) the role of inflammasome and, (ii) the interplay of the different cell types involved (Kupfer cells, recruited/resident macrophages). Regarding point (i) the reviewer provided specific proposal and references for consideration:

AOP144:



Proposed revision:



- Editorial comments, more specific and revised wording.
- Better alignment with AOP38 was suggested again, if possible, at this stage but certainly in the future.

3. Summary record of the teleconference

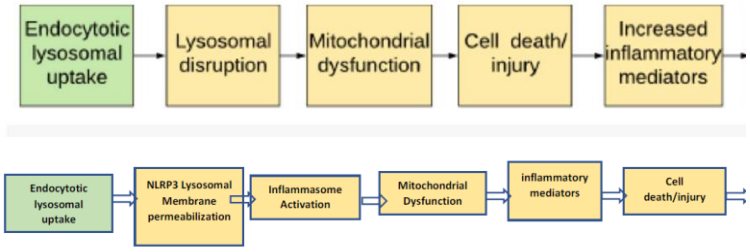
End-of-review teleconference (TC) was attended by four out of the five reviewers. All identified issues were discussed and the recording of the discussion was provided to all reviewers together with the draft summary. Three out of the four co-authors were present at the TC (absentee highlighted grey in Annex 1). Agenda for the TC was provided to all before the TC for comments and agreement.

3.1. TC agenda

Agenda for AOP 144 end-of-review teleconference discussion

24 April 2021, 9am-11:30 am Paris time

1. Introductions		1-2 min each
	All participants will introduce themselves (background/expertise/interests)	
2. Short introduction by Review Manager		3-5 min
	RM will briefly revisit the review process in the context of the OECD EAGMST and WNT/WPHA & outline the purpose of the TC ***The participants are invited to ask Qs and can seek clarifications.***	
3. CQ 1 - Scientific Quality		10 min
	• **Inclusion of more recent literature** ***Reviewers*** are invited to reiterate their observations on this issue ***Authors*** are invited to propose how and to what extent they can address it (above principle *applicable to all agenda items below*)	
CQ 2 - Weight of Evidence assessment and justification		(ideally 1.5 hours, 10 min per item)
4	**AOP144 overall (including additional observations)**	
4a	• **Highlight uncertainties** – compensatory mechanisms (as in AOP38) – quantitative/dose-response aspects	

4b	• **Coverage of the inflammatory process** – Incorporate evidence on inflammasome activation – Revisit description of the interplay and specific roles of different cell types involved (Kupfer cells, recruited/resident macrophages) ***The participants are invited to discuss these issues in the context of overall AOP assessment keeping in mind that some overlapping aspects are part of agenda items 5, 9 and 10 (which deal with specific KERs WoE).***	
4c	• **Discuss NMs dynamics** in the body i.e. how the stressors access hepatic and inflammatory cells.	
4d	• **Reconsider** inclusion of **ROS as a stressor** in the AOP summary **(page 3) or?**	Clarify understanding of comment made in context of KER363
5	***KER 993: Disruption, Lysosome leads to N/A, Mitochondrial dysfunction 1***	High WoE not agreed by all
	• **To discuss** – Is the WoE challenge based on questioning the essentiality of mitochondrial dysfunction for the AOP or occurrence of the next KE (cell death/injury), in which case the uncertainty of the WoE relates to KER 363. – More detailed description of relevant aspects of mitochondrial dysfunction e.g. inhibition/uncoupling of the respiratory chain – Possibility to incorporate evidence on inflammasome, ER stress and apoptosis within existing KEs and KERs **OR** consider Alternative series of KEs  **RM Q:** could indirect KER between Mitochondrial dysfunction and increased inflammatory mediators help	

	address the issue in the current outline?	
6	***KER 363: Mitochondrial dysfunction 1 leads to Cell injury/death***	Suggestion to upgrade WoE from moderate to high
	• **To discuss** – Would "high" WoE be justified for this particular KER regardless there may be other KERs converging onto the <u>downstream</u> KE *Cell injury/death* – Include liver specific evidence (neuronal tissue only covered currently)	
7	***KER 1775: Endocytosis leads to Disruption, Lysosome***	WoE agreed
	• **To clarify/be more specific** – Types of uptake mechanisms covered – Elaborate/define the process of autophagy – Applicability domain (cell types)	
8	***KER 1776: Cell injury/death leads to Increased pro-inflammatory mediators***	WoE agreed
	• **Confirm agreement on WoE call** – Potential discussion on further alignment with AOP38 discussed under agenda item 4a (here specifically role of resident cell/macrophage activation and whether evidence can be incorporated in the existing AOP outline?)	
9	***KER 1777: Increased pro-inflammatory mediators leads to Leukocyte recruitment/activation***	WoE agreed
	• **Consider** – Inclusion of liver specific evidence for leukocyte recruitment (relevant also to next KER1778) – Uncertainty due to evidence on the role of activation of Kupffer cells and subsequent effect on HSC activation (discuss under item 10) [RM Q: role in generation of proinflammatory mediators?]	
10	***KER1778: Leukocyte recruitment/activation leads to Activation, Stellate cells***	WoE agreed, suggestions made to

		strengthen justification
	• **Improve the description of the stellate cell activation** – Consider two-step process: an initiation phase e.g. by injured hepatocytes, ROS or Kupffer cells (resident cells), and a subsequent stimulation phase [recruited macrophages, leukocytes?]. – Discuss the role of TGF-β1 specifically (link to AOP38 was pointed out again). – Discuss the role of recruited and residential macrophages in HSC activation via NF-κB. • **Include additional evidence for leukocyte recruitment, if further discussion needed following discussion on agenda item 9.**	
11. Any other issues not covered		
	If applicable	
12. Overview of agreed actions		
	Group invited to comment/agree	

3.2. Main issues and responses during the call

Reviewers agreed that the TC agenda covered all the issues raised with the initial written comments. Each agenda item was introduced by the RM, discussed by all and action items (Section 3.3) were agreed as a way forward to revise the AOP144 before submission to the EAGMST.

Agenda item 3 - CQ1: Scientific Quality

The initial comments pointed out that it may be useful to update the AOP with more recent literature and some specific references were suggested.

Authors agreed to review and include more recent literature but questioned whether well studied basic biological processes need updated literature.

After further discussion it was agreed that in the past 10 years there has been significant new literature addressing specific mechanisms relevant to (i) lysosomal membrane permeabilization (LMP) and mitochondrial dysfunction; (ii) specific molecular mechanisms (e.g. NLRP3[1] inflammasome activation) relevant and

[1] NOD-, LRR- and pyrin domain-containing protein 3

leading to different types of cell death (e.g. apoptosis, necrosis...); (iii) role of Kupfer cells in the inflammatory process specific to liver.

Including illustrations was also encouraged to help readers with understanding the different molecular mechanisms and the sequence of events even within individual KEs descriptions.

Authors agreed to consider the newer references already provided and those that will be sent after the TC and include them, either in the same outline of KEs or slightly modify the sequence considering the new evidence (see discussion below).

Authors emphasised that mitochondrial dysfunction particularly is a hot topic and the KE describing it is likely to be further modified by other authors who currently work on AOPs including the wide range of aspects of mitochondrial dysfunction. Nevertheless, it was agreed to consider the newer literature relevant to liver fibrosis.

One reviewer also pointed out that there have been more recent advancements in the methods for measuring aspects related to mitochondrial dysfunction which may need to be included (e.g. mitochondrial respiration, mitophagy, mitochondrial oxidative stress with ROS-indicators).

Agenda item 4 – WoE overall for AOP144

Item 4a - *alignment with AOP38* **and 4b** *representation of the inflammatory hub KEs in AOP38 vs AOP144* were discussed together

Authors agreed that there is a need to align the AOPs (i.e. merge the inflammatory hub KE). The apparent discrepancy between AOP144 and AOP38 is a result of the timing of the development of the particular AOPs and the workshop on representing the inflammatory hub in a number of different AOPs (Villeneuve et. al., 2018: 10.1093/toxsci/kfy047). The KE: Tissue resident cell activation will be added downstream of KE: Cell death/injury.

Also, the limitation of the AOP144 in quantitative understanding was acknowledged and will be highlighted more in the overall AOP assessment.

Inclusion of LMP and NLRP3 inflammasome activation was discussed extensively. It was agreed that evidence for NLRP3 inflammasome activation in LMP will be included in the KE: lysosomal disruption, as it may be considered an aspect of the disruption. Information for describing this aspect of lysosomal disruption will be taken from an OECD report[2] examining the ability of nanomaterials inducing LMP and also references provided by reviewers.

For the suggested KE *inflammasome activation,* authors informed that they are involved in a separate project where such KE is being developed for AOP relevant to COVID19 pathogenesis and they will include the relevant evidence in AOP144. The evidence may be included as a separate KE or within a KER leading from

[2] No. 92 - Ability of biopersistent/biodurable manufactured nanomaterials (MNs) to induce lysosomal membrane permeabilization (LMP) as a prediction of their long-term toxic effects

lysosomal disruption to mitochondrial dysfunction, depending on the weight of the measurable evidence relevant to AOP144 and liver toxicity. Reviewers agreed that this is a very good approach and solution for AOP144. Caution was raised that there may not be high WoE relevant for this AOP, but the additional references would be explored by authors to support potential KER for a new KE: inflammasome activation in AOP144.

Review manager suggested that if there is no sufficient evidence to include new KE, then to capture the limited evidence in the KER between the updated KE: lysosomal disruption and KE: mitochondrial dysfunction, as a gap or uncertainty.

Role of different cells in progressing the inflammatory process leading to fibrosis in the liver: it was agreed that inclusion of the KE: Tissue resident cell activation and alignment with AOP38 will help clarify the role of different cells as key drivers of the AOP.

Item 4c – *dynamics of nanoparticles in reaching liver*

It was agreed that it would be useful to include information supporting how nanoparticles reach the liver, the organ that is in the focus of this AOP. Authors informed that they already identified literature to support and reviewers may also send additional references.

Item 4d – *ROS as a stressor for this AOP*

It was agreed that ROS is an important element of AOP144 but not an initiating stressor and needs to be removed from the stressor list on the AOP main page.

Agenda Item 5 – KER 993: Disruption, Lysosome leads to N/A, Mitochondrial dysfunction 1

The panel agreed that high WoE for this KER would be justified and even further strengthened by the addition of the evidence discussed under the previous agenda items and by the actions 1, 3 and 4 (Section 3.3).

Agenda Item 6 – KER 363: Mitochondrial dysfunction 1 leads to Cell injury/death

All reviewers agreed that the WoE for this KER, particularly in the liver is very strong and authors were asked to explain their moderate call. It was recalled that the moderate call is a result of reusing the KER from a neuro-based AOP where evidence may not have been high at the time. Discussion followed that the evidence is particularly high for the link between LMP and mitochondrial dysfunction via cathepsins (to be included as a result of Action 3 (Section 3.3)) even bypassing inflammasome activation.

This discussion highlighted the difficulty in managing evidence and WoE calls in the AOP Wiki/KB, for KERs between KEs with same biological mechanisms in different tissues/organs. Authors of AOP144 and the authors of the neuro-based AOP that is the basis for KER363 are from the same organisation and will discuss how to best add the liver specific evidence in the KER and upgrade the WoE. However, the issue

of WoE for tissue/organ specific KERs and overall KER may need to be discussed also at the EAGMST level or at within its Knowledge Base development subgroup.

In addition, it was recognised that the KE: mitochondrial dysfunction is complex and may cover many aspects of dysfunction not all of which are relevant to each KER or pathway. However, it was recognised that it may be difficult to resolve the complexity without potentially losing important interactions between the different molecular aspects of mitochondrial dysfunction. Authors informed the group that there is a project underway within the OECD that develops a network of AOPs around the different aspects of mitochondrial dysfunction which may provide or inform a practical solution to address the issue of representation of complex KEs and KERs in the future.

Agenda Item 7 – KER 1775: Endocytosis leads to Disruption, Lysosome

WoE for this KER was agreed. However it was discussed that the applicability domain needed additional clarification in regard to relevant stressors (type of chemicals/materials) and cell types critical for progression of the pathway.

Also correction of the type of mechanisms involved in the upstream event (MIE) uptake by endocytosis, not autophagy.

See action 8 in the Actions list (Section 3.3).

Agenda Item 8 – KER 1776: Cell injury/death leads to Increased pro-inflammatory mediators

WoE for this KER was agreed.

Panel agreed that the points raised for this KER in the initial comments (Annex 2) would be addressed by the alignment of AOP 144 with AOP 38 discussed and agreed under agenda item 4 (Section 3.2) with actions 1 and 2 (section 3.3).

Agenda Item 9 – KER 1777: Increased pro-inflammatory mediators leads to Leukocyte recruitment/activation

WoE for this KER was agreed.

As above, it was reiterated that the points raised for this KER in the initial comments (Annex 2) would be addressed by the alignment with AOP 38 discussed and agreed under agenda item 4 (Section 3.2) with actions 1 and 2 (section 3.3).

In addition, applicability domain in relation to cell types relevant for this particular KER would need to be updated with liver specific aspects (action 8 in section 3.3).

Agenda Item 10 – KER 1778: Leukocyte recruitment/activation leads to Activation, Stellate cells

WoE for this KER was agreed. However, the role of persistency of the inflammation in driving the adversity was emphasised and it was agreed that it would be good to include a mention/discussion on it in this or the previous KER.

Authors pointed out again that, as with the previous two KER discussions, this point would be addressed by the alignment with AOP 38 where emphasis was on the inflammation process that when persisting and not resolving leads to adversity, while the emphasis of AOP 144 was the initiation process of lysosomal uptake.

It was agreed that alignment of AOP 144 with AOP38 as discussed above would be sufficient to address comments on KE1778.

Agenda Item 11 – Any other issues not covered

Reviewer made a point about the importance of ER stress in relation to nanoparticles but recognised that this is a very complex process that may need an entire dedicated AOP. At present ER stress is just briefly mentioned in KER363 and KE: mitochondrial dysfunction, but not sufficiently elaborated.

Given the complexity and the main focus of the current AOP 144, it was agreed that ER stress need not be further elaborated in this AOP, but only identified as an important gap to potentially address in the future. The reviewer supported this by additional references (Section 4) to be considered for future development within OECD AOP programme or by outside developers.

Similarly, recent developments in the research and understanding of the role of metabolism of immune cells is lacking in AOP 144 and may be a good ground for future iteration of lysosomal uptake leading to liver fibrosis or other networking AOPs, e.g. those dealing with specific aspects of mitochondrial dysfunction.

3.3. Action list for revision of AOP144 before submission for approval

The action list below was developed at the end-of review teleconference by reviewers and agreed as a guide for planned revisions by the authors.

For Authors

1. Consider and include newer literature on (i) LMP and mitochondrial dysfunction; (ii) molecular mechanisms of cell death; (iii) role of Kupffer cells in liver inflammatory process

   For inflammasome activation, Mitochondrial damage, Cell death:
   - Zheng, Z.; Li, G. Mechanisms and Therapeutic Regulation of Pyroptosis in Inflammatory Diseases and Cancer. Int. J. Mol. Sci. 2020, 21, 1456. https://doi.org/10.3390/ijms21041456

   For the NLRP3 inflammasome plays an important role in liver fibrosis development:
   - Inzaugarat ME, Johnson CD, Holtmann TM, et al. NLR Family Pyrin Domain-Containing 3 Inflammasome Activation in Hepatic Stellate Cells Induces Liver Fibrosis in Mice. *Hepatology*. 2019;69(2):845-859. doi:10.1002/hep.30252)

   For the uncontrolled activation of the immune response and natural aging process, which may aggravate liver inflammation and fibrosis:
   - Paloma Gallego 1,y, Beatriz Castejón-Vega 2,y, José A. del Campo 3,* and Mario D. Cordero The Absence of NLRP3-inflammasome Modulates Hepatic Fibrosis Progression, Lipid Metabolism, and Inflammation in KO NLRP3 Mice during Aging. Cells 2020, 9, 2148; doi:10.3390/cells9102148 www.mdpi.com/journal/cells).

   For the extent of lysosomal membrane permeabilization appears to regulate NLRP3, as limited permeabilization facilitates NLRP3–inflammasome activation, whereas complete

loss of lysosomal integrity inhibits activation:

- Katsnelson et al., 2016). Katsnelson, M.A., K.M. Lozada-Soto, H.M. Russo, B.A. Miller, and G.R. Dubyak. 2016. NLRP3 inflammasome signaling is activated by low-level lysosome disruption but inhibited by extensive lysosome disruption: roles for K+ efflux and Ca2+ influx. Am. J. Physiol. Cell Physiol. 311:C83–C100. https://doi.org/10.1152/ajpcell.00298.2015

For lysosomal membrane permeabilization (LMP) has been proposed to precede nanoparticle induced macrophage injury and NLRP3 inflammasome activation; however, the underlying mechanism(s) of LMP is unknown. We propose that nanoparticle-induced lysosomal hyperpolarization triggers LMP.

- Tahereh Ziglari 1 , Zifan Wang 2 and Andrij Holian. Contribution of Particle-Induced Lysosomal Membrane Hyperpolarization to Lysosomal Membrane Permeabilization. Int. J. Mol. Sci. 2021, 22, 2277. https://doi.org/10.3390/ijms22052277

Role of hepatic macrophages, including Kupffer cells in the fibrinogenic process

- Pradere et al, 2013; doi: 10.1002/hep.26429).

2. Align AOP144 with AOP38 by adding KE: Resident cell activation after KE: Cell death/injury.
   - Also, similar to AOP38 discuss the uncertainties for AOP144 related to compensatory mechanisms to adversity
   - Highlight the gap in quantitative understanding of the KERs and the AOP overall
3. Update KE898: lysosomal disruption to include link of NLRP3 inflammasome activation to LMP
4. Include evidence for NLRP3 inflammasome activation either as a separate KE or within a KER between KE: lysosomal disruption and mitochondrial dysfunction.
   - References considered in the OECD Report [2]
   - Relevant references from Action 1 above
   - References supporting WoE between LMP and inflammasome activation listed above
5. Include in the AOP background a short discussion and references regarding the dynamics of the nanoparticles reaching the liver.
6. Remove ROS from the stressor list on the initiating AOP page
7. Consider revision of KER363 with liver relevant evidence and upgrade WoE call to ‘high’.
8. Include more recent methods for measuring aspects related to mitochondrial dysfunction (e.g. mitochondrial respiration, mitophagy, mitochondrial oxidative stress with ROS-indicators)
9. For KER 1775: Endocytosis leads to Disruption, Lysosome
   - Applicability domain: clarify and include supporting references on which **chemicals/materials** are within and which are out of the domain (e.g. chemicals that passively enter lysosomes or do not enter lysosomes and do not cause their disruption or LMP)
   - Applicability domain: clarify and include supporting references for the **cell types** critical for the KER in liver. This action is also applicable to KER1777 and 1778. (e.g. Kupffer cells, infiltrating macrophages, which types of inflammatory cells)
   - Correction on page 20: “endocytosis” instead of “autophagy” in the MIE description

For EAGMST

10. Note the discussion under agenda item 6 and the need to:

    - optimise within the Wiki/KB the representation and re-use of KERs within different tissues/organs from complex and re-usable KEs applicable to different tissue/organs
    - develop additional guidance in the Users' Handbook (or where relevant) for development and re-use of complex KEs such as mitochondrial dysfunction or other similar examples

11. Encourage future developments of AOP144 and related networking AOPs to consider and include: (i) the role of ER stress for nanoparticle related toxicity and, (ii) the role of metabolism in immune cell function (see further discussion, Section 4).

4. Further discussion

Following the TC, reviewer provided the following references to support the discussion under agenda item 11 and future developments:

Huo et al., 2015 (doi: 10.1016/j.biomaterials.2015.05.029): Silver nanoparticles activate endoplasmic reticulum stress signalling pathway in cell and mouse models: The role in toxicity evaluation

Simard et al., 2015 (doi: 10.1074/jbc.M114.61089) Silver nanoparticles induce degradation of the endoplasmic reticulum stress sensor activating transcription factor-6 leading to activation of the NLRP-3 inflammasome.

Xia et al., 2007 (doi: 10.1021/nn700256c) Cationic Polystyrene Nanosphere Toxicity Depends on Cell-Specific Endocytic and Mitochondrial Injury Pathways

O'Neill et al., 2016 (doi: 10.1038/nri.2016.70) A guide to immunometabolism for immunologists

5. Outcome of the external review

Authors undertake to revise the AOP144: *Endocytic Lysosomal Uptake Leading to Liver Fibrosis* following the action list in section 3.3, before submission to the OECD EAGMST for approval. They will provide the revised AOP together with list of revisions against the action list. This report contains authors' responses (Annex 2) to the initial written review comments, also taking into account the discussion at the end-of-review TC (Section 3).

Reviewers agree that the revised AOP will represent a significant contribution to the OECD AOP Programme, and together with AOP38 provides a solid scaffold for expanding a network of knowledge, testing methods and causal linkages for liver toxicity.

Annex 1: Table of review participants

Reviewers[3]	Affiliation
Seiichi Ishida	Division of Applied Life Science, Graduate School of Engineering, Sojo University, Kumamto, Japan
Laura Suter-Dick	School of Life Sciences (Institute of Chemistry and Bioanalytics), University of Applied Sciences Northwestern Switzerland, Switzerland
Albert Braeuning	German Federal Institute for Risk Assessment (BfR) Berlin, Germany
Rob Vandebriel	Centre for Health Protection National Institute Of Public Health & Environment (RIVM) Bilthoven, the Netherlands
Mary Gulumian	National Institute for Occupational Health (NIOH) Johannesburg, South Africa

Authors	Affiliation
Marina Kuburic	Independent Consultant
Kirsten Gerloff	Independent Consultant
Brigitte Landesmann	European Commission, Joint Research Centre (JRC) Chemical Safety and Alternative Methods, and EURL ECVAM, Ispra, Italy
Alicia Paini	European Commission, Joint Research Centre (JRC) Chemical Safety and Alternative Methods, and EURL ECVAM, Ispra, Italy

Review Manager	Affiliation
Julija Filipovska	Independent Consultant

[3] Order of reviewers in this list does not correspond to the order of review number in the comments table in Annex2.

Based on the analysis of the declaration of interest from the review manager, the OECD Secretariat, organiser of the review, can confirm that the review manager has no potential conflict of interest (COI). Based on the analysis of the declarations of interest made by the reviewers, the review manager can confirm that there are no potential COIs of reviewers.

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

Charge Question 1: Scientific quality:		**Author responses**
Does the AOP incorporate the critical scientific literature and evidence?		
Reviewer 1	Yes	
Reviewer 2	My overall impression is that the references are relatively old. It is preferable to refer more recent literatures.	
Reviewer 3	Yes/No. The authors have done a good job in collecting many relevant studies and clustering them per KE/KER. I acknowledge and thank them for this big piece of work. Overall, the report does a good job in substantiating the AOP. However, there is concern that the bulk of the scientific literature incorporated dates back before 2011. See also below.	references will be updated
Reviewer 4	Yes but the inclusion of more recent publications should be considered	
Reviewer 5	No comment	
Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?		
Reviewer 1	Yes and See comments below	
Reviewer 1	No comment	
Reviewer 3	Partially. Although I did not make a quantitative analysis of the references, it is obvious that only a small percentage of the references stems from the last decade. This may set back the report in two ways: (1) in the past decade, clearly more insight in mechanisms underlying inflammation has been gained, (2) many studies on nanomaterial (NM) hazard, especially in-depth mechanistic studies, have been published in the last decade.	With update of literature we will gain more insight into new scientific work, to address the points raised by this review. We will evaluate the information available to include the inflammasome as KE.
Reviewer 4	It certainly does specially the involvement of lysosomal disruption is certainly commendable.	
Reviewer 5	No comment	
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.		
KER 1775: Endocytosis leads to Disruption, Lysosome		
Reviewer 1	Yes. The MIE (1539) defines by title endocytosis as the mechanism of uptake of the toxic compounds; the MIE description also mentions passive diffusion of compounds into the lysosome. While the link from endocytosis to lysosomal disruption appears causal (given sufficient quantity of uptake of a toxic compound), it is not entirely clear whether the MIE is meant to also include lysosomal accumulation of compounds not caused by endocytosis. This should be clarified.	MIE is not meant to include lysosomal accumulation of compounds not caused by endocytosis. will be corrected
Reviewer 2	Yes. P. 21, More specific staining,,,: Target proteins and their antibodies should be described.	More specific staining can be achieved by staining with anti-LAMP1, monoclonal antibodies against lysosome-associated membrane protein 1 (Kroemer and Jäättelä, 2005). will be added
Reviewer 3	Yes. LMP is a well-established effect of NM uptake, so - with the provision described above- I agree with high weight of evidence.	
Reviewer 3	On page 16, the authors state that caveolin-mediated uptake does not lead to localization in lysosomes. This does mean some types or sizes of NM do not fit this KER?	Caveolae-dependent pathways can bypass lysosomes (e.g.Parton and Simons, 2007). However, some studies, Wu et al. (2019) found that organically modified silica nanoparticles uptaken by caveolae mediated endocytosis accumulated in lysosomes. I think more studies are needed on this. will be added
Reviewer 3	On page 20, the authors state that after being taken up, NM are transported to the lysosome by autophagy. This should be elaborated, at least autophagy should be explained.	Should be phagocytosis, not autophagy. will be added
Reviewer 4	Well described and justified.	
Reviewer 5	MIE: Endocytic lysosomal uptake of chemicals: The limitation of the AOP to substances that are actively uptaken into the lysosome (such as NPs) should be clearly stated. It would be useful to know if the uptake is mainly by the hepatocyte of by the Kupffer cell.	Agree. Sadauskas et al. (2007), Tsoi et al. (2016) found that NPs accumulate in the Kupffer cells. Kermanizadeh et al. (2012) found that hepatocytes are able to internalize NMs in large quantities. Cheng et al. (2012) proved that positively charged silica NPs were significantly uptaken by hepatocytes, while negatively charged by Kupffer cells. It is prevailing assumption is that Kupffer cells are responsible for nanomaterial uptake by the liver, however there are some discrepancies and more studies on

		factors which determine cells that will uptake NMs are needed. will be added
KER 993: Disruption, Lysosome leads to N/A, Mitochondrial dysfunction 1		
Reviewer 1	Yes	
Reviewer 2	Yes. P. 47, “LMP” should be spelled out and its definition should be required.	lysosomal membrane permeabilization (LMP) is spelt out on the page before 46
Reviewer 3	NO. In my view, the relationship between KE lysosomal disruption and KE mitochondrial dysfunction is a difficult one, especially since mitochondrial dysfunction is a key player in many cellular processes. I would rank the weight of evidence as moderate.	All reviewers agreed that the WoE for this KER, particularly in the liver is very strong. It will be discussed with the authors of the other AOPs involved how to best add the liver specific evidence in the KER and upgrade the WoE.
Reviewer 3	The past 10 years, the close relationship between immune cell function and metabolism has become evident. Possibly, this may help to better evaluate the consequences of e.g. inhibition of the respiratory chain (page 23). This inhibition may be linked to a more pro-inflammatory status of the cell.	will be mentioned in the description
Reviewer 3	Several NM have been shown to induce ER stress. On page 24, it is stated that ER stress can induce mitochondrial dysfunction, and vice versa. Are the authors convinced that ER stress can’t be seen as a KE upstream or downstream of the KE mitochondrial dysfunction?	it was agreed that ER stress need not be further elaborated in this AOP, but only identified as an important gap to potentially address in the future.
Reviewer 4	A revised version is proposed as below	
Reviewer 5	Link between lysosomal disruption and mitochondrial dysfunction: Mitochondrial dysfunction is not necessarily synonym with MPTP-opening leading to apoptosis. Mitochondrial dysfunction could also refer to uncoupling, or other alterations in the respiratory chain. I suggest to be more specific here, as the phenomenon is the intrinsic apoptotic pathway.	newer literature relevant to liver fibrosis will be added, including the OECD document KE mitochondrial dysfunction is complex and may cover many aspects of dysfunction not all of which are relevant to each KER or pathway.
KER 363: Mitochondrial dysfunction 1 leads to Cell injury/death		
Reviewer 1	Yes. WoE considered moderate by the authors; appears nonetheless plausible.	
Reviewer 2	Yes. P. 25, (GSSH + NADPH + H+ à 2 GSH + NADP+). should be corrected.	will be done
Reviewer 2	p. 50, Are there any cases in liver? Most cases described in this section are relating to nerves.	liver examples will be added
Reviewer 3	Yes. I agree with a moderate weight of evidence	
Reviewer 4	A revised version is proposed as below.	
Reviewer 5	KER: mitochondrial dysfunction => cell injury/death: The evidence supporting this link is “high”, even if cell death can be achieved through alternatives pathways (e.g.	We are investigating how we can change this to have more selective WOE for different organs. The KE

	extrinsic apoptosis or necrosis). Stressors: Please, reconsider ROS as a stressor. ROS in this context are generated intracellularly mainly by detoxification, or by the mitochondria, or by the lysosome. As a stessor causal to the MIE perhaps inappropriate	was developed for MD (AOP3) in neuron thus the WOE was considered at the time of development moderate due to the evidence available. ROS will be removed from the stressor list on the AOP main page.
KER 1776: Cell injury/death leads to Increased pro-inflammatory mediators		
Reviewer 1	Yes. The KE/KER is spanning the whole process from cell death of hepatocytes to the increase of pro-inflammatory mediators caused by tissue-resident macrophages. I wonder why this AOP has not used the approach of AOP 38 (protein alkylation leading to liver fibrosis), where activation of macrophages (KE 1492) is present as an additional KE between KE 55 (cell death) and KE 1493 (increase in inflammatory mediators). See also general comment below.	alignment with AOP 38 will be done
Reviewer 2	Yes. P.55, There is evidence that the immune system has…: References should be cited.	reference will be added
Reviewer 3	Yes. I agree with a high weight of evidence	
Reviewer 4	A revised version is proposed as below	
Reviewer 5	No comment	
KER 1777: Increased pro-inflammatory mediators leads to Leukocyte recruitment/activation		
Reviewer 1	Yes. KE 1494 is not part of the existing AOP 38 (protein alkylation leading to liver fibrosis), which describes the same biology as this AOP. Preferably, both AOPs should be aligned.	alignment with AOP 38 will be done
Reviewer 2	P. 32, LIVER: should be removed.	This is a shared hub KE description and therefore explanations and references for various tissues are given, following a general description on how the KE works Therefore, liver is mentioned here.
	P. 61, There is essential role of interleukins, but…: References should be cited.	reference will be added
Reviewer 3	Yes. I agree with a high weight of evidence	
Reviewer 4	Well described and justified.	
Reviewer 5	In this particular context, I am not sure that the weight of evidence supports the recruitment of leukocytes. Resident macrophages (Kupffer cells) may be sufficient to elicit fibrotic phenotype. Based on literature activated Kupffer cells may be sufficient to elicit the required level of HSC activation (indeed, the lack of quantitative data makes this a difficult statement to prove).	alignment with AOP 38 will be done

	Recruitment of leukocytes occurs, but may not be absolutely necessary to elicit the response. At least based on the arguments provided and the current literature. Evidence of infiltrating cells is provided, but mainly referring to other tissues; this probably also occurs in the liver, but it is not proven that is absolutely necessary for the development of fibrosis.	
KER1778: Leukocyte recruitment/activation leads to Activation, Stellate cells		
Reviewer 1	Yes. In KER 1778 it is described that stellate cell activation depends on two steps, an initiation phase consisting of HSC activation e.g. by injured hepatocytes, ROS or Kupffer cells, as well as a subsequent stimulation phase. It might be discussed whether this can be included in the AOP.	will be included
Reviewer 2	Yes. P. 37, Recruited monocytes recruited mature into… should be corrected.	will be corrected
	P. 37, There are two “references” section.	will be corrected
Reviewer 3	Yes I agree with a high weight of evidence	
Reviewer 4	Well described and justified.	
Reviewer 5	Essentiality of KE5: The logic supporting this Key event as a stand-alone event is unclear to me. Evidence shows that lack of Kupffer cells prevent fibrosis what would point to Kupffer cell activation as the essential KE. The recruitment of Leucocytes (circulating cells) is not specifically addressed here and not supported by the scientific evidence provided. Additional evidence suggests that recruited macrophages work together with Kupffer cells in the fibrinogenic process: Hepatic macrophages, including Kupffer cells and recruited macrophages, also enhance liver fibrosis by promoting the survival of activated HSCs in a NF-κB–dependent manner; this reference could be added: Pradere et al, 2013; doi: 10.1002/hep.26429).	5th KE= in this AOP Increased Pro-inflammatory mediators will be solved by alignment with AOP 38
Reviewer 5	Relationship between inflammation and HSC activation: As the authors already state, TGF-β1 is a strong promoter of fibrosis. This is also a KE in AOP #38. Question is, why is TGF-β1 release not a key event leading to HSC-activation in this AOP?	TGF-β1is included in KE Increased Pro-inflammatory mediators the AOP (also 38) has been updated to correspond to the agreed hub-KEs to represent inflammation
KER295: Activation, Stellate cells leads to Accumulation, Collagen		
Reviewer 1	yes	
Reviewer 2	Yes. P. 69, Increasing matrix stiffness is a stimulus for HSC activation…: References should be cited.	reference will be added
Reviewer 3	I agree with a high weight of evidence	
Reviewer 4	Well described and justified.	
Reviewer 5	No comment	

KER 82: Accumulation, Collagen leads to N/A, Liver fibrosis		
Reviewer 1	yes	
Reviewer 2	No comment	
Reviewer 3	I agree with a high weight of evidence	
Reviewer 4	Well described and justified.	
Reviewer 5	No comment	
WoE for AOP144 as a whole (Biological plausibility, Concordance, Uncertainties)		
Reviewer 1	In general, I consider the WoE for this AOP good. General points to be addressed, are as follows: This AOP contains a sequence of KE which is more or less identical to AOP 38 (protein alkylation leading to liver fibrosis). As noted above, however, there are some discrepancies between the two AOPs with respect to some KE (1492 not present in this AOP, 1494 only present here but not in AOP 38). Ideally, this should be aligned, as the underlying biology is the same in both AOPs. A number of weaknesses of the existing AOP 38 have been highlighted in a paper by Leist et al. (Arch Toxicol [2017] 91(11), 3477-3505). The criticisms e.g. comprise the fact that anti-fibrotic mechanisms are not considered in the uni-directional AOP, and that some KE may happen without leading to the AO, due to e.g. counter-balancing mechanisms and/or the necessity for multiple stimulations of a mechanisms. The criticisms address the KE and KER common to AOP 38 and this AOP. Even though it may not be possible yet to incorporate the above into the present AOP, I recommend that the critique should nonetheless be mentioned in the general AOP description.	Will be mentioned in more detail in the general AOP description, that they do not include repair processes, regeneration and counter-regulation, exposure and timing conditions, as for most other AOPs. Suggestion is that this descriptive AOP in the future is converted in the quantitative AOP.
Reviewer 2	Yes. The description of NMs dynamics in the body would be preferable to show their interaction to hepatic or inflammation relating cells. The authors pointed out that the importance of the route of exposure (i.e. P. 2), but did not show clearly the evidence of their distribution to liver. I think the authors should indicate the reference(s) which analyze their distribution in the body.	Distribution depends on the multiple factors, such as size, surface charge, modification, administration route and opsonization (Lin et al. 2014). The liver is described as the primary organ for the distribution of Ag NP, no matter if the administration route is intravenous (Park et al. 2011), oral (Loeschner et al. 2011), subcutaneous (Tang et al. 2008) or through inhalation (Takaneka et al. 2001). Sadauskas et al. (2009), showed that intravenously administered gold nanoparticles accumulate in the liver in mice. Liver was proved to be the primary organ for TiO2 NP as well (Xie et

		al.2011). will be added
Reviewer 3	To me, there is sufficient biological plausibility for AOP 144 as a whole.	
Reviewer 3	Regarding concordance, the authors cite studies that establish for two KE which one is upstream, and which is one downstream. Only few studies on temporal and dose-response concordance are cited, but this can be explained by the fact that mechanistic studies are often limited in this respect.	Agreed, we stressed this in the overall assessment of AOP P4.
Reviewer 3	In my view the by far largest uncertainty is between KE 898 lysosomal disruption and KE 55 cell injury/death. While I agree that these two KE's are linked, the KE or KE's in-between are difficult to judge. This of course has to do with the many mechanisms involved. The authors should e.g. clarify whether ER stress is involved in this AOP.	it was agreed that ER stress need not be further elaborated in this AOP, but only identified as an important gap to potentially address in the future.
Reviewer 4	With the revised version, the Proposed AOP will satisfy the required biological plausibility, concordance and uncertainties.	
Reviewer 5	AO: Please, revisit the wording. The accumulation and modification of composition of ECM for an extended period is the definition of fibrosis (= AO of this AOP).	agreed and will be corrected
Additional/general observations		
Reviewer 1	General comment: as also stated by the authors, quantitative aspects are not yet considered in the AOP and future research is needed to generate appropriate data here. As the quantity of alteration with respect to the MIE and KE is considered crucial for the development of the AO, integration of quantitative aspects in future versions of this AOP is expected to be of great value.	Agree
Reviewer 4	Although inflammasome is mentioned number of times in the document AOP144-snapshot for scientific review circulated earlier, no reason was given as why this aspect was not included as an important KER in the proposed AOP.	as discussed at the TC, inflammasome will be included either as a separate KE or within a KER leading from lysosomal disruption to mitochondrial dysfunction, depending on the available WoE.
Reviewer 4	Please see below for additional/general observations.	
Reviewer 5	The authors may consider merging with AOP #38, already endorsed. Relationship with AOP #38 (liver fibrosis) is unclear: they should represent converging AOPs with different MIE, and from the point of hepatic cell death (=> inflammation, HSC activation, ECM-deposition).	the AOP will be merged with AOP 38
Reviewer 5	Editorial suggestions and specific points also commented in the attachment pdf AOP snapshot.	will be done
Reviewer 5	In general, some statements should be more specific. This refers to the wording of the abstract as well as some KE-descriptions (see specific comments below and on the attachment). Also, several KE refer to cellular responses, but the cell type is not specified. As the authors correctly state, fibrosis requires the interplay of several cell types, this	the critical cell types for the KERs in liver will be clarified.

	information is key to correctly capture the sequence of events. One of the main points in this respect that requires further clarification is the process of “inflammation” which is insufficiently	