Organisation for Economic Co-operation and Development

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Adverse Outcome Pathway External Review Report

AOP131: Aryl hydrocarbon receptor activation leading to uroporphyria

Short Title: AHR activation-uroporphyria

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1. Introduction and background to specific AOP

Background

The project for development of the AOP 131: *Aryl hydrocarbon receptor activation leading to uroporphyria* sprung out of a broader project submitted to the AOPs Development Programme in 2012 (project 1.7) to develop *the Adverse Outcome Pathways for Sustained Activation of the Aryl Hydrocarbon Receptor leading to a Range of Species-Specific Effects* led by BIAC and Canada.

The initial proposal was revised to cover two individual AOPs that were accepted in the AOP Workplan in 2013. One of these two individual AOPs led by Canada, the *AOP for Aryl Hydrocarbon Receptor 1 Activation Leading to Developmental Abnormalities and Embryolethality in Birds* was additionally broken down in two smaller linear AOPs:

- AOP 131: AhR activation leading to uroporphyria, and
- AOP 150: Aryl hydrocarbon receptor activation leading to embryolethality via cardiotoxicity[1]

AOP131 has undergone an internal review and modifications in early 2017 (Internal review AOP 131). Based on these, the Extended Advisory Group for Molecular Screening and Toxicogenomics (EAGMST) agreed at its June 2017 meeting, that the AOP131 draft [PDF] was ready for external expert review.

A scientific review panel (Annex1) was selected by an independent review manager in accordance with the Standard Operation Procedure (SOP) for Adverse Outcome Pathway Scientific Review (v.7 December 2017).

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 and outlined in the SOP:

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:

- Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?

[1] This AOP was renamed after its external review to Aryl hydrocarbon receptor activation leading to early life stage mortality, via reduced VEGF (Vascular Endothelial Growth Factor)

CQ3 Regulatory applicability:

- Considering the strength of evidence and current gaps / weaknesses, what would be the regulatory applicability of this AOP, in your opinion?

CQ4 Conclusion:

- What are your overall conclusions of the assessment of this AOP?

The review was conducted during December 2017 and April 2018. Based on the initial responses to the charge questions (Annex 2) main issues (Section 2) were discussed at a teleconference on 26 February 2018 (Section 3). Based on the discussion at the teleconference further written discussion and actions (Section 4), authors outlined a summary of planned revisions (Section 5) to include in the AOP before its submission to the EAGMST.

Introduction

AOP 131: *Aryl hydrocarbon receptor activation leading to uroporphyria* (short title: AHR activation-uroporphyria) includes the description and assessment of the critical elements of the pathway initiated by sustained activation of the aryl hydrocarbon receptor (AhR) leading to accumulation of highly carboxylated porphyrins (HCP) in various organs (Uroporphyria).

AOP131 can be initiated by a range of aromatic hydrocarbons, including dibenzo-p-dioxin, some polychlorinated biphenyls (non-ortho substituted congeners), and hexachlorobenzene. Iron was also included as a stressor in this AOP draft, as iron overload is strongly associated with porphyrin accumulation in mice. However, this effect is not mediated via the initiating event of this pathway.

The Molecular Initiating Event (MIE) of AOP131 (Figure 1) is the binding of stressors to AhR with relatively high affinity that leads to AhR activation, nuclear translocation and interaction with the aryl hydrocarbon receptor nuclear translocator (ARNT). The nuclear AhR/ARNT complex, in concert with other co-regulators, stimulates the transcription of Cyp1A2 (in mammals) and Cyp1A5 (in birds) via the xenobiotic response elements (XRE) in their promotors. Consequently the synthesis of the corresponding P450 enzymes, the monooxygenases CYP1A2/5 is increased resulting in oxidation of uroporphyrinogen III to uroporphyrin III. Accumulation of uroporphyrin III and other HCPs lead to the adverse effects of porphyria.

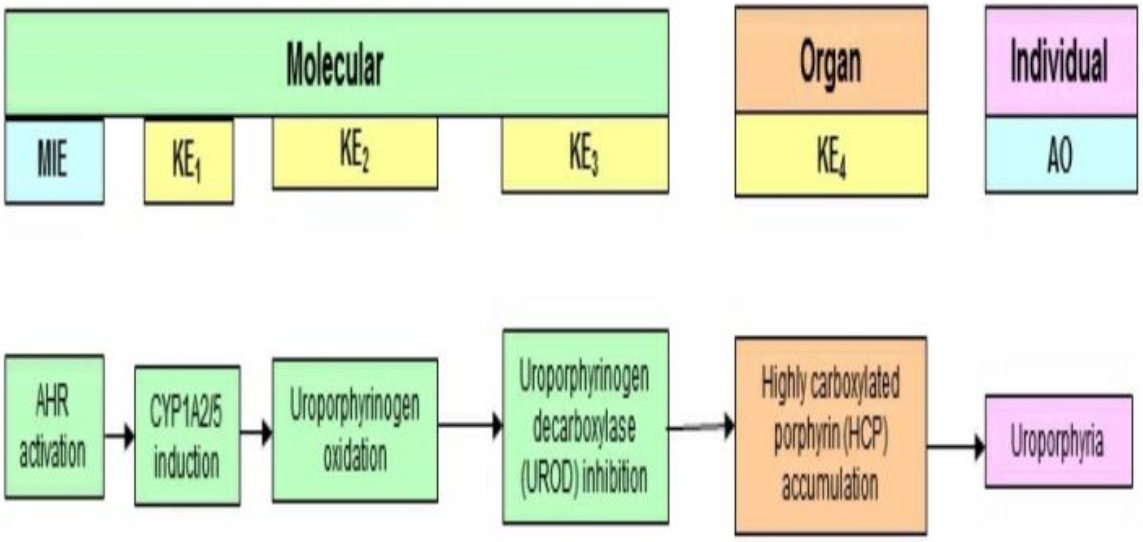


Figure1: Graphical representation of the components of AOP131.

In the absence of stressors (AhR inducers), uroporphyrinogen III is converted to coproporphyrinogen III by uroporphyrinogen decarboxylase (UROD) and enters the heme synthesis pathway. Therefore, this AOP is closely related to perturbation of the heme synthesis pathway.

In the presence of stressors, oxidation of uroporphyrinogen III is also associated with inhibition of UROD activity. UROD inhibition is possibly mediated by a partially oxidised uroporphyrinogen III intermediate. This inhibition could further potentiate accumulation of uroporphyrinogen III, its preferential oxidation, and accumulation of uroporphyrin III and other HCP (i.e. Uroporphyria).

Therefore, it could be argued that specific to this pathway is the complex interplay between the inhibition of UROD-catalysed uroporphyrinogen decarboxylation and the oxidation of uroporphyrinogen by CYP1A leading to the accumulation of uroporphyrin III and other HCPs causing the adverse effects of uroporphyria.

Number of uncertainties has been outlined for this AOP. For example, UROD inhibition is not always observed and/or is less pronounced in avian models of porphyria. In addition, evidence exists that AhR binding stressors under certain conditions (high acute exposure) do not lead to the adverse effect, particularly in some mammalian strains. Considering these uncertainties, authors argue that the development of AOP131 and its future applications should drive better understanding of all the complex aspects of occurrence of uroporphyria as an adverse effect.

Overall taxonomic applicability and consequently regulatory utility for AOP131 include juvenile and adult mammals (rodents and humans) and birds. Interspecies differences in sensitivity have been observed.

2. Synthesis of main issues of the review

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

Overall, AOP 131 was assessed as a good attempt at a very complex scenario which at first sight may seem relatively simple. Given the complexity, the AOP description was assessed as clear and solid construct based on the appropriate scientific literature. However, all reviewers thought that it needs to be presented in the wider context. To address this at a general level, inclusion of description of the heme synthesis pathway and porphyrias in the background section (now missing) was recommended.

Other more specific comments focused around:

- the need to discuss in more detail the nature of stressors in terms of their affinity for AhR and possible induction of porphyria through alternate pathways linked to some of the KE in AOP131
- uncertainty with the essentiality of CYP1A2/5 for AhR-induced uroporphyria
- the role of iron as a modifying factor in experimentally induced uroporphyria
- uncertainty with the identity of the UROD inhibitor and the mechanism by which it is generated

Reviewers also commented on the limitations regarding readability of the pdf format.

Summary of responses to CQ 1 - Scientific Quality

There was a general agreement that the AOP incorporates the most important scientific literature and current scientific knowledge in this field. List of additional references was suggested for consideration.

However, uncertainties were stressed relating to the current interpretation of the evidence, mostly relating to the role and essentiality of CYP1A2/5. The question of essentiality of cyp1a/2 induction was extensively discussed at the end of review teleconference.

In addition, further consideration of the iron metabolism/loading as a modifying factor or even a potential KE of this AOP was suggested by the reviewers.

The issue of uncertainty with the nature/identity of the UROD inhibitor, based on current literature, was also highlighted.

Summary of responses to CQ 2 - Weight of Evidence

Reviewers generally agreed with the scoring of the weight of evidence (WoE) for the KEs and KERs. Some clarifications and additional considerations were requested for:

- KER868: KE1 to KE2, related to uncertainty with essentiality of induction of CYP1A2/5 (KE1) for UROX[2] (KE2) and the role of iron for induction of UROX (KE2) in some experimental models.
- KER865: KE2 (Oxidation, Uroporphyrinogen) leads to KE3 (UROD inhibition), related to uncertainty with the identity of the UROD inhibitor and also the process/pathway in which the inhibitor is generated. KER865 was identified as the "weakest link" in this pathway by one reviewer.
- KER1070: KE4 to KE5, related to the essentiality of UROD inhibition (KE4) for accumulation of HCPs (KE5) in some avian models
- Uncertainty with AhR activation (MIE) by high acute doses of stressor (e.g. Tetrachlorodibenzo-p-dioxin (TCDD)) in some models e.g. Sprague Dawley (SD) rats.

Summary of responses to CQ3 - Regulatory Applicability

Some reviewers thought that the utility of this AOP for regulatory application for human toxicity assessment may be questionable at this stage, given the uncertainties and gaps in our understanding of the molecular mechanisms of AhR activation and uroporphyria in humans. However, there was also an opinion that the finding that a reduction in UROD activity of at least 70% is required to lead to uroporphyria in humans, may be significant in this respect.

Less uncertainty was associated with the applicability of this AOP in the environmental regulatory context.

Specific suggestions for regulatory applicability of AOP 131 given the current evidence and knowledge gaps/weaknesses, included:

- to inform the development and the prioritisation of validation for tests targeting KEs along this AOP and the wider heme synthesis pathway
- for screening level hazard assessment when there is a suspicion about porphyrinogenic effects of a chemical/mixture under study
- in long term, to facilitate development of battery approaches for assessment of uroporphyric potential of substances

Summary of responses to CQ4 - Overall conclusions of the assessment of AOP131

Reviewers agreed that in general, AOP131 represents a clear and solid assessment of the scientific literature related to porphyria as an adverse outcome in a number of species.

[2] UROX – uroporphyrinogen oxidation

However, there was also a general view that this AOP needs to incorporate (e.g. in the Background) and/or be presented in the wider context of the heme synthesis pathway (i.e. further development of a network of "porphyria AOPs").

There was also an opinion that the AOP131 represents the simplest interpretation of the evidence from the most susceptible mouse strain (C57BL/6), AhR-/- and Cyp1A2-/- knockout mice, and *in vitro* assays using either rodent or avian microsome systems. Whether or not the interpretations from these experimental systems can be extrapolated to intra-and inter-species is not clear. While it is recognised that the simplification may be the intent of the AOP concept to provide solid platforms to further mechanistic understanding of particular toxicity pathways, more extensive consideration/discussion of the body of seemingly inconsistent observations within and between taxa may provide important insights into the relationship between AhR activation and uroporphyria and even strengthen the overall weight of evidence.

In this context, it was suggested to consider and strengthen the discussion of the:

- differences in the effects of chronic and acute to TCDD exposure for progression to the AO
- CYP1A-independent AhR mediated uroporphyria cases
- nature of UROD inhibition (direct/indirect/by what)
- antioxidant capacity of cells, oxidative stress inducers
- effect of ascorbic acid levels on CYP12A activity

3. Summary record of the teleconference

End-of-review teleconference (TC) was attended by all reviewers, the 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 six main aspects of the AOP outlined in the figure below.

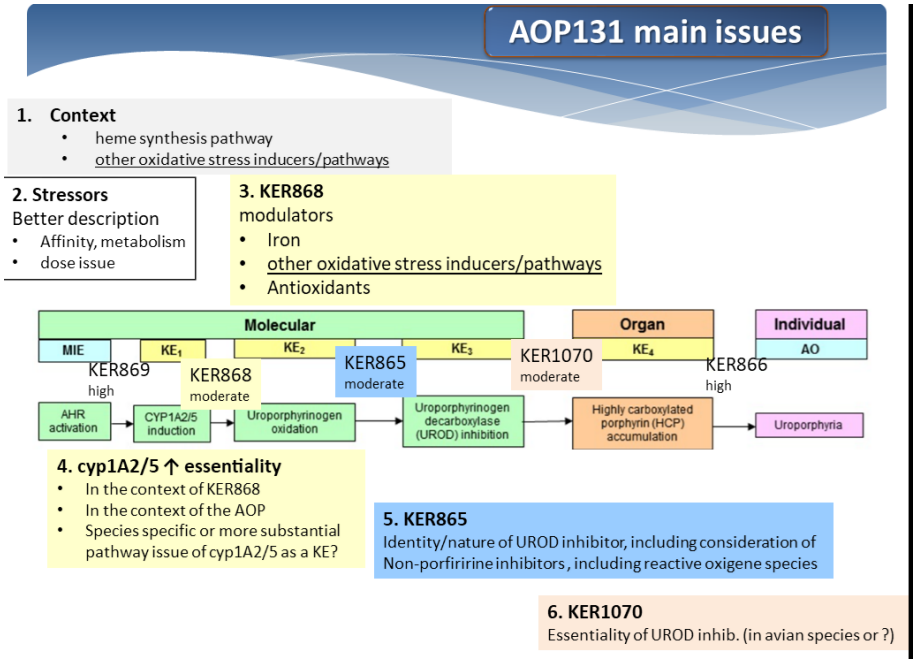


Figure 2: Main issues digest for the TC

In this context, specific comments (as numbered in Annex 2) were grouped and discussed according to the agenda (section 3.1) keeping in mind that AOPs should be simplification of complex biology and that they represent living documents.

3.1. TC agenda

Agenda for end-of-review teleconference AOP 131

26 February 2018, 3pm Paris time

1. Introduction of participants

2. Short introduction of main issues by RM and outline of TC

   SCIENTIFIC ISSUES:

3. General: the **context** of the AOP (comments no: 1, 2, 3, 4, 5, 7, 19, 25, 27, 28)

4. Role of **iron** in this AOP (comments no: 7, 10)

   - other **modulators** of AOP131 (comm. No: 7, 18, 28)

5. **Activation of the pathway** related to chronic vs acute exposure to stressors and other pharmaco- dynamic and kinetic considerations

   5a. Uncertainty with AhR activation (MIE) leading to AO via CYP1A2 induction by **high acute doses** of stressor (e.g. TCDD) **in some models** e.g. SD rats (comments no: 15, 16)

   5b. Stressors: enhance their description in terms of affinity for AhR and other aspects relevant for induction of porphyria (comments no: 3, 15, 28)

6. **Essentiality of CYP1A2/5** for AhR-induced uroporphyria and KE1 to K2 WoE score: interpretation of the cases of CYP1A2-independent, iron-dependent UROX (comments no: 3, 6, 10, 11c, 13a, 14, 17)

7. **KER865**

   7a. Essentiality of UROX for UROD inhibition: contribution of non-porphyrine oxidised species generated by CYP or other CYP independent pathways (comments no: 13b, 14)
   7b. UROD inhibitor (comments no: 6, 10, 18, 28)

8. **Essentiality of UROD inhibition** (KE4) for accumulation of HCPs (KE5) KER1070 in some avian models (comments no: 13c)

9. **Technical issues,** additional **references** and **errors**

   - How UROD is measured (comment no: 11b)
   - Link between porphyria and neuropsychiatric symptoms (comment no: 11d)
   - Addition of references (comments no: 9, 30)

- Abstract statement on number of enzymes involved in hepatic uroporphyria (comments no: 11a)

10. Regulatory applicability/significance discussion (comments no: 21-24)

    - For human health assessment
    - For environmental safety assessment

11. Overall conclusion about AOP131 – open discussion guided by the initial written comments

    - Simple or simplistic? (also related to the discussion under agenda item 3a.)
    - Format/readability (comments no: 2, 12, 20)

3.2. Main issues and responses during the call

For each issue there was a short introduction by the RM followed by reviewers emphasising and/or clarifying the point of their comment. Authors' initial responses and further discussion lead to agreement on way forward to best address the particular issue.

Agenda item 3: All reviewers were of the opinion that appropriate background context is missing and AOP131 needs to be put into the context of other interacting pathways, particularly the heme synthesis pathway and porphyrias in general.

Response: Authors pointed out, that relevant interactions are referenced to at various places throughout the pathway description. However, it was agreed that it would be good to include a Background section, which would contain an integrated discussion of the interaction of AOP131 elements with the heme synthesis pathway.

Agenda item 4: Iron has been included in the list of AOP specific stressors in the summary of this AOP. However, some comments raised the issue of better description of the role of iron in the progression of AOP131 and generally in porphyria.

Response:
Authors considered that the issue of the role of iron overload as well as some inconsistencies of experimental data regarding essentiality of cyp1a2/5 induction in AOP131 may be addressed better if iron is removed from the stressor list and included in this AOP as modulating factor. It was agreed that evidence demonstrating modulation of specific KER in AOP131 by iron and/or other modulating factors (e.g. activation of other oxidative pathways and their products and antioxidants) should be examined and included in the relevant KER's section as modulating factors at least in qualitative terms.
There was some uncertainty as to where specifically to include this information and this remained to be determined. However, it was indicated by one of the authors that the updated AOP Wiki 2.2 version should allow for easy inclusion of modulating factors.

Agenda item 5a: Related to the observation that high acute doses of high affinity stressor (e.g. TCDD) fails to elicit the AO in SD rats, it was questioned whether additional

discussion is needed to provide more comprehensive overview of relevant uncertainties that could be informative for the potential users, particularly regulators. See also comments 15 and 16 in Annex 2.

This initiated a robust discussion particularly regarding the evidence that cyp1a induction is not directly proportional to the level of uroporphyria, which has been an interesting conundrum in the area of porphyria.

Response:
It was agreed that it is important to address uncertainties and inconsistencies across the existing evidence related to the pathway leading from AhR activation to uroporphyria. In fact one for the goals of the AOP development is to identify knowledge gaps and focus questions for future investigation.

Authors indicated that, since the initial comments, they have looked more closely at the data related to pharmacokinetic aspects for TCDD in mice versus rats. However, data show that TCDD clearance in mice and rats were comparable and could not help explain the observation of the differences between chronic and acute doses in SD rats.

They also pointed out that the AOP already includes discussion of the evidence that mild uroporphyria is observed even in the absence of cyp1a induction but not in cyp1a knock-outs, indicating that while basal CYP1a level is needed for strong uroporphyric effect, cyp1a induction is not essential for low levels of porphyria when other uroporphyrinogen oxidative processes may be involved.

This was supported by one reviewer comments pointing out to earlier genetic studies showing different susceptibility loci, other than AhR, that contribute to susceptibility to porphyria in mice. However, these studies have not been examined in more detail up to date.

Finally it was agreed that, while it is useful to keep the AOPs simple, it is very important to make the uncertainties and inconsistencies easily accessible for different users. This initiated discussion about the best format to make it possible, considering specific KERs or the AOP specific section.

It was noted that, while info was included in the discussion under relevant KER and KE uncertainties, it may have been difficult to access it at all stages of the review when using the pdf version of the snapshot. Authors pointed out the plans for the future updates within the AOP knowledge base that will allow easy access to all linked pathways and creation of relevant networks that will allow easy access to all relevant uncertainties and inconsistences.

To address this issue in the immediate term, the authors undertook to summarise significant uncertainties and inconsistencies for this AOP on the main AOP131 specific page.

Agenda item 5b: Some comments indicated that more information is needed in the description of the stressors in the AOP specific part.

This initiated discussion as to what kind of information would be useful here and whether Polycyclic aromatic hydrocarbons (PAHs) should be included in the AOP specific part. One of the reviewers pointed out to evidence where non-chlorinated compounds have induced porphyria under iron overloading diet exposure conditions.

Response:
It was agreed to keep the PAHs as AOP131 stressors and include the relevant references pointed out by one of the reviewers. These references would also be considered in the discussion of the iron as a modulating factor for AOP131 (see agenda item 4 discussion above).

It was also agreed that additional information in the AOP main page should relate to the specific aspects of chemicals groups inducing uroporphyria rather than chemical specific considerations.

Agenda item 6: Essentiality of cyp1a2/5 induction, discussed in relation to the AO under agenda item 4, was also discussed in the context of uncertainties related to KER868 (KE1 to KE2). Reviewers have questioned the interpretation of the evidence that in the context of AhR-/- mice phenotype, UROX (KE2) is not dependent on cyp induction (KE1).

Response:
It was recognised by the authors that the context of AhR-null mice is not most appropriate to infer about the essentiality of cyp1a induction for downstream KEs, particularly taking into account that the evidence comes from conditions of iron overload, potentially modulating elements of AOP131 downstream of the MIE, as discussed above in agenda item 4. Therefore, they agreed to revisit the interpretation of the observations of porphyria in AhR-null mice in the context of KER868.

However, they pointed out that the call for KER868 evidence was ‘moderate’ and for quantitative understanding ‘low’, taking into account the uncertainties of the observations in the AhR-null mice and also the evidence that UROX activity in human liver microsomes was not correlated with CYP1A2 content.

Agenda item 7a: Review rose the issue that the evidence supporting direct link between UROX and UROD is not convincing. In particular, it is not clear whether CYP1A2 directly or indirectly produces an UROD inhibitor via uroporphyrinogen oxidation, or reactive oxygen species generated from iron overload or other induced pathways can also potentially induce UROX. It was pointed out that the evidence discussed to support KER865 comes mostly from experiments with microsomes *in vitro* and there is no evidence *in vivo* addressing this point in particular.
Reviewers discussed that uroporphyrinogen does not appear as a “typical” CYP1A2 substrate and that in fact it is not associated with the microsomal but with the cytosolic fraction, bringing again the discussion to the essentiality of CYP1A for UROX.

Given that the uncertainty is well emphasised, reviewers agreed with the “Moderate” evidence call for this KER.

Response:
Authors recognised this uncertainty but pointed out, and everybody agreed, that it is very difficult to address this point experimentally *in vivo*. However, it was agreed that

additional consideration should be included in the *Biological Plausibility* section for KER868 to address the: (a) the issue of lack of evidence *in vivo* and (b) the related uncertainty more prominently.

Agenda item 7b: All reviewers agreed that unequivocal identification of the chemical identity of the UROD inhibitor would greatly benefit the understanding of AhR-mediated uroporphyria. One reviewer questioned whether without this information, the evidence for KER865 can be considered moderate.

It was pointed out that there have been considerable efforts to identify the chemical nature of the inhibitor and that most evidence points to an oxidative metabolite of porphyrin type substrate. The critique of the study identifying uroporphomethene as the inhibitor candidate was perceived, at least by one reviewer, as probably not well justified. However it was recognised that the evidence is not yet conclusive.

Nevertheless, all reviewers agreed that there is a clear correlation between KE2 and KE3 and that the current moderate call may only become strong and also informative for quantitative analysis, if evidence is generated that unequivocally identifies the UROD inhibitor.

Response:
Authors pointed out that they have discussed the uncertainty about the evidence relating to the identification of the UROD inhibitor in the KER's *Biological Plausibility* section by stating that "*Early reports confirmed the presence of a UROD inhibitor in porphyric animal models that was not present in animals resistant to chemical-porphyria under the same conditions. The identity of this UROD inhibitor is not yet agreed upon, but there is a general consensus among the scientific community that it is an oxidation product of uroporphyrinogen or hydroxymethylbilane (the tetrapyrrole precursor of uroporphyrinogen)*" and identifying the relevant references.

Reviewers considered this again and agreed that the above statement adequately reflects the current knowledge and related uncertainties.

All agreed that the uncertainty discussion in the evidence for this KER865, serves well one of the objectives of the AOP development i.e. identifies current knowledge gap, which is critical to fill to improve mechanistic and quantitative understanding of the pathways leading to uroporphyria.

Agenda item 8: One reviewer questioned the evidence call 'moderate' for KER1070: KE4 to KE5 based on evidence in the study of Lambrecht et al. showing accumulation of porphyrins in vitro in chicken embryo hepatocytes and in vivo in Japanese quail liver without a decrease in UROD activity.

Response:
It was important to clarify whether the issue is related to the taxonomic applicability or the overall WoE for the KER. Reviewer indicated that at least for Japanese quail and chicken the evidence appears low.

It was noted by other reviewers that evidence for AhR induced porphyria and oxidation of uroporphyrinogen is strong and chicken embryos have been used as model system

extensively, however UROD inhibition has not been shown and is not likely to be essential at least in this bird species.

Authors agreed that there is a need to revisit the taxonomic applicability of KER1070, particularly in avian species. This should also be reflected accordingly in the conclusions about the taxonomic applicability to "birds" as formulated in the overall taxonomic applicability.

A point was raised by a reviewer that the AOP may be applicable to fish. Authors consider that even though accumulation of HCPs has been observed in fish in highly contaminated areas, they have not seen mechanistic evidence supporting this AOP in fish.

It was also noted that currently, taxonomic applicability is based mostly on positive, inclusion criteria. Based on the issues raised in the discussion above, it was suggested that for future AOP development guidance, consideration should be made also clearly identify species to which AOP or KER are not applicable.

Agenda item 9: Additional HPLC method was recommended for inclusion in KE845: Inhibition, UROD. In addition, a number of references were recommended for consideration to include in the AOP as appropriate and some errors of statements were noted.

Response:

- The high performance liquid chromatography (HPLC) method for UROD activity measurement will be included following provision of references by the reviewer.
- The statement on the link of porphyrins and neuropsychiatric symptoms will be removed based on the reviewer's advice that existing evidence links ALA rather than porphyrins to neuropsychiatric symptoms. Authors recognise that they have made the statement about the link of porphyrins and neuropsychiatric symptoms based on only one study and will remove the statement.
- Additional references will be considered as appropriate for the updated AOP.
- Abstract will be modified to better reflect the wider context of porphyrias and the enzymes involved. In their initial written response author suggested to use the modified version of the statement already included in the AOP131 specific main page "Porphyria is a disorder in which the disturbance of heme biosynthesis results in accumulation and excretion of porphyrins. A variety of porphyrias exist depending on which enzyme in the pathway is deficient"

Agenda item 10: Reviewers addressed the potential for regulatory applicability/usefulness in their initial comments.

At the TC discussion focused on the implications of the inter- and even intra-species differences observed at different steps of the pathway, for inferring applicability to humans. Given that understanding of the quantitative aspects of the other KER along the pathway is not clear in mammals and humans, AOP131 may have limited applicability for human health safety assessment. However, it may be useful for drug safety screening through the measurement of cyp1 induction *in vitro*.

It was agreed by all that at the current level of AOP131 development can be useful in focusing on key measures of aspects of porphyria toxicity along the pathway before overt porphyria appears in humans. In addition, clearly identifying the uncertainties and gaps in the knowledge is very useful to inform future efforts that would lead to better quantitative understanding of the pathway, including the development of AOP networks within the heme synthesis pathway.

The potential utility of this AOP in the environmental safety context was assessed as more significant at this stage. In this context, one of the reviewers emphasised the on-going efforts within EURL-ECVAM on tests for measurement receptor-mediated cyp1a2 induction.

Response:
Authors agreed to consider reflecting the discussion, particularly regarding the uncertainties with the usefulness for human health safety assessment in the updated AOP.

Agenda item 11: Overall assessment of the AOP131

Reviewers agreed that notwithstanding the changes needed to improve the presentation of the wider context, AOP131 represents a good construct of the porphyria pathway. It was particularly pointed out that identification of uncertainties and knowledge gaps is particularly valuable. This will be improved by the changes discussed in agenda item 5.

The issue of the readability of the pdf version was brought up several times during the TC and it is agreed that EAGMAST should consider changes/improvements for future reviews. Authors indicated that, compared to the pdf version, AOP Wiki gives better overview and easier links to uncertainties that may help the review process.

Response:
Authors appreciated the overall assessment and agreed that the readability issue should be addressed by Wiki developers following consideration of the EAGMST.

3.3. Action list

1. Include Background section addressing the context of the heme synthesis pathway and other relevant pathways/modulators.

2. Examine the evidence demonstrating that AhR induced uroporphyria is modulated by iron, other cellular pathways (e.g. estrogen activation, other oxidative stress pathways) and antioxidants (ascorbic acid), and
   - reviewers to provide specific references to be considered
   - authors assess which KERs are most appropriate to include this evidence (most likely KE1 to KE2, but also others) and modify the AOP accordingly.

3. Present a summary of the significant uncertainties and inconsistencies for this AOP on the main AOP131 specific page.

4. Revisit the interpretation of the observations of porphyria in AhR-null mice in the context of KER868. Discussion on iron as a modulating factor would also be useful in this context.

5. Authors to propose changes in the Biological Plausibility section of KER868 and/or KER865 that would include the uncertainty regarding in vivo evidence for uroporphyrinogen oxidation leading to UROD inhibition.

6. Revisit the literature relevant to applicability of KER1070 in various avian species and modify the relevant sections accordingly.

7. Reviewer to provide authors with the reference(s): (a) for description of HPLC methods for measurement of UROD activity, and (b) to support the change in the current paragraph that porphyrins are responsible for neuropsychiatric symptoms of porphyria. Authors to include the info in the AOP as appropriate.

8. Authors to consider including references provided by reviewers that would help support evidence throughout the AOP and ensure all critical aspects have been sufficiently covered.

9. Abstract will be modified to better reflect the wider context of porphyrias and the enzymes involved.

10. Authors to modify the Regulatory Applicability section to reflect the discussion under agenda item 10.

11. EAGMST to consider the future formatting of AOPs for external review.

12. EAGMSTG and Wiki developers to consider including information on NON-applicability to particular species.

4. Further Discussion

Following the TC authors confirmed that errors in Table 3 (comment number 14) will be corrected as suggested by the reviewer.

In addition the reviewer provided further discussion on the interspecies differences observed in mice and implications for the overall WoE assessment, related to the TC discussion under agenda item 10 (above):

With regards to WOE call for KE1 (i.e. Comment [AF30] in Annex 3b):

Given my understanding of the literature, I am comfortable with the statement:

"Cyp1A2 is necessary but not sufficient for AhR-mediated urophorphyria."

I am less comfortable with the phrase:

"Induction of Cyp1A2 by AhR activation is necessary but not sufficient for AhR-mediated urophorphyria."

I am not sure if we discussed how to frame/tackle this issue of "susceptible" vs. "resistant" species/strains during the teleconference. For C57BL/6J, evidence for KE1 is strong. For DBA/2 and perhaps other mouse strains as well as in humans and other species, the evidence is weak (I have not had the time to look over Reviewer's 1 CYP validation report). Even for rats, the evidence is moderate to weak [consider that resistant rat strain Han/Wistar showed induction of Cyp1A2 without uroporphyria (Watson et al., 2014)]. Unfortunately, I don't have an answer as to how best to convey this type of information in the context of AOPs.

One important consideration is which scenario should we emphasize? According to OECD Handbook on AOPs, the following is stated on Pg. 12:

"Importantly, AOPs do not describe every detail of the biology but instead focus on describing **critical steps or check-points** along the path to adversity, which are both measurable and have **potential predictive value**."

One can thus ask, does KE1 (CYP1A2 induction) have any "predictive value"? I am not sure if it does. For C57BL/6J and some other "susceptible" strains, perhaps it does. But the observation of "induction of Cyp1A2" by TCCD in of itself does not predict uroporphyria, unless we take into account the species/strain and other confounders such as iron overload. On the other hand, KE2-KE4 have reasonable predictive value, in my opinion. For example, if it can be shown that UROX is induced or UROD is inhibited or HCP are shown to accumulate, uroporphyria is likely to occur.

The reason Davies et al. (2008) study caught my eye is that they showed that the resistant properties of DBA/2 to TCDD-dependent uroporphyria cannot simply be due to **lower ligand-affinity of AhR if Cyp1A2 induction was essential in the pathway**. In fact, their observation suggests the possibility that another, yet unidentified AhR dependent gene(s) not induced in TCDD-exposed DBA/2 mice may be an equally likely culprit. While Watson et al. (2014) observed that Cyp1a2 showed differential induction between

susceptible and resistant rat strains, they implied that there are at least 6 other candidate genes that may mediate TCDD toxicity (they did not specifically look for uroporphyria endpoints, however).

So the question remains: under what context is Cyp1A2 considered essential & has potential predictive value? One can hypothesize that for unknown reasons, DBA/2 is an exception. For example, one can speculate that the induced CYP1A2 protein in DBA/2 mice cannot efficiently oxidize uroporphyrinogen. But one can also turn that around and speculate that **C57BL/6J might be the exception and not the norm**. Of note, there is some evidence for the latter. The particular substrain of C57BL (i.e. 6J substrain) used by Davies et al. (2008) has a mutation in nicotinamide nucleotide transhydrogenase (Nnt) gene (Mekada et al., 2009). This gene product is a component of the mitochondria whose deletion leads to a dysregulation of redox homeostasis in mitochondria (Ronchi et al., 2013). C57BL/6J is often used as a model strain for diet-induced obesity, and it has been hypothesized that susceptibility of C57BL/6J to metabolic syndrome (compared to other strains of mice) is due to its susceptibility to oxidative stress (Fontaine and Davis, 2016; Freeman et al., 2006).

With this as a backdrop, considering that:

a) there are susceptible and resistant strains of rats and mice to TCDD induced uroporphyria;

b) studies often cited for evidence of essentiality of Cyp1A2 [i.e.(Gorman et al., 2002; Phillips et al., 2011; Sinclair et al., 2000; Smith et al., 2001)] all used mice with C57BL/6J background;

c) Cyp1A2 induction does not appear to be essential in humans;

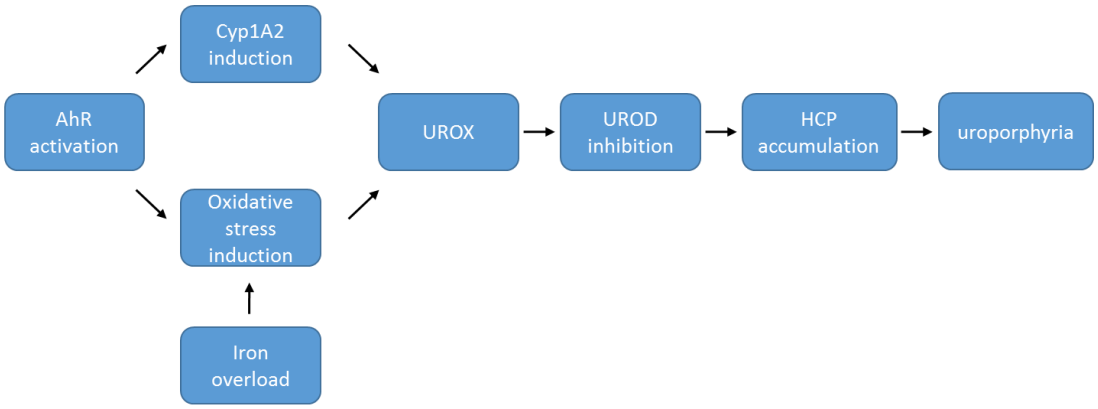
d) susceptible/resistant strains in rats appear to correlate with levels of ascorbic acid (a known antioxidant) (Sinclair et al., 1993);

e) iron overload, often results in induction of oxidative stress (Smith et al., 1998); and

f) induction of oxidative stress through AhR activation has been documented (Aly and Domenech, 2009; Reichard et al., 2006; Senft et al., 2002),

I submit for your consideration that the "essentiality" of Cyp1A2 induction to TCDD-induced uroporphyria only applies to susceptible strains/species in which the antioxidant capacity is low or reduced, such as C57BL/6J.

In the context of regulatory setting in which we often make decisions on animals (& humans) "out in the wild" through inference from studies in laboratory animals, a pathway with a little more nuance/qualifier may be more useful (such as one shown below). I recognize that this may add too much complexity to the simple linear building blocks for AOP131 and that sufficient quantitative evidence for AhR-dependent oxidative stress may not exist, but I thought I would throw it out there.



Aly, H.A., and Domenech, O. (2009). Cytotoxicity and mitochondrial dysfunction of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in isolated rat hepatocytes. Toxicol Lett *191*, 79-87.

Davies, R., Clothier, B., Robinson, S.W., Edwards, R.E., Greaves, P., Luo, J., Gant, T.W., Chernova, T., and Smith, A.G. (2008). Essential role of the AH receptor in the dysfunction of heme metabolism induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Chem Res Toxicol *21*, 330-340.

Fontaine, D.A., and Davis, D.B. (2016). Attention to Background Strain Is Essential for Metabolic Research: C57BL/6 and the International Knockout Mouse Consortium. Diabetes *65*, 25-33.

Freeman, H., Shimomura, K., Cox, R.D., and Ashcroft, F.M. (2006). Nicotinamide nucleotide transhydrogenase: a link between insulin secretion, glucose metabolism and oxidative stress. Biochem Soc Trans *34*, 806-810.

Gorman, N., Ross, K.L., Walton, H.S., Bement, W.J., Szakacs, J.G., Gerhard, G.S., Dalton, T.P., Nebert, D.W., Eisenstein, R.S., Sinclair, J.F., *et al.* (2002). Uroporphyria in mice: thresholds for hepatic CYP1A2 and iron. Hepatology *35*, 912-921.

Mekada, K., Abe, K., Murakami, A., Nakamura, S., Nakata, H., Moriwaki, K., Obata, Y., and Yoshiki, A. (2009). Genetic differences among C57BL/6 substrains. Exp Anim *58*, 141-149.

Phillips, J.D., Kushner, J.P., Bergonia, H.A., and Franklin, M.R. (2011). Uroporphyria in the Cyp1a2-/- mouse. Blood Cells Mol Dis *47*, 249-254.

Reichard, J.F., Dalton, T.P., Shertzer, H.G., and Puga, A. (2006). Induction of oxidative stress responses by dioxin and other ligands of the aryl hydrocarbon receptor. Dose Response *3*, 306-331.

Ronchi, J.A., Figueira, T.R., Ravagnani, F.G., Oliveira, H.C., Vercesi, A.E., and Castilho, R.F. (2013). A spontaneous mutation in the nicotinamide nucleotide transhydrogenase gene of C57BL/6J mice results in mitochondrial redox abnormalities. Free Radic Biol Med *63*, 446-456.

Senft, A.P., Dalton, T.P., Nebert, D.W., Genter, M.B., Puga, A., Hutchinson, R.J., Kerzee, J.K., Uno, S., and Shertzer, H.G. (2002). Mitochondrial reactive oxygen

production is dependent on the aromatic hydrocarbon receptor. Free Radic Biol Med *33*, 1268-1278.

Sinclair, P.R., Gorman, N., Walton, H.S., Bement, W.J., Dalton, T.P., Sinclair, J.F., Smith, A.G., and Nebert, D.W. (2000). CYP1A2 is essential in murine uroporphyria caused by hexachlorobenzene and iron. Toxicol Appl Pharmacol *162*, 60-67.

Sinclair, P.R., Gorman, N., Walton, H.S., Bement, W.J., Jacobs, J.M., and Sinclair, J.F. (1993). Ascorbic acid inhibition of cytochrome P450-catalyzed uroporphyrin accumulation. Arch Biochem Biophys *304*, 464-470.

Smith, A.G., Clothier, B., Carthew, P., Childs, N.L., Sinclair, P.R., Nebert, D.W., and Dalton, T.P. (2001). Protection of the Cyp1a2(-/-) null mouse against uroporphyria and hepatic injury following exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol Appl Pharmacol *173*, 89-98.

Smith, A.G., Clothier, B., Robinson, S., Scullion, M.J., Carthew, P., Edwards, R., Luo, J., Lim, C.K., and Toledano, M. (1998). Interaction between iron metabolism and 2,3,7,8-tetrachlorodibenzo-p-dioxin in mice with variants of the Ahr gene: a hepatic oxidative mechanism. Mol Pharmacol *53*, 52-61.

Watson, J.D., Prokopec, S.D., Smith, A.B., Okey, A.B., Pohjanvirta, R., and Boutros, P.C. (2014). TCDD dysregulation of 13 AHR-target genes in rat liver. Toxicol Appl Pharmacol *274*, 445-454.

Another reviewer commented in writing in relation to the discussion above. They agree that induction of CYP1A2 rather than sufficient expression level may not be the most critical element of the AhR induced porphyria. However C57BL/6 results are not easily explained by just the levels of antioxidants as a consequence of Nnt mutation in this strain. Quantitative trait locus (QTL) analyses for porphyria with crosses between C57BL/6 and DBA/2 have not shown a susceptibility locus on Chr 13 corresponding to the Nnt gene. There are unknown genes present on other chromosomes that strongly influence porphyria development but these are not regulated by AhR

5. Summary of planned revisions

Action Item (Section 3.3)	Intended revisions
1) Include Background section addressing the context of the heme synthesis pathway and other relevant pathways/modulators.	• Add more context to background by describing where UROD lies within the heme biosynthesis pathway (similar to KE369 page) • Consider using wording from comment # 4 in Annex 2 (clinical vs. toxicological view)
2) Examine the evidence demonstrating that AhR induced uroporphyria is modulated by iron, other cellular pathways (e.g. estrogen activation, other oxidative stress pathways) and antioxidants (ascorbic acid),	• Remove iron as a stressor and add as a modulating factor in KER868 • Include other potential modulating factors in discussion o Levels of ascorbic acid negatively correlated with CYP1A2 induction(/activity?) (see text from comment #28)→ may help-explain diff. in strain susceptibility o antioxidant capacity within a hepatic cell may be a confounding factor as to why some animals and humans are susceptible or resistant to ArH inducers o General increase in oxidative stress [i.e. lipid peroxidation, reactive oxygen species (ROS) and oxidized glutathione] due to TCDD is not dependent on functional CYP1A2 (Comment 28d), and may potentiate UROX.
3) Present a summary of the significant uncertainties and inconsistencies for this AOP on the main AOP131 specific page.	• Include summary of uncertainties on main AOP page that combines issues mentioned throughout KERs (specifically CYP1A2 essentiality/induction in humans, UROD inhibition in birds and potential alternate pathways) o These will remain on individual KER pages, and simply summarized on the AOP main page. • Add PAHs to main AOP page as stressor o Add more details about stressors including characteristics of strong vs low affinity AHR agonists
4) Revisit the interpretation of the observations of porphyria in AhR-null mice in the context of KER868 Discussion on iron as a modulating factor would also be useful in this context.	(KER868=CYP1A2 Induc→UROX) • Elaborate on conditions of experiment in which mild porphyria observed in AHR-null mice (what were the potential driving forces?) o Iron over-load + genetic predisposition in UROD gene (lower inherent enzyme activity) • Re-word to clarify that the CYP1A2 enzyme is necessary, but its induction is not. (i.e. not alternate CYP1A2-

	independent pathway, but alternate pathway independent if CYP1A2 induction→ may not be an alternate pathway at all, but the same pathway potentiated by iron; which will make more sense once iron is added as a modulating factor)

5) Authors to propose changes in the Biological Plausibility section of KER865 that would include the uncertainty regarding in vivo evidence for uroporphyrinogen oxidation leading to UROD inhibition.	(865=UROX→UROD inhib.)
	• Expand on uncertainties
	  ○ Not clear whether CYP1A2 directly or indirectly produces an UROD inhibitor via uroporphyrinogen oxidation, or reactive oxygen species generated from iron overload or other induced pathways can also potentially induce UROX.
	  ○ Lack of evidence in vivo, in which other pathways may be more relevant

6) Revisit the literature relevant to applicability of KER1070 (UROD inhib.→ HCP acc.) in various avian species and modify the relevant sections accordingly.	• Change WOE call for chicken to low or medium (revisit literature to determine) • Expand on text about possibility that this KER is not applicable to Quail (and potentially other birds) based on in Lambrecht study • Also consider re-visiting overall WOE call for birds on AOP main page (and expand in taxonomic applicability text).
7) Reviewer to provide authors with the reference(s): (a) for description of HPLC methods for measurement of UROD activity, and (b) to support the change in the current paragraph that porphyrins are responsible for neuropsychiatric symptoms of porphyria. Authors to include the info in the AOP as appropriate.	• HPLC ref. received → add to methods section (UROD activity) Francis and Smith (1983) Analytical Biochemistry 138:404-410 • Statement linking porphyrins to neuropsychiatric symptoms has been removed from KER866 (HCP accumulation→uroporphyria) and references updated accordingly.
8) Authors to consider including references provided by reviewers that would help support evidence throughout the AOP	• References have been received from reviewers • Francis and Smith (1987) Biochem and biophys research comm. 146(1):13-20 • Urquhart et al (1988) Biochem J. 253: 357-362 • Smith and Chernova (2006) Disruption of heme synthesis by polyhalogenated aromatics. Advances in Molecular toxicology Vol 3 Ch 6.CYP Induction EURL ECVAM Validation project report → Upon reading these references, it will be decided if and where they should be included throughout the AOP.
9) Abstract will be modified to better reflect the wider context of porphyrias and the enzymes involved.	• Include summary statement on context of UROD within heme biosynthesis pathway in Absract.

10) Authors to modify the Regulatory Applicability section to reflect the discussion under agenda item 10.	• Uncertainties in relevance for human risk assessment (CYP1A2 involvement, sig. drop in UROD activity required to view clinical symptoms (70%)…inherent activity could play a large role dictating how much inhibition necessary) • Consider on-going efforts within EURL-ECVAM on tests for measurement receptor-mediated cyp1a2 induction. o Report sent by reviewer to primary author o Decision to include pending review of document
11) EAGMSTG to consider the future formatting of AOPs for external review.	• None
12) EAGMSTG and Wiki developers to consider including information on NON-applicability to particular species.	• None
Comment # (Annex 2)	**Intended or completed changes**
11a) Abstract: Second sentence is incorrect. There are 8 enzymes of the pathway in liver and hepatic uroporphyria is really the consequence of only inhibition of UROD	• Corrected by addressing TC action items 1 and 9.
14) Table 3, Temporal concordance: Davies at al. 2008 results depicted incorrectly	• Add the corrected CYP1A2 values and include the relative porphyrin levels in the last column (0, 56, 677). o The 20 and 270 refer to absolute values of porphyrins (from Figure 1A) and the text states that this is a 56 and 677-fold change, so effectively give the same information. • The UROD column will be left blank.
28) What is unclear from cited studies is how CYP1A2 induction and UROX ultimately lead to UROD inhibition? Is this a direct or indirect effect?	• Describe positive feedback loop in AOP main page (maybe in abstract, or in separate section) o Add qualitative positive feedback loop in graphic representation of AOP (simply for explanatory purposes; will not involve the addition of any new KERs) o Careful to mention uncertainty in UROD inhib for birds…ie. CYP1A2 induction enough to drive UROX. • Also include discussion of positive feedback look in appropriate KERs under the new section titled "Known Feedforward/Feedback loops influencing this KER" o Mainly under KER1070 (UROD inhib.→ HCP acc.) since this isn't a direct relationship o Potentially under KER865 and 868
28) TCDD did elicit AhR-dependent, CYP1A1/A2-independent mitochondrial ROS production in mice suggesting that general oxidative stress	• Add this possibility under uncertainties/inconsistencies section in KER868

induced independently of CYP1A2 induction may contribute to the resulting overall UROX by TCDD (Senft et al., 2002).	

6. Outcome of the external review

AOP131 represents scientifically solid description of the AhR induced uroporphyria pathway.

AOP131 also identifies and outlines the uncertainties and knowledge gaps necessitating further investigation. The particular role of cyp1a2 was a major point of discussion during and after the TC.

Given the above, AOP131 provides a systematically organised and transparent analysis to support screening level assessment of AhR binding, cyp1a inducing and UROD inhibiting potential of chemicals in different species. In the long term, it could inform the development and prioritisation of the validation for tests targeting KEs along this AOP and the wider heme synthesis pathway, ultimately facilitating the development of battery approaches for assessment of uroporphyric potential of substances.

AOP131 modified as per the authors plan outlined in Section 5, will be a valuable addition to the AOP-KB. It provides the basis for future efforts towards a better quantitative understanding of the porphyria pathway and for potential development of heme synthesis AOP networks.

Annex 1: List of Reviewers, Authors and Review manager

Reviewer	Affiliation	Representing country
Olavi Pelkonen	Department of Pharmacology and Toxicology, University of Oulu, Oulu	Finland
Andy Smith	MRC Integrative Toxicology Training Partnership (ITTP) MRC Leicester	UK
Ad Peijnenburg	RIKILT Wageningen University Wageningen	Netherlands
Kotaro Kaneko	Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, College Park, MD	USA

Author	Affiliation	Representing country
Amani Farhat	Environment and Climate Change Canada	Canada
Jason O'Brien	Environment and Climate Change Canada	Canada

Review Manager	Affiliation
Julija Filipovska	Independent Consultant

Annex 2: Individual reviewers' comments

General		N°:
Reviewer 1	Overall, I found the AOP a logical and well-founded construct and – within its rather restricted scope – based on an adequately comprehensive survey of literature. Taking into account the restricted scope and the complexity of porphyrias, it would have been beneficial to provide some succinct background (which is now empty) to put the AOP on the wider context. Actually there are some general information in some parts of the AOP, e.g. in the description of the AO.	1
	Internal review has been pretty thorough and reading it was very useful when preparing for the external review. I share some concerns of the author regarding the structure and presentation of the AOP and snapshot, but maybe this concern belongs to "the realm of newcomer's confusion".	2
	Although the AOP as such is very good, there are number of points that may require some further discussion, e.g. the nature of stressors and adequacy of focusing on CYP1A2/5, possible links and interactions with other parts of the heme synthesis pathway.	3
Reviewer 4	As a scheme for this phenomenon I think the AOP is a good logical attempt for an outcome which is quite specific and species variable. Besides particular scientific points addressed below, there are some wider contextual difficulties however, which may be the consequence of the toxicological view and approach of an AOP compared to medical experience. This contrast cannot be unique to this endpoint though. It may be helpful to illustrate that hepatic uroporphyria is viewed somewhat differently by clinicians and toxicologists. **For the former** it is mostly a sporadic disease (porphyria cutanea tarda; PCT) occurring sometimes in patients exposed to a variety of insults such as alcohol, estrogens, hepatitis viruses, HIV and on dialysis. Importantly, very early on it was found that lowering body iron stores by bleeding or now chelators causes remission. In some northern European and US patients, carrying the hemochromatosis mutation is a risk factor but in other patients other iron susceptibility genes may contribute. Carrying a UROD mutation (lowering activity) is also a risk factor but still dependent on other susceptibility factors to see porphyria. To reproduce these findings experimentally has proved challenging but now possible. **For toxicologists** hepatic uroporphyria has mostly been seen as a toxic, but unique and curious endpoint of polychlorinated ligands of the AHR. Experimentally, TCDD in mice is the most potent agent consistent with AHR mode of action but is more difficult in rats and other organisms. Hexachlorobenzene (HCB) has been greatly studied for its porphyria-inducing abilities and a large incident of porphyria in some young people in Turkey 60 years ago was ascribed to susceptible individuals who had consumed HCB. It is controversial whether HCB is a weak AHR ligand. Evidence of porphyria in people exposed accidentally or occupationally to accepted AHR ligands such as TCDD and PCBs is thin. Importantly, iron status can profoundly modify experimental uroporphyria induced by these chemicals especially in mice. In fact iron overload alone of mice will eventually produce a strong hepatic uroporphyria which is markedly genetically determined and toxicity can be ameliorated by chelators resembling PCT. Thus hepatic porphyria could alternatively be viewed as an iron AOP. At an overall level hepatic uroporphyria in animals and patients is the outcome of complex genetic traits and external stimuli in which in some traditional toxicological circumstances binding of a chemical to the AHR may have a major contribution but in others may not.	4
Charge Question 1: 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?		

Reviewer 1	I have performed PubMed literature searches on various search terms pertaining to key events and their relationships: I did not identify any additional crucial publications. I'd think that the AOP131 in the narrow context as it stands now (which is probably also the goal of concept developers) is based on the solid and appropriate scientific literature. As said above, the focus of this AOP is strictly upon AHR activation, CYP1A2 induction and production UROD inhibitor(s) and the most crucial studies performed in AHR-compromised (polymorphic strains or gene knockouts) mouse strains. In this scenario, the evidence is pretty strong that AHR activation is the MIE. However, *there is an interesting observation of Davies et al: The induction of CYP1A2 is not crucial for chemical-induced porphyria, but a basal level of expression is absolutely essential*. This may imply that CYP1A2 activity for the production of UROD inhibitor(s) in itself is the most important KE (even perhaps the MIE) and induction is a modifying factor for this KE. **This is not to state that the current AOP is not correct, but just to point out that there are alternative ways to view various events and processes in the construct.** Some of these problems are presented in the recent extensive review on the AOP concept (Leist et al 2017 Arch Toxicol), as well as the role of the concept as a knowledge management tool (Vinken et al 2017 Arch Toxicol). Focussing on AHR and CYP1A2 and their more or less specific stressors seems to be a valid scenario, but what about other (nuclear) receptors, their activations and other CYP enzymes. I did only sporadic literature searches about whether these potentially additive possibilities were studied. For example, many PCB mixtures and individual compounds are known to be ligands of nuclear receptors (e.g. PXR, CAR), as well as hexachlorobenzene. Also CYP1A2 is known to be induced by other inducers than DLC or PAHs. To place AOP131 into a wider context, the authors show the whole pathway of heme synthesis and various porphyrias. It may be hypothesised that various disturbances in the pathway could modify the AOP 131, e.g. phenobarbital induction of ALA synthetase will increase the flow of pathway until UROD and decrease the threshold for overt uroporphyria. Some modifying factors have been mentioned, such as Fe and ALA, which may cause (mild) uroporphyria in the absence of AHR activation.	5 6 7
Reviewer 2	This is a thorough AOP that incorporates the most important scientific literature and current scientific knowledge in this field.	8
Reviewer 3	For the most part, yes (see below list of references)	9
Reviewer 4	A role of CYP1A2 is strong but it is not clear at the present time why this P450 above others in the liver. Whether direct oxidation of uroporphyrinogen by CYP1A2 in vivo occurs or some other mechanism of CYP1A2 has not been shown. Only a modest level of CYP1A2 may be required (Gorman et al 2007). Hepatic CYP1A2 binds TCDD and HCB etc (for unknown reasons) and this is being used in modelling of internal 'dioxin' exposure and body burden. Stimulated uncoupling of CYP1A2 may be a key event not just expression. At high doses non chlorinated AHR polycyclic ligands administered to AHRb mice with iron overload will develop a marked hepatic uroporphyria which incidentally, should be distinguished from a uroporphyrinuria observed in some animal models (Francis 1987). Both experimental and clinical evidence suggest that an aspect of Fe metabolism could be seen as a KE. The ultimate MIE of uroporphyria could be the inhibition of UROD but by what? A major paper has identified a uroporphomethene, the first oxidation product of uroporphyrinogen, as an inhibitor but there are problems with this. Uroporphyrinogen is oxidised to other non porphyrin products both in vitro and in vivo which may be more pertinent. Experimentally (and with clinical samples) the enzyme activity cannot be recovered.	10

	In birds, the evidence suggests that oxidation of uroporphyrinogen may occur but not so much to a potent inhibitor.	
	Other particular comments:	11
	Abstract. Second sentence is incorrect. There are 8 enzymes of the pathway in liver and hepatic uroporphyria is really the consequence of only inhibition of UROD although genetic variants of UROS may have clinical consequences with some similarities. Only homozygous mutations of UROD lead to a hepatic uroporphyria.	11a
	Measurement of UROD. Small amounts of uroporphyrinogen can be prepared easily and cleanly by reduction and buffering without need for purification. Product porphyrins from UROD assays are measured by reverse phase HPLC without methylation for the last 30 years (see Lim papers and Francis 1983).	11b
	On page 27 and uncertainties in humans, it may be worth recording that genetic variants of CYP1A2 do not strongly correlate with PCT patients. It may be that oxidation of uroporphyrinogen occurs by other mechanisms. At a chemical physical properties level it is also curious why uroporphyrinogen would be a substrate for CYP1A2.	11c
	On page 32 concerning consequences of porphyrin accumulation there is a reference to porphyrins causing neuropsychiatric symptoms. The evidence for this is poor and in hepatic acute intermittent porphyria it is established that it is ALA the first precursor not porphyrins that is responsible.	11d
Charge Question 2: Weight of evidence: Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?		
Reviewer 1	Although the WOE judgements were not always easy to dig out in the snapshot (Wiki layout seemed easier), I'd think that scoring calls for KEs, KERs and the overall AOP are pretty well defendable.	12
Reviewer 2	I agree with the scoring that the AOP developers have assigned to the various KEs and KERs.	13
	As also indicated by the developers one of the uncertainties of the AOP concerns the relationship between KE1 (induction of CYP1A2/CYP1A5) and KE2 (oxidation of uroporphyrinogen to uroporphyrin; UROX). It appears that UROX can occur in the absence of the CYP1A2 enzyme. Furthermore, CYP1A2 seems to be less significant in humans during the development of porphyria cutanea tarda.	13a
	Other uncertainties concern the relationship between KE2 (oxidation of uroporphyrinogen) and KE3 (inhibition of UROD). CYP-mediated UROX leads to the generation of metabolites that are suggested to function as inhibitor of UROD. The identity of the inhibitor is still under debate but one study defined uroporphomethene as the inhibitor of UROD. Thus, the AOP would be benefitted by an unequivocal identification of the metabolite responsible for inhibition of UROD.	13b
	UROD inhibition prevents the conversion of uroporphyrinogen to coproporphyrinogen and as such (further) increases UROX, subsequently leading to KE4 (accumulation of HCPs). However, for some avian models it has been shown that accumulation of porphyrins occurs without a decrease in UROD activity.	13c
Reviewer 3	**In Table 3/Temporal Concordance, the summary of Davies et al. (2008)** is not depicted correctly as some of the numbers are in the wrong column. It should be as follows:	14

Strain	Time (weeks)	CYP1A2 expression fold change	UROD (nmol/g liver)	Relative Uroporphyrin level
DBA/2	0.5	~39	<1	0
	2	~27	<1	0
	5	~15	<1	0
C57BL/6J	0.5	~49	<1	0
	2	~50	20	56
	5	~25	270	677

Thus, according to Davies et al. (2008), the relationship between CYP1A2 induction by 75µg/kg TCDD and UROD is not straightforward. Even though DBA/2 expresses AhR with much lower affinity to TCDD, the dose used clearly results in induction of Cyp1A2 mRNA in DBA/2 without up-regulation of UROD. This suggests that activation of AhR transcriptional activity is not sufficient to induce uroprophyria. For further discussion, please see below.

MI Event (AhR activation): evidence for essentiality appears strong, although Davies et al. (2008) study suggests it is not sufficient to induce uroporphyria in some rodent strains as stated in the text. One caveat is in rats (Sprague-Dawley), chronic exposure, but not acute exposure to TCDD, results in the induction of uroporphyria. Presumably, there is no reason to believe that high acute dose of TCDD would not activate AhR; thus, sustained AhR activation appears to be required. It is also possible that since TCDD can bind and inhibit CYP1A2 activity (Staskal et al., 2005), high acute dose of TCDD could effectively inhibit CYP1A2's downstream effects (i.e. UROX). 15

KE1 (CYP1A2/Cyp1A5 induction): the main evidence for this key event is based on Cyp1A2-/- mice, corroborated by association of CYP1A2/Cyp1A5 induction levels with susceptibility or severity of uroporphyria in mice and avian species. However, the relevance of Cyp1A2 induction for the downstream events in humans and resistant rodent strains is not known. Although not mentioned in the text, CYP1A2 can bind and sequester TCDD in the liver, possibly potentiating TCDD's effects (Hakk et al., 2009). 16

KE2 (uroporphyrinogen oxidation/UROX): Although in vitro assays showed that rodent CYP1A2 and avian CYP1A5 can oxidize uroporphyrinogen into uroporphyrins, human CYP1A2 has low such UROX activity. Thus in humans, how UROX induction is initiated via AhR is unclear. Furthermore, chemically induced porphyria in mice absolutely requires iron even when CYP1A2 is induced (Nakano et al., 2009); and CYP1A2-independent, iron-dependent UROX pathways has been also proposed (Phillips et al., 2011). 17

KE3 (uroporphyrinogen decarboxylase/UROD inhibition): Although evidence appears strong that inhibition of UROD is involved in chemically induced uroporphyria, linkage with upstream KE2 is unclear. This is the weakest link between MIE (AhR activation) and urophorphyria, in my opinion. Since the exact UROX product that leads to inhibition of uroporphyrinogen decarboxylase (UROD) is not known, molecular connection between CYP1A2-induced UROX and UROD inhibition is unclear. For example, it is not clear whether CYP1A2 directly or indirectly produces a UROD inhibitor through oxidation of uroporphyrinogen. As stated above and implied in Figure 1 (https://aopwiki.org/wiki/index.php/File:UROD_inhibition.jpg), 18

	reactive oxygen species generated from iron overload or other induced pathways can also potentially induce UROX.	
	KE4 [highly carboxylated porphyrin (HCP) accumulation]: This KE matches what is normally observed in hereditary forms of human porphyria, in which various enzymes along the heme biosynthetic pathway are affected.	19
Reviewer 4	Given all the uncertainties which the authors have drawn attention to, I consider the scoring calls not unreasonable. The AOP are not easy to digest though.	20
Charge Question 3: Regulatory applicability: Considering the strength of evidence and current gaps / weaknesses, what would be the regulatory applicability of this AOP, in your opinion?		
Reviewer 1	By scope, the AOP131 is rather restricted, but it may be of use when there is a suspicion about porphyrinogenic effects of a chemical/mixture under study. I would think that – given the complexity of the heme synthesis pathway and linked porphyrias – porphyria AOP network would be an ideal tool. Currently, rodent in vivo studies do not contain any tests specific for disturbances of the heme synthesis pathway. I am not very familiar with exotoxicological test systems, but naturally examples of avian or fish problems may point at least to further studies in this field to clarify the usefulness of this AOP in ecotoxicological assessment.	21
Reviewer 2	Assays representative for a number of KEs (MIE, KE1, KE2 and KE3) are available. Extensive validation and use of these assays as part of a battery approach would facilitate regulatory applicability on the long term. Important is the quantitative understanding of the outcome of the assays. In that respect, the finding that a reduction in UROD activity of at least 70% is required to lead to uroporphyria (in mammals) is an important finding. The authors of the AOP indicate that UROD inhibition is not always observed in avian models of porphyria. This emphasises the use of a battery of tools, rather than individual assays, to test for porphyria.	22
Reviewer 3	For agencies concerned with environmental impact including effects on wildlife, this AOP will be quite useful. However, for agencies that deal specifically with human health, the usefulness of this AOP is questionable, given the uncertainties and gaps in our understanding of the molecular mechanisms of AhR activation and uroporphyria in humans. Sinclair et al. conclude that human CYP1A2 can oxidize uroporphyrinogen, but has lower activity compared to murine homologue; in addition, they conclude that most of UROX activity found in human liver microsomes is not due to CYP1A2 (Sinclair et al., 1998). In addition, evidence of either a direct or associative link between TCDD exposure and human porphyria cutanea tarda is weak (Calvert et al., 1994; Fader and Zacharewski, 2017).	23
Reviewer 4	I am not sure it is particularly useful for regulatory use for mammals and health particularly as this is a complex scenario going beyond AHR as illustrated. However, the comparisons with other non mammalian species, especially birds, may well be very useful illustrating unwanted environmental exposure to AHR ligands.	24
Conclusion: What are your overall conclusions of the assessment of this AOP?		
Reviewer 1	This AOP seems to be a very clear and solid construct based on the appropriate scientific literature. It could be useful to present this AOP in a wider context, i.e. by describing the heme synthesis pathway and porphyrias in the background section or pondering upon some potential interactions within this wider context. Considerations on the appropriate context are certainly difficult and not easily solved, but at least some	25

	remarks could be useful for further development of a network of "porphyria AOPs".	
Reviewer 2	My overall conclusion is that the AOP is quite solid and complete and well-supported with references The authors have provided detailed information on the biological plausibility, empirical support, and quantitative understanding of the KEs and KERs. They also have indicated the pitfalls of the AOP. Some knowledge/data gaps still have to be tackled, before the AOP can be endorsed for regulatory application.	26
Reviewer 3	AOP is nicely laid out conceptually, and the description of each KEs and MIE are well documented. The proposed pathway relies mostly on evidence from the most susceptible mouse strain (C57BL/6), AhR-/- and Cyp1A2-/-knockout mice, and in vitro assays using either rodent or avian microsome systems, and perhaps is the simplest interpretation of those data. While the simplest AOP, as a building unit, is worthwhile, I believe that the AOP requires proper context to be widely useful. It is widely acknowledged that the toxicological effects of AhR activators display, qualitatively and quantitatively, tremendous inter- and intra-species variations (Reichard et al., 2006). These inter- or intra-species differences may provide important insights into the relationship between AhR activation and uroporphyria. While the authors of the AOP allude to some of the data that are not in-line with the proposed AOP, I believe more extensive discussion of these seemingly inconsistent observations would be helpful to put the proposed AOP in context. The following are some suggestions that are worth discussing as a side-note.	27
	o The levels of ascorbic acid negatively correlate with CYP1A2 activity. Ascorbic acid was shown to be a competitive inhibitor of CYP1A2's UROX activity in vitro (Sinclair et al., 1993); this inhibitory activity by ascorbic acid was observed in vivo using Osteogenic Disorder Shionogi (ODS) mutant rat, which lacks the ability to synthesize ascorbic acid: in a dose-dependent manner, ascorbic acid prevented uroporphyrin accumulation in ODS mutant rats treated with either 3-methylcholanthrene (MC) and 5-aminolevulinate (ALA) or hexachlorobenzene (Sinclair et al., 1993). In the same study, MC/ALA treatment did not result in uroporphyrin accumulation in Fischer 344 rats; this was ascribed to these rats showing much higher hepatic ascorbic acid levels compared to ODS rats. Interestingly, in both strains treated with MC and ALA, CYP1A2 was induced, suggesting that ascorbic acid modulates CYP1A2 activity rather than its expression. These studies lend credence to the involvement of CYP1A2 activity in uroporphyria; furthermore, some of the observed intra- and inter-species differences on the susceptibility to AhR activators on uroporphyria induction may be explained by differences in the hepatic levels of ascorbic acid in those animals. o The requirement of chronic, instead of acute, TCDD exposure in Sprague-Dawley rats requires discussion, especially in light of the fact that a single 75µg/kg dose of TCDD in "susceptible" C57BL/6 mice resulted in accumulation of hepatic porphyrins (Smith et al., 1981). o Although CYP1A2 clearly appears to be the main downstream target gene in AhR activation, other AhR-dependent genes may also modulate the magnitude of adverse effects leading to uroporphyria. For example, CYP1A1-/- mice show reduced uroporphyria upon TCDD exposure suggesting that CYP1A1 also contributes to production of porphyrins (Rifkind, 2006; Uno et al., 2004). In fact, Davies et al. (2008) conclude that other genes induced by TCDD, including those involved in glucose and iron metabolism as well as oxidative stress response, may contribute to TCDD-dependent uroporphyria in C57BL mice. o What is unclear from cited studies is how CYP1A2 induction and UROX ultimately lead to UROD inhibition? Is this a direct or indirect effect?	28

	o Given that other contributors to etiology of uroporphyria (iron overload and alcohol) are also well-known oxidative stressors, antioxidant capacity within a hepatic cell may be a confounding factor as to why some animals and humans are susceptible or resistant to ArH inducers. General increase in oxidative stress [i.e. lipid peroxidation, reactive oxygen species (ROS) and oxidized glutathione] due to TCDD is not dependent on functional CYP1A2 (Slezak et al., 1999); however, TCDD did elicit AhR-dependent, CYP1A1/A2-independent mitochondrial ROS production in mice suggesting that general oxidative stress induced independently of CYP1A2 induction may contribute to the resulting overall UROX by TCDD (Senft et al., 2002).	
Reviewer 4	This is a good attempt at a very complex scenario which at first sight seems relatively simple. Despite considerable research proven toxic endpoints relevant to significant human adverse outcomes from AHR ligands are few and it is important that this AOP documents and systemizes this both what is known, the uncertainties and the limitations.	29
List of references		
Reviewer 3	Calvert, G.M., Sweeney, M.H., Fingerhut, M.A., Hornung, R.W., and Halperin, W.E. (1994). Evaluation of porphyria cutanea tarda in U.S. workers exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Am J Ind Med *25*, 559-571. Davies, R., Clothier, B., Robinson, S.W., Edwards, R.E., Greaves, P., Luo, J., Gant, T.W., Chernova, T., and Smith, A.G. (2008). Essential role of the AH receptor in the dysfunction of heme metabolism induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Chem Res Toxicol *21*, 330-340. Fader, K.A., and Zacharewski, T.R. (2017). Beyond the Aryl Hydrocarbon Receptor: Pathway Interactions in the Hepatotoxicity of 2,3,7,8-Tetrachlorodibenzo-p-dioxin and Related Compounds. Curr Opin Toxicol *2*, 36-41. Hakk, H., Diliberto, J.J., and Birnbaum, L.S. (2009). The effect of dose on 2,3,7,8-TCDD tissue distribution, metabolism and elimination in CYP1A2 (-/-) knockout and C57BL/6N parental strains of mice. Toxicol Appl Pharmacol *241*, 119-126. Nakano, K., Ishizuka, M., Sakamoto, K.Q., and Fujita, S. (2009). Absolute requirement for iron in the development of chemically induced uroporphyria in mice treated with 3-methylcholanthrene and 5-aminolevulinate. Biometals *22*, 345-351. Phillips, J.D., Kushner, J.P., Bergonia, H.A., and Franklin, M.R. (2011). Uroporphyria in the Cyp1a2-/- mouse. Blood Cells Mol Dis *47*, 249-254. Reichard, J.F., Dalton, T.P., Shertzer, H.G., and Puga, A. (2006). Induction of oxidative stress responses by dioxin and other ligands of the aryl hydrocarbon receptor. Dose Response *3*, 306-331. Rifkind, A.B. (2006). CYP1A in TCDD toxicity and in physiology-with particular reference to CYP dependent arachidonic acid metabolism and other endogenous substrates. Drug Metab Rev *38*, 291-335. Senft, A.P., Dalton, T.P., Nebert, D.W., Genter, M.B., Puga, A., Hutchinson, R.J., Kerzee, J.K., Uno, S., and Shertzer, H.G. (2002). Mitochondrial reactive oxygen production is dependent on the aromatic hydrocarbon receptor. Free Radic Biol Med *33*, 1268-1278.	30

Sinclair, P.R., Gorman, N., Tsyrlov, I.B., Fuhr, U., Walton, H.S., and Sinclair, J.F. (1998). Uroporphyrinogen oxidation catalyzed by human cytochromes P450. Drug Metab Dispos *26*, 1019-1025.

Sinclair, P.R., Gorman, N., Walton, H.S., Bement, W.J., Jacobs, J.M., and Sinclair, J.F. (1993). Ascorbic acid inhibition of cytochrome P450-catalyzed uroporphyrin accumulation. Arch Biochem Biophys *304*, 464-470.

Slezak, B.P., Diliberto, J.J., and Birnbaum, L.S. (1999). 2,3,7,8-Tetrachlorodibenzo-p-dioxin-mediated oxidative stress in CYP1A2 knockout (CYP1A2-/-) mice. Biochem Biophys Res Commun *264*, 376-379.

Smith, A.G., Francis, J.E., Kay, S.J., and Greig, J.B. (1981). Hepatic toxicity and uroporphyrinogen decarboxylase activity following a single dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin to mice. Biochem Pharmacol *30*, 2825-2830.

Staskal, D.F., Diliberto, J.J., Devito, M.J., and Birnbaum, L.S. (2005). Inhibition of human and rat CYP1A2 by TCDD and dioxin-like chemicals. Toxicol Sci *84*, 225-231.

Uno, S., Dalton, T.P., Sinclair, P.R., Gorman, N., Wang, B., Smith, A.G., Miller, M.L., Shertzer, H.G., and Nebert, D.W. (2004). Cyp1a1(-/-) male mice: protection against high-dose TCDD-induced lethality and wasting syndrome, and resistance to intrahepatocyte lipid accumulation and uroporphyria. Toxicol Appl Pharmacol *196*, 410-421.

Annex 3: Written response from the authors in preparation for the end of review Teleconference

Annex 3a: Authors' Overall Response

We would like to thank the reviewers and review manager for their thorough assessment of this AOP, and truly appreciate the constructive comments. We have responded to individual comments within the review document, but noticed a theme throughout the review that we feel should be addressed with a few overall statements. Please consider these points during our upcoming meeting.

1. Restrictions of the AOP framework: A number of the comments stress the fact that this particular AOP does not consider a number of potential modulating factors and alternate pathways, which would help to put the AOP in context and better illustrate the inherent uncertainties. We completely agree that this AOP is not the only possible pathway to uroporphyria, and appreciate that biology is complex and not linear. However, the purpose of an AOP is to depict a single possible pathway that is biologically plausible and supported as much as possible with empirical evidence. So when considering the WOE calls for each KER, and the AOP as a whole, one must ask: is there sufficient evidence supporting the indicated adverse outcome (uroporphyria) through this particular sequence of events? The potential of other completely independent or partially related pathways should not affect this WOE call.

The reviewers stress the importance of viewing the biology as a whole, which is certainly powerful, but is not the intent of a single AOP. It seems what they desire is better represented by AOP network, which is an alternate goal of AOP developers, and is in-fact the functional unit of the framework. There are numerous efforts in the development of algorithms to interpret AOP networks, which show all the possible pathways to an AO. These networks are comprised of multiple linear AOPs that share at least one key event. In order to create meaningful AOP networks, we must first focus on preparing solid singular AOPs.

2. Essentiality of KEs: For a KE to be considered strongly essential, it must be demonstrated that the downstream events would not occur in its absence; it is not a condition to show that they will necessarily occur in its presence (i.e. it is essential but not sufficient). The essentiality of CYP1A2 has been criticized throughout the review, but no new evidence was presented. In other words, the reviewers believe that the KE is not strongly essential based on the same inconsistencies/uncertainties that the authors have already considered. But it seems that this stems from the potential of alternate pathways to the AO, or the presence of modulating factors (primarily iron).

I propose that the essentiality of CYP1A2 induction remains strong, and that iron is removed as a "stressor" (since it doesn't interact with the MIE); rather it should be included as a modulating factor in the oxidation of uroporphyrinogen (either in the KE, of KER between CYP and UROX, or both). CYP1A2 is a hemoprotein that contains a heme-iron center in its active site, so it makes sense that iron deficiency would suppress this pathway. Would the reviewers agree? Let's discuss.

3. WOE calls for KERs: Similar to point 2, the links between various KEs have been criticized; again, not much new evidence was introduced, so the

inconsistencies/uncertainties discussed have already been considered by the authors. Keeping in mind point 1, do the reviewers suggest changes to some of the WOE calls? If so, please be prepared to discuss with specific examples.

4. Domain of applicability: The strength of the AOP was questioned based on the uncertainties that lie between species differences. For example, UROD inhibition is less strongly correlated with uroporphyrin accumulation in birds. Also, the role or essentiality of CYP1A2 in humans is questionable. These things considered, we propose that an explicit discussion be added to the main AOP page to expand on the "domain of applicability section". In short, it will state the following, with supporting citations:

a. The AOP is most strongly applicable to rodents. (This is true, even in the case of resistant strains; since the resistance is often attributed to differences in the AHR gene).

b. Although UROD inhibition plays a pivitol role in mammalian development of uroporphyria, the relationship is more complex in birds. UROD inhibition is often observed in avian cases of uroporphyria, however, the reduction is not as pronounced as it is in mammals. Elevated porphyrins have also been observed without the inhibition of UROD in vitro.

c. The essentiality of CYP1A2 induction in human porphyria cutanea tarda is unclear. UROX activity in human liver microsomes was not correlated with CYP1A2 content. There is contradictory evidence regarding the association between CYP1A2 polymorphism and susceptibility to porphyria cutanea tarda. It may be possible that in patients with a genetic variation in UROD causing an inherent reduction in activity, the activity of CYP1A2 is less important.

Annex 3b: Authors' Response and Questions to individual comments

A: corresponding (main) author; B: co-author

General		
Reviewer 1	Overall, I found the AOP a logical and well-founded construct and – within its rather restricted scope – based on an adequately comprehensive survey of literature. Taking into account the restricted scope and the complexity of porphyrias, it would have been beneficial to provide some succinct background (which is now empty) to put the AOP on the wider context. Actually there are some general information in some parts of the AOP, e.g. in the description of the AO.	A: Can easily provide info on the different types of porphyria and where this one lies…similar to the AO page. Maybe in the background section?
	Internal review has been pretty thorough and reading it was very useful when preparing for the external review. I share some concerns of the author regarding the structure and presentation of the AOP and snapshot, but maybe this concern belongs to "the realm of newcomer's confusion".	
	Although the AOP as such is very good, there are number of points that may require some further discussion, e.g. the nature of stressors and adequacy of focusing on CYP1A2/5, possible links and interactions with other parts of the heme synthesis pathway.	A: Suggested alternatives? Under which KER would these go? We don't want to branch to start branching out. B: This would represent constructing of additional AOPs that converge with the AO (or other KEs) in this AOP (i.e. an AOP network). It is not the goal of an AOP to describe ALL possible paths and interactions, but merely one discrete route that contributes to the AO.
Reviewer 4	As a scheme for this phenomenon I think the AOP is a good logical attempt for an outcome which is quite specific and species variable. Besides particular scientific points addressed below, there are some wider contextual difficulties however, which may be the consequence of the toxicological view and approach of an AOP compared to medical experience. This contrast cannot be unique to this endpoint though. It may be helpful to illustrate that hepatic uroporphyria is viewed somewhat	

	differently by clinicians and toxicologists. **For the former** it is mostly a sporadic disease (porphyria cutanea tarda; PCT) occurring sometimes in patients exposed to a variety of insults such as alcohol, estrogens, hepatitis viruses, HIV and on dialysis. Importantly, very early on it was found that lowering body iron stores by bleeding or now chelators causes remission. In some northern European and US patients, carrying the hemochromatosis mutation is a risk factor but in other patients other iron susceptibility genes may contribute. Carrying a UROD mutation (lowering activity) is also a risk factor but still dependent on other susceptibility factors to see porphyria. To reproduce these findings experimentally has proved challenging but now possible. **For toxicologists** hepatic uroporphyria has mostly been seen as a toxic, but unique and curious endpoint of polychlorinated ligands of the AHR. Experimentally, TCDD in mice is the most potent agent consistent with AHR mode of action but is more difficult in rats and other organisms. Hexachlorobenzene (HCB) has been greatly studied for its porphyria-inducing abilities and a large incident of porphyria in some young people in Turkey 60 years ago was ascribed to susceptible individuals who had consumed HCB. It is controversial whether HCB is a weak AHR ligand. Evidence of porphyria in people exposed accidentally or occupationally to accepted AHR ligands such as TCDD and PCBs is thin. Importantly, iron status can profoundly modify experimental uroporphyria induced by these chemicals especially in mice. In fact iron overload alone of mice will eventually produce a strong hepatic uroporphyria which is markedly genetically determined and toxicity can be ameliorated by chelators resembling PCT. Thus hepatic porphyria could alternatively be viewed as an iron AOP. At an overall level hepatic uroporphyria in animals and patients is the outcome of complex genetic traits and external stimuli in which in some traditional toxicological circumstances binding of a chemical to the AHR may have a major contribution but in others may not.	A: Interesting. So should these be contrasted in the AO page? A: Iron is included as a stressor in the AOP main page, and a brief statement is made confirming the association between iron overload and porphyrin accumulation…I'm leaning towards removing it as a stressor (because it doesn't interact with the AHR) and adding it as a modulating factor. Iron-overload is mentioned as a potential alternate, CYP1A2 independent, pathway under KE Relationship: 868 (Induction, CYP1A2/CYP1A5 leads to Oxidation, Uroporphyrinogen)….although I'm still not sure this is completely independent of the CYP1A2 protein. It is true that hepatic porphyria could alternatively be viewed as an iron AOP, but since it follows an alternate pathway, it would be an AOP independent of this one (potentially sharing

		some of the same KEs) I'm curious to know if these are CYP1A2 knockouts, or if they have a genetic predisposition to reduced UROD already. Can you please provide a citation?
Charge Question 1: 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?		
Reviewer 1	I have performed PubMed literature searches on various search terms pertaining to key events and their relationships: I did not identify any additional crucial publications. I'd think that the AOP131 in the narrow context as it stands now (which is probably also the goal of concept developers) is based on the solid and appropriate scientific literature. As said above, the focus of this AOP is strictly upon AHR activation, CYP1A2 induction and production UROD inhibitor(s) and the most crucial studies performed in AHR-compromised (polymorphic strains or gene knockouts) mouse strains. In this scenario, the evidence is pretty strong that AHR activation is the MIE. However, *there is an interesting observation of Davies et al: The induction of CYP1A2 is not crucial for chemical-induced porphyria, but a basal level of expression is absolutely essential*. This may imply that CYP1A2 activity for the production of UROD inhibitor(s) in itself is the most important KE (even perhaps the MIE) and induction is a modifying factor for this KE. **This is not to state that the current AOP is not correct, but just to point out that there are alternative ways to view various events and processes in the construct.** Some of these problems are presented in the recent extensive review on the AOP concept (Leist et al 2017 Arch Toxicol), as well as the role of the concept as a knowledge management tool (Vinken et al 2017 Arch Toxicol). F ocussing on AHR and CYP1A2 and their more or less specific stressors seems to be a valid scenario, but what about other (nuclear) receptors, their activations and other CYP enzymes. I did only sporadic literature searches about whether these potentially additive possibilities were studied. For example, many PCB	A; Interesting perspective. A: This is all very true, but the intent of an AOP is to describe one single possible pathway to an adverse outcome, not to encompass all possibilities.

	mixtures and individual compounds are known to be ligands of nuclear receptors (e.g. PXR, CAR), as well as hexachlorobenzene. Also CYP1A2 is known to be induced by other inducers than DLC or PAHs. To place AOP131 into a wider context, the authors show the whole pathway of heme synthesis and various porphyrias. It may be hypothesised that various disturbances in the pathway could modify the AOP 131, e.g. phenobarbital induction of ALA synthetase will increase the flow of pathway until UROD and decrease the threshold for overt uroporphyria. Some modifying factors have been mentioned, such as Fe and ALA, which may cause (mild) uroporphyria in the absence of AHR activation.	What you describe is the need for multiple AOPs that feed into each other; there is significant effort being put into the analysis of AOP networks, which I believe is what you're referring to. This function will only be strengthened with time and the development of more "singular" AOPs. B: This would involve making many new AOP that form an AOP network. A great idea! But not required for AOP 131.
Reviewer 2	This is a thorough AOP that incorporates the most important scientific literature and current scientific knowledge in this field.	
Reviewer 3	For the most part, yes (see below list of references)	
Reviewer 4	A role of CYP1A2 is strong but it is not clear at the present time why this P450 above others in the liver. Whether direct oxidation of uroporphyrinogen by CYP1A2 in vivo occurs or some other mechanism of CYP1A2 has not been shown. Only a modest level of CYP1A2 may be required (Gorman et al 2007). Hepatic CYP1A2 binds TCDD and HCB etc (for unknown reasons) and this is being used in modelling of internal 'dioxin' exposure and body burden. Stimulated uncoupling of CYP1A2 may be a key event not just expression. At high doses non chlorinated AHR polycyclic ligands administered to AHRb mice with iron overload will develop a marked hepatic uroporphyria which incidentally, should be distinguished from a uroporphyrinuria observed in some animal models (Francis 1987).	A: Regarding issue of "P450 above others": I'm not sure what to make of this. I thought the WOE section gave sufficient evident for the essentiality of CYP1A2. I am not an expert in this field, but based on the definitive statements made in the literature (e.g. "The oxidation of uroporphyrinogen to uroporphyrin (UROX) has been demonstrated to be catalyzed by CYP1A2") I assumed this was agreed upon. The following citations make similar statements: Jacobs et al. (1989) Biochem. J 258 (1), 247-253. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1138347/ Lambrecht et al. (1992). Arch. Biochem. Biophys. 294 (2), 504-510. https://doi.org/10.1016/0003-9861(92)90717-B Sinclair et al. (1997). Drug Metab. Dispos. 25 (7), 779-783. http://dmd.aspetjournals.org/content/25/7/779.long I am not sure what "Stimulated uncoupling of CYP1A2 may

		be a key event not just expression" means.
	Both experimental and clinical evidence suggest that an aspect of Fe metabolism could be seen as a KE.	A: Regarding: "aspect of Fe metabolism could be seen as a KE." : Should It be added as a modulating factor in KER 868 (Induction, CYP1A2/CYP1A5 leads to Oxidation, Uroporphyrinogen)?
	The ultimate MIE of uroporphyria could be the inhibition of UROD but by what? A major paper has identified a uroporphomethene, the first oxidation product of uroporphyrinogen, as an inhibitor but there are problems with this. Uroporphyrinogen is oxidised to other non porphyrin products both in vitro and in vivo which may be more pertinent. Experimentally (and with clinical samples) the enzyme activity cannot be recovered. In birds, the evidence suggests that oxidation of uroporphyrinogen may occur but not so much to a potent inhibitor.	A: Regarding UROD inhibition issue: This was initially discussed as an option when the AOP was first being developed (2014), but considering the "stressor" does not directly interact with UROD, by definition, it cannot be the MIE. If Uroporphomethane is the inhibiting factor, it is produced as a consequence of AHR activation-> CYP1A induction (or whatever path produces the inhibitor), making it a result of preceding events, and not the initiator of the cascade.
	Other particular comments:	
	Abstract. Second sentence is incorrect. There are 8 enzymes of the pathway in liver and hepatic uroporphyria is really the consequence of only inhibition of UROD although genetic variants of UROS may have clinical consequences with some similarities. Only homozygous mutations of UROD lead to a hepatic uroporphyria.	A: Thank you for catching this mistake. It will be corrected. I think it's better described in the AO page: "Porphyria is a disorder in which the disturbance of heme biosynthesis results in accumulation and excretion of porphyrins[1]. A variety of porphyrias exist depending on which enzyme in the pathway is deficient In the case of chemically induced urporphyria, uroporphyrinogen decarboxylase (UROD), which converts uroporphyrinogen to coproporphyrinogen, is inhibited." A similar statement will be made in the abstract. Reviewer 1 previously mentioned the benefit of including background information. Maybe the diagram indicating the different enzymes and corresponding disorders (Fig 1. KE 369:

		Uroporphyria) should go here?
	Measurement of UROD. Small amounts of uroporphyrinogen can be prepared easily and cleanly by reduction and buffering without need for purification. Product porphyrins from UROD assays are measured by reverse phase HPLC without methylation for the last 30 years (see Lim papers and Francis 1983).	A: Please provide specific citation; I wasn't able to find this method. If this is the method you're referring to "Lim and Peters (1986) High-Performance Liquid-Chromatography Of Uroporphyrin And Coproporphyrin Isomers. *Methods In Enzymology* Volume: 123, Pages: 383-389" I would really appreciate a PDF version, as I do not have access to it. Do you suggest it's added as an additional method or should it replace those mentioned?
	On page 27 and uncertainties in humans, it may be worth recording that genetic variants of CYP1A2 do not strongly correlate with PCT patients. It may be that oxidation of uroporphyrinogen occurs by other mechanisms. At a chemical physical properties level it is also curious why uroporphyrinogen would be a substrate for CYP1A2.	A: I found a French study supporting this (Tchernitchko et al (2012) Comprehensive cytochrome P450 CYP1A2 gene analysis in French caucasian patients with familial and sporadic porphyria cutanea tarda. BRITISH JOURNAL OF DERMATOLOGY Volume: 166 Issue: 2 Pages: 425-429) But also a Danish case study that contradicts it (Christiansen et al (2000) Association between CYP1A2 polymorphism and susceptibility to porphyria cutanea tarda. Human Genetics. Volume 107, Issue 6, pp 612–614) Can you suggested any reviews to cite? If I recall correctly genetic variation in the AHR altering binding affinity better correlates with sensitivity. Is it possible that, due to the promiscuity of CYP1A2, multiple variants can have similar Oxidative activities? Or have these variants been shown to have low oxidative capacity?
	On page 32 concerning consequences of porphyrin accumulation there is a	A: True this was only demonstrated in one study that I came

	reference to porphyrins causing neuropsychiatric symptoms. The evidence for this is poor and in hepatic acute intermittent porphyria it is established that it is ALA the first precursor not porphyrins that is responsible.	across. Do you suggest this paragraph is removed?
Charge Question 2: Weight of evidence: Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?		
Reviewer 1	Although the WOE judgements were not always easy to dig out in the snapshot (Wiki layout seemed easier), I'd think that scoring calls for KEs, KERs and the overall AOP are pretty well defendable.	
Reviewer 2	I agree with the scoring that the AOP developers have assigned to the various KEs and KERs. As also indicated by the developers one of the uncertainties of the AOP concerns the relationship between KE1 (induction of CYP1A2/CYP1A5) and KE2 (oxidation of uroporphyrinogen to uroporphyrin; UROX). It appears that UROX can occur in the absence of the CYP1A2 enzyme. Furthermore, CYP1A2 seems to be less significant in humans during the development of porphyria cutanea tarda. Other uncertainties concern the relationship between KE2 (oxidation of uroporphyrinogen) and KE3 (inhibition of UROD). CYP-mediated UROX leads to the generation of metabolites that are suggested to function as inhibitor of UROD. The identity of the inhibitor is still under debate but one study defined uroporphomethene as the inhibitor of UROD. Thus, the AOP would be benefitted by an unequivocal identification of the metabolite responsible for inhibition of UROD. UROD inhibition prevents the conversion of uroporphyrinogen to coproporphyrinogen and as such (further) increases UROX, subsequently	A: If this statement is based on the literature cited in this AOP, then I'm not sure it is true. We mention that oxidation occurs in the absence of CYP1A2 **induction**, but that a basal level of expression is still necessary (since CYP1A2 knockout prevents porphyrin accumulation but under certain conditions, mild uroporphyria was observed in AHR KO mice). B: Again… our AOP does not disallow the possibility of other pathways that lead to UROX. Will the review agree that CYP1A2 is ONE way that can lead to UROX? B: We agree that "Thus, the AOP would be benefitted by an unequivocal identification of the metabolite responsible for inhibition of UROD". Is the review suggesting that the WoE call for this KER be changed to "Low" until this evidence is produced? B: This should be added in the inconsistencies section. Can

	leading to KE4 (accumulation of HCPs). However, for some avian models it has been shown that accumulation of porphyrins occurs without a decrease in UROD activity.	the reviewer please provide the relevant reference.This should be added in the inconsistencies section. Can the reviewer please provide the relevant reference. A: It has been stated and referenced under inconsistencies in KER 1070: Inhibition, UROD leads to Accumulation, Highly carboxylated porphyrins, but with only 1 in vitro reference (Lambrecht et al 1988). More studies do show a reduction in URD activity (although often less than 50%), which is why the WOE call was reduced to moderate for birds (I didn't think one contradictory study warranted a call of weak).
Reviewer 3	**In Table 3/Temporal Concordance, the summary of Davies et al. (2008)** is not depicted correctly as some of the numbers are in the wrong column. It should be as follows: Thus, according to Davies et al. (2008), the relationship between CYP1A2 induction by 75µg/kg TCDD and UROD is not straightforward. Even though	A: Thank you for noticing, there is certainly an error in the table, but I think the corrected version should be a combination of our two. I looked through the paper again and it doesn't report UROD levels. The 20 and 270 refer to absolute values of porphyrins (from Figure 1A) and the text states that this is a 56 and 677-fold change (so effectively give the same information). I will add the corrected CYP1A2 values and include the relative porphyrin levels in the last column (0, 56, 677). The UROD column will be left blank. The updated table will be an excellent example of temporal concordance evidence! B: I looked at the Davies paper. You are correct. They did not report UROD. You can put relative amounts in the last column (and if you want you can include the nmol/g amounts in brackets in that last column) A: It has been established that the expression and levels of UROD are not changed, or do not correlate with CYP1A2

Strain	Time (weeks)	CYP1A2 expression fold change	UROD (nmol/g liver)	Relative Uroporphyrin level
DBA/2	0.5	~39	<1	0
	2	~27	<1	0
	5	~15	<1	0
C57BL/6J	0.5	~49	<1	0
	2	~50	20	56
	5	~25	270	677

	DBA/2 expresses AhR with much lower affinity to TCDD, the dose used clearly results in induction of Cyp1A2 mRNA in DBA/2 without up-regulation of UROD. This suggests that activation of AhR transcriptional activity is not sufficient to induce uroprophyria. For further discussion, please see below.	expression. It is in fact the activity of UROD that is affected (Although levels remain the same, there is competitive binding of the active site inhibiting it's normal function). Neither UROD protein levels nor activity are measured in this paper. In relation to the suggestion that "*activation of AhR transcriptional activity is not sufficient to induce uroprophyria*".: This is true. A KE must be proven essential to be included in an AOP (i.e. the cascade of events specified does not occur in its absence), but does not necessarily have to be sufficient on its own.
	MI Event (AhR activation): evidence for essentiality appears strong, although Davies et al. (2008) study suggests it is not sufficient to induce uroporphyria in some rodent strains as stated in the text. One caveat is in rats (Sprague-Dawley), chronic exposure, but not acute exposure to TCDD, results in the induction of uroporphyria. Presumably, there is no reason to believe that high acute dose of TCDD would not activate AhR; thus, sustained AhR activation appears to be required. It is also possible that since TCDD can bind and inhibit CYP1A2 activity (Staskal et al., 2005), high acute dose of TCDD could effectively inhibit CYP1A2's downstream effects (i.e. UROX).	A: The AOP specifically states that transient activation of the AHR is not sufficient to induce porphyria.
	KE1 (CYP1A2/Cyp1A5 induction): the main evidence for this key event is based on Cyp1A2-/- mice, corroborated by association of CYP1A2/Cyp1A5 induction levels with susceptibility or severity of uroporphyria in mice and avian species. However, the relevance of Cyp1A2 induction for the downstream events in humans and resistant rodent strains is not known. Although not mentioned in the text, CYP1A2 can bind and sequester TCDD in the liver, possibly potentiating TCDD's effects (Hakk et al., 2009).	A: True. Would you say these uncertainties are enough to change the WOE call from Strong to Moderate? B: Supporting evidence for KE1 is still strong for susceptible species and strains. Perhaps we just need to make that distinction A: Thank you for the interesting paper. So the sequestration of TCDD by CYP1A2 prevents its binding to the active CYP1A1, and therefore keeps it around longer. Possibly potentiating it's effects… But a similar argument could be made, in that if it's binding to CYP1A2 it results in competition with uroporphyrinogens thereby inhibiting potential oxidation…

		Maybe a point of discussion? B: Agree. This might mean that if CYP1A2 is bound to TCDD, it would not be available for UROX…
	KE2 (uroporphyrinogen oxidation/UROX): Although in vitro assays showed that rodent CYP1A2 and avian CYP1A5 can oxidize uroporphyrinogen into uroporphyrins, human CYP1A2 has low such UROX activity. Thus in humans, how UROX induction is initiated via AhR is unclear. Furthermore, chemically induced porphyria in mice absolutely requires iron even when CYP1A2 is induced (Nakano et al., 2009); and CYP1A2-independent, iron-dependent UROX pathways has been also proposed (Phillips et al., 2011).	A: All these things are mentioned within the AOP. A KE must only be proven to be essential for this specific pathway (which it has) but not necessarily sufficient to induce the AO. So I don't think the WOE call should be changed. Maybe sufficient iron levels should be added as a modulating factor in KER 868 (Induction, CYP1A2/CYP1A5 leads to Oxidation, Uroporphyrinogen)? B: Agreed
	KE3 (uroporphyrinogen decarboxylase/UROD inhibition): Although evidence appears strong that inhibition of UROD is involved in chemically induced uroporphyria, linkage with upstream KE2 is unclear. This is the weakest link between MIE (AhR activation) and urophorphyria, in my opinion. Since the exact UROX product that leads to inhibition of uroporphyrinogen decarboxylase (UROD) is not known, molecular connection between CYP1A2-induced UROX and UROD inhibition is unclear. For example, it is not clear whether CYP1A2 directly or indirectly produces a UROD inhibitor through oxidation of uroporphyrinogen. As stated above and implied in Figure 1 (https://aopwiki.org/wiki/index.php/File:UROD_inhibition.jpg), reactive oxygen species generated from iron overload or other induced pathways can	Although this is all true, it does not affect the call for the KE itself (It may be relevant to the WOE of the CYP1A2->UROX KER). UROD inhibition is certainly essential in mammalian development of uroporphyria; potentially less so in birds. Do you think this species difference is enough to change the WOE call to moderate (I wouldn't go as far as labelling it weak)? B: I agree that they seem to be questiononig the KER... and not the essentiality of the KE. can ask for clarification at TC

	also potentially induce UROX.	
	KE4 [highly carboxylated porphyrin (HCP) accumulation]: This KE matches what is normally observed in hereditary forms of human porphyria, in which various enzymes along the heme biosynthetic pathway are affected.	
Reviewer 4	Given all the uncertainties which the authors have drawn attention to, I consider the scoring calls not unreasonable. The AOP are not easy to digest though.	
Charge Question 3: Regulatory applicability: Considering the strength of evidence and current gaps / weaknesses, what would be the regulatory applicability of this AOP, in your opinion?		
Reviewer 1	By scope, the AOP131 is rather restricted, but it may be of use when there is a suspicion about porphyrinogenic effects of a chemical/mixture under study. I would think that – given the complexity of the heme synthesis pathway and linked porphyrias – porphyria AOP network would be an ideal tool. Currently, rodent in vivo studies do not contain any tests specific for disturbances of the heme synthesis pathway. I am not very familiar with exotoxicological test systems, but naturally examples of avian or fish problems may point at least to further studies in this field to clarify the usefulness of this AOP in ecotoxicological assessment.	B: Agreed that porphyria AOP network would be an ideal tool
Reviewer 2	Assays representative for a number of KEs (MIE, KE1, KE2 and KE3) are available. Extensive validation and use of these assays as part of a battery approach would facilitate regulatory applicability on the long term. Important is the quantitative understanding of the outcome of the assays. In that respect, the finding that a reduction in UROD activity of at least 70% is required to lead to uroporphyria (in mammals) is an important finding. The authors of the AOP indicate that UROD inhibition is not always observed in avian models of porphyria. This emphasises the use of a battery of tools, rather than individual assays, to test for porphyria.	
Reviewer 3	For agencies concerned with environmental impact including effects on	

	wildlife, this AOP will be quite useful. However, for agencies that deal specifically with human health, the usefulness of this AOP is questionable, given the uncertainties and gaps in our understanding of the molecular mechanisms of AhR activation and uroporphyria in humans. Sinclair et al. conclude that human CYP1A2 can oxidize uroporphyrinogen, but has lower activity compared to murine homologue; in addition, they conclude that most of UROX activity found in human liver microsomes is not due to CYP1A2 (Sinclair et al., 1998). In addition, evidence of either a direct or associative link between TCDD exposure and human porphyria cutanea tarda is weak (Calvert et al., 1994; Fader and Zacharewski, 2017).	
Reviewer 4	I am not sure it is particularly useful for regulatory use for mammals and health particularly as this is a complex scenario going beyond AHR as illustrated. However, the comparisons with other non mammalian species, especially birds, may well be very useful illustrating unwanted environmental exposure to AHR ligands.	
Conclusion: What are your overall conclusions of the assessment of this AOP?		
Reviewer 1	This AOP seems to be a very clear and solid construct based on the appropriate scientific literature. It could be useful to present this AOP in a wider context, i.e. by describing the heme synthesis pathway and porphyrias in the background section or pondering upon some potential interactions within this wider context. Considerations on the appropriate context are certainly difficult and not easily solved, but at least some remarks could be useful for further development of a network of "porphyria AOPs".	A: Will add more information of the main page regarding where UROD lies within the heme biosynthesis pathway.
Reviewer 2	My overall conclusion is that the AOP is quite solid and complete and well-supported with references The authors have provided detailed information on the biological plausibility, empirical support, and quantitative understanding of the KEs and KERs. They also have indicated the pitfalls of the AOP. Some knowledge/data gaps still have to be tackled, before the AOP can be endorsed for regulatory application.	
Reviewer	AOP is nicely laid out conceptually, and the description of each KEs and MIE	A: There are quite a few AHR AOPs available on the

3	are well documented. The proposed pathway relies mostly on evidence from the most susceptible mouse strain (C57BL/6), AhR-/- and Cyp1A2-/-knockout mice, and in vitro assays using either rodent or avian microsome systems, and perhaps is the simplest interpretation of those data. While the simplest AOP, as a building unit, is worthwhile, I believe that the AOP requires proper context to be widely useful. It is widely acknowledged that the toxicological effects of AhR activators display, qualitatively and quantitatively, tremendous inter- and intra-species variations (Reichard et al., 2006). These inter- or intra-species differences may provide important insights into the relationship between AhR activation and uroporphyria. While the authors of the AOP allude to some of the data that are not in-line with the proposed AOP, I believe more extensive discussion of these seemingly inconsistent observations would be helpful to put the proposed AOP in context. The following are some suggestions that are worth discussing as a side-note.	wiki…even this AOP itself started out as a branched network in its early conception. But the AOP concept was later refined to focus on linear pathways, that automatically generate AOP networks…and it is the network that is indeed the functional unit of the AOP. So I completely agree that context and complexity of cross-talk and interactions is important, it is not meant to be captured in a single AOP…but rather by the network that is generated. As a side note, species differences are somewhat confusing for the AO of uroporphyria, but are very well understood in the context of embryonic AHR activation leading to mortality (See AOP 150).
	o The levels of ascorbic acid negatively correlate with CYP1A2 activity. Ascorbic acid was shown to be a competitive inhibitor of CYP1A2's UROX activity in vitro (Sinclair et al., 1993); this inhibitory activity by ascorbic acid was observed in vivo using Osteogenic Disorder Shionogi (ODS) mutant rat, which lacks the ability to synthesize ascorbic acid: in a dose-dependent manner, ascorbic acid prevented uroporphyrin accumulation in ODS mutant rats treated with either 3-methylcholanthrene (MC) and 5-aminolevulinate (ALA) or hexachlorobenzene (Sinclair et al., 1993). In the same study, MC/ALA treatment did not result in uroporphyrin accumulation in Fischer 344 rats; this was ascribed to these rats showing much higher hepatic ascorbic acid levels compared to ODS rats. Interestingly, in both strains treated with MC and ALA, CYP1A2 was induced, suggesting that ascorbic acid modulates CYP1A2 activity rather than its expression. These studies lend credence to the involvement of CYP1A2 activity in uroporphyria; furthermore, some of the observed intra- and inter-species differences on the susceptibility to AhR activators on uroporphyria induction may be explained by differences in the hepatic levels of ascorbic acid in those animals.	A: Thank you for this
	o The requirement of chronic, instead of acute, TCDD exposure in Sprague-Dawley rats requires discussion, especially in light of the fact that a single 75μg/kg dose of TCDD in "susceptible" C57BL/6 mice resulted in accumulation of hepatic porphyrins (Smith et al., 1981).	A: How so? And where would such a discussion be incorporated? I'm not sure what I would say on this issue. It can't be

		explained based on the pharmacokinetics of TCDD, as the half-life is 30 days in rats and 8 days in mice. Any suggestions on the direction of the discussion? B: I am not sure how to address this either. Lets discuss at TC
	o Although CYP1A2 clearly appears to be the main downstream target gene in AhR activation, other AhR-dependent genes may also modulate the magnitude of adverse effects leading to uroporphyria. For example, CYP1A1-/- mice show reduced uroporphyria upon TCDD exposure suggesting that CYP1A1 also contributes to production of porphyrins (Rifkind, 2006; Uno et al., 2004). In fact, Davies et al. (2008) conclude that other genes induced by TCDD, including those involved in glucose and iron metabolism as well as oxidative stress response, may contribute to TCDD-dependent uroporphyria in C57BL mice.	A: True, and I think it's generally understood that an AOP is not independent of other potential factors at play (the initial AOP contained both CYP1A1 and 1A2); but in light of the linear nature of the AOP framework, the focus was placed on CYP1A2 as it has been demonstrated to be the main target. I don't know whether there is sufficient information on the other pathways mentioned (other than iron) to include them as modulators in the AOP.
	o What is unclear from cited studies is how CYP1A2 induction and UROX ultimately lead to UROD inhibition? Is this a direct or indirect effect?	A: This link was tricky to depict within the restrictive Wiki framework. It was initially displayed as a feedback loop in which: •CYP1A2 induction makes it more available and better able to compete with UROD to oxidize uroporphyrinogen. •One (or more?) of the oxidation products is believed to be a competitive inhibitor of UROD • UROD inhibition potentiates the oxidation of uroporphyrinogens by CYP1A2 to porphyrins leading to accumulation I tried my best to describe this relationship, but it's kind of spread over two KER pages (KER 868: Induction, CYP1A2/CYP1A5 leads to Oxidation, Uroporphyrinogen AND KER 865 :Oxidation, Uroporphyrinogen leads to Inhibition, UROD). Any suggestions on making this more

		clear? B: Seems to be a point of contention here… Is there not sufficent evidence that this is via Uroporphomethane? If the evidence is strong enough, could we change the title of KE to oxidation of uroporphrynogen to uroporphomethane? We may have to consider reassessing the WoE of this KER. A: There definitely isn't enough evidence to make that change. There is only one paper that identifies the inhibitor as uroporphomethene, and it has been criticized. So although the identity f the inhibitor is not agreed upon, there is plenty of evidence that shows that a UROD inhibitor is generated.
	o Given that other contributors to etiology of uroporphyria (iron overload and alcohol) are also well-known oxidative stressors, antioxidant capacity within a hepatic cell may be a confounding factor as to why some animals and humans are susceptible or resistant to ArH inducers. General increase in oxidative stress [i.e. lipid peroxidation, reactive oxygen species (ROS) and oxidized glutathione] due to TCDD is not dependent on functional CYP1A2 (Slezak et al., 1999); however, TCDD did elicit AhR-dependent, CYP1A1/A2-independent mitochondrial ROS production in mice suggesting that general oxidative stress induced independently of CYP1A2 induction may contribute to the resulting overall UROX by TCDD (Senft et al., 2002).	B: This citation will be incorporated into KER 868 under "Uncertainties and Inconsistencies" in addition to the existing discussion.
Reviewer 4	This is a good attempt at a very complex scenario which at first sight seems relatively simple. Despite considerable research proven toxic endpoints relevant to significant human adverse outcomes from AHR ligands are few and it is important that this AOP documents and systemizes this both what is known, the uncertainties and the limitations.	
List of references		
Reviewer 3	Calvert, G.M., Sweeney, M.H., Fingerhut, M.A., Hornung, R.W., and Halperin,	A: Your thoroughness is greatly appreciated!

W.E. (1994). Evaluation of porphyria cutanea tarda in U.S. workers exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Am J Ind Med *25*, 559-571.

Davies, R., Clothier, B., Robinson, S.W., Edwards, R.E., Greaves, P., Luo, J., Gant, T.W., Chernova, T., and Smith, A.G. (2008). Essential role of the AH receptor in the dysfunction of heme metabolism induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Chem Res Toxicol *21*, 330-340.

Fader, K.A., and Zacharewski, T.R. (2017). Beyond the Aryl Hydrocarbon Receptor: Pathway Interactions in the Hepatotoxicity of 2,3,7,8-Tetrachlorodibenzo-p-dioxin and Related Compounds. Curr Opin Toxicol *2*, 36-41.

Hakk, H., Diliberto, J.J., and Birnbaum, L.S. (2009). The effect of dose on 2,3,7,8-TCDD tissue distribution, metabolism and elimination in CYP1A2 (-/-) knockout and C57BL/6N parental strains of mice. Toxicol Appl Pharmacol *241*, 119-126.

Nakano, K., Ishizuka, M., Sakamoto, K.Q., and Fujita, S. (2009). Absolute requirement for iron in the development of chemically induced uroporphyria in mice treated with 3-methylcholanthrene and 5-aminolevulinate. Biometals *22*, 345-351.

Phillips, J.D., Kushner, J.P., Bergonia, H.A., and Franklin, M.R. (2011). Uroporphyria in the Cyp1a2-/- mouse. Blood Cells Mol Dis *47*, 249-254.

Reichard, J.F., Dalton, T.P., Shertzer, H.G., and Puga, A. (2006). Induction of oxidative stress responses by dioxin and other ligands of the aryl hydrocarbon receptor. Dose Response *3*, 306-331.

Rifkind, A.B. (2006). CYP1A in TCDD toxicity and in physiology-with particular reference to CYP dependent arachidonic acid metabolism and other endogenous substrates. Drug Metab Rev *38*, 291-335.

Senft, A.P., Dalton, T.P., Nebert, D.W., Genter, M.B., Puga, A., Hutchinson, R.J., Kerzee, J.K., Uno, S., and Shertzer, H.G. (2002). Mitochondrial

reactive oxygen production is dependent on the aromatic hydrocarbon receptor. Free Radic Biol Med *33*, 1268-1278.

Sinclair, P.R., Gorman, N., Tsyrlov, I.B., Fuhr, U., Walton, H.S., and Sinclair, J.F. (1998). Uroporphyrinogen oxidation catalyzed by human cytochromes P450. Drug Metab Dispos *26*, 1019-1025.

Sinclair, P.R., Gorman, N., Walton, H.S., Bement, W.J., Jacobs, J.M., and Sinclair, J.F. (1993). Ascorbic acid inhibition of cytochrome P450-catalyzed uroporphyrin accumulation. Arch Biochem Biophys *304*, 464-470.

Slezak, B.P., Diliberto, J.J., and Birnbaum, L.S. (1999). 2,3,7,8-Tetrachlorodibenzo-p-dioxin-mediated oxidative stress in CYP1A2 knockout (CYP1A2-/-) mice. Biochem Biophys Res Commun *264*, 376-379.

Smith, A.G., Francis, J.E., Kay, S.J., and Greig, J.B. (1981). Hepatic toxicity and uroporphyrinogen decarboxylase activity following a single dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin to mice. Biochem Pharmacol *30*, 2825-2830.

Staskal, D.F., Diliberto, J.J., Devito, M.J., and Birnbaum, L.S. (2005). Inhibition of human and rat CYP1A2 by TCDD and dioxin-like chemicals. Toxicol Sci *84*, 225-231.

Uno, S., Dalton, T.P., Sinclair, P.R., Gorman, N., Wang, B., Smith, A.G., Miller, M.L., Shertzer, H.G., and Nebert, D.W. (2004). Cyp1a1(-/-) male mice: protection against high-dose TCDD-induced lethality and wasting syndrome, and resistance to intrahepatocyte lipid accumulation and uroporphyria. Toxicol Appl Pharmacol *196*, 410-421.