AOP 439 Review - Summary Evaluation

AOP Title: *Activation of the AhR leading to metastatic breast cancer*

Review Team:

Name	***Affiliation***	***Role***
William Bisson	Oregon Health & Science University, US	Reviewer
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Approach and summary:

The review team completed the evaluation of AOP 439 "Activation of the AhR leading to metastatic breast cancer" authored by Benoit et al. considering both the February 3, 2025 AOP Wiki version (https://aopwiki.org/aops/439) as well as the manuscript authored by Benoit et al. (2022) in two phases. During the initial phase, the entire team reviewed the complete AOP and provided high level comments regarding overall structure, biological context and specificity, and alignment with OECD guidelines. Subsequently, two sub-groups formed that evaluated specific elements of the AOP based on their expertise. Sub-group 1 consisting of experts in AhR signaling focused on the MIE and subsequent molecular KEs and associated KERs. Sub-group 2 consisting of experts in mechanisms of (breast) cancer initiation and progression focused on KEs and KERs linking cellular/organ level events to AOs.

Overall, the review team felt that AOP 439 was well-aligned with OECD guidance on developing AOPs, and that this AOP constitutes an important contribution to the AOP Wiki and the AOP Series on Adverse Outcome Pathways. However, several questions were raised concerning the need for greater specificity of the MIE and select KE descriptions to inform the specific AO (metastatic breast cancer), the conditions under which this AOP applies (and under which it does not apply), as well as the reliance solely on *in vitro* methods and studies that could limit its regulatory adoption or relevance without *in vivo* information. Furthermore, there were concerns that the AOP represents an oversimplification of the complex, dynamic, and sometimes conflicting roles of AhR activation in cancer progression. Specifically, the review team felt that it would be important to discuss and provide examples of the specific conditions under which AhR activation leads (or does not lead) to breast cancer given the promiscuity of the receptor (i.e. under which conditions does this AOP apply and under which it does not).

A few technical items were also raised including the concern that this AOP represents a network rather than a linear AOP, some bias that seems to have been introduced by using AI-supported text mining, and the need to expand the literature considered in support of the AOP to include *in vivo* studies.

A detailed discussion of these points can be found below:

Specific Comments:

1. *Overall Framework*

- The conceptual AOP structure is well-aligned with OECD guidelines, but the causal evidence for linking AhR activation to breast cancer-related death remains heterogeneous and sometimes indirect.

    - There is missing context regarding the role of cellular plasticity, cancer subtypes, and the tumor microenvironment (TME)
        - The AOP mostly does not consider the influence of AhR on TME factors, including immune cells (e.g., T- cells), stromal interactions, and metabolic changes, which play a critical role in breast cancer progression.
        - Interaction of the AhR with estrogen receptors (ER), hypoxia, and metabolic pathways influence downstream outcomes, but the involvement of other receptors is not uniform for all AhR activation. Some of this is briefly mentioned but not sufficiently integrated into the AOP.
        - Differences in breast cancer subtypes (e.g., ER-positive, PR-positive or negative, Her2-positive, triple negative) and their responses to AhR activation are not acknowledged and considered. The ER was listed in keyword search, but it is not discussed elsewhere in the AOP. Much of the evidence the authors cite throughout is for ER+ *or* TNBC cell lines, but concordant evidence for both is not consistently provided, and Her2 tumors are not discussed.
- Key events (KEs) and their weight of evidence is moderate
    - Several KERs (key event relationships) are supported only by correlative rather than causative data.
    - Inflammation, apoptosis resistance, and angiogenesis are important in cancer progression, but their link to AhR activation per se is not rigorously demonstrated.
    - The evidence strength for some KEs (e.g., endothelial migration, tumor invasion) is rated high based on only a few selected studies, which makes the conclusions less robust.
- The AOP presents AhR activation as a molecular initiating event of breast cancer progression to be metastatic, while in the literature its role in cancer promotion vs suppression is very conflicting and depends on specific conditions.
    - Pro-tumorigenic effects (e.g., through inflammation, angiogenesis).
    - Anti-tumorigenic effects (e.g., involvement in differentiation and tumor suppression under certain conditions).
    - While the AOP development does not require a comprehensive view of relevant evidence, it will benefit the readers greatly to include the specific conditions or scenarios when this AOP does apply. Otherwise, leaving out information on when this AOP does not apply (e.g., Benoit methods) might be seen as intentionally leaving out conflicting evidence.
    - The authors describe conflicting evidence in some places but do not sufficiently address how this affects the overall AOP interpretation. E.g., second to last paragraph of AOP description on page 18 of pdf: “In human breast cancer, the **AhR is thought to be responsible of its progression** (Goode, Kanno, Optiz, Novikov, Hall, Subramaniam, Barhoover). In human mammary benign cells, Brooks et al. noted that a high level of AhR was associated with a modified cell cycle (with a 50% **increase in population doubling time** in cells expressing the AhR by more than 3-fold) and EMT including increased cell migration. Narasimnhan et al. found that **suppression of the AhR pathway had a pro-tumorigenic effect *in vitro* (EMT, tumor migration) in triple negative breast cancer**.
    - Excluding events with contradictory evidence raises concerns about literature selection bias (see AI comments below).

- *Recommendation: AOP should specify conditions under which it applies, and discuss conditions where it does not*

- There are concerns that the AOP represents a network rather than linear AOP. Do all the upstream KEs listed in parallel have to occur together to trigger the downstream KE? If not, then this AOP represents a network. Also, there seems to be some conflation between tumor growth and invasion, which are two separate processes.
- Consider moderating the discussion of implications for regulatory use based on actual confidence and uncertainties associated with the AOP.
    - While the paper suggests strong confidence in the AOP, it lacks **confirmation from experimental results** (e.g., knockdown studies in relevant models, dose-response studies).
    - The authors acknowledge that an *in vitro* validation is needed, but until then, the AOP remains a theoretical construct rather than a validated regulatory tool. Also, *in vitro* confirmation alone is not sufficient at this point. Authors should point out that *in vivo* confirmation or strong evidence that *in vitro* is informative of *in vivo* outcomes are also required for an AOP to be used as a base of regulation standard setting
    - The OECD AOP framework requires strong evidence for each KE and KER, yet some key transitions (e.g., from immune surveillance to evasion, increased invasion to metastasis) are weakly supported. Additional evidence supporting these key event relationships is needed.
- There are some concerns regarding the predominant focus on *in vitro* methods listed for measuring KEs/AO. *In vivo* assays are critical for interpretation of *in vitro* results. Similarly, most of the discussions and supporting sections refer to the *in vitro* literature, and these descriptions should be expanded by including relevant *in vivo* literature.
- The reliance on AI-driven text mining (AOP-helpFinder, PubTator) is interesting but raises concerns about data selection bias and the depth of biological validation.
    - The use of AOP-helpFinder suggests a large dataset was analyzed, but manual curation reduced the number of relevant studies to 113, which is a relatively small number given the complexity of breast cancer pathways. As per our next comment we recommend that the authors should consider a broader range of studies to avoid any potential bias.
    - The exclusion of contradictory evidence (e.g., AhR's role in differentiation and apoptosis) suggests a bias toward pro-tumorigenic findings. This is nature in the development process. Given the many and diverse downstream effects of AhR activation, it becomes critical to provide information on under which conditions this AOP applies in the final product. The paper does not discuss false positives or potential errors introduced by AI-driven keyword-based searches, which could lead to misinterpretation of data. Could the authors include references on the performance of these tools and include additional analysis and discussion of these concerns?

2. *MIE*

- Ligand-specific effects are not sufficiently explored: The AOP assumes all AhR activators will lead to the same outcome, which is a major oversimplification.
    - Ligand-specific effects of AhR activation: TCDD (potent AhR agonist) is emphasized, but AhR also binds to endogenous and dietary ligands, which can trigger distinct signaling pathways. Also, what evidence is there for prototypic AhR ligands such as TCDD listed in this AOP to actually trigger the AO? PAHs are different from dioxins etc., but AOPs are supposed to be agent-agnostic.

The AhR has a protective role supporting compensation/maintenance of homeostasis as well. In order to trigger an adverse effect (the AO of metastatic breast cancer in this case) these compensatory responses need to be overwhelmed. Again, a more thorough discussion of the complex dynamics involving the AhR in these processes should be discussed/considered.

3. *KE-specific comments*

- KE 1982 Metastatic breast cancer
    - Please add *in vivo* assays and citations
- KE 1196 Increased, invasion
    - Please add *in vitro* assays and citations for both *in vivo* and *in vitro* assays
- KE 1971 Increased, tumor growth:
    - If the emphasis is on increased growth rate, consider "increased, tumor growth rate" as the title of this KE. This would make sense for KER, increased angiogenesis, to be from this KE. For increased growth rate, all description and assays need to be updated accordingly.
    - If this KE is more about "Increased, tumor size (volume)", consider naming it so. It is true that larger tumor at the time of diagnosis is a risk factor for metastasis, but the connection to metastatic breast cancer via KER 3137 (angiogenesis) seems to be skipping over a step.
    - Additionally, current text for assays is about tumor size with no reference to time points. Please add a general description of comparing results from different time points.
    - Bi-directional interaction: KE1213 and KE1971 influence each other, and there is no fixed order for them to occur.
- KER 3137 is listed for both increased tumor growth -> metastatic BC and for angiogenesis --> metastatic BC in AOP diagram and WoE summary. In the AOP-wiki, 3137 is angiogenesis KER and tumor growth should be KER 3139.