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## Review article

# Adverse outcome pathway from activation of the AhR to breast cancer-related death

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## ABSTRACT

Adverse outcome pathways (AOPs) are formalized and structured linear concepts that connect one molecular initiating event (MIE) to an adverse outcome (AO) via different key events (KE) through key event relationships (KER). They are mainly used in eco-toxicology toxicology, and regulatory health issues. AOPs must respond to specific guidelines from the Organization for Economic Co-operation and Development (OECD) to weight the evidence between each KE.

Breast cancer is the deadliest cancer in women with a poor prognosis in case of metastatic breast cancer. The role of the environments in the formation of metastasis has been suggested. We hypothesized that activation of the AhR (MIE), a xenobiotic receptor, could lead to breast cancer related death (AO), through different KEs, constituting a new AOP.

An artificial intelligence tool (AOP-helper), which screens the available literature, was used to collect all existing scientific abstracts to build a novel AOP, using a list of key words. Four hundred and seven abstracts were found containing at least a word from our MIE list and either one word from our AO or KE list. A manual curation retained 113 pertinent articles, which were also screened using PubTator. From these analyses, an AOP was created linking the activation of the AhR to breast cancer related death through decreased apoptosis, inflammation, endothelial cell migration, angiogenesis, and invasion. These KEs promote an increased tumor growth, angiogenesis and migration which leads to breast cancer metastasis and breast cancer related death.

The evidence of the proposed AOP was weighted using the tailored Bradford Hill criteria and the OECD guidelines. The confidence in our AOP was considered strong. An *in vitro* validation must be carried out, but our review proposes a strong relationship between AhR activation and breast cancer-related death with an innovative use of an artificial intelligence literature search.

## 1. Introduction

Breast cancer is a frequent disease, responsible of 2 262 419 new cases and 684 996 deaths in 2020 in the world, making it the deadliest female cancer (Bray et al., 2018). In 70% of cases, the disease is localized, and the prognosis is favorable with a 5-year survival of 99%. However, once the disease spreads (lymph nodes, metastasis), survival is

severely altered with a 5-year survival rate of 26% in case of metastasis (Henley et al., 2020). It is therefore of paramount importance to understand the mechanisms of metastasis in breast cancer.

Amongst risk factors clearly established, including obesity, genetic mutations and hormonal exposure, the importance of the role of the environment is currently emerging (Koual et al., 2020 Nov 17). In an epidemiologic study, we found a positive association between the

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concentrations of 2,3,7,8-TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxine) in the adipose tissue surrounding the tumors, and breast cancer metastasis in overweight and obese patients (Koual et al., 2019). Moreover, we have shown that, using both *in vivo* and *in vitro* models, TCDD exposure could promote an aggressive phenotype to breast cancer cells, thus favoring the formation of metastatic cells (Koual et al., 2021). TCDD is a potent ligand of the aryl hydrocarbon receptor (AhR), a transcriptional factor involved notably in the metabolism of xenobiotics (Larigot et al., 2022). Hence, the impact of the environment on breast cancer aggressiveness could be mediated by the activation of the AhR.

Interest is growing on the role of the AhR in breast cancer. First, the AhR is often overexpressed in different breast cancer cell lines (Zudaire et al., 2008; Kim et al., 2000 Nov 16; Li et al., 2014). Interestingly, the level of expression can be correlated to the stage or the molecular subtype of the disease (Zudaire et al., 2008; Zhao et al., 2013). Second, the AhR pathway has been associated with different pro-metastatic features in breast cancer, such as resistance to apoptosis, invasiveness, modified cell cycle, migration and proliferation (Zudaire et al., 2008; Goode et al., 2013 Dec 15; Kanno et al., 2006). Triple negative cell lines, breast cancer cell lines with the worse prognosis (not over-expressing Her2 receptor or hormonal receptors), over-expressing the AhR seem to develop stem-like characteristics, favoring epithelial-mesenchymal transition (EMT) and thus metastasis (Stanford et al., 2016). Thirdly, the AhR could be involved in the resistance of breast cancer to treatments (Goode et al., 2013 Dec 15; Goode et al., 2014): after AhR knockout, Goode et al. found enhanced sensitivity of paclitaxel (a drug targeting cancer cells) in triple negative breast cancer, a cancer particularly difficult to treat (Goode et al., 2014). Breast cancer patients expressing estrogen receptors (ER-positive) in their cancer cells, can benefit from an efficient endocrine therapy, which greatly improves their survival. Activation of the AhR can lead to the loss of expression of the ER alpha and therefore to the loss of a potential therapeutic target (Safe et al., 2000 Jul).

The mechanisms linking the activation of the AhR to breast cancer aggressiveness are still unclear. Adverse outcome pathways (AOP) are formalized and structured linear concepts that connect one molecular initiating event (MIE) to an adverse outcome (AO) via different key events (KE) through key event relationships (KER) (Leist et al., 2017). More than 400 AOPs have currently been proposed, although only a small fraction (Carvaillo et al., 2019) as of the April 2022) have fully cleared the OECDs review process (AOP, 2021). Their aim is to support the assessment of health risks by describing evidence that links KEs to an apical AO. They can then be used in human toxicology, ecotoxicology, and to support the development of Integrated Approach to Testing and Assessment (IATA) that can be used for regulatory purposes. AOPs are stressors-agnostic. Building an AOP is complex and must ideally follow the OECD handbook guidelines with several quality assessments (OECD, 2018). Based on the AOP-wiki database (<https://aopwiki.org/>, last accessed March 2022), the central repository for AOPs, the AhR has already been proposed in several AOPs, but never in one characterized by the AO breast cancer related to death. Likewise, an AOP linking an MIE to breast cancer aggressiveness has never been proposed.

From our expertise and available knowledge, we hypothesize that the activation of the AhR could be a MIE leading to breast cancer related death (AO) through different KEs and KERs.

## 2. Material and methods

### 2.1. Data input, automatic identification, and extraction of the available knowledge

An artificial intelligence based method, named AOP-helpFinder (<https://aop-helpfinder.u-paris-sciences.fr/index.php>), was used to scope the available relevant literature (Carvaillo et al., 2019; Zgheib et al., 2021; Rugard et al., 2020 Jan 1; Jornod et al., 2020; Jornod et al., 2022). This tool screens the contents of abstracts from the PubMed database

using lists of key words, including stressors, MIEs, KEs and AOs. It then automatically finds, and extracts links co-mentioned in abstracts between pairs of events (MIE/KE/AO) and ranks them using graph theory. In the proposed study, the key words included MIEs (AhR ...), KEs (migration, hypoxia, colony...) and an AO (breast cancer, mammary tumor...). All biological events used for the screening were defined using AOP-wiki database and personal keywords. We first scanned the list of KE and KER proposed by AOP wiki and retained words and concepts which concerned cancer aggressivity. Personal keywords were added if necessary (ex: EMT). We reviewed the list of words as a team in order to be as extensive as possible. The key words used can be found in Supplementary Table S1.

The obtained results (i.e., abstracts co-mentioning KE and AO) were manually curated to refine them, and keep the most significant knowledge. First only the abstracts were read and if the article was kept, the full text was reviewed. A manual curation decision tree was created to only include articles that concerned: i) the AhR, ii) breast cancer, iii) cancer progression and not initiation and iv) reviews were excluded (details in results §1 AOP-helpFinder text mining tool).

The newly established version of the AOP-helpFinder tool was applied on the more than 30 million available abstracts from PubMed database, which allowed us to use the two proposed options: i) the refinement filter based on machine learning method (lemmatization process of text) combined with deletion of words of context in order to improve the accuracy of the outputs, and ii) the reduced search option which allow to screen only a part of the abstracts (Jornod et al., 2022). This allows to not screen the introductory part of an abstract which usually refer to a study hypothesis, with the aim to decrease false positives.

In order to cross-check the selected concepts, and to support the evidence in favor of the proposed AOP, all selected articles (after text mining and manual curation) were run through the PubTator Central tool (<https://www.ncbi.nlm.nih.gov/research/pubtator/>). PubTator is a web-based system that screens PubMed abstracts and PMC full-text articles for biomedical concepts (genes, diseases, mutations, species, cell lines) (Wei et al., 2013; Wei et al., 2019 Jul 2). The PMIDs of the selected articles were used, and PubTator scanned their abstracts. The system overview is detailed by Wei et al. here (Wei et al., 2019 Jul 2). The retrieved chemical, disease, species, cell line and gene concepts were used to support the developed AOP.

### 2.2. AOP – Knowledge integration and weight of evidence assessment

The AOP was then developed using all the previously extracted data, using the OECD handbook (OECD, 2018). The graphical linear flow diagram was used to represent our proposed AOP.

The evidence supporting the AOP was organized and evaluated according to the tailored Bradford-Hill criteria, as described by OECD guidance (OECD, 2018; Hill, 1965). Briefly, these criteria are a group of minimal conditions for providing adequate evidence of a causal relationship between 2 events.

First, the OECD guidelines were used to evaluate the weight of evidence (WoE) between each KE. The biological applicability domain of each KE was assessed for sex, life stage and taxa. Due to the heterogeneity of breast cancer, another category was added, namely whether the KE is present only in certain types of cancer (ER-positive, triple negative, Her2 overexpression). The types of assays used to measure the KE were also recorded. Then, the confidence in the essentiality of each KE was evaluated using the system proposed by the OECD (high, moderate, low) and detailed in the guidebook.

Second, the tailored Bradford-Hill criteria modified to fit the AOP evidence assessment were used as proposed previously to evaluate each KER (Vinken et al., 2013; Becker et al., 2015): i) the concordance of dose-response relationships (ex: is there more migration with higher dosage AhR activators?), ii) the temporal concordance among the KER and AO (ex: is there cell migration before activation of the AhR?), iii)

strength, consistency, and specificity of association (ex: use of specific AhR antagonists, silencing or knock out), iv) biological plausibility, coherence, and consistency of the experimental evidence (ex: number of studies finding the association, concordance amongst cell lines), and v) alternative mechanisms that logically present themselves and the extent to which they may distract from the postulated AOP (ex: use of different stressors).

Third, the OECD guidelines were used to evaluate the KERs: i) biological plausibility (mechanistic approach), ii) empirical support (dependent change and the experimental data present), iii) evidence and finally iv) quantitative evidence (closely linked to the tailored Bradford-Hill criteria). These were evaluated using the system proposed by the OECD (high, moderate, low) and detailed in the guidebook.

Finally, the AOP was overall assessed using the three primary considerations: the biological plausibility, the essentiality of each KE and the empirical support of KERs. Since the detail of the evaluation of the overall weight assessment of the AOP is not described in the OECD guidebook, this evaluation was subjective and decided by our team using all the different elements studied before.

### 3. Results

#### 3.1. AOP-helpFinder text mining tool

A total of 17,760 articles related to the MIE, AhR or aryl hydrocarbon receptor, were identified in the PubMed database (as of December 2021). This list of AhR-related abstracts was used to screen the ones concerning the putative AOP. All abstracts had to mention at least one event from the AO list (breast cancer or synonym) and one event from the KE list (KEs leading to metastasis: invasion, stemness...). The list of key words used for searching AOs and KEs can be found in Supplementary Table 1. After merging the identified abstract using their PubMed identifications (PMID), only 407 abstracts were kept from the AhR list with both a key event from our AO list and our KE list (Fig. 1).

Four steps were used for the manual curation, that was performed by

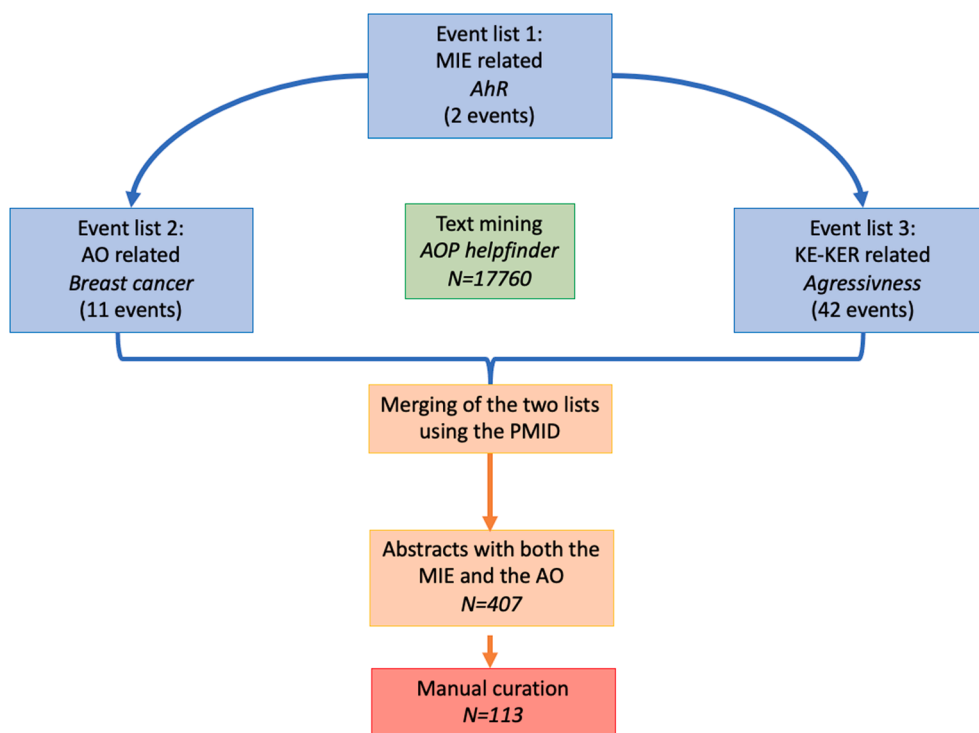
several experts. First, all the abstracts were screened to check if it indeed concerned the AhR. One hundred and thirty-four abstracts were excluded mainly because the retrieved “ahr” do not concern the aryl hydrocarbon receptor but the adjusted hazards ratio. Moreover, “ahr” sometimes referred to the last name of an author (Ex: Andre Ahr). Then, only abstracts concerning breast cancer were included. Eighteen abstracts were excluded because they did not directly concern breast cancer. One hundred and nine articles were not selected because they concerned breast cancer initiation and not aggressiveness / metastasis. Finally, 33 articles were reviews and not original works. In total, 113 articles were considered to develop our AOP (Fig. 2).

#### 3.2. AOP-development

The 113 articles were then classified by category in the metastatic process (migration, invasion...). Each category represented a hallmark of cancer and was associated with the corresponding key words that were used to explore Pubmed using AOP-helpFinder. The number of abstracts for each event and category is detailed in Table 1.

“Proliferation” was the most frequently found category (83 articles) followed by “estrogen” (Degner et al., 2009 Jan); “in vivo” articles (Hsieh et al., 2012 Feb); migration (Li et al., 2012) and invasion (Jornod et al., 2022). Even though some categories were found frequently, the evidence was contradictory between articles (ex: activation of the AhR-increase or decrease of proliferation) and were therefore not selected. Articles with contradicting evidence were read and analyzed but the KE concerned were not selected in our AOP to keep it as simple as possible using only causal relationship.

The 113 articles were also run through the PubTator Central tool to screen for biomedical concepts. The most frequently found disease was breast cancer, followed by toxicity and concepts concerning metastasis and aggressivity. TCDD (or dioxin, or 2,3,7,8-tetrachlorodibenzo-p-dioxin) was by far the most used chemical followed by estrogen, indole-3-carbinol, alpha-naphthoflavone, polycyclic aromatic hydrocarbons and hexachlorobenzene, mainly ligands of the AhR. Most



**Fig. 1.** Workflow of the text mining procedure to identify all abstracts related to the events searched for implementation of our AOP. First, the AOP-helpFinder tool screens the available literature in the Pubmed database using the key word “AhR” (our MIE). It then selects the abstracts containing both an AO (breast cancer) and a KE (aggressiveness). The two obtained 2 lists are merged, and a manual curation is carried out.

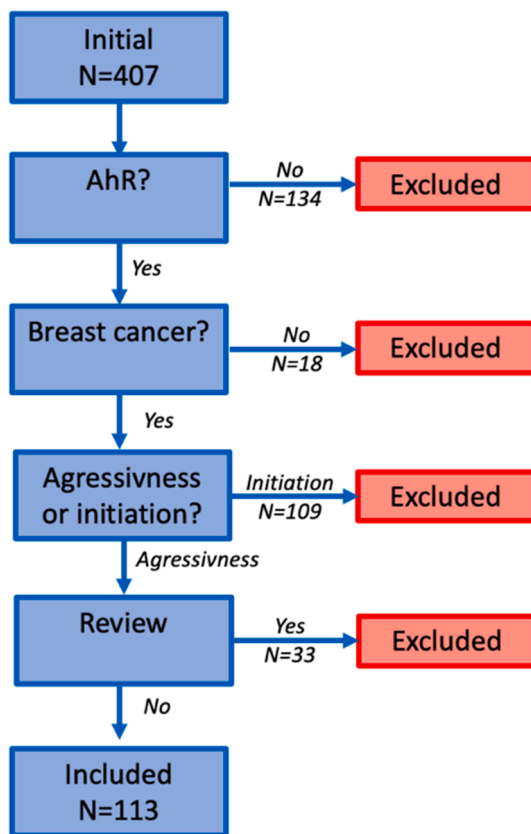


Fig. 2. Manual curation decision tree of the articles selected by AOP-helpFinder.

Table 1

Key word list and number of studies concerning each category of key word included after manual curation.

Category	Key words	Number of articles	
Migration	Migrate, migration	25	37
	Colony formation	7	
	Spheroids	3	
	Anchorage-independent growth	2	
Invasion	Invasive	23	23
	Invasion		
Inflammatory	Inflammation, inflammatory	11	11
Proliferation	Proliferation	44	83
	Cell death / apoptosis	11	
	Cell cycle	8	
	Viability	7	
	Cell growth	13	
Stemness	Stemness, stem cells	8	16
	Cancer stem cells	5	
	Aldehyde dehydrogenase	3	
Epithelial mesenchymal transition	EMT	4	4
Angiogenesis	Angiogenesis, neo angiogenesis	4	4
Estrogen	ER, estrogen, estrogen receptor	61	61
Oxidative stress	reactive oxygen specie	5	5
Resistance to chemotherapy	Resistance to chemotherapy	7	7
<i>In vivo</i>	Tumor growth	13	55
	Survival	16	
	Metastasis	21	
	Mortality	2	
	Necrosis	3	

identified studies concerned humans or mice, except one on zebrafish. The ER-positive cell line MCF-7 was the most frequently studied, followed by the aggressive MDA-MB-231 (triple negative) cell line. Without surprise, the AhR was the “gene concept” the most assessed followed by the estrogen receptor, CYP1A1 and CYP1B1. All results are displayed in [Supplementary data S2](#). These results guided the concepts used in our AOP and the biological applicability domain.

After a careful read of the selected articles by all experts and based on their expertise, an adverse outcome network (AON) using 6 different linear AOPs was proposed and is presented in [Fig. 3](#).

### 3.3. AOP: KE and KER justification

Tables summarizing the confidence in each KE and KER can be found [Table 2](#) and [3](#), respectively.

#### 3.3.1. KER 1 and KER 2: Activation of the AhR leads to decreased apoptosis which promotes tumor growth

Several studies have found that the activation of the AhR by stressors such as TCDD, can promote a decrease in apoptosis (KER1), which is a deleterious event with regards to cancer ([Al-Dhfyhan et al., 2017 Jan 19](#); [Bekki et al., 2015](#)). Additionally, an increase in cell death was found when blocking the AhR pathway using AhR silencing (RNA interference or knock-out), knockout cell lines or antagonists (CH223191 or alpha-naphthoflavone) ([Goode et al., 2013 Dec 15](#); [Al-Dhfyhan et al., 2017 Jan 19](#); [Bekki et al., 2015](#); [Regan Anderson et al., 2018](#)). The most frequently used assay to evaluate apoptosis was cytometry with the use of Annexin V: this was performed with ER-positive cells lines (MCF-7, T-47D), triple negative cell lines (MDA-MB-231, HS 578), cells over-expressing the Her2 (SK-BR-3) and cells lines derived from cancer samples from patients ([Goode et al., 2013 Dec 15](#); [Al-Dhfyhan et al., 2017 Jan 19](#); [Bekki et al., 2015](#); [Regan Anderson et al., 2018](#); [Fujisawa et al., 2011](#)).

For KER 2, *in vivo*, Goode et al. showed that the knockout of the AhR in mice reduced tumor growth through an increase of cell apoptosis ([Goode et al., 2013 Dec 15](#)).

The concordance of the evidence was classified as “moderate” since the aim of most studies was to evaluate the capacity to survive in an apoptosis-promoting environment (i.e., chemotherapeutic drugs). Indeed, they assessed the resistance to chemotherapy agents such as doxorubicin and paclitaxel and found that the concomitant inactivation of the AhR pathway could decrease the resistance to these chemotherapy agents through an increase in cell death when compared to cells with a functional (or expressed at sufficient levels) AhR ([Goode et al., 2013 Dec 15](#); [Al-Dhfyhan et al., 2017 Jan 19](#); [Bekki et al., 2015](#); [Regan Anderson et al., 2018](#); [Fujisawa et al., 2011](#)). Since the environment was modified by the presence of chemotherapy, the hypothesis of an alternative pathway cannot be completely discarded. It must be noticed that the exact biological mechanisms linking the activation of the AhR to the decrease in apoptosis remains unclear. Indeed, Anderson *et al.* suggested that the AhR interacts with the glucocorticoid receptor (GR) and the hypoxia inducible factor-2 $\alpha$  (HIF-2 $\alpha$ ) ([Regan Anderson et al., 2018](#)). The presence of the GR is associated with a poor prognosis, notably in triple negative breast cancer ([Pan et al., 2011](#); [Moran et al., 2000 Feb 15](#)). Indeed, this receptor is involved in survival and resistance to chemotherapy through up-regulation of c-myc, Bcl2 and Kruppel-like factor 5 ([Pan et al., 2011](#); [Wu et al., 2004](#); [Li et al., 2017](#)). Both GR and HIF 2 $\alpha$  could be up regulated by the AhR. They then activate Brk (also known as PTK6), a ligand of EGFR (epidermal growth factor receptor), involved in the inhibition of apoptosis ([Regan Anderson et al., 2018](#); [Li et al., 2012](#)). Another possible mechanism suggested by Bekki et al. is that the decrease in apoptosis was caused by the induction of cyclooxygenase 2 (COX-2) and the NF- $\kappa$ B subunit RelB ([Bekki et al., 2015](#)). They both prevent apoptosis through induction of Bcl2, an anti-apoptotic factor ([Tsujii and DuBois, 1995](#); [Vogel et al., 2007](#); [Thomas et al., 2020](#); [Baud and Jacque, 2008 Dec](#); [Demiccio et al., 2005 Nov](#); [Wang et al., 2007 Apr](#);



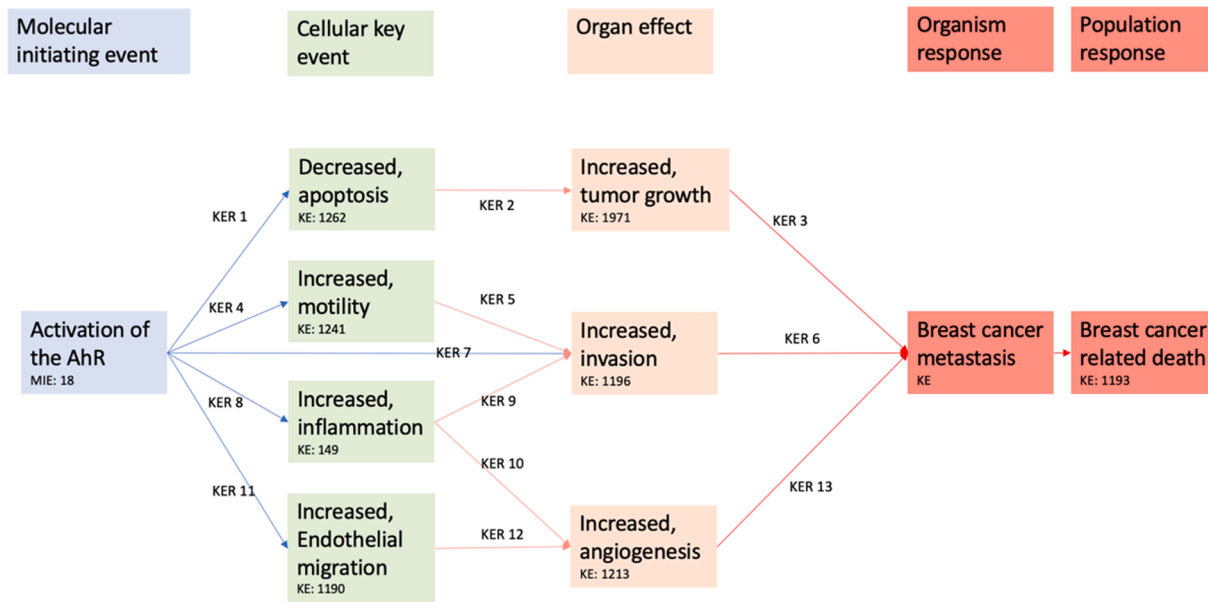


Fig. 3. Adverse outcome pathway presenting the relationship between the activation of the AhR and breast cancer related death through different key events (KE) and key event relationships (KER).

Table 2

Table of key events (KE): type, biological domain, measurement, and weight of evidence according to the OECD guidelines.

Key event	Key event in AOP wiki	Found in AOP	Domain of applicability				Measurement	Confidence in the supporting data for essentiality of KEs within the AOP is considered
			Sex	Life stage	Taxa	Type of breast cancer		
KE1: Decreased, apoptosis	KE 1262	205, 212, 419, 207, 285	Female and male	Any	All species	ER+ ER-	Annexin V	Moderate
KE2: Increased, motility	KE 1241	200	Female and male	Any	All species	ER+ ER-	Scratch wound, Boyden chamber, organoid branching	High
KE3: Increased, inflammation	KE 149	115, 206, 27, 280	Female and male	Any	All species	ER+ ER-	Cytokines	Moderate
KE4: Increased, Endothelial migration	KE 1190	200	Female and male	Any	Vertebrae	ER+ ER-	In vivo, Matrigel	Moderate
KE5: Tumor growth	KE 1971		Female and male	Any	Vertebrae	ER+ ER-	In vivo, colony	High
KE6: Increased, invasion	KE 1196	200	Female and male	Any	Vertebrae	ER+ ER-	Matrigel, MMP	High
KE7: Increased, angiogenesis	KE 1213	200	Female and male	Any	Vertebrae	ER+ ER-	In vivo	Moderate

Legend:

For the confidence in the essentiality of each key event:

- **High** if there is direct evidence from specifically designed experimental studies illustrating prevention or corresponding impact on downstream KEs and/or the AO if upstream KEs are blocked or modified (e.g., via stop exposure/reversibility studies, antagonism, knock out models, etc.).
  - **Moderate** if there is indirect evidence that modification of one or more upstream KEs is associated with a corresponding (increase or decrease) in the magnitude or frequency of downstream KEs [e.g., augmentation of proliferative response (KE upstream) leading to increase in tumor formation (KE downstream or AO)].
  - **Low**: No or contradictory experimental evidence of the essentiality of any of the KEs.
- ER+: hormonal receptor positive breast cancer, ER- hormonal receptor negative breast cancer.

Liu et al., 2001 May 25).

The relationship between decreased apoptosis and increase in tumor growth (KER 2) is not detailed here due to extensive evidence in the scientific literature (Hanahan and Weinberg, 2011 Mar 4).

Bibliographic details concerning KERs are presented in Supplementary Table S3.

3.3.2. KER 4: Activation of the AhR leads to an increased cell motility

The activation of the AhR can modulate cell motility in different

types of breast cancers such as: ER-positive cells lines (MCF-7, T-47D, ZR-75-1), triple negative (MDA-MB-231, MDA-MB-435, HS-578-T, SUM149), and cells overexpressing the Her2 (SK-BR-3) (Goode et al., 2013 Dec 15; Regan Anderson et al., 2018; Parks et al., 2014 Nov; Pontillo et al., 2011 Apr; Qin et al., 2011 Oct 20; Nguyen et al., 2016 Nov 15; Novikov et al., 2016 Nov; Miret et al., 2016 Jul; Shan et al., 2020 Nov; Dwyer et al., 2021 Feb; Narasimhan et al., 2018 May 7; Hsieh et al., 2012 Feb). Activation of the AhR with TCDD, butyl-benzyl phthalate, di-n-butyl phthalate, hexachlorobenzene, and benzo[a]pyrene can

**Table 3**

Table of key events relations (KER): name and weight of evidence according to the OECD guidelines and Bradford Hill modified criteria.

KER n°	KER	Biological plausibility of KERs  Is there a mechanistic (i.e., structural, or functional) relationship between KE up and KE down consistent with established biological knowledge?	Modified Bradford Hill criteria to AOP					Empirical Support for Each KER	Evidence	Quantitative understanding
			Concordance of dose-response relationships	Temporal concordance among the key events and adverse outcome	Strength, consistency, and specificity of association	Biological plausibility, coherence, and consistency of the experimental evidence	Alternative mechanisms that logically present themselves and the extent to which they may distract from the postulated AOP.			
1	Activation of the AhR leads to Decreased apoptosis	Moderate	Moderate	High	High	High	Moderate	Moderate	Moderate	Moderate
2	Decreased apoptosis leads to Tumor growth	High	High	High	High	High	Moderate	High	High	High
3	Tumor growth leads to Breast cancer related death	High	High	High	High	High	Moderate	High	High	High
4	Activation of the AhR leads to Increased motility	High	Moderate	High	High	High	Moderate	High	High	High
5	Increased motility leads to Increased invasion	High	High	High	High	High	Moderate	High	High	High
6	Increased invasion leads to Breast cancer related death	High	High	High	High	High	Moderate	High	High	High
7	Activation of the AhR leads to Increased invasion	High	Moderate	High	High	High	Moderate	High	High	High
8	Activation of the AhR leads to Increased inflammation	Moderate	Moderate	High	High	High	Moderate	Moderate	Moderate	Moderate
9	Increased inflammation leads to Increased invasion	High	High	High	High	High	Moderate	High	High	High
10	Increased inflammation leads to Increased angiogenesis	High	High	High	High	Moderate	Moderate	Moderate	Moderate	Moderate
11	Activation of the AhR leads to Increased endothelial proliferation	Moderate	Moderate	High	High	Moderate	Moderate	Moderate	Moderate	Moderate
12	Increased endothelial migration leads to increased angiogenesis	High	High	High	High	High	Moderate	High	High	High
13	Increased angiogenesis leads to Breast cancer related death	High	High	High	High	High	Moderate	High	High	High

**Legend:**

For the Biological plausibility of KERs.

- **High:** Extensive understanding based on extensive previous documentation and broad acceptance -Established mechanistic basis.
  - **Moderate:** The KER is plausible based on analogy to accepted biological relationships, but scientific understanding is not completely established.
  - **Low:** There is empirical support for a statistical association between KEs (See 3.), but the structural or functional relationship between them is not understood.
- For the Empirical Support for Each KER.
- **High:** if there is dependent change in both events following exposure to a wide range of specific stressors (extensive evidence for temporal, dose–response, and incidence concordance) and no or few data gaps or conflicting data.
  - **Moderate:** if there is demonstrated dependent change in both events following exposure to a small number of specific stressors and some evidence inconsistent with the expected pattern that can be explained by factors such as experimental design, technical considerations, differences among laboratories, etc.
  - **Low:** if there are limited or no studies reporting dependent change in both events following exposure to a specific stressor (i.e., endpoints never measured in the same study or not at all), and/or lacking evidence of temporal or dose–response concordance, or identification of significant inconsistencies in empirical support across taxa and species that don't align with the expected pattern for the hypothesized AOP.

promote cell migration in different assays (Parks et al., 2014 Nov; Pontillo et al., 2011 Apr; Qin et al., 2011 Oct 20; Novikov et al., 2016 Nov; Miret et al., 2016 Jul; Shan et al., 2020 Nov; Narasimhan et al., 2018 May 7; Hsieh et al., 2012 Feb). On the other hand, the use of AhR antagonists, AhR silencing or AhR knockout reversed this effect (Goode et al., 2013 Dec 15; Regan Anderson et al., 2018; Parks et al., 2014 Nov; Pontillo et al., 2011 Apr; Qin et al., 2011 Oct 20; Novikov et al., 2016 Nov; Shan et al., 2020 Nov; Narasimhan et al., 2018 May 7; Hsieh et al., 2012 Feb). The most frequently used assays for evaluating cell migration were the scratch wound assay and the transwell chamber assay. Only three works evaluated the dose–response concordance of AhR activation with stressors and cell migration (Pontillo et al., 2011 Apr; Miret et al., 2016 Jul; Shan et al., 2020 Nov). The evidence was therefore classified as “moderate”.

Bibliographic details concerning KERs are presented in Supplementary Table S3.

### 3.3.3. KER 4 and 5 (existing KER 1306): Activation of the AhR leads to an increased cell motility which promotes organ invasion

The relation between cell migration and organ invasion has already been shown (KER-1306, <https://aopwiki.org/relationships/1306>). Since the 2 are closely linked, most articles studied both cell migration (chemo-tactic) and the capacity to invade the extra-cellular matrix. Cell invasion is indeed defined as the capacity of a cell to migrate and degrade/invade the extracellular matrix. *In vitro*, this process was evaluated mostly using transwell chamber with Matrigel® and the presence of matrix metalloproteinases (MMP). This effect was found in ER-positive cells, triple negative cell lines and cells overexpressing the Her2.

Due to the extensive robust and concordant literature of the link between activation of the AhR-increased cell motility-increased invasion-breast cancer progression, the confidence in these key events was rated as high. However, due to the use of ligands to activate the AhR, it cannot be completely ruled out that alternative pathways (independent of the AhR) can also contribute to these features. For instance, 2 main pathways seem to explain this increase in migration and invasion: the c-Src/HER1/STAT5b, and ERK1/2 pathways. Yet, these pathways seem only to explain the relation between the AhR activation and cell migration / invasion, when the ligand used is hexachlorobenzene, an organochlorinated pesticide (Pontillo et al., 2011 Apr; Miret et al., 2016 Jul; Pontillo et al., 2013 May 1). Even though alternative mechanisms may present themselves, all studies blocked the AhR pathway and found a decrease in cell migration/invasion. The evidence for alternative mechanisms was therefore classified as “moderate” and the biological plausibility of KER was also classified as “moderate”.

Bibliographic details concerning KERs are presented in Supplementary Table S3.

### 3.3.4. KER 7: Activation of the AhR leads to an increased invasion

The activation of the AhR through the use of different ligands (benzophenone, butyl benzyl phthalate, di-n-butyl phthalate, hexachlorobenzene, chlorpyrifos, TCDD) or the blockage of the AhR (silencing, KO or antagonism) increased or decreased cell invasion, respectively (Parks et al., 2014 Nov; Qin et al., 2011 Oct 20; Nguyen

et al., 2016 Nov 15; Miret et al., 2016 Jul; Shan et al., 2020 Nov; Narasimhan et al., 2018 May 7; Hsieh et al., 2012 Feb; Pontillo et al., 2013 May 1; Miller et al., 2005; Belguise et al., 2007 Dec 15; Yamashita et al., 2018 May 1; Miret et al., 2020 May). The dose–response concordance for cell invasion was demonstrated using increasing doses of hexachlorobenzene, benzo[a]pyrene, chlorpyrifos and TCDD (Miret et al., 2016 Jul; Shan et al., 2020 Nov; Pontillo et al., 2013 May 1; Miller et al., 2005; Miret et al., 2020 May). To further explore cell invasion, Nguyen et al. created a model of a lymphatic barrier using a three-dimensional lymph endothelial cell as a monolayer co-cultured with spheroids of MDA-MB231 cells (Nguyen et al., 2016 Nov 15). They found that silencing or antagonizing the AhR (DIM) or activating the AhR (FICZ) respectively decreased or increased invasion of the lymphatic barrier.

On an organ level, *in vivo*, an increase in metastasis has been found in mice and zebrafish after the activation of the AhR with different ligands (butyl benzyl phthalate, di-n-butyl phthalate, hexachlorobenzene, TCDD) (Goode et al., 2014; Shan et al., 2020 Nov; Narasimhan et al., 2018 May 7; Hsieh et al., 2012 Feb; Pontillo et al., 2013 May 1). In the zebrafish model, Narasimhan et al. treated the animals either with triple negative MDA-MB-231 cells only (untreated) or with MDA-MB-231 cells treated with an AhR inhibitor (CB7993113 or CH22319) (Narasimhan et al., 2018 May 7). Untreated fish had significantly more metastasis (OR = 9, IC95%=3–35). Similar results were found using mice models (Goode et al., 2014; Shan et al., 2020 Nov; Narasimhan et al., 2018 May 7; Hsieh et al., 2012 Feb; Pontillo et al., 2013 May 1).

Bibliographic details concerning KERs are presented in Supplementary Table S3.

### 3.3.5. KER 8: Activation of the AhR leads to an increased inflammation (existing in AOP 21)

In triple negative breast cell lines (MDA-MB436, MDA-MB-231) and ER-positive cell lines, it has been shown that the activation of the AhR can lead to an increase in inflammation. (Bekki et al., 2015; Miller et al., 2005; Yamashita et al., 2018 May 1; Degner et al., 2009 Jan; Vogel et al., 2011 Aug 1; Kolasa et al., 2013 Apr 25; Vacher et al., 2018; Malik et al., 2019 Oct). The stressors mainly used to activate the AhR were TCDD followed by benzo[a]pyrene and 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhiP). After AhR inhibition (KO or antagonists), a decrease in inflammation biomarkers was found (Miller et al., 2005; Yamashita et al., 2018 May 1; Degner et al., 2009 Jan; Vogel et al., 2011 Aug 1; Kolasa et al., 2013 Apr 25). Assays evaluating cell inflammation were quantitative dosages of IL-6, IL-8 and Cox2 activity/expression. Cox-2 and IL-8 were amongst the top “gene concepts” retrieved by the PubTator Central tool, likewise, “inflammation” was frequently found as a disease concept. The most consensual pathway linking the AhR activation to cell inflammation was the NF-κB pathway (Vogel et al., 2011 Aug 1; Kolasa et al., 2013 Apr 25). Only half of the studies found a dose–response relationship (Miller et al., 2005; Kolasa et al., 2013 Apr 25; Malik et al., 2019 Oct). No studies were carried out *in vivo* for breast cancer and therefore the concordance and evidence were classified as “moderate”.

AOP 21 also found the association between AhR activation and inflammation via COX 2 (Aryl hydrocarbon receptor activation leading to early life stage mortality, via increased COX-2) with a weight of



evidence classified as “high”. Indeed, the AhR/ARNT heterodimer links to the dioxin responsive elements which in turn up-regulates COX-2 (66,67).

Bibliographic details concerning KERs are presented in Supplementary Table S3.

### 3.3.6. KER 9: Inflammation promotes organ invasion

In the specific setting of AhR activation, only 2 studies showed the continuum between AhR activation – increased inflammation – increased invasion (Miller et al., 2005; Yamashita et al., 2018 May 1). However, in general, there is extensive knowledge on the relationship between cell inflammation and organ invasion. First, COX-2 is expressed at higher levels in triple negative invasive breast cancers than in less aggressive ER-positive cancers (Gilhooly and Rose, 1999 Aug; Liu and Rose, 1996 Nov 15). COX-2 catalyzes the conversion of arachidonic acid into prostaglandin H<sub>2</sub>, a pro-inflammatory factor, and is therefore considered as a prognosis factor in breast cancer (Ristimäki et al., 2002 Feb 1; Parrett et al., 1997 Mar). Transfection with COX-2 triple negative MDA-MB-435 cells increased cell migration 2-fold compared to control cells in a transwell-Matrigel® assay. Antagonism of COX-2 through an inhibitor (NS-398) reversed this action in a dose-dependent way (Singh et al., 2005 May). Second, *in vivo*, the use of anti-inflammatory treatments such as celecoxib (COX-2 inhibitor) can reduce tumor growth and spread (Harris et al., 2000 Apr 15). Finally, epidemiologic evidence suggests that inflammatory breast cancers have the worse prognosis. Indeed, the median overall survival of patients with inflammatory breast cancer compared with those with non-inflammatory breast cancer tumors is 4.75 years versus 13.40 years for stage III disease and 2.27 years versus 3.40 years for stage IV disease (Schlichting et al., 2012 Aug; Fouad et al., 2017 Apr).

The mechanism of action of COX-2 are consensual. COX-2 promotes cell invasion through upregulation of MMPs (notably 2 and 9) (Takahashi et al., 1999 Oct 22; Sivula et al., 2005 Feb; Larkins et al., 2006 Jul). Moreover, COX-2 could also activate the urokinase plasminogen activator (uPA) which degrades the basal membrane of epithelia (Singh et al., 2005 May; Takahashi et al., 1999 Oct 22; Larkins et al., 2006 Jul; Guyton et al., 2000 Mar).

The relationship between inflammation and invasion is well documented therefore the evidence was classified as “strong”.

Bibliographic details concerning KERs are presented in Supplementary Table S3.

### 3.3.7. KER 10: Inflammation promotes angiogenesis

Likewise, two studies evaluated the specific continuum AhR activation – increased inflammation – increased angiogenesis (Pontillo et al., 2015 Nov 19; Zárate et al., 2020 Aug). As previously mentioned, the AhR activation increases inflammation, notably through an increase in COX 2 (Bekki et al., 2015; Miller et al., 2005; Degner et al., 2009 Jan; Pontillo et al., 2015 Nov 19; Zárate et al., 2020 Aug).

COX-2 can promote angiogenesis through an increase in VEGF (Vascular endothelial growth factor) (Harris et al., 2014 Oct 10; Kirkpatrick et al., 2002). In a pathologic study characterizing 46 breast cancer specimen using immunohistochemistry, it was found that the density of microvessels was significantly higher in patients with COX-2 expression than in those without expression ( $p = 0.03$ ) (Costa et al., 2002 Jun). The relationship between COX-2 and angiogenesis has also been shown in gastric and colorectal cancer (Tsuji et al., 1998 May 29; Uefuji et al., 2000 Jan). Indeed, colon carcinoma cells overexpressing COX-2 produce proangiogenic factors (VEGF, bFGF, TGF- $\beta$ , PDGF, and endothelin-1), and stimulate endothelial migration and the formation of tube vessels. These effects were reversed by an inhibitor (NS-398). *In vivo*, Diclofenac, a COX-2 inhibitor, decreased angiogenesis in mice presenting a colorectal cancer (Seed et al., 1997 May 1). Likewise, in a murine model of breast cancer, celecoxib (a selective COX-2 inhibitor) reduced metastasis and tumor burden through a decrease of micro vessel density and VEGF (Yoshinaka et al., 2006 Dec; Zhang et al., 2004 Sep).

In clinical studies, patients with inflammatory breast cancers have increased levels of genes involved in angiogenesis such as VEGF (Van der Auwera et al., 2004 Dec 1). Patients with an inflammatory breast cancer benefit the most from anti-angiogenic treatment bevacizumab (Pierga et al., 2012 Apr).

The evidence was classified as “moderate” due to the lack of dose response studies.

Bibliographic details concerning KERs are presented in Supplementary Table S3.

### 3.3.8. KER 11 and 12 (existing KER 1266 and 1267): Activation of the AhR leads to an increased endothelial migration which promotes angiogenesis

The activation of the AhR can lead to an increased endothelial cell migration. This was found when HMEC-1 or EA.hy926 cells were co-cultured with ER-positive MCF-7 cells and triple negative MDA-MB-231 cells (Pontillo et al., 2015 Nov 19; Zárate et al., 2020 Aug). The assay mainly used was the Matrigel® / tube formation assay. Only one study found an increase in endothelial cell proliferation and not migration, therefore it was not kept as a KE (Pontillo et al., 2015 Nov 19). The main pathway explaining this relationship was again related to the activation of COX2 and subsequently to the increase in VEGF. The association between the activation of the AhR and endothelial cell migration was classified as “weak” since only 2 studies explored this feature, and both used hexachlorobenzene as a stressor. However, these works were robust with strong evidence, and both found a reversed association after AhR blockage. No contradicting results were found in the scientific literature.

As opposed to our work, another AOP displayed a link between AhR activation and angiogenesis (AOP 150) and found that activation of the receptor could decrease VEGF production with moderate evidence and quantitative understanding. It must be noted that these AOPs applied only to chicken, zebrafish, and certain rodents whereas our AOP concerns humans. As detailed further, the AhR presents a variability between species which must be considered.

For KER 12, Pontillo et al. treated mice with increasing doses of hexachlorobenzene and then calculated the vessel density in mammary fat pads (Pontillo et al., 2015 Nov 19). They found that mice treated with hexachlorobenzene had a higher vessel density with a dose–response concordance. Treatment by AhR antagonists completely reversed this association (Pontillo et al., 2015 Nov 19; Zárate et al., 2020 Aug). The relationship between endothelial migration and angiogenesis was not detailed here since there is existing extensive knowledge (Lamallice et al., 2007 Mar 30; Norton and Popel, 2016 Nov 14; Ausprunk and Folkman, 1977 Jul 1). The KER 12 was considered as “strong”.

Bibliographic details concerning KERs are presented in Supplementary Table S3.

### 3.3.9. KER 3, 6 and 13: Increased tumor growth, increased invasion, and increased angiogenesis lead to breast cancer metastasis

Due to extensive data in the scientific literature and the empirical evidence in favor of these KERs, these KERs were not detailed here.

## 3.4. AOP overall assessment

The *biological applicability domain* of the putative AOP concerned mainly females of menstrual or post-menopausal age. Indeed, existing cell lines were derived from women of menstrual or post-menopausal age and *in vivo*, studies were performed on mice of reproductive age. Only one study used the zebra fish larvae (Narasimhan et al., 2018 May 7). However, it could be extrapolated to men. Indeed, breast cancers in men present similar tumor characteristics and no work has found diverging functions of the AhR between men and women. Moreover, no difference in AhR expression has been characterized between men and women. Furthermore, our AOP concerns ER-positive and triple negative cell lines.

Studies were carried out in humans, mice, and zebrafish (xenotransplant studies, no mammary gland) (i.e. PubTator results) and it can be hypothesized that this AOP is conserved across mammals. Indeed, the AhR is a very conserved and ancient protein (Hahn, 2002 Sep 20). However, since the sensitivity to adverse events are variable among taxa, we can only postulate this AOP in human and mice (Korkalainen et al., 2001 Aug 3; Cohen-Barnhouse et al., 2011 Jan; Doering et al., 2013 Mar).

The *biological plausibility* of KERs is defined by the OECD as the « understanding of the fundamental biological processes involved and whether they are consistent with the causal relationship being proposed in the AOP ». The biological plausibility is strong due to the presence of overwhelming evidence present in different studies. A minor setback would be the difficulty to dismiss alternative mechanisms caused by the ligands used for AhR activation. This is detailed in the discussion.

The *essentiality of KEs* refers to « experimental data for whether or not downstream KEs or the AO are prevented or modified if an upstream event is blocked ». The essentiality of KEs is strong: most works use suppression or inhibition of the AhR (knock out, antagonists and/or silencing) with results coherent with our findings.

Finally, the *empirical support* of KERs, is often « based on toxicological data derived by one or more reference chemicals where dose–response and temporal concordance for the KE pair can be assessed ». The overall assessment of the empirical support of our KERs is also strong. There is evidence in human cell lines and mice showing a dose–response and temporal concordance for severity of our KE and the presence of metastasis.

#### 4. Discussion

There is a recent growing interest for the development of AOPs, mainly due to the search for new tools to improve toxicological regulations. Indeed, these sequential chains of linked events can be used as a basis for risk assessment in health and ecotoxicology. We propose here an AOP in which activation of the AhR (MIE) could promote breast cancer-related death (AO) through a series of key events (KE) with mostly, strong evidence. The main strength of the developed AOP lies in the use of artificial intelligence tools (AOP-help finder, PubTator) which helped us to create a new pathway using all the available knowledge published in the scientific literature and extract the most robust concepts. The presented AOP was elaborated without preconceived ideas (artificial intelligence) and was then supported using our complementary expertise and the knowledge extracted from relevant studies.

##### 4.1. Applicability domain: Breast cancer progression

The AhR is a fascinating yet complex receptor since its activation is ligand and cell dependent. To avoid more bias, we decided to limit our AOP to breast cancer. First, this cancer is the most frequent female malignancy, which makes it a major public health concern. Second, this illness is hormonal-dependent and therefore the impact of the environment, through the AhR, can be strongly suggested. However, we have reasons to believe this AOP could be extrapolated to other cancers which share common regulatory pathways (Larigot et al., 2022). The AhR is overexpressed not only in breast cancer but also in lung, liver, stomach, head & neck, cervix, and ovarian cancer (Stanford et al., 2016; DiNatale et al., 2010 Aug 6; Liu et al., 2013 Aug; Stanford et al., 2016 Aug). Moreover, in these cancers, the level of expression is correlated to the stage of the disease (Zudaire et al., 2008; Koliopoulos et al., 2002 Sep 5; Chang et al., 2007 Jan 1). Additionally, Moenniks et al. found that mice with constitutively active AhR had more liver tumors than wild type mice (55% versus 6%) (Moennikes et al., 2004 Jul 15). *In vitro* evidence suggests that the AhR activation could promote a more aggressive phenotype to renal, lung, head and neck, and urothelial cancer through an increase in invasion, migration, and resistance to apoptosis which constitute representative key events of our AOP (Zudaire et al., 2008;

Stanford et al., 2016 Aug; Ishida et al., 2015 Jul 15; Ishida et al., 2010 Feb; Diry et al., 2006 Sep 7; John et al., 2014 Oct). Besides, an AOP associating AhR activation and lung cancer initiation is currently under development (AOP, 2021) (<https://aopwiki.org/aops/417>, accessed May 2022).

Likewise, our AOP covers only breast cancer progression and not initiation. The mechanisms of breast cancer initiation are different from the metastatic pathway, but the AhR could also be involved in breast cancer initiation. *In vitro*, it was noted that human mammary benign cells with a high level of AhR had an increase in cell proliferation, and migration, and potentially display EMT-like features (Brooks and Eltom, 2011 Jun). *In vivo*, mice fed with 7,12-dimethylbenz[a]anthracene (DMBA, an AhR activator and a potent mutagen) had an increased risk of mammary tumors, with higher AhR expression (Currier et al., 2005). Strangely in regard of the deadly outcomes associated with aggressive breast tumors, the number of studies focusing on this specific aspect of mammary carcinogenesis is limited and therefore, epidemiological data on the effects of the exposome in breast cancer aggressiveness is scarce. Indeed, occupational exposure is difficult to quantify, and patients are usually exposed to a mixture of pollutants and not a single pollutant in a chronic way. A memory bias cannot be excluded since the half-life of TCDD, for instance, is 7–11 years (Pirkle et al., 1989). Industrial accidents, such as the Seveso incident, studied the increase in breast cancer incidence but did not record breast cancer aggressiveness since it is more complex to quantify. At an early stage, breast cancer has a favorable prognosis whereas the therapeutic challenge lies in the treatment of breast cancer metastases. Therefore, even though epidemiologic and cell evidence suggests that exposure to pollutants and AhR activation could promote breast cancer initiation, we chose to study breast cancer progression, the most complex situation (Pesatori et al., 2009 Sep; Warner et al., 2002 Jul).

##### 4.2. Gaps of knowledge, uncertainties: Pitfalls of the AhR

The activation of the AhR is a MIE that has been found in 3 other endorsed AOPs: mortality (AOP 21 and 150) and uroporphyrin (AOP 131) (AOP, 2021). The AhR is a basic Helix Loop Helix/Period ARNT single-minded (bHLH/PAS) transcription factor identified in 1976 (Poland et al., 1976 Aug 25). Two main activation pathways have been described: the genomic and non-genomic. For the former, once activated by a ligand, the AhR acts as transcription factors regulating the expression of genes encoding for xenobiotic metabolizing enzymes (XMEs). The AhR is localized in the cytoplasm of most cells, complexed to several chaperone proteins (Heat shock protein 90 (HSP 90), p23 and X-associated protein (XAP)) (Petrucci and Perdew, 2002 Sep 20). These proteins 1) maintain the correct folding of the AhR, allowing a good recognition of the ligand by the receptor and 2) protect it from its degradation (Larigot et al., 2018 Dec; Guyot et al., 2013 May). After ligand binding, the AhR is translocated into the nucleus and binds to the AhR Nuclear Translocator (ARNT) to form an active heterodimer. The latter binds to specific DNA sequences, called XREs (Xenobiotic Responsive Elements) and interacts with co-regulators allowing the recruitment of RNA polymerase II and the regulation of transcription of target genes containing these XREs in their promoters. The first identified target genes encode for proteins involved in xenobiotic metabolism such as CYP1A1, CYP1B1, glutathione transferases or aldehyde dehydrogenases (ALDH). After being exported from the nucleus, AhR is rapidly degraded in the cytoplasmic compartment by the proteasome (Davarinos and Pollenz, 1999 Oct 1). Several non-genomic pathways have also been identified (Larigot et al., 2018 Dec), involving other transcriptional regulators or signal transducers such as c-Src,  $\beta$ -catenin, NF- $\kappa$ B or the estrogen receptor alpha (Tian et al., 1999 Jan 1; Faust et al., 2013 Apr; Procházková et al., 2011 Aug).

The exogenous ligands of the AhR are numerous (Larigot et al., 2018 Dec). The most common are xenobiotics, such as aromatic hydrocarbons. TCDD has the highest affinity for the AhR and is classified by the

International Agency for Research on Cancer (IARC) in their group 1 (carcinogenic to humans). Natural components of food such as flavonoids (quercetin and resveratrol) and indole-3-carbinol can also link to the AhR. Several endogenous ligands can also activate the AhR: FICZ (formylindolo [3,2-b] carbazole), indirubin, indigo, metabolites of arachidonic acid or kynurenine pathway metabolites. Chronic and acute activation of the AhR can also have diverging effects (Larigot et al., 2022).

One of the main limitations of our AOP is the existence of these diverse ligands and pathways, complexifying the definition of 'AhR activation' (Larigot et al., 2022; Narasimhan et al., 2018 May 7). Using PubTator, we found that TCDD was by far the most used chemical followed by I3C, alpha-naphthoflavone, polycyclic aromatic hydrocarbons and hexachlorobenzene, all ligands of the AhR. These ligands can activate different pathways after AhR binding and we therefore assumed that these compounds were AhR agonists. It can be difficult to dismiss alternative mechanisms caused by the ligands used for AhR activation. However, the AhR is the only characterized target of TCDD for example, and studies which use several ligands including TCDD, display similar results using the other modulators. Moreover, the concordance of studies using various ligands and the coherence with the AhR inhibition are in favor of the robustness of the proposed AOP. Indeed, to obtain the most accurate AOP possible, the KEs selected had to be present, no matter the ligand used by the study.

Another minor setback of using the AhR, is that the dose response concordance is a non-monotonous curve for several ligands (Gardner, 2000 Mar 1; Kayajanian, 2002 Jan). Therefore, the tailored Bradford-Hill criteria could sometimes not be fulfilled.

#### 4.3. Gaps of knowledge, uncertainties: Pathways not chosen

There are other pathways by which the activation of the AhR could promote breast cancer metastasis. The objective of an AOP is not to offer the most extensive knowledge on a relationship between MIE and AO. An AOP must clearly and simply explain the process between an MIE and an AO using causal relations (AOP, 2021). AOP-helpFinder retrieved the most articles under the category "cell proliferation" and "estrogen". However, due to the presence of diverging evidence on the activation of the AhR and cell proliferation, we chose not to include these in our AOP. Indeed, on one hand, activation of the AhR through ligands such as NK150460, ANI-7, emodine or derivatives of revesterol decrease cell proliferation in ER-positive and ER-negative breast cancer cell lines (Fukasawa et al., 2015 Feb; van den Brand et al., 2019 Jun; Gilbert et al., 2018 Feb; Zhang et al., 2020). TCDD has been found to promote cell cycle arrest through phosphorylation of the retinoblastoma protein which binds to E2F (Bar Hoover et al., 2010 Feb; Puga et al., 2000 Jan 28; Greenlee et al., 2001 Feb). In ER-positive cell lines, beta-naphthoflavone mediated cell cycle arrest through an upregulation of P21 (Wang et al., 2014 Mar). On the other hand, AhR activation could promote cell proliferation. Pearce et al. found that MCDF (6-methyl-1,3,8-trichlorodibenzofuran), an AhR agonist could stimulate cell proliferation with a dose-response concordance (Pearce et al., 2004 Apr 15). Likewise, I3C, HCB, CPF and licorice could also promote cell proliferation (Xue et al., 2005 Apr 15; Riby et al., 2000 Feb 8; García et al., 2010 Feb 1; Chu et al., 2014; Moyano et al., 2020 Jul). However, it seems that this cell proliferation is ER-dependent (Pearce et al., 2004 Apr 15; Xue et al., 2005 Apr 15; Riby et al., 2000 Feb 8; García et al., 2010 Feb 1; Chu et al., 2014; Moyano et al., 2020 Jul; Cirillo et al., 2019 Aug 1; Kanaya et al., 2019 Jun 1; Gearhart-Serna et al., 2020 Dec 31). Indeed, these ligands induced cell proliferation only in ER-positive cells lines with an effect dependent on the level of estrogen present in the medium. Whether this increase in ER-dependent cell proliferation can be independent of the AhR remains unclear (Shipley and Waxman, 2006 Jun 1). This increase in proliferation could also be mediated by the association of the RelA subunit of NF-kappaB with the AhR resulting in the activation of c-myc gene transcription in breast cancer cells (Kim et al., 2000 Nov 16; Tian

et al., 1999 Jan 1). This would explain why Rodriguez et al. found that proliferation was modulated by the CYP1A1, independently of an exogenous ligand activation of the AhR (Rodriguez and Potter, 2013 Jul). These complex effects, highly dependent on the context (cell types, medium content, type of ligand...) were therefore not included in our AOP despite the strong evidence.

Another interesting hall marker of cancer frequently found by AOP help-finder was oxidative stress. Indeed, several works have found that AhR activation could lead to an increase in oxidative stress in ER-positive and ER-negative breast cancer cells (Lin et al., 2007; Lin et al., 2008; Chen et al., 2013; McLean et al., 2015). The AhR could increase the formation of reactive oxygen species which could lead to the accumulation of DNA damage and therefore promote breast cancer. However, promoting breast cancer is different than progressing into a metastatic state, and the evidence was classified as poor since most studies found these results only using TCDD. An alternative mechanism could not be ruled out. Likewise, stemness could also be a key event associating AhR activation to breast cancer-related death but the evidence present was low (Stanford et al., 2016; Al-Dhfyhan et al., 2017 Jan 19). In triple negative inflammatory breast cancer lines, cells presented cancer stem like characteristics after overexpression of the AhR (increased expression of stem-like cell markers, ALDH1 high, Sox2, Snai1, Twist 1, Twist2, Tgfb1, and Vim) (Stanford et al., 2016).

Finally, in our initial search strategy, abstracts retrieved had to concern the MIE, the AO and a KE. This unusual approach permitted a focused and targeted search to initially elaborate our AOP. However, a manual search of existing scientific databases could be carried out afterwards to weigh the evidence of several KEs and KERs.

## 5. Conclusion

In conclusion, we propose a simple and robust AOP associating activation of the AhR and breast cancer related death through migration, invasion, inflammation, and neo-angiogenesis. The main limitation of our AOP is the variability of the pathways activated by the AhR ligands and the possibility of alternative pathways. To overcome these limitations, our KE had to be present no matter the ligand and in a consistent way. Moreover, the originality of our work lies in the use of artificial intelligence too such as AOP-helpfinder, which enables a thoroughly search of existing knowledge in the PubMed database and PubTator (Carvaillo et al., 2019; Zgheib et al., 2021; Rugard et al., 2020 Jan 1). Therefore, our literature review was complete and evidence in favor of our proposed AOP was overwhelming. We plan to validate our proposed AOP in a quantitative *in vitro* work using Integrated Approaches to Testing and Assessment (IATA).

### CRedit authorship contribution statement

**Louise Benoit:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Florence Jornod:** Data curation, Formal analysis, Methodology, Software. **Elias Zgheib:** Data curation, Formal analysis, Methodology, Software. **Celine Tomkiewicz:** Writing – review & editing. **Meriem Koual:** Writing – review & editing, Supervision. **Thibaut Coustillet:** Data curation, Formal analysis, Methodology, Software. **Robert Barouki:** Writing – review & editing, Supervision. **Karine Audouze:** Data curation, Formal analysis, Methodology, Software, Writing – review & editing, Supervision. **Mathieu Vinken:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing. **Xavier Coumoul:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envint.2022.107323>.

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