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A pragmatic workflow for human relevance assessment of toxicological pathways and associated new approach methodologies

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ABSTRACT

Currently, safety assessments of chemical substances are predominantly based on animal data. Multiple considerations call for the use of alternative testing strategies that are based on new approach methodologies (NAMs). However, the human relevance of these testing strategies is usually uncertain. This necessitates a harmonized and accepted workflow for assessing their applicability for regulatory purposes. This report proposes such a workflow, applicable for assessing the human relevance of a toxicological pathway and the relevance of NAMs related to the different components of the pathway. The workflow starts with an established toxicological pathway, of which the adverse outcome is relevant for human health risk assessment and that has sufficient weight of evidence. Human relevance is assessed through three main questions, related to the different components (steps) of the pathway, the pathology of human syndromes that have a similar adverse outcome, and quantitative aspects. The latter comprise both interspecies differences and *in vitro – in vivo* differences. The combined evidence is scored as 'strong', 'moderate' or 'weak' support of human relevance, based on expert judgement. The workflow developed was tested in a case study, through application to an AOP describing craniofacial malformations after *in utero* exposure to triazoles. Based on evidence collected for two of the three main questions, the case study provided moderate to strong support for human relevance of both the various components of the AOP and its associated NAMs. Furthermore, it demonstrated that the workflow is a promising approach that allows for a more transparent scientific evaluation of human relevance of toxicological pathways and associated NAMs. Therefore, despite some areas for improvement, we consider the workflow an important step forward for application of AOPs and related NAMs in human health risk assessment.

1. Introduction

The current approaches to human health risk assessment of chemical substances heavily rely on information obtained from toxicity studies in experimental animals. These are, however, associated with substantial concerns related to scientific, ethical, financial and legal limitations. The ensuing ambition to reduce, refine and replace experiments in laboratory animals (3Rs principle) has resulted in the development of new approach methodologies (NAMs), *i.e.* any technology, methodology, approach, or combination thereof that avoids the direct use of experimental animals and can be used for hazard characterization of chemicals (Van Mulders et al., 2020). However, data obtained from NAMs are not easily extrapolated to the human *in vivo* situation. Uncertainty about the representativity of NAMs for the toxicological mechanisms in humans necessitates a validated and accepted workflow for assessing their applicability for regulatory purposes.

An attempt to address extrapolation of NAM-derived conclusions to humans comes from a framework for analysis of mode of action (MOA) and related human relevance, which has been developed in initiatives of the International Programme on Chemical Safety (IPCS) of the World Health Organization (WHO) (Sonich-Mullin et al. 2001; Boobis et al., 2006) and the International Life Sciences Institute Risk Sciences Institute (ILSI-RSI) (Meek et al., 2003; Seed et al., 2005). In this framework, human relevance evaluation considers whether a proposed MOA that has sufficient weight of evidence in laboratory animals, can occur in humans at relevant levels of exposure based on qualitative and quantitative similarities in biological processes between humans and test animals. More recently, the framework has been updated to reflect the

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experience acquired in its application and to extend its scope to enable integration of information at different levels of biological organization, including data from NAMs (Meek et al., 2014a). This valuable modification to the previous versions of the framework connects well to the ongoing developments to modernize toxicity testing through implementation of NAMs. In essence, the framework addresses two key questions: a) are there sufficient data to hypothesize a MOA for the health effects observed, and b) if so, is it likely that this MOA will operate in humans at relevant exposure levels? The latter is referred to as species concordance analysis. The MOA analysis relies on biological plausibility and coherence, and the weight of evidence is evaluated using modified Bradford Hill criteria (Meek et al., 2014b).

In spite of instructions and templates and case studies (Meek et al. 2014a, 2014b), application of the WHO/IPCS framework in practice, including the analysis of species concordance, remains challenging. How to best address the different questions in practice, where to find valuable information, etc.? Also, the framework is focused on the human relevance of a MOA but does not include assessment of the relevance of NAMs associated with the MOA. Therefore, we developed a pragmatic workflow for human relevance assessment of toxicological pathways. It uses a human-specific toxicological pathway paradigm, whereas the MOA-based approach compares biological processes among species and models. Thereby, it should be applicable for assessing both the human relevance of a toxicological pathway and the relevance of NAMs related to the key events (KEs) or key event relationships (KERs) of the pathway under study.

The workflow was thus developed using an Adverse Outcome Pathway (AOP; Ankley et al., 2010; OECD, 2017; Edwards et al., 2016) as a starting point, of which the adverse outcome is considered of relevance for human health risk assessment. AOPs are frameworks for organizing knowledge of biological pathways, *i.e.* they describe sequential events leading from a molecular initiating event (MIE) to an adverse outcome (AO) at the individual or population level. Using a well-described AOP for developing the workflow allows for multiple starting points in the pathway for the assessment of human relevance, while it does not exclude human relevance when evidence for an intermediate step of the AOP is missing. Furthermore, the AOP allows for assessment of both adjacent and non-adjacent KERs, and to link adjacent or more distant KEs, including the MIE and AO. In order to be useful for human health risk assessment purposes, an AOP is to be assessed for the weight of evidence, supporting the AOP as such, as well as its relevance to humans, in which issues relating to study quality, relevance and strength are evaluated (Meek et al., 2014b; Dekant et al., 2017; ECHA, 2011). At present, the weight of evidence supporting published (draft) AOPs, such as those available through the OECD AOP wiki (Ives et al., 2017; https://aopwiki.org), is evaluated using the modified Bradford Hill criteria (Meek et al., 2014b; OECD, 2018). Additionally, taxonomic applicability is evaluated as part of the biological domain (OECD, 2018), but currently this does not involve a specific human relevance assessment.

In the following, our proposed workflow for assessment of human relevance is tested in a case study, through application to an AOP that was already evaluated for weight of evidence elsewhere (Menegola et al., 2021). This AOP is deemed suitable for the purpose because it is well studied, which allows for a more detailed evaluation of the workflow. Findings from the case study are presented and discussed, together with aspects that need further effort.

2. Workflow design

The goal of this study was to outline a pragmatic workflow for assessing human relevance of a toxicological pathway and for assessing the relevance of NAMs for measuring KEs and/or KERs. The starting point of the workflow is a toxicological pathway such as an AOP, of which the endpoint (AO) is considered relevant for human health risk assessment and for which the overall evidence - based on the modified Bradford Hill criteria (Meek et al., 2014b; OECD, 2018) - is considered sufficient.

The workflow consists of a structured series of questions, targeted at each of the KE(R)s in the toxicological pathway. The questions are similar, but not identical, to the series of questions defined in the WHO/IPCS framework (Meek et al., 2014a). Considerations are provided for each of the questions in the workflow, including possible tools and sources of information. The questions were drafted, tested, and revised along the case study AOP 'Disruption of retinoic acid metabolism leading to developmental craniofacial defects', relying exclusively on NAM-based or human data. It should be noted that the workflow is applicable for all kinds of data, including data from animal studies. We tested the workflow using solely NAM-based or human data because this should be the ultimate goal in modern risk assessment. The final generic workflow, resulting from iterative refinements in the drafting process, is presented here (Fig. 1).

2.1. Human relevance of the toxicological pathway

The assessment of human relevance starts with a qualitative assessment of the toxicological pathway underlying the defined AO with relevance for humans. The first question (Q1), to be answered for each of the components (steps) of the pathway, is defined as '*Is the event likely to occur in humans?*' (Fig. 1). In addressing this question, it is necessary to consider target organ, sensitive time window(s) or life stage(s), possible differential expression between sexes, and expression and essentiality of different isoforms of proteins involved in the pathway (Table 1). In case of insufficient data and/or knowledge, an additional question (Q1.1) may be needed: '*Is the event likely to be evolutionary conserved?*'. Data on evolutionary conservation across a wide(r) range of species could be used to make an assumption about human relevance. Evidence from human diseases or syndromes with a similar AO (*i.e.* pathological phenotype), which may have a similar (hypothesized) pathogenesis, could further support human relevance of the pathway. Therefore, Q2 is defined as '*Do human diseases with a similar AO have a similar pathogenesis?*'. Considerations associated with this question include whether (epi)genetic factors are of relevance to the aetiology of the disease, whether it is plausible that the events of the pathway play a role in the disease, and whether alternative pathways have been hypothesized for the disease (Table 1). The next question (Q3) is aimed at assessing possible quantitative differences between information derived from animal-based models and humans, and between NAMs and the human *in vivo* situation, due to which human relevance can be reasonably excluded: '*Is human relevance unlikely in view of quantitative differences following differential regulation of (biological) processes between species, or following (technical) issues related to different in vitro – in vivo conditions?*'.

For each of the questions, evidence for human relevance is evaluated along specific considerations (Table 1), as detailed as available data allow, and subsequently scored as 'strong', 'moderate' or 'weak', based on expert judgement and detailed criteria (see next section). Integration of the different lines of evidence leads to a human relevance score for each individual element of the toxicological pathway. The combined score for all elements represents the overall support for human relevance of the pathway.

2.2. Relevance of NAMs in the context of AOPs

Each NAM used as source of information is assessed for its power to support human relevance of the pathway, through assessment of the relevance of a NAM in relation to measurement of a KE and/or KER in the pathway. According to OECD Guidance Document 34, "(…) the relevance of a test method describes the relationship between the test and the effect in the target species and whether the test method is meaningful and useful for a defined purpose, with the limitations identified" (OECD, 2005; update ongoing). It should be noted that for regulatory use not only the relevance but also the reliability of a test

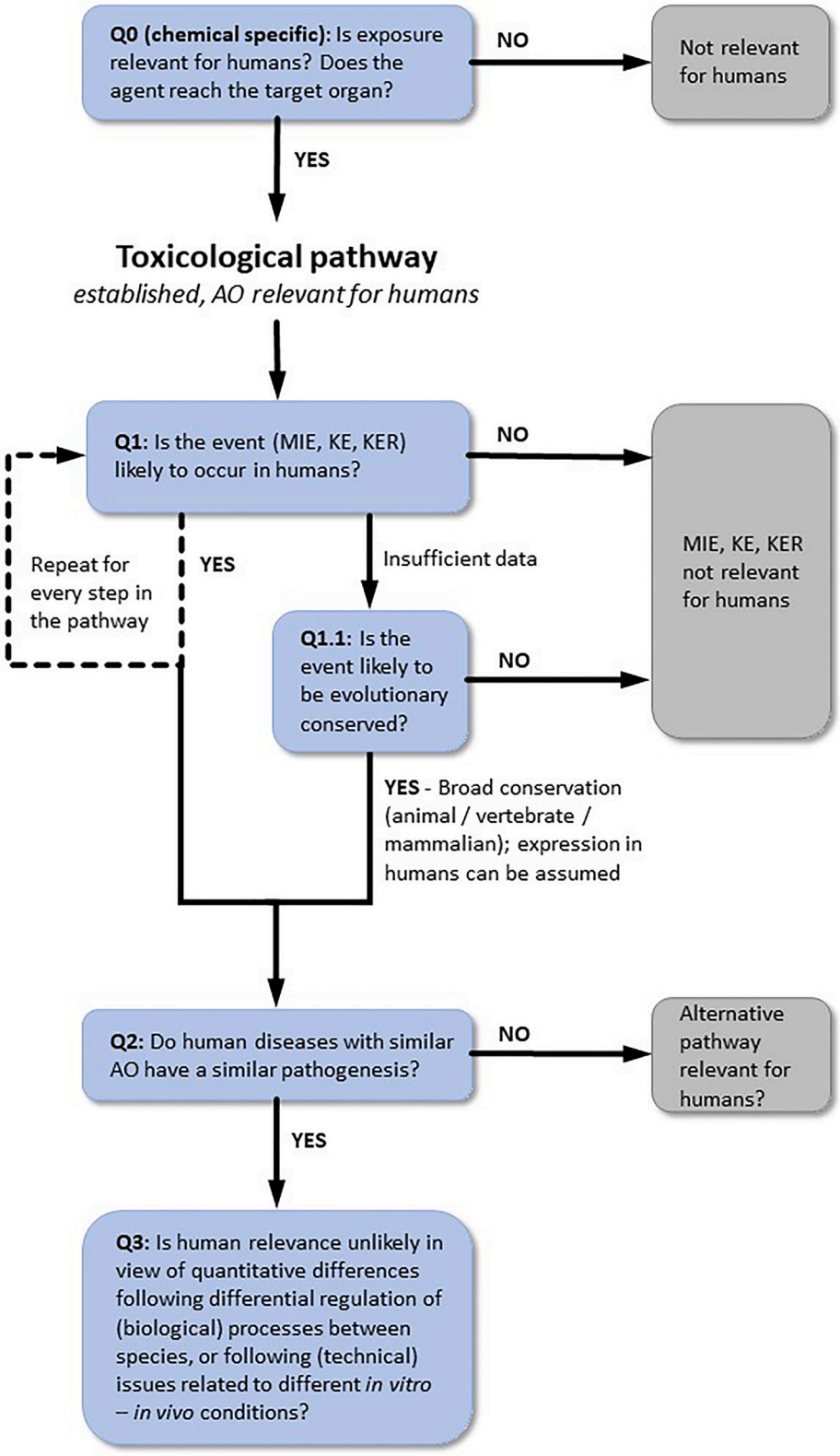

Fig. 1. Workflow for assessing human relevance of a toxicological pathway and associated NAMs. AO, adverse outcome; MIE, molecular initiating event; KE, key event; KER, key event relationship. Considerations for each of the questions are listed in Table 1. Human relevance assessment systematically assesses the different elements of an established toxicological pathway such as an AOP, of which the AO is considered relevant for human health risk assessment.

Table 1
Considerations for the different questions of the workflow for human relevance assessment.

Question[a]	Considerations	Tools	References[b]
1	MIE/KEs/KERs/AO		
	Expression/occurrence in target organ(s)	Expression atlas[c]; ENCODE[d]	EMBL-EBI (2024)
	Expression/occurrence specific to a sensitive time-window(s); and/or specific to life-stage	Expression atlas; textbooks; scientific literature	(Lee et al., 1992; Pursley et al., 1996; Aleck and Bartley, 1997; Lopez-Rangel and Van Allen, 2005; Colleoni et al., 2011; Zimmer et al., 2014; EMBL-EBI (2024); Lippmann et al., 2015; Huang et al., 2016; Dou et al., 2016)
	Expression/occurrence specific to gender	Expression atlas; textbooks; scientific literature	EMBL-EBI (2024)
	Expression of isoforms	Expression atlas	EMBL-EBI (2024)
	Polymorphisms that may affect expression	dbSNP database[e]; scientific literature	Lee et al. (2007)
	Enzymes/metabolism that may affect the relationship/event	Scientific literature	(Laue et al., 2011; Browne et al., 2012; Topa et al., 2020; Guo et al., 2020)
	Relevant case reports, pharmacovigilance (clinical data)	Drug registries (e.g. European Medicines Agency, WHO Vigibase), scientific literature	
2	Clinical data, genetics/epigenetics of disease	Disease registry (e.g., OMIM[f]); textbook; scientific literature	(Sant'anna 2006; Fukami and Ogata, 2014; Lambert et al., 2018)
	Function of AOP events in the disease	Scientific literature	Schoenwolf et al. (2009)
	Hypothesized MOA	Scientific literature	(Sant'anna 2006; Schoenwolf et al., 2009; Fukami and Ogata, 2014)
3	Interspecies differences; *in vitro-in vivo* differences	Response-response comparisons across species; Pharmacokinetic/toxicokinetic data, human biomonitoring, QIVIVE[g] models	
0	Human exposure/biomonitoring data and/or estimates	IPCheM[h]; NHANES[i]; scientific literature	
	Pharmacokinetic modeling		
	System-dependent parameters: absorption, metabolism, volume of distribution, elimination half-life	Drugbank[j]/Pharmacological reference, computational tools (e.g. BioTransformer, Meteor Nexus, ADMET Predictor, Simcyp)	(U.S. EPA, 2020; Knox et al., 2024; Kim et al., 2025)
	Substance-dependent parameters: logP, pKa, molecular weight, protein binding, etc.	PubChem[k]/Drugbank	
	Lipinski's rule of five (likeliness of bioavailability): HH-bond donors (5 or less), H-bond acceptors (10 or less), molecular mass (<500 Da), LogK_{OW} (<5)	PubChem	(Kim et al., 2025)

[a] Numbers relate to the questions of the workflow (Fig. 1)
[b] References relate to the case study
[c] https://www.ebi.ac.uk/gxa/home (Papatheodorou et al. 2018, 2020).
[d] https://www.encodeproject.org/(Dunham et al., 2012; Davis et al., 2018).
[e] https://www.ncbi.nlm.nih.gov/snp/(Sherry et al., 2001).
[f] https://www.omim.org/.
[g] QIVIVE = quantitative *in vitro* to *in vivo* extrapolation.
[h] https://ipchem.jrc.ec.europa.eu/.
[i] https://www.cdc.gov/nchs/nhanes.
[j] https://www.drugbank.com/.
[k] https://pubchem.ncbi.nlm.nih.gov/.

method is of importance. The workflow for human relevance assessment includes evaluation of the relevance of a NAM, but not its reliability. The relevance assessment is done in a systematic way, implementing a number of considerations, including species and/or taxonomic class from which the NAM originated (human, mammal, non-mammalian), representativity of the test system for the evaluated process (organ/-tissue, developmental/life stage), complexity of the test system (ranging from *e.g.* simple cell line up to whole body), the physiological state of cell cultures (primary versus immortalized, wild-type versus genetically modified, grade of pathological transformation), directness of evidence and robustness of the NAM. Each of the considerations is scored by expert judgment for its supportive power to the evidence of human relevance (weak, moderate or strong), and for its estimated contribution (importance) to the overall assessment relative to other considerations. Robustness scores of the NAMs are based on their established use and level of validation and/or suitability for the measured response, but not explained in detail in the below. The combined assessments of evidence and importance for each NAM provide together the overall support to the human relevance of the pathway. As an example, the NAM measuring retinoic acid degradation in MCF-7 human breast cancer cells after exposure to a triazole (see Table 2) is scored "strong" for its human origin, "weak" for representativity, "weak" for its complexity/physiological state of the cells, "moderate" for the directness of the evidence, and "moderate" for its robustness. The overall support for human relevance from this NAM in the AOP was evaluated (by expert judgement involving the authors and a few of their colleagues) as "strong", because in this case the first criterion was considered more important than the other criteria (see section on the case study for more details). The combined relevance scores of all NAMs for a certain level of biological complexity in the AOP provide the overall evidence for that level of biological organization.

2.3. Chemical-specific application of the workflow

Perturbation of a toxicological pathway begins with interaction of the biological system with a stressor, which, in case of an AOP, is undefined. However, when the AOP is applied to a specific substance, an initial question is whether this substance will be available for the biomolecule(s) that is(are) involved in the MIE of that AOP. The workflow described above is then preceded by the question *'Is the substance (stressor) bioavailable in humans?'*. Strictly speaking, this is not part of the assessment of human relevance of the toxicological pathway; therefore, this question is defined as Q0 (Fig. 1). Q0 can be addressed in detail through biochemical analysis and pharmacokinetic/toxicokinetic

Table 2
Empirical evidence for human relevance of the MIE.

Stressor	NAM	Response	Ref.	Considerations	Evidence	Importance	Relevance[c]
Test azoles[a], liarozole	MCF-7 cells	RA degradation	(Pautus et al., 2006; Gomaa et al. 2012a, 2012b)	Species: human	strong	high	strong
				Representativity[b]: adult, breast cancer	weak	low	
				Complexity, cell status: immortalized transformed cells	weak	moderate	
				Directness of evidence	moderate	low	
				Robustness	moderate	moderate	
Talarozole	Human AoSMCs	RA degradation, expression of RA responsive genes	Ocaya et al. (2010)	Species: human	strong	high	strong
				Representativity: adult, aortic smooth muscle cells	weak	low	
				Complexity, cell status: normal primary cells	strong	moderate	
				Directness of evidence	moderate	low	
				Robustness	moderate	moderate	

[a] Series of triazole- and imidazole-derivatives, in drug development tested for retinoic acid metabolism blocking activity.
[b] Life stage and/or tissue represented by the test system.
[c] Relevance is the integrated assessment of evidence and importance of all considerations, thus providing the overall level of evidence of human relevance of the generated response of the specific test method (NAM); the combined relevance scores produce the overall evidence for the stage in the toxicological pathway (summarized in Table 11).

modelling. Such analysis may reveal potency differences between different stressors; for example, structure differences among agricultural triazoles may give a different magnitude in CYP inhibition. Alternatively, bioavailability of a stressor can be (roughly) estimated on the basis of the physicochemical properties of the substance. For the latter, in cases where more detailed information is lacking, Lipinski's rule of five provides a pragmatic tool, which states that chemicals can be considered more likely to be orally active if they have a molecular mass less than 500 Da, an octanol-water partition coefficient below five, no more than five hydrogen bond donors, and no more than ten hydrogen bond acceptors (Lipinski et al., 2001).

2.4. Case study AOP: application of the workflow

In order to demonstrate how the workflow for human relevance assessment could be applied for regulatory purposes, we used the AOP 'Disruption of retinoic acid metabolism leading to developmental craniofacial defects' (Fig. 2; Menegola et al., 2021) as a case study. The AO is considered relevant for human health risk assessment, because craniofacial malformations have been observed in infants after exposure to triazoles (Lee et al., 1992; Pursley et al., 1996; Aleck and Bartley, 1997; Lopez-Rangel and Van Allen, 2005). A graphical representation of this AOP is shown in Fig. 2, while its KEs and KERs are listed in Table 3. Craniofacial development starts with the morphogenesis of cranial tissues leading to the formation of the pharyngeal arches (also referred to as branchial arches) which, in humans, takes place between Carnegie stages 9–13 corresponding to day 20–31 of gestation (GD20-31). This process is evolutionary extremely conserved, and it is tightly regulated by morphogenetic molecules, including retinoic acid (RA). RA is a small soluble molecule secreted by caudal regions expressing synthetic enzymes (particularly retinaldehyde dehydrogenase, *RALDH* and its variants) and degraded by refractory regions expressing catabolic enzymes (particularly cytochrome P450 26, *CYP26*). The balance between synthesis and degradation allows the morphogenetic RA gradient formation

Table 3
Events in the AOP 'Disruption of retinoic acid metabolism leading to developmental craniofacial defects'.

Event		Level of biological organization
MIE	Inhibition of CYP26 activity	Molecular
KER1	CYP26 inhibition leads to reduced clearance of retinoic acid	
KE1	Increased retinoic acid levels	Cellular
KER2	Increased retinoic acid levels alter HOX expression	
KE2	Altered HOX gene expression	Tissue
KER3	Altered HOX expression disrupts NCC specification, migration and differentiation	
KE3	Disrupted NCC specification, migration, differentiation	Tissue
KER4	Disrupted NCC specification, migration and differentiation leads to dysmorphology of branchial arches	
KE4	Dysmorphology of branchial arches in the developing embryo	Organism
KER5	Dysmorphology of branchial arches in the developing embryo leads to craniofacial malformations in the newborn	
AO	Craniofacial malformations in the newborn	Organism

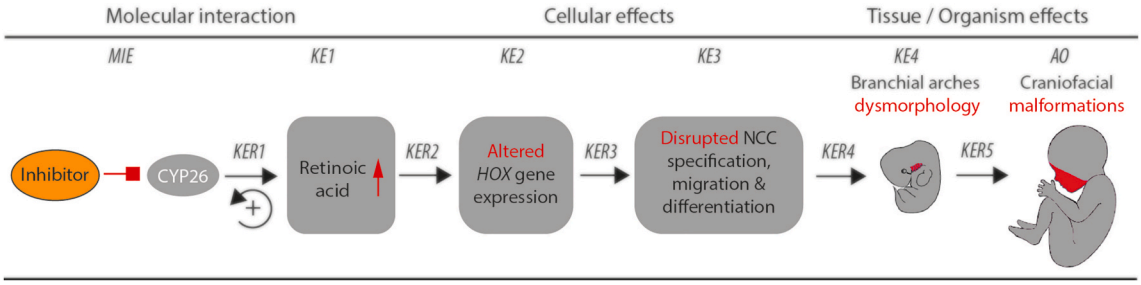

Fig. 2. Graphical representation of the AOP 'Disruption of retinoic acid metabolism leading to developmental craniofacial defects'. Inhibition of CYP26 isoforms by specific stressors (particularly triazoles) leads to reduced catabolism of retinoic acid. Consequently, retinoic acid accumulates, altering HOX gene expression, which in turn leads to altered regional specification of neural crest cells (NCCs), and subsequently impaired migration and differentiation of these cells. In the developing embryo, this leads to dysmorphology of the branchial arches, which is the basis for craniofacial malformations in the newborn.

(Dubey et al., 2018). As far as the craniofacial formation is concerned, the early established RA gradient induces brain-derived neural crest cell (NCC) specification and their migration at the level both of the head and of the pharyngeal regions, where they condense and differentiate into craniofacial elements (Cordero et al., 2011).

The RA morphogenetic signal regulates expression of genes with particular roles in different embryonic processes, including cranial NCC specification. Relevantly, RA regulates *homeobox* (*HOX*)-code genes expression, inducing NCCs to be specified and to migrate to dedicated regions in the pharyngeal arches, where they differentiate to contribute to specific craniofacial structures, which are established in week 4–10 after gestation (Schoenwolf et al., 2009). Consequently, NCCs are major determinants of the basic morphology of the face.

Prototypic stressors of the AOP are triazoles, which are organic heterocyclic compounds with numerous applications, including pharmaceutical and agrochemical fungicide, corrosion inhibitor, dyestuff, optical brightener, fluorescent whitener, and photo stabilizer of polymers. The pharmacological target of triazole fungicides is inhibition of CYP51 in mold, modifying ergosterol/lanosterol metabolism, subsequently impairing the formation of the fungal cell wall and thus fungus growth (Giavini and Menegola, 2012). Triazoles can also inhibit mammalian CYP51, modifying cholesterol synthesis, leading to accumulation of lanosterol and altered regulation of sonic hedgehog signalling (Krakowiak et al., 2003; Keber et al., 2011; Giavini and Menegola, 2012). Defects in cholesterol synthesis and related impaired sonic hedgehog signalling underlay an Antley-Bixler syndrome-like phenotype (Giavini and Menegola, 2012), which is characterized by craniosynostosis and midface hypoplasia (Fukami and Ogata, 2014). However, triazoles can also affect various other CYP enzymes in off-target species (Giavini and Menegola, 2010), and CYP26 is particularly sensitive to inhibition by triazole agents. Inhibition of CYP26 results in increased retinoic acid levels (Fig. 2; Giavini and Menegola, 2012), and subsequent dysregulation of *HOX* gene expression results in aberrant regional identity of NCCs (disrupted specification), affecting their migration and differentiation. In the developing embryo, this leads to dysmorphology of the branchial arches, which is the basis for craniofacial defects in the newborn.

Human exposure to triazoles occurs after pharmaceutical treatment of fungal infections, which is typically with high doses of limited duration. Human exposure can also occur chronically at low doses, after contamination of food with agricultural triazoles used for crop protection (Boon et al., 2015).

3. Evaluation of human relevance

3.1. Molecular initiating event

3.1.1. Background

The MIE of the AOP is defined as 'Inhibition of CYP26 activity' (Fig. 2; Table 3). Since the AO is induced during development, considerations such as sensitive time window and life stage are of relevance here. The cytochrome P450 enzymes *CYP26A1* and *CYP26B1* are expressed in all analysed tissues of the human embryo between the earliest and the latest sampling time reported (*i.e.* 4-19 weeks post conception), whereas *CYP26C1* expression is mainly observed in the brain, during week 4–16 post conception (EMBL-EBI, 2024; Cardoso--Moreira et al., 2019). *CYP26* expression is similar in males and females (EMBL-EBI, 2024). Regarding evolutionary conservation (Q1.1), both *CYP26A1* and *CYP26B1* are shown to be essential in murine embryo development, whereas *CYP26C1* knockout mice are viable (Thatcher and Isoherranen, 2009). Homologs of *CYP26* have been isolated from human, mouse, chick, zebrafish, and *Xenopus* with all the genes exhibiting a high degree of sequence conservation (Loudig et al., 2000; Rodríguez-Marí et al., 2013).

In silico modelling indicates that triazole compounds can interact with the active site of CYP26A1 (Ren et al., 2008), inhibiting CYP26 activity. This analysis (called molecular docking) has shown that triazoles may bind similarly to CYP enzymes in humans, rats and zebrafish (Table 4). These results support binding of triazoles to human CYP26 and suggest utility of rat whole embryo culture (WEC) and zebrafish embryo results to model effects of triazole-CYP26 interaction in humans.

3.1.2. Evidence assessment

Table 2 lists two NAMs that could be used for measuring CYP26 inhibition: MCF-7 breast cancer cells and Aortic Smooth Muscle cells (AoSMCs). Multiple triazoles have been demonstrated to inhibit CYP26 activity in MCF-7 cells and AoSMCs (Pautus et al., 2006; Ocaya et al., 2010; Gomaa et al. 2012a, 2012b). We consider evidence obtained from both MCF-7 cells and AoSMCs strong support for human relevance of this event, despite the fact that these test systems are not directly representative of the period in which facial morphology is determined in the developing embryo, nor for the target tissue. However, CYP26 is known to be also expressed in adult humans, in fully matured cells, and the first criterion is therefore considered most important. Thus, given the biological plausibility of the MIE, together with the evidence obtained from the two test models, we consider the human relevance of the MIE to be strong.

3.2. Key events, key event relationships, and adverse outcome

3.2.1. KER1/KE1: Reduced clearance of retinoic acid leads to increased levels of retinoic acid

3.2.1.1. Background. KER1 is described as 'CYP26 inhibition leads to reduced clearance of retinoic acid', while KE1 is described as 'Increased retinoic acid levels'. Q1 ('*Is the event likely to occur in humans?*') can be answered affirmative, because it is known that, similar to other vertebrates, during human embryonic development a retinoic acid gradient across the hindbrain is formed, resulting from differential expression of *RALDH* and *CYP26* enzymes (Dubey et al., 2018). Since CYP26 isoenzymes have been characterized as the main enzymes responsible for retinoic acid clearance, retinoic acid concentrations are low at the site of *CYP26* expression (Schoenwolf et al., 2009). Hence, inhibition of CYP26 (MIE) leads to a decreased clearance of retinoic acid (KER1), which results in increased retinoic acid levels at the active site (KE1). There are no known differences in retinoic acid regulation between males and females in relation to craniofacial malformations. Of the three isoforms, CYP26A1 is the most efficient P450 enzyme in retinoic acid clearance in

Table 4
Binding energy [a] of triazoles with CYP26 enzymes in human, rat and zebrafish.

Stressor	Human		Rat		Zebrafish	
	CYP26A1	CYP26B1	CYP26A1	CYP26B1	Cyp26a1	Cyp26b1
Cyproconazole	−8.5	−8.5	−8.7	−8.0	−8.1	−8.2
Prochloraz	−7.7	−7.9	−8.0	−7.6	−7.9	−7.8
Propiconazole	−8.3	−8.3	−8.0	−7.8	−8.2	−7.5
Triadimenol	−9.0	−7.7	−8.3	−8.4	−7.7	−8.3

[a] The unit of the free binding energy is kcal/mol. Only a few triazoles are shown as examples, but the binding energy is similar for many triazoles. Data obtained from (Heusinkveld et al., 2020; Metruccio et al., 2020).

isolated human liver microsomes (Thatcher et al., 2010). Several single nucleotide polymorphisms have been reported for *CYP26A1* and *CYP26B1* (Lee et al., 2007; Krivospitskaya et al., 2012; Fransén et al., 2013; Wu et al., 2015), of which two SNPs in *CYP26A1* exhibited significantly lower metabolism (Lee et al., 2007). Retinoic acid metabolism by CYP26 is conserved among humans (Thatcher et al., 2010), mice (Han et al., 2010), rats (Xia et al., 2010), zebrafish (White et al., 1996) and *Xenopus* (Hollemann et al., 1998).

3.2.1.2. Evidence assessment. NAMs that are potentially relevant to KER1/KE1 are listed in Table 5. Different triazoles have been indirectly shown to affect the levels of retinoic acid in human keratinocytes, by measuring the expression of retinoic acid responsive genes (Giltaire et al., 2009; Pavez Loriè et al., 2009b). In HUES-1 (human embryonic stem cells) or human keratinocytes, retinoic acid was shown to upregulate *CYP26B1* (Pavez Loriè et al., 2009a; Colleoni et al., 2011). This data is indirect evidence for KER1, because *CYP26B1* induction results from the feedback mechanism to tightly control retinoic acid levels. Nevertheless, the evidence supporting human relevance of KE1/KER1, although indirect, is considered strong, because both cell types are untransformed and of human origin, and HUES-1 cells are representative of the developing embryo during the period in which facial morphology is determined. In promyelocytic leukemia patients treated with fluconazole, elevated levels of retinoic acid were found (Schwartz et al., 1995; Vanier et al., 2003), proving that retinoic acid metabolism is affected in humans, although without specifying that this is due to inhibition of CYP26 enzymes. Based on the biological plausibility and the empirical evidence for KER1/KE1 in this AOP (Menegola et al., 2021), in combination with indirect measurements of effects of CYP26 inhibitors in human cell models and the whole human body, we consider the human relevance of KER1 and KE1 as strong.

3.2.2. KER2/KE2: Increased levels of retinoic acid leading to altered HOX gene expression

3.2.2.1. Background. Retinoic acid binds retinoic acid receptors to activate genes containing retinoic acid responsive elements (Schoenwolf et al., 2009), including body plan regulating *HOX* genes. Typically, local regulation of *HOX* gene expression by retinoic acid is a strict and specific process: some *HOX* genes are highly responsive and activated by low local retinoic acid levels, whereas activation of many other *HOX* genes is progressive, requiring higher retinoic acid concentrations. This balanced *HOX* gene response to increasing retinoic acid concentrations produces the so-called HOX-code (Glover et al., 2006), and aberrantly high levels of retinoic acid lead to a spatial shift in the expression of *HOX* genes, producing HOX-code dysregulation (Menegola et al., 2021). *HOX* genes are expressed in tissues of both male and female human embryos between the earliest and latest stage of sampling reported (*i.e.* 4- 19 weeks post conception) (EMBL-EBI, 2024).

3.2.2.2. Evidence assessment. Table 6 lists empirical evidence for KER2/KE2. Retinoic acid has been shown to induce/alter *HOX* expression in human embryonic cells and embryonal carcinoma cells (Simeone et al., 1991; Colleoni et al., 2011; Lippmann et al., 2015; Dou et al., 2016). The evidence from measurements conducted in normal embryonic stem cells for support of human relevance is considered strong, because the test models are untransformed cells of human origin representing the developing embryo during the period in which facial morphology is determined, and the evidence is direct. The evidence from test systems using embryonal carcinoma cells is also considered strong, despite the fact that these are transformed cells. In HepG2 liver cells, the measurements of RAR (retinoic acid receptor) activation after exposure to triazoles is considered moderate, because this empirical evidence is indirect evidence for changes in *HOX* gene expression. Moreover HepG2 cells are transformed liver cells and not representative of the life stage of interest (Seeger et al., 2019; U.S. EPA, 2020). Finally, increased

Table 5
Empirical evidence for human relevance of KER1/KE1.

Stressor	NAM/model	Response	Ref.	Considerations	Evidence	Importance	Relevance[b]
Talarozole, Liarozole, Ketoconazole	Human keratinocytes	Altered expression retinoic acid responsive genes	(Giltaire et al., 2009; Pavez Loriè et al., 2009b)	Species: human	strong	high	Strong
				Representativity[a]: adult, skin	weak	low	
				Complexity, cell status: Normal primary cells	strong	moderate	
				Directness of evidence	moderate	low	
				Robustness	moderate	moderate	
Retinoic acid	Human HUES-1[c] cells	*CYP26B1* upregulated	Colleoni et al. (2011)	Species: human	strong	high	Strong
				Representativity: embryo, stem cells	strong	low	
				Complexity, cell status: untransformed	moderate	high	
				immortalized cells	moderate	low	
				Directness of evidence	strong	moderate	
				Robustness	moderate	moderate	
Retinoic acid	Human keratinocytes	Altered expression retinoic acid responsive genes	Pavez Loriè et al. (2009a)	Species: human	strong	high	Strong
				Representativity: adult, skin	weak	low	
				Complexity, cell status: Normal primary cells	strong	moderate	
				Directness of evidence	moderate	low	
				Robustness	moderate	moderate	
Fluconazole	Human blood samples	Increased levels of retinoic acid	(Schwartz et al., 1995; Vanier et al., 2003)	Species: human	strong	high	Strong
				Representativity: adult system	weak	low	
				Complexity: whole body	strong	moderate	
				Directness of evidence	weak	low	
				Robustness	moderate	moderate	

[a] Life stage and/or tissue represented by the test system.
[b] Relevance is the integrated assessment of evidence and importance of all considerations, thus providing the overall level of evidence of human relevance of the generated response of the specific test method (NAM); the combined relevance scores produce the overall evidence for the stage in the toxicological pathway (summarized in Table 11).
[c] HUES, human embryonic stem cells.

Table 6
Empirical evidence for human relevance of KER2/KE2.

Stressor	NAM	Response	Ref.	Considerations	Evidence	Importance	Relevance [b]
Retinoic acid	Human HUES-1 cells	Increased expression of *HOX* genes, incl. *HOXA1*, *HOXA3*, *HOXB1*, *HOXB4*	Colleoni et al. (2011)	Species: human	strong	high	Strong
				Representativity[a]: embryo, stem cells	strong	low	
				Complexity, cell status: untransformed	moderate	moderate	
				immortalized cells	strong	high	
				Directness of evidence	strong	moderate	
				Robustness	strong	moderate	
Retinoic acid	Human H9 cells	Increased expression of *HOXA1*, *HOXB4, HOXC5*	Lippmann et al. (2015)	Species: human	strong	high	Strong
				Representativity[a]: embryo, stem cells	strong	low	
				Complexity, cell status: untransformed	moderate	moderate	
				immortalized cells	strong	high	
				Directness of evidence	strong	moderate	
				Robustness	strong	moderate	
Retinoic acid	Human H1 ESCs[c]	Increased expression of *HOXA* cluster	Dou et al. (2016)	Species: human	strong	high	Strong
				Representativity: embryo, stem cells	strong	low	
				Complexity, cell status: untransformed	moderate	moderate	
				immortalized cells	strong	high	
				Directness of evidence	strong	moderate	
				Robustness	strong	moderate	
Retinoic acid	Human embryonal carcinoma cells	Downregulation of *HOX3* and *HOX4*	Simeone et al. (1991)	Species: human	strong	high	Strong
				Representativity: embryo, tissue undefined	strong	low	
				Complexity, cell status: transformed immortalized cells	weak	moderate	
				Directness of evidence	strong	high	
				Robustness	weak	moderate	
Flusilazole Propiconazole Metconazole Tetraconazole	Human HepG2 cells	Activation Retinoic Acid Receptor	(Seeger et al., 2019; U.S. EPA, 2020)	Species: human	strong	high	Moderate
				Representativity: adult, liver	weak	low	
				Complexity, cell status: transformed immortalized cells	weak	moderate	
				Directness of evidence	moderate	high	
				Robustness	moderate	moderate	
Cyproconazole Flusilazole Hexaconazole Triadimefon	Zebrafish embryo, 5dpf[d]	Increased expression of *hoxb1a*	Heusinkveld et al. (2020)	Species: zebrafish	weak	high	Moderate
				Representativity: 5dpf[d] embryo, whole system	moderate	moderate	
				Complexity: whole organism	strong	moderate	
				Directness of evidence	moderate	high	
				Robustness	moderate	moderate	

[a] Life stage, tissue, represented by the test system.
[b] Relevance is the integrated assessment of evidence and importance of all considerations, thus providing the overall level of evidence of human relevance of the generated response of the specific test method (NAM); the combined relevance scores produce the overall evidence for the stage in the toxicological pathway (summarized in Table 11).
[c] ESCs: Embryonic Stem Cells.
[d] dpf, days post fertilization, i.e. after completion of neural crest cell specification, migration, and differentiation.

expression of *hoxb1a* in zebrafish embryos after exposure to triazoles (Heusinkveld et al., 2020) is also considered moderate evidence, because of limited directness and the used species, which are the most important considerations. Based on the biological plausibility and the empirical evidence collected of KER2 and KE2 in this AOP (Menegola et al., 2021), in combination with measurements of changes in the expression of *HOX* genes in human embryonic cells as the most relevant test method, the human relevance of KER2/KE2 is considered strong.

3.2.3. *KER3/KE3: Altered HOX gene expression disrupts NCC specification, migration and differentiation*

3.2.3.1. Background. Similarly to development in other vertebrates (Kulesa et al., 2004; Menegola et al., 2021) NCCs originating from the hindbrain in the human embryo migrate to the pharyngeal arches according to their specification, and differentiate according to their final pharyngeal arch location (Schoenwolf et al., 2009). Changes in HOX-code expression cause dysregulation of the NCC specification and abnormal NCC migration patterns (Parker et al., 2018).

3.2.3.2. Evidence assessment. Empirical support for human relevance of KER3/KE3 is listed in Table 7. Differentiating human ESCs (embryonic stem cells), have been shown to have increased expression of HOX genes (Atkinson et al., 2008; Lee et al., 2011). This evidence for KER3/KE3 is considered moderate, because the test system comprises untransformed cells of human origin, representing the life stage of interest, but the evidence is indirect and the cells are immortalized. By contrast, the evidence provided by measurements of NCC migration (Zimmer et al., 2014) or specification (Huang et al., 2016) using human ESCs and iPSCs (induced pluripotent stem cells) is direct, which contributed to

Table 7
Empirical evidence for human relevance of KER3/KE3.

Stressor	NAM	Response	Ref.	Considerations	Evidence	Importance	Relevance[b]
HOX	Human ESCs[c]	Differentiating cells have increased *HOX* expression	(Atkinson et al., 2008; Lee et al., 2011)	Species: human	strong	high	Moderate
				Representativity[a]: embryo, stem cells	strong	low	
				Complexity, cell status: Untransformed immortalized cells	moderate	moderate	
				Directness of evidence	weak	moderate	
				Robustness	moderate	moderate	
Triadimefon Cyproconazole	Human H9 cells	Inhibition NCC migration	Zimmer et al. (2014)	Species: human	strong	high	Strong
				Representativity: embryo, stem cells	strong	low	
				Complexity, cell status: Untransformed immortalized cells	moderate	moderate	
				Directness of evidence	strong	moderate	
				Robustness	moderate	moderate	
Retinoic acid	Human ESCs and iPSCs[d]	increased *HOX* expression of A/B/C/D clusters, specification towards trunk NCCs	Huang et al. (2016)	Species: human	strong	high	Strong
				Representativity: embryo, stem cells	strong	low	
				Complexity, cell status: Untransformed immortalized cells	moderate	moderate	
				Directness of evidence	strong	moderate	
				Robustness	moderate	moderate	
Retinoic acid	Rat, whole embryo culture	Disrupted NCC migration	Lee et al. (1995)	Species: rat	moderate	high	Moderate
				Representativity: embryo, whole system	strong	moderate	
				Complexity: whole organism	strong	high	
				Directness of evidence	moderate	moderate	
				Robustness	strong	moderate	

[a] Life stage and/or tissue represented by the test system.
[b] Relevance is the integrated assessment of evidence and importance of all considerations, thus providing the overall level of evidence of human relevance of the generated response of the specific test method (NAM); the combined relevance scores produce the overall evidence for the stage in the toxicological pathway (summarized in Table 11).
[c] ESCs: Embryonic Stem Cells.
[d] iPSCs: induced pluripotent stem cells.

considering this empirical evidence as strong. Evidence of disrupted NCC migration in rat whole embryo cultures after exposure to retinoic acid (Lee et al., 1995) is also direct, but the species difference turns the relevance of this assay to moderate. Overall, based on the biological plausibility and the empirical evidence collected for KER3 and KE3 of this AOP (Menegola et al., 2021), combined with measurements related to changes in specification, migration and/or differentiation of NCCs in human ESCs and in related models, the evidence for human relevance of KER3/KE3 is considered to be strong.

3.2.4. KER4/KE4: Disrupted NCC specification, migration and differentiation leads to dysmorphology of branchial arches in the developing embryo

3.2.4.1. Background. As described above, the pathway leading to facial morphogenesis is determined by proper specification, migration, and differentiation of NCCs, which contribute to the development of branchial arches (Schoenwolf et al., 2009), and it is evolutionary conserved across vertebrates (Menegola et al., 2006b).

Table 8
Empirical evidence for human relevance of KER4/KE4.

Stressor	NAM	Response	Ref.	Considerations	Evidence	Importance	Relevance[b]
Retinoic acid	Rat; whole embryo culture	Disrupted NCC migration	Lee et al. (1995)	Species: rat	moderate	high	Moderate
				Representativity[a]: embryo, whole system	strong	low	
				Complexity: whole organism	strong	moderate	
				Directness of evidence	moderate	low	
				Robustness	strong	moderate	
Fluconazole Retinoic acid Imidazole Ketoconazole Enilconazole	Rat; whole embryo culture	Branchial arch dysmorphology	(Menegola et al. 2003, 2004, 2006a)	Species: rat	moderate	high	Moderate
				Representativity: embryo, whole system	strong	low	
				Complexity: whole organism	strong	moderate	
				Directness of evidence	weak	low	
				Robustness	strong	moderate	

[a] Life stage and/or tissue represented by the test system.
[b] Relevance is the integrated assessment of evidence and importance of all considerations, thus providing the overall level of evidence of human relevance of the generated response of the specific test method (NAM); the combined relevance scores produce the overall evidence for the stage in the toxicological pathway (summarized in Table 11).

3.2.4.2. Evidence assessment. Appropriate *in vitro* test methods for generating empirical evidence for KER4/KE4 do not exist (yet); empirical support for KER4/KE4 is provided by studies using for example cultured embryos from rat (Table 8) (Lee et al., 1995; Menegola et al. 2003, 2004, 2006a). There is no empirical evidence supporting the relationship between disrupted NCC specification, migration, and/or differentiation and branchial arch anomalies in humans. At present, imaging techniques of the human embryo in the first trimester are insufficient for proper diagnosis of facial defects (Mak and Leung, 2019). Additionally, large scale *ex vivo* human embryo analyses are descriptive, not providing causes for described anomalies (Gilbert-Barness and Debich-Spicer, 2005; Nakano et al., 2017). Hence, the evidence supporting human relevance of KER4/KE4 relies on evolutionary conservation, which is considered moderate.

3.2.5. KER5/AO: Dysmorphology of branchial arches in the developing embryo results in craniofacial malformations in the newborn

3.2.5.1. Background. Craniofacial structures develop from branchial arches by remodelling of these transient embryonic organs in a species-specific manner, building on a general skeletal developmental plan which is common to all vertebrates. By consequence, upon maturation, dysmorphology of the branchial arches in the developing embryo results in craniofacial malformations in the newborn (Passos-Bueno et al., 2009; Johnson et al., 2011). Triazoles have been demonstrated to induce branchial arch abnormalities in studies using rat whole embryo cultures (Menegola et al., 1999; Di Renzo et al. 2007, 2011c; Battistoni et al., 2019). Furthermore, triazoles and retinoic acid have been reported to induce craniofacial malformations in humans, zebrafish, *Xenopus*, rat, and mouse (Lee et al., 1992; Pursley et al., 1996; Aleck and Bartley, 1997; Lopez-Rangel and Van Allen, 2005; Menegola et al., 2005; Di Renzo et al. 2007, 2011a, 2011b; Mondal et al., 2017; Zoupa et al., 2020). However, in view of focus on NAMs here, the *in vivo* studies in rat and mouse are only included as background knowledge and excluded from the human relevance assessment (Table 9).

3.2.5.2. Evidence assessment. Empirical support for human relevance of the AO is listed in Table 9. There are several case reports of craniofacial malformations in humans after *in utero* exposure to fluconazole, a CYP26 inhibitor (Lee et al., 1992; Pursley et al., 1996; Aleck and Bartley, 1997; Lopez-Rangel and Van Allen, 2005). This contributes strong evidence in view of the human *in vivo* observations, whereas the indirectness of the effect provides only weak support, which however is considered of lesser importance. On the other hand, craniofacial malformation was not identified as an extra risk of oral fluconazole early in pregnancy in a recent large cohort study (Bérard et al., 2019). *In utero* exposure during early embryogenesis to isotretinoin (a synthetic vitamin A) has also been reported to induce craniofacial malformations, as part of a set of highly variable morphological and neural lesions collectively named “retinoic acid embryopathies” (Lammer et al., 1985; Coberly et al., 1996; Mondal et al., 2017). Although all these lines of evidence are indirect, they are all considered strong, because the evidence consists of actual case

Table 9
Empirical evidence for human relevance of KER5/AO.

Stressor	NAM/model	Response	Ref.	Considerations	Evidence	Importance	Relevance[b]
Fluconazole	Human; whole body	Craniofacial, skeletal and heart defects	(Lee et al., 1992; Pursley et al., 1996; Aleck and Bartley, 1997; Lopez-Rangel and Van Allen, 2005; Bérard et al., 2019)	Species: human	strong	high	Strong
				Representativity[a]: birth, whole system	strong	high	
				Complexity: whole organism	strong	high	
				Directness of evidence	weak	moderate	
				Robustness	n.a.		
Isotretinoin	Human; whole body	Craniofacial defects	(Lammer et al., 1985; Coberly et al., 1996; Mondal et al., 2017)	Species: human	strong	high	Strong
				Representativity: birth, whole system	strong	high	
				Complexity: whole organism	strong	high	
				Directness of evidence	moderate	moderate	
				Robustness	n.a.		
Alcohol	Human; whole body	Craniofacial defects	Sant’anna (2006)	Species: human	strong	high	Weak
				Representativity: birth, whole system	strong	high	
				Complexity: whole organism	strong	high	
				Directness of evidence	weak	High	
				Robustness	n.a.		
Fluconazole	*Xenopus laevis* embryo	Craniofacial defects	Di Renzo et al. (2011a)	Species: Xenopus	weak	high	Moderate
				Representativity: embryo,	strong	moderate	
				whole system	strong	high	
				Complexity: whole organism	moderate	low	
				Directness of evidence	strong	moderate	
				Robustness	strong	moderate	
Cyproconazole Flusilazole Triadimefon	Zebrafish embryo	Craniofacial defects	Zoupa et al. (2020)	Species: zebrafish	weak	high	Moderate
				Representativity: embryo,	strong	moderate	
				whole system	strong	high	
				Complexity: whole organism	moderate	low	
				Directness of evidence	strong	moderate	
				Robustness	strong	moderate	

[a] Life stage represented by the test system.
[b] Relevance is the integrated assessment of evidence and importance of all considerations, thus providing the overall level of evidence of human relevance of the generated response of the specific model or test method (NAM); the combined relevance scores produce the overall evidence for the stage in the toxicological pathway (summarized in Table 11); n.a., not applicable.

reports. Similarly, foetal alcohol syndrome, caused by maternal alcohol consumption during pregnancy, is characterized by a distinct pattern of craniofacial malformations. One of the hypothesized modes of action is that the maternal alcohol consumption leads to retinoic acid imbalance and altered *HOX* expression, thereby causing craniofacial malformations (Sant'anna 2006). This hypothesis supports the relevance of the AOP, although only weakly because of the indirectness of the effect, which is of high importance in this case. Further evidence comes from experimental triazole exposures in *Xenopus* and zebrafish, which leads to craniofacial malformations (Di Renzo et al., 2011a; Zoupa et al., 2020). Despite the robustness of these models, human relevance is considered moderate in view of the species differences.

3.3. Evidence from human syndromes/diseases

Further support for human relevance of the AOP could be obtained from human syndromes or diseases with a similar AO, which may have a similar (hypothesized) pathogenesis (Fig. 1). Hence, Q2 is defined as '*Do human diseases with a similar AO have a similar pathogenesis?*'. Considerations to be taken into account for this question include whether (epi) genetic factors play a role in the aetiology of the disease, whether it is plausible that the events of the AOP play a role in the disease, and whether alternative pathway(s) have been hypothesized for the disease (Table 1).

Syndromes that are potentially relevant for this case study include the DiGeorge syndrome and the Antley-Bixler syndrome (Table 10). DiGeorge syndrome is characterized by palatal abnormalities and phenocopied by the loss of NCCs. Patients suffering from this syndrome have a depletion on the long arm of chromosome 22, at position 11.2 (Lambert et al., 2018). Due to the depletion two genes are lost, causing DiGeorge syndrome: *TBX1* (T-Box Transcription Factor 1) and *CRKL* (CT10 Regulator of Kinase (CRK)-like protein). *TBX1* is a transcription factor expressed in the pharyngeal endoderm and mesoderm. Loss of *TBX1* and/or *CRKL* has been linked to increased retinoic acid signalling in the cranial pharyngeal arches (Schoenwolf et al., 2009), providing support for human relevance of the AOP. One of the clinical features of the Antley-Bixler syndrome is midface hypoplasia (Fukami and Ogata, 2014). The Antley-Bixler syndrome is characterized by a deficiency of the cytochrome P450 oxidoreductase (POR) enzyme. This deficiency in POR is caused by mutations in several cytochrome P450 enzymes, such as CYP26A1, CYP26B1 and CYP26C1 (Fukami and Ogata, 2014), providing further support for human relevance of the AOP. Human null and hypomorphic mutations in the *CYP26B1* gene have been reported to lead to skeletal and craniofacial malformations, resulting in a phenotype that is similar to the Antley-Bixler syndrome (Laue et al., 2011; Morton et al., 2016). Genetic mutations in other key events, such as retinoic acid receptor beta and *HOX* genes, have also been associated with craniofacial defects (Tischfield et al., 2005; Bosley et al., 2008; Srour et al., 2016). Taken together, the evidence from these syndromes for human relevance of the AOP is considered strong.

3.4. Quantitative differences due to which human relevance might be excluded

Quantitative differences could affect the proportionality of the response-response relationships across the AOP and thus downgrade the human relevance of an AOP. We consider two different types of quantitative differences of relevance here: interspecies differences as well as differences between *in vitro* test methods and the human *in vivo* situation. Q3 is thus aimed to assess whether such differences apply and to estimate the order of magnitude, to address the question whether human relevance of the observed effect might be reasonably excluded because of these quantitative differences. Interspecies differences relate to information obtained from toxicity tests conducted in experimental animals as well as information from NAMs using materials of non-human origin. Quantitative differences between species may be limited for a single KER in an AOP; however, the sum of such differences across an entire AOP could become significant for the assessment of human relevance. For the AOP studied here, data used to assess the human relevance of the AOP was derived from humans or NAMs. Therefore, quantitative differences between NAMs were considered the most important issue here. Quantitative *in vitro – in vivo* differences can be manifold, including differences in intracellular concentrations due to toxicokinetic differences, differences in sensitivity of receptor binding, differences in polymorphisms that affect the biological response etc. Such differences could affect the relevance of the NAMs evaluated for the AOP under study. For the case study presented here, we considered it too early to conduct such an assessment; rather the criteria for evaluating the relevance of NAMs should first be further developed. When assessing data for either interspecies differences or differences between *in vitro* test methods and the human *in vivo* situation it is important to take into account the possibility that dose-dependent transitions in the principal mechanism of toxicity may have occurred. With increasing dose or concentration kinetic and/or dynamic factors related to the pathway under study may change, which impacts on the relationship of the response rate as a function of dose or concentration (Slikker et al., 2004a, 2004b).

Table 10
Evidence from human diseases.

Stressor	Response[a]	Ref.	Considerations[b]	Evidence	Importance	Relevance[c]
loss of *TBX1/CRKL (*DiGeorge syndrome)	Palatal abnormalities	Lambert et al. (2018)	Complexity: whole organism	strong	high	Strong
			Directness of evidence	weak	moderate	
CYP26XX mutation (Antley-Bixler syndrome	Skeletal malformations, craniofacial defects	Fukami and Ogata (2014)	Complexity: whole organism	strong	high	Strong
			Directness of evidence	moderate	moderate	
CYP26B1 mutation	Skeletal malformations Craniofacial defects	(Morton et al., 2016; Laue et al., 2011)	Complexity: whole organism	strong	high	Strong
			Directness of evidence	moderate	moderate	
RARB mutation	Craniofacial, skeletal and heart defects	Srour et al. (2016)	Complexity: whole organism	strong	high	Strong
			Directness of evidence	moderate	moderate	
HOXA1 mutation	Craniofacial defects	(Tischfield et al., 2005; Bosley et al., 2008)	Complexity: whole organism	strong	high	Strong
			Directness of evidence	moderate	moderate	

[a] Observed in human, whole body.
[b] Considerations are adapted to avoid fallacy of reasoning in Q2.
[c] Relevance is the integrated assessment of evidence and importance of all considerations, thus providing the overall level of evidence of human relevance of the observation; the combined relevance scores produce the overall evidence for the stage in the toxicological pathway (summarized in Table 11).

Table 11
Overall evidence for human relevance of the AOP.

Different elements of the workflow	Evidence
Q1:	
MIE	Strong
KER1/KE1	Strong
KER2/KE2	Strong
KER3/KE3)	Strong
KER4/KE4	Moderate
KER5/AO	Strong
Q2: Similar pathogenesis	Strong
Q3: Proportionality not affected	n.a.
Provisional conclusion	Strong

n.a., not assessed.

3.5. Overall support for human relevance of the AOP

The final step of the workflow is to combine and evaluate the evidence identified for each of the questions, in order to conclude on the overall support for human relevance of the pathway under study as well as its associated NAMs. An overview of the evidence for the case study AOP is presented in Table 11. Summarizing the evidence from its different components, the support for human relevance of the AOP obtained for Q1 is considered strong. The evidence from syndromes with a similar (hypothesized) pathogenesis (Q2) is also considered strong. Q3 did not indicate relevant interspecies differences; however, this question was not applied yet to the NAMs evaluated for Q1. Therefore, the evidence for Q3 could not be evaluated yet and only a provisional conclusion on the overall support for human relevance of the AOP could be drawn. Based on the available evidence, the weight of evidence supporting the human relevance of the AOP 'Disruption of retinoic acid metabolism leading to developmental craniofacial defects' is considered strong.

3.6. Chemical-specific application

Besides using the workflow for evaluation of the human relevance of an AOP *per se* and the relevance of a NAM for that AOP, one could also use the workflow in a chemical-specific fashion. For such an application, the assessment of the AOP would be preceded by questions regarding human exposure, such as: '*What are the conditions under which humans might be exposed*?', '*Is a specific population group of concern*?', etc.

For the AOP under study, exposure to women in their early pregnancy are of concern. The target is defined as the fate of NCCs and related developing facial structures in the embryo; the sensitive time window in humans ranges from GD20-31. Pharmacokinetic analysis of triazoles used for treatment of fungal infections shows that these drugs have an oral bioavailability that exceeds 90 %, and can even be close to 100 %, and have a good distribution to organs. Triazoles are metabolized in the liver by various cytochrome P450 enzymes (CYP2C19, CYP2C9, CYP3A4, CYP3A5) and parent compounds generally have a higher or similar toxicity as their metabolites (EFSA PPR Panel 2009). The elimination half-time ranges from 30 to over 100 h. Although data on bioavailability is poor for triazoles for agricultural use, estimation of the oral bioavailability using Lipinski's rule of five shows that such compounds follow all above mentioned criteria and are therefore expected to have a high oral bioavailability (Kim et al., 2025). Together with the moderate to long elimination half-time, this leads to the conclusion that exposure to triazoles via food is considered relevant to humans. For the AOP under study, triazoles in a toxicologically active form need to reach the embryo. It is well-known that diffusion across the human placenta is dependent on molecular weight, lipid solubility, degree of ionization and protein binding (Griffiths and Campbell, 2015). These criteria partly overlap with those for oral bioavailability and thus the molecular weight of triazole compounds is low enough (<500 Da; Griffiths and Campbell, 2015) to enable passage over the placenta, their lipophilicity is generally in a range to allow diffusion across the lipid membranes of the placenta, and their low pK_a probably sustains a non-ionized state in the blood, thus allowing placenta passage (Kim et al., 2025). On the other hand, protein binding of pharmaceutical triazoles, with the exception of fluconazole, is generally high (Knox et al., 2024), which may hamper availability for placenta passage. Overall, triazoles follow most criteria to cross the placenta, and in addition, triazoles have been shown to cross the placenta in mice, rats and rabbits (Pilmis et al., 2014). Further indirect evidence of placenta passaging and subsequent *in utero* exposure of fluconazole comes from several case reports of children with craniofacial anomalies after maternal treatment with this compound (Lee et al., 1992; Pursley et al., 1996; Aleck and Bartley, 1997; Lopez-Rangel and Van Allen, 2005).

For quantitative comparison between effective concentrations observed in the test models evaluated above and effective concentrations in the human body (in blood or at the target), chemical analysis or pharmacokinetic modelling is required. An extensive analysis is beyond the scope of this exercise. However, as an indication, an average steady-state peak serum concentration of 91.8 mg/L (~300 μM) was measured in patients receiving a high dose of fluconazole (2000 mg/day) for treatment of invasive mold infections (Anaissie et al., 1995), and in a range of 20.2–34.9 mg/L (~66–114 μM) in patients receiving 200–800 mg fluconazole/day (Schiave et al., 2018). Irrespective of potency differences, such concentrations exceed effective concentrations in the NAMs evaluated for the different components of the AOP, which are in the range of >1–50 μM (Supplementary Table S1), and the results from these NAMs are therefore estimated to be relevant for activation of the AOP.

A rough estimate of serum concentrations can also be made for chronic oral exposure to food contaminating conazole residues used for crop protection. Starting with residue concentrations in food of a relevant list of conazole contaminants in food, these can be converted to a probable oral dose (Sprong et al., 2020). For the purpose of illustrating the principle of determining the human relevance of this chemical specific oral dose, the values of a selection of 12 conazoles (epoxiconazole, triadimenol, propiconazole, prothioconazole, metconazole, cyproconazole, tebuconazole, flusilazole, paclobutrazol, uniconazole, flutriafol, triadimefon) were added, not accounting for different contributions or potency differences. This addition leads to a total oral intake of approximately 8 μg/kg body weight/day (Sprong et al., 2020). When calculating with a body weight of 60 kg, and similar pharmacokinetic behaviour as fluconazole (see above) but ignoring physicochemical differences, this would lead to an estimated added serum concentration of 0.17 μM. Comparison of this value to effective nominal concentrations of 1–50 μM in the listed NAMs leads to a lower margin of a factor 5.9, and the summed exposure of these compounds is therefore considered as relevant for humans. This justifies more precise calculations, considering exact individual compound characteristics and potencies, and pharmacokinetic details.

Thus, exposure to triazoles is relevant in humans, both related to intended pharmacological use and unintended consumption as food contaminants. Steady-state blood concentrations after pharmaceutical use are high when compared to effective doses in NAMs, and therefore human risk is also relevant in a quantitative way; this is supported by case reports linking maternal treatment with triazoles to craniofacial malformations in the infant. A rough estimate of the margin of exposure to an effective concentration in the listed NAMs also supports the relevance of human consumption of food contaminating triazoles in a quantitative way, although the lowest effective dose in humans for the induction of craniofacial malformations is not known.

The above described example is presented to illustrate how the workflow could be applied in a chemical-specific fashion. The exact needs in terms of data and information for a human health risk assessment strongly depend on the problem formulation at hand. There certainly is not a default need for NAM-based data for all KEs in the AOP. In case of sufficient understanding of the quantitative relationships

between the KEs in the AOP, data from NAMs for earlier KEs (*i.e.* at a lower level of biological complexity) could be sufficient to address the regulatory questions defined in the problem formulation, including the derivation of a point-of-departure, provided the level of uncertainty is considered acceptable. It should be noted that quantitation of the KERs could also involve indirect KERs, and would not necessarily require quantitation of all KERs in an AOP.

4. Discussion

The present manuscript describes a first effort to develop a pragmatic workflow for assessing human relevance of a defined AOP and associated NAMs in a transparent and scientifically sound manner. The workflow was developed using the existing WHO/IPCS framework for the assessment of human relevance as a reference (Cohen et al., 2003; Meek et al. 2003, 2014a; Seed et al., 2005; Boobis et al., 2006) and evaluated by using the AOP of 'Disruption of retinoic acid metabolism leading to developmental craniofacial defects' as a case study, for which a comprehensive (but not necessarily all-inclusive) set of NAMs was collected and assessed.

The case study showed that the workflow provides a promising tool for application for regulatory purposes, although further refinement and improvements may be achieved through additional case studies for both human relevant AOPs and non-human relevant AOPs. The current workflow consists of three main questions, related to the components of an AOP (Q1), epidemiological and/or clinical data (Q2), and quantitative aspects (Q3). The order of the questions was driven by the rationale that a quantitative assessment, which might be more challenging than a qualitative assessment, is redundant if an AOP would be considered non-relevant to humans in a qualitative manner. However, assessment of quantitative aspects is highly important since quantitative differences between species and/or between *in vitro* test methods and the human *in vivo* situation could downgrade the human relevance of an AOP. It should be noted that the quantitative assessment is different from developing a quantitative AOP, although quantification of KERs in different model systems with evolutionary conservation of the AOP could be a basis for extrapolation to humans. Such extrapolation across model systems should be done with a careful consideration of exposure conditions, sensitive time windows and toxicokinetic properties. Further elaboration of the workflow for assessing quantitative aspects is needed to produce an improved workflow.

Another important aspect of the workflow that needs further improvement is the evaluation of the evidence collected, both for assessing the human relevance of an AOP and for assessing the relevance of its associated NAMs. Evidence supporting human relevance of an AOP is evaluated according to the modified Bradford Hill criteria, with biological plausibility carrying more weight than empirical evidence (Meek et al., 2014b; Becker et al., 2015; Collier et al., 2016; Becker et al., 2017). In the current workflow, evidence was first evaluated separately for each component of an AOP (MIE, KEs/KERs, AO) and then integrated for the overall AOP. In the case study presented, we explicitly chose to rely solely on NAMs and human data where possible, with the aim to challenge the workflow. In practice, existing animal data that are relevant to the AOP under study should certainly be taken into account; thus, appropriate sources for information (Table 1) should be added to a further version of the workflow. The present assessment was based on defined criteria with related terms of evaluation and weighing of relative importance of each criterion for the assessed event in the AOP, with ensuing scores of evidence as 'strong', 'moderate' or 'weak'. Although scoring thus followed a defined template, expert judgement is still an important factor in the weighing, and this may be paired with the risk of (unintentional) bias. Although this can be defeated at least in part through a multi-evaluator effort, further objectivation of criteria definitions would increase the transparency of the evaluation. Another aspect to be included in further development is how to take into account evidence indicating non-relevance to humans. Furthermore, regarding the overall assessment, discussions between subject-area experts are needed to determine whether or not some components of the AOP should carry more weight than others, for example those at a higher level of biological complexity.

Assessment of the relevance of NAMs associated with an AOP was included in the workflow, because we consider this of utmost importance. Firstly, because this is essential for regulatory applications, as described in OECD Guidance Document 34 (OECD, 2005; update ongoing). Furthermore, having insight into the degree to which NAMs (ranging from simple *in vitro* tests to complex variants such as high-content imaging) bear relevance to the toxicological pathway operating in humans allows us to actually benefit from the knowledge gained from a species concordance analysis. After all, chemical safety assessments are conducted based on data in the context of our current knowledge on toxicology. Insight into the relevance of a NAM for a certain KE will greatly enhance its acceptance and implementation for regulatory purposes. This does not mean that NAMs which cannot be directly linked to a specific KE are not potentially useful for regulatory use. For example, transcriptomics approaches using *in vitro* test systems can be highly useful in revealing which toxicological pathways are perturbed upon chemical exposure; such NAMs can be linked to multiple KEs in different AOPs. It should also be noted that it is not needed to have relevant NAMs in place for all KEs in an AOP for a NAM to become useful for regulatory application.

We drafted a set of criteria for assessing the relevance of NAMs. It should be noted that the relevance is always assessed in the context of an AOP. Hence, the same NAM may be considered as highly relevant for one AOP, but not for another AOP. The criteria include species of origin of the test system, representativity of the test system for the evaluated process (organ/tissue, developmental/life stage), complexity of the model (one cell type culture up to whole organism), the physiological state of cell cultures (primary *versus* immortalized, wild-type *versus* genetically modified, grade of pathological transformation), and finally the directness of evidence provided and robustness of the test model (*e.g.* validated OECD test model, abundant literature, etc.). Each of the criteria was scored for its supportive power to the evidence of human relevance (weak, moderate or strong), and for its estimated contribution to the overall assessment relative to other criteria (importance). Additional case studies and discussions with experts in the field are needed to reach consensus on the criteria to be included. These criteria should be accompanied by a guidance for evaluating the evidence supporting the relevance of a NAM. Not all criteria are always applicable to each NAM and their weight may differ, dependent on the KE under study and the response measured or modelled.

It should be noted that assessment of the relevance of a NAM does not include its validation. In the context of regulatory application, validation of a NAM should preferably precede assessment of its relevance to a particular KE(s). Validation of NAMs requires a more flexible and knowledge-based approach compared to validation of traditional test methods (Eskes and Whelan, 2016; Burgdorf et al., 2019). Principles for validation of NAMs have been described in OECD Guidance Document 34 (OECD, 2005; update ongoing) and discussions on appropriate criteria are ongoing (Parish et al., 2020; Patterson et al., 2021).

Future case studies will help to further refine the workflow presented. Such case studies allow for verification of the aspects of the workflow that are truly necessary to assess human relevance of an AOP and the relevance of associated NAMs and reveal the aspects that may be discarded or should be improved. Aspects that prove to be essential should be given more weight in the overall evaluation. These aspects might be considered mandatory for a human relevance assessment: if case appropriate data are lacking for the essential aspects, data should first be generated before conducting the assessment. Lastly, additional case studies may lead to successful examples of how the workflow could be used in practice for human health risk assessment, based on well-defined and validated AOPs and NAMs.

Still, despite some areas for improvement, in view of its protocolized

assessment of human relevance of toxicological data, the described workflow represents an important step forward for application of AOPs and related NAMs in human health risk assessment.

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CRediT authorship contribution statement

Christina H.J. Veltman: Writing – original draft, Visualization, Methodology, Conceptualization. **Leo T.M. van der Ven:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Elena Menegola:** Writing – review & editing, Methodology, Investigation. **Mirjam Luijten:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mirjam Luijten reports financial support was provided by the Dutch Ministry of Health, Welfare, and Sports. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yrtph.2025.105828.

Appendix 1. Selected NAMs to illustrate effective concentrations

Agent	Model system	Conc.	Time point	Effect	Reference
MIE (CYP26 inhibition)					
Triazoles	Human MCF7 cells	0.5 nM-20 μM	Adult cells	Determination IC_{50} CYP26	(Pautus et al., 2006; Gomaa et al. 2012a, 2012b)
Talarozole	Human AOSMCs	1 μM	Adult cells	Effects are similar as upon *CYP26* silencing	Ocaya et al. (2010)
KE1 (increased retinoic acid levels) and KER1 (CYP26 inhibition leads to increased retinoic acid levels)					
RA	Human HUES-1 cells	200 nM	Blastocyst	*CYP26B1* is upregulated	Colleoni et al. (2011)
Talarozole	Human keratinocytes	1 nM–1 μM	Adult cells	Altered expression RA responsive genes	(Pavez Loriè et al., 2009a; Giltaire et al., 2009; Pavez Loriè et al., 2009b; Pavez Loriè et al. 2009c)
Fluconazole	Human blood samples	400 mg + 200 mg/day	Adult	Increased RA levels	(Schwartz et al., 1995; Vanier et al., 2003)
KE2 (altered HOX expression) and KER2 (increased retinoic acid levels alter HOX expression)					
Retinoic acid	Human H9 cells	1 μM	Embryo cells	*HOXA1*, *HOXB4* and *HOXC5* induced expression	Lippmann et al. (2015)
Retinoic acid	Human ESCs	1 μM	Embryo cells	*HOXA* cluster induced expression	Dou et al. (2016)
Retinoic acid	Human HUES-1 cells	2 nM	Blastocyst	Increased *HOX* expression	Colleoni et al. (2011)
Retinoic acid	Human embryonal carcinoma cells	0.1 μM	Embryo cells	*HOX3* and *HOX4* downregulation	Simeone et al. (1991)
Flusilazole, Propiconazole Metconazole Tetraconazole	Human HepG2 cells	10–20 μM	Adult cells	Activation RAR	(U.S. EPA, 2020; Seeger et al., 2019)
Retinoic acid	Human primary myeloid leukemia cells	22.5 mg/m^2 twice/day	Adult cells	Modulation *HOX* activity	Reikvam et al. (2017)
KE3 (disrupted NCC specification, migration and differentiation) and KER3 (altered HOX expression disrupts NCC specification, migration and differentiation)					
HOX	Human H9 cells	n.a.	Embryo	Differentiating cells have increased *HOX* expression	EMBL-EBI (2024)
Triadimefon	Human H9 cells	50 μM	Embryo	Inhibition NCC migration	Zimmer et al. (2014)
Cyproconazole		5 μM			
Retinoic acid	Human ESCs and iPSCs	1 μM	Embryo	Cranial NCCs specify as trunk NCCS	Huang et al. (2016)

Appendix 2. Estimation of dietary exposures to conazole residues

For the purpose of illustrating the principle of determining the human relevance of chronic oral exposure to food contaminating conazole residues used for crop protection, calculations of exposure estimates were performed for a selection of 12 conazoles, *i.e.* epoxiconazole, triadimenol, propiconazole, prothioconazole, metconazole, cyproconazole, tebuconazole, flusilazole, paclobutrazol, uniconazole, flutriafol, and triadimefon. For this, a database developed in the Horizon2020 project 'EuroMix' was used (Crépet et al., 2019). This 'EuroMix database' contains data on pesticide and other contaminant concentrations in food items from 2010 to 2014 from eight European countries. Calculations were performed using the Monte Carlo Risk Assessment tool (MCRA; version 8.2; https://mcra.rivm.nl), using food consumption data for the Dutch population aged 7–69 years (van Rossum et al., 2011) and concentration data (Sprong et al., 2020). Exposure calculations were performed for an upper bound scenario, which assumes that non-detects at the level of detection (LOD) or level of quantification (LOQ) equal the value of the LOD or LOQ. For the present study, p95 values for the

12 substances were summed to estimate a total chronic oral intake (Sprong et al., 2020). Based on these exposure estimates, serum concentrations were estimated assuming a body weight of 60 kg, and a similar pharmacokinetic behavior as fluconazole but ignoring physicochemical differences.

Data availability

No data was used for the research described in the article.

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