NAMs Use in the Development of AOPs and the Application of Systematic Review Practices for Hazard Evaluation and Predictive Toxicology

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DISCLAIMER: The views expressed are those of the presenters and do not reflect Agency policy.
A large number of systematic review slides and/or content are provided courtesy of Xabier Arzuaga.

Tox21 CPP-13 Participants

Predictive Toxicology of the Retinoid Signaling Pathway

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NCTR/FDA
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Miao Li

Tox21 contact
Nisha Sipes, CCTE

CPP-13 Aims and Deliverables

Aim 1. Formalize an Adverse Outcome Pathway (AOP) framework for the retinoid system.

Aim 2. Map high-throughput screening data from relevant assays in ToxCast/Tox21 profiles to the AOP framework.

Aim 3. Build and test computational models for quantitative disruption of ATRA signaling.

Knowledgebase
(skeletal development)

AOP-WIKI
(limb defects)

HTS-based signatures
(ToxPi classifier)

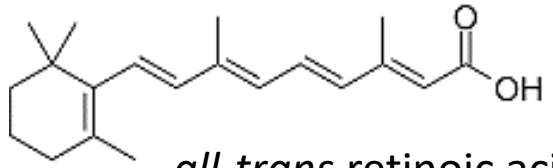
HTS data analysis
(ToxCast/Tox21/ChEMBL)

Pregnancy IVIVE models
(targeted case studies)

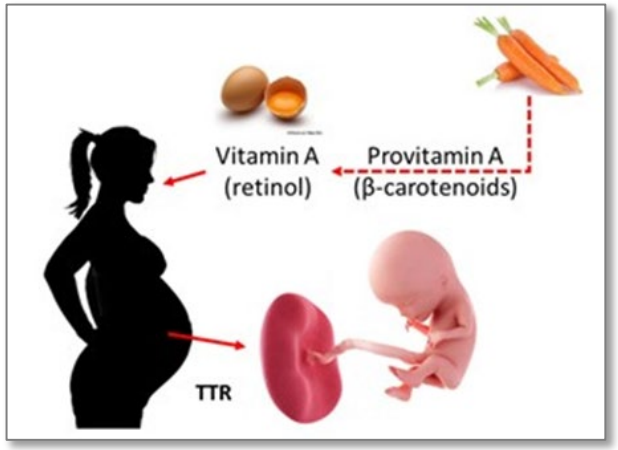
Performance-based prediction
(ATRA pathway in Devtox)

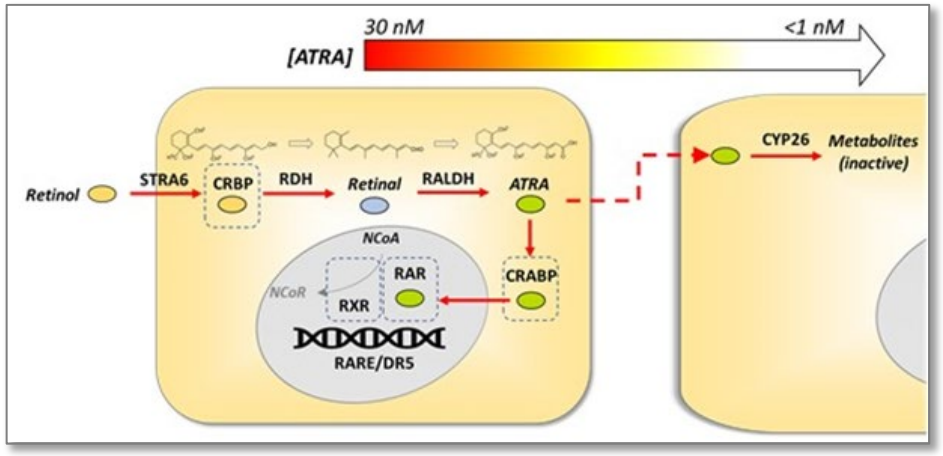
Morphoregulatory simulation
(Limb ABM)

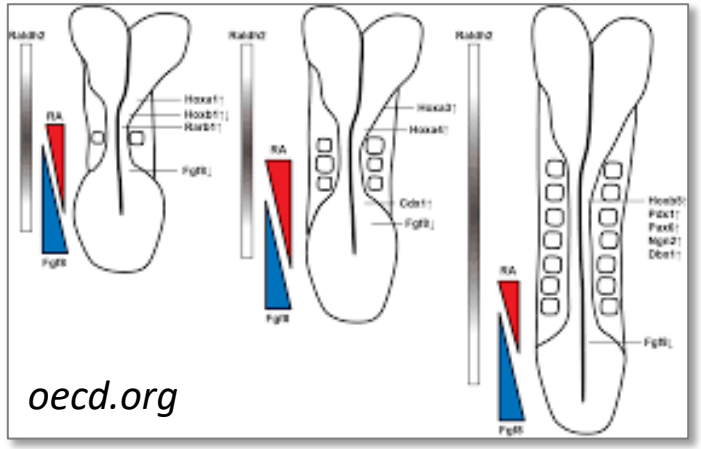
Retinoid signaling pathway



all-trans retinoic acid (ATRA)

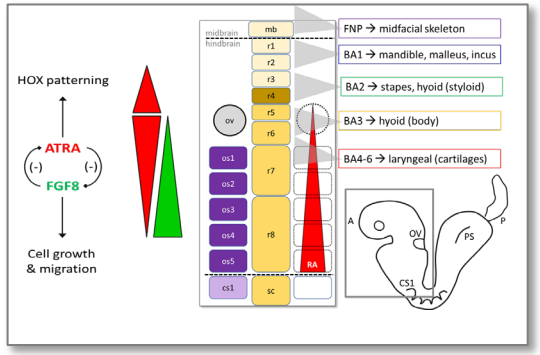




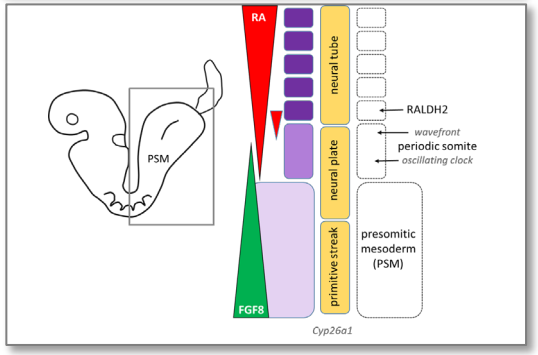


- ATRA is locally regulated by a complex network of enzymes, molecular transporters, and nuclear receptors (RARs) determined by cell-specific expression.
- ATRA gradients collaborate with some of the most powerful morphogenetic signals that shape embryonic growth and development (e.g., FGF, BMP, SHH, WNT, …).
- Local regulation of ATRA homeostasis and its disruption may be captured in diverse AOP frameworks linking molecular initiating events (MIEs) to adverse developmental outcomes.

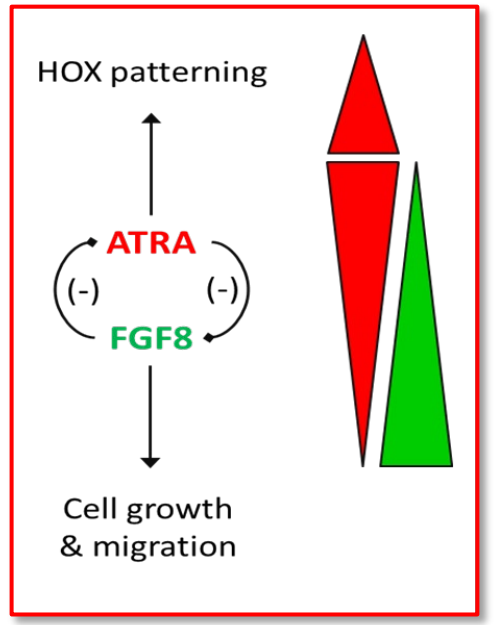
Regional domains for ATRA-dependent skeletal patterning

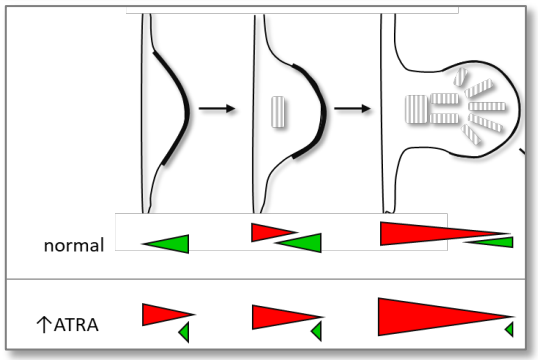


Facial skeleton: positional information of premigratory neural crest cells destined for branchial arches (5- to 11 somite stage).



Vertebral skeleton: size, alignment, and specification of somites giving rise to individual vertebrae/ribs (0- to 36 somite stage).





Appendicular skeleton: limb-bud initiation, outgrowth, patterning, and differentiation (12- to 36+ somite stage).

Knudsen et al., Reprod Toxicol (2021) – special issue devoted to retinoid signaling (Guest Editor: H Håkansson).

New Approach Methods (NAMs)

- Any technology, methodology, approach (including models), or combination thereof that can be used to provide information on chemical hazard and risk assessment that avoids the use of intact animals. For the purposes of TSCA, EPA recognizes this new term (i.e., NAMs) as encompassing any...

"[A]lternative test methods and strategies to reduce, refine, or replace vertebrate animals." (U.S. EPA)

Multi-Database Workflow

Toxicity Reference Database (ToxRefDB v1)
https://github.com/USEPA/CompTox-ToxRefDB
(2,946 prenatal developmental toxicity studies with adverse skeletal outcome)

ToxRefDB Skeletal Defect Studies and Associated Chemicals
57,198 composite skeletal defects across 363 chemicals (rat (31,1661), mouse (1,232), rabbit (16,375), chinchilla (368), other/unspecified (7562))

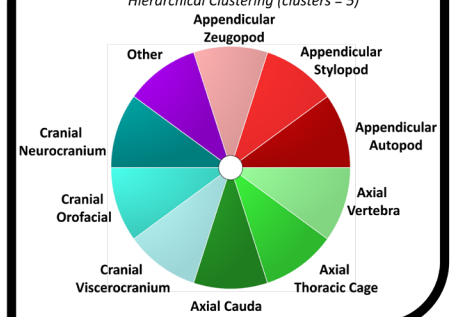
Retinoid Pathway Reference Chemicals
220 candidate reference chemicals were culled from Protein Data Bank, ChEMBL, ToxCast, and biomedical literature in PubMed. AbstractSifter tool identified the biological effects as consistent with retinoid pathway disruptions

Extraction of ToxCast Chemicals
AbstractSifter (Baker *et al.*, 2017) deduced 7 non-ToxRefDB chemicals of 42 benchmark (Zurlinden *et al.*, 2020) ToxCast chemicals demonstrate connection to skeletal defects

Regional Annotation for 370 chemicals
Appendicular (8,611): autopod (7,310), stylopod (969), zeugopod (332);
Axial (34,122): cauda (2,224), thoracic cage (19,132), vertebra (12,766);
Cranial (7,658): neurocranium (5,037), orofacial (2,426), viscerocranium (195);
Other (6,807): unspecified (6,807)

CompTox Chemicals Dashboard
https://comptox.epa.gov/dashboard
374 chemicals (of 8,079 tested chemicals) selected due to bioactivity across 1 or more of 10 assays for relevant ATRA pathway nodes (Knudsen *et al.*, 2020); Criteria for positivity called active based on efficacy and potency

Toxicity Prioritization Score (ToxPi v.2.3)
K-means Clustering by nearest centroid (k=4)
Hierarchical Clustering (clusters = 5)



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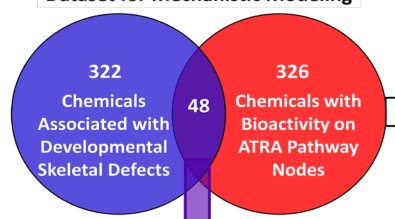
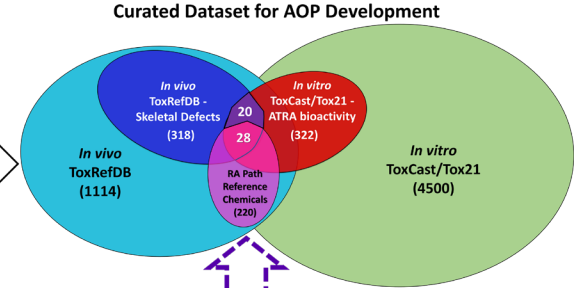
Dataset for Mechanistic Modeling



Curated Dataset for AOP Development



AOP Elucidation

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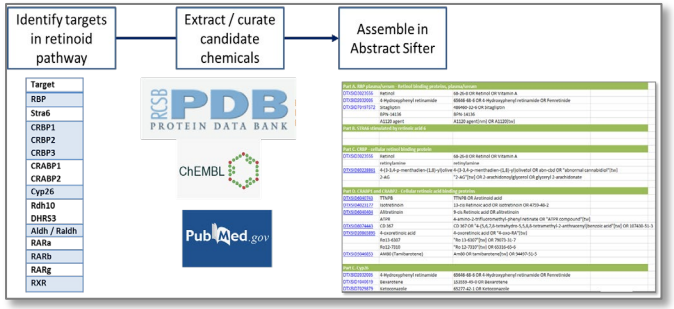
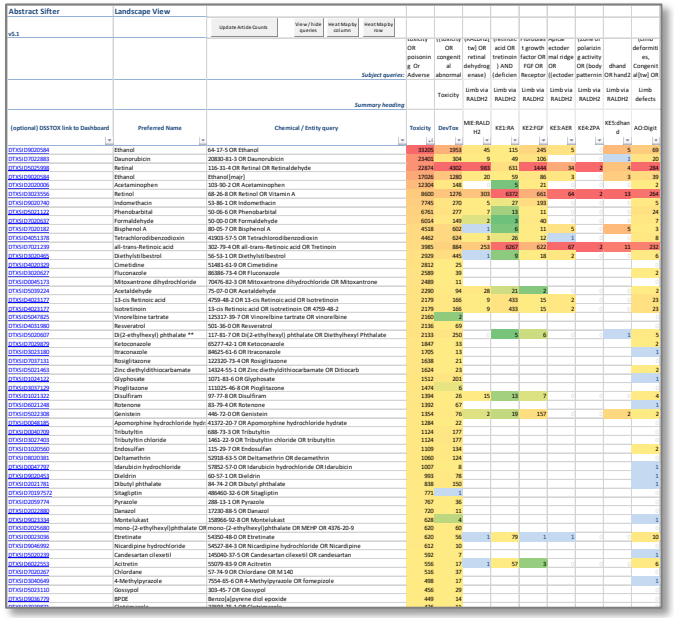
Reference chemicals for the ATRA system





>167 compounds

Retinoid pathway targets
RBP - Retinol binding protein – plasma
CRBP – cellular retinol binding protein
STRA6 – stimulated by retinoic acid
ADH - alcohol dehydrogenase
RALDH – retinal dehydrogenase
CRABP – cellular retinoic acid binding protein
CYP26 – cytochrome P450 family 26
RAR – retinoic acid receptor alpha, beta, gamma

- **Assemble candidate chemical list for each target:**
  - search databases (Protein Data Bank, ChEMBL, ToxCast/Tox21, …);
  - advanced literature mining to define bioactivity at each target.
- **For each candidate chemical:**
  - mine literature for MIEs, key events, and adverse outcomes;
  - e.g., skeletal defects, CNS defects, cardiovascular defects, ...
- **Compile top candidate chemicals for each target:**
  - detailed analysis of the Tox21 qHTS 10K dataset;
  - consult with domain experts.

Although HTS data exists for modeling downstream RAR/RXR responses, the availability of direct assays for upstream ATRA metabolism (eg, RDH10, RALDH2, CYP26a/b/c) and molecular transporters (eg, STRA6, CRABP1, CRABP2) are limited.

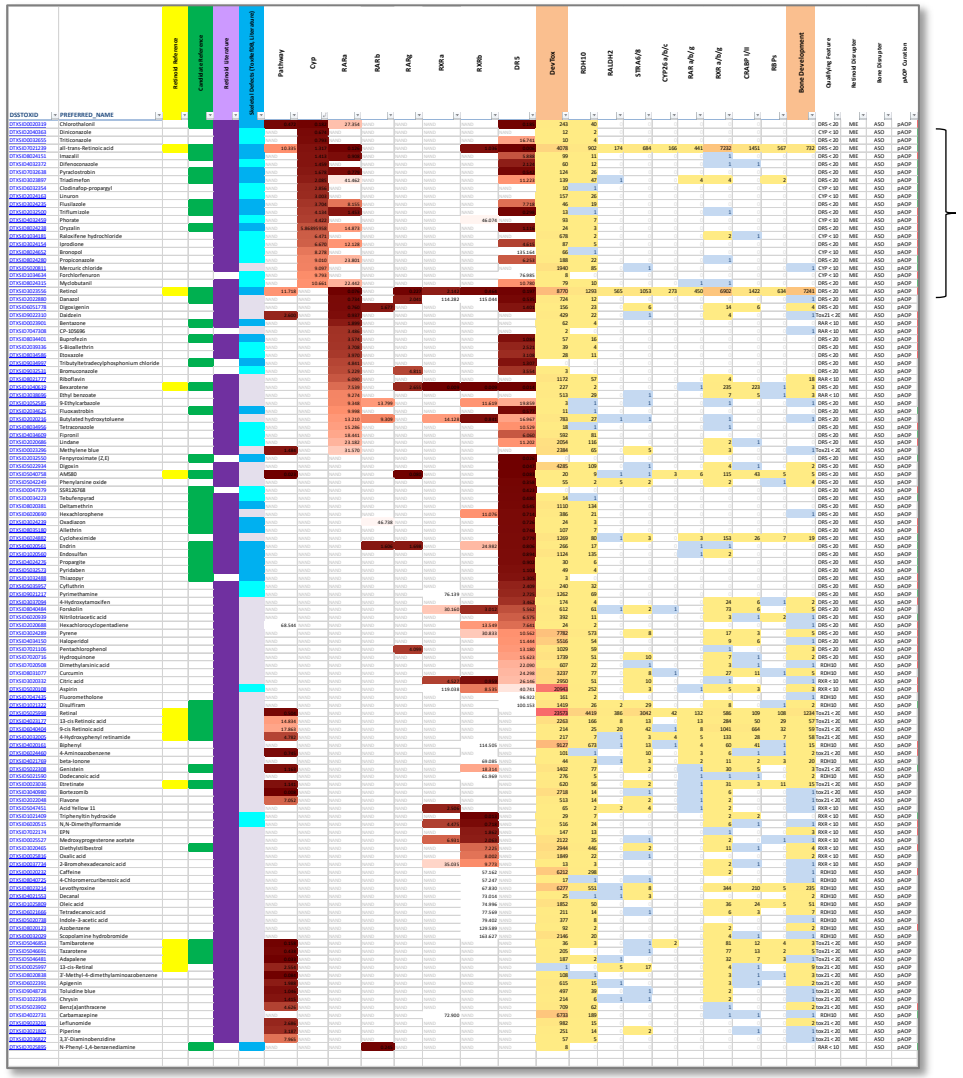
Baker et al. (manuscript under technical review)

Mapping potential MIEs to ASOs in the ATRA pathway

Chemicals (n=117)

In vitro bioactivity (k=8 assays)

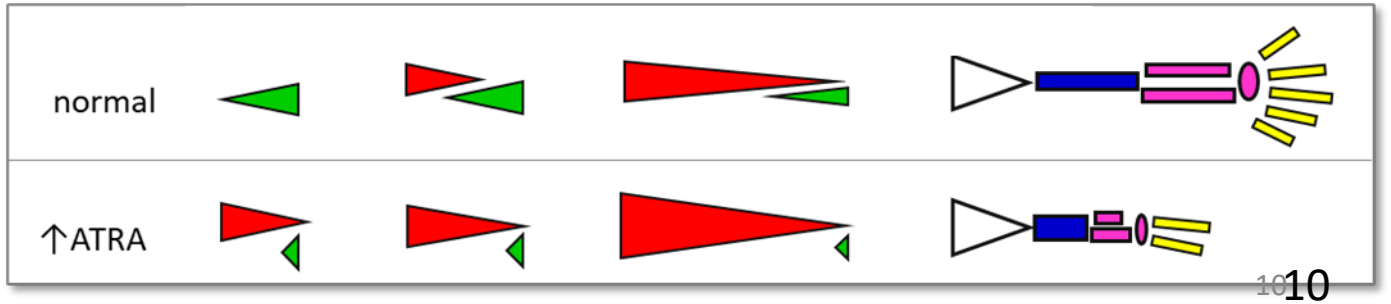
Literature (k=8 assays)



PREFERRED_NAME	Retinoid Reference	Candidate Reference	Retinoid Literature	Skeletal Defects (ToxRefDB, Literature)	Pathway	Cyp	RARa	RARb	RARg	RXRa	RXRb	DR5	DevTox	RDH10
Chlorothalonil					0.472	0.18192163	27.354	NAND	NAND	NAND	NAND	0.185	243	40
all-trans-Retinoic acid					10.335	1.317	0.126	NAND	NAND	NAND	1.036	0.006	4078	902
Imazalil					NAND	1.413	0.908	NAND	NAND	NAND	NAND	5.888	99	11
Pyraclostrobin					NAND	1.678	0.779	NAND	NAND	NAND	NAND	0.542	124	26
Triadimefon					NAND	2.085	41.462	NAND	NAND	NAND	NAND	11.223	139	47
Flusilazole					NAND	3.704	8.155	NAND	NAND	NAND	NAND	7.718	46	19
Triflumizole					NAND	4.134	1.453	NAND	NAND	NAND	NAND	0.298	13	1
Oryzalin					NAND	5.86895958	14.873	NAND	NAND	NAND	NAND	1.116	24	3
Retinol					11.718	NAND	0.076	NAND	0.227	2.142	0.464	0.197	8770	1293

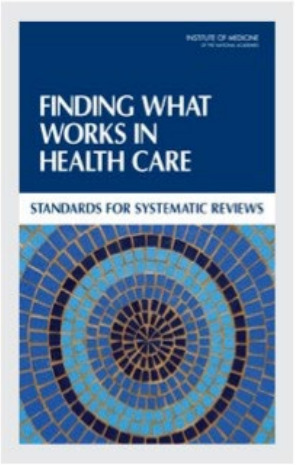
Limb-Bud Mesoderm	Hyperactivation of the RAR/RXR heterodimer	Underextension FGF8 signaling from the AER	Dysregulation of *Meis1/2* and *Hox* gene expression	Proximalization of the limb-bud mesenchyme	Mis-specification of precartilage blastema	Malformed cartilaginous bone rudiment	Phocomelia

Example: phocomelia can result when a 30-somite embryo experiences ATRA overload.



Systematic Review

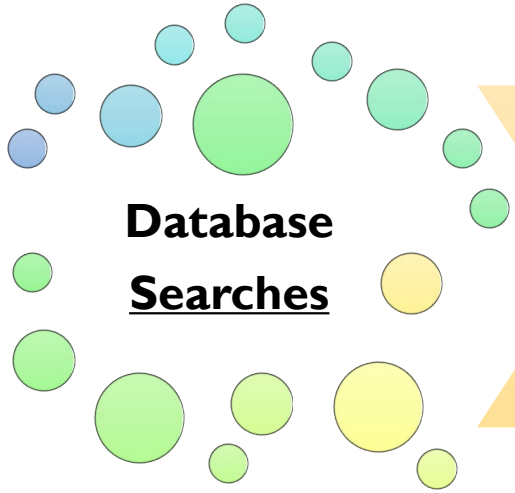
A structured and documented process for transparent literature review[1]



"As defined by IOM [Institute of Medicine], systematic review 'is a scientific investigation that focuses on a specific question and uses explicit, pre-specified scientific methods to identify, select, assess, and summarize the findings of similar but separate studies.'" [p. 4] (National Research Council [NRC], 2014)

[1] Institute of Medicine. Finding What works in Health Care: Standards for Systematic Reviews. p.13-34. The National Academies Press. Washington, D.C. 2011

Routine Evidence Identification Processes



- Identify peer-reviewed and "gray" (unpublished) literature
- PubMed, ToxLine, and Web of Science are standard (others can be included as needed)
- Conduct regular search updates
- Details of search strategy, dates, and retrieved records are presented in protocols and assessments

Screening

1. Title/abstract
2. Full text

- Use manual and automated approaches
- ≥ 2 screeners
- Tag studies as excluded, meeting PECO criteria, or supplemental information
- Screening decisions available in HERO
- Typically do not apply language-restrictions
- Review reference list of included studies and relevant reviews to identify studies missed from database searches
- Share list of included studies with public to further ensure all relevant studies included

Inventories

Health Outcome & PBPK Studies

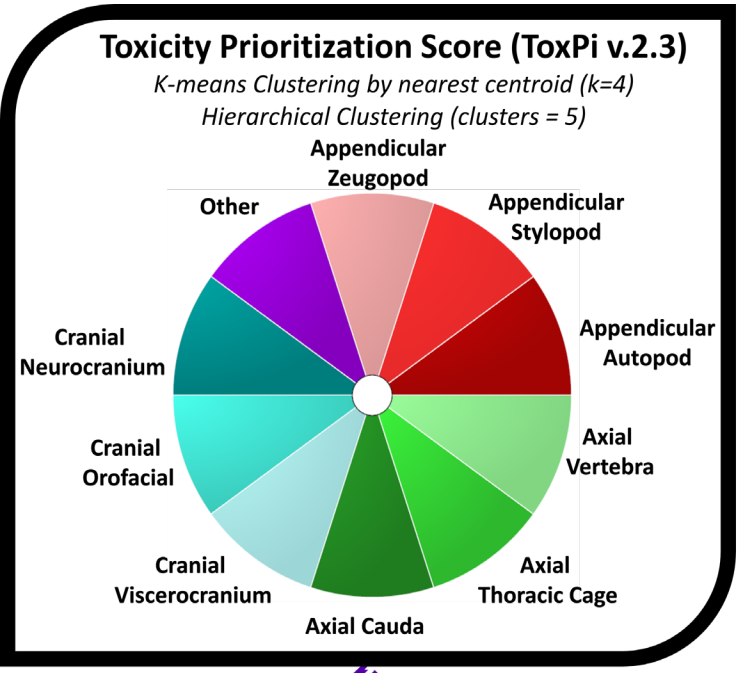
- Tag studies by line of evidence and outcome
- Distribute to disciplinary experts for review

Supplemental Studies

- Includes in vitro and other mechanistic evidence (e.g., non-PECO exposure route; non-PECO animal model; toxicokinetic data)

- Inventories contain basic study methods for evaluation and prioritization decisions

Moving Towards Qualitative and Quantitative AOPs



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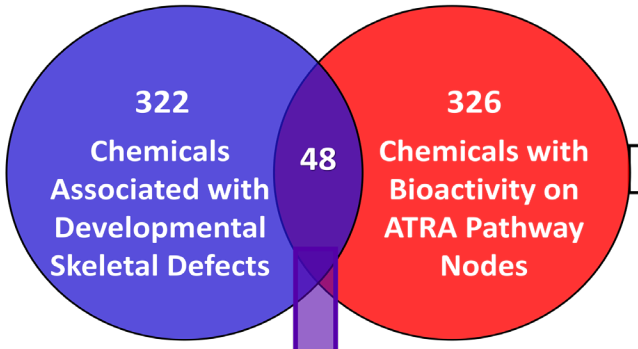

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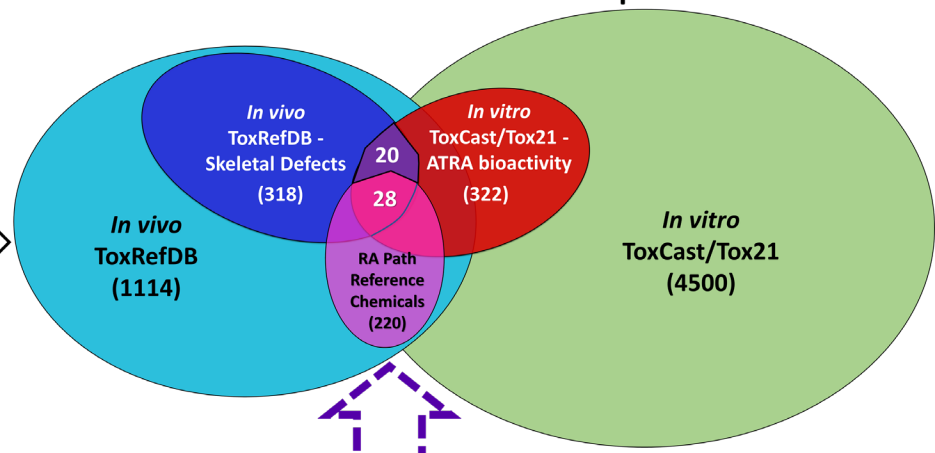
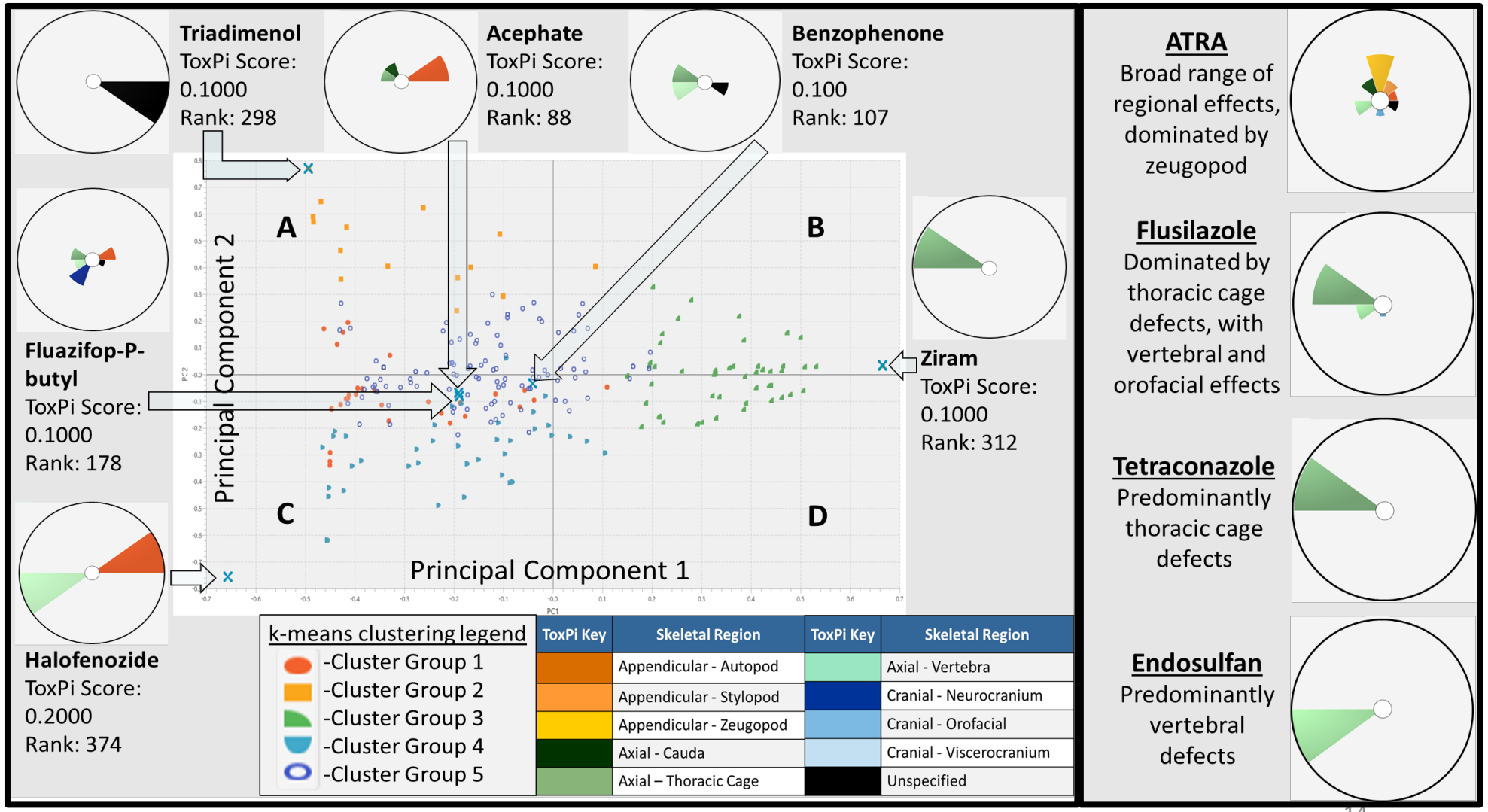


AOP Elucidation

Chemicals (375) clustered by ToxPi phenotype (k=5)

- **Group 1** – Primarily driven by autopod defects
- **Group 2** – Primarily unspecified skeletal defects
- **Group 3**– Primarily driven by axial defects
- **Group 4** – Primarily driven by vertebral and thoracic and other
- **Group 5** – Broad regional effects dominated by neurocranial



Results

- **48 hit one or more HTS assay mapping to the ATRA pathway**

- **Starting point… likely other chemicals with developmental skeletal effects that did not meet requisites of this list**

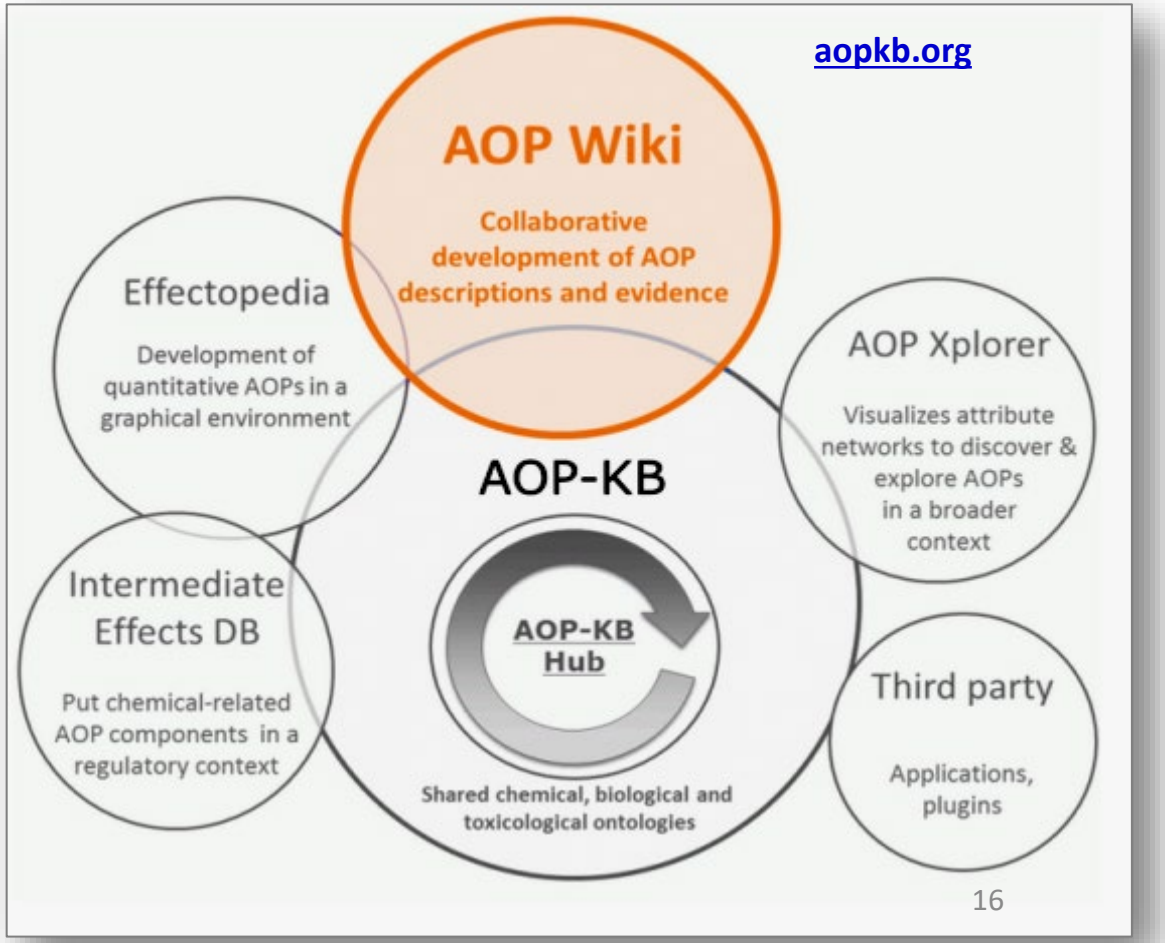
Allethrin	Diniconazole	Linuron	Raloxifene hydrochloride
All-Trans Retinoic Acid	Endosulfan	Myclobutanil	Retinol
Aspirin	Endrin	N,N-Dimethylformamide	S-Bioallethrin
Bentazone	Etoxazole	N-Phenyl-1,4-benzenediamine	SSR126768
Bromuconazole	Fenpyroximate (Z,E)	Oryzalin	Tebufenpyrad
Bronopol	Fipronil	Oxadiazon	Tetraconazole
Buprofezin	Fluoxastrobin	Phorate	Thiazopyr
Chlorothalonil	Flusilazole	Propargite	Triadimefon
Clodinafop-propargyl	Forchlorfenuron	Propiconazole	Tributyltetradecylphosphonium chloride
Cyfluthrin	Imazalil	Pyraclostrobin	Triflumizole
Deltamethrin	Iprodione	Pyridaben	Triphenyltin hydroxide
Difenoconazole	Lindane	Pyrimethamine	Triticonazole

Adverse Outcome Pathways (AOPs)

With HTS we can test the majority of chemicals in commerce within the decade but using the New Approach Methods (NAMs) for *in vitro* profiling and assessing toxicity is a challenge.

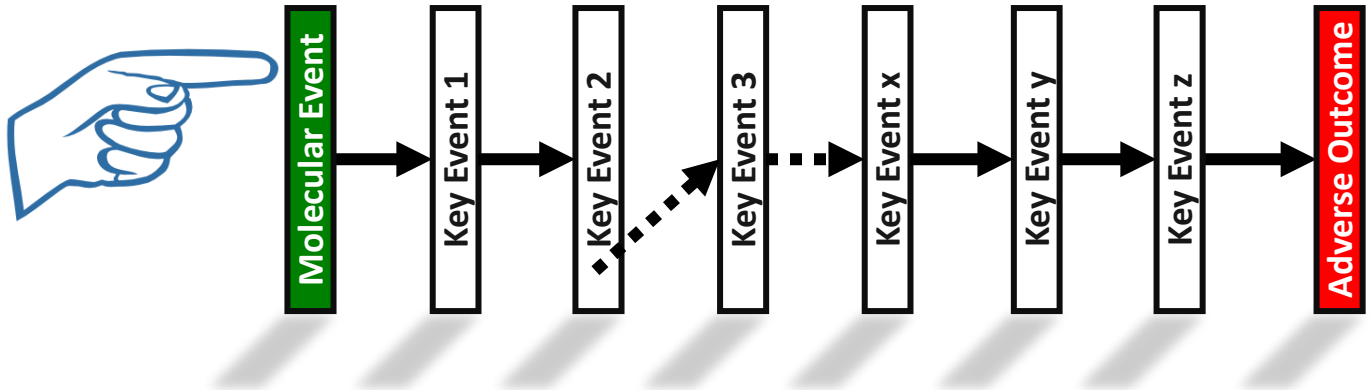
AOP: says "*here is a biological perturbation that can lead to a specific adverse outcome, and here is how we think it happens*" - hypothesis

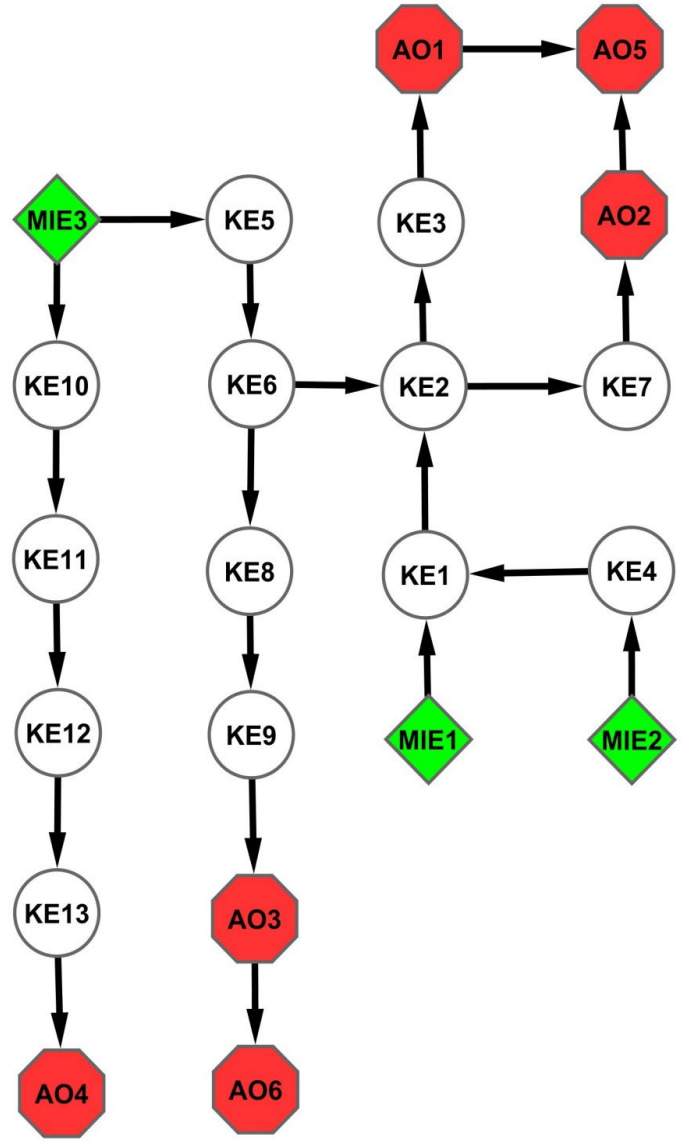
AOP-Knowledgebase (KB): compendium of curated AOPs with demonstrated relevance connecting a molecular perturbation to adverse outcome.



SOURCE: Dan Villeneuve, USEPA/CCTE

Principles for Building an AOP

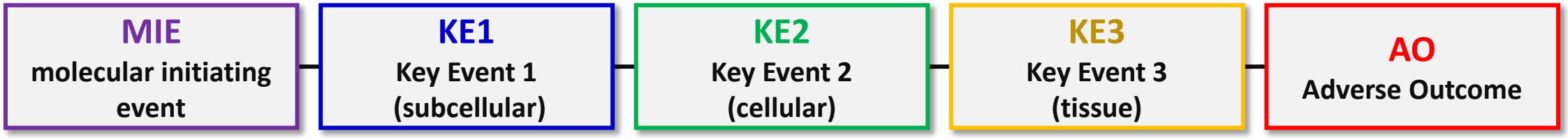




1. ***AOPs are not chemical-specific*** (based on biological motifs of failure)
2. ***AOPs are modular*** (individual relationships based on weight of evidence)
3. ***Individual AOPs are a pragmatic simplification*** (linearized sequence of biology)
4. ***AOP networks are the functional unit of prediction*** (in most cases)
5. ***AOPs are living documents*** (evolve as knowledge grows)

SOURCE: Villeneuve et al. (2014) Toxicol Sci

Putative AOPs for ATRA-dependent skeletal embryopathy

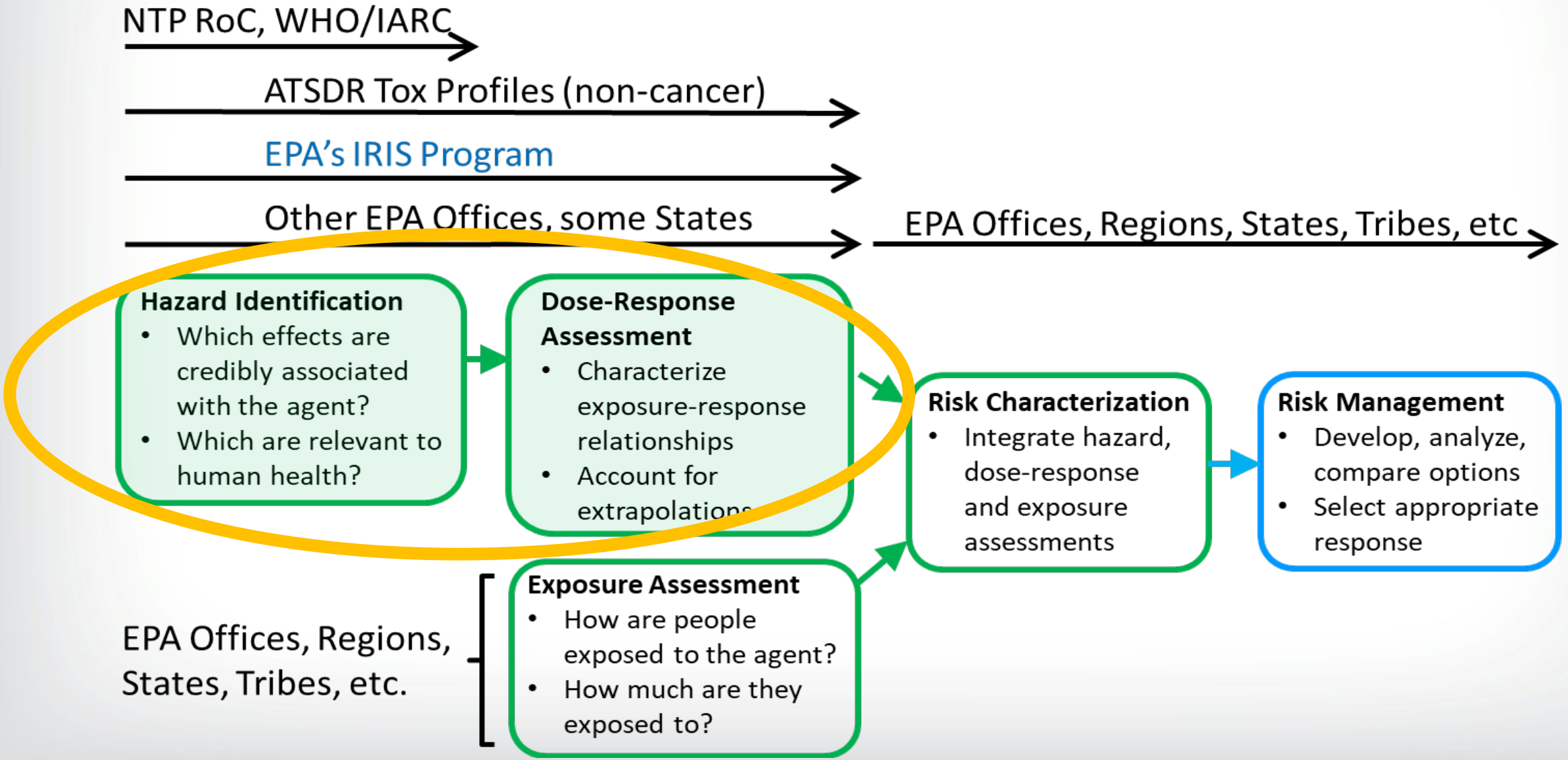


REGION	*Molecular Initiating Event (MIE)*	*Key Event 1 (KE1)*	*KE2*	*KE3*	*KE4*	*KE5*	*Adverse Outcome (AO)*
Anterior Neural Tube	Inhibition of CYP26A1 enzymatic activity	Local increase in endogenous ATRA levels	Hyperactivation of the RAR/RXR heterodimer	Repression of Fgf8 limits FGF8 signaling	Mis-specification of CNC cell fate and behavior	Maxillary arch dysplasia alters palatal outgrowth	Cleft palate
Paraxial Mesoderm	Reduction in RDH/RALDH2 activity	Local decrease in endogenous ATRA levels	Hypoactivation of the RAR/RXR heterodimer	Overextension of FGF8 signaling	Disruption of the periodic somitic wavefront	Altered somite number, shape, and alignment	Hemivertebra
Limb-bud	Hyperactivation of the RAR/RXR heterodimer	Underextension FGF8 signaling from the AER	Dysregulation of Meis1/2 and Hox gene expression	Proximalization of the limb-bud mesenchyme	Mis-specification of precartilage blastema	Malformed cartilaginous bone rudiment	Phocomelia

Knudsen et al., Reprod Toxicol (2021)

Although it is not clear which AOPs may be attributable exclusively to a retinoid-related mechanism, harmonized protocols assessing retinoid signaling can aid developmental hazard prediction.

Where AOPs Fit for Public Health Agencies



Systematic Review's Current Situation

- Any literature-based analysis requires searching for existing evidence
- Use of systematic review methods to identify evidence brings transparency and rigor to the process
- Use of defined workflows and specialized software to identify literature makes the process efficient, i.e., unclear if process takes longer than non-systematic methods
- More discussion and method development warranted for study evaluation and evidence synthesis/integration for NAM-based analyses

Mechanistic Evidence

"Mechanistic data represent a wide variety of studies not intended to identify an adverse outcome." (NRC, 2014)

"When human data are nonexistent, are mixed, or consistently show no association and an animal study finds a positive association, the importance of mechanistic data is increased..." (NRC, 2014)

- When evaluating mechanistic evidence, the scope is larger than "*in vitro*" data
- **Mechanistic inventories collected at earlier stages may include:**
  - *In vivo, In vitro* or *ex vivo*, Non-animal or non-mammalian alternative animal models, Big data, "Intervention" studies

"…there might be hundreds of *in vitro* and other mechanistic studies of a given chemical…" (NRC, 2014) OR NONE!!

"For a given chemical, multiple mechanisms might be involved in a given end point, and it might not be evident how different mechanisms interact in different species to cause the adverse outcome." (NRC, 2014)

Quantitative AOP (qAOP)

- qAOP:

"[A]n assembly of KEs supported by descriptions of how the KEs can be measured and the accuracy and precision with which the measurements are made along with KERs supported by quantitative understanding of what magnitude and/or duration of change in the upstream KE is needed to evoke some magnitude of change in the downstream KE…"

- OECD Guidance document on the use of AOPs in IATA, 2016

Common Features of qAOP Models

Common feature	Criteria
Problem formulation	• Question addressed and/or purpose of modelling • AO studied, developed, validated, and usable
Mechanistic knowledge and associated data	• Presence of the AOP in the OECD AOP-Wiki • Type of AOP: linear or network • Type of chemical model applied to (single chemical(s)/mixtures) • Type of data: in vivo, in vitro, in silico and/or other variables • Dose/concentration–responses • (D/C–R) and time–responses (T–R) • Adjacency of KERs: adjacency and non-adjacency • Biological levels: cellular, tissue, organ, organism, population
Quantitative approach	• Type of quantitative approach
Regulatory applicability	• Human health/ecological risk assessment
Additional considerations	• Cross species extrapolation; Modulating factors; Uncertainty evaluation; • Positive/negative feedback loops; Sensitivity analysis • Compensatory mechanisms; Kinetics; Exposure assessment

From: Spinu, N., Cronin, M.T.D., Enoch, S.J. *et al.* Quantitative adverse outcome pathway (qAOP) models for toxicity prediction. *Arch Toxicol* **94**, 1497–1510 (2020). https://doi.org/10.1007/s00204-020-02774-7

Questions?