Identification of Chemicals Associated with Retinoid Signaling Pathway Disturbance and Skeletal Dysmorphogenesis Through Predictive Computational Toxicology Models

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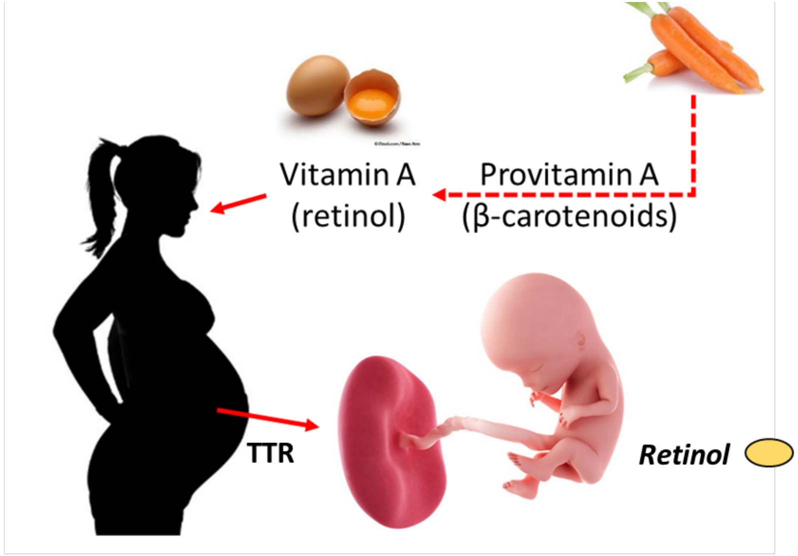
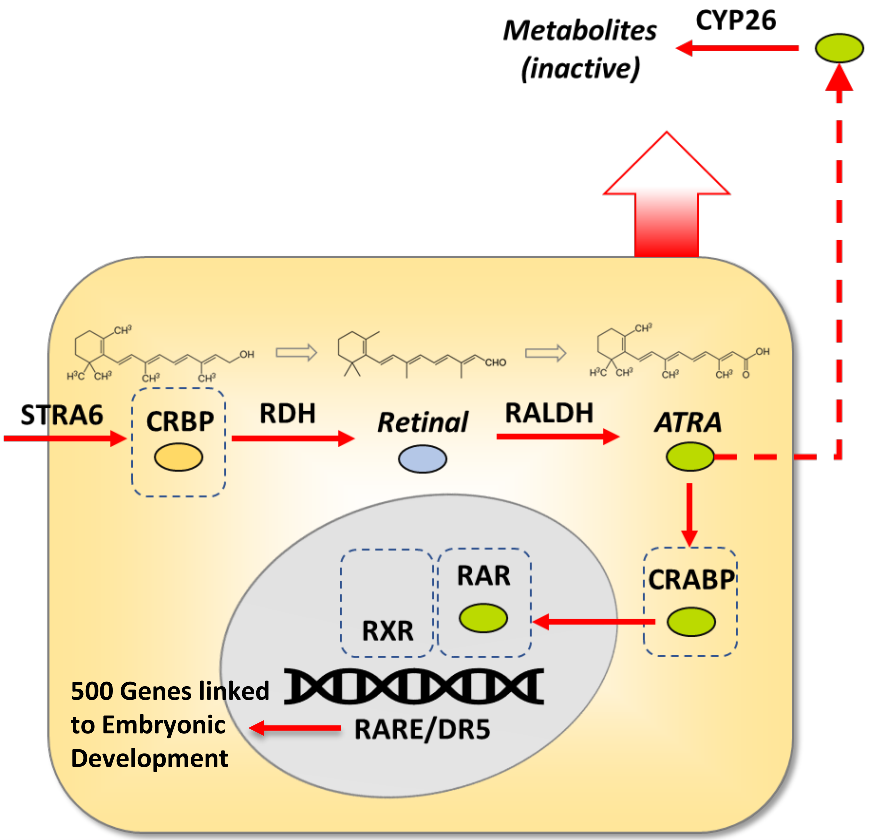
Introduction

- All-Trans Retinoic Acid (ATRA) is biologically active form of retinol (Vitamin A) and necessary to normal development in all tissues
- ATRA was the first signal characterized as a morphogenetic signal in vertebrate embryos
- ATRA has cross-talk with key morpho-regulatory pathways (SHH, FGF, WNT, TGFbeta, RTKs, …) and can be disrupted by genetic or environmental factors
- Over 500 ATRA-responsive genes regulate diverse biological processes important for development at the cellular, tissue and organ levels

ATRA Signaling Pathway

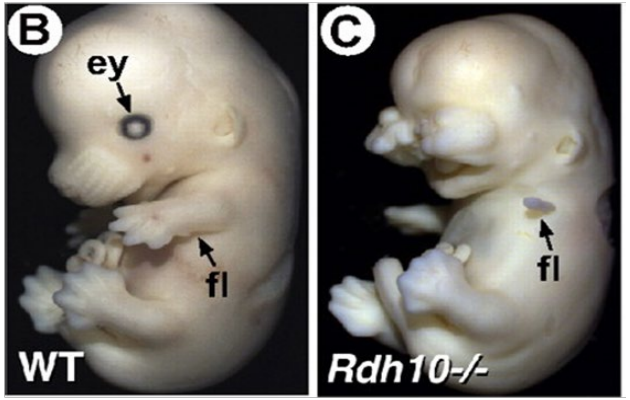


Adapted from Niederreither and Dolle, 2008



In Vivo Results of ATRA Excess and Deficiency

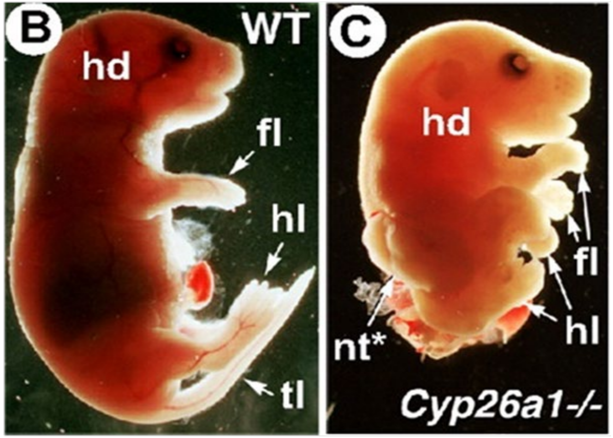
Deficient ATRA production



SOURCE: Rhinn et al. (2011) PNAS

- Without Retinoic Acid (RA) primarily have defects in anterior features
- RA needed for face and upper limb development

Deficient ATRA breakdown



SOURCE: Rhinn and Dolle (2012)

- Excess RA results in posterior defects
- Caudal deficiencies

ATRA Thresholds: *Teratogenesis and Morphogenetic Signaling*


Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Retinoid signaling in skeletal development: Scoping the system for predictive toxicology

Thomas B. Knudsen, Jocylin D. Pierro, Nancy C. Baker

Dosimetric	Conc.	Indication	Reference
baseline ATRA (5 somite zebrafish embryo)	< 1 nM	non-morphogenetic	(Shimozono, Iimura et al. 2013)
maternal serum (animal study)	1.7 nM	non-teratogenic	(Daston, Beyer et al. 2014)
devTOX^{qp} assay (pluripotent hESC)	3.0 nM	teratogenic threshold	(Zurlinden, Saili et al. 2020)
normal plasma concentration	5.0 nM	physiological (adult)	(Napoli, Posch et al. 1991)
axial gradient (5 somite zebrafish embryo)	6.0 nM	morphogenetic signal	(Shimozono, Iimura et al. 2013)
endodermal differentiation (h-iPSC)	17 nM	toxicological tipping point	(Saili, Antonijevic et al. 2019)
devTOX^{qp} assay (pluripotent h-iPSC)	19 nM	DevTox potential	(Palmer, Smith et al. 2017)
genetic perturbation (mouse)	30 nM	altered homeostasis	(Helms, Thaller et al. 1994)
maternal serum (animal study)	30 nM	teratogenic potential	(Daston, Beyer et al. 2014)
limb-bud (GD 10.5 mouse embryo)	30 nM	physiological (embryo)	(Horton and Maden 1995)
pharmacological kinetics	1,000 nM	efficacious (therapeutic)	(Helms, Thaller et al. 1994)
limb-bud (GD 11 mouse embryo)	1,500 nM	weakly teratogenic dose	(Satre and Kochhar 1989)
limb-bud (GD 10.5 mouse embryo)	12,500 nM	fully teratogenic dose	(Horton and Maden 1995)

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

Hypothesis

- Number of assays in retinoid pathway available in ToxCast/Tox21 → New Approach Methods (NAMs)
- Number of skeletal defects can be defined from the ToxRefDB and Literature → Adverse Outcome Pathways (AOPs)

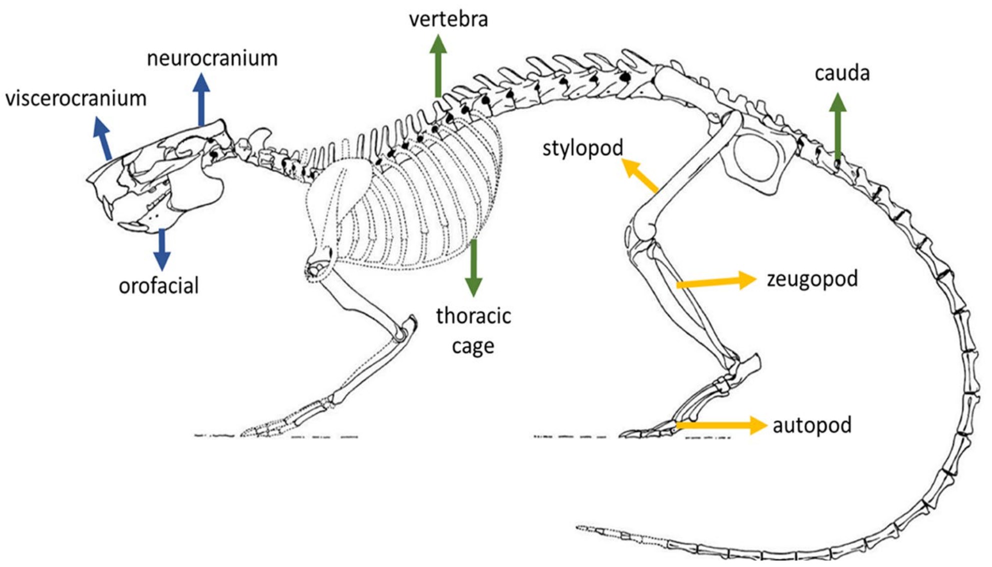
- <u>NAM-based AOPs provide a weight-of-evidence approach for predictive toxicology of ATRA-dependent skeletal dysmorphogenesis</u>

Approach:

Derive multi-database models from ToxCast, Tox21, ToxRefDB, literature mining, and AOP frameworks for

chemical disruption of retinoid signaling on altered skeletal development

Workflow



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

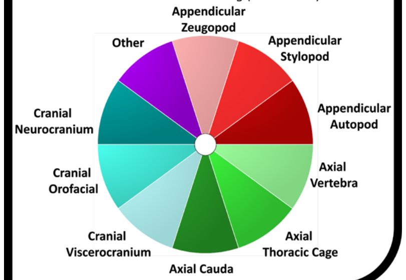
ToxRefDB Skeletal Defect Studies and Associated Chemicals
57,198 composite skeletal defects across 363 chemicals
(rat (31,1661), mouse (1,232), rabbit (16,375), chinchilla (368), other/unspecified (7562))

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

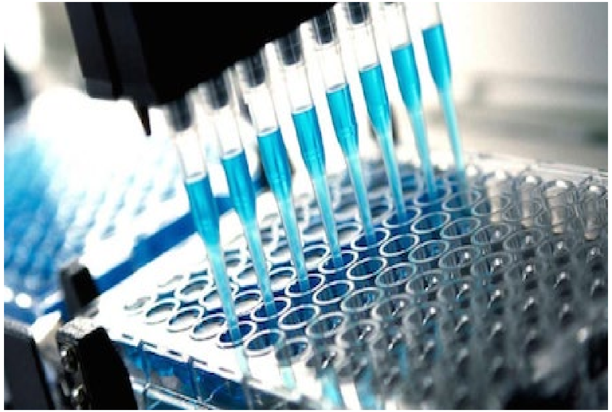
Regional Annotation for 370 chemicals
Appendicular (8,611): autopod (7,310), stylopod (969), zeugopod (332);
Axial (34,122): cauda (2,224), thoracic cage (19,132), vertebra (12,766);
Cranial (7,658): neurocranium (5,037), orofacial (2,426), viscerocranium (195);
Other (6,807): unspecified (6,807)

CompTox Chemicals Dashboard
https://comptox.epa.gov/dashboard
374 chemicals (of 8,079 tested chemicals) selected due to bioactivity across 1 or more of 13 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)



Mapping HTS Data



Toxicity Prioritization Score (ToxPi v.2.3)

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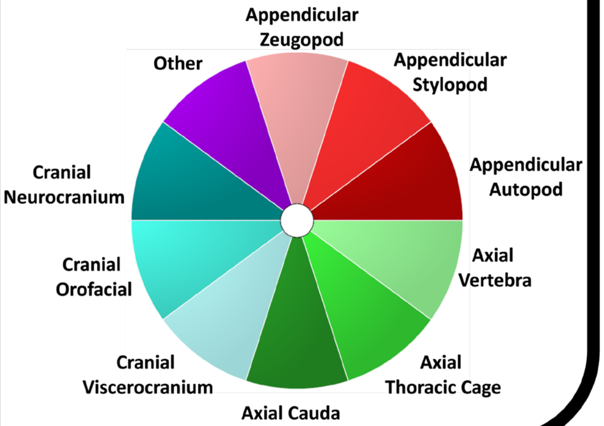
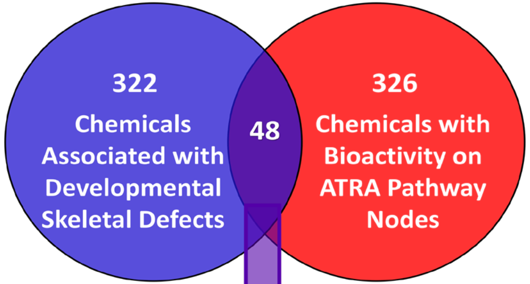


Comparison of Data Sets' Chemicals

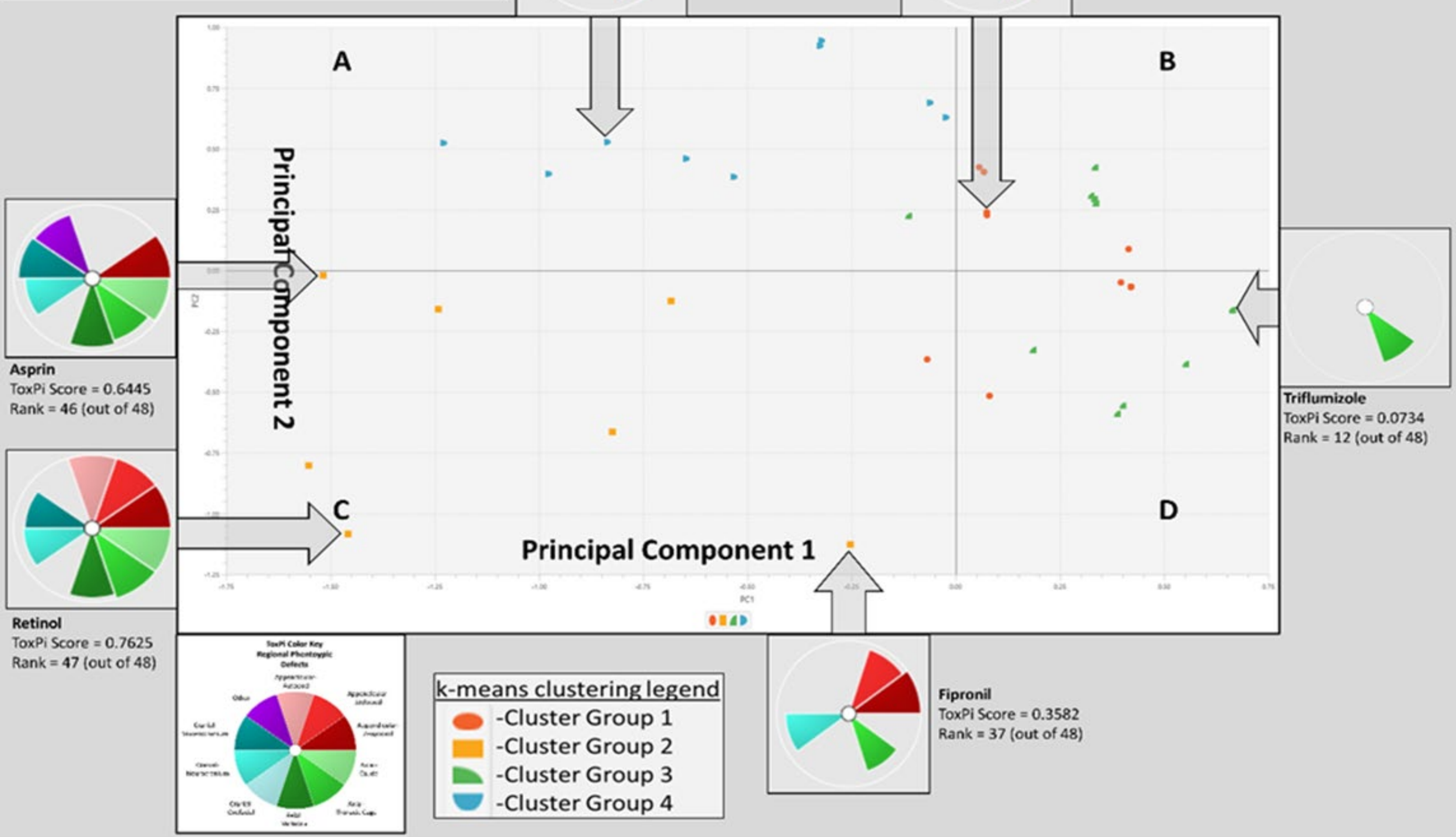
Compared 374 compounds that induced bioactivity in ATRA pathways and 363 ToxRefDB or 7 Benchmark ToxCast chemicals associated with skeletal defects to ascertain common chemicals

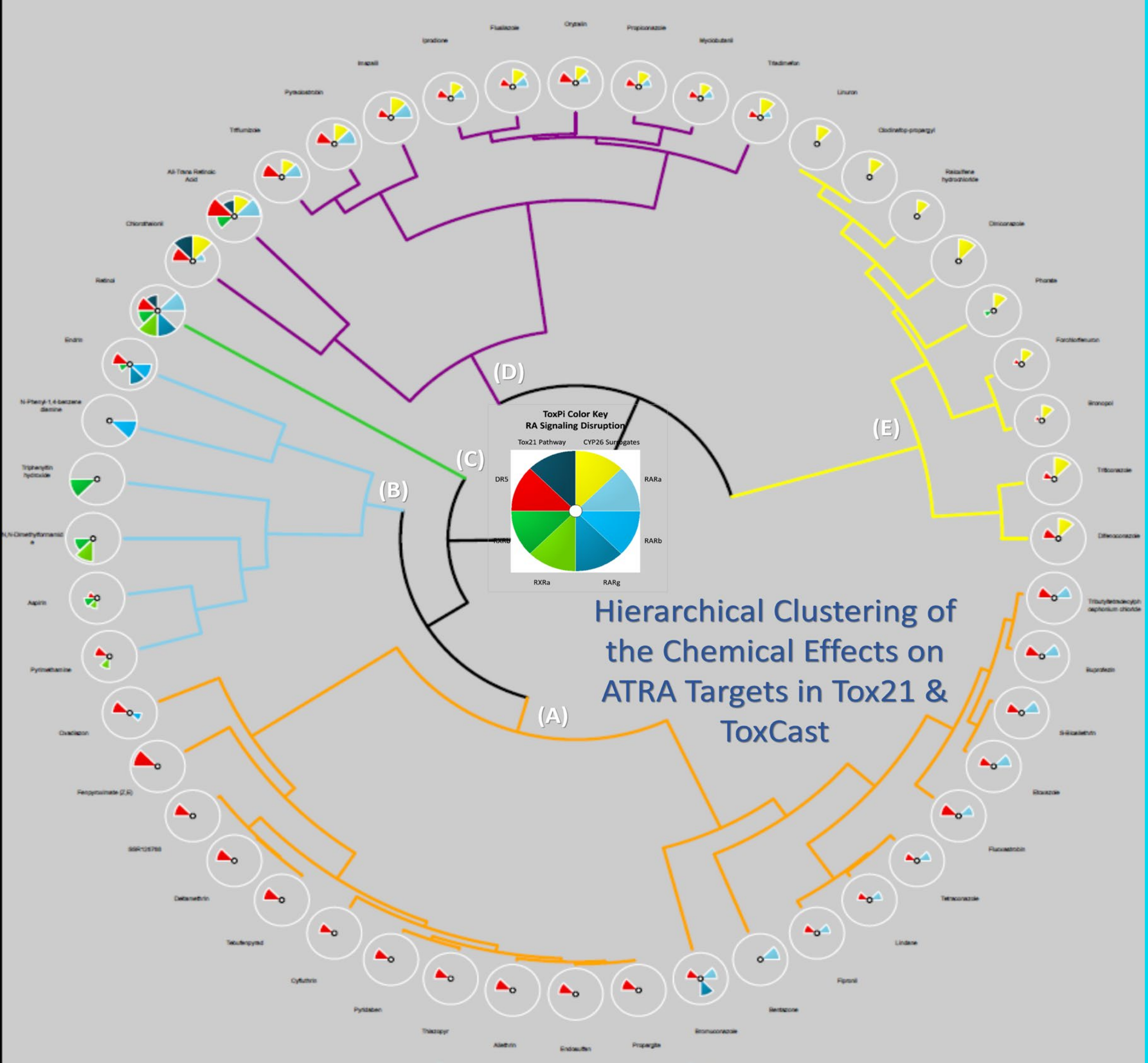
Dataset for Mechanistic Modeling



AOP Elucidation

K-means Clustering Skeletal Defects

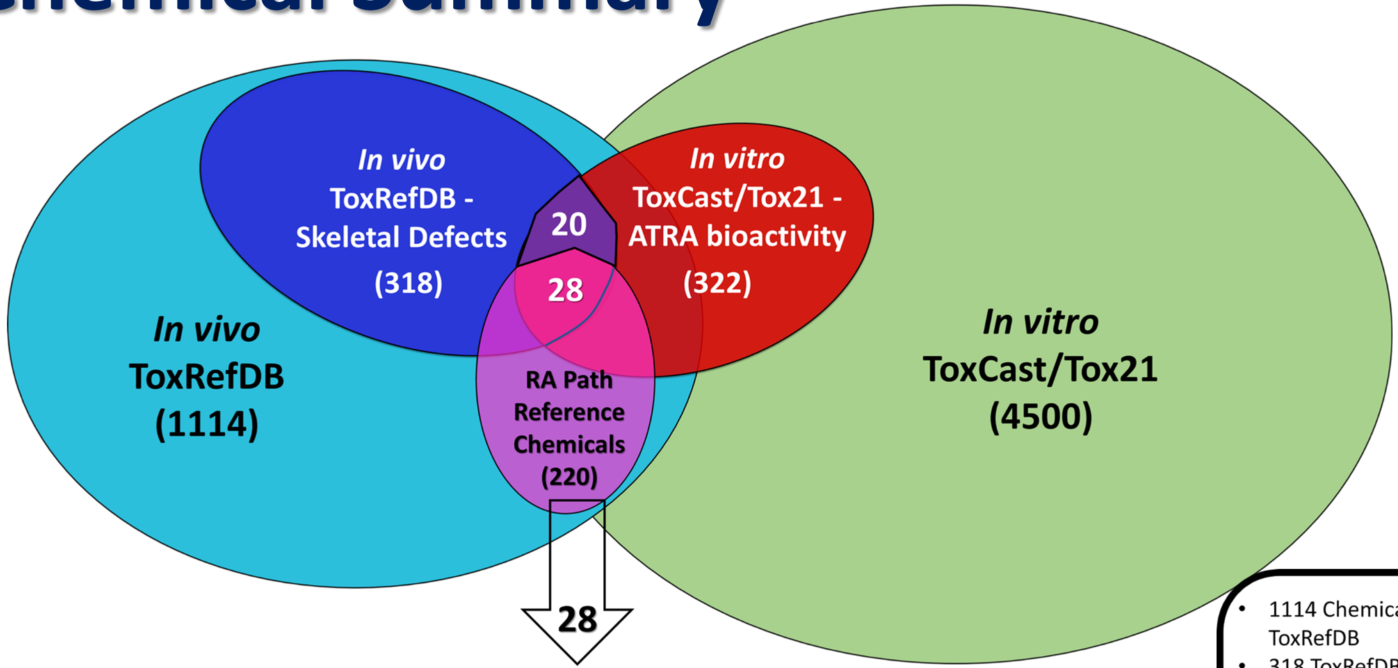




Hierarchical Clustering Clockwise Activity

- 3-5:30 o'clock DR5 & RARA (Cluster A)
- 5:30-8 o'clock DR5 (Cluster A)
- 8:30-9:30 o'clock RXRA/B and RARA/B (Cluster B)
- 10 o'clock Tox21, RARA/B, RXRA/B, and DR5 (Cluster C)
- 10-1 o'clock CYP, DR5, and RARA (Cluster D)
- 1-3 o'clock CYP (Cluster E)

Chemical Summary



28 Retinoid Pathway Reference Chemicals from Protein Data Bank, ChEMBL, ToxCast, and biomedical literature in PubMed were consistent with other databases.

- 1114 Chemicals Tested in vivo recorded in ToxRefDB
- 318 ToxRefDB chemicals associated with *in vivo* skeletal defects
- 4500 Chemicals Tested *in vitro* recorded in ToxCast/Tox21
- 322 ToxCast & ToxRefDB chemicals associated with *in vitro* ATRA pathway bioactivity
- 28 chemicals found in 3 databases establishing association with skeletal defects and ATRA path bioactivity

20 Unique Chemicals	28 Chemicals in Lit	
Triphenyltin hydroxide	Allethrin	Retinol
Raloxifene hydrochloride	All-Trans Retinoic Acid	SSR126768
Forchlorfenuron	Bentazone	Tebufenpyrad
Lindane	Buprofezin	Thiazopyr
Linuron	Chlorothalonil	Triadimefon
S-Bioallethrin	Deltamethrin	Tributyltetradecylphosphonium chloride
Iprodione	Difenoconazole	Triflumizole
Phorate	Diniconazole	Triticonazole
Fipronil	Endosulfan	
Aspirin	Endrin	
Cyfluthrin	Fenpyroximate (Z,E)	
N,N-Dimethylformamide	Fluoxastrobin	
Clodinafop-propargyl	Flusilazole	
Propiconazole	Imazalil	
Myclobutanil	N-Phenyl-1,4-benzenediamine	
Bronopol	Oryzalin	
Etoxazole	Oxadiazon	
Tetraconazole	Propargite	
Pyrimethamine	Pyraclostrobin	
Bromuconazole	Pyridaben	

48 Chemicals

Potential AOPs for ATRA-Skeletal Defects

MIE	KE1	KE2	KE3	KE4	AO
Loss of CYP26 activity	Increase in ATRA	Decrease in FGF8 signaling	Modification of axial patterning genes	Alterations in cartilage, rudiments, and ossification	Misshapen, poorly ossified, and missing thoracic cage 48% Thoracic Cage
Overactivation of RARs	Increased transactivation of RARE	Downregulation of FGF8 expression in the apical ectoderm	Activation of apoptotic pathway	Excessive interdigital cell death (ICD)	Truncation of the autopod 6% Autopod

These do not reflect complete manifestation of genetic models; these are partial effects on the system

Cross-Species Extrapolation

- *In vitro* to *in vivo* extrapolation (IVIVE)
  - High-Throughput Toxicokinetics (httk)

  [Chang et al. 2022; Wambaugh 2016 with updates]
- Human extrapolation required for *in vivo* data
- Uncertainty factors – to incorporate, or not?
- Retinoid pathway well conserved, human likely consistent

Summary and Conclusions

- 48 ToxCast/Tox21/ToxRefDB chemicals were identified with potent effects on ATRA pathway assays and fetal skeletal defects
- Hierarchy of *in vitro* effects: DR5 (bioindicator of ATRA transactivation) → CYP (CYP26 surrogate inhibition) → RAR-dependent transactivation
- Thoracic cage defects was the first and most frequent skeletal outcomes in this model, followed by other axial defects (vertebra and cauda), cranial and limb defects
- This data supports the hypothesis that NAM-based AOPs provide a weight-of-evidence approach for predictive toxicology of ATRA-dependent skeletal dysmorphogenesis

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Questions?

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