8 September 2024 | Copenhagen

ECETOC & RSA EUROTOX 2024 Satellite Symposium:

TESTING, ASSESSMENT AND CLASSIFICATION OF THYROID HORMONE DISRUPTORS

Workshop background and objectives:

- **Review the state of the science of thyroid hormone disruption mediated developmental neurotoxicity and its current application in regulatory decision-making.**
- **Propose options for improving the evaluation of thyroid hormone related neurodevelopmental toxicity.**
- **Discuss the proposed ECETOC testing and assessment scheme.**

CRITICAL REVIEWS IN TOXICOLOGY
https://doi.org/10.1080/10408444.2020.1839380

Part 1

Taylor & Francis
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REVIEW ARTICLE

OPEN ACCESS Check for updates

Toward a science-based testing strategy to identify maternal thyroid hormone imbalance and neurodevelopmental effects in the progeny – part I: which parameters from human studies are most relevant for toxicological assessments?

Ursula G. Sauer[a], Alex Asiimwe[b], Philip A. Botham[c], Alex Charlton[c], Nina Hallmark[d], Sylvia Jacobi[e], Sue Marty[f], Stephanie Melching-Kollmuss[g], Joana A. Palha[h,i,j], Volker Strauss[g], Bennard van Ravenzwaay[g] and Gerard Swaen[k]

CRITICAL REVIEWS IN TOXICOLOGY
2021, VOL. 51, NO. 4, 328–358
https://doi.org/10.1080/10408444.2021.1910625

Part 2

Taylor & Francis
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REVIEW ARTICLE

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Towards a science-based testing strategy to identify maternal thyroid hormone imbalance and neurodevelopmental effects in the progeny – part II: how can key events of relevant adverse outcome pathways be addressed in toxicological assessments?

Sue Marty[a], Manon Beekhuijzen[b], Alex Charlton[c], Nina Hallmark[d], Bethany R. Hannas[e], Sylvia Jacobi[f], Stephanie Melching-Kollmuss[g], Ursula G. Sauer[h], Larry P. Sheets[i], Volker Strauss[g], Daniel Urbisch[g], Philip A. Botham[c] and Bennard van Ravenzwaay[g]

CRITICAL REVIEWS IN TOXICOLOGY
2022, VOL. 52, NO. 7, 546–617
https://doi.org/10.1080/10408444.2022.2130166

Part 3

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REVIEW ARTICLE

OPEN ACCESS Check for updates

Towards a science-based testing strategy to identify maternal thyroid hormone imbalance and neurodevelopmental effects in the progeny—part III: how is substance-mediated thyroid hormone imbalance in pregnant/lactating rats or their progeny related to neurodevelopmental effects?

M. Sue Marty[a], Ursula G. Sauer[b], Alex Charlton[c], Rashin Ghaffari[d], Davy Guignard[e*], Nina Hallmark[f], Bethany R. Hannas[d*], Sylvia Jacobi[g], Heike-Antje Marxfeld[h], Stephanie Melching-Kollmuss[i], Larry P. Sheets[j], Daniel Urbisch[i*], Philip A. Botham[c] and Bennard van Ravenzwaay[k]

CRITICAL REVIEWS IN TOXICOLOGY
https://doi.org/10.1080/10408444.2023.2231033

Part 4

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REVIEW ARTICLE

OPEN ACCESS Check for updates

Towards a science-based testing strategy to identify maternal thyroid hormone imbalance and neurodevelopmental effects in the progeny – Part IV: the ECETOC and CLE Proposal for a Thyroid Function-Related Neurodevelopmental Toxicity Testing and Assessment Scheme (Thyroid-NDT-TAS)

Stephanie Melching-Kollmuss[a], Kathrin Bothe[b], Alex Charlton[c], Babunilayam Gangadharan[d], Rashin Ghaffari[e], Sylvia Jacobi[f], Sue Marty[g], Heike-Antje Marxfeld[h], Elizabeth F. McInnes[i], Ursula G. Sauer[j], Larry P. Sheets[k], Christian Strupp[l], Helen Tinwell[d], Christiane Wiemann[m], Philip A. Botham[i] and Bennard van Ravenzwaay[n]

https://www.ecetoc.org/task-force/special-t4-task-force/

Item	Start	End	Agenda item	Who
1	11:30 AM	12:00 PM	Arrival and registration at the Copenhagen Island Hotel	Organising committee
2	12:00 PM	12:30 PM	**Welcome Lunch** (Registration to continue through lunch)	
3	12:30 PM	12:40 PM	**Welcome, introduction and symposium objectives**	Bennard van Ravenzwaay (ECETOC) Christine Walter (RSA)
4	12:40 PM	1:00 PM	**Testing and assessment of thyroid-hormone related neurodevelopmental toxicity – the ECETOC-CLE Thyroid NDT-TAS**	Stephanie Melching-Kollmuss (BASF)
5	1:00 PM	1:20 PM	**Experiences with Thyroid ED assessment – EFSA ED Database**	Martina Panzarea (EFSA)
6	1:20 PM	1:40 PM	**Experiences with Thyroid ED assessment & new CLP ED Criteria**	Niklas Andersson (ECHA)
7	1:40 PM	2:00 PM	**In vitro and in vivo investigation of pesticide effects on the T-axis**	Philip Marx-Stoelting (BfR)
8	2:00 PM	2:20 PM	***Coffee break***	
9	2:20 PM	2:40 PM	**Use of the ECETOC-CLE Thyroid NDT-TAS to Support Identification and Classification of Thyroid Hormone Disruptors**	Helen Tinwell (Bayer)
10	2:40 PM	3:00 PM	**How to identify adverse neurodevelopmental toxicity in laboratory animals**	Heike-Antje Marxfeld (BASF)
11	3:00 PM	3:20 PM	**Decisive new endpoints indicative of abnormal neurodevelopment**	Katie O'Shaugnessy (US EPA)
12	3:20 PM	3:40 PM	**Assessment of thyroid hormone alterations in rat brain and plasma**	Christiane Hindrichs (BASF Metabolome Solutions)
13	3:40 PM	4:00 PM	**Species-specific thyroxine (T4) metabolism and response to nuclear-receptor activators in long-term cultured hepatocytes**	Lysiane Richert (KaLy-Cell) & Laure Asselin (KaLy-Cell)
14	4:00 PM	4:15 PM	**Summarise and closing remarks**	Bennard van Ravenzwaay (ECETOC) Christine Walter (RSA)
15	4:15 PM	4:45 PM	***30 min to EUROTOX opening ceremony***	
16	4:45 PM	6:45 PM	**EUROTOX Congress Opening Ceremony (Tivoli Hotel & Congress Center)**	

Testing and assessment of thyroid-hormone related neurodevelopmental toxicity – the ECETOC-CLE Thyroid NDT-TAS

8th September 2024

Satellite to Eurotox

Dr. Stephanie Melching-Kollmuss, BASF SE, on behalf of the ECETOC Thyroxine (T4) Task Force

The question(s)

- How is maternal thyroid hormone imbalance related to neurodevelopmental outcomes?
- What are the most sensitive parameters for human / rat neurodevelopment?
- Are there most sensitive timepoints for thyroid hormone measurements?
- Are there thresholds?

Overall Goal: Develop a testing and assessment scheme: Thyroid-NDT-TAS

ECETOC T4 TF work at a glance

Towards a science-based testing strategy to identify maternal thyroid hormone imbalance and neurodevelopmental effects in the progeny:

Which parameters from **human** studies are most relevant for toxicological assessment? (Sauer et al., **2020**)

How can key events of relevant **adverse outcome pathways (AOPs)** be addressed in toxicological assessments? (Marty et al., **2021**)

How is substance-mediated thyroid hormone imbalance in pregnant / lactating rats or their progeny related to neurodevelopmental effects? (Marty et al., **2022**)

Testing and assessment scheme (Thyroid-NDT-TAS) (Melching-Kollmuss et al., **2023**)

Endocrine Disruption Criteria

Applied for
Pesticide Active Ingredient
& Biocide Registrations

"An endocrine disruptor is an exogenous substance or mixture that alters the function (s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations." (WHO/IPCS, 2002)

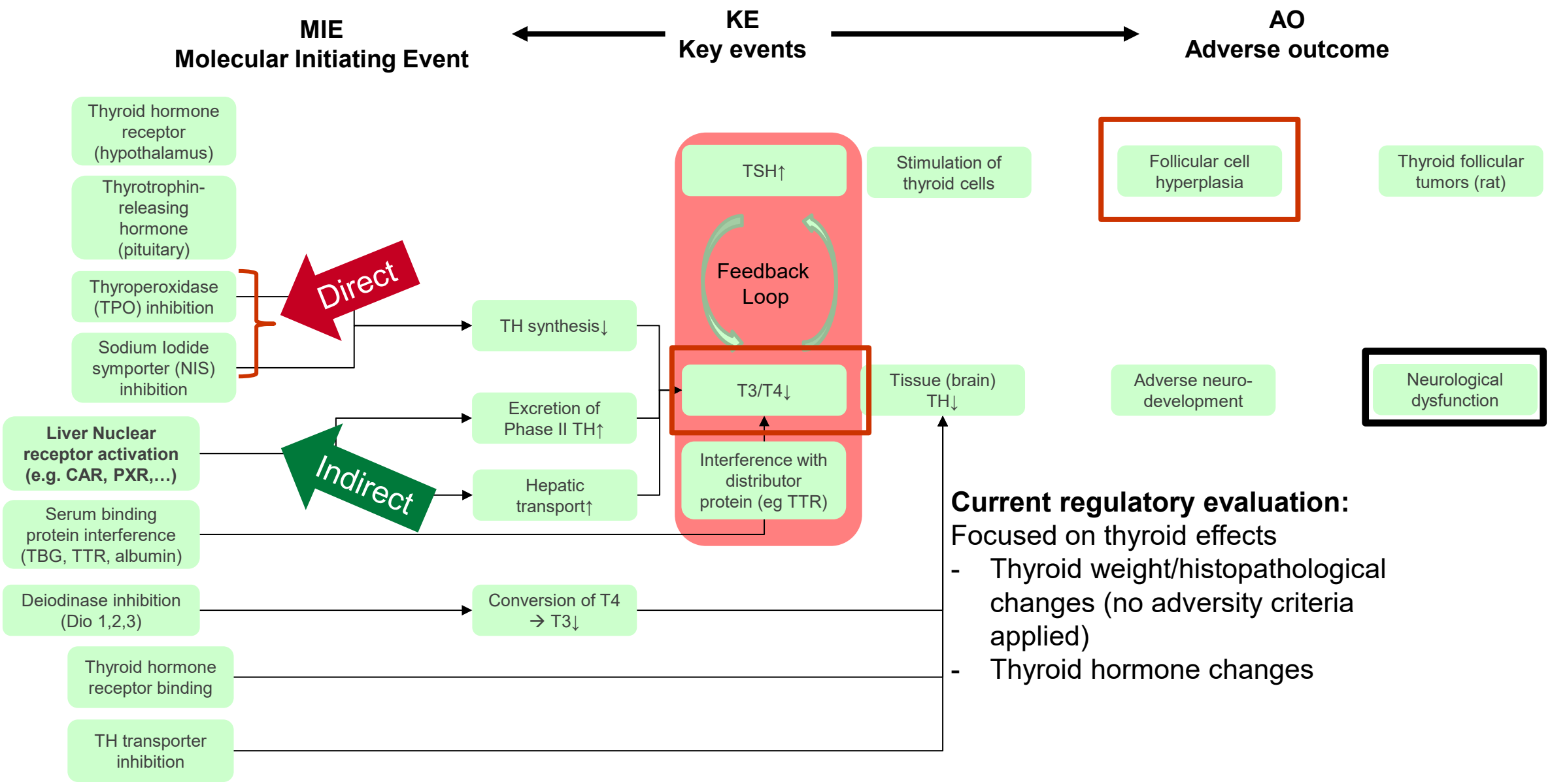
(Human-relevant) Adverse Effects

(Human-relevant) Endocrine Activity
Estrogen
Androgen
Thyroid
Steroidogenesis*

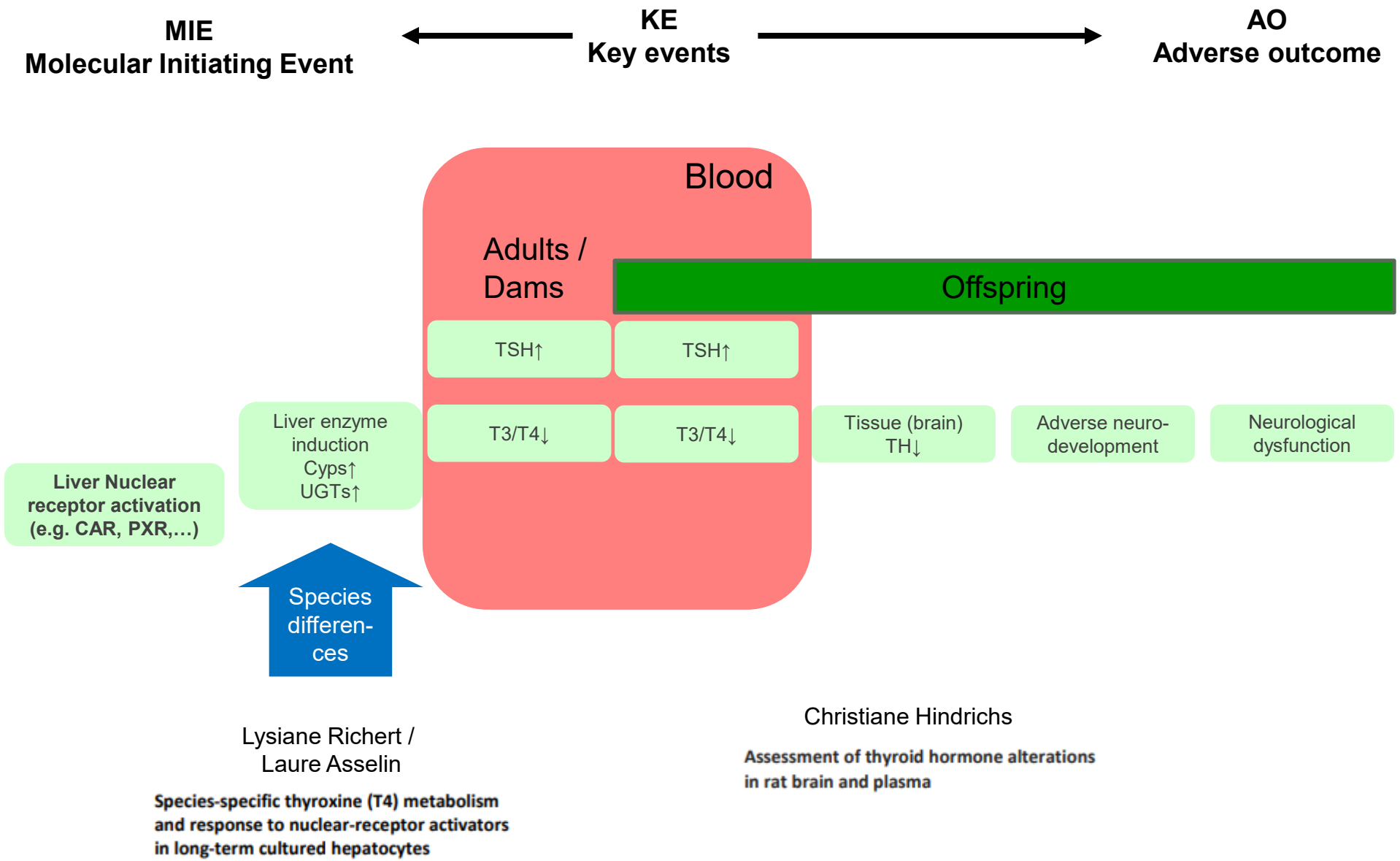
Plausible link between adverse effect and activity

*according to ECHA/EFSA Guidance, 2018

In future: Endocrine Disruption under CLP
ED HH Cat 1
ED HH Cat 2

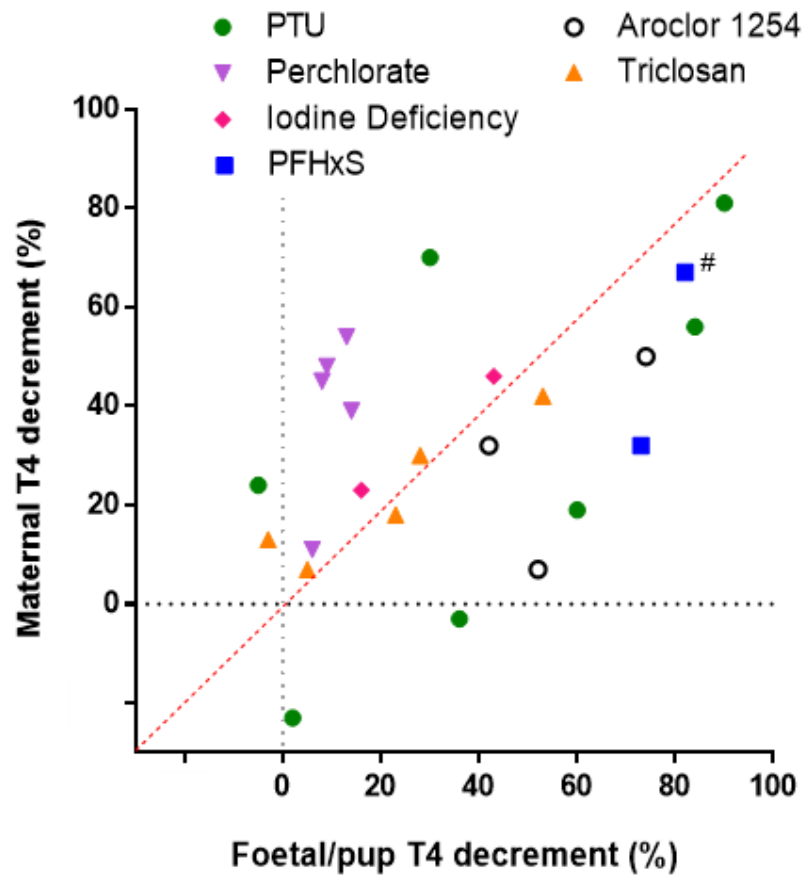


See Noyes et al., 2019


MIE
Molecular Initiating Event
KE
Key events
AO
Adverse outcome
Blood
Adults / Dams
Offspring
TSH↑
TSH↑
T3/T4↓
T3/T4↓
Liver enzyme induction
Cyps↑
UGTs↑
Tissue (brain)
TH↓
Adverse neuro-development
Neurological dysfunction
Liver Nuclear receptor activation (e.g. CAR, PXR,…)
Species differen-ces
Lysiane Richert / Laure Asselin
Species-specific thyroxine (T4) metabolism and response to nuclear-receptor activators in long-term cultured hepatocytes
Christiane Hindrichs
Assessment of thyroid hormone alterations in rat brain and plasma

Maternal vs offspring T4 hormone decrements

Figure 1: Relationship between maternal serum T4 decrements measured on GD 20 – GD 21 and foetal / pup serum T4 decrements measured on GD 20 – PND 0



No good correlation between maternal and offspringTH levels

Marty et al., 2022
DOI: 10.1080/10408444.2022.2130166

Brain-related parameters (assessed in rats)

- Functional changes in **late-stage key events** (eg alterations in electrophysiology or auditory signaling)
- **Neurobehavioural effects** on the organism level (motor activity, acoustic starles response, learning and memory)
- **Structural changes in brain** (e.g. periventricular heterotopia, decreased volume or thickness of specific brain layers, altered glial cell labelling / cell density)
- Changes in **expression** of **brain genes** and brain-related proteins

Assessed in 4 case studies
1: TPO inhibitors
2: NIS inhibitors / Iodine deficiency
3: Liver enzyme inducers / Interaction with serum binding proteins
4: Dio 1 inhibitors

Marty et al., 2022
DOI: 10.1080/10408444.2022.2130166

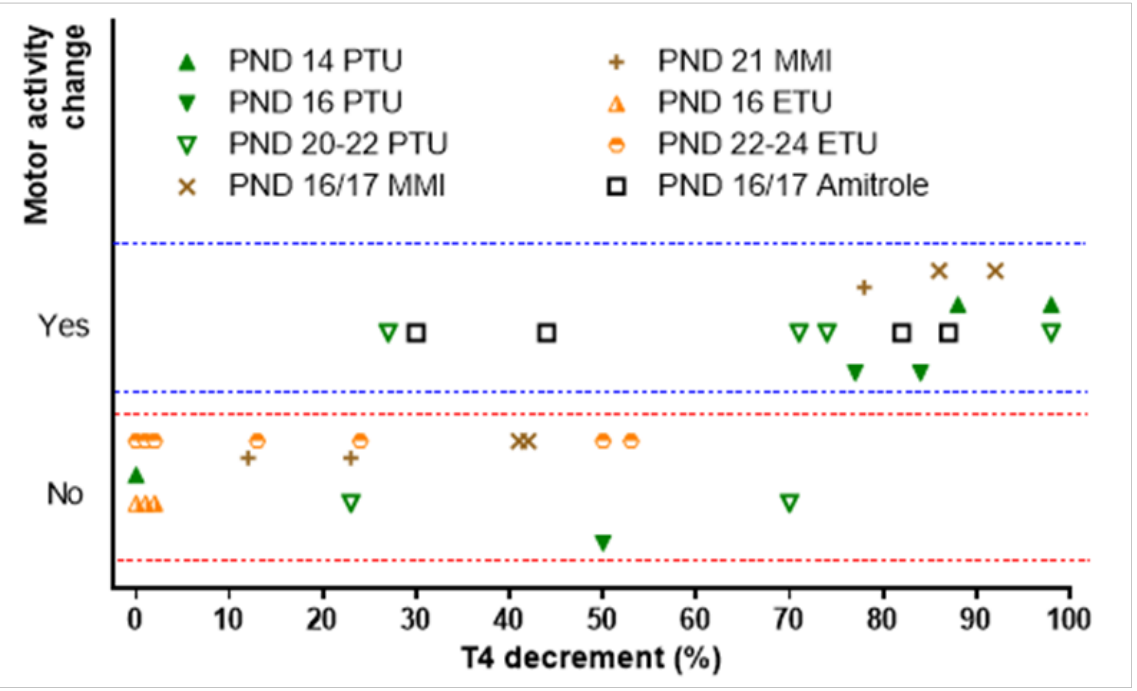
TPO – Thyroid Peroxidase
NIS – Sodium Iodide Symporter
Dio - Deiodinase

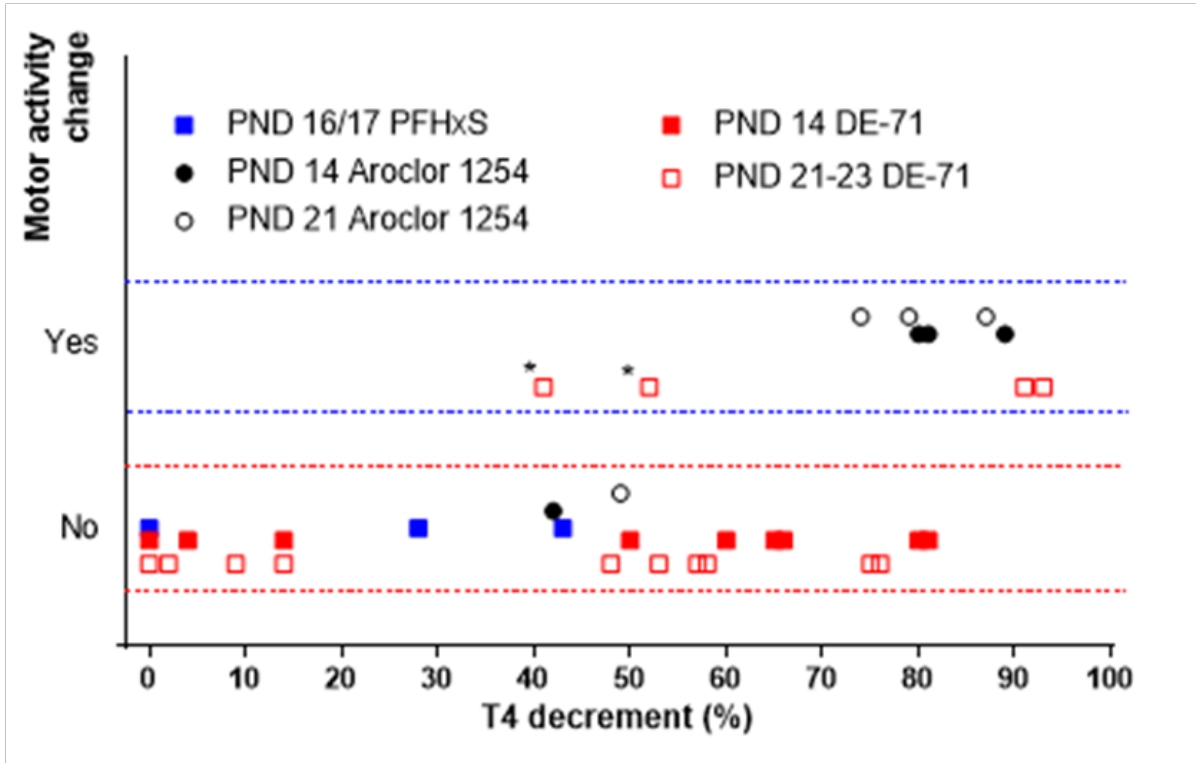
Association T4/T3 decrement in offspring and motor activity

Case study 1

PND 14 – PND 30

Case study 3

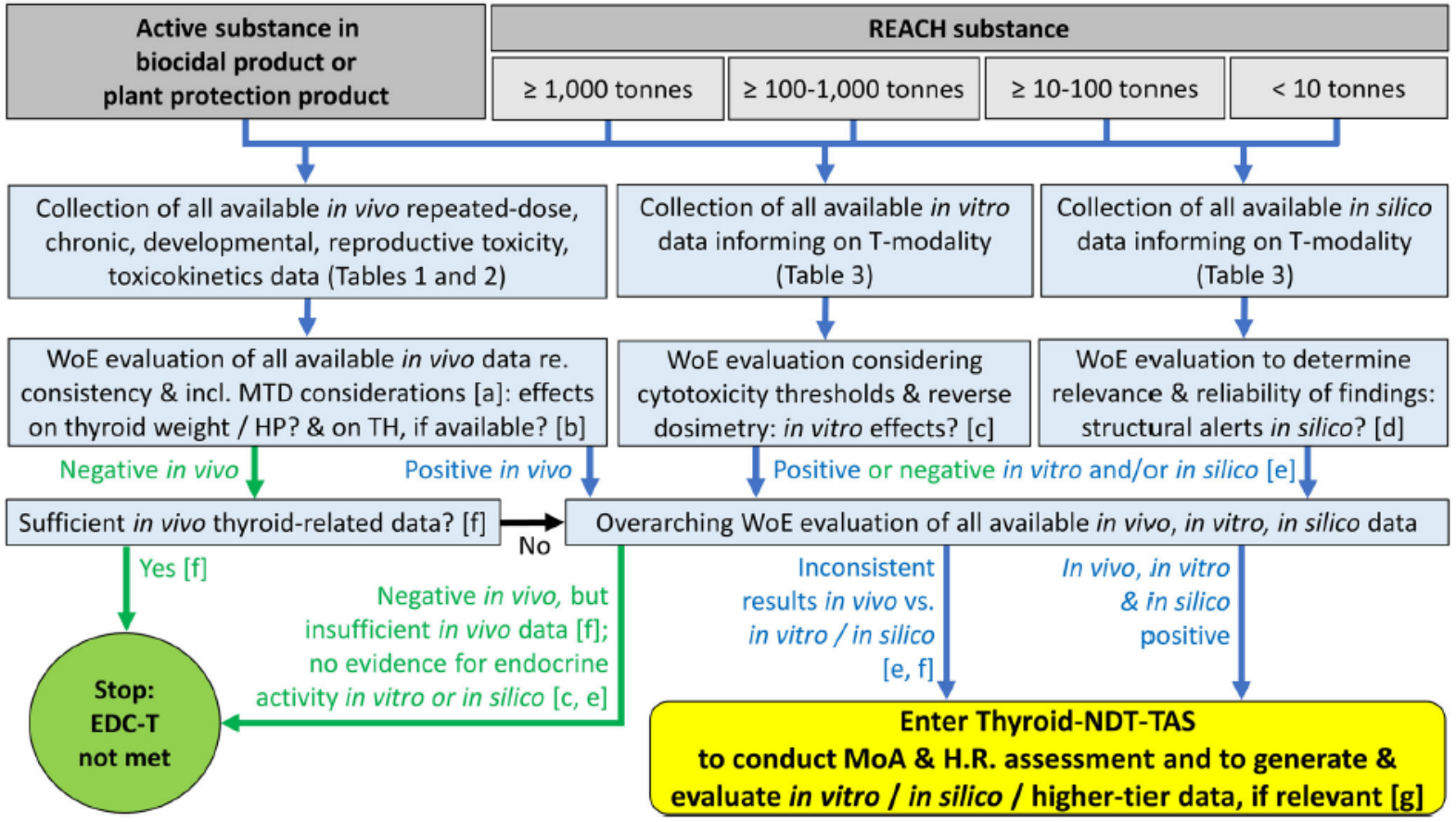




Marty et al., 2022
DOI: 10.1080/10408444.2022.2130166

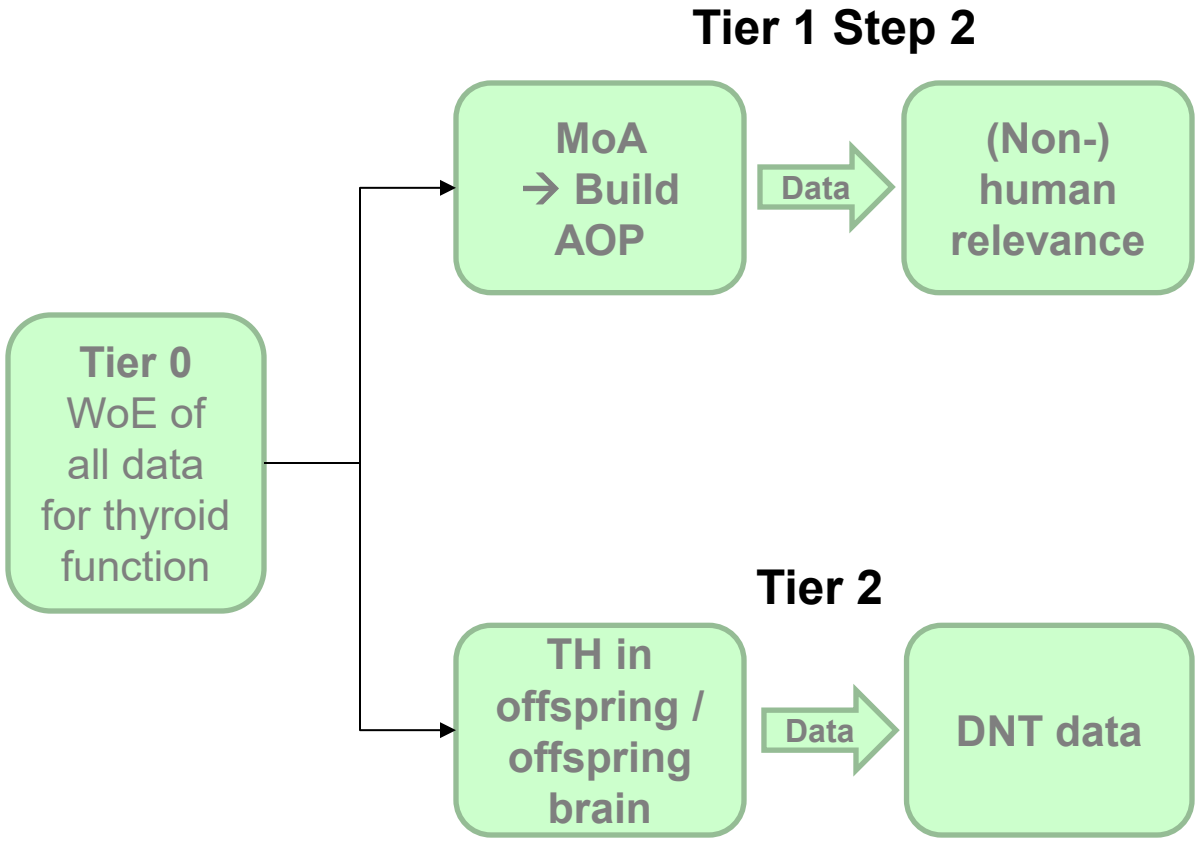
Thyroid Function-related **N**eurodevelopmental **T**oxicity **T**esting and **A**ssessment **S**cheme (Thyroid-NDT-TAS)

This works with data-rich and data-poor substances!



Melching-Kollmuss et al., 2023
DOI: 10.1080/10408444.2023.2231033

Thyroid Function-related **N**eurodevelopmental **T**oxicity **T**esting and **A**ssessment **S**cheme (Thyroid-NDT-TAS)



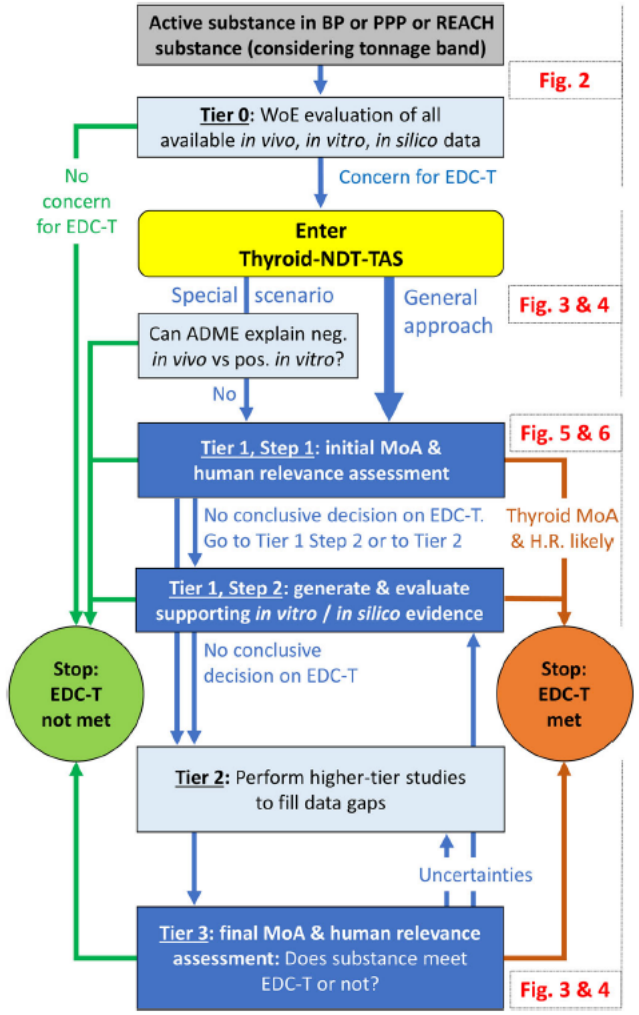
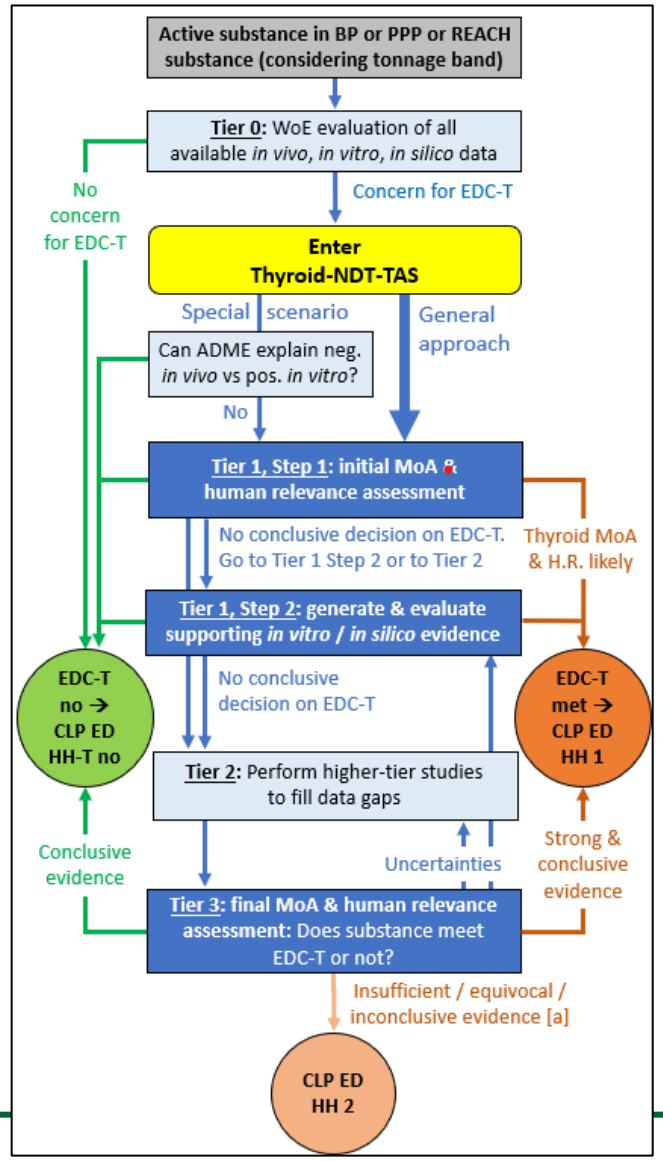


Figure 1. Overview of the ECETOC and CLE Thyroid-NDT-TAS (see Figures 2–6 for details). BP: biocidal product; EDC-T: endocrine disruptor criteria for the thyroid modality; MoA: mode-of-action; PPP: plant protection product; REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals; WoE: weight-of-evidence.

Melching-Kollmuss et al., 2023
DOI: 10.1080/10408444.2023.2231033

Follow-up work from the ECETOC / CLE Task



- Develop four Thyroid Case Studies
- Propose criteria to differentiate between ED HH Cat 1 and Cat 2
- Helen Tinwell

Use of the ECETOC-CLE Thyroid NDT-TAS to Support Identification and Classification of Thyroid Hormone Disruptors

Authors of the CLE / ECETOC Testing scheme

- Stephanie Melching-Kollmuss, BASF SE
- Kathrin Bothe, Bayer Crop Science
- Alex Charlton, Syngenta Crop Protection
- Babunilayam Gangadharan, Bayer Crop Science
- Rashin Ghaffari, Corteva Agriscience
- Sylvia Jacobi, SJ-Consult
- Sue Marty, Dow Inc
- Heike-Antje Marxfeld, BASF SE
- Elizabeth McInnes, Syngenta Crop Protection
- Ursula Sauer, Scientific Consultancy – Animal Welfare
- Larry Sheets, Bayer Crop Science
- Christian Strupp, Gowan Crop Protection
- Helen Tinwell, Bayer Crop Science
- Christiane Wiemann, BASF SE
- Phil Botham, Syngenta Crop Protection, ECETOC Steward
- Bennard van Ravenzwaay, Environmental Sciences Consulting, ECETOC Steward

THANK YOU FOR YOUR ATTENTION

EXPERIENCES WITH THYROID ED ASSESSMENT

EFSA ED DATABASE

Martina Panzarea, Scientific Officer PREV Unit EFSA

EUROPEAN FOOD SAFETY AUTHORITY

DISCLAIMER

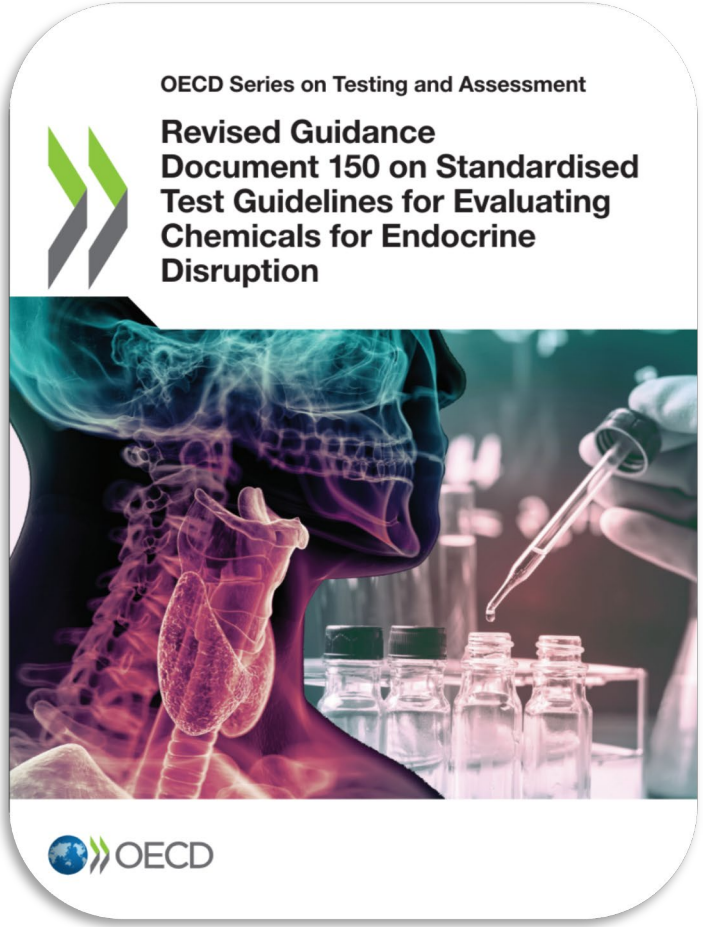
The positions and opinions presented in these slides are those of the presenter alone and do not necessarily represent the views/any official position or scientific works of EFSA.

LEGISLATIVE BACKGROUND

Endocrine Disruptor criteria laid down in Commission Delegated Regulation (EU) No 2017/2100 for Biocidal Products (BPs) and **Commission Regulation (EU) No 2018/605** for Plant Protection Products (PPPs).

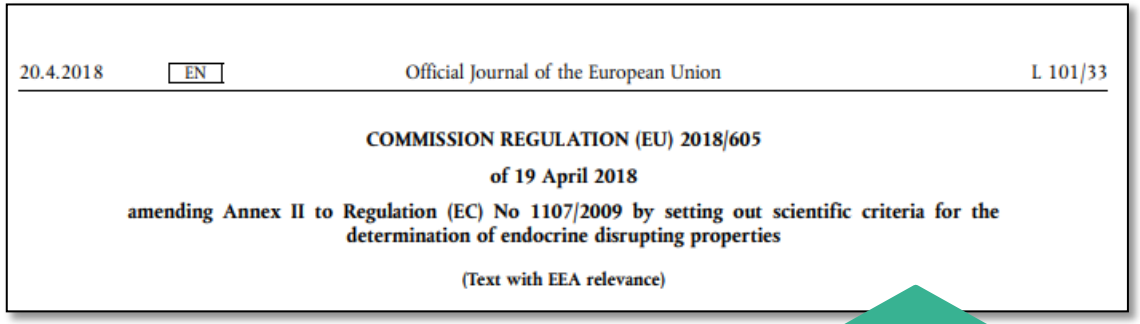
EFSA and ECHA were mandated to provide **technical guidance** (**ECHA-EFSA Guidance, 2018**) on the implementation of the ED criteria applicable in the context of the BP and PPP Regulations, respectively.

The assessment is based on the **OECD Guidance Document No 150** for classification of the endpoints (endpoints endocrine mediated vs. endpoints sensitive).



OECD (2018), Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption.

LEGISLATIVE BACKGROUND



20.4.2018 EN Official Journal of the European Union L 101/33

COMMISSION REGULATION (EU) 2018/605

of 19 April 2018

amending Annex II to Regulation (EC) No 1107/2009 by setting out scientific criteria for the determination of endocrine disrupting properties

(Text with EEA relevance)

a) it shows an **adverse effect** in an intact organism or its progeny, which is a change in the morphology, physiology, growth, development, reproduction or life span of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences;
b) it has an **endocrine mode of action**, i.e. it alters the function(s) of the endocrine system; and
c) the adverse effect **is a consequence** of the endocrine mode of action.

Endocrine mode of action = endocrine activity.

Term 'endocrine mode of action' in point **(c)** includes both the endocrine activity, the adverse effect(s) and a biologically plausible link between.

Points **(b) and (c)** should be understood as:

(b) it shows endocrine activity, i.e. it has the potential to alter the function(s) of the endocrine system;
(c) the substance has an endocrine disrupting mode of action, i.e. there is a biologically plausible link between the adverse effect and the endocrine activity.

INFORMATION SOURCES FOR ED (T-MODALITY) IDENTIFICATION

In vitro mechanistic (OECD CF level 2)

At the moment, there are no level 2 OECD TG studies available for T-modality.

Information on in vitro mechanistic endpoints is retrieved from non-guideline in vitro assays e.g., thyroperoxidase inhibition assay, Sodium Iodide Symporter inhibition assay, Deiodinase 1-3 inhibition assays.

1

In vivo mechanistic (OECD CF level 3)

At the moment, there are no level 3 OECD TG studies available for T-modality.

Information on in vivo mechanistic endpoints i.e.. THs and TSH is retrieved from repeated-dose toxicity studies conducted in accordance with OECD TGs.

2

Provide information on endocrine activity

T-mediated (OECD CF levels 4 & 5)

T-mediated parameters e.g. thyroid histopathology, thyroid weight, HDL/LDL ratio, liver weight, measured in the repeated-dose in vivo toxicity studies.

3

Sensitive to, but not diagnostic of, T (OECD CF levels 4 & 5)

Endpoints e.g. learning and memory in offspring, measured in the repeated-dose in vivo toxicity studies. These are effects that may provide indications of an endocrine MoA that might warrant further investigation.

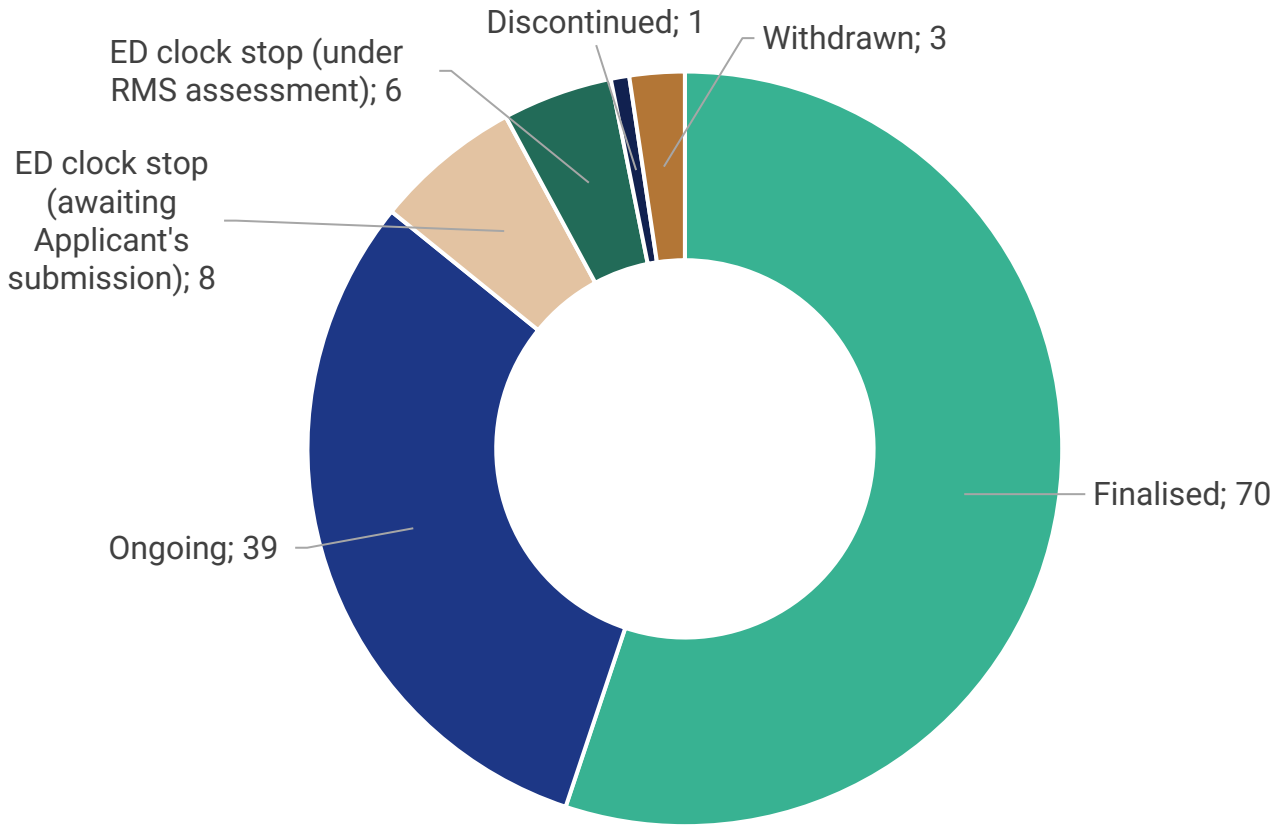
4

Provide information on potentially adverse effects

ED DATABASE

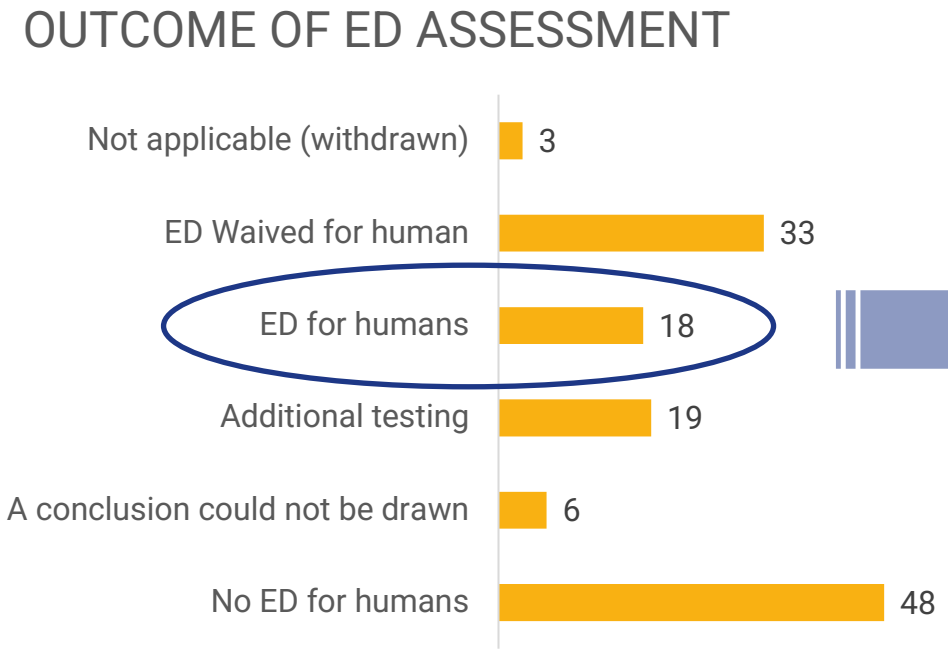
127

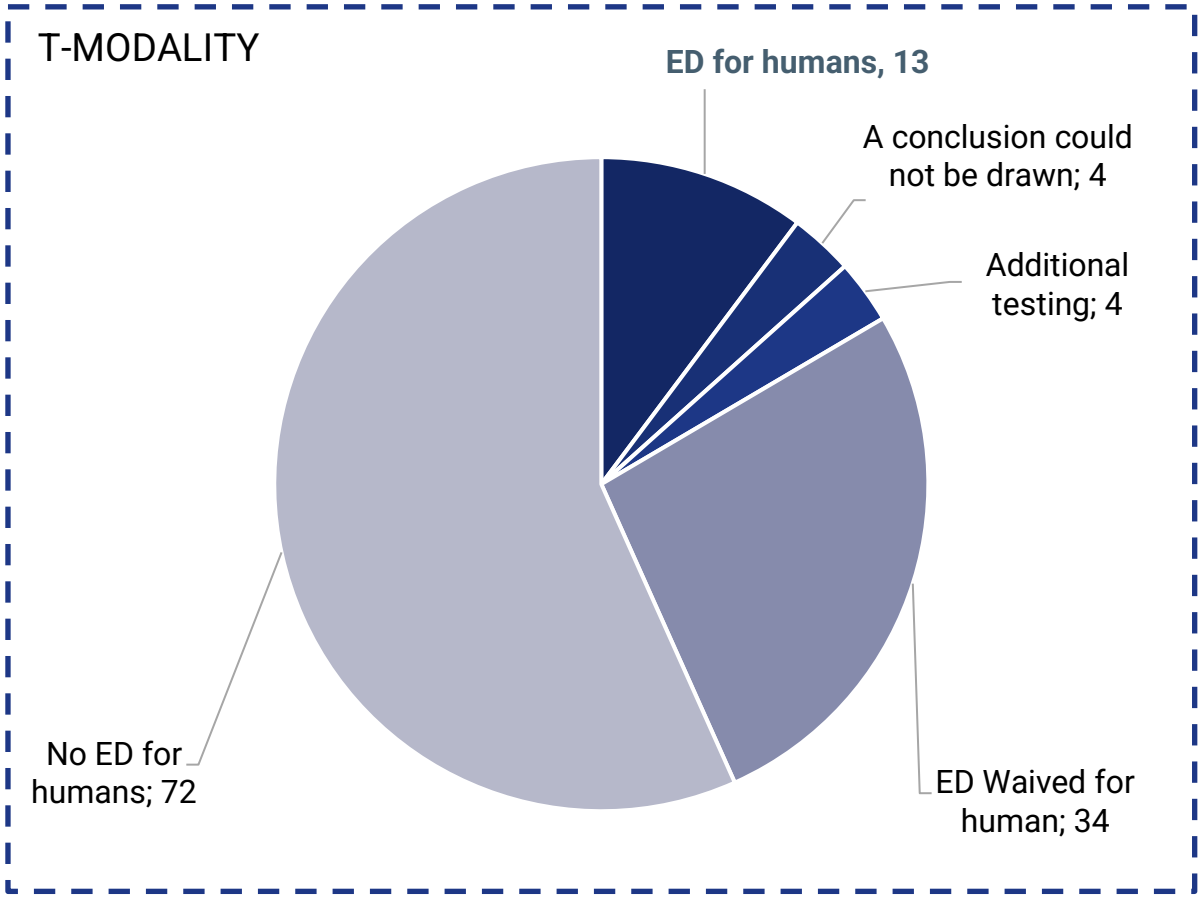
Substances assessed since the entering in force of the ECHA/EFSA ED Guidance (Nov 2018- August 2024)*



- 43 substances in 2020 (see EFSA (European Food Safety Authority), 2020. Technical report on the outcome of the pesticides peer review meeting on general recurring issues in mammalian toxicology. EFSA supporting publication 2020: 17(4):EN-1837. 26 pp. doi: 10.2903/sp.efsa.2020.EN-1837).
- These data are publicly available in the EFSA website: https://www.efsa.europa.eu/en/applications/pesticides .

ED DATABASE: OUTCOME OF THE ED ASSESSMENT FOR HUMANS





- The conclusion was based on the identification of **EATS mediated adversity.**
- Data on **endocrine activity (including hormonal levels)** were used to substantiate the MoA.

ED CRITERIA MET

Overview of data availability for the 13 substances meeting the ED criteria for T-modality.

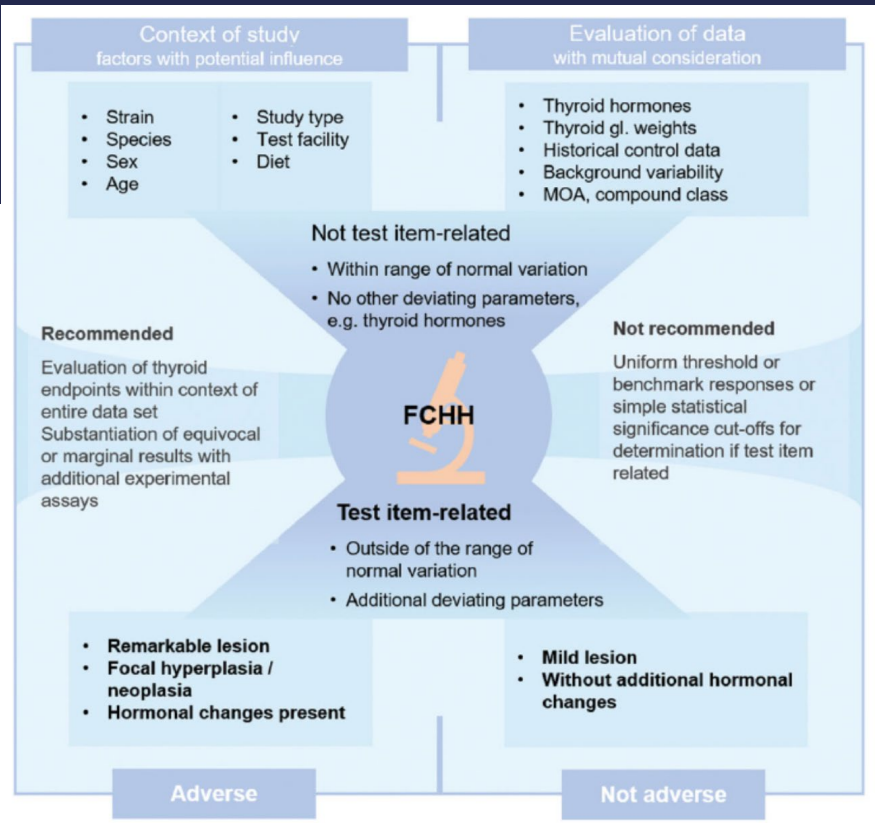
Substance N.	WoE for Adversity (outcome)	rat	mouse	dog	DNT study (OECD TG 426) or DNT Cohort	CTA study	WoE for Activity (outcome)	T4	T3	TSH	TPO	NIS	DIO	TR	OTHER KEs	MoA analysis	Most plausible MoA(s)	Conclusion T-m
1	Yes	H	H	↑ OW	N/A	N/A	Yes	↓29%	↓ ↑	↑ 14%		N/A	N/A	N/A	↑ UDPGT	Y	CAR/PXR	ED
2	Yes	H, ↑ OW	H, ↑ OW	H, ↑ OW		N/A	Yes	↓52%		↑ 169%		N/A	N/A		N/A	Y	TPO inhibition	ED
3	Yes	H, ↑ OW	H, ↑ OW	H, ↑ OW	N/A	N/A	Yes	↓35%	↑	↑ 121%		N/A	N/A		N/A	Y	TPO inhibition	ED
4	Yes	H	H		N/A	N/A	Yes	↓18%		↑ 16%	N/A	N/A	N/A	N/A	↑ UDPGT, P450	Y	CAR/PXR	ED
5	Yes	H	H	H, ↑ OW	Morphology	Y	Yes	↓ 72%	↓ 38%	↑ ↓	N/A		DIO 3	N/A	↑ UDPGT, P450	Y	CAR/PXR - DIO	ED
6	Yes	H			N/A	N/A	Yes	↓ ↑	↓ 25%	↑ 109%			N/A	N/A	↑ UDPGT	Y	CAR/PXR	ED
7	Yes	H		H, ↑ OW	N/A	N/A	Yes	↓ 22%	↓ 46%	↑ 20%		N/A	N/A	N/A	N/A	Y	TPO inhibition	ED
8	Yes	H			N/A	Y	Yes	↓ 70%	↓ 48%	↑ 270%			DIO1-3		↑ UDPGT	Y	CAR/PXR	ED
9	Yes	H		H	N/A	Not Acc.	Yes	↓	↓ 19%	↑ 189%					↑ UDPGT	Y	CAR/PXR	ED
10	Yes	H, ↑ OW		↑ OW	N/A	Y	Yes	↓ 46%	↓ 46%	↑ 236%					↑ UDPGT	Y	CAR/PXR	ED
11	Yes	H, ↑ OW	↑ OW	H, ↑ OW	N/A	N/A	Yes	↓ 47%	↓ 25%	↑140% F		N/A	N/A		↑ UDPGT	Y	TPO - CAR/PXR	ED
12	Yes	H			N/A	N/A	Yes	↓ 52%	↑ 73%	↑ 339%			N/A	N/A	↑ UDPGT	Y	CAR/PXR	ED
13	Yes	H	H		N/A	N/A	Yes	↓46.5	↓ 33%	↑ 130%			DIO1		↑ UDPGT	Y	CAR/PXR - DIO	ED

N/A = not available; H =histology; OW = Organ weight; DNT= developmental neurotoxicity; CTA =comparative thyroid assay;

ED CRITERIA MET

Thyroid adversity:

- **Thyroid follicular cell hypertrophy** and/or **hyperplasia/adenomas/carcinomas** were observed in the different species, most of the time in adult **rats (with data from short-term toxicity studies).**
  - Attention should be focused on how the diagnosis is performed e.g., inclusion of severity grade and comparison vs. the control is crucial.
- **Organ weight** (OW) was never observed as stand-alone endpoint.



Huisinga et al. 2020

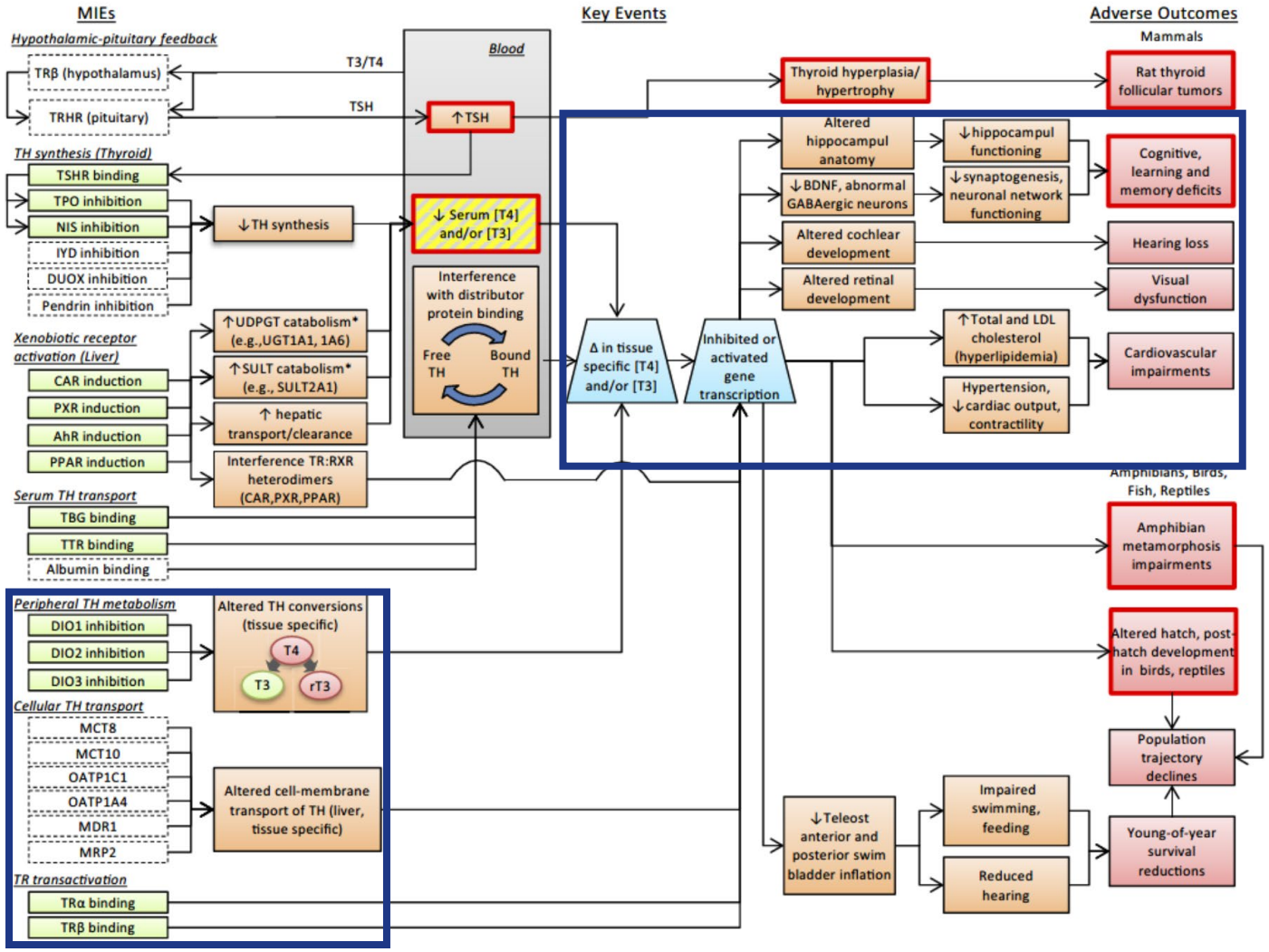
THs/TSH:

- In accordance with recent publications **reductions > 20% of serum T4** in adult animals were considered a concern. 20% drop in serum T4 in dams GD20 were associated to brain malformation (Hassan et al. 2017).
  - Range of changes observed in the database > T4: ↓18-72%; T3: ↓ 19-48%; TSH: 16-339%.
  - Independently from the analytical method applied, most of the time the quality of data in the study report is low with no information on the method validation and no HCD from the performing laboratory. The **quality of experimental data is crucial**.
- Few cases where THs increases were observed. This remain a concern since it is currently not known the effect of on the developing brain.

ED CRITERIA MET

MIE:

- **CAR/PXR activation** mode of action (MoA) is the most investigated;
  - Currently insufficient empirical data to dispute that this mode of action is of less concern for DNT effect (Li et al. 2019).
  - Lack of human relevance is difficult to be demonstrated because there is **no validated test methodology for phase I and phase II liver enzyme induction**.
- For the **other MIEs**, **ToxCast database** remains the main source of information.
  - A concordance analysis between the **in vitro assays** and the AO is still premature whereas the same level of uncertainty is not true for the hormonal analysis. **This represents a concern especially for the MIEs where no changes in circulating THs is expected.**



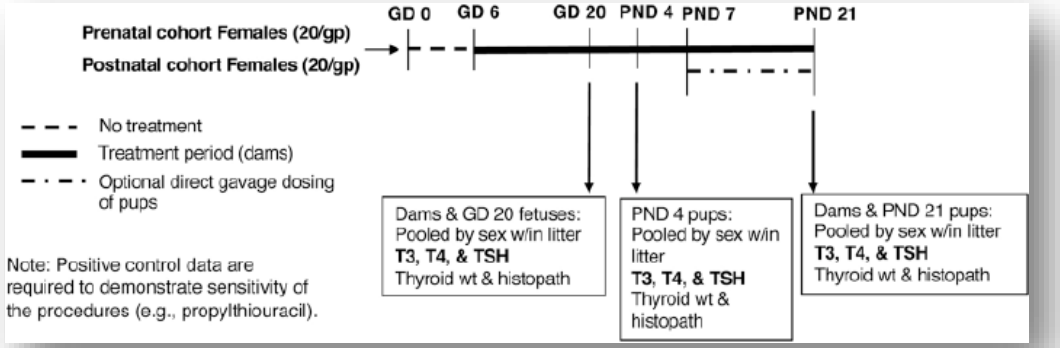
Noyes et al. 2019

COMPARATIVE THYROID ASSAY

Substance N.	WoE for Adversity	rat	mouse	dog	DNT effects	CTA study	WoE for Activity	T4	T3	TSH	TPO	NIS	DIO	TR	OTHER	MoA analysis	Conclusion T-m
1	Yes	H	H	H, OW	y	Y	Yes	↓ 72%	↓ 38%	↑ ↓			DIO3		↑ UDPGT	DIO - CAR/PXR	ED
2	Yes	H			N/A	Y	Yes	↓ 70%	↓ 48%	↑			DIO1-3		↑ UDPGT	CAR/PXR	ED
3	Yes	H		OW	Y	R	Yes	↓ 27%		↑ 60%	N/A		N/A	N/A	↑ UDPGT	Not performed	AT
4	Yes	H			Y	R	Yes	N/A	N/A	N/A	N/A		N/A	N/A	↑ UDPGT	Not performed	AT
5	Yes	H		H	N/A	Not Acc.	Yes	↓	↓ 19%	↑					↑ UDPGT	CAR/PXR	ED
6	Yes	H, OW		OW	N/A	Y	Yes	↓ 46%	↓ 46%	↑ 236					↑ UDPGT	CAR/PXR	ED

N/A = not available; H =histology; OW = Organ weight; R =requested; AT= Additional Testing

- Comparative Thyroid Assay (CTA) is expected to be conducted based on the results of study(ies) in adult animals that provide evidence that a substance produces effects on thyroid function.
- In the overall WoE, CTA study has a higher impact compared to other studies.
- CTA study designs allow to maximize the ability to capture thyroid adversity in target population.



Li et al. 2019

* US EPA (United States Environmental Protection Agency), 2005. Guidance for Thyroid Assays in Pregnant Animals,Fetuses and Postnatal Animals, and Adult Animals. In. US EPA, Office of Pesticide Programs, Health EffectsDivision, Washington (DC). 12 pp. Available online: https://www.epa.gov/sites/production/files/2015-06/documents/thyroid_guidance_assay.pdf

DNT

WoE for Adversity (outcome)	rat	mouse	dog	DNT study (OECD TG 426) or DNT Cohort	CTA study	WoE for Activity	T4	T3	TSH	TPO	NIS	DIO	TR	OTHER	MoA(s) formulated	Conclusion T-m
Yes	H	H	↑ OW	N/A	N/A	Yes	↓29%	↓ ↑	↑ 14%		N/A	N/A	N/A	↑ UDPGT	CAR/PXR	ED
Yes	H, OW	H, OW	H, OW	N/A	N/A	Yes	↓35%	↑	↑ 121%		N/A	N/A		N/A	TPO inhibition	ED
Yes	H	H		N/A	N/A	Yes	↓18%		↑ 16%	N/A	N/A	N/A	N/A	↑ UDPGT	?	ED
Yes	H			N/A	N/A	Yes	N/A	N/A	N/A			DIO3		IYD	CAR/PXR, NIS and DIO	I
Yes	H	H	H, OW	Morph.	Y	Yes	↓ 72%	↓ 38%	↑ ↓			DIO3		↑ UDPGT	DIO - CAR/PXR	ED
Yes	H			N/A	N/A	Yes	↓ ↑	↓ 25%	↑ 109%			N/A	N/A	↑ UDPGT	CAR/PXR	ED
Yes	H		H, OW	N/A	N/A	Yes	↓ 22%	↓ 46%	↑ 20%		N/A	N/A	N/A	N/A	TPO inhibition	ED
No	> MTD			Morph., ASR	N/A	No								↑ UDPGT	Not performed	No ED
Yes	H			N/A	Y	Yes	↓ 70%	↓ 48%	↑ 270%			DIO1-3		↑ UDPGT	CAR/PXR	ED
Yes	H		OW	ASR, NP, FOB	R	Yes	↓ 27%		↑ 60%	N/A		N/A	N/A	↑ UDPGT	N/A	AT
Yes				ASR, NP	N/A	Yes	N/A	N/A	N/A		N/A	DIO1-3		N/A	N/A	I
Yes	H			ASR, NP	R	Yes	N/A	N/A	N/A	N/A		N/A	N/A	↑ UDPGT	N/A	AT
Yes	H			N/A	N/A	Yes	↓ 10%	N/A	↑ 167%			N/A	N/A	↑ UDPGT	N/A	I
Yes	H		H	N/A	Not Acc.	Yes	↓	↓ 19%	↑ 189%					↑ UDPGT	CAR/PXR	ED
Yes	H, OW		OW	N/A	Y	Yes	↓ 46%	↓ 46%	↑ 236					↑ UDPGT	CAR/PXR	ED
Yes	H, OW	OW	H, OW	N/A	N/A	Yes	↓ 47%	↓ 25%	↑140%		N/A	N/A		↑ UDPGT	TPO - CAR/PXR	ED
Yes	H			N/A	N/A	Yes	↓ 52%	↑ 73%	↑ 339%			N/A	N/A	↑ UDPGT	CAR/PXR	ED
No				N/A	N/A	Yes	↓ 48%	N/A	↑ 36%			DIO 1-2		↑ UDPGT	N/A	AT
Yes	H	H		N/A	N/A	Yes	↓46.5%	↓ 33%	↑ 130%			DIO 1		↑ UDPGT	DIO - CAR/PXR	ED

ASR = auditory startle response; NP = neuropathology; FOB = functional observation battery, Morph = morphometry

- It is difficult to contextualise DNT effect for those MIEs (e.g., DIO, cellular TH transport) that are not going through changes in TSH/THs in circulating blood as intermediate KEs.
- Moreover, the only endpoint that is thyroid specific is the **cortical heterotopia** that is observed in rat pups born to hypothyroid dams (Hassan et al. 2017).

SUMMARY

- **Assessment of thyroid disrupting chemicals is still mainly based on animal studies and on thyroid histological findings.**
  - It is recommended to conduct the diagnosis in accordance with Huisinga et al. 2020.
  - Future directions on how to use the IVB of the EU NETVAL to avoid in vivo studies will be needed.
- **The CTA study is the gold standard: it measures relevant endpoint in sensitive population e.g. foetuses, pups and pregnant dams.**
- **THs / TSH are always affected when the substance is concluded to be ED:**
  - the doses at which the effects are observed were always in the same range of the doses at which thyroid histopathological findings are reported.
  - A gold-standard on how to conduct the analysis is missing. From EFSA's experience the following information is crucial : coefficient of variations (CVs) (25-30% for THs and 35% for TSH) and reference to the validation of the study, including LOQ-LOD.
  - **TH increase** is also considered a concern for the developing brain. Regulatory experience on how to handle qualitative and quantitative increase in THs is still representing an uncertainty. Only one case is reported in the database, however it was a unique case because the increase in THs at low doses was followed by decrease at higher doses (accompanied by histopathological correlates).
- **Increase in cholesterol (i.e. HDL/LDL ratio) is a recognized endpoint in hypothyroidism syndrome in humans and it is known to occur also in experimental animals.**
  - However, the impact of cholesterol in the overall analyses of thyroid disrupting chemicals is currently not included in the ED database curated by EFSA.
- **MIEs with no changes in circulating TSH/THs as intermediate KEs (i.e. DIOs inhibition and cellular transporter) will represent a real concern for DNT.**
  - **Measuring brain concentration for TH** could be relevant for DNT adverse outcome, although this remains a methodological challenge.

THANK YOU FOR YOUR ATTENTION

FOR ANY QUESTION:
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ACKNOWLEDGEMENT:
THANKS TO ANDREA TERRON AND TO THE MAMMALIAN TOXICOLOGY TEAM OF EFSA PREV UNIT

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Experiences with Thyroid ED Assessment & the new CLP ED Criteria

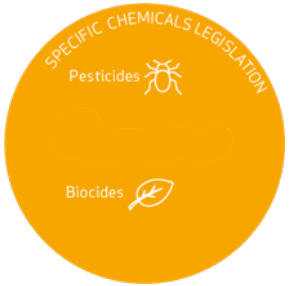
ECETOC & RSA Symposium "Testing, Assessment and Classification of Thyroid Hormone Disruptors”

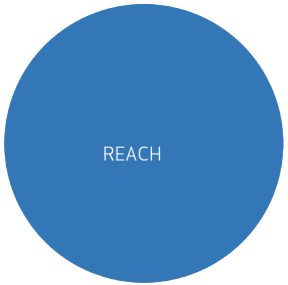
8 September 2024

Niklas Andersson
Scientific Area Leader Endocrine Disruption
European Chemicals Agency

Experiences with Thyroid ED Assessments

→ EU Chemicals legislation framework for EDs 2018-2023





Biocides and Pesticides

→ ED criteria

→ ED specific information requirements

→ ECHA/EFSA Guidance
- Appendix A

REACH

Dossier evaluation:

→ Approx. 400 EOGRTS requested
- 25% include request to extend Cohort 1B; 60 cases have an ED-based trigger for F1 (about half due to thyroid effects)
- 26% include request to include the DNT; (about half due to thyroid effects)

Candidate List of substances of very high concern for Authorisation

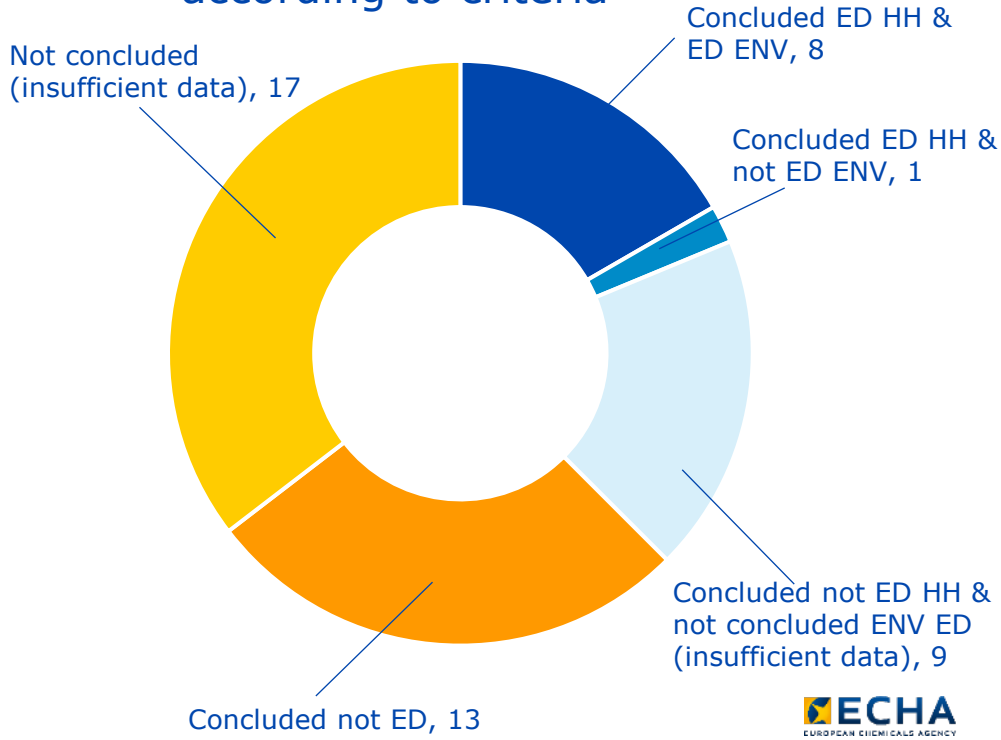
→ 22 (out of 241) entries include endocrine disrupting properties (Article 57(f) human health or environment)

ED assessments under BPR 2018-

193

Pending assessments

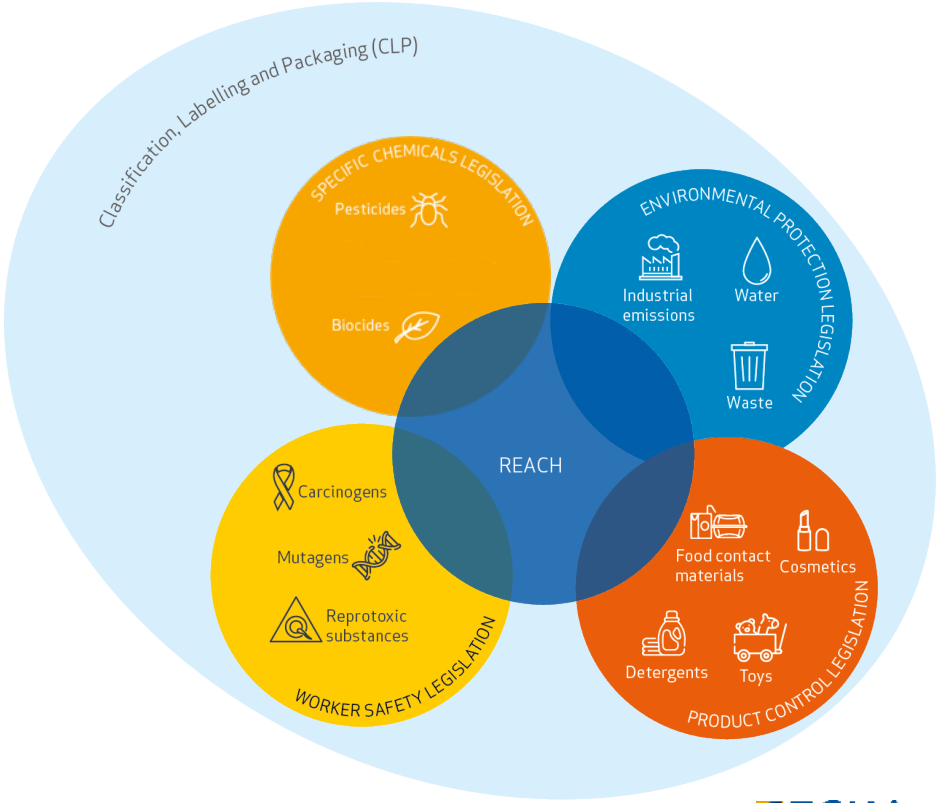
48 active substances assessed according to criteria



New CLP Criteria

→ EU Chemicals legislation framework for EDs from 2023

New hazard classes in CLP

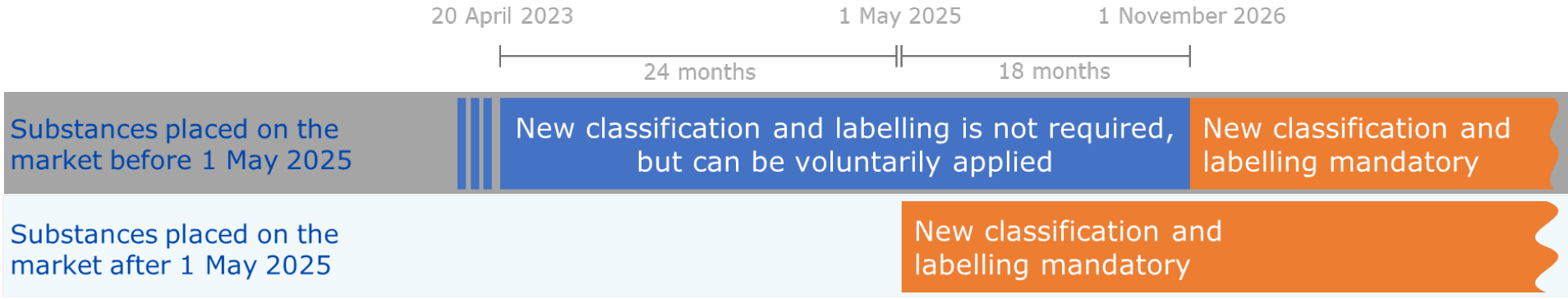


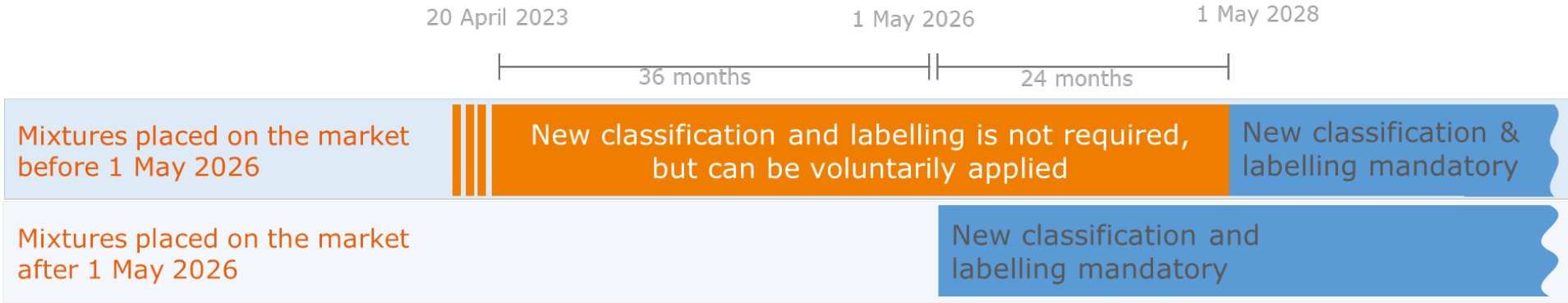
CLP regulation update - DA (Annex I) – What's in

	New criteria for Endocrine Disruptors for humans and environment (ED HH & ED ENV)	EDs Cat 1 (known/presumed) and Cat 2 (suspected)
	Persistent, Bio-accumulative and Toxic (PBT), very Persistent, very Bioaccumulative (vPvB)	no subcategories
	Persistent, Mobile and Toxic (PMT), very Persistent and very Mobile (vPvM)	no subcategories

https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32023R0707

Application dates for the new hazard classes





https://echa.europa.eu/new-hazard-classes-2023

ED criteria for Human health and Environment

Substance is classified when

i) *endocrine activity* and

ii) *adverse effect* and

iii) *biologically plausible link between* adversity and endocrine activity is established

Cat 1 – Known or presumed endocrine disruptors

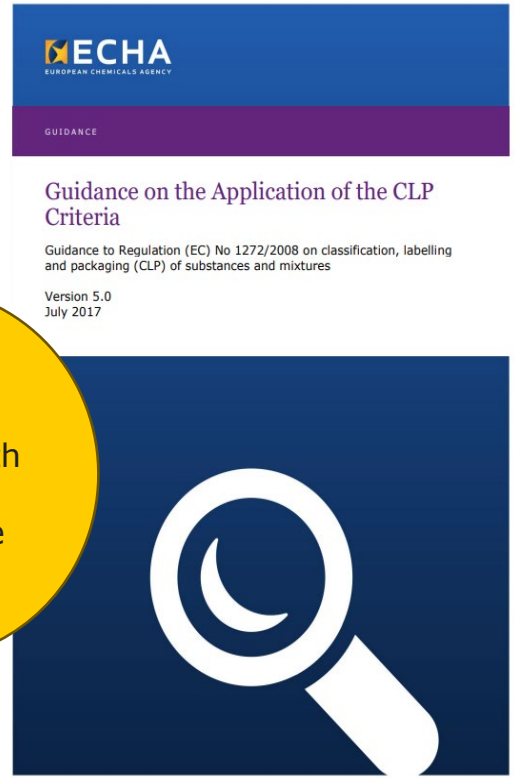
Cat 2 – Suspected endocrine disruptors

Component classified as:	Generic concentration limits triggering classification of a mixture as:	
	Category 1	Category 2
Category 1	≥ 0,1 %	
Category 2		≥ 1 %

**The concentration limits in this Table apply to solids and liquids (w/w units) as well as gases (v/v units).*

→ Guidance update

- ’Guidance on the Application of the CLP Criteria’
  - New classes and criteria
  - Part 3
    - 3.11 ED for HH
  - Part 4
    - 4.2 ED for ENV
    - (4.3 PBT/vPvB & PMT/vPvM)



Further updates with more experience

ED guidance publication: October 2024

Before publication, EFSA/ECHA Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and EC No 1107/2009 may be used for Cat 1
https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2018.5311

Substances already concluded as EDs under PPPR, BPR or REACH (SVHC)

→ An ED conclusion based on the ED criteria for BPR or PPPR correspond to *Category 1 under CLP* – direct transfer foreseen

→ An active substance concluded not to meet the ED criteria under BPR or PPPR

- Can be Category 1, Category 2 or No classification under CLP, depending on data available when re-assessed

→ ED SVHCs correspond to *Category 1 under CLP* – direct transfer foreseen

Thyroid assessment – Two guidance documents developed for different contexts

ECHA/EFSA Guidance, Appendix A

→ How to generate the data needed to be able to conclude

- Investigate thyroid MoAs do the extent possible
- Offered advice on how to investigate of increase of thyroid hormone metabolism in the liver
- Offered advice on how to follow-up perturbations of circulating thyroid hormone in the absence of histological changes in adults

CLP Guidance

→ How to conclude on the ED criteria

- All thyroid related MoAs are relevant to humans
- Classification is warranted when a '***pattern of thyroid-related effects lead to the overall conclusion that they constitute an adverse effect***'
- When adverse effects are observed on the thyroid gland, additional mechanistic information is not necessarily required to meet the ED criteria.

Thank you

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German Federal Institute for Risk Assessment

In vitro in vivo investigation of pesticide effects on the T-axis

08.09.2024, Copenhagen

Dr. Philip Marx-Stölting
Testing and Assessment Strategies
Pesticides Safety

www.nature.com/scientificreports

scientific reports

Check for updates

OPEN

In vitro and in vivo investigation of a thyroid hormone system-specific interaction with triazoles

Asya Kadic[1], Patricia Oles[2], Benjamin Christian Fischer[1], Anne Elisabeth Reetz[1,3], Boubacar Sidiki Sylla[1], Katreece Feiertag[1], Vera Ritz[1], Tanja Heise[1], Philip Marx-Stoelting[1], Tewes Tralau[1], Kostja Renko[2]✉ & Marize de Lourdes Marzo Solano[1]✉

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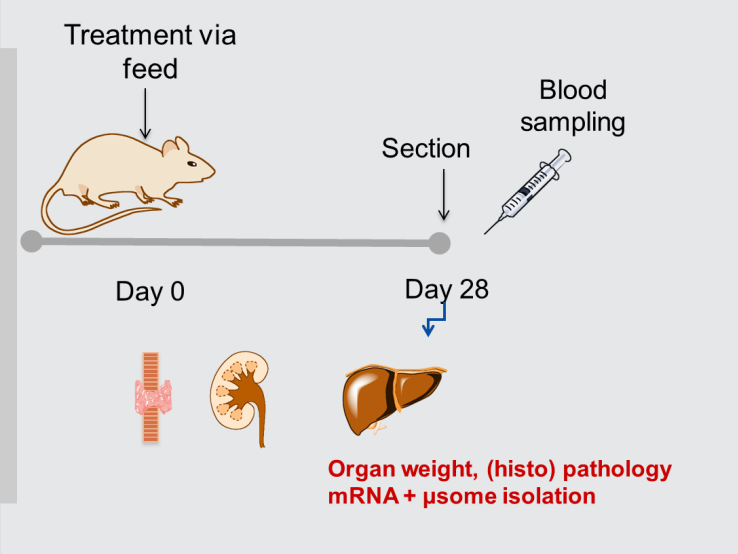
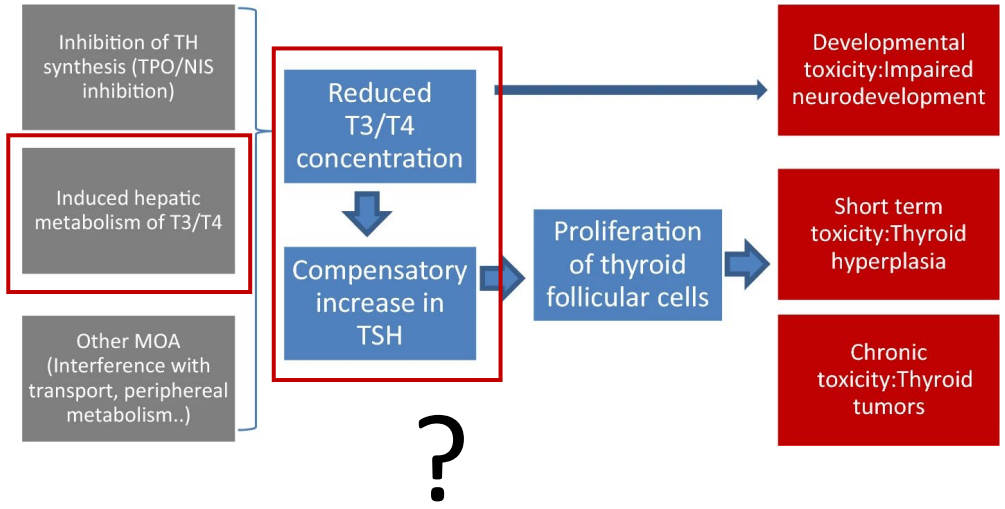
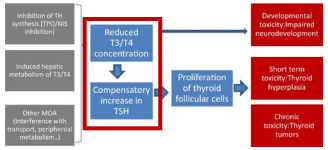
Study design

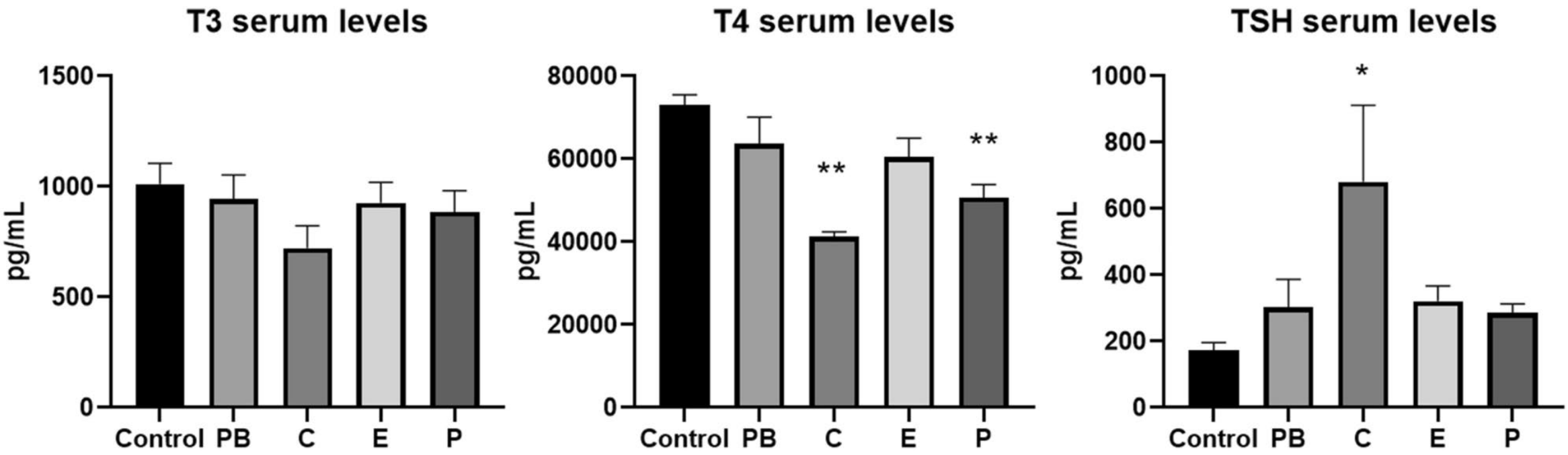


Cyproconazole, 1 – 1000ppm
Epoxiconazole, 0.9 – 900ppm
Prochloraz, 1 – 1000ppm
Penobarbital, 500ppm
Mainly liver effects (hypertrophy, vacuolization)
T- related effects at high dose levels only

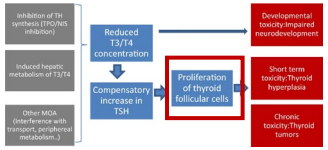


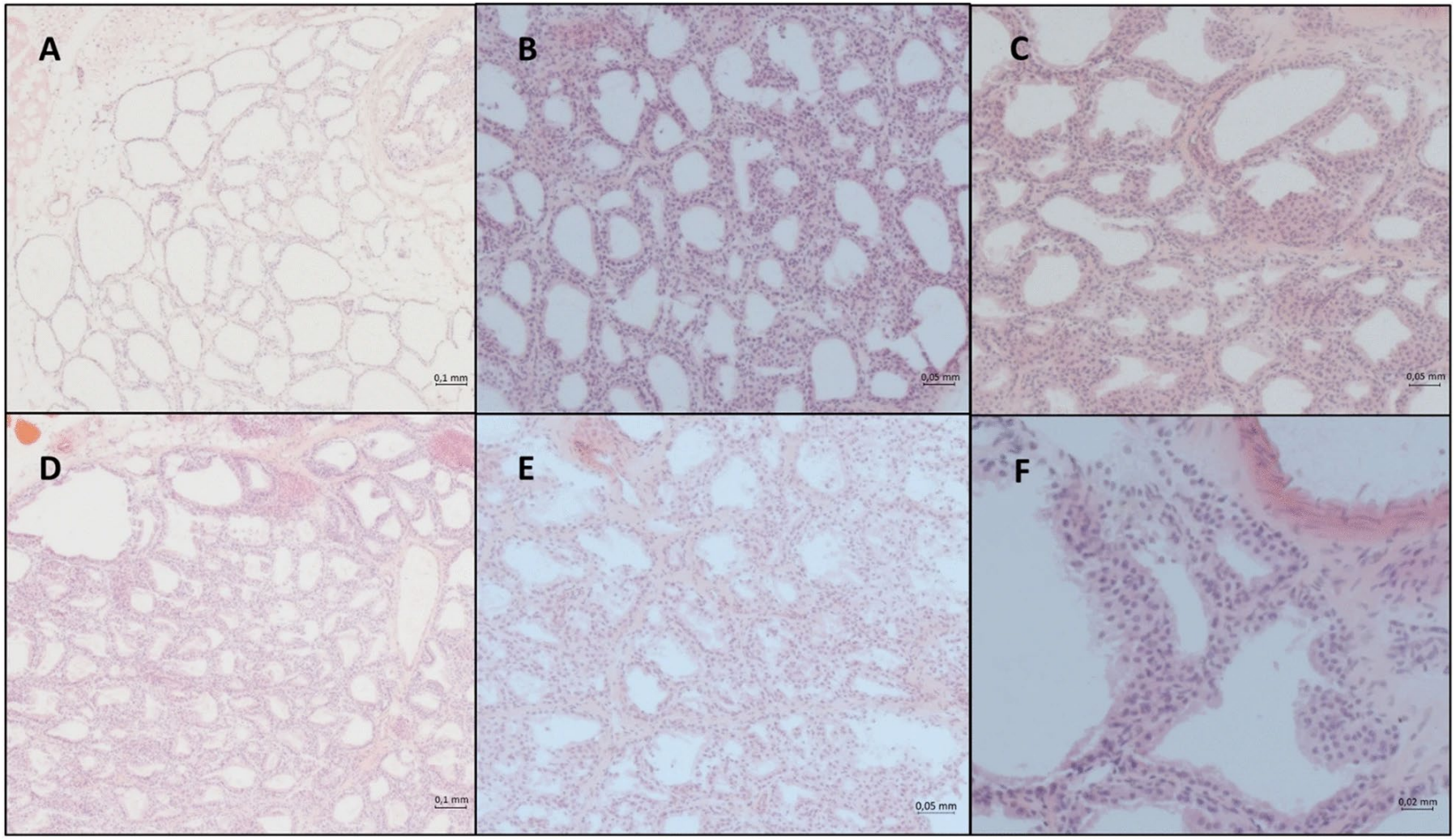
Thyroid hormone levels



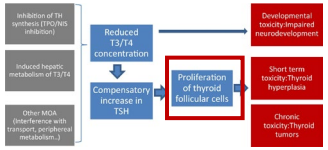


Histopathology





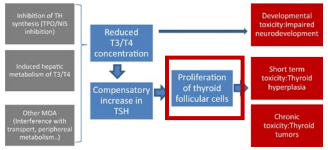
Histopathology - results



Incidence	Number of animals observed	Follicular hypertrophy	Follicular hyperplasia	Follicular dilation	No visible lesions
Control negative	6	0	0	4 (67%)	2 (33%)
Phenobarbital 500 ppm	5	5 (100%)*	5 (100%)*	5 (100%)	0
Cyproconazole 1000 ppm	5	5 (100%)*	4 (80%)*	5 (100%)	0
Epoxiconazole 900 ppm	5	5 (100%)*	5 (100%)*	5 (100%)	0
Prochloraz 1000 ppm	4	4 (100%)*	4 (100%)*	4 (100%)	0

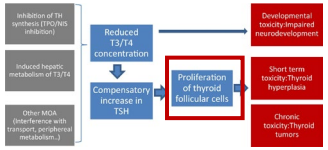
*($p \leq 0.05$), Fisher's Exact Test.

Histopathology – results

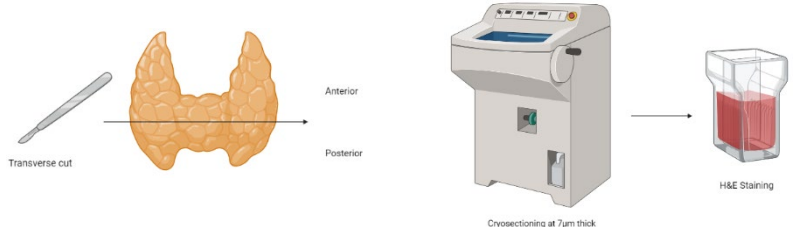


SEVERITY	Control negative	Phenobarbital 500ppm	Cyproconazole 1000ppm	Epoxiconazole 900ppm	Prochloraz 1000ppm
Number of animals observed	6	5	5	5	4
Follicular hypertrophy	0	XXXXX	XXXXX	XXXXX	XXXX
total follicular hypertrophy score	0	10	6	9	4
Follicular hyperplasia	0	XXXXX	XXXX	XXXXX	XXXX
total follicular hyperplasia score	0	9	5	5	7
Follicular dilation	XXXX	XXXXX	XXXXX	XXXXX	XXXX
total follicular dilation score	4	10	7	8	6

Morphometric analysis

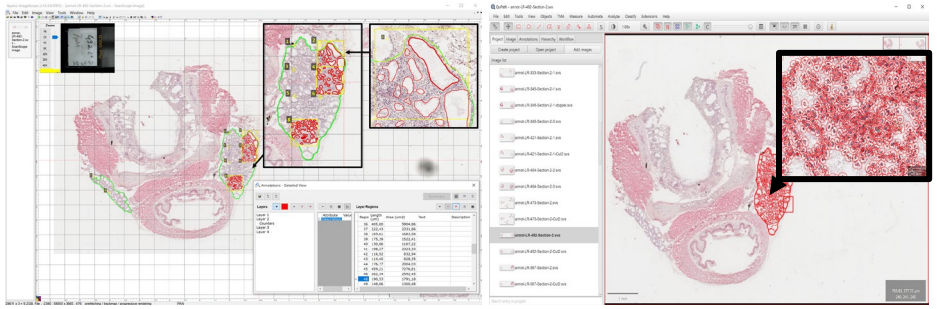


- Cryosectioning and H&E staining



- Digital scanning at 400x magnification

- ImageScope & QuPath for annotations



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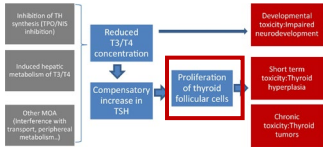
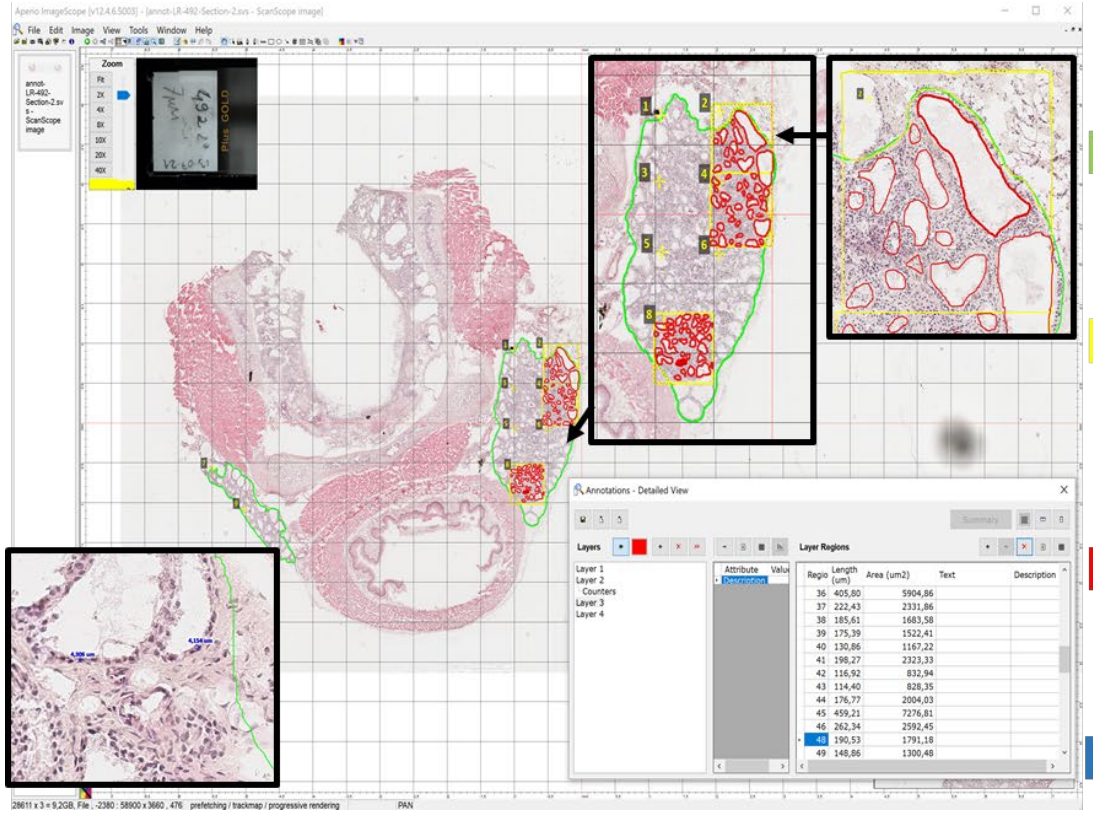
Semi-automated morphometric analysis



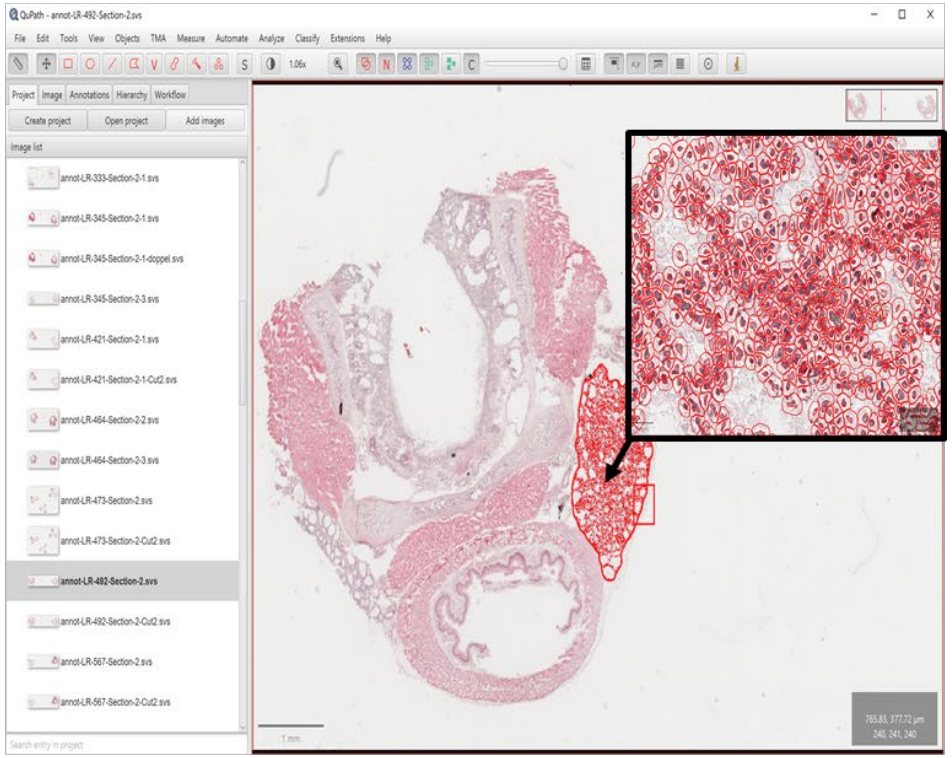


Thyroid Area (L1)

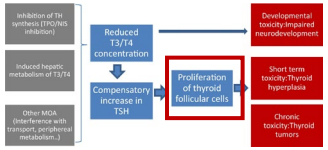
High Power Field (HPF) Thyroid Area (L2)

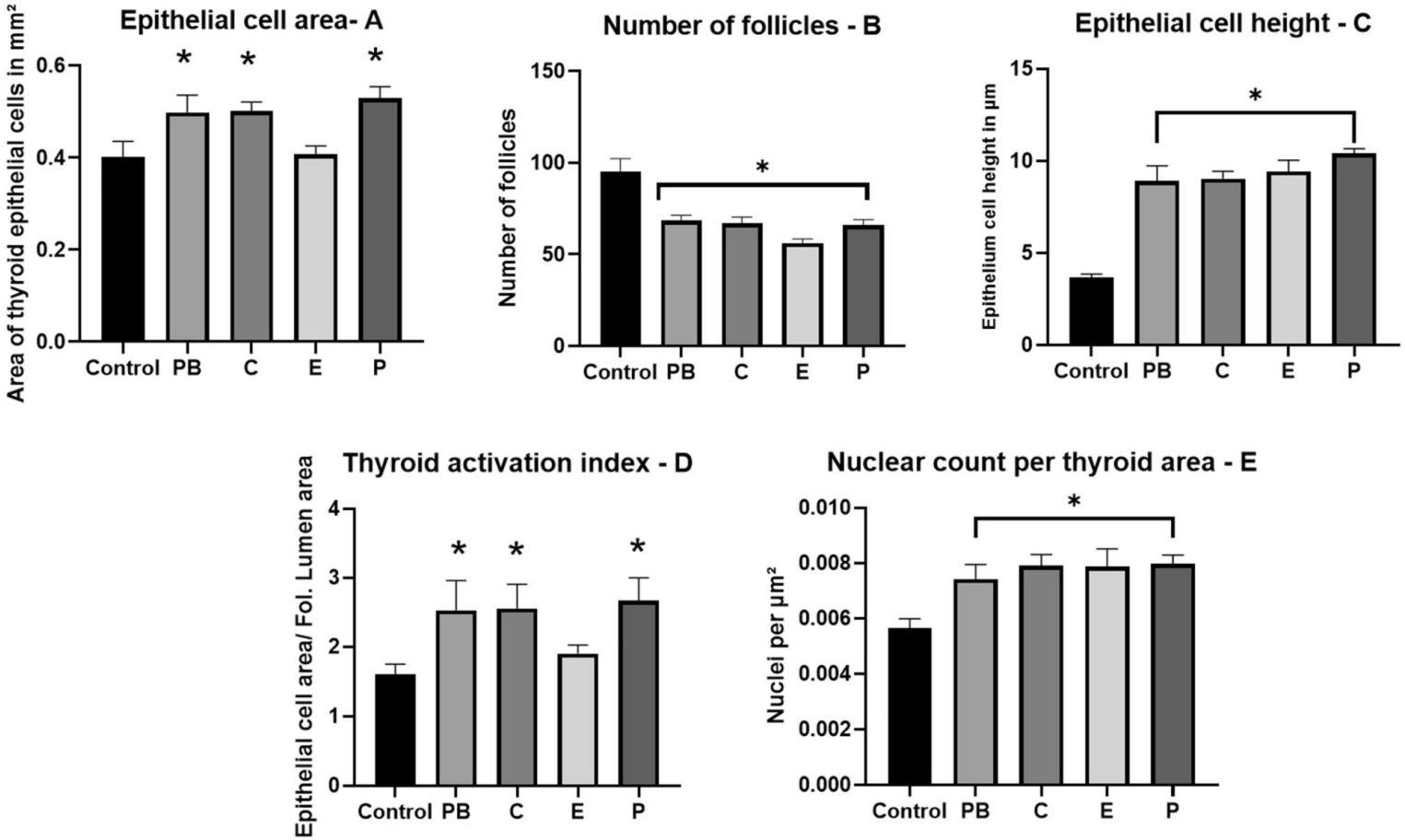
Follicle Lumen Area (L3)

Cell height

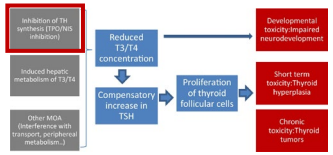


Morphometry - results





In vitro / ex vivo mechanistic investigations



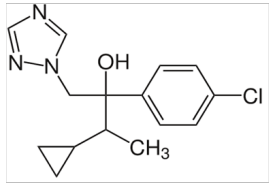
In vivo

[2]

Male Wistar rats, treated for 28d
via the diet with Cypro (up to 1000ppm)

KeyEvents

TSH ↑ T4↓

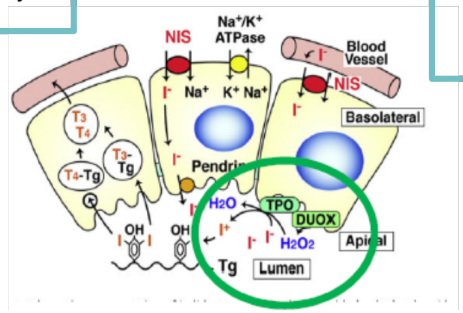


Cyproconazole
Agricultural fungicide
CAR-inducer

unchanged — Thyroidal Dio1 & Iyd activtiy

unchanged — Hepatic Dio1 & Iyd activtiy, mRNAs

unchanged — Renal Dio1 & Iyd activtiy

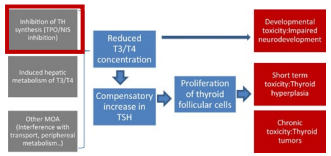


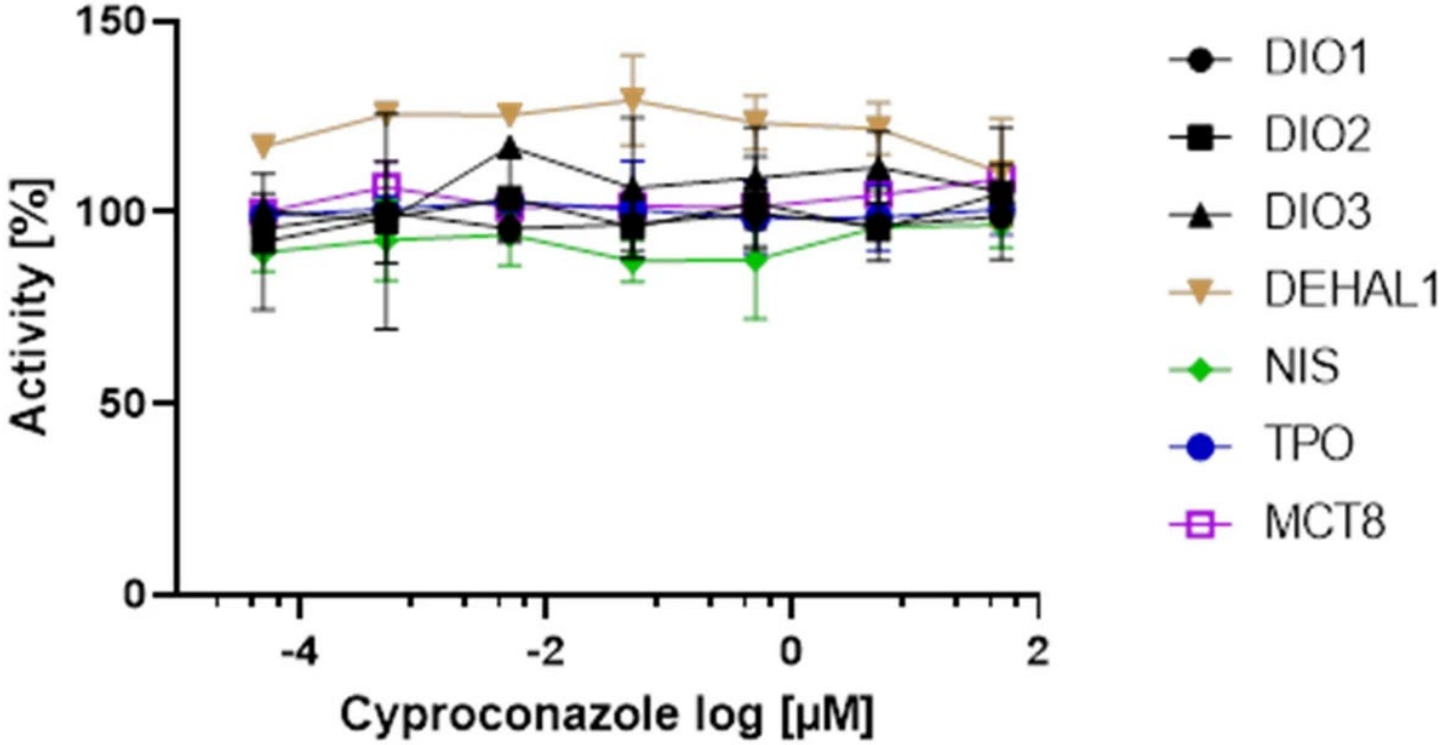
In vitro & in chemico
„humanized test systems“

Thyroidal iodide uptake (NIS-transfected HEK293) [3]	not affected
TH biosynthesis (in chemico, TPO inhibiton) [4]	not affected
TH deiodination (in chemico, D1/D2/D3 inhibiton)[5]	not affected
Iodide recycling (in chemico, IYD inhibiton) [6]	not affected
TH cellular uptake (in vitro, MCT8 inhibiton) [7]	unclear/unspecific (cell toxicity)

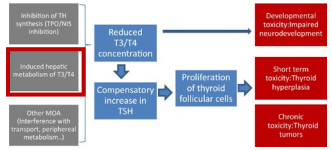
BfR

Example in vitro dose response curves: cyproconazole





In vitro / ex vivo mechanistic investigations



In vivo

[2]

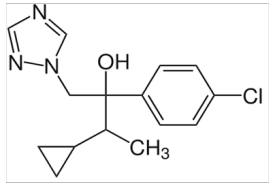
Male Wistar rats, treated for 28d
via the diet with Cypro (up to 1000ppm)

UGTs↑ mRNA	SULTs(?) mRNA

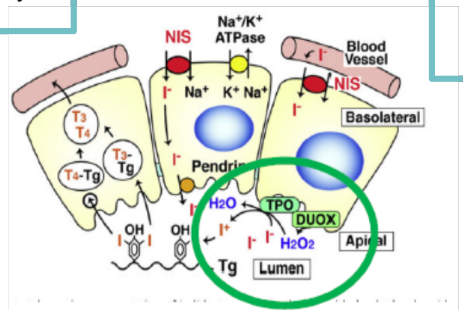
unchanged	Thyroidal Dio1 & Iyd activtiy
unchanged	Hepatic Dio1 & Iyd activtiy, mRNAs
unchanged	Renal Dio1 & Iyd activtiy

KeyEvents

TSH ↑ T4↓



Cyproconazole
Agricultural fungicide
CAR-inducer



In vitro & in chemico
„humanized test systems“

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TH cellular uptake (in vitro, MCT8 inhibiton) [7]	unclear/unspecific (cell toxicity)

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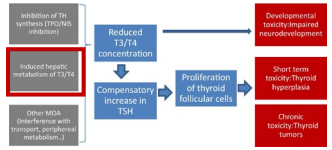
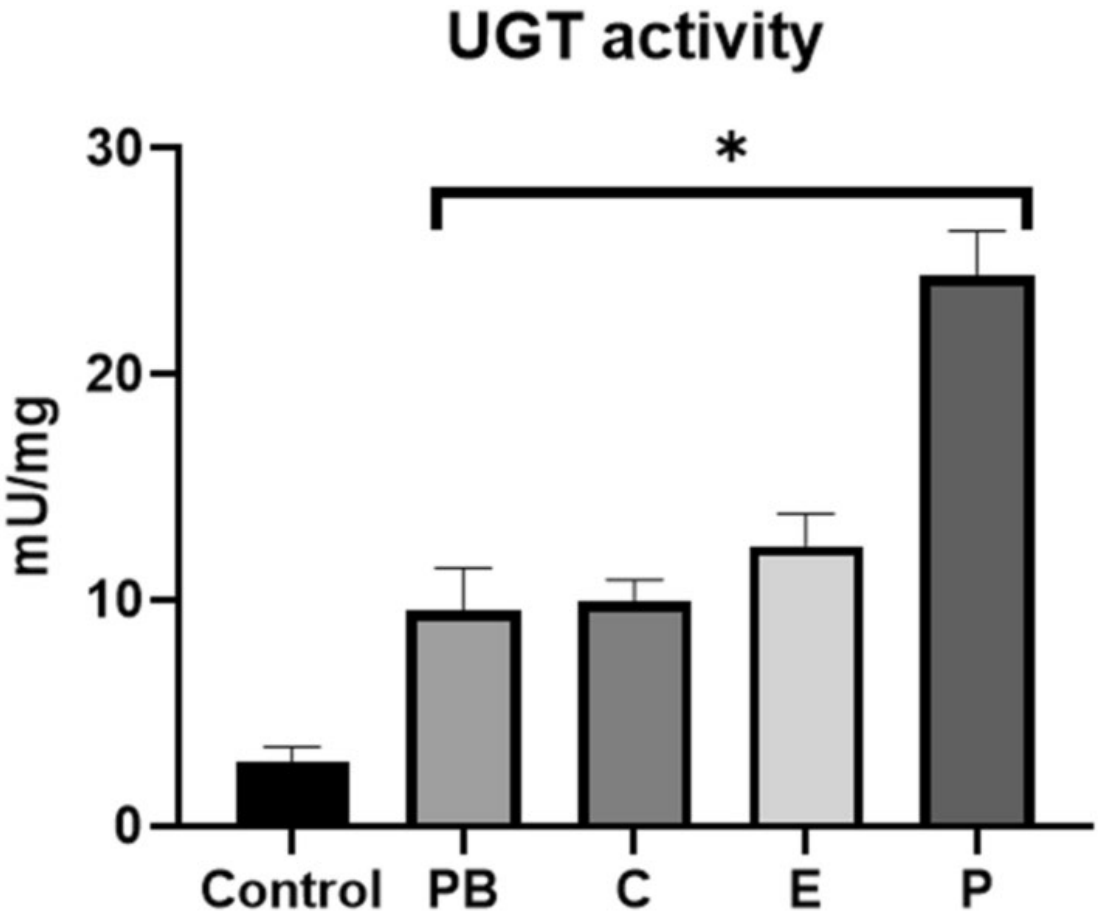
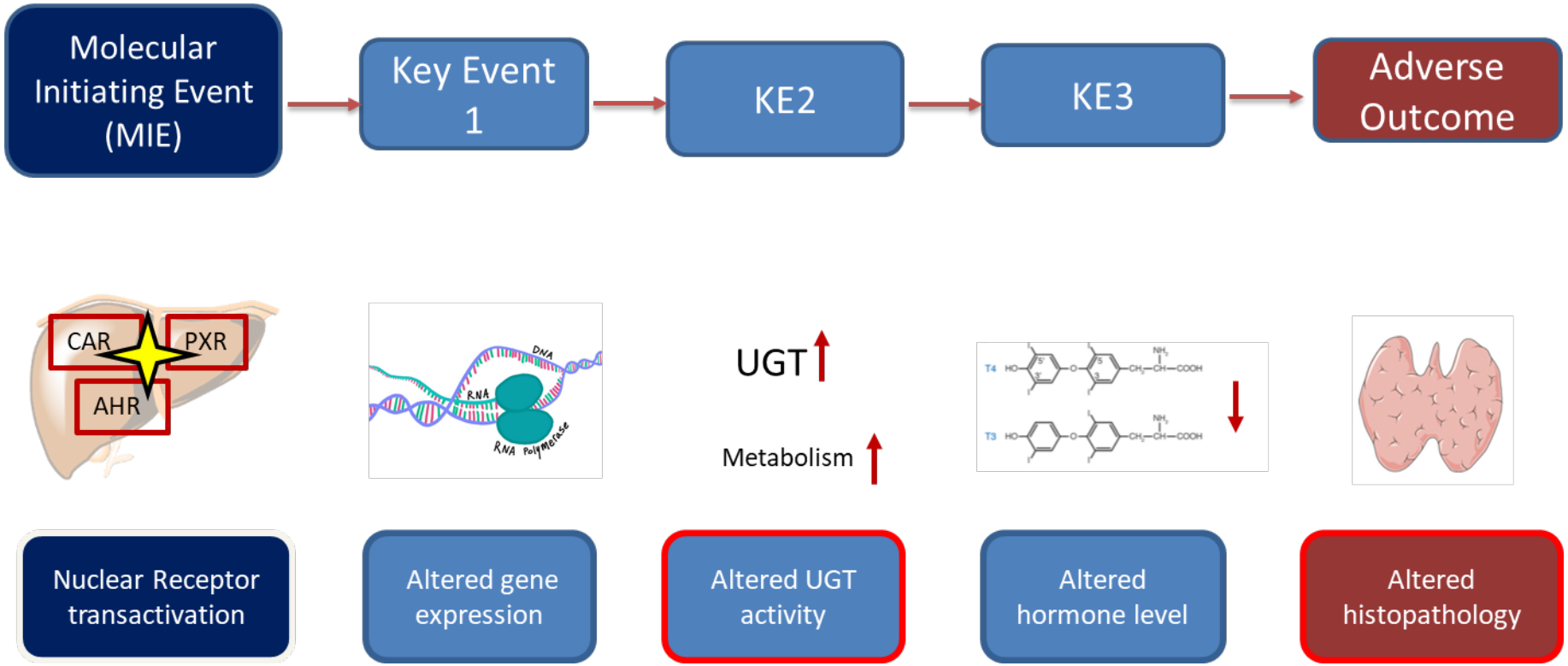
Ex vivo UGT activity significantly altered as compared to control






Molecular Initiating Event (MIE)
Key Event 1
KE2
KE3
Adverse Outcome
CAR
PXR
AHR
DNA
RNA
RNA Polymerase
UGT
Metabolism
T4
T3
Nuclear Receptor transactivation
Altered gene expression
Altered UGT activity
Altered hormone level
Altered histopathology

Summary 1

The substances investigated caused alterations in the thyroid at high dose levels due to hepatic enzyme induction.

Enzyme induction was the only mechanism confirmed.

Related questions:

- Is this relevant to humans?

- Do EDC have a threshold?

Is this relevant for humans?

- Differences in regulation make it on a quantitative level less likely that effects observed in the rat would affect humans

- On the level of T3/T4 storage via TBG

Research Article

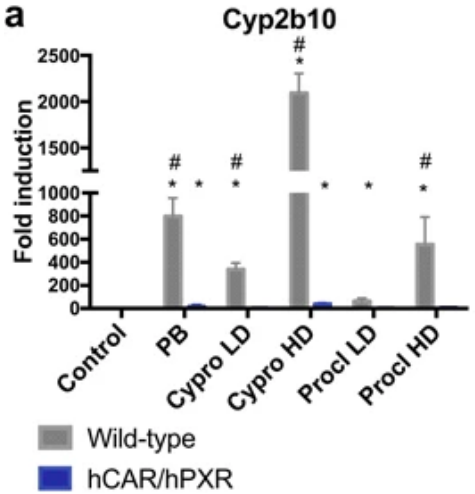
Thiazopyr and Thyroid Disruption: Case Study Within the Context of the 2006 IPCS Human Relevance Framework for Analysis of a Cancer Mode of Action

Vicki L. Dellarco, Douglas McGregor, Sir Colin Berry, Samuel M. Cohen & Alan R. Boobis

Pages 793-801 | Published online: 10 Oct 2008

- On the level of enzyme induction in the liver

- work in humanized mice / comparative in vitro analysis



Archives of Toxicology (2021) 95:117–133
https://doi.org/10.1007/s00204-020-02939-4

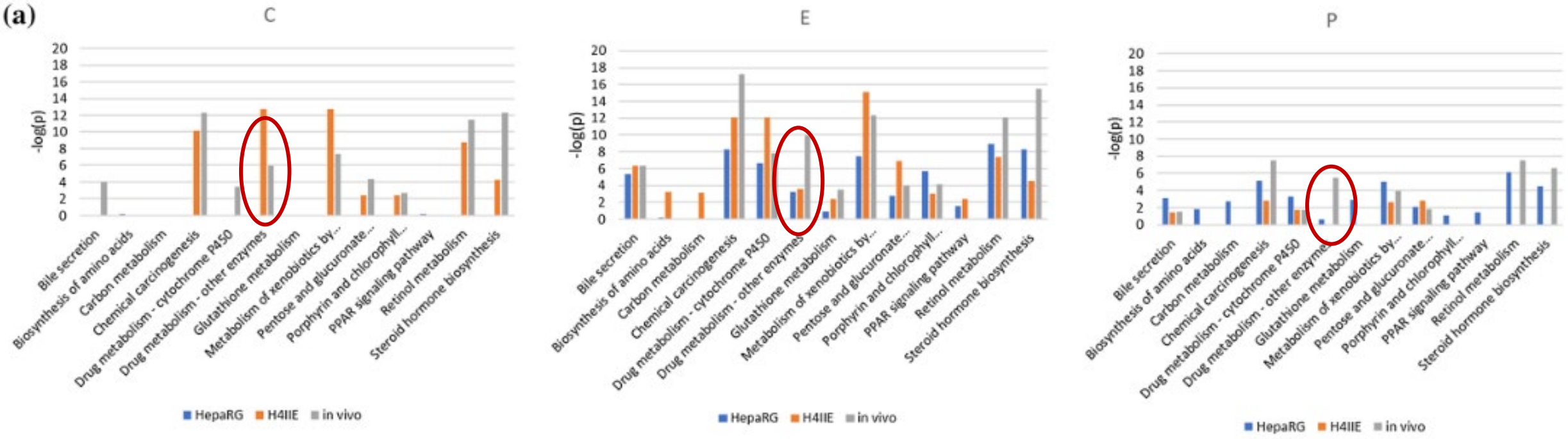
TOXICOKINETICS AND METABOLISM

Cross-species analysis of hepatic cytochrome P450 and transport protein expression

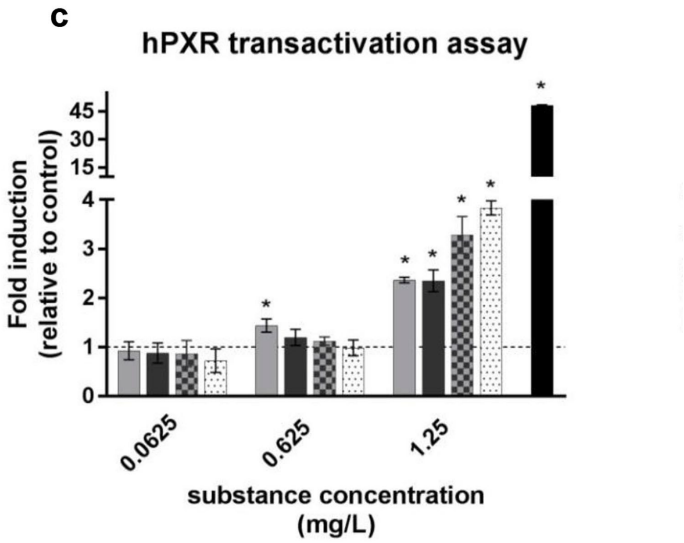
Helen Hammer[1] · Felix Schmidt[1] · Philip Marx-Stoelting[2] · Oliver Pötz[1] · Albert Braeuning[3]

Work in progress

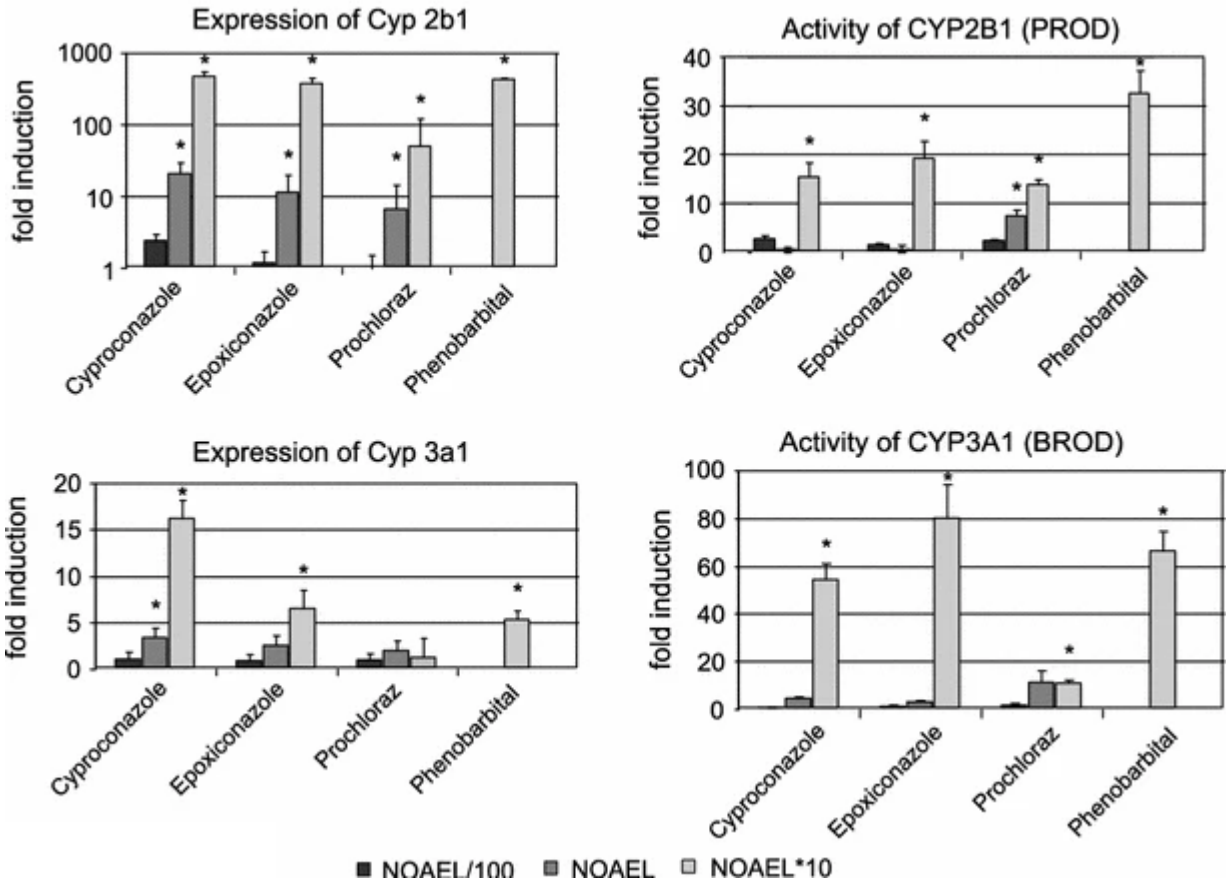
Comparative analysis in vitro (human, rat) and ex vivo



Do EDCs have thresholds?



Zahn et al. 2017; Heise et al. 2015



Archives of Toxicology (2024) 98:1795–1807
https://doi.org/10.1007/s00204-024-03723-4

MOLECULAR TOXICOLOGY

Check for updates

The physiological and biochemical basis of potency thresholds modeled using human estrogen receptor alpha: implications for identifying endocrine disruptors

Christopher J. Borgert[1] · Lyle D. Burgoon[2] · John C. Matthews[3]

BfR

Do EDCs have thresholds?

Archives of Toxicology (2024) 98:2019–2045
https://doi.org/10.1007/s00204-024-03748-9

REGULATORY TOXICOLOGY

Check for updates

Thresholds of adversity for endocrine disrupting substances: a conceptual case study

Judy Choi[1] · Stefanie Rotter[1] · Vera Ritz[1] · Carsten Kneuer[1] · Philip Marx-Stoelting[1] · Marize de Lourdes Marzo Solano[1] · Angelika Oertel[1] · Susanne Rudzok[1] · Andrea Ziková-Kloas[1] · Tewes Tralau[1] · Andreas Hensel[1]

BfR

Chemical name	CAS no	Status of review	Selected for screening	Data sources
EFSA				
Asulam	3337–71-1	Final	Yes	EFSA Conclusion (EFSA 2021d): RAR and ED assessment: https://www.efsa.europa.eu/en/consultations/call/public-consultation-active-substance-asulam-regards-assessments
Benthiavalicarb isopropyl	413615–35-7	Final	Yes	EFSA Conclusion (EFSA 2021b): https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2021.6833
Clofentezine	74115–24-5	Final	Yes	EFSA Conclusion (EFSA 2021c): RAR: https://www.efsa.europa.eu/sites/default/files/consultation/consultation/Clofentezine_revised_RAR_EDNegl_exp_August_2020_public.zip
Dimethomorph	110488–70-5	Ongoing	Yes	EFSA Conclusion (EFSA 2023a): RAR and ED Assessment: https://connect.efsa.europa.eu/RM/s/publicconsultation2/a0l7U0000011ZX5/pc0175
Mancozeb	8018–01-7	Final	Yes	EFSA Conclusion (EFSA 2020): https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2020.5755
Metiram	9006–42-2	Final	Yes	EFSA Conclusion (EC 2023; EFSA 2023b): RAR and ED assessment: https://open.efsa.europa.eu/questions/EFSA-Q-2015-00589
Metribuzin	21087–64-9	Ongoing	Yes	EFSA Conclusion (EFSA 2023c): https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2023.8140
Thiabendazole	148–79-8	Final	Yes	EFSA Conclusion (EFSA 2022b): https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2022.7212
Triflusulfuron-methyl	126535–15-7	Final	Yes	EFSA Conclusion (EFSA 2022c): https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2022.7303
ECHA				
1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1H-1,2,4-triazole (Propiconazole)	60207–90-1	Final	Yes	Opinion of the Biocidal Products Committee on the application for renewal of the approval of the active substance propiconazole for product type 8 (ECHA 2022): https://echa.europa.eu/documents/10162/2b615a3d-38d2-0087-31b6-dda6cfea6902
2,2-dibromo-2-cyanoacetamide (DBNPA)	10222–01-2	Final	Yes	Opinion of the Biocidal Products Committee on the application for approval of the active substance 2,2-Dibromo-2-cyanoacetamide (DBNPA) for product type 4 (ECHA 2021a): https://echa.europa.eu/documents/10162/085a4896-b067-bdbc-e38c-8f794e60e4f3
Cyanamide	420–04-2	Final	Yes	Opinion of the Biocidal Products Committee on the application for approval of the active substance Cyanamide for product type 3 (ECHA 2021b): https://echa.europa.eu/documents/10162/f5e04e73-afe6-4595-abda-864931b167bb

Criterion	2,2-Dibromo-2-cyanoacetamide	Asulam	Benthiavalicarb	Clofentezine	Cyanamide	Dimethomorph	Mancozeb	Metiram	Metribuzin	Propiconazole	Thiabendazole	Triflusulfuron-methyl
Sufficient investigation of endocrine activity(ies) with positive findings	p	p	N	Y	Y	p	N	Y	Y	Y	N	Y
Sufficient investigation of endocrine adversity(ies) with positive findings	p	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Dose–response relationship of endocrine adverse effect(s)	p	p	p	p	p	Y	Y	Y	Y	Y	p	Y
Effect(s) observed in more than one study	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Effect(s) observed in multiple species	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y
The biological plausibility between endocrine activity/activities and adversity is well-established	Y	p	p	Y	Y	Y	Y	Y	p	Y	p	p
Threshold level for the endocrine adversity could be determined	Y	p	Y	p	Y	Y	Y	Y	Y	Y	Y	Y
Degree of uncertainties[a]	+	+ + +	+ + +	+ +	+ +	+	+	+	+ +	+	+ + +	+ +

Y yes; *N* no; p: partially (This applies when there are differences or uncertainties in the assessment, e.g., dose–response relationship observed for one effect but not for another effect.)

[a] + + +: high; + +: moderate; +: low

Summary 2

Some MoA may be of limited or no relevance for humans due to significant species differences

Decisions will always be made case by case based on a weight of evidence approach

Receptor mediated toxicity generally has an effect-threshold below which no adverse effects could be observed

Thanks to the team

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Judy Choi

Carsten Kneuer

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- soundcloud.com/risikobewertung

Decisive new endpoints indicative of abnormal neurodevelopment

Katherine (Katie) L. O'Shaughnessy, PhD

The United States Environmental Protection Agency

Center for Public Health and Environmental Assessment

Public Health and Integrated Toxicology Division

Neurological and Endocrine Toxicology Branch

Disclosure Statement

I have no conflicts of interest to declare.

This work is supported by the US Environmental Protection Agency's Office of Research and Development. The contents of this presentation do not reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. The interpretations presented here are solely of the authors and do not represent an official position of the US EPA.

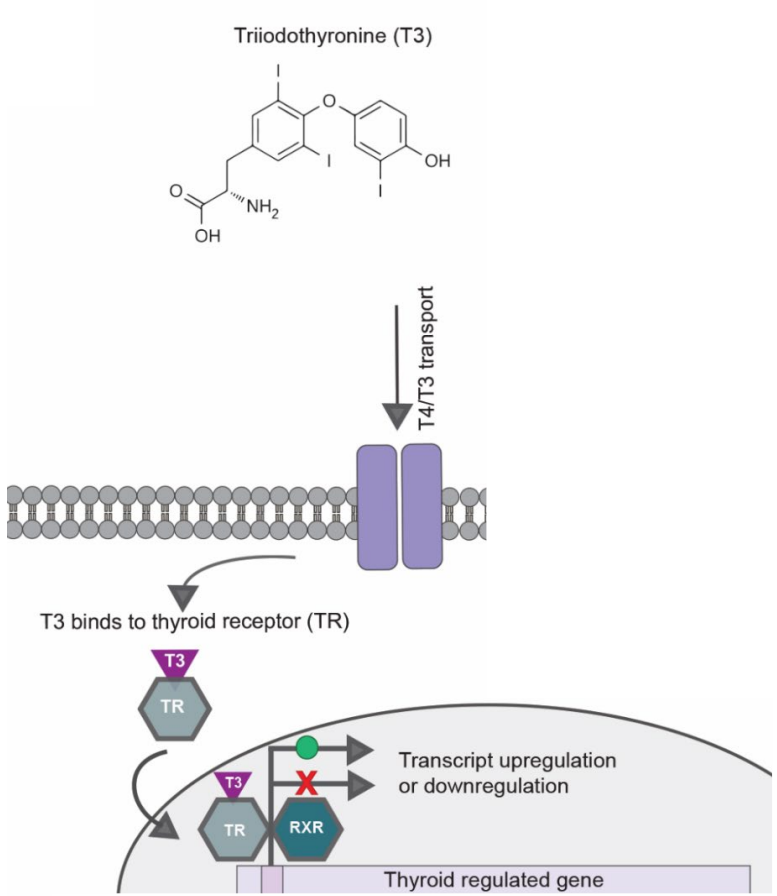
We know that thyroid hormones (THs) are crucial to brain development.

Women aged 17-20 years with cretinism.

Image courtesy of www.endotext.org, August 2014

But when is a serum TH reduction adverse?

Thyroid Action Is Mediated by Multiple Pathways



Thyroid action refers to signal transduction within cells.

O'Shaughnessy and Gilbert 2020, doi: 10.1016/j.mce.2019.110663

The Issues When Evaluating Chemicals for Thyroid Disrupting Activity

*"Antithyroid activity is the capability of a chemical to suppress **the action** of a natural thyroid hormone (e.g. T3) in an organism." - OECD*

Assay	OECD TG 414 - Prenatal Developmental Toxicity	OECD TG 421 – Reproduction/Developmental Toxicity	OECD TG 422 - Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test	OECD TG 443 - Extended One-Generation Reproductive Toxicity Study	US EPA OPP Guidance for Thyroid Assays in Pregnant Animals, Fetuses, Postnatal Animals, and Adult Animals[a]
Serum total T4	Dams: Required at time of euthanasia on GD20-21 Fetuses: Not required	Dams: Not required, but an optional measurement on PN13 Pups: Not required during the early postnatal period but an optional measurement on PN4. Required measurement on PN13 (two littermate samples pooled)	Dams: Not required but an optional measurement on PN13 Pups: Not required during the early postnatal period but an optional measurement on PN4. Required measurement on PN13 (two littermate samples pooled)	Dams: Required at termination Pups (Cohort 1A): An optional measurement on PN4 and required on PN22 (two littermates samples pooled)	Dams: Suggested at time of euthanasia on GD20 or PN21 Fetuses: Suggested in the GD20 fetus - (all fetal blood pooled within the litter) Pups: Suggested on PN4 and PN21 (two littermate samples pooled)

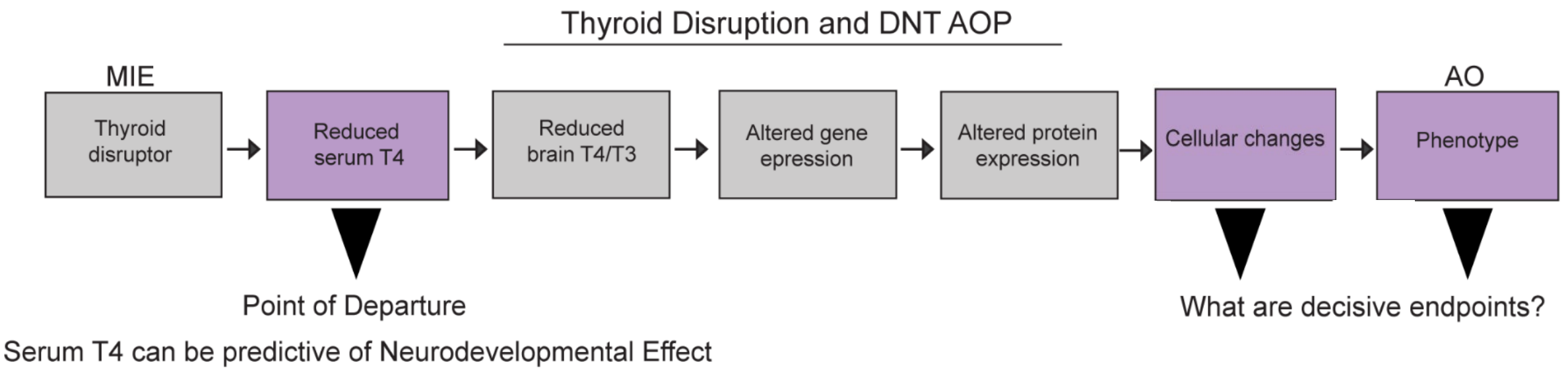
O'Shaughnessy and Gilbert 2020, doi: 10.1016/j.mce.2019.110663

OECD/EPA studies that require/suggest serum TH measurements

Only OECD TG 443 has neurodevelopmental endpoints

Even if serum T4 is reduced, what does it mean to the brain?

What are clear endpoints indicative of TH disruption?

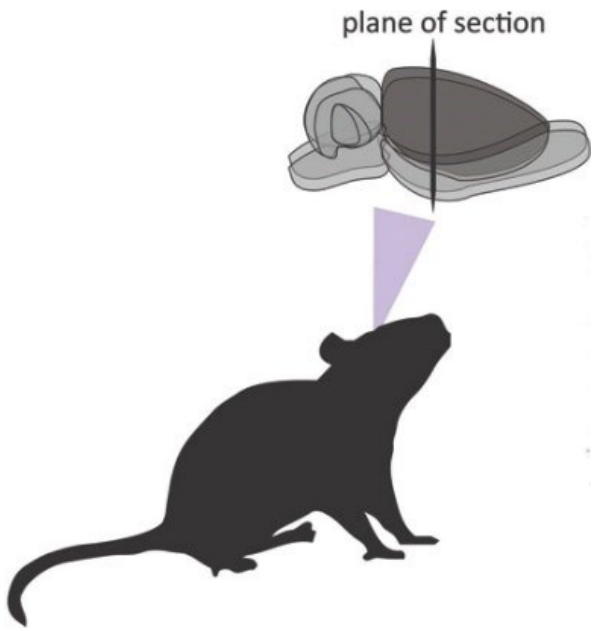


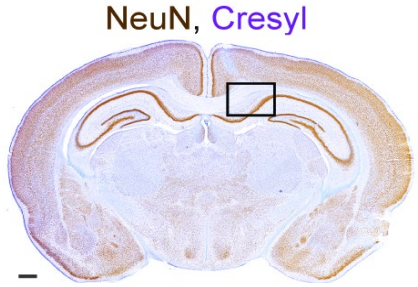
The endpoint should:

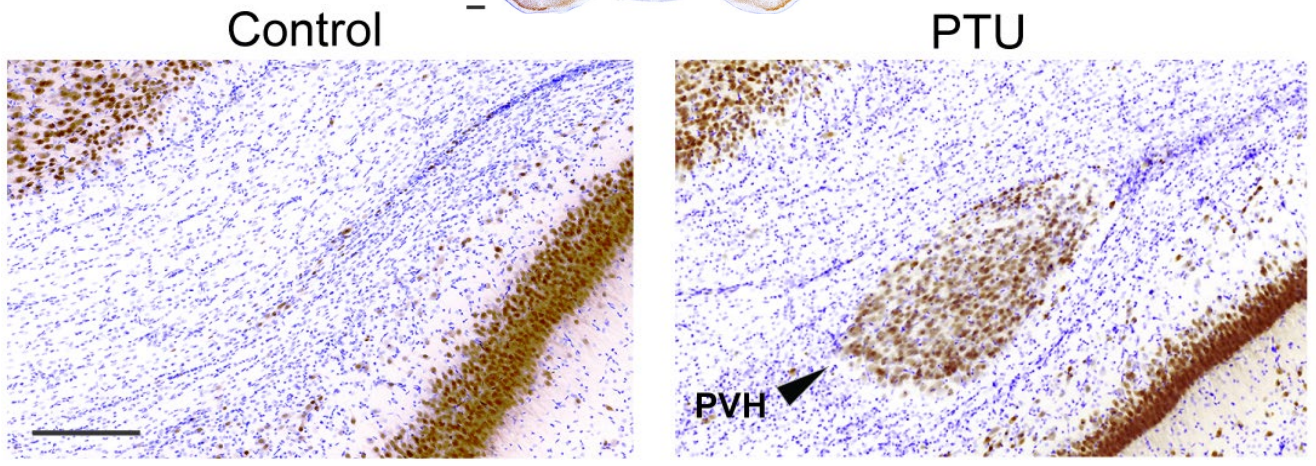
1) Correlate to brain TH levels and/or action**
2) Be quantitative
3) Be reproducible and applicable to humans
4) Be measurable in developing animals (ideally) using translatable methods

See Melching-Kollmuss et al. 2023 for additional information

A Rat Model of Complex Neurodevelopmental Disorders







Induced by maternal exposure

Periventricular heterotopia (pictured, morphological)

Decreased seizure threshold

Neurobehavioral deficits, including learning

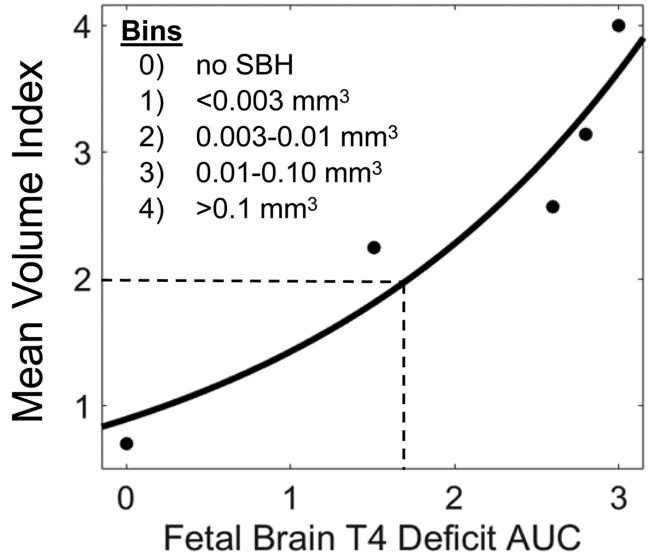
Neurotransmission Impairments

Shift in the ratio of excitatory/inhibitory neurons

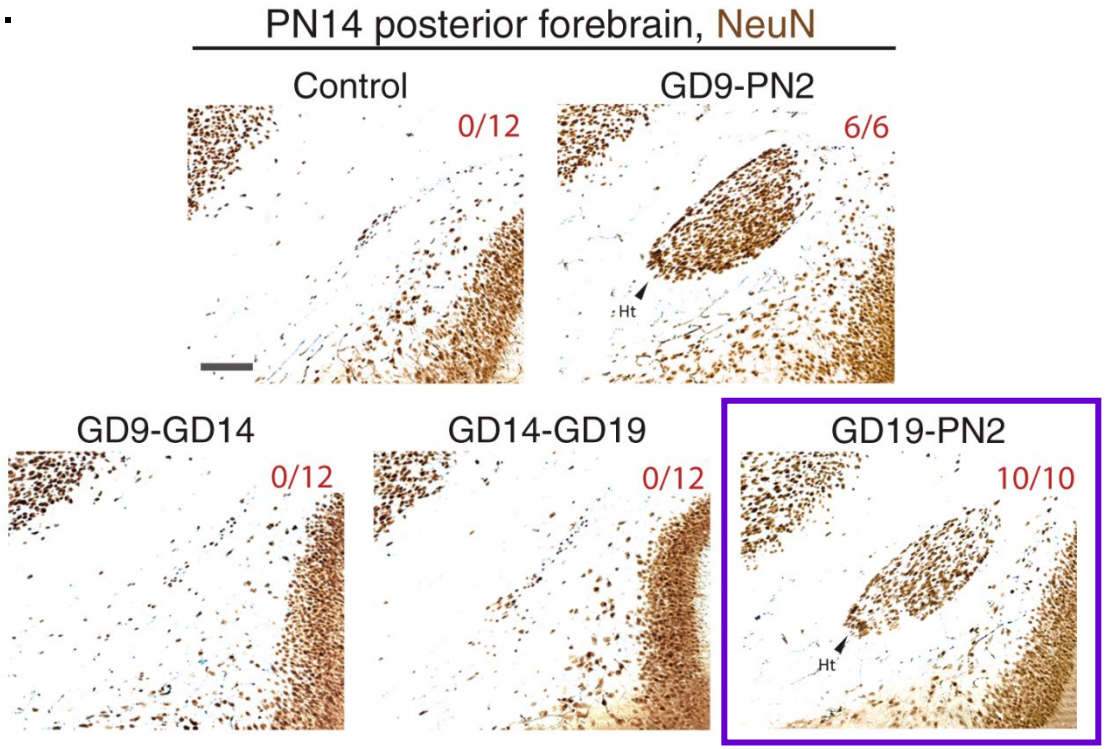
What Do We Know About the Heterotopia?

1) Correlates to serum and brain THs in the rat.
2) Dose-dependent (larger heterotopia with more severe hypothyroidism).
3) It is reproducible. Other instances of heterotopic neurons have also been reported.
4) It is permanent, but dependent *only* on perinatal TH deficiency.
5) Can be induced by environmental contaminants.

Fetal brain T4 deficit vs heterotopia (KER4)



Hassan *et al.* 2017, doi:10.1093/toxsci/kfx163.

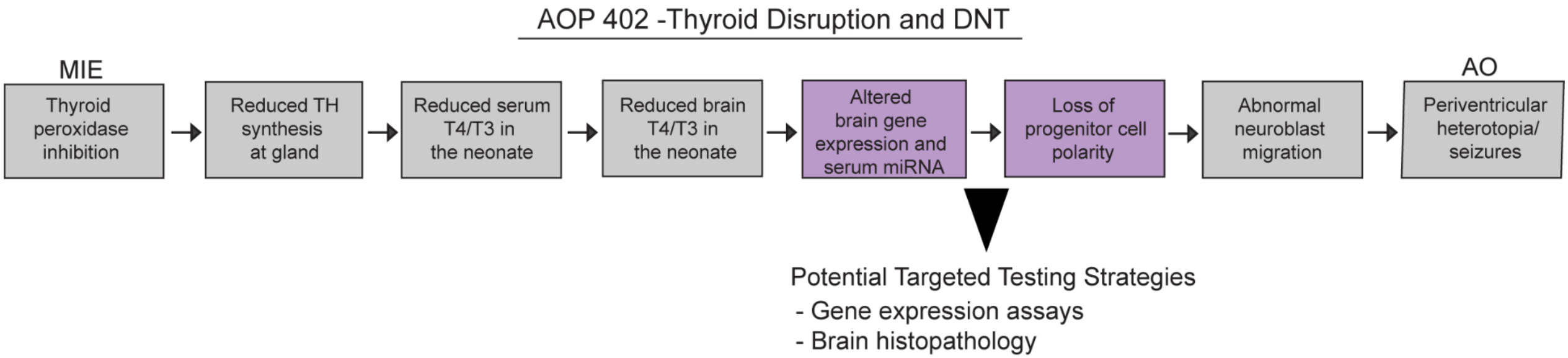


O'Shaughnessy *et al.* 2019, doi:10.1038/s41598-019-40249-7.

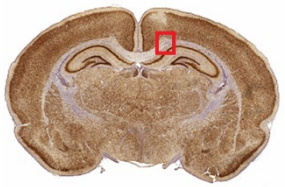
The Limitations of Heterotopia Analysis for Toxicology

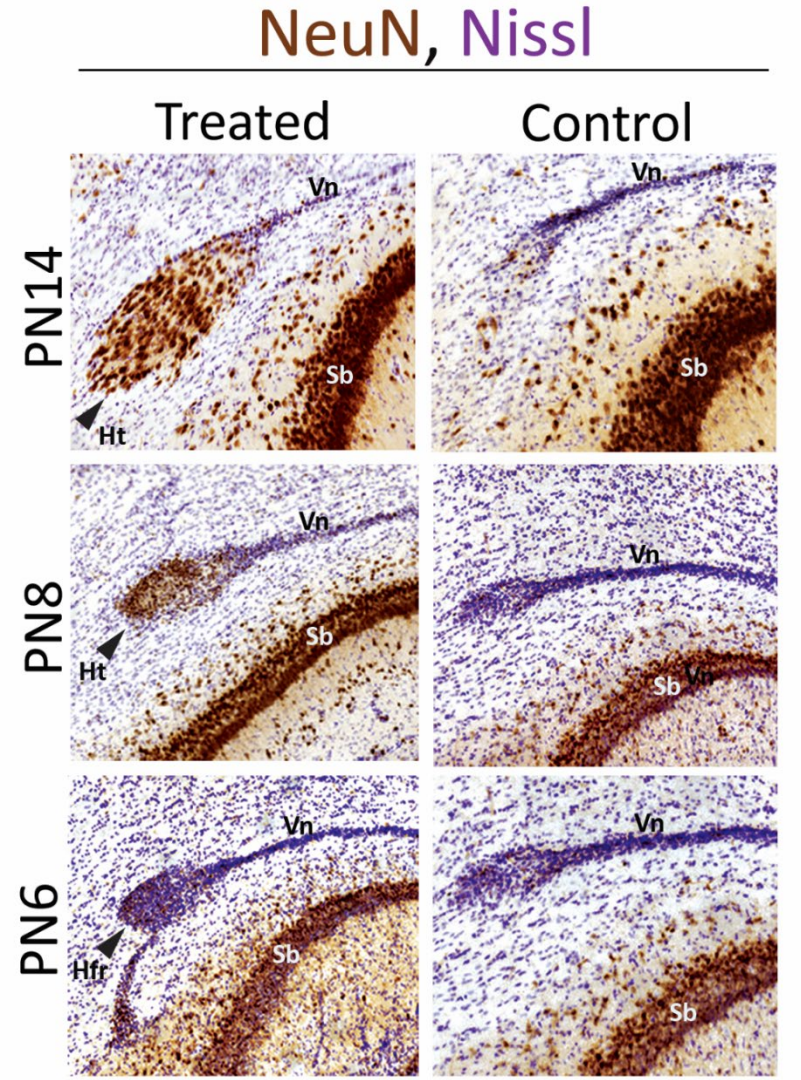
1) It is dependent *only* on perinatal TH deficiency (O'Shaughnessy et al. 2019).
2) Thus far only observed with NIS and TPO inhibitors (many papers, EPA and beyond).
3) Not completely reproducible in mice (Ramhøj et al. 2023).
4) In humans, heterotopias are considered a *severe* phenotype.

Even if this endpoint has its limitations, the mechanism of its formation is reliable and conserved across species.



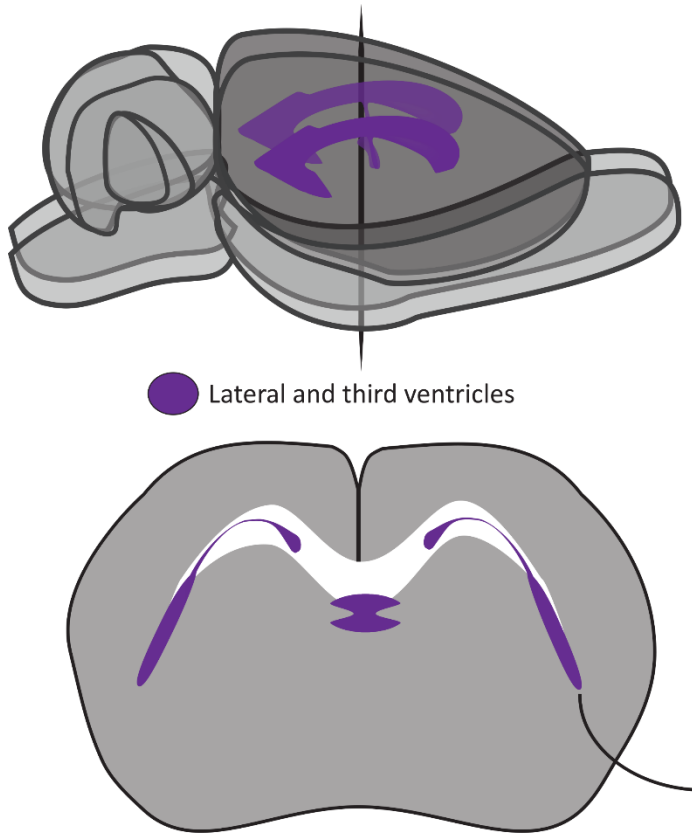
The Heterotopia Forms Due to Abnormal Cell Migration

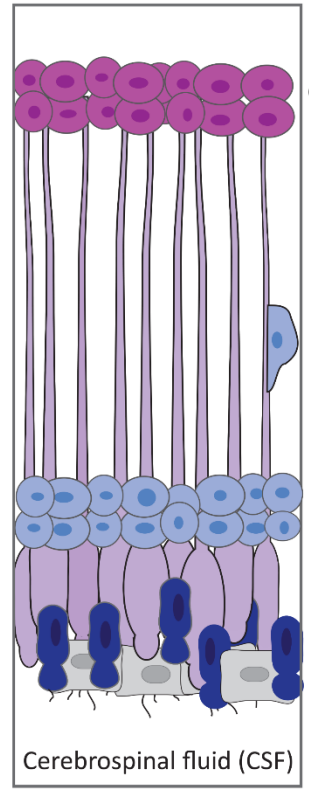




For PN14 animal:
Bregma -3.40 mm
Lambda 2.80 mm

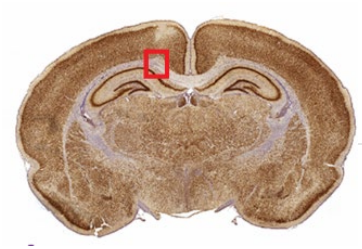
The Ventricular Zone is a Stem Cell Niche and a "Brain Barrier"

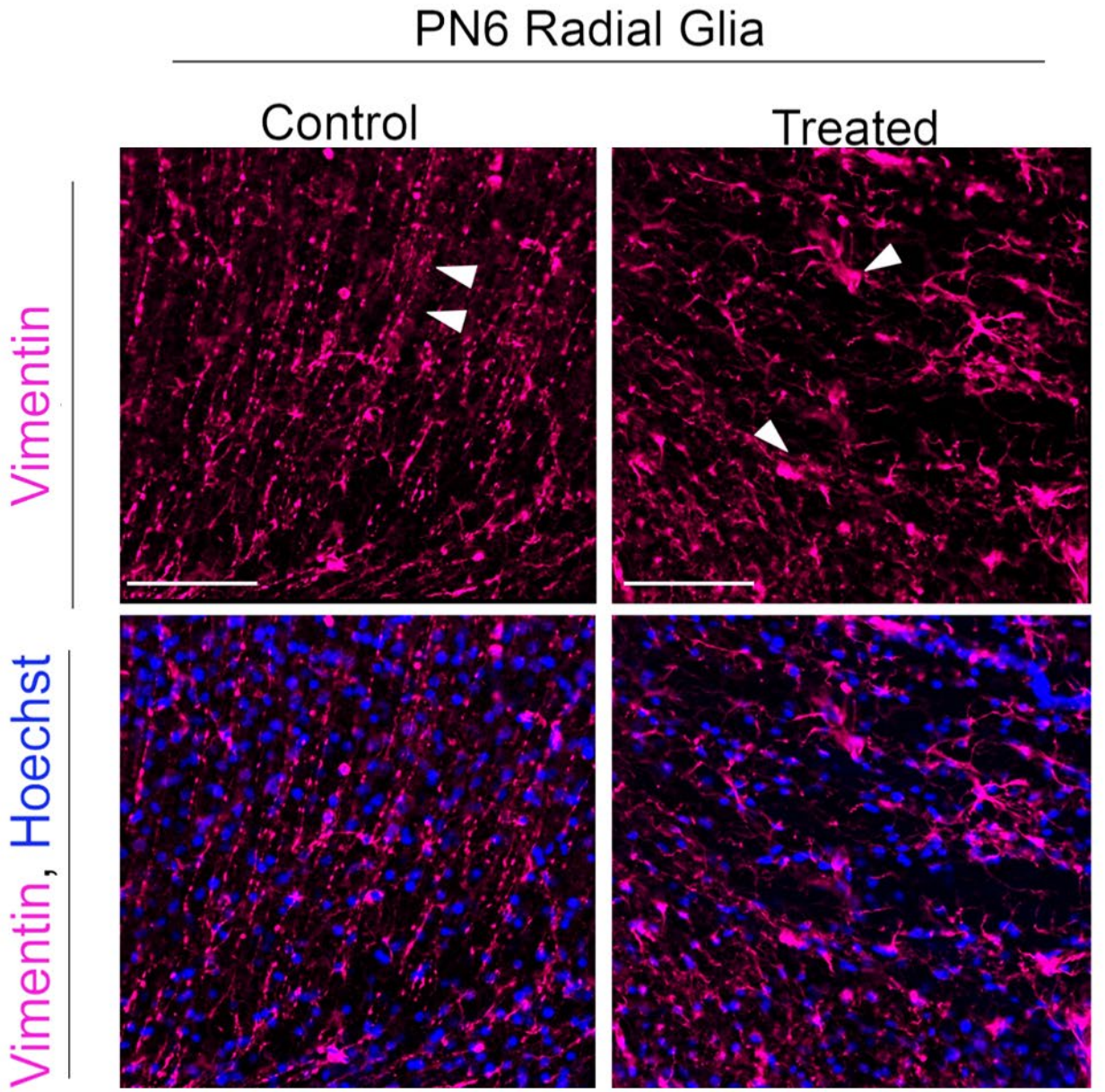




O'Shaughnessy *et al.* 2019, doi: 10.1038/s41598-019-40249-7.

Hypothyroidism Disrupts the Radial Glial Scaffolding

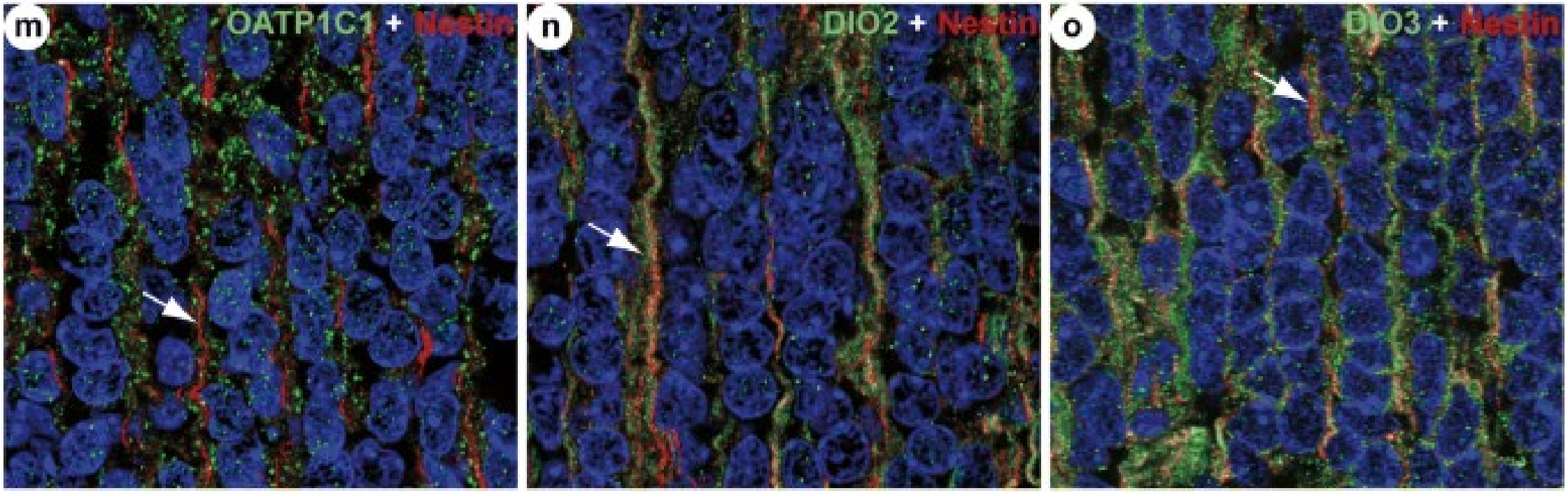




O'Shaughnessy *et al.* 2019, doi:10.1038/s41598-019-40249-7.

Radial Glia Cells Regulate Local TH Economy

These data are from GW16−19 human fetal neocortex analyzed by single cell RNA-Seq (Diaz et al. 2021), or GW 14-38 fetal brains analyzed by IHC (Lopez-Espindola et al. 2019).

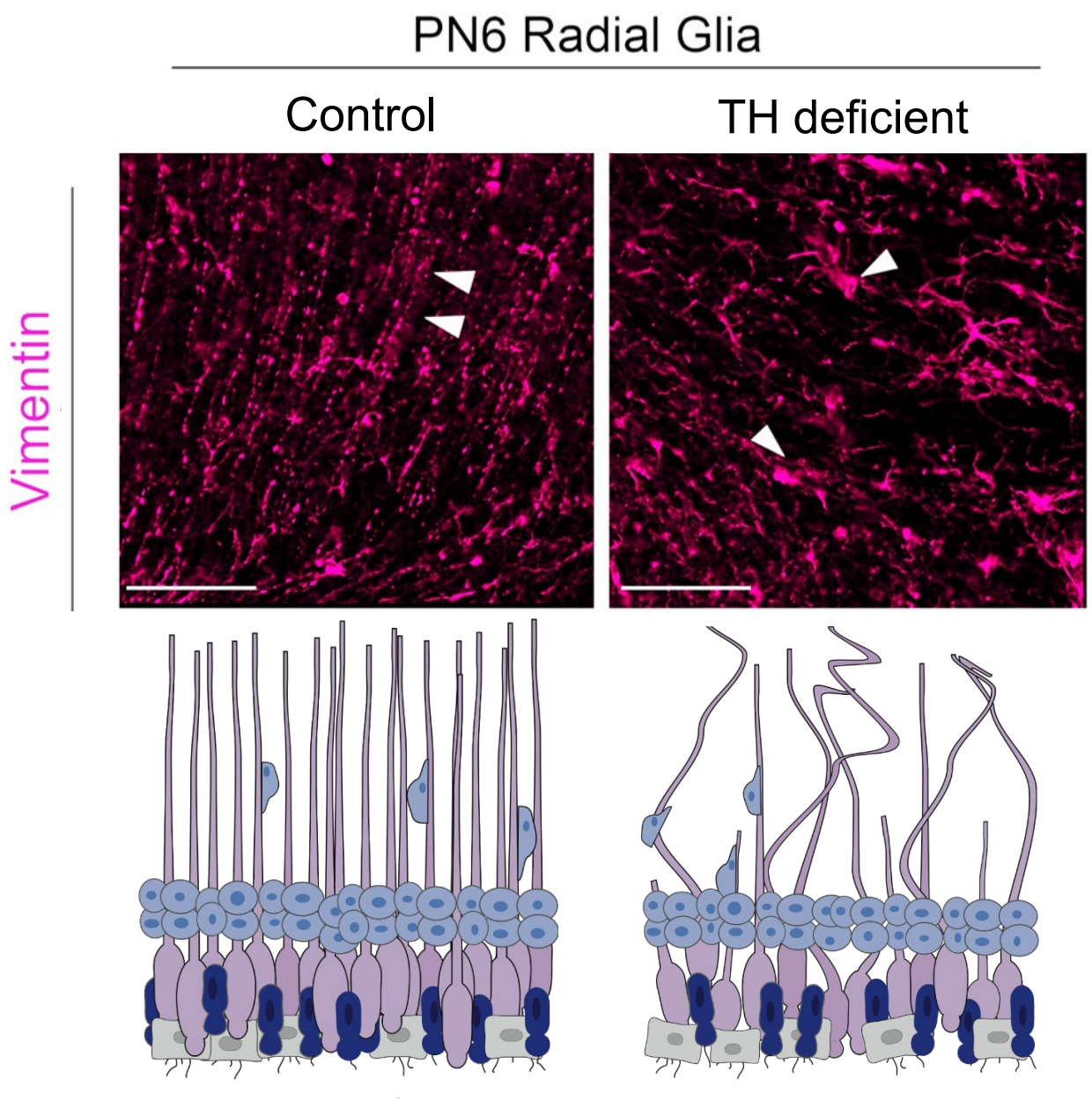


Lopez-Espindola et al. 2019, doi: 10.1007/s00429-019-01896-8.

Radial glia co-express *SLCO1C1* and *DIO2,* indicating close cooperation between the T4 transporter OATP1C1 and DIO in local T3 formation (Diaz et al. 2021).

Diez et al. 2021, https://doi.org/10.1089/thy.2021.0057

How Can We Quantify Radial Glia Malformations?



O'Shaughnessy *et al.* 2019, doi: 10.1038/s41598-019-40249-7.

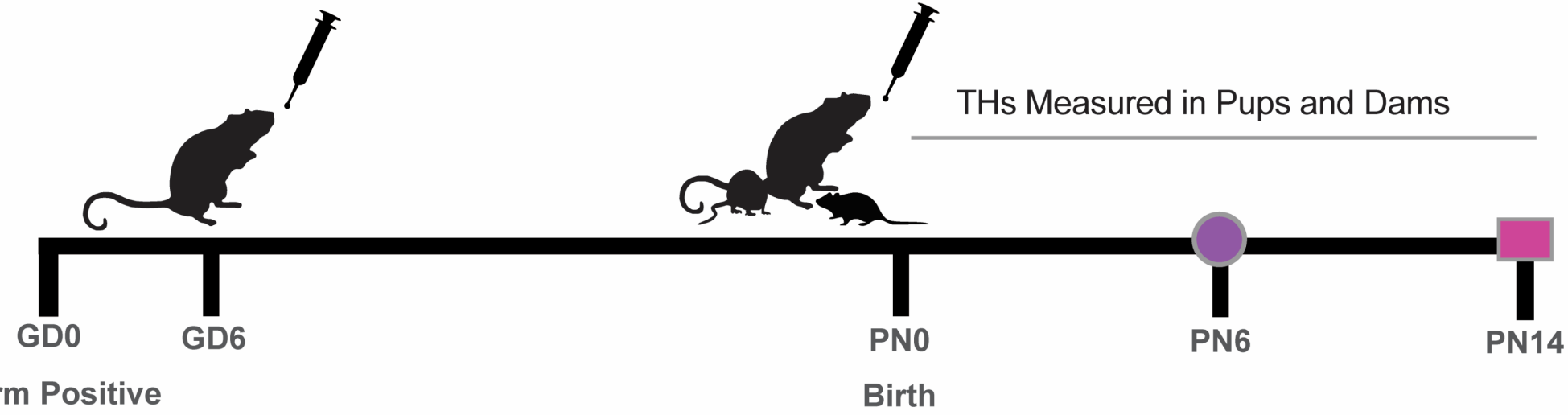
The Experiment

Administered Doses of PFHxS: 0 (Control), 17, or 50 mg/kg/d from GD6-PN14+

Administered Doses of PTU: 0.875 mg/kg/d from GD6-PN14+

Radial glia cell morphology and brain RNA-Seq

Heterotopia quantification



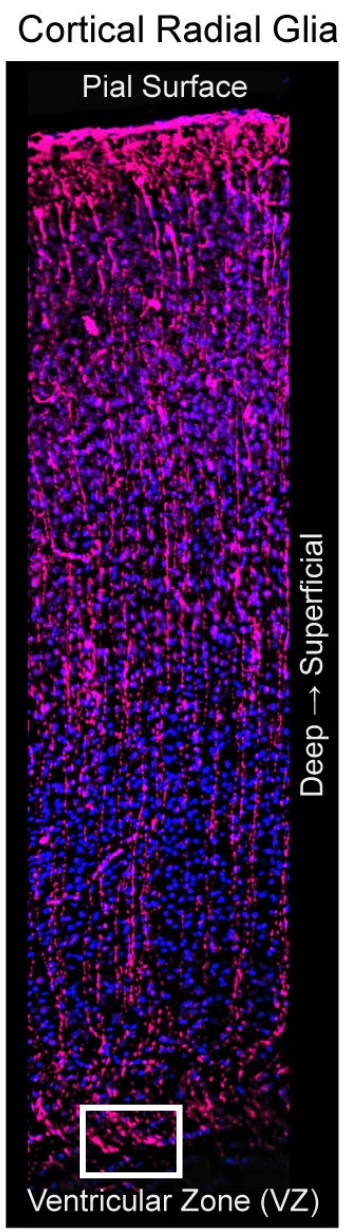
PTU = TPO Inhibitor

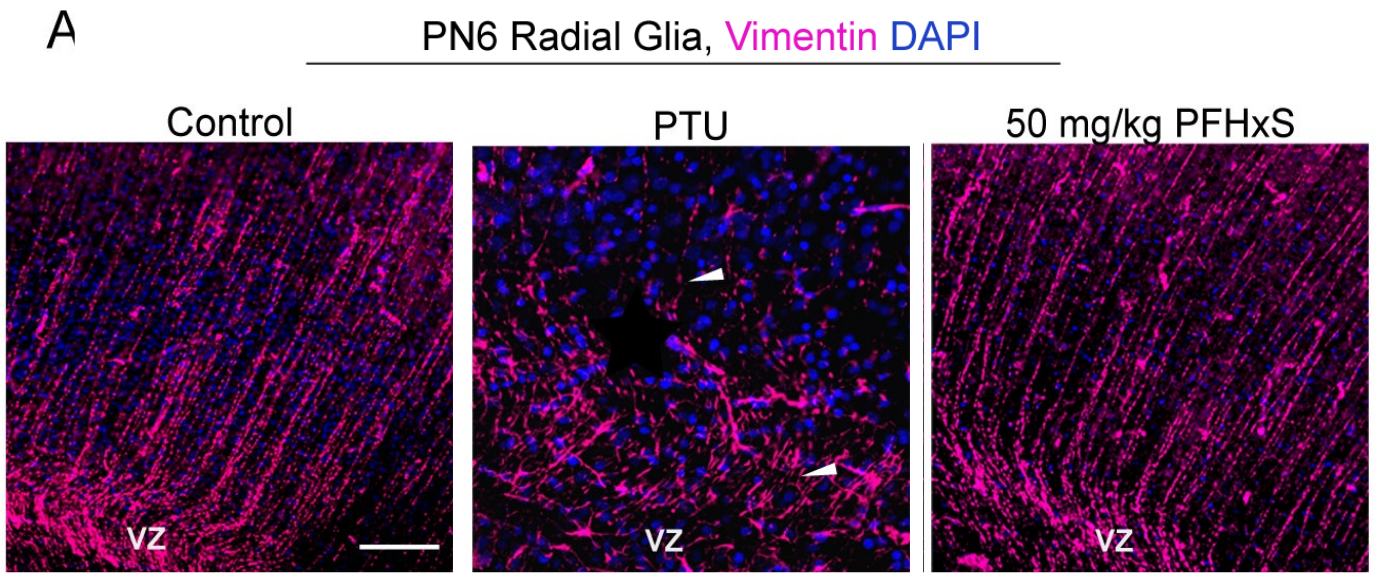
PFHxS = Perfluorohexane sulfonate, binds competitively to transthyretin

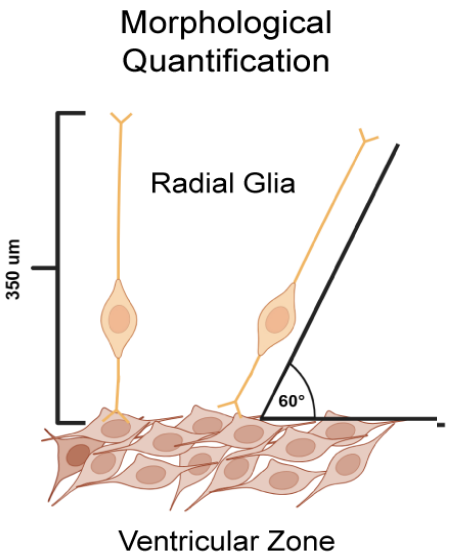
O'Shaughnessy *et al.* 2019, doi: 10.1038/s41598-019-40249-7.

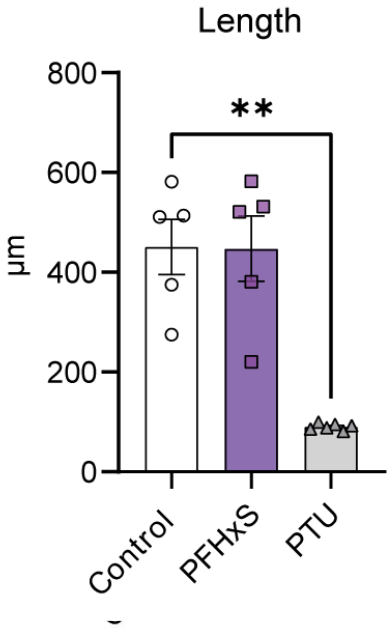
Quantification of Radial Glia Disturbances

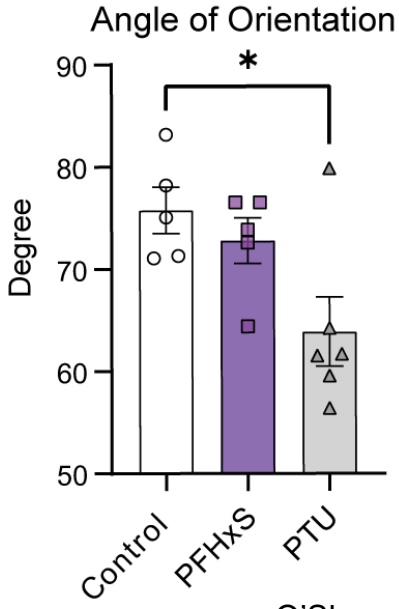
Radial glia dysfunction causes heterotopia and other neurodevelopmental effects (i.e., it is upstream).

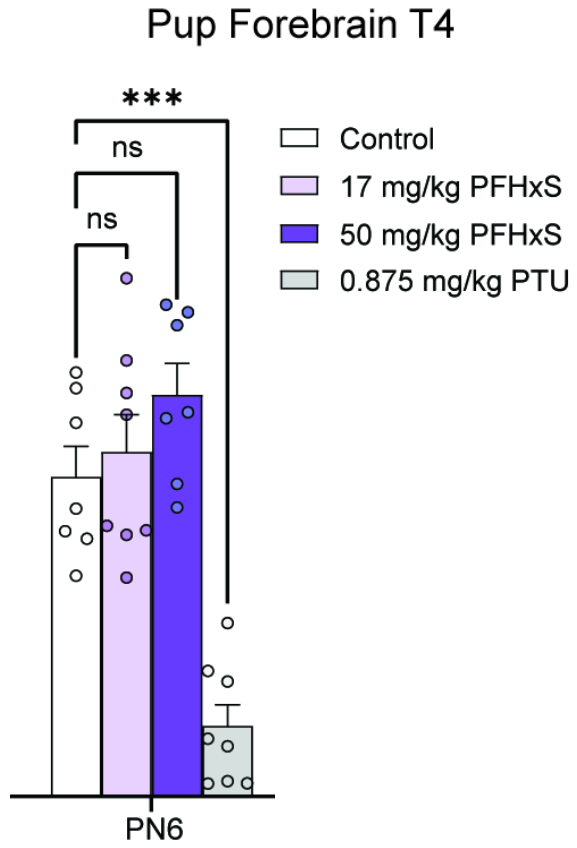












O'Shaughnessy *et al.* 2024, doi: 10.1016/j.envint.2024.108838.

Benefits of Measuring Radial Glia

1) Correlates to brain TH levels
2) Quantitative
3) Reproducible and applicable to humans.
4) Measured in young animals (≤PN8).
5) Measured in a small number of thin sections.
   - Cryo or paraffin
   - Vimentin immunostaining using calorimetric reaction (fluorescence not necessary).
6) We used imaging technology commonplace in pathology labs.

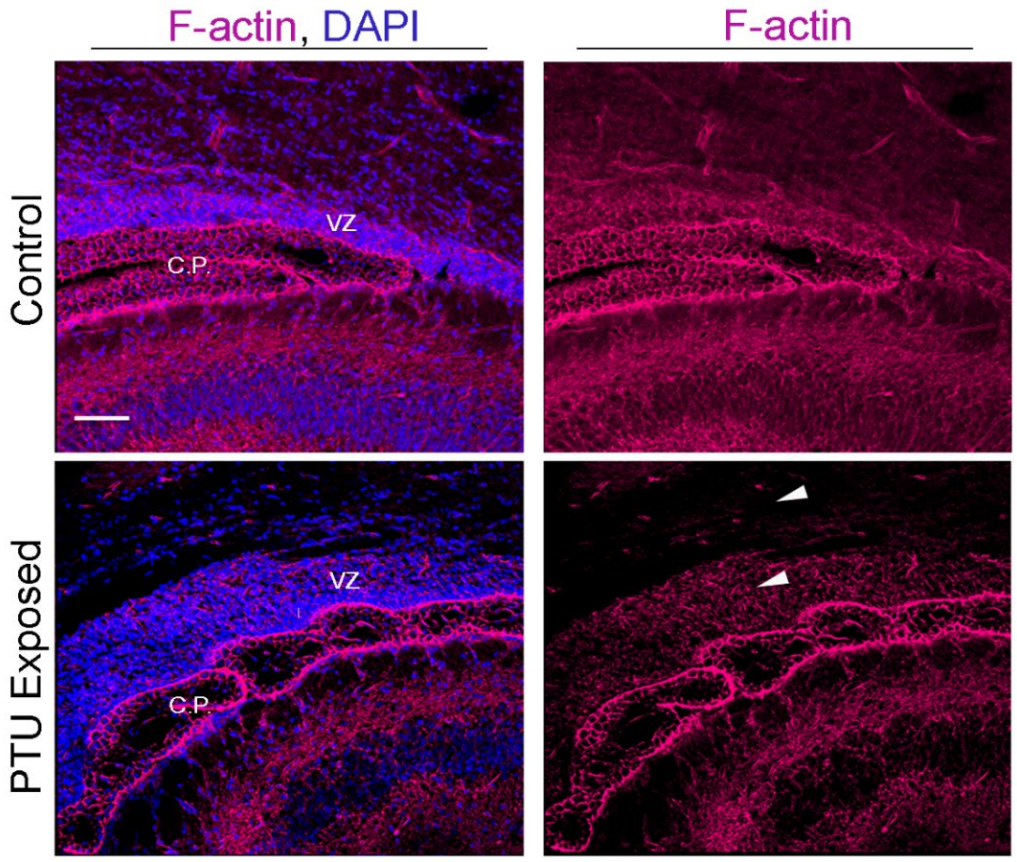
We are currently assessing the sensitivity of this assay.

Thyroid Action Affects Cell Migration and Cell Junctions

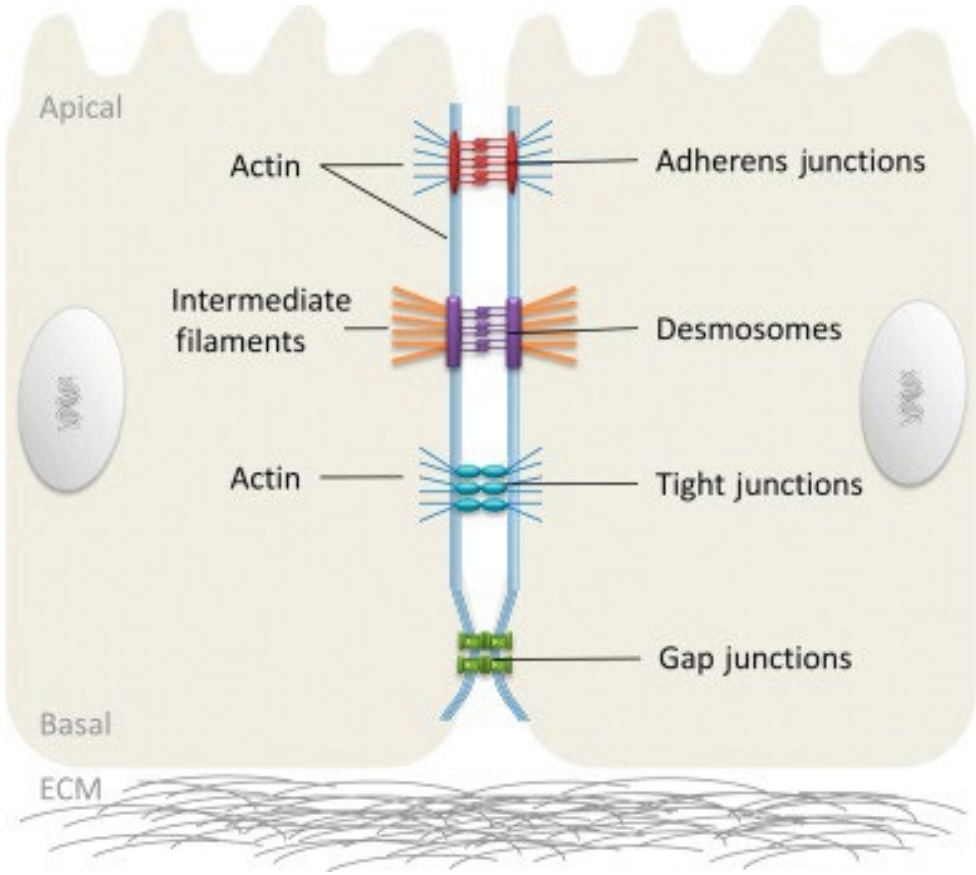
There are <u>significant supporting data in humans and rodents</u>.

1. T4 (*<u>not T3</u>*) regulates actin polymerization in the brain. Radial glia are “strapped down” by actin.

2. THs also regulate tight and adherens junction proteins.



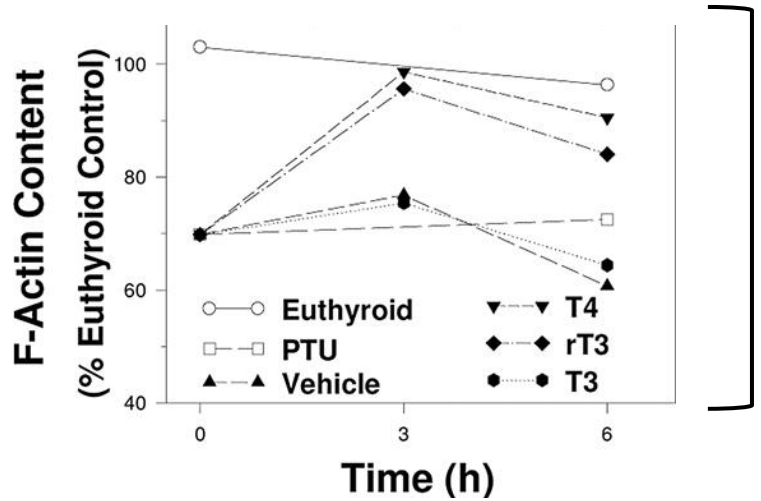
O’Shaughnessy et al. 2023, 10.3389/fendo.2023.1090081



Related Opportunities: Measuring Actin Dynamics

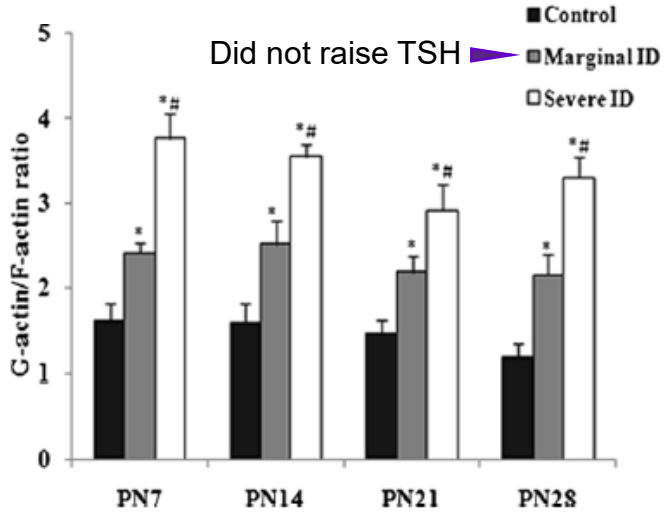
Actin is regulated by T4 in the brain and crucial to cell migration/junctions.

Actin Quantification by Western Blotting



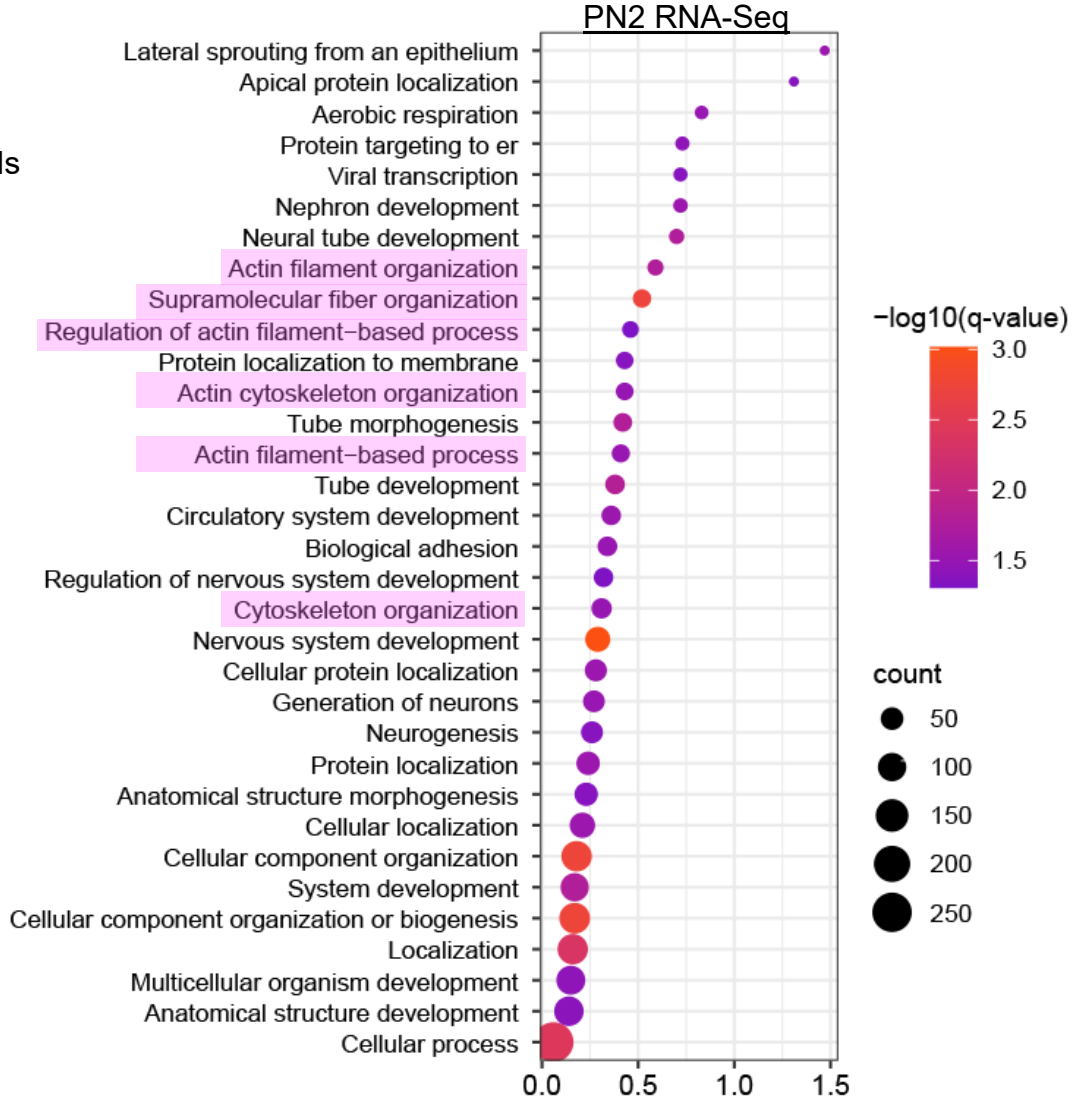
F-actin responds to brain T4/rT3

Farwell et al. 2006, doi: 10.1210/en.2005-1272.



Min et al. 2016, doi: 10.1007/s12035-015-9657-5.

Actin Quantification by Gene Expression



O'Shaughnessy et al. 2023, doi: 10.3389/fendo.2023.1090081.

More Information on Targeted Testing Strategies

Molecular and Cellular Endocrinology 518 (2020) 110663

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

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

Review

Thyroid disrupting chemicals and developmental neurotoxicity – New tools and approaches to evaluate hormone action

Katherine L. O'Shaughnessy*, Mary E. Gilbert

United States Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Toxicity Assessment Division, Endocrine Toxicology Branch, Research Triangle Park, NC, 27711, USA

Other Biomarkers of DNT will be discussed on Wednesday.

S24: *New approach methods for risk assessment of thyroid disrupting chemicals*

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Co-Authors and Contributors (All US EPA)

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Cal D. Riutta*
Susan E. Thomas*
Luke Ford*
Stephanie S. Spring*

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ORISE Fellowships*

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oshaughnessy.katie@epa.gov

GBAGROUP

Species-specific thyroxine (T4) metabolism and response to nuclear-receptor activators in sw-cultured hepatocytes

September 8, 2024

Dr. Prof. Lysiane Richert
Dr. Laure Asselin

Kaly-cell.com
Pharmacelsus.com

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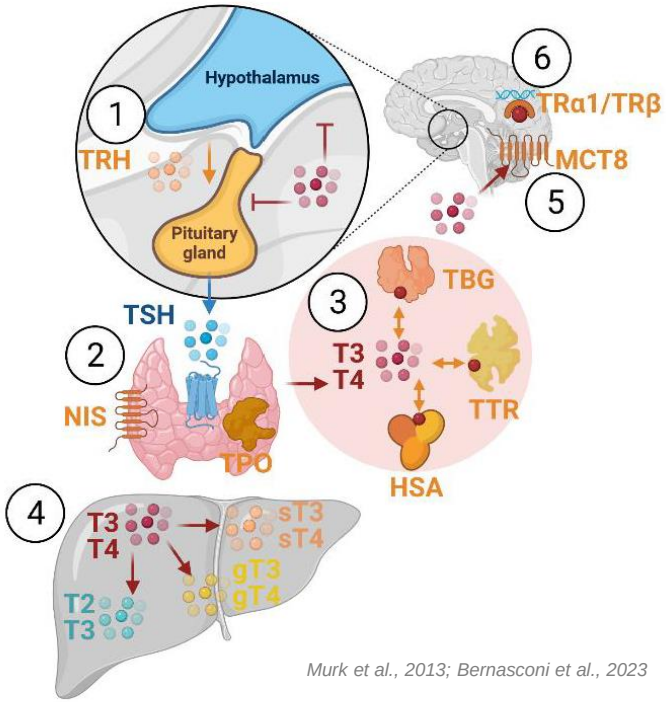
PHARMACELSUS

Thyroid hormone system and targets for TH disruption



Murk et al., 2013; Bernasconi et al., 2023

1. **Central regulation** via the HPT axis
2. **TH synthesis** (NIS, TPO, DIO1-2) → NIS, TPO, DIO1-2-3 inhibition assays
   - **Asselin et al., 2024 (in revision)**
3. **Binding and transport in serum** (TTR, TBG)
4. **Metabolism and excretion** (Glucuronidation, sulfation, deiodination) → **Today's talk**
5. **Intracellular transport** (MCT8)
6. **Cellular responses** (activation of nuclear receptors TR)

Performance of the hepatocyte sw culture model

for metabolism & toxicity evaluations and species-comparison

Toxicology in Vitro 30 (2015) 79–94

Contents lists available at ScienceDirect

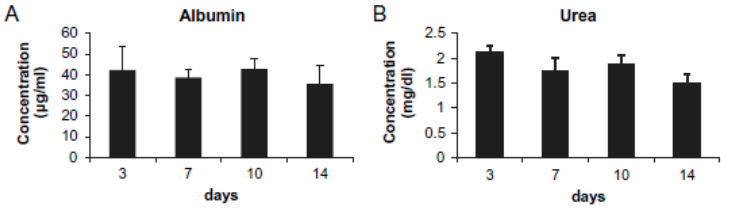
Toxicology in Vitro

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

Determination of liver specific toxicities in rat hepatocytes by high content imaging during 2-week multiple treatment

Davide Germano, Marianne Uteng, Francois Pognan, Salah-Dine Chibout, Armin Wolf *

Discovery and Investigative Safety, Preclinical Safety, Novartis Institutes for Biomedical Research, CH-4057 Basel, Switzerland



Toxicology and Applied Pharmacology 274 (2014) 124–136

Contents lists available at ScienceDirect

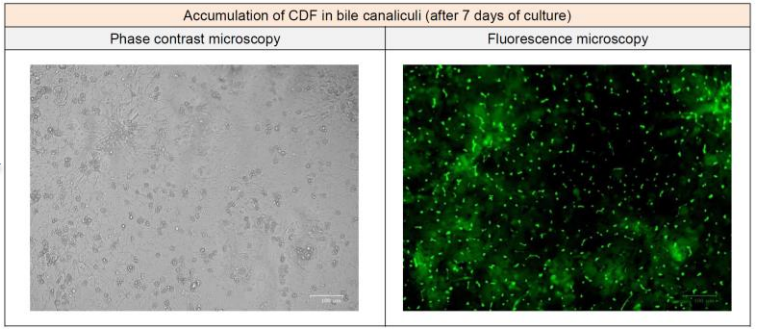
Toxicology and Applied Pharmacology

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

Hepatocyte-based in vitro model for assessment of drug-induced cholestasis

Sagnik Chatterjee [a], Lysiane Richert [b], Patrick Augustijns [a], Pieter Annaert [a,*]

[a] *Drug Delivery and Disposition, KU Leuven Department of Pharmaceutical and Pharmacological Sciences, O&N2, Herestraat 49 – bus 921, 3000 Leuven, Belgium*
[b] *KaLy-Cell, 20A rue du Général Leclerc, 67115 Plobsheim, France*

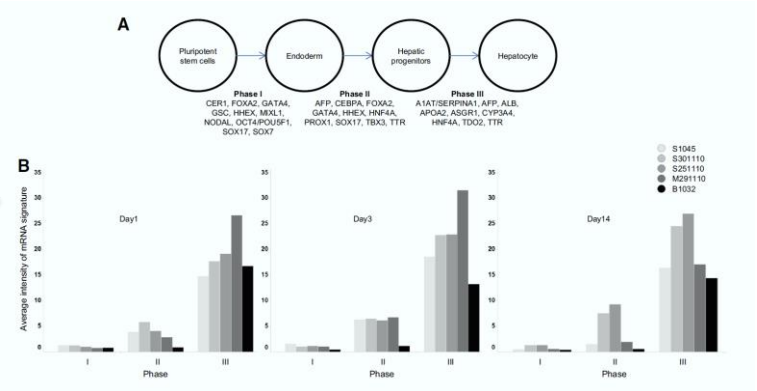


Arch Toxicol (2017) 91:2879–2893
DOI 10.1007/s00204-017-1930-0

ORGAN TOXICITY AND MECHANISMS

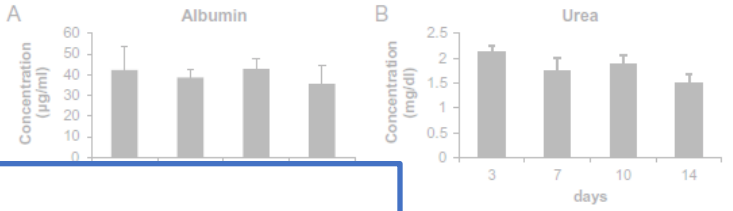
Evaluation of transcriptomic signature as a valuable tool to study drug-induced cholestasis in primary human hepatocytes

Céline Parmentier[1] · Philippe Couttet[2] · Armin Wolf[2] · Thomas Zaccharias[3] · Bruno Heyd[4] · Philippe Bachellier[5] · Marianne Uteng[2] · Lysiane Richert[1,6]



Performance of the hepatocyte sw culture model

for metabolism & toxicity evaluations and species-comparison



Determination of liver specific toxicitie
content imaging during 2-week multip

Davide Germano, Marianne Uteng, Francois Pogn

Discovery and Investigative Safety, Preclinical Safety, Novartis Institutes for Biomed

Toxicology and Applied

Contents lists a

Toxicology and A

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journal homepage: ww

Hepatocyte-based in vitro model for asse
drug-induced cholestasis

Sagnik Chatterjee [a], Lysiane Richert [b], Patrick August

[a] *Drug Delivery and Disposition, KU Leuven Department of Pharmaceutical and Pharmaco*
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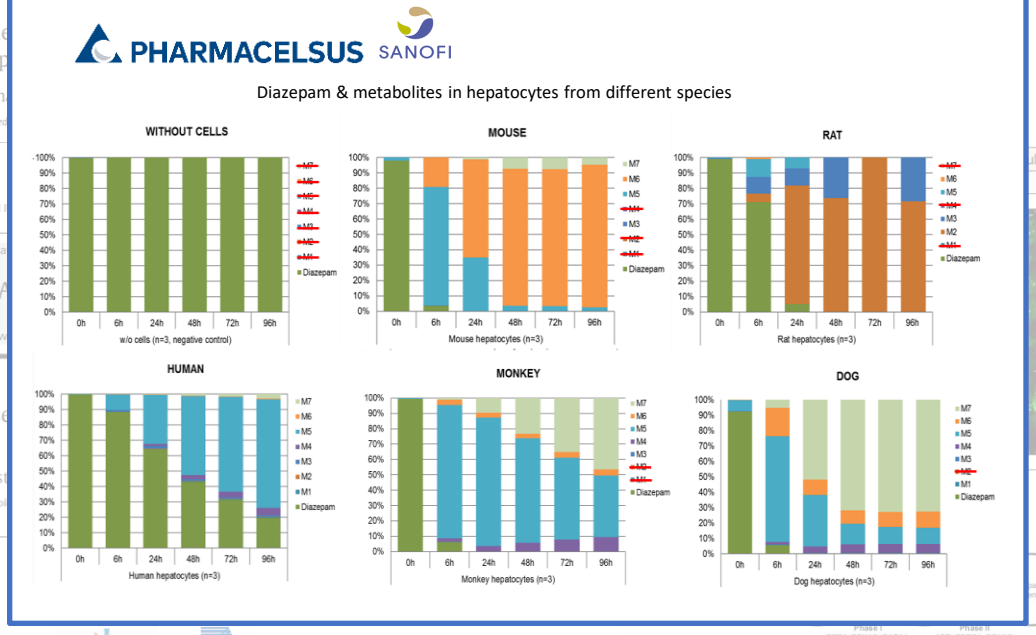

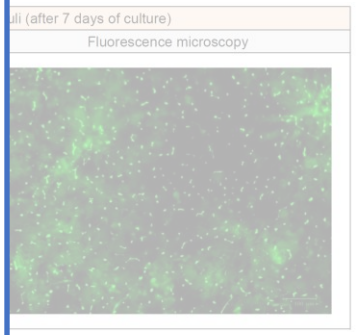

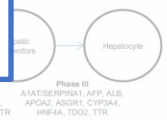

Arch Toxicol (2017) 91:2879–2893
DOI 10.1007/s00204-017-1930-0

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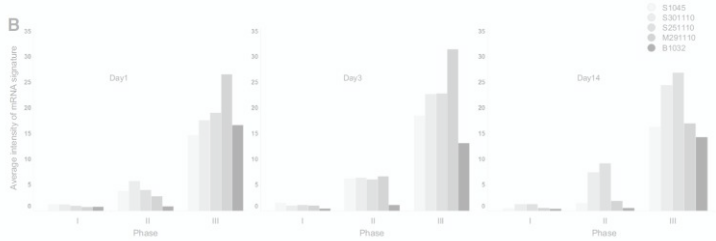
ORGAN TOXICITY AND MECHANISMS

Evaluation of transcriptomic signature as a valuable tool to study drug-induced cholestasis in primary human hepatocytes

Céline Parmentier[1] · Philippe Couttet[2] · Armin Wolf[2] · Thomas Zaccharias[3] · Bruno Heyd[4] · Philippe Bachellier[5] · Marianne Uteng[2] · Lysiane Richert[1,6]



Performance of the hepatocyte sw culture model

for metabolism & toxicity evaluations and species-comparison

Contents lists available at ScienceDirect

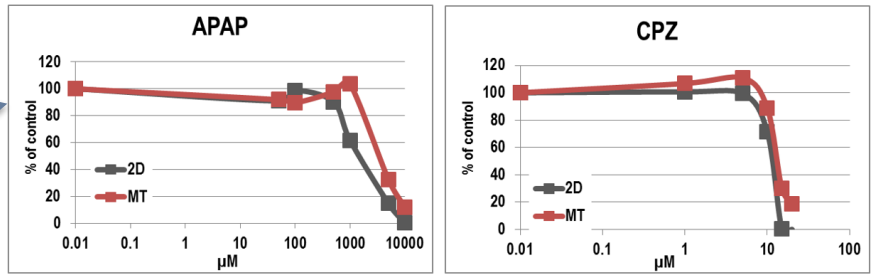
Toxicology Letters

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

Cytotoxicity evaluation using cryopreserved primary human hepatocytes in various culture formats

Lysiane Richert[a,b,*], Audrey Baze[a], Céline Parmentier[a], Helga H.J. Gerets[b], Rowena Sison-Young[c], Martina Dorau[d], Cerys Lovatt[e], Andreas Czich[d], Christopher Goldring[c], B. Kevin Park[c], Satu Juhila[f], Alison J. Foster[g], Dominic P. Williams[g]

Cytotoxicity profiles 2D *versus* MT



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ORGAN TOXICITY AND MECHANISMS

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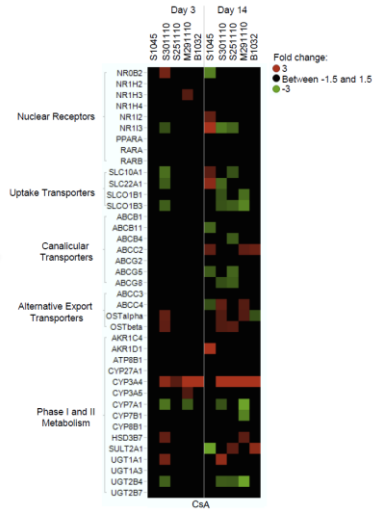
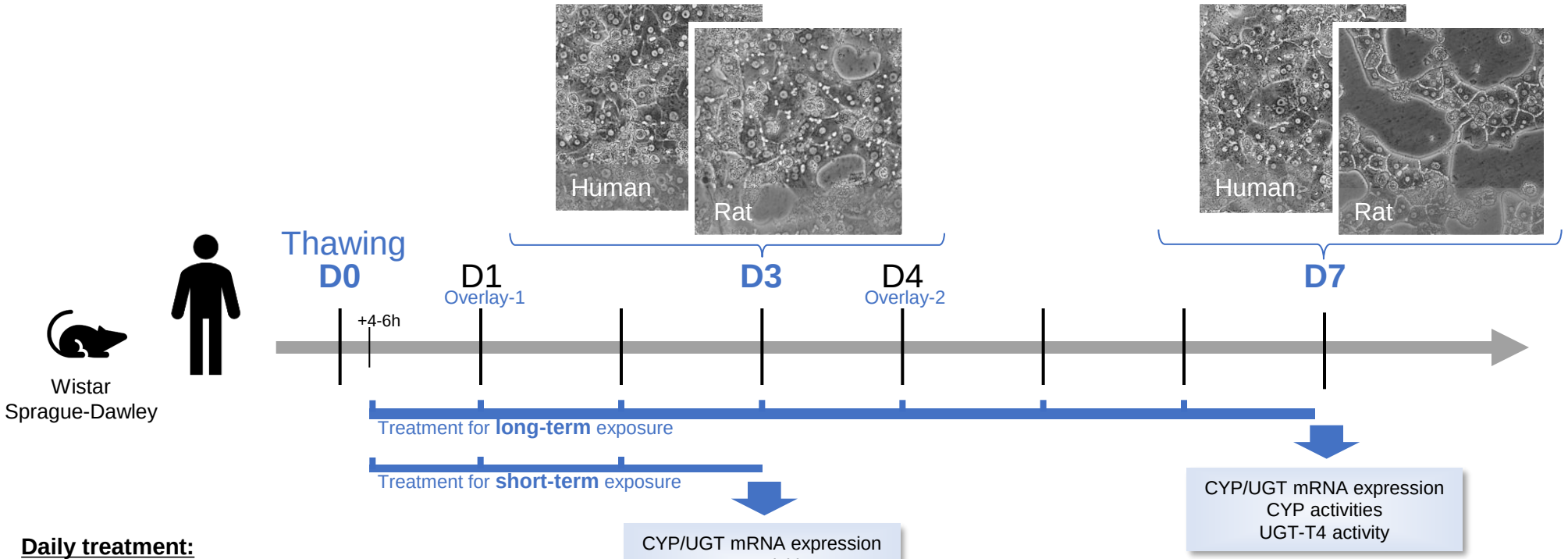


Protocol overview:

gene expression and enzyme activities in rat and human hepatocytes, after 3 or 7 days of exposure



Daily treatment:

BNF (rat & human AhR activator): 5 µM

PCN (rat-specific PXR activator): 6 µM

RIF (human-specific PXR/CAR activator): 15 µM

PB (rat and human CAR/PXR activator): 1000 µM

- *mRNA ⇒ RNA extraction and quantification, RT-qPCR*
- *Activities:*
  - *CYP ⇒ incubation for **30min** with a **cocktail of substrates** ⇒ supernatants frozen until analysis (**Pharmacelsus**)*
  - *UGT-T4 ⇒ incubation for **4h** with **T4 at 50µM** ⇒ supernatants frozen until analysis (**Pharmacelsus**)*

BNF: Beta-naphthoflavone
PB: Phenobarbital
PCN: 5-pregnen-3β-ol-20-one 16a-carbonitrile
RIF: Rifampicin

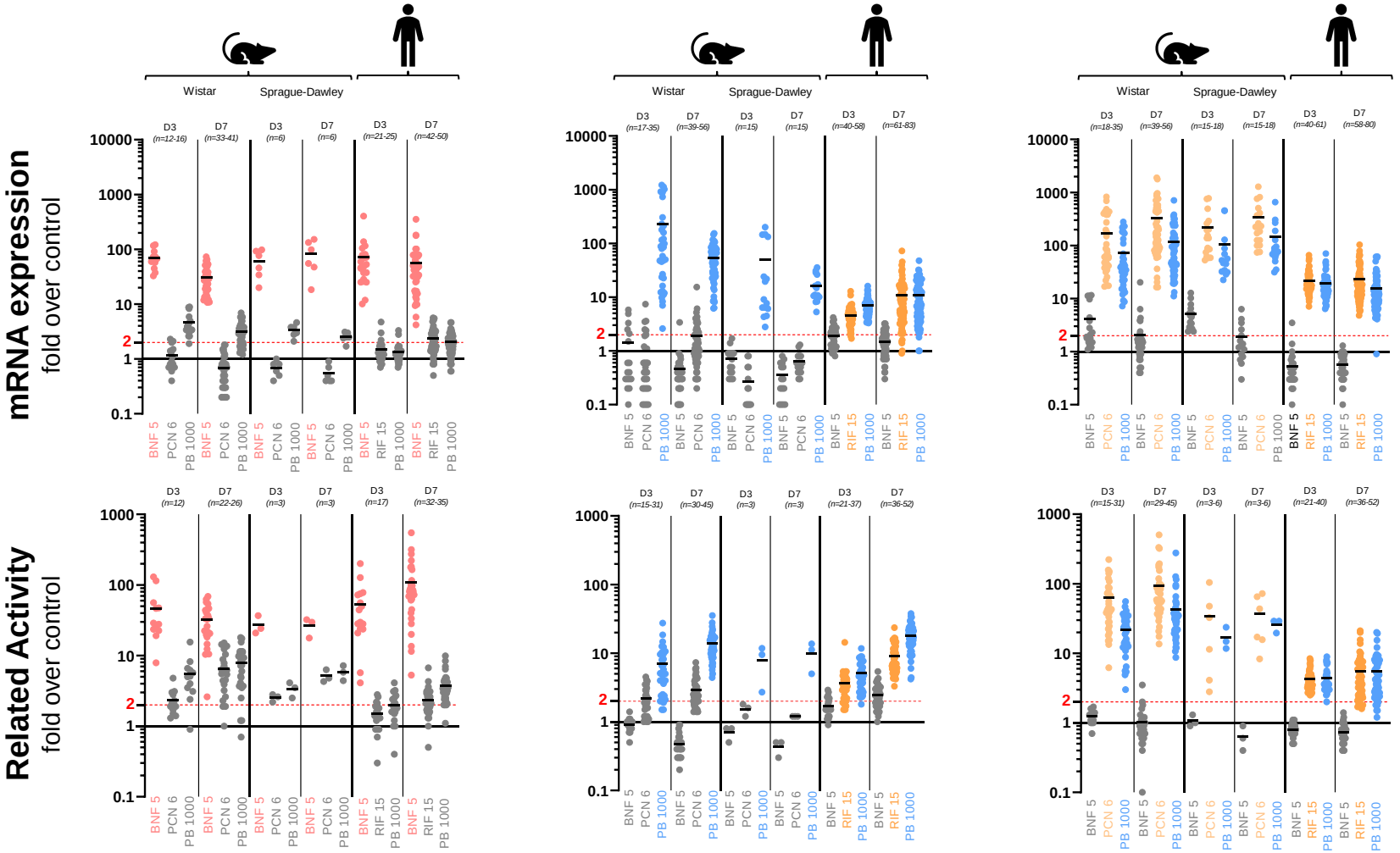
Cyp/CYP450 mRNA expression & related activities

in response to liver reference inducers in rat and human hepatocytes, after 3 or 7 days of exposure

Cyp1a2/CYP1A2

Cyp2b1/CYP2B6

Cyp3a1/CYP3A4



→ **Expected responses: Day 3 = Day 7** &

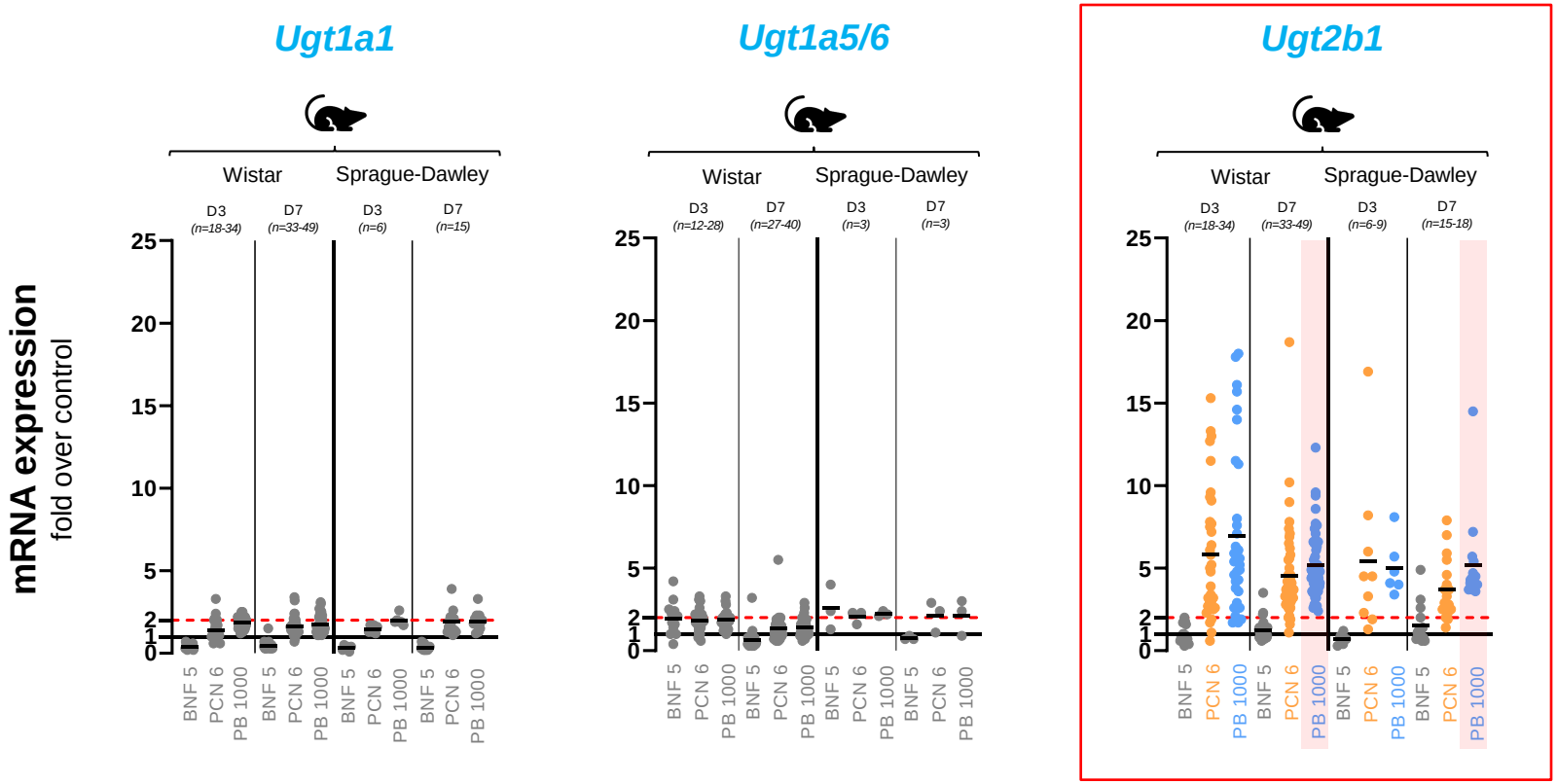
D: day
···· : 2-fold threshold
BNF: Beta-naphthoflavone;
PCN: 5-pregnen-3β-ol-20-one 16a-carbonitrile; RIF: Rifampicin; PB: Phenobarbital. Concentrations in µM.

Ugt mRNA expression in rat hepatocytes

in response to liver inducers, after 3 or 7 days of exposure

Choice of relevant UGT isoforms **based on literature** *(Vansell and Klaassen, 2002; Al Khansa et al., 2010; Shelby and Klaassen 2006)*

- ***Ugt1a1***, ***Ugt1a5/6***: Implicated in T4-glucuronidation & Induced by AhR, CAR/PXR activators
- ***Ugt2b1:*** Induced by AhR, CAR/PXR activators



→ **Most induced isoform: *Ugt2b1* (by PCN and PB)**

→ **Responses Day 3 = Day 7** (>2-fold on Day 7 with PB)

In line with *Parmentier et al., 2022; Wiemann et al., 2022; Baze et al., 2024*

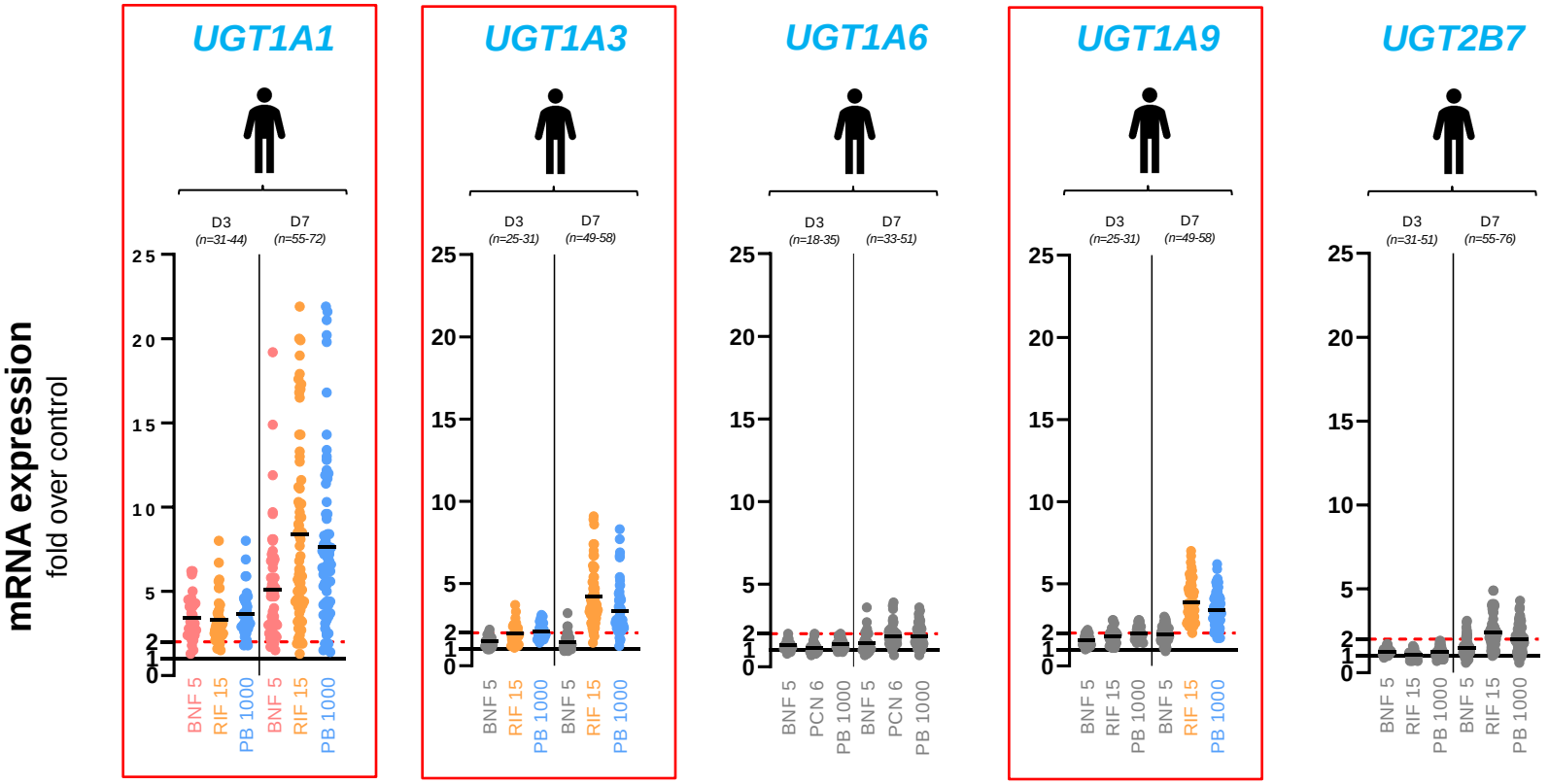
D: day
···· : 2-fold threshold
BNF: Beta-naphthoflavone;
PCN: 5-pregnen-3β-ol-20-one 16a-carbonitrile; RIF: Rifampicin; PB: Phenobarbital. Concentrations in µM.

UGT mRNA expression in human hepatocytes

in response to liver inducers, after 3 or 7 days of exposure

Choice of relevant UGT isoforms **based on literature** *(Kato et al. 2008; Yamanaka et al. 2007; Tong et al. 2007; Findlay et al. 2000; Bock et al., 2010)*

- ***UGT1A1*** (major isoform), ***1A3*** and ***1A9*****:** Implicated in T4-glucuronidation & induced by AhR, CAR/PXR activators
- ***UGT2B7*** (induced in rats)



→ **Most induced isoforms:*UGT1A1, 1A3* and *1A9***

→ **Responses Day 7 > Day 3**

In line with *Parmentier et al., 2022; Wiemann et al., 2022; Baze et al., 2024*

D: day
···· : 2-fold threshold
BNF: Beta-naphthoflavone;
PCN: 5-pregnen-3β-ol-20-one 16a-carbonitrile; RIF: Rifampicin; PB: Phenobarbital. Concentrations in µM.

In vivo / in vitro comparison of *Ugt* mRNA expression in rats

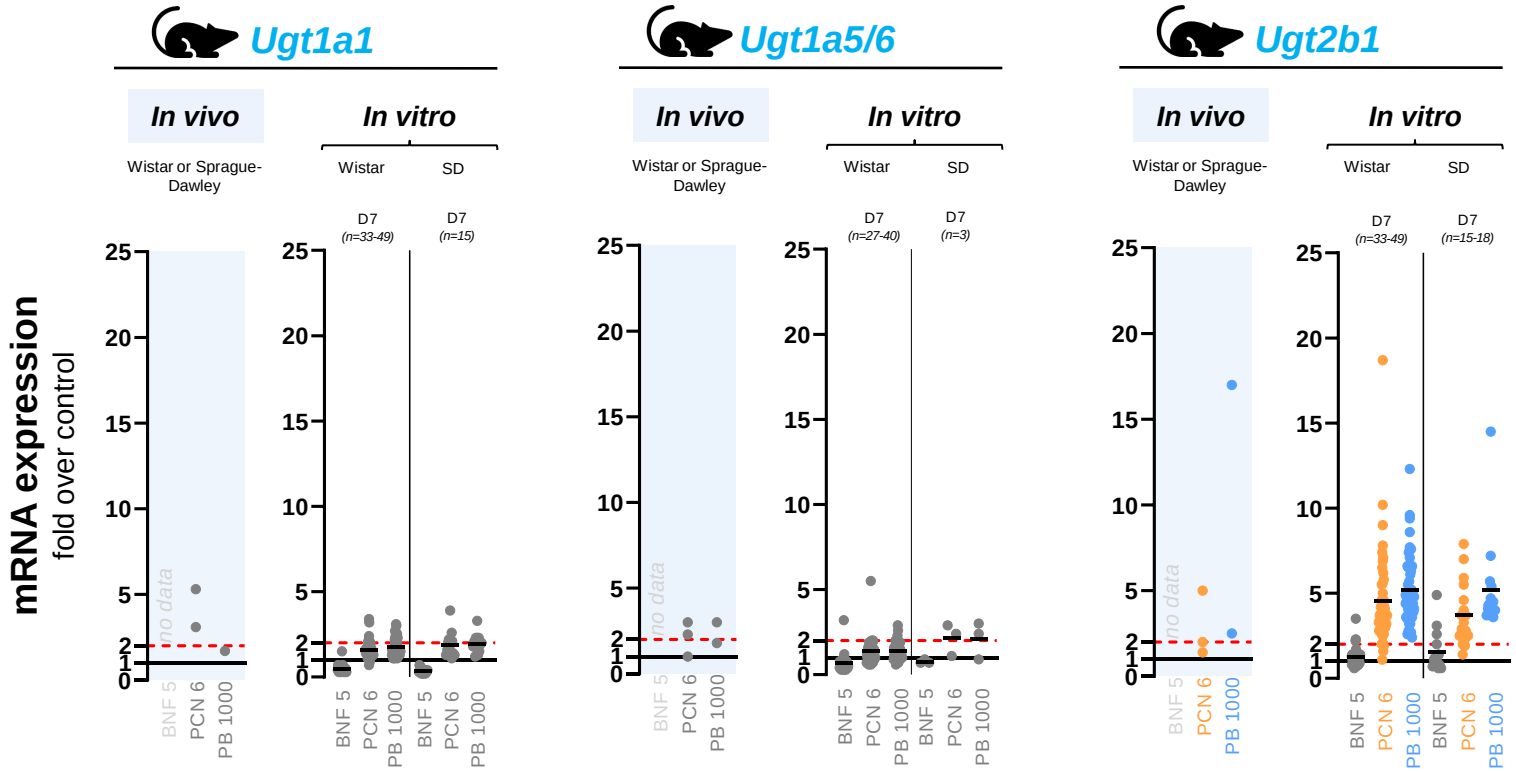
in response to liver inducers

In vivo data (based on literature):

- **PCN** administration = 50-125mg/kg/day for **4-7 days** by ip, gavage or diet; or 1000-1600ppm for **7-10 days** by diet
- **PB** administration = 1-100mg/kg administrated once or daily for **3-7 days** by ip, gavage or diet; or 1200-2400ppm for **7-10 days** by diet

(no data found for BNF)

Refs: *Tavoloni et al., 1983; Watkins and Klaassen, 1982; Kato et al., 2005; Bock and Bock-Henning, 2010; Barter and Klaassen, 1991; Saito et al., 1991; Koster et al., 1986; Hood and Klaassen, 2000; Vansell and Klaassen, 2002; Shelby and Klaassen, 2006; Al Khansa et al., 2010; Personal comm: Bayer CropScience (Helen Tinwell, Rémi Bars).*



→ ***In vitro*** **data coherent with** ***in vivo*** **data**

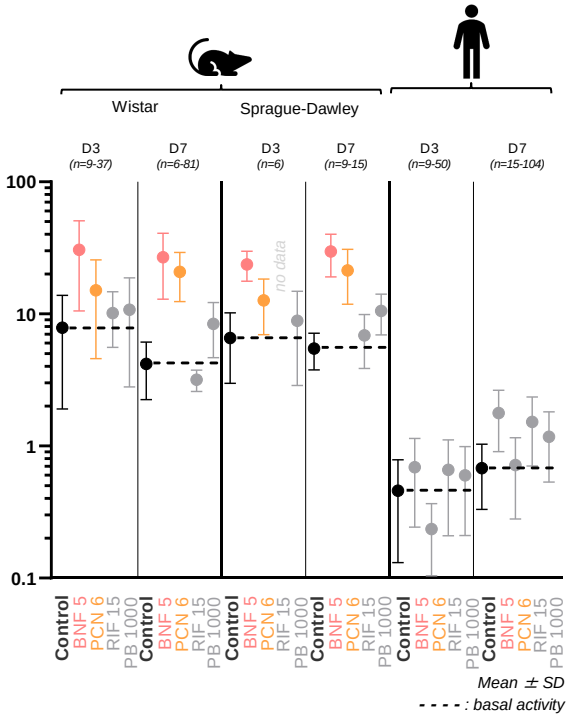
D: day
···· : 2-fold threshold
BNF: Beta-naphthoflavone;
PCN: 5-pregnen-3β-ol-20-one 16a-carbonitrile; RIF: Rifampicin; PB: Phenobarbital. Concentrations in µM.

UGT-T4 activity in rat and human hepatocytes

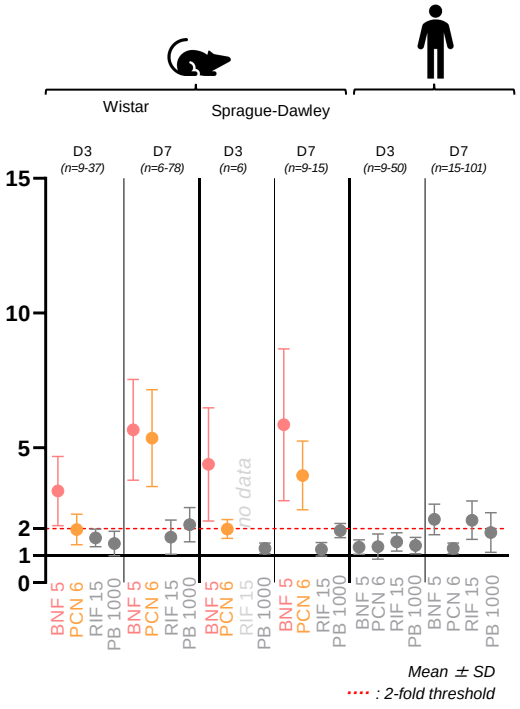
and in response to liver inducers, after 3 or 7 days of exposure

Absolute activity

pmol/min/mg cell protein

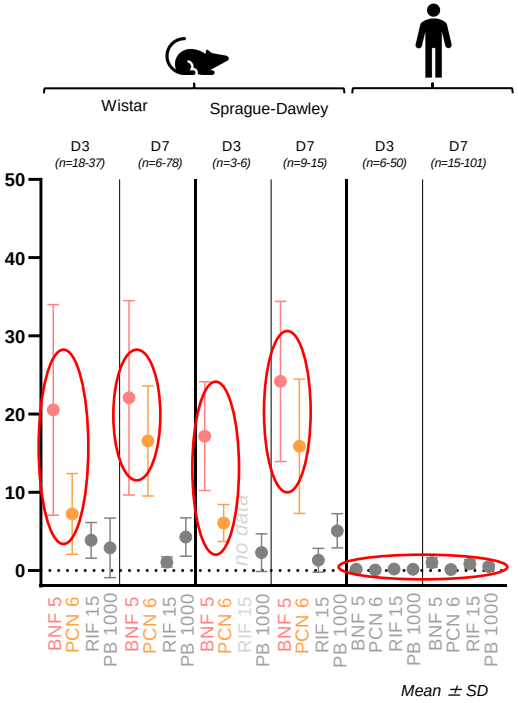


fold over control activity



Relative activity = Delta

pmol/min/mg cell protein



→ **Activity increase:**

Day 3 = Day 7 > Day 7 > Day 3

→ **Control activity:** (≥ 6x)

→ **Delta activity** >>>

BNF and PCN

In line with *Baze et al., 2024 & Baze, Ory et al., in preparation*

In vivo / in vitro comparison of UGT-T4 activity in rats

in response to liver inducers

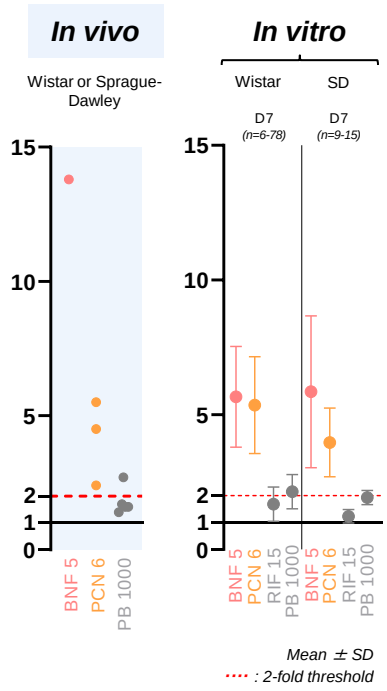
In vivo data (based on literature):

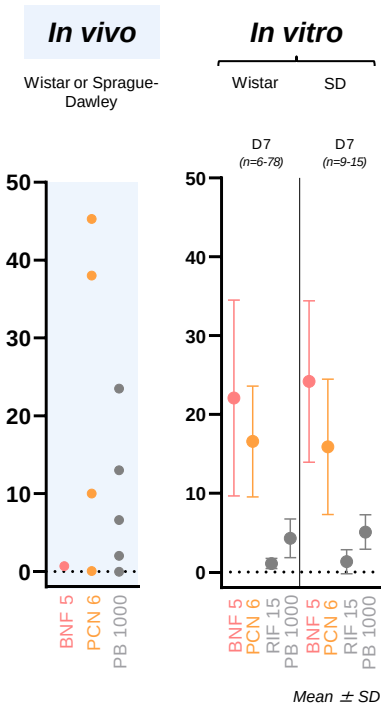
- **BNF** administration = 75mg/kg/day for **4 days** by ip
- **PCN** administration = 100mg/kg/day for **4-7 days** by ip or gavage; or 1000-1600ppm for **7-10 days** by diet
- **PB** administration = 75-80mg/kg/day for **4-7 days** by ip or gavage; or 1200-2400ppm for **7-10 days** by diet

Refs: Kato et al., 2005; Barter and Klaassen, 1991; Saito et al., 1991; Hood and Klaassen, 2000; Personal comm: Bayer CropScience (Helen Tinwell, Rémi Bars).

Absolute activity
fold over control activity

Relative activity = Delta
pmol/min/mg cell protein



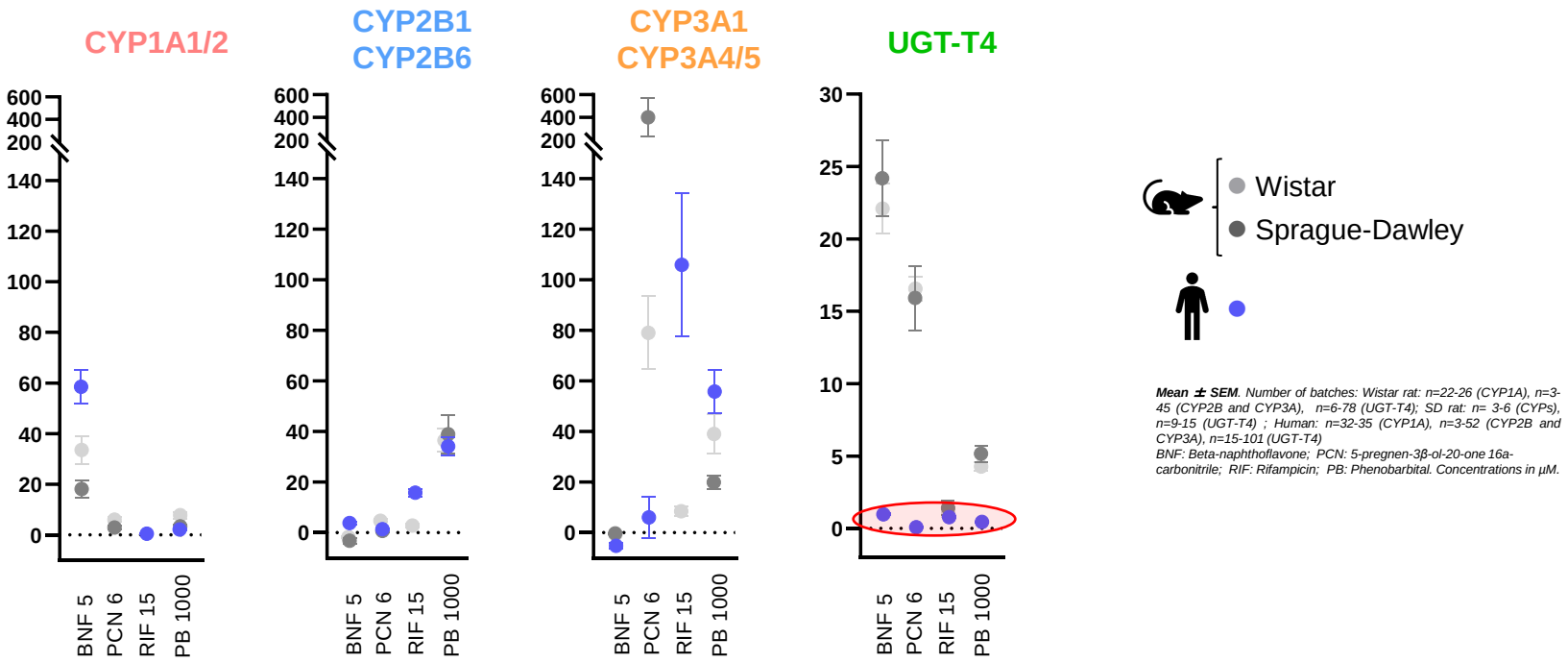


→ ***In vitro*** **data coherent with** ***in vivo*** **data**

Comparison of delta CYP and UGT-T4 activities

in response to liver inducers, in rat and human hepatocytes, after 7 days of exposure

Relative activity = Delta
pmol/min/mg cell protein



Mean ± SEM. *Number of batches: Wistar rat: n=22-26 (CYP1A), n=3-45 (CYP2B and CYP3A), n=6-78 (UGT-T4); SD rat: n= 3-6 (CYPs), n=9-15 (UGT-T4) ; Human: n=32-35 (CYP1A), n=3-52 (CYP2B and CYP3A), n=15-101 (UGT-T4)*
BNF: Beta-naphthoflavone; PCN: 5-pregnen-3β-ol-20-one 16a-carbonitrile; RIF: Rifampicin; PB: Phenobarbital. Concentrations in µM.

Delta CYP450: =

- → **BNF** increased CYP1A1/2 (both species)
- → **PB** increased CYP2B and CYP3A (both species)
- → **PCN** strongly increased CYP3A1 (rat)
- → **RIF** increased CYP2B6 and CYP3A4/5 (human)

Delta UGT-T4: only

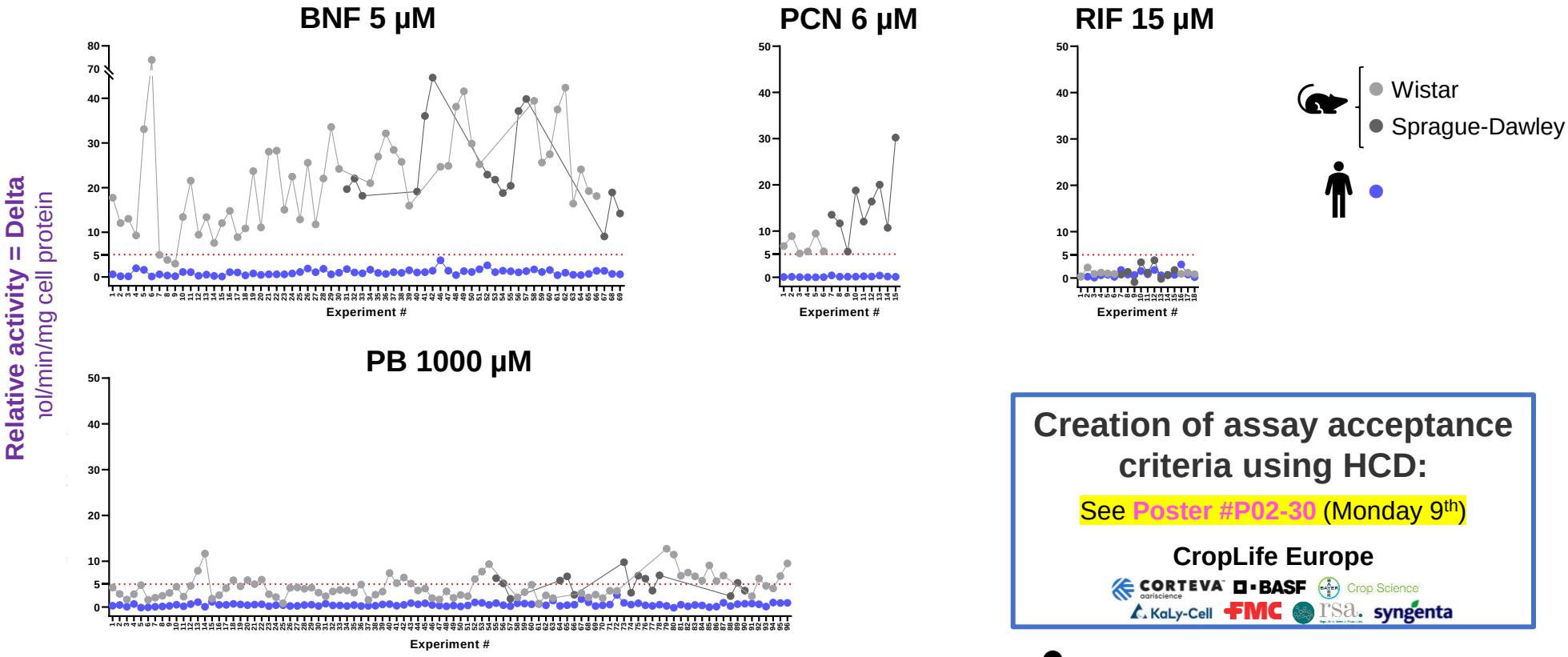
- → **Strongly by BNF and PCN**
- → Slightly by PB
- → No effect of RIF

No/very low delta UGT-T4 in → "non-responsive species"

Baze, Ory et al., in preparation

Comparison of UGT-T4 activities – experiment per experiment

in response to liver inducers, in rat and human hepatocytes, after 7 days of exposure



Creation of assay acceptance criteria using HCD:

See Poster #P02-30 (Monday 9th)

CropLife Europe

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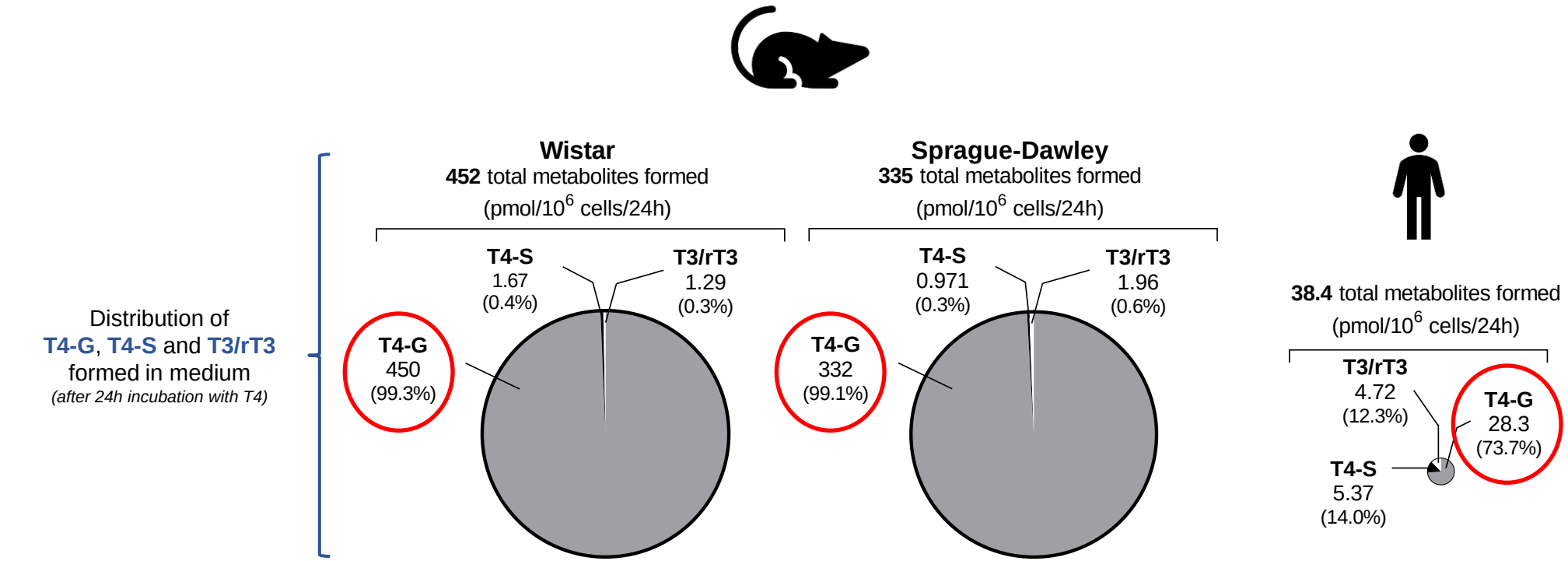
Delta UGT-T4 activity (pmol/min/mg)

BNF (n=69)	**≥ 5**	(66 → 96%)	**< 5**	(69 → 100%)	→ **PC**
PCN (n=15)	**≥ 5**	(15 → 100%)	**< 5**	(15 → 100%)	
RIF (n=18)	**< 5**	(18 → 100%)	**< 5**	(18 → 100%)	→ **NC**
PB (n=96)	**≥ 5**	(32 → 33%)	**< 5**	(96 → 100%)	→ **Not a PC**

T4 metabolism:

Distribution of T4 metabolites, in control rat and human hepatocytes, after 7 days of culture



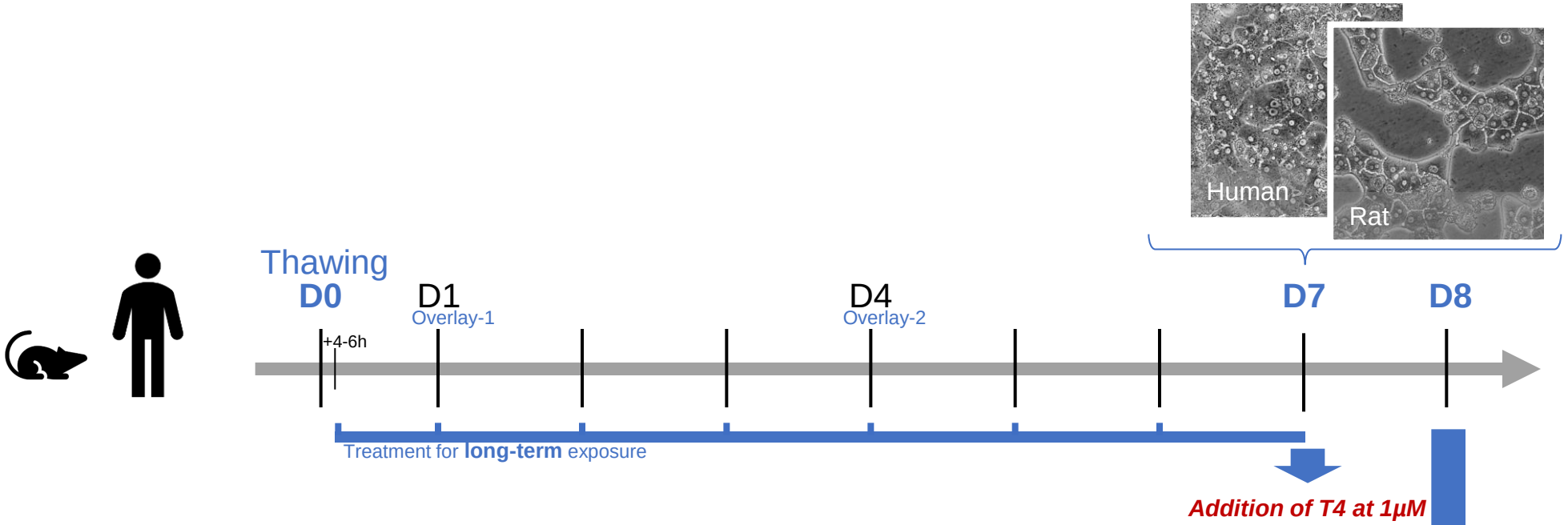
→ **T4 metabolites:** (~9x) >>>

T4-G 99% **T4-G 70%,** T4-S, T3/rT3

Baze et al., 2024; Baze, Ory et al., in preparation

Protocol overview:

T4 metabolism in rat and human hepatocytes, after 7 days of exposure



Daily treatment:

BNF (rat & human AhR activator): 5 µM

PCN (rat-specific PXR activator): 6 µM

RIF (human-specific PXR/CAR activator): 15 µM

PB (rat and human CAR/PXR activator): 1000 µM

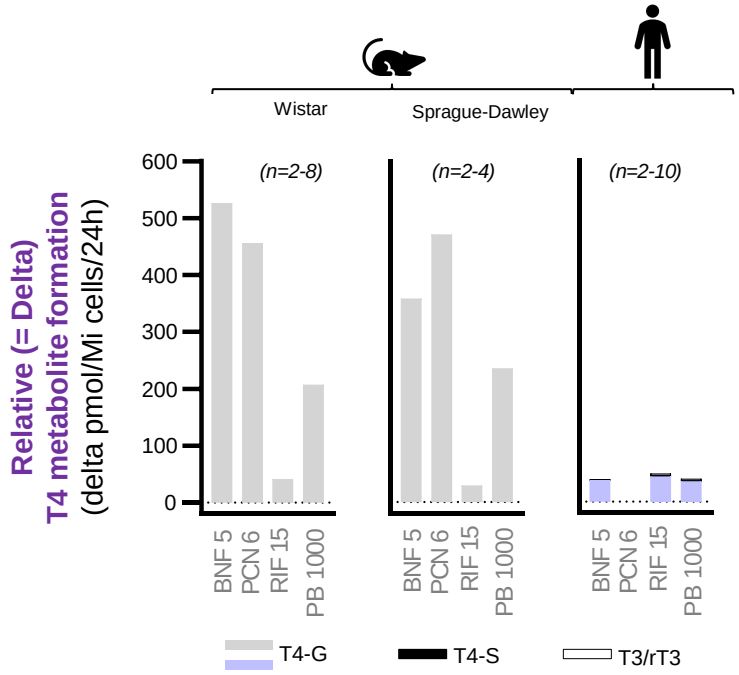
T4 metabolites after 24h:
- T4-glucuronide
- T4-sufate
- T3/rT3

(Pharmacelsus)

BNF: Beta-naphthoflavone
PB: Phenobarbital
PCN: 5-pregnen-3β-ol-20-one 16a-carbonitrile
RIF: Rifampicin

T4 metabolism

in response to liver inducers, in rat and human hepatocytes, after 7 days of exposure



→ **Increased in total T4 metabolites:**

T4-G only **>>>** **T4-G >>>** T4-S, T3/rT3

Baze et al., 2024; Baze, Ory et al., in preparation

Take-home message:

T4 metabolism and excretion

- ***Ugt* mRNA induction** & **UGT-T4 activity** in response to liver reference inducers:  ***In vivo* ⇔ *in vitro***
- **Species-specific differences**:
  - *Ugt/UGT* mRNA induction by reference inducers:
    -  ***Ugt2b1*** (Day 3 = Day 7)
    -  ***UGT1A1/3/9*** (Day 7 > Day 3)
  - Control condition:
    - Absolute **T4-UGT activity**:  > (≥ 6x) 
    - **T4 metabolism over 24h**:
      - T4 metabolites:  T4-G 99% >>>  T4-G 70%, T4-S, T3/rT3
  - Responses to liver reference inducers:
    - **Relative CYP450 activity increase**:  = 
    - **Relative T4-UGT activity increase**:  >>> 
    - **Increased total T4 metabolites over 24h:**  >>> 
      -  T4-G only
      -  T4-G >>> T4-S = T3/rT3

- **Assay acceptance criteria** (see **Poster #P02-30**) **and interpretation criteria** (Kent et al., in preparation)
- **Formal validation of the assays for regulatory acceptance**

Thank you for your attention ☺

Special thanks to:

The **KaLy-Cell** team !

Pharmacelsus

Our **collaborators** ☺

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Poster

#P02-30 on Monday 9th

(Assay acceptance criteria using HCD)