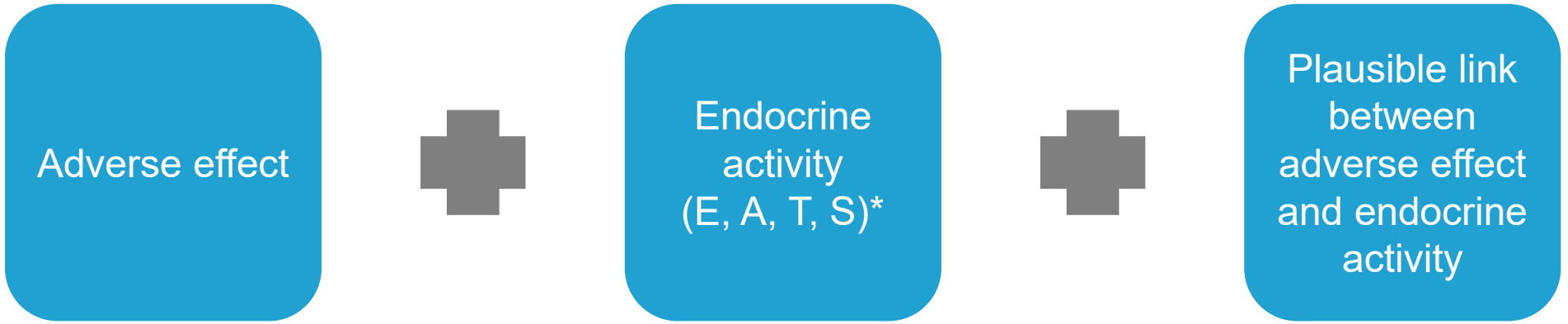
Available / accepted methods Strategies

Stephanie Melching-Kollmuss, BASF SE

13th March 2024

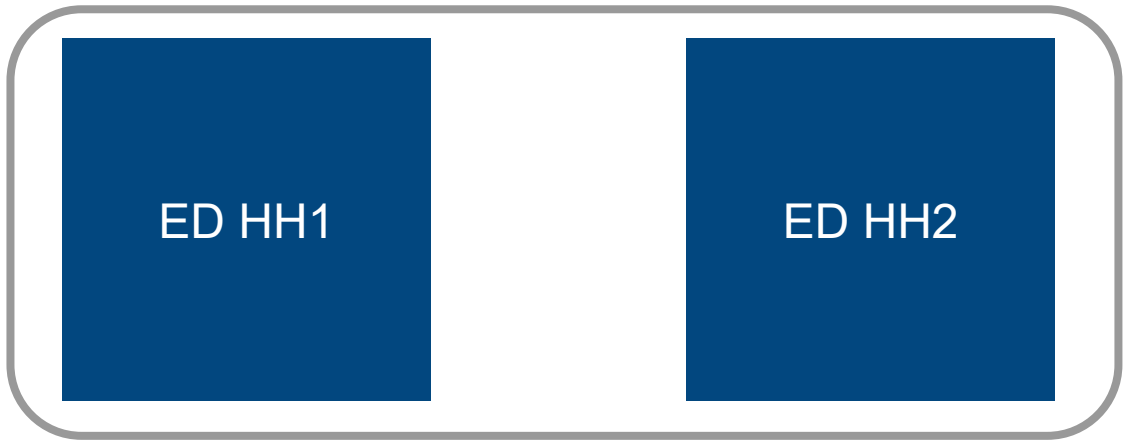
Endocrine Disruption Criteria



*according to ECHA/EFSA Guidance, 2018

Agrochemicals & biocides

EC 605/2018
2100/2017



Guidance under development

All chemicals

EC 1272/2008 (Classification & Labelling)

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Gather all relevant information / data

Relevant tests in accordance to OECD GD 150 and Table 9 of ECHA/EFSA Guidance Document:

In vitro **mechanistic -** **OECD Level 2**

e.g. – Estrogen or androgen receptor binding affinity
– Estrogen receptor transactivation, yeast estrogen screen (**OECD 455**)
– Androgen receptor transactivation (**OECD 458**)
– Steroidogenesis *in vitro* (**OECD 456**)
– Aromatase assay (OPPTS)
– Thyroid disruption assays (e.g. thyroperoxidase inhibition, transthyretin binding)
– Retinoid receptor transactivation assays

In vivo **mechanistic -** **OECD Level 3**

– Uterotrophic Assay (**OECD 440**)
– Hershberger assay (**OECD 441**)
– Changes in hormone levels *in vivo* OECD level 4 and 5 assays

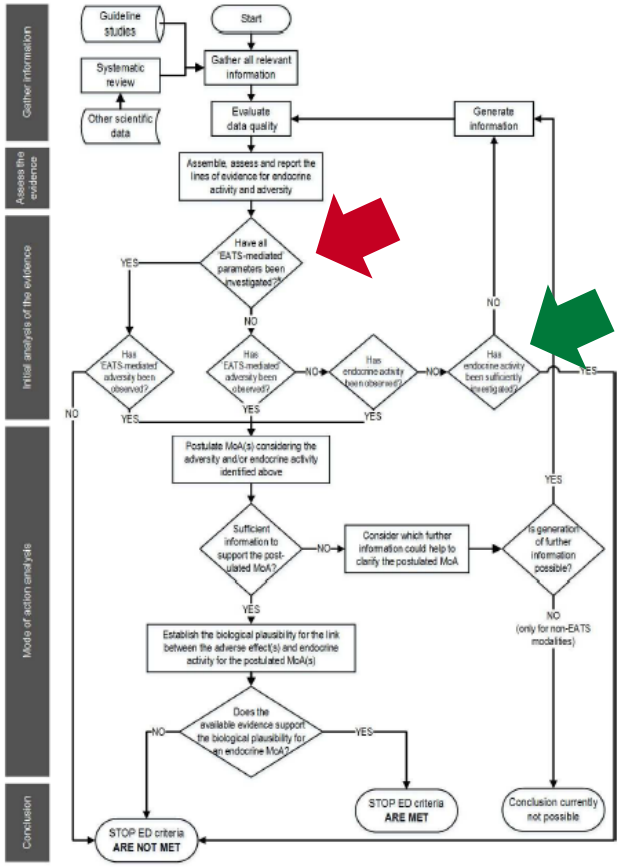
In vivo **adversity** of **ED-related** effects - **OECD Level 4**

e.g.– Repeated dose toxicity (28-, 90-day studies, chronic studies) (**OECD 407, 408, 409, 451, 452, 453**)
– Prenatal developmental toxicity study rats or rabbits (**OECD 414**)
– Pubertal development and thyroid function assay in peripubertal rats
– Developmental neurotoxicity study (**OECD 426**)

In vivo **adversity** of **ED-relevant** effects - **OECD Level 5**

– Extended one-generation reproductive toxicity study (EOGRTS) (**OECD 443**)
– Two-generation reproduction toxicity study (**OECD 416**, **2001 update**)

Figure 1. Flowchart illustrating the ED assessment strategy



Flow scheme
ECHA/EFSA Guidance, 2018

What we do?

- We look at Estrogen (E), Androgen (A), Thyroid (T), Steroidogenesis (S) only
- We usually have a large adversity data set
- A weight-of-evidence assessment can be done – pattern of effects can be identified (more specifically for EAS modalities)
- Systemic toxicity is only taken into account in terms of „confounding“ toxicity
- Plausible link is in most cases only qualitatively assessed
- Human relevance is the default

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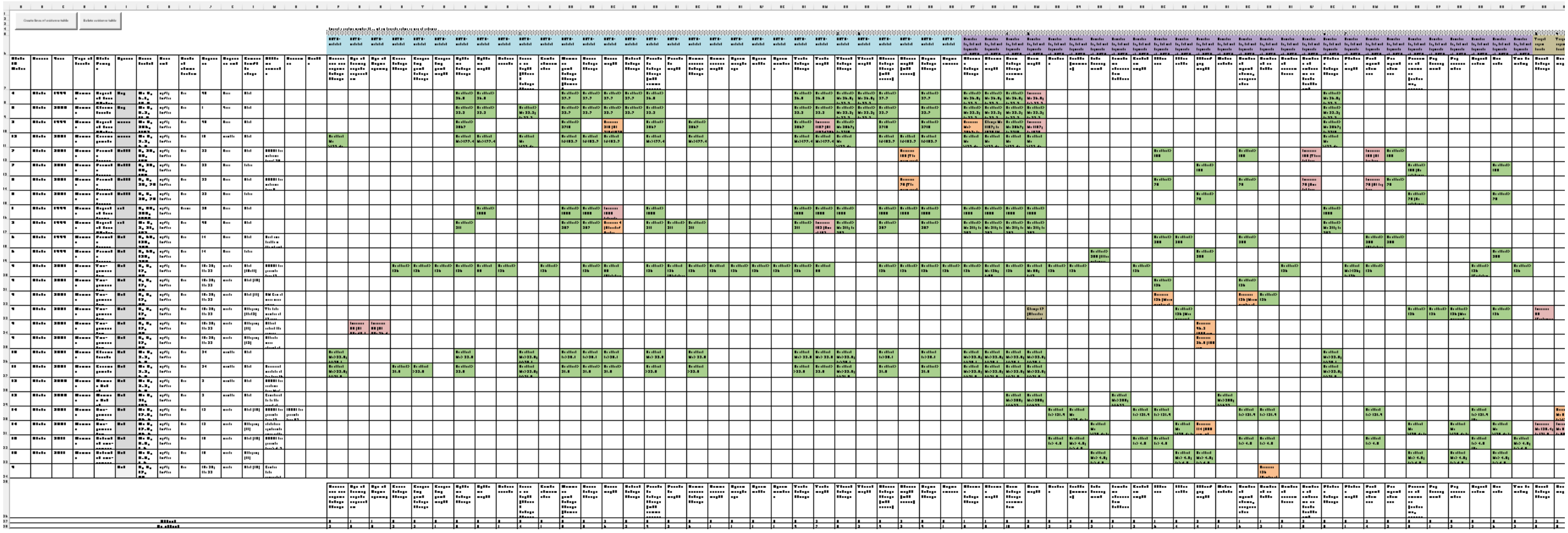
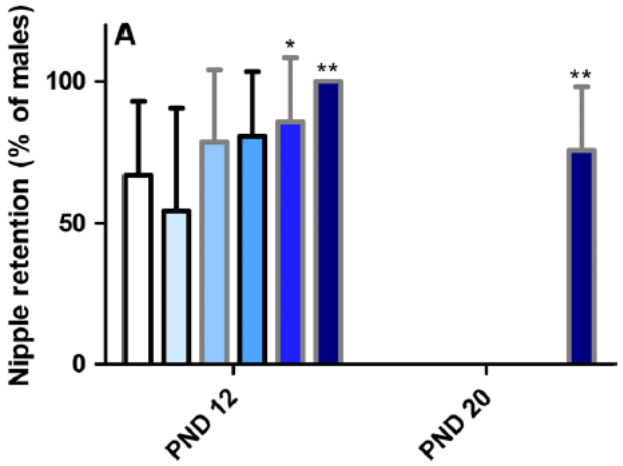
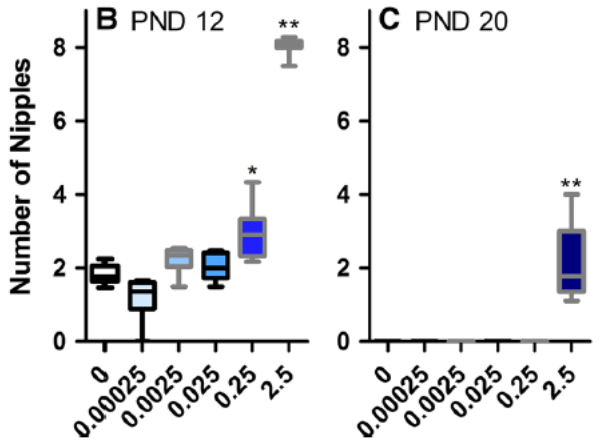
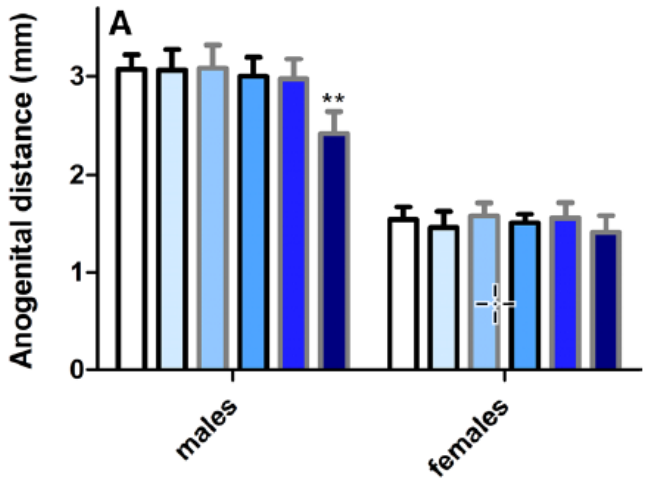
Practical Example: All data at a glance – weight of evidence; pattern of effects

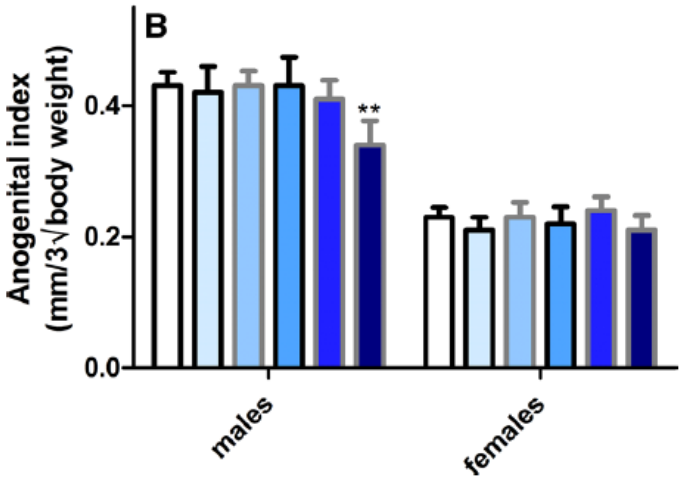


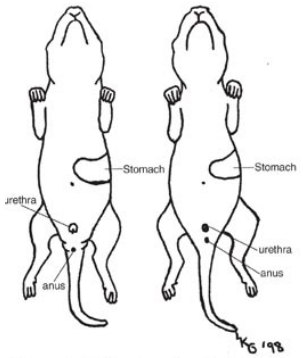
Reproduction toxicity study results suitable to examine a pattern of effects - focus on antiandrogenicity



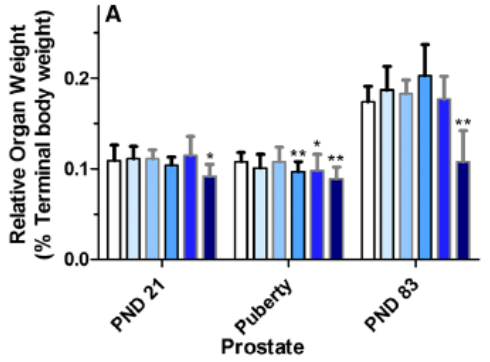


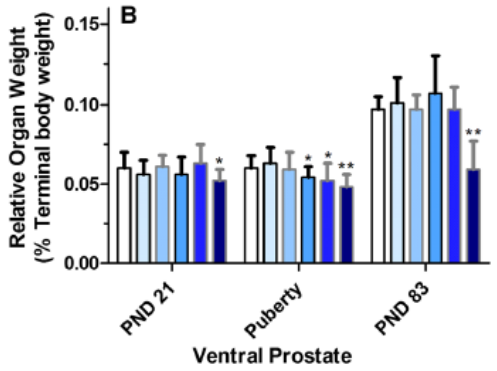


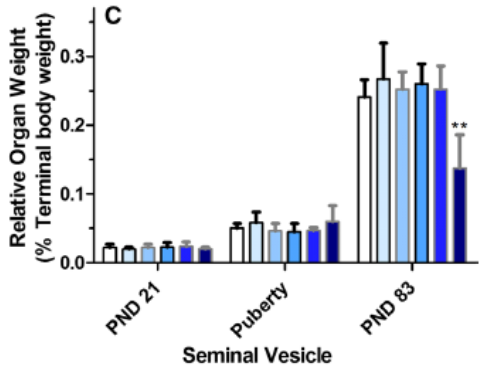




Male on the left, female on the right. Notice the distance is farther on the male between the anus and the urethra.

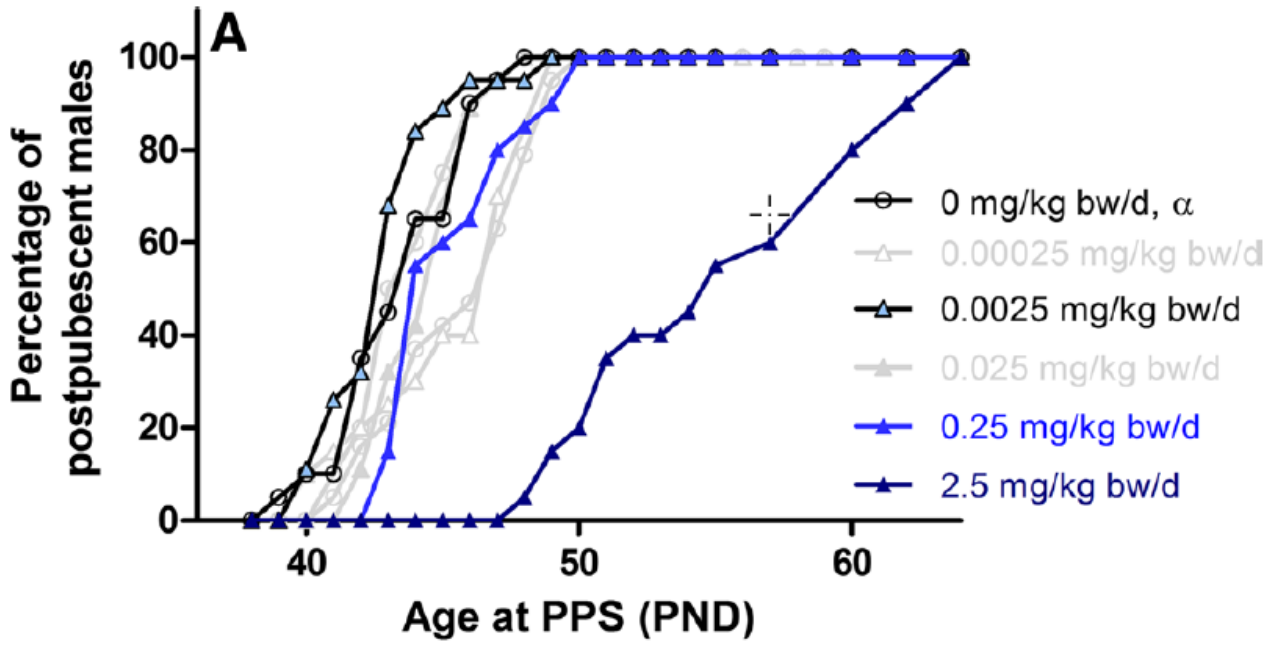


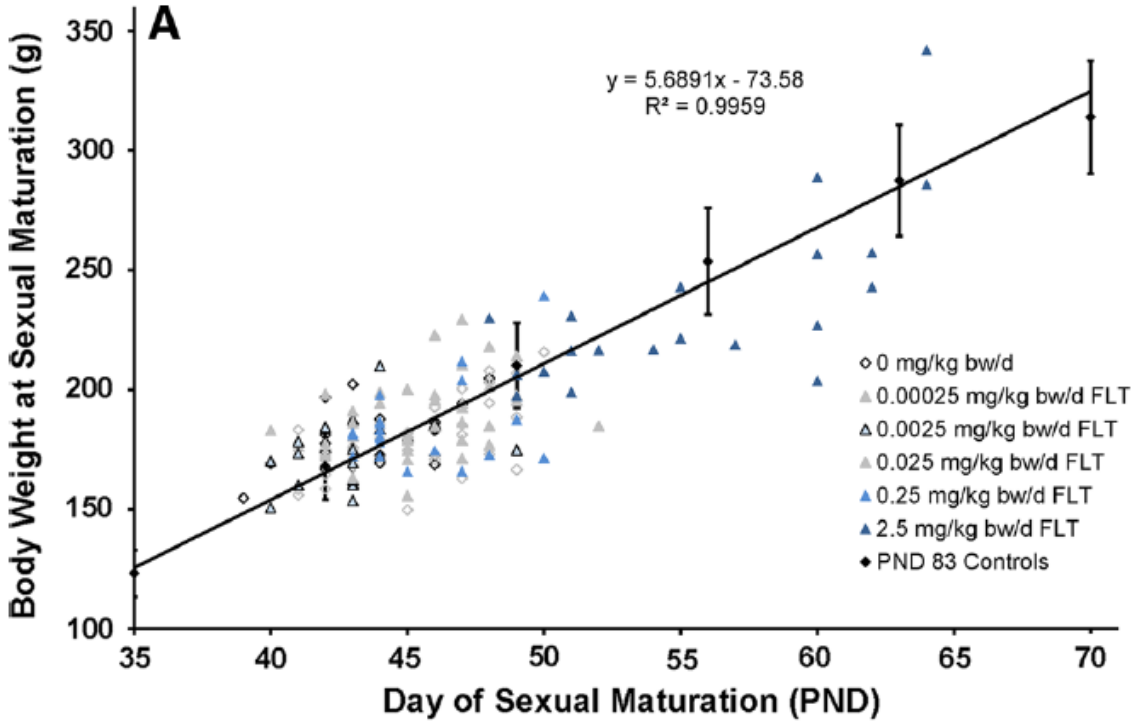




Fussell et al., 2015
Antiandrogenicity and low dose
- Flutamide

Reproduction toxicity study results suitable to examine a pattern of effects - focus on antiandrogenicity





Fussell et al., 2015

LRI Long-Range Research Initiative

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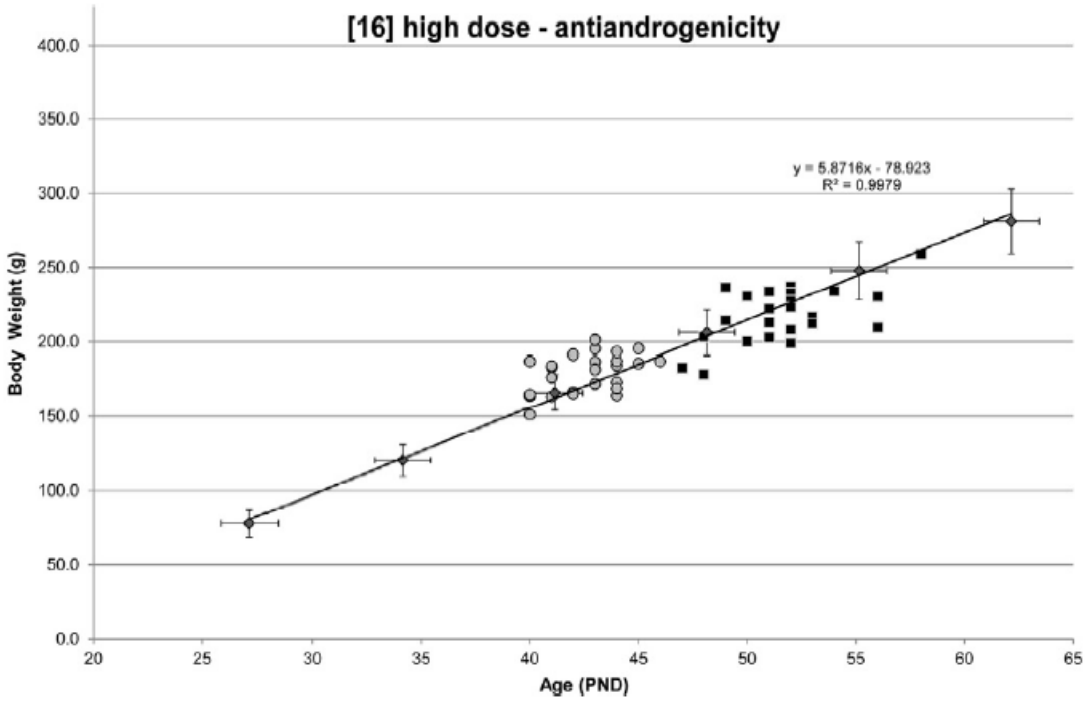
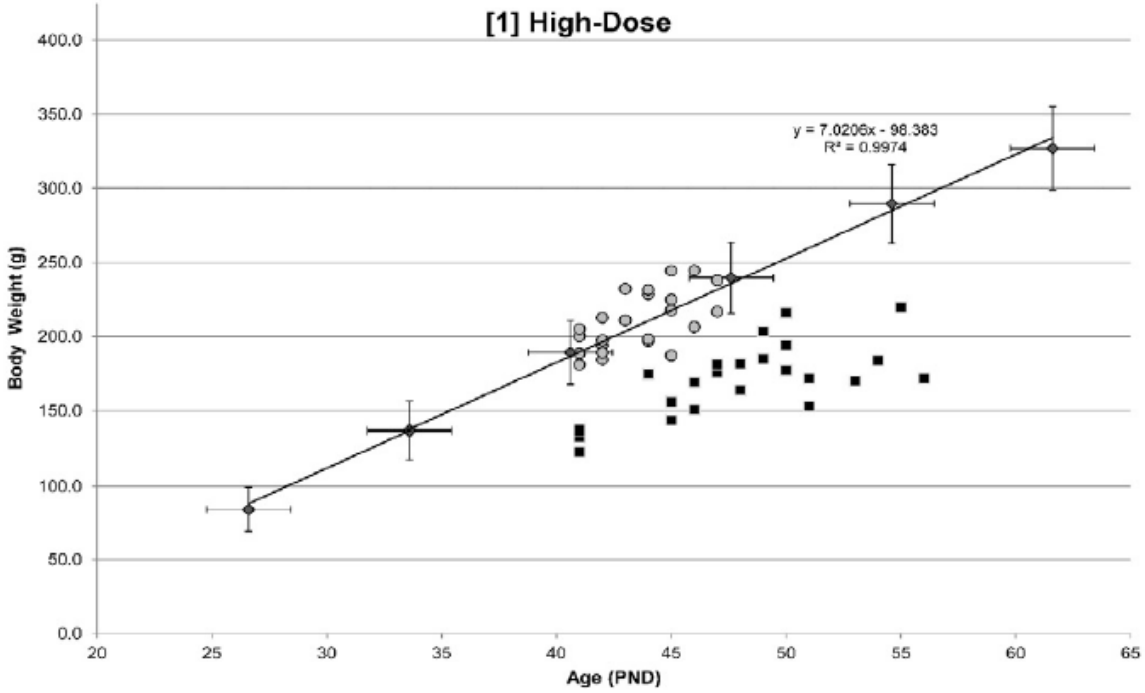
Non-specific „EATS-mediated parameters“ vs specific ED effects

Age at balanopreputial separation
- Delay ca be caused by antiandrogenic mode of action / lower body weight
Similar phenomenon in females – delayed vaginal opening





Melching-Kollmuss, 2014

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Non-specific vs ED-mediated effects

Parameter	Non-specific cause	Reference
Estrous cycle disruption	Severely lower body weight; stress	Frisch_1987; Everds_2013; Witorsch_2016
Post-implantation losses	Feed restriction	Harazono_1998, Terry_2005
Reproduction organ weight decreases / atrophy	Severely lower body weights; stress	Everds_2013, DePeyster & Mihaich_2014, Pellegrini_1998
Adrenal weight changes	Stress	Harvey & Sutcliffe_2010
Puberty onset delayed	Lower offspring body weight	Carney_2004; many others
Thyroid hormone status	Stress	Döhler_1979
Serum hormone levels	Stress	Everds_2013; Witorsch_2016

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Human relevance

- Rat vs human receptor binding / inhibition → IC_{50} concentrations largely different
  - Qualitative vs quantitative
- Physiological differences in parturition processes / hormone levels
- Differences in hormone transport
  - Thyroid hormones: Thyroid Binding Globulin (TBG) vs albumin
  - Sex hormones: SHBG vs albumin
- Differences in ADME properties

Generally: Very challenging to prove qualitative species differences relevant for C & L

What we could do

- Take quantitative aspects into account – develop quantitative AOPs
- Thresholds for Key Event Relationships (KERs)
- Use In vivo to in vitro extrapolations (IVIVE) approaches
- Work together on robust in vitro assays to understand modes of actions / to measure species differences
- Develop intelligent / integrated testing approaches with less animals
- Use quantitative / PBPK Modelling

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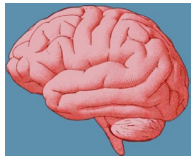
Simplified thyroid – related Adverse Outcome Pathway



TSH↑

Stimulation of thyroid cells

Follicular cell hypertrophy/ hyperplasia

Thyroid tumors

Thyroperoxidase (TPO) inhibition

Sodium Iodide symporter (NIS) inhibition

TH synthesis↓

T3/T4↓

Tissue TH↓

Adverse neuro-development

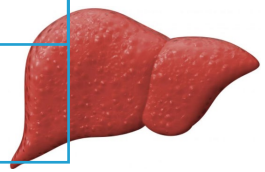
Neurological dysfunction

Endpoint of concern

Nuclear receptor activation (e.g. CAR, PXR,…)

Excretion of Phase II TH↑

Hepatic transport↑



Serum binding protein interference (TBG, TTR, albumin)

Excretion of Phase II TH↑

Deiodinase inhibition (Dio 1,2,3)

Conversion of T4 → T3↓

Currently: Thyroid hormone / Thyroid histopathological changes are considered indicative for neurodevelopmental toxicity

Currently: Exclude other modes of actions mainly NIS, TPO, Dio

HPT axis: How are thyroid hormones produced?

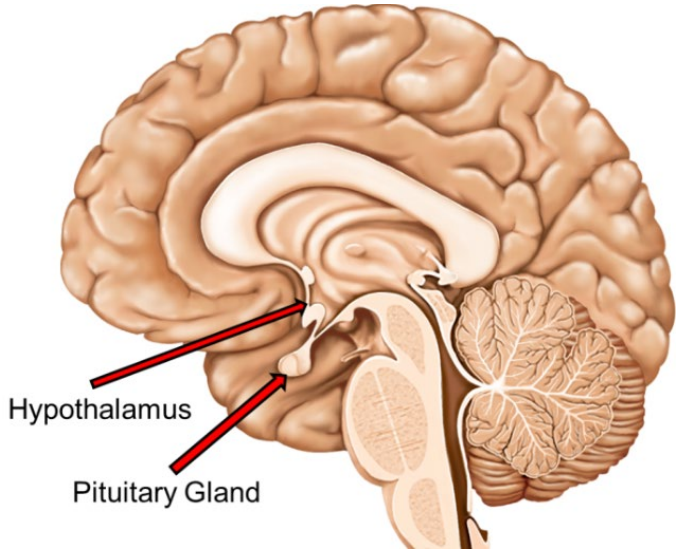
Hypothalamus
(paraventricular nuclei)

TRH

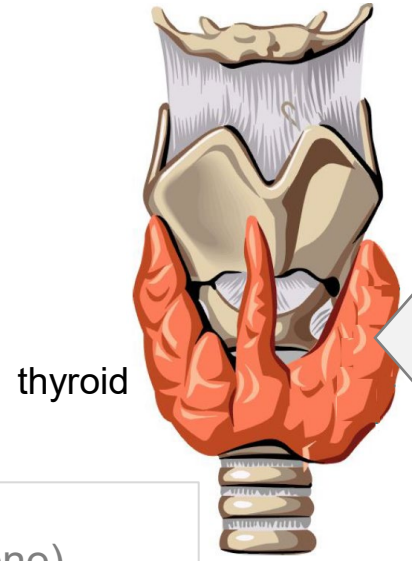
Pituitary
(thyrotroph)

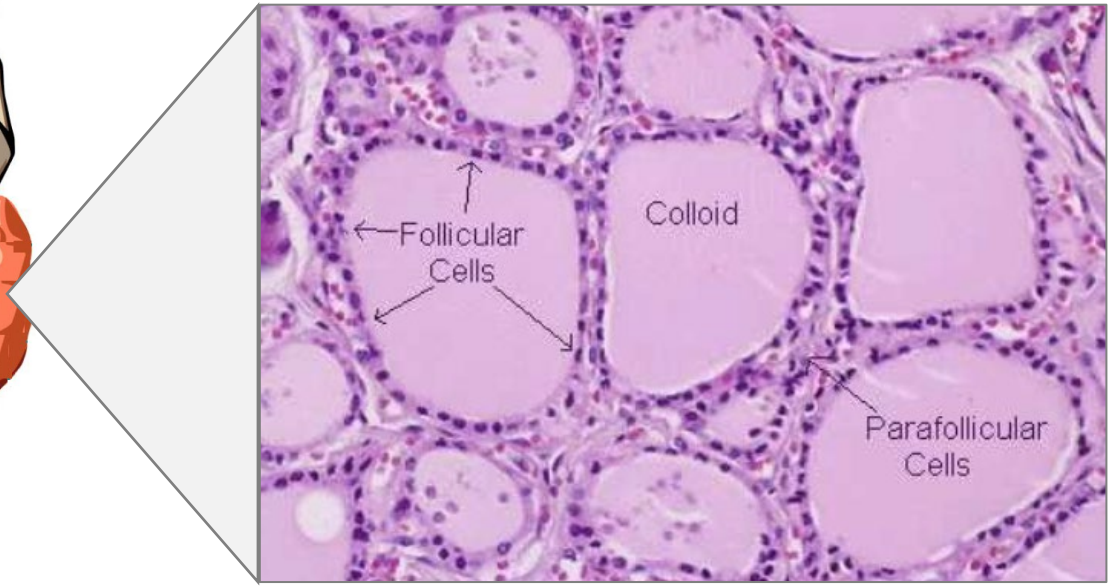
TSH

Thyroid
(follicle)



Parafollicular cells (C-cells) → Calcitonin (calcium homeostasis)
Follicular cells → functional unit / thyroid hormones

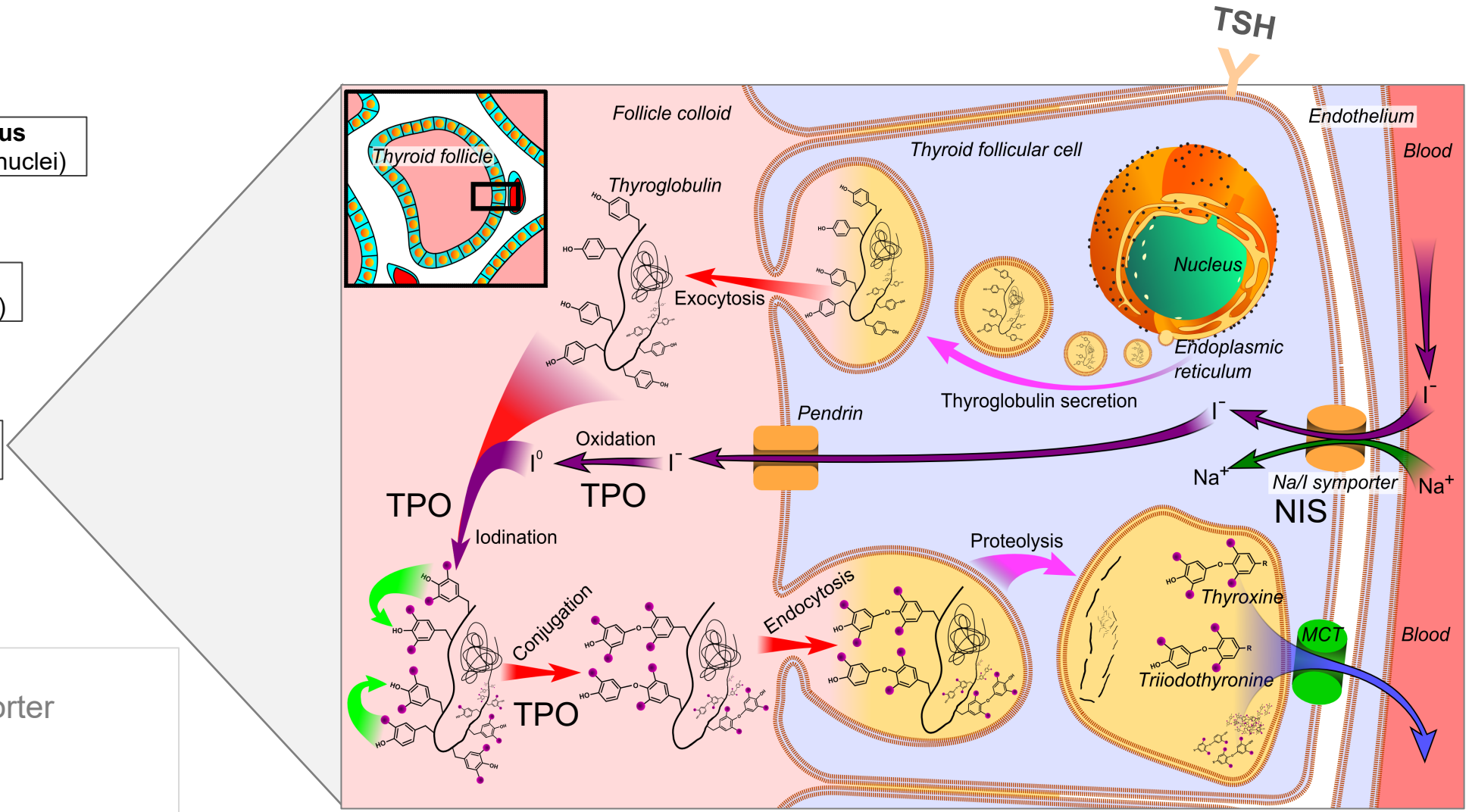




TRH: thyrotropin-releasing hormone
TSH: thyrotropin (thyroid-stimulating hormone)

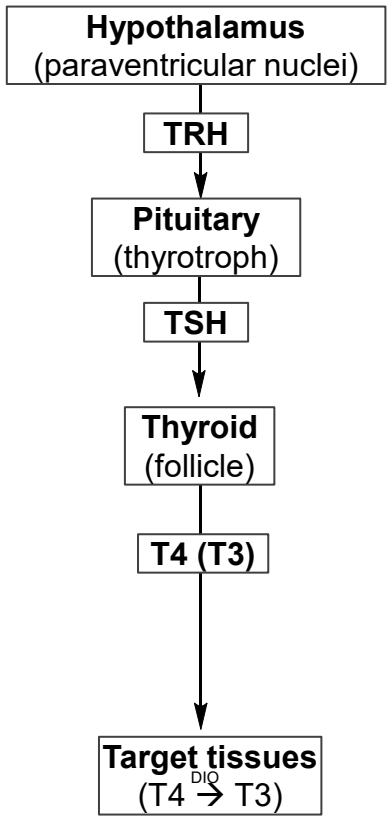
HPT axis: How are thyroid hormones produced?

Hypothalamus
(paraventricular nuclei)
↓ **TRH**
Pituitary
(thyrotroph)
↓ **TSH**
Thyroid
(follicle)
↓ **T4 (T3)**



TPO: thyreoperoxidase
NIS: sodium-iodine symporter
MIT: mono-iodine tyrosine
DIT: di-iodine tyrosine
T4: thyroxine
T3: triiodothyronine
MCT: monocarboxylattransporter

Thyroid hormones: Transport

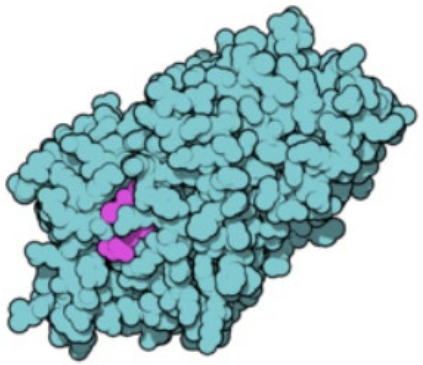


Thyroid hormones in blood:

- reversible complex of T4 (& T3) with liver-derived transport/binding-proteins
  - TBG (thyroid hormone binding globulin) → high affinity
  - TTR (transthyretin) → moderate affinity
  - Albumin → low affinity
- these three proteins bind 99.97% of T4 and 99.70% of T3
- buffering system maintaining the concentration of free TH constant
- transport to target tissues

TH affinities [M^{-1}]: albumin < TTR < TBG (Robbins and Edelhoch, 1986)

- Albumin: affinity for T4 (7.0×10^5) & T3 (1.0×10^5) → 10% of TH in human blood
- TTR: affinity for T4 (7.0×10^7) & T3 (1.4×10^7) → 15% of TH in human blood
- TBG: affinity for T4 (1.0×10^{10}) & T3 (4.6×10^8) → 75% of TH in human blood



TBG-T4
(T4 (pink) bound to Thyroxin-binding globulin (blue))

Identify species differences: e.g. comparative rat/human *in vitro* liver enzyme induction

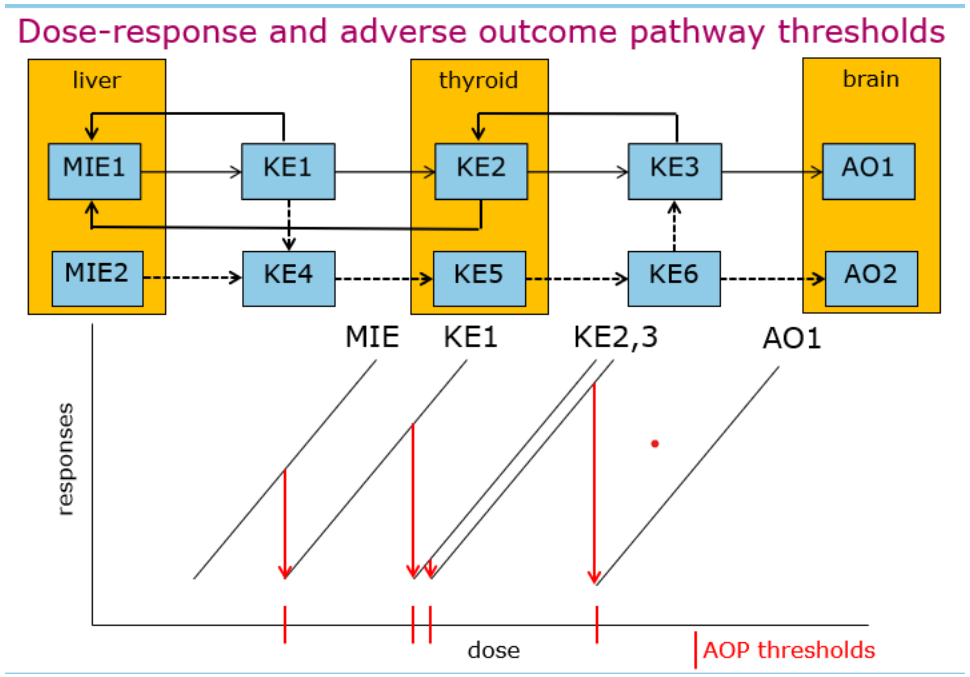
Endpoint/Parameter	Human	Rat	Reference
Binding of thyroid hormones	High affinity thyroxin binding globulin (TBG)	Low-affinity albumin	Bartsch et al., 2018 Foster et al., 2021
Half-lives of T4 in plasma	5 – 9 days	12 – 24 h	Bartsch et al., 2018 Foster et al., 2021
Half-lives of T3 in plasma	1 day	6 h	Bartsch et al., 2018 Foster et al., 2021
Levels of total T4	4200 – 12500 ng/dl	3400 – 6000 ng/dl	Bartsch et al., 2018
Levels of total T3	78 – 201 ng/dl	60 – 80 ng/dl	Bartsch et al., 2018
Levels of free T3	0.23 – 0.48 ng/dl	0.17 – 0.18 ng/dl	Bartsch et al., 2018
Amount of T4 supplementation required in absence of functioning thyroid	2.2 mg/kg bw/day	20 mg/kg bw/day	Foster et al., 2021

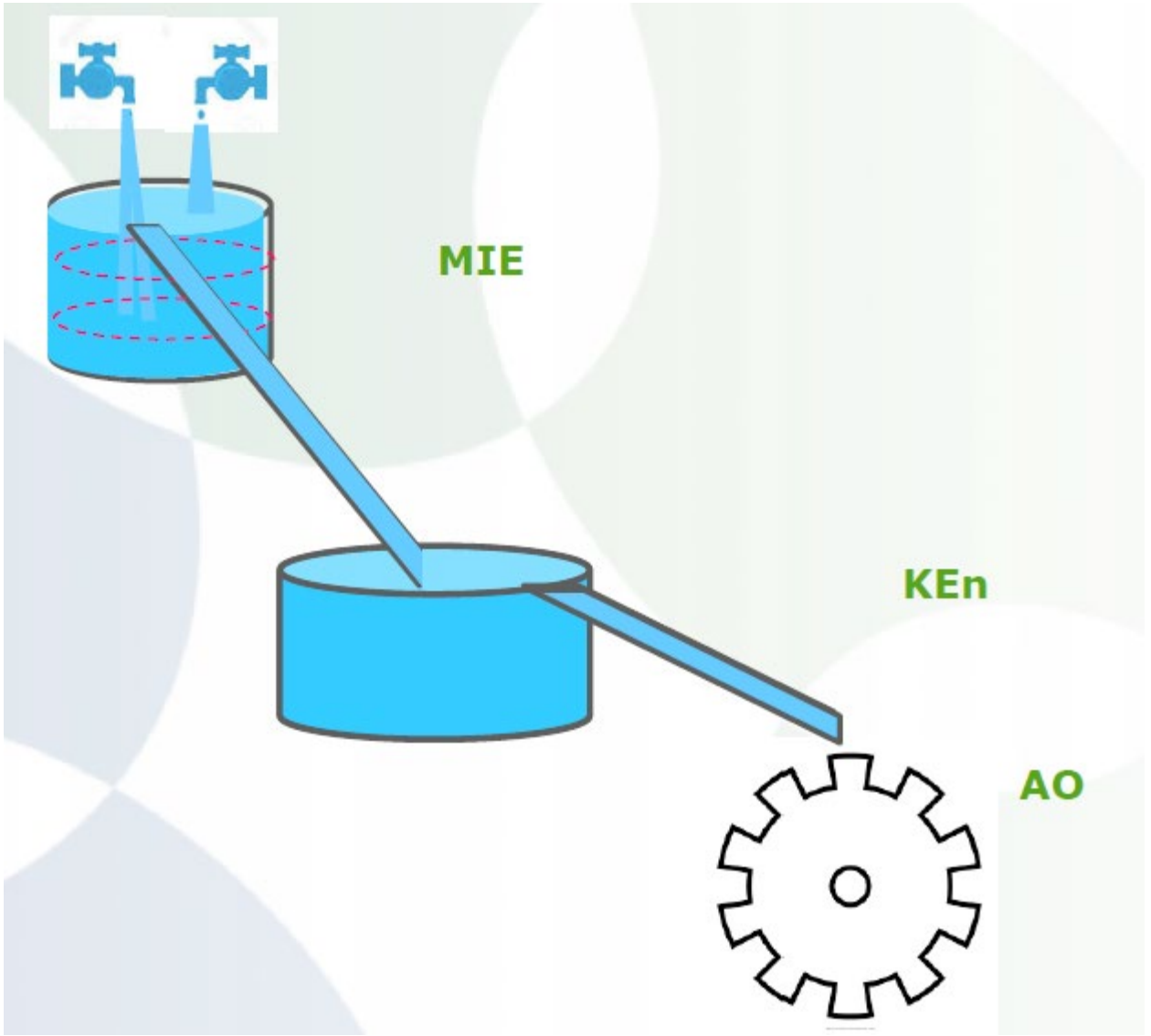
- Number of known species differences between rats and humans, but limited assays to prove it on a substance base

Quantitative aspects

There are thresholds, that MIEs or KEs become relevant for the development of an adverse outcome.

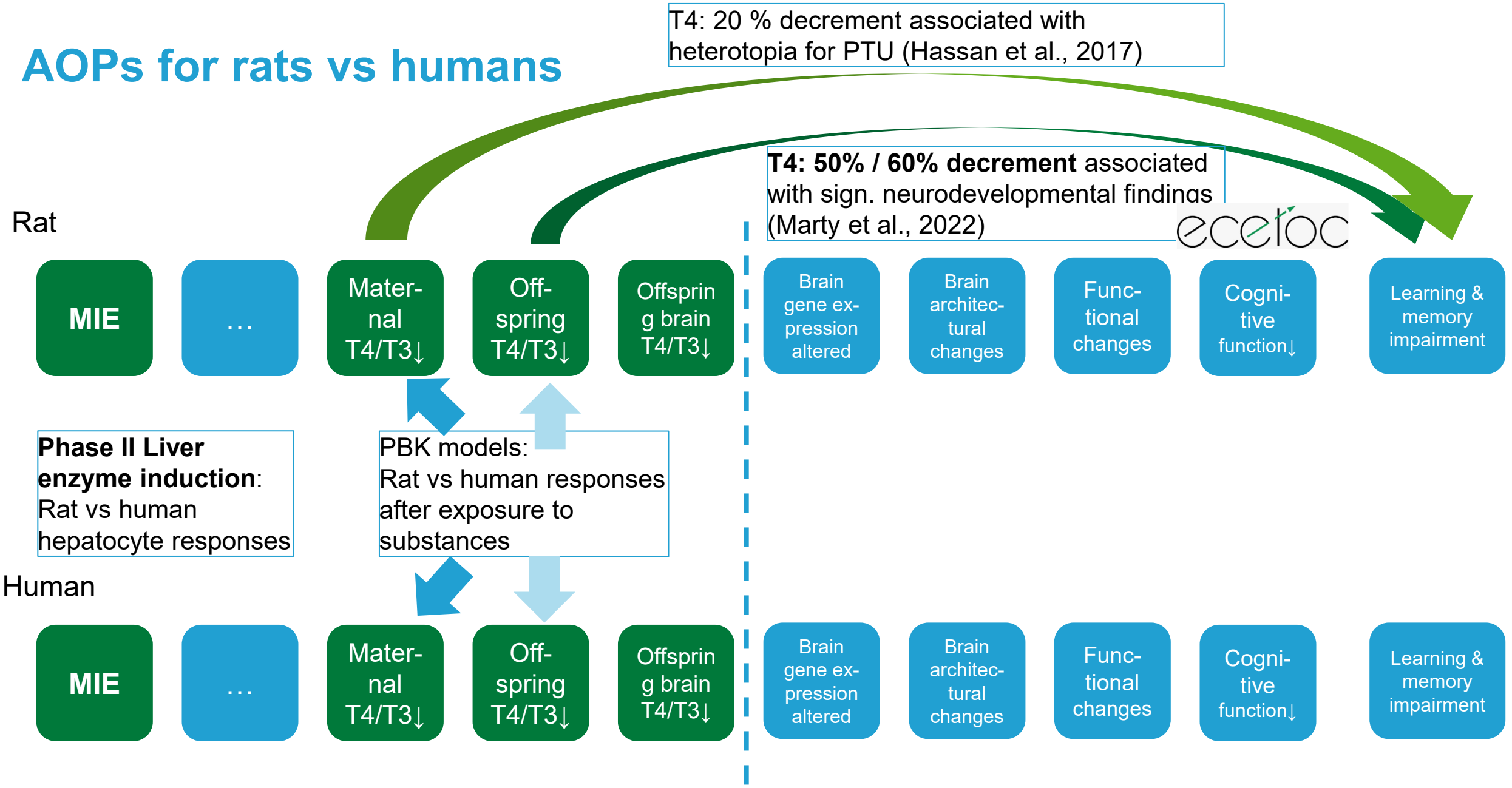
National Institute for Public Health and the Environment
Ministry of Health, Welfare and Sport





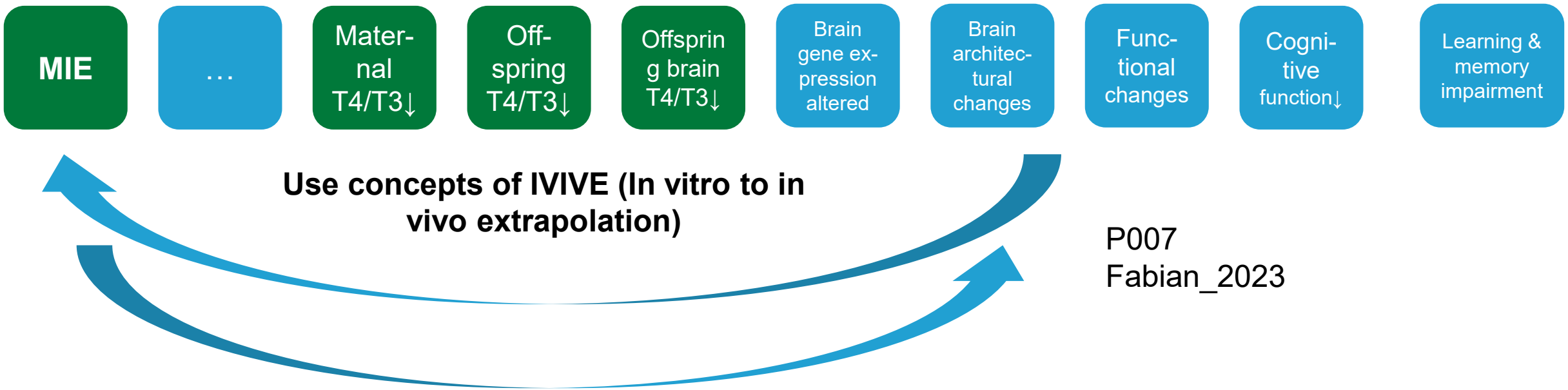
Cefic LRI EMSG59

AOPs for rats vs humans



AOPs for rats vs humans – and not to forget

Consider toxicodynamics:
Ideally internal doses are used based on
- Measure plasma levels
- Apply bioavailability considerations



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Fabian_2023

Comparative liver enzyme induction – Phase I activity

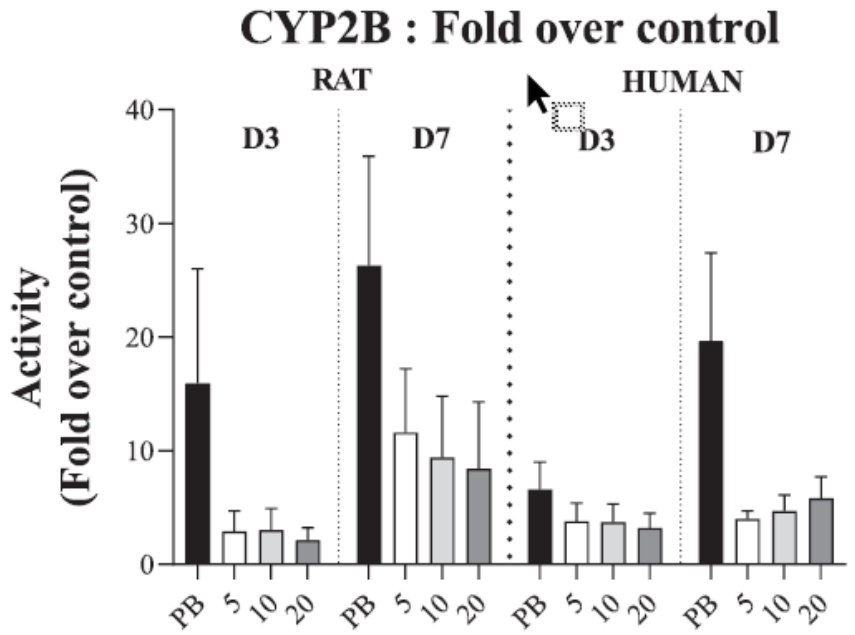
Rat

MIE …

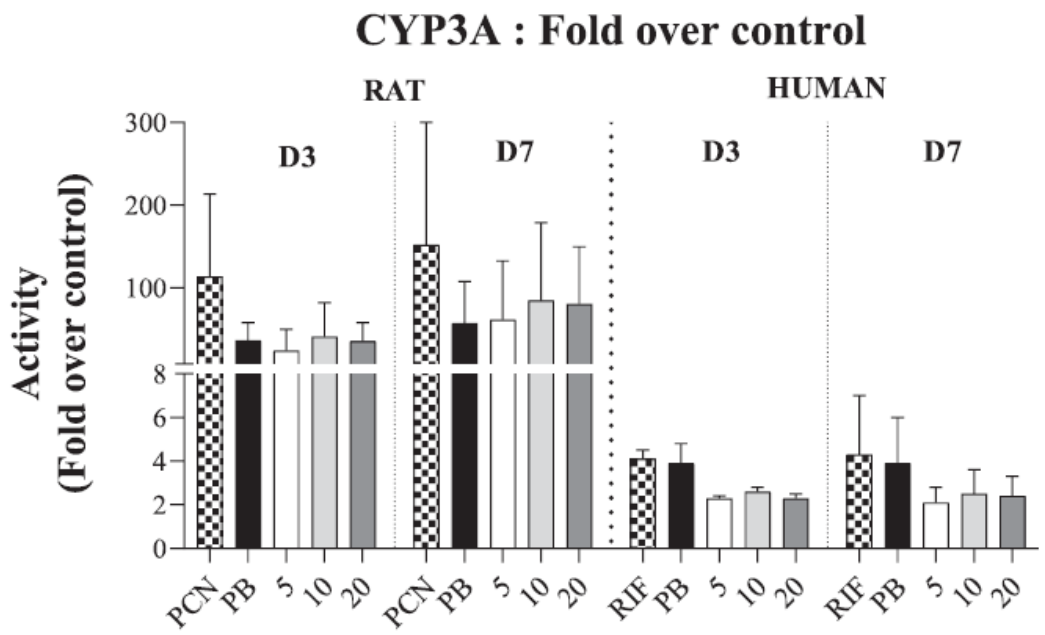
Phase II Liver enzyme induction: Rat vs human hepatocyte responses

Human

MIE …



mean individual values ± sd ; n=9 for rat and n=9 for human hepatocytes



mean individual values ± sd ; n=9 for rat and n=9 for human hepatocytes

PB – phenobarbinal
PCN – Pregnenolone carbonitrile
RIF - Rifampicin

Wiemann_2023

Comparative liver enzyme induction – Phase II liver enzyme induction - mRNA

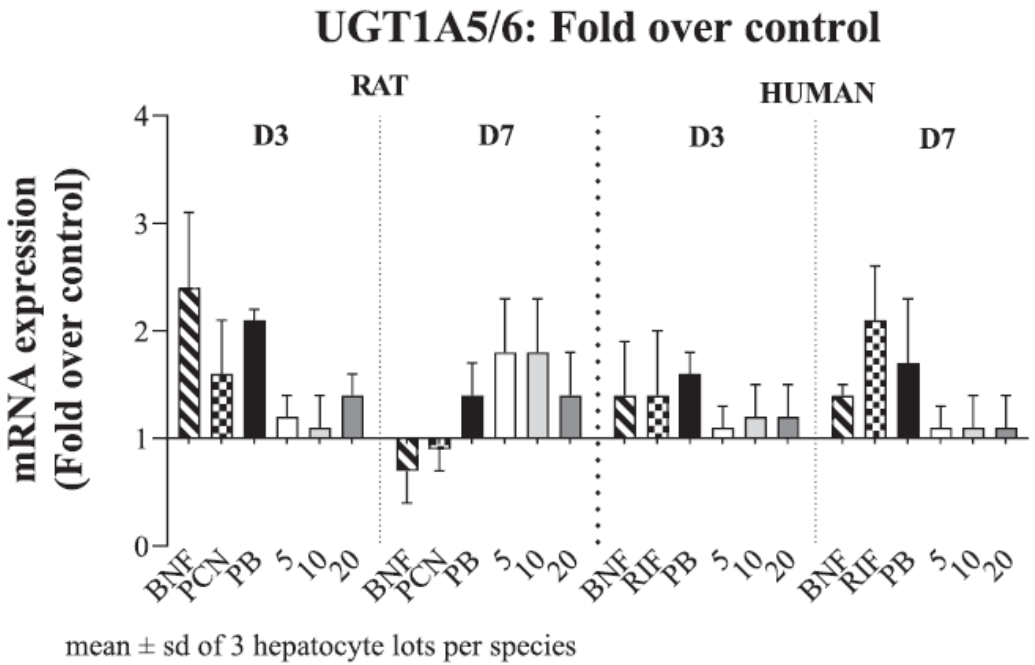
Rat

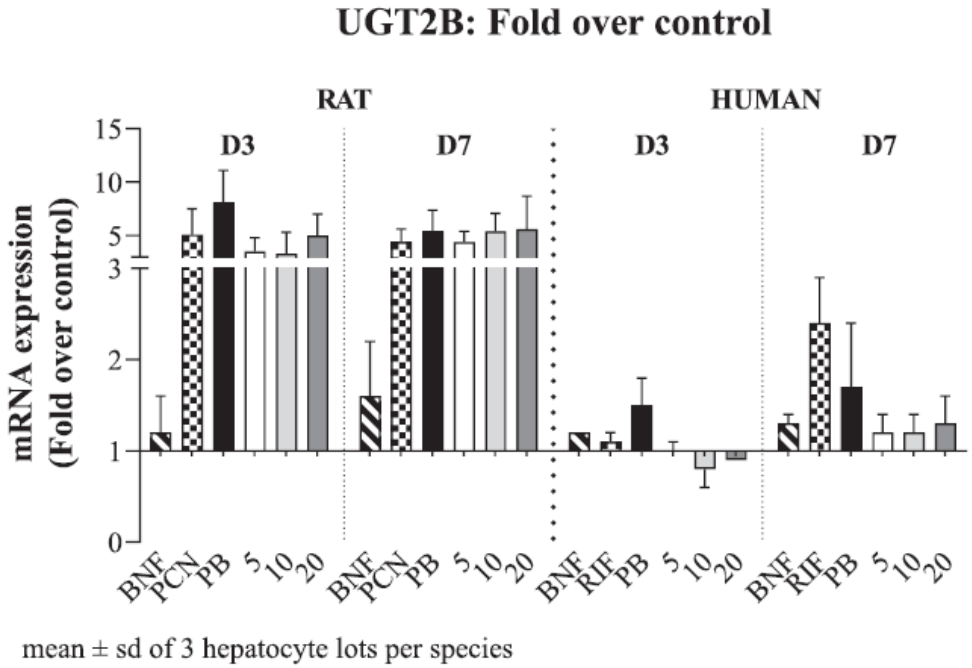
MIE …

Phase II Liver enzyme induction: Rat vs human hepatocyte responses

Human

MIE …





BNF – beta-Napthoflavone
PB – phenobarbinal
PCN – Pregnenolone carbonitrile
RIF - Rifampicin

Wiemann_2023

T4-glucuronidation activity – delta activities

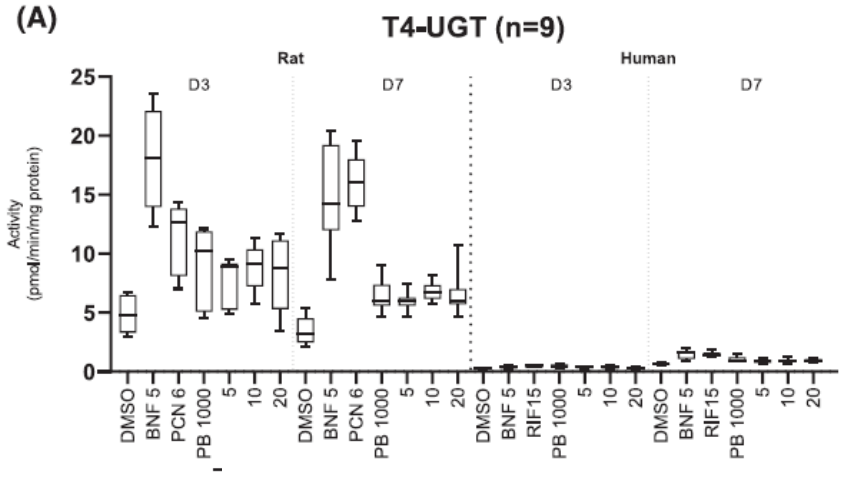
Rat

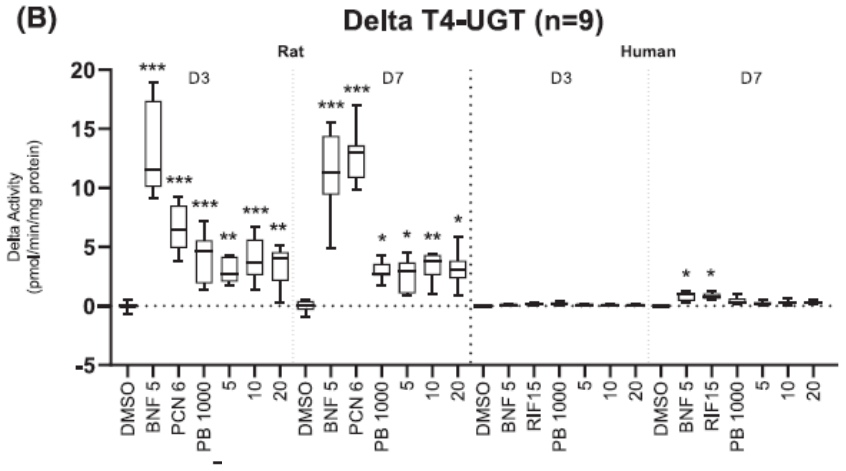
MIE …

Phase II Liver enzyme induction: Rat vs human hepatocyte responses

Human

MIE …





Delta activites (Relative activity increase compared with basal activities)

- Subtract mean DMSO control activity from each individual replicate activity of this lot

- Given for each individual hepatocyte culture (n = 9)

→ „Normalized“ species-specific increases

→ Results are given as statistically significant results

BNF – beta-Napthoflavone
PB – phenobarbinal
PCN – Pregnenolone carbonitrile
RIF - Rifampicin

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Comparative liver enzyme induction – historical controls

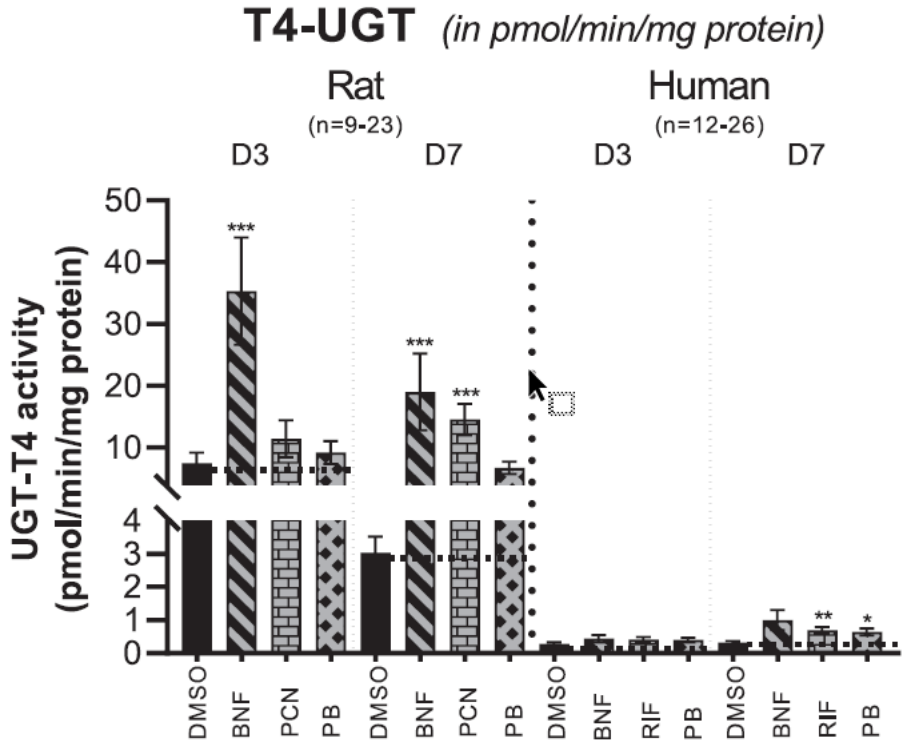


FIGURE 3 Historical control data for T4-UGT activity in primary rat and human hepatocytes. Values are a mean + standard error mean.

Wiemann_2023

Reference compound data of 28 studies are evaluated

Extent of Variability / what is acceptable

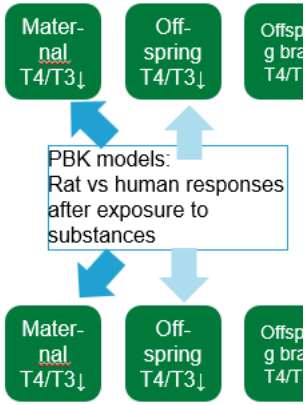
Reproducibility (same donor)

3 days vs 7 days timepoint

Evaluation criteria (delta activity; biologically relevant response)

Publication planned in Q3 2024

Thyroid hormone (action) modelling in various species



- GitHub - Open-Systems-Pharmacology/Thyroid-Hormones-PB-QSP-Model: Physiology-based systems pharmacology model of thyroid hormones regulation in rat and human
  - Bundle the activities started in the different Companies and CROs
  - Thyroid hormone modelling in adult plasma / offspring plasma
  - Thyroid hormone concentration modelling in offspring brain
  - Brain receptor occupancy modelling
  - QIVIVE
- Publication of a workshop ongoing – Seek for collaboration / approach alignment with regulators / academia)

Testing strategy using *in vitro* methods (I)

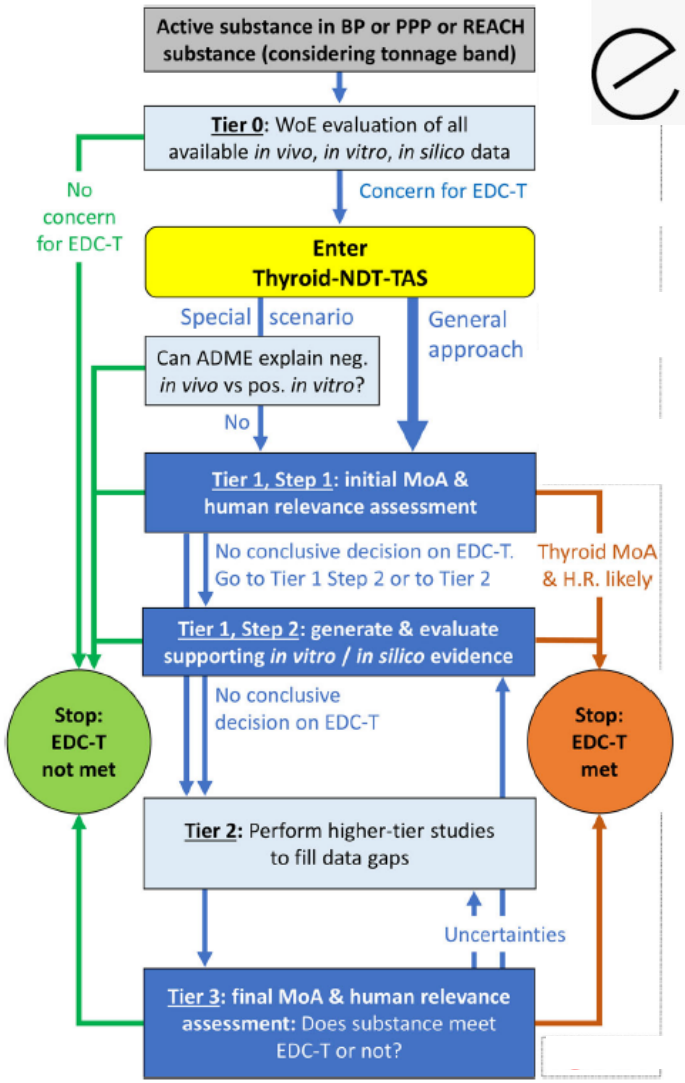


Figure 1. Overview of the ECETOC and CLE Thyroid-NDT-TAS (see Figures 2–6 for details). BP: biocidal product; EDC-T: endocrine disruptor criteria for the thyroid modality; MoA: mode-of-action; PPP: plant protection product; REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals;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

Thyroid histopathological or hormone effects

Either

In vivo follow up:
TH in offspring
TH in offspring
brain
DNT / EOGRTS

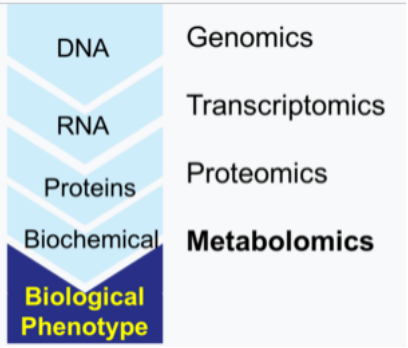
Or

Confirm mode of action
Exclude other MoAs
Prove non-human relevance

No evidence for thyroid hormone changes in humans → no concern for neurodevelopmental toxicity

Melching-Kollmuss_2023

Testing strategy using *in vitro* methods (II)



„Patterns of common metabolite changes of thyroid effects were established.“

„Metabolites separating **indirect** and **direct** thyroid effects were identified“

Liver enzyme inducers

TPO inhibitors

Montoya_2014

Concept

Indirect mechanisms

Liver enzyme inducers

Interacting with Serum binding proteins

Direct mechanisms

Iodine deficiency

NIS inhibitors

Adapt follow up testing strategy

Comparative liver enzyme induction

Species-specific THBP

P003

Complete the metabolome patterns and Adapt the testing strategy

Applicability of *in vitro* studies in context of endocrine disruption assessment / substance screening

Assay	Test Guideline	OECD Type	Modality	Comments / Application
ToxCast Model	n.a.		E, A, T, S, +++	ER, AR Bioactivity Models; thyroid and liver assays
Aromatase	OPPTS 890.1200	Level 2	Steroidogenesis	Inhibition of aromatase (Cyp 19) – one enzyme in steroid pathway
H295R assay	456	Level 2	Steroidogenesis	Maximum dose testing; evaluation criteria → New Test Guideline version 2023
ERTA / ARTA	455 / 458	*Level 2*	Estrogen/Androgen	
TPO	n.a.	*Level 2*	Thyroid	Assess activity at non-cytotoxic concentrations
NIS	n.a.	*Level 2*	Thyroid	
Dio 1	n.a.	*Level 2*	Thyroid	In vivo Phenotype for Dio 1?
Interaction with serum binding proteins (TTR)	n.a.	*Level 2*	Thyroid	Thyroid hormones are transported bound to serum binding proteins (albumin and TTR in rats, TTR and TBG in humans) Potential to study species differences **(P003)**
In vivo to *in vitro* extrapolation IVIVE	n.a.			Relevance of *in vitro* results for *in vivo* concentrations Maximum dose used in *in vitro* testing
Comparative liver enzyme induction			Liver – thyroid	Species difference in Phase II liver enzyme induction (rat vs human hepatocytes)

TPO – Thyroid peroxidase
NIS – Sodium Iodid Symporter
Dio - Deiodinase

Take home messages

- The Endocrine Disruption assessment for pesticide active ingredients is built on adverse effects
- In vitro ED studies are done in case of insufficient data sets or to confirm plausible links (qualitatively)
- Non-animal and integrated approaches are used for screening
  - QSAR for E, A, T
  - ER, AR, steroidogenesis for EAS modality
  - T modality: Early changes in short term rat studies & read-across (metabolome); targeted T mechanistic studies, combine with ecotox studies (e.g. XETA)
- IVIVE considerations are rarely used / accepted
- Suggest to built concepts to integrate quantitative aspects / threshold considerations / species differences – for use in regulatory assessments

Toxicology
safe use — safe people

BASF
We create chemistry

We create chemistry