

# A Testing Strategy for the Identification of mammalian Endocrine Disruptors with particular focus on steroids

Tzutzuy Ramirez, Robert Landsiedel, Susanne Kolle, Hennicke Kamp, Bennard van Ravenzwaay

# Our understanding of endocrine disruptors

**Weybridge** 1996:

“An endocrine disrupter is an exogenous substance that causes **adverse health effects** in an intact organism, or its progeny, **secondary (consequent) to changes in endocrine function**”.

**WHO** 2002:

“An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes **adverse** effects in an **intact organism**, or its progeny, or (sub)populations.”



„Endocrine disruption“ is potentially a cut-off criterion without any risk assessment

# 1989: The beginning

## Study case: Vinclozolin

### Anti-androgenic effects in:

- prenatal developmental toxicity study: reduced ano-genital distance

### 2-generation study:

- a syndrome of changes resulting in a feminization of male rat offspring
  - reduced prostate & seminal vesicle weight and activity
  - hypospadias
  - vaginal pouch
  - delayed / incomplete testicular descent

can be explained by several modes of action:

- 1) Reduction of hormone synthesis
- 2) Increased breakdown of hormones
- 3) Receptor block

# Overview of the Tier-1 EDSP battery

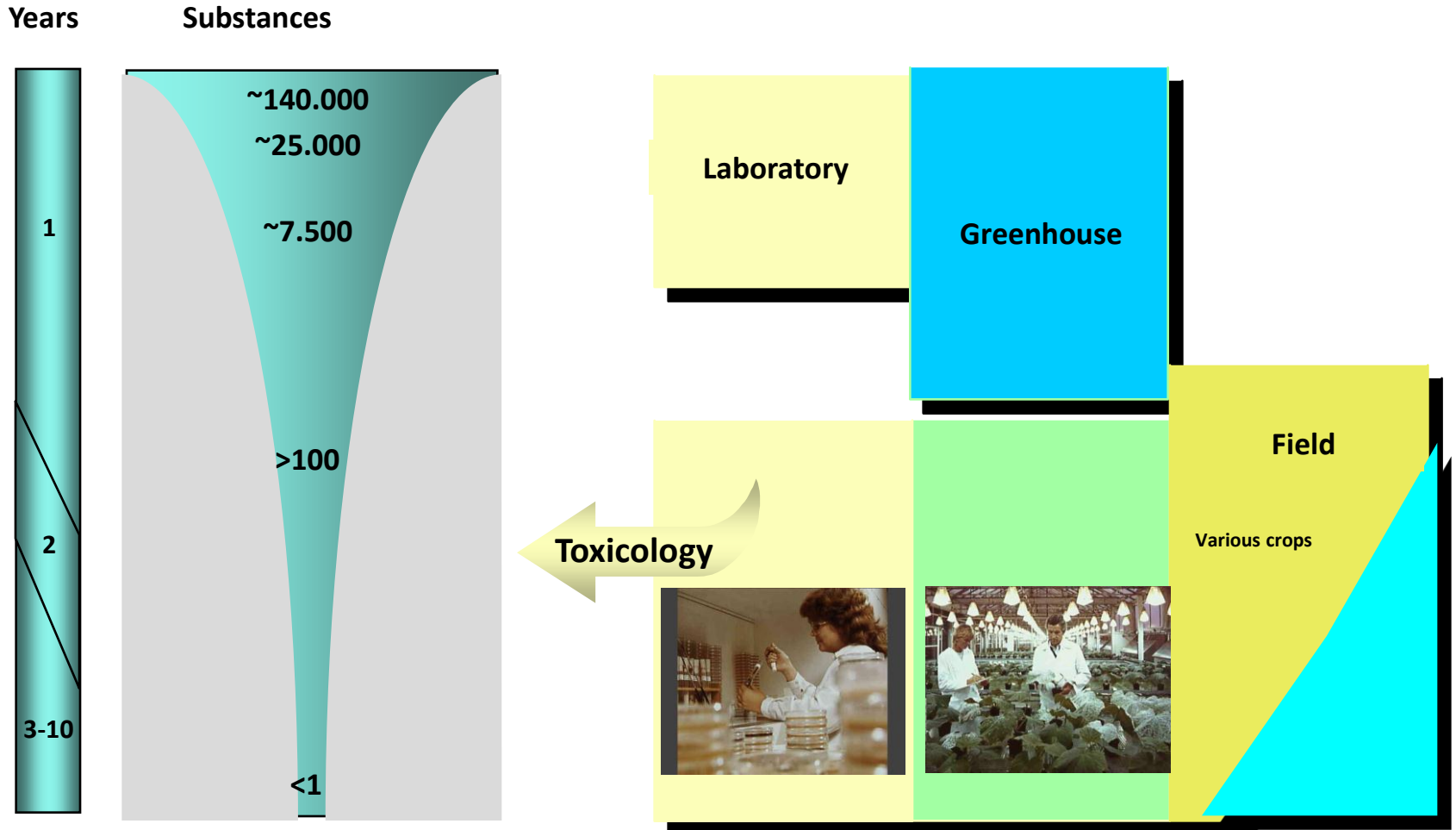
## *In vitro*

- Androgen receptor (AR) binding -rat prostate cytosol
- Estrogen receptor (ER) binding - rat uterine cytosol
- Aromatase - Human recombinant aromatase
- human estrogen receptor a transcriptional activation in HeLa-9903 line (OECD 455)
- Steroidogenesis in H295R line (OECD 456)

## *In vivo*

- Uterotrophic (rat) (OECD 440)
- Hershberger (rat) (OECD 441)
- Pubertal female (rat)
- Pubertal male (rat)
- Amphibian metamorphosis (frog)
- Fish short-term reproduction

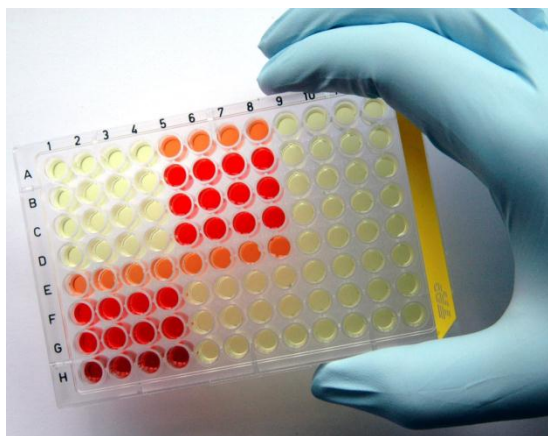
# Development and Registration of Agrochemicals: Screening and Selection



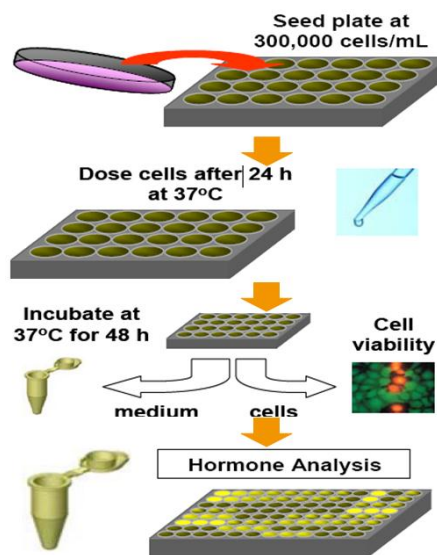
# An integrated strategy for detection of Endocrine Modes of Action under 3Rs TIER 1

## In vitro

### Receptor dependent



### Hormone synthesis



## Refined in vivo

### Metabolic profiling

MetaMap\_TOX - Microsoft Internet Explorer

Selected Metabolites: Find Compounds

Direction	Method	Grade	Metabolite	th7	th14	th28	th7
up	GC polar	SO	Metabolite 1	1.27	1.37	1.38	1.49
up	GC polar	SO	Metabolite 2	1.44	1.39	1.45	1.46
up	GC polar	SO	Metabolite 3	1.15	1.22	1.22	1.16
down	GC polar	SO	Metabolite 4	0.7	0.74	1.09	0.5
down	GC polar	SO	Metabolite 5	0.78	0.69	0.76	0.78
down	LC polar	SO	Metabolite 6	0.95	0.68	0.31	0.6
down	LC lipid	SO	Metabolite 7	0.83	0.89	0.91	0.71
down	LC lipid	SO	Metabolite 8	0.18	0.3	0.22	0.24
down	LC lipid	SO	Metabolite 9	0.21	0.35	0.29	0.24
down	LC lipid	SO	Metabolite 10	0.12	0.27	0.24	0.2
down	LC lipid	SO	Metabolite 11	0.21	0.41	0.42	0.33
down	LC lipid	SO	Metabolite 12	0.19	0.32	0.38	0.32
down	LC lipid	SO	Metabolite 13	0.19	0.62	0.39	0.2
down	LC lipid	SO	Metabolite 14	0.15	0.34	0.37	0.15
down	LC lipid	SO	Metabolite 15	0.19	0.26	0.29	0.11
down	LC lipid	SO	Metabolite 16	0.46	0.28	0.44	0.18
up	LC lipid	SO	Metabolite 17	1.05	3.16	1.98	1.46
down	LC lipid	SO	Metabolite 18	0.39	0.51	0.55	0.38
down	LC lipid	SO	Metabolite 19	0.34	0.42	0.48	0.3
down	LC lipid	SO	Metabolite 20	0.24	0.17	0.31	0.29
down	LC lipid	SO	Metabolite 21	0.25	0.48	0.38	0.3
down	LC lipid	SO	Metabolite 22	0.28	0.42	0.5	0.38
up	LC lipid	SO	Metabolite 23	2.36	5.95	3.25	1.35
up	LC lipid	SO	Metabolite 24	1.52	2.65	1.52	1.13
down	LC catecholamine	SO	Metabolite 25	1.03	0.67	0.56	0.63

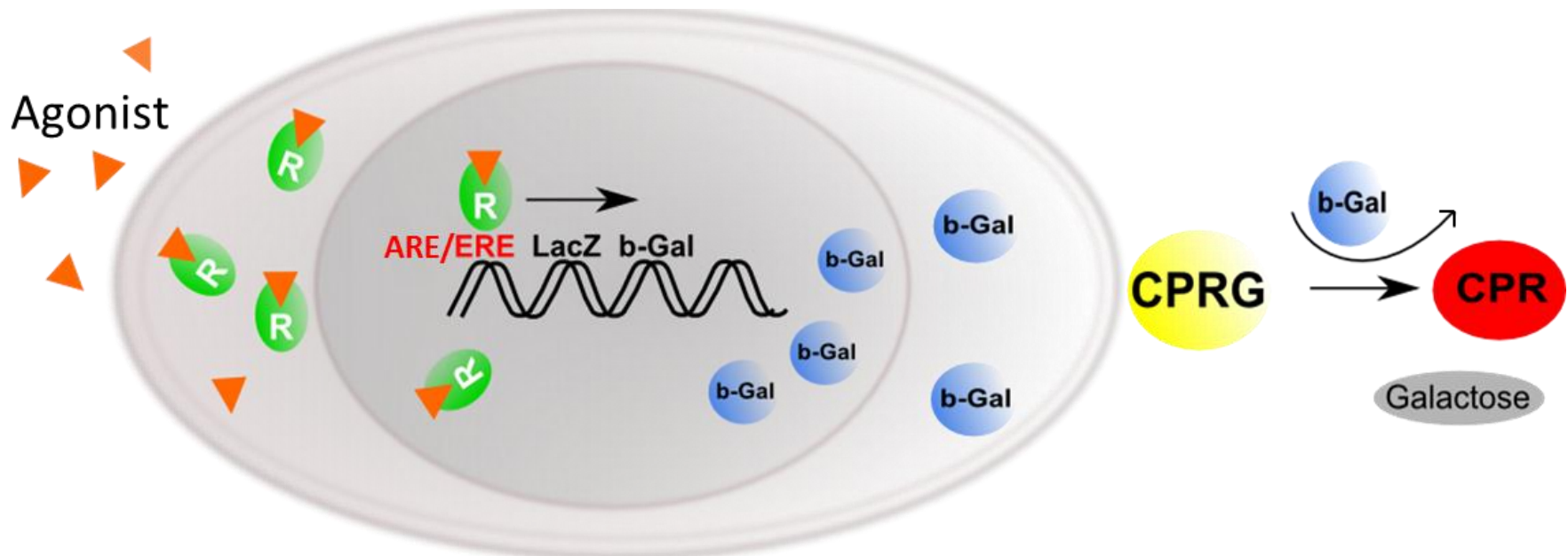
Selected Metabolites: Find Compounds

logout

1 (C) Metanomics 2005-2008, Version 3.1 (3912)   
 [Home](#)

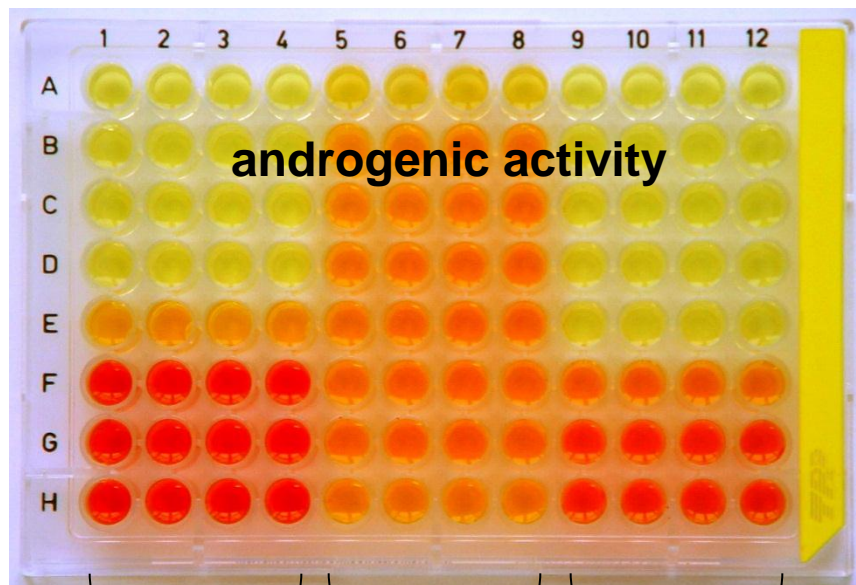
## Assessment for Mammalian Endocrine Activity

# Tier 1: Yeast Estrogen/ Androgen Screening Assays (YES/YAS)



- Yeast estrogen/ androgen screening assay
- Recombinant yeast strains expressing **human ER** or **AR** and reporter gene.
- **Agonistic** and **antagonistic** effects measurable
- Determination of effect by **reporter assay**

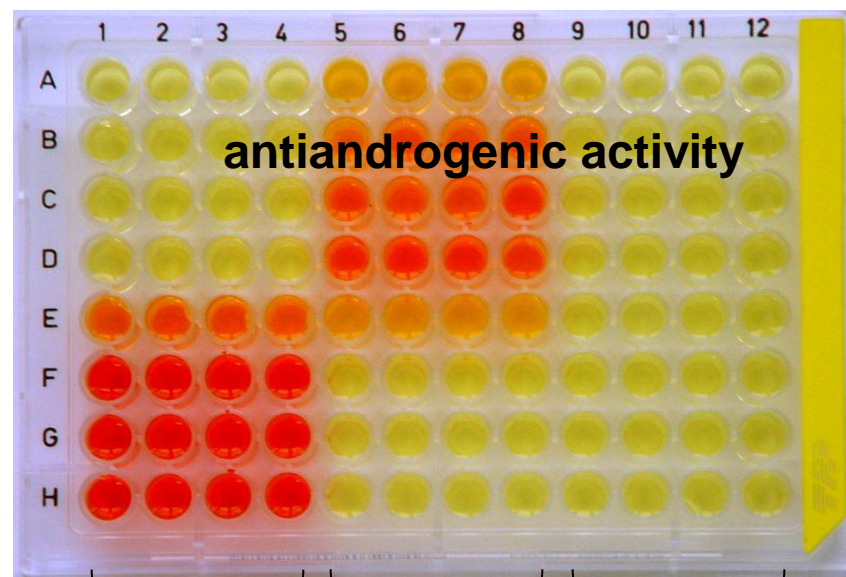
# Tier 1: Yeast Estrogen/ Androgen Screening Assays (YES/YAS)



Positive  
Control

Test subs  
+ PC

Test subs



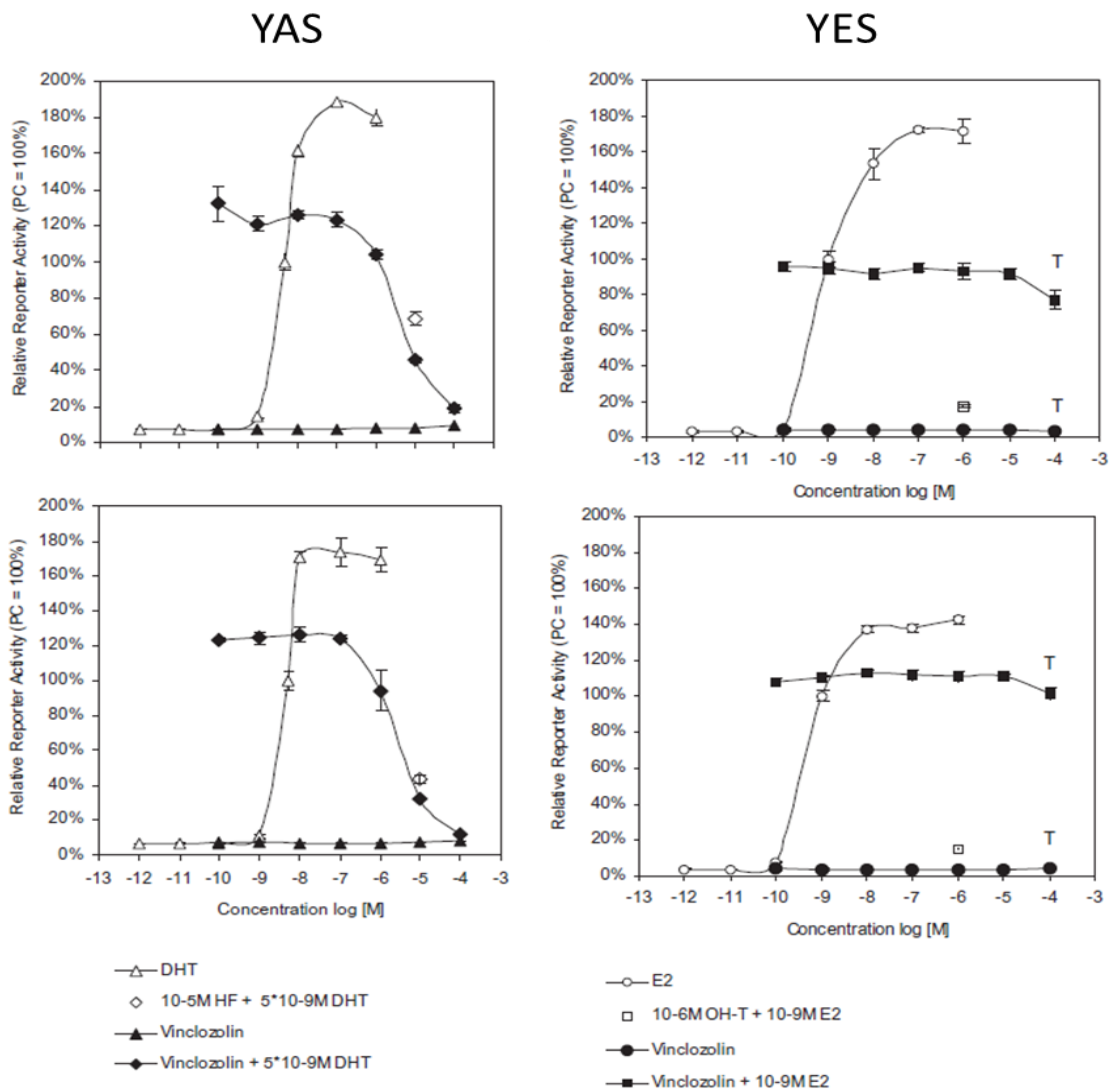
Positive  
Control

Test subs  
+ PC

Test subs



# Tier 1: Yeast Estrogen/ Androgen Screening Assays (YES/YAS)



# In house validation of the YES/YAS with 105 substances

Androgens/antiandrogens: reported activity and test results.<sup>a,b</sup>

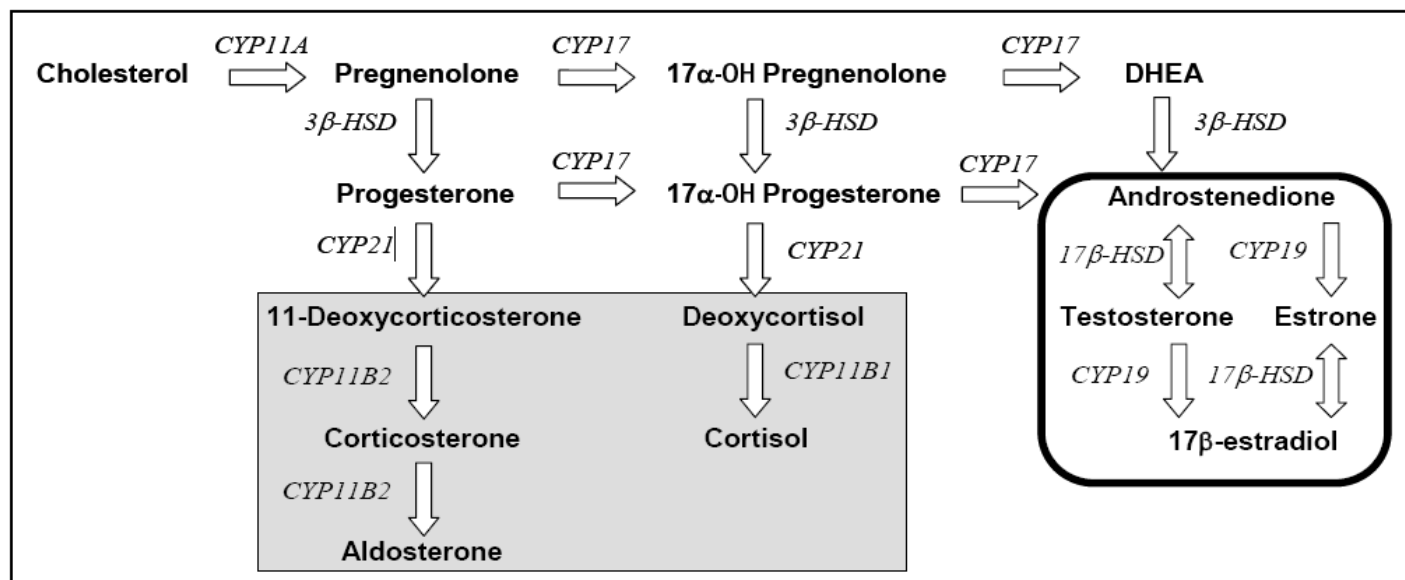
Name	CAS	Class	YES/YAS Result				Literature			
			E	AE	A	AA	E	AE	A	AA
1a,25-Dihydroxyvitamin D3 (Calcitriol)	32222-06-3	Other chemicals	-	+	-	+	o	+	o	+
2,4-Dihydroxybenzo-phenone	131-56-6	Antiandrogen	+	<sup>d</sup> [-]	-	+	+	+	-	+
4,4'-Biphenol	92-88-6	Antiandrogen	+	-	(+)	+	+	+	-	+
4,4'-DDD (Dichlorodiphenyl-dichlorethane)	72-54-8	Antiandrogen	-	-	-	+	o	o	o	+
4-Androstene-3,17-dione	63-05-8	Other chemicals	-	+	+	-	-	o	+	-
4- <i>n</i> -Octylphenol	1806-26-4	Other chemicals	-	-	-	(+)	-	-	-	+
4- <i>tert</i> -Octylphenol	140-66-9	Other chemicals	+	<sup>d</sup> [-]	-	+	+	+	-	+
6 $\alpha$ -Methyl-17 $\alpha$ hydroxy-progesterone(Medroxyprogesterone)	520-85-4	Androgen	-	+	+	-	-	+	-	+
Apigenin	520-36-5	Estrogen	(+)	-	-	-	+	-	o	+
Benzophenone	119-61-9	Antiandrogen	(+)	(+)	-	+	(+)	o	o	+
Benzylbutylphthalate	85-68-7	Antiandrogen	(+)	-	-	+	+	-	-	+
Bicalutamide	90357-06-5	Antiandrogen	-	+	+	<sup>d</sup> [-]	+	o	+	+
Bis(2-ethylhexyl) phthalate (DEHP)	117-81-7	Other chemicals	-	-	-	-	o	o	+	-
Bisphenol A	80-05-7	Estrogen	+	-	-	+	+	-	-	+
Bisphenol A-dimethacrylate	3253-39-2	Estrogen	(+)	-	-	-	+	-	o	+
Corticosterone	50-22-6	Other chemicals	-	(+)	-	+	-	+	-	+
Cyproteronacetate	427-51-0	Antiandrogen	-	+	+	(+)	-	o	+	+
Dibenz[a,h]anthracene	53-70-3	Other chemicals	-	-	-	-	-	+	+	-
<sup>c</sup> Dibutylphthalate	84-74-2	Other chemicals	+	-	-	+	-	-	-	+
Dihydrotestosterone	521-18-6	Androgen	+	-	+	-	+	o	+	-
Dihydroxymethoxychlor (HPTE)	2971-36-0	Estrogen	+	-	-	+	+	-	-	+
Diisononylphthalate (DINP)	28553-12-068515-48-0	Other chemicals	-	-	-	-	-	-	-	+
Di- <i>n</i> -amylphthalatedipentyl phthalate	131-18-0	Antiandrogen	-	+	-	+	-	-	-	+
Estrone	53-16-7	Estrogen	+	-	+	-	+	-	+	-
Ethinylestradiol	57-63-6	Estrogen	+	-	(+)	+	+	o	-	+
Fenarimol	60168-88-9	Estrogen	+	-	-	<sup>d</sup> [-]	+	-	o	+

**Detection of estrogenic compounds with accuracy of 87%, antiestrogenic with 90%, androgenic with 95% and antiandrogenic with 85%.**

(Kolle, et al., *Toxicol. In Vitro* 24(7), 2030-2040).

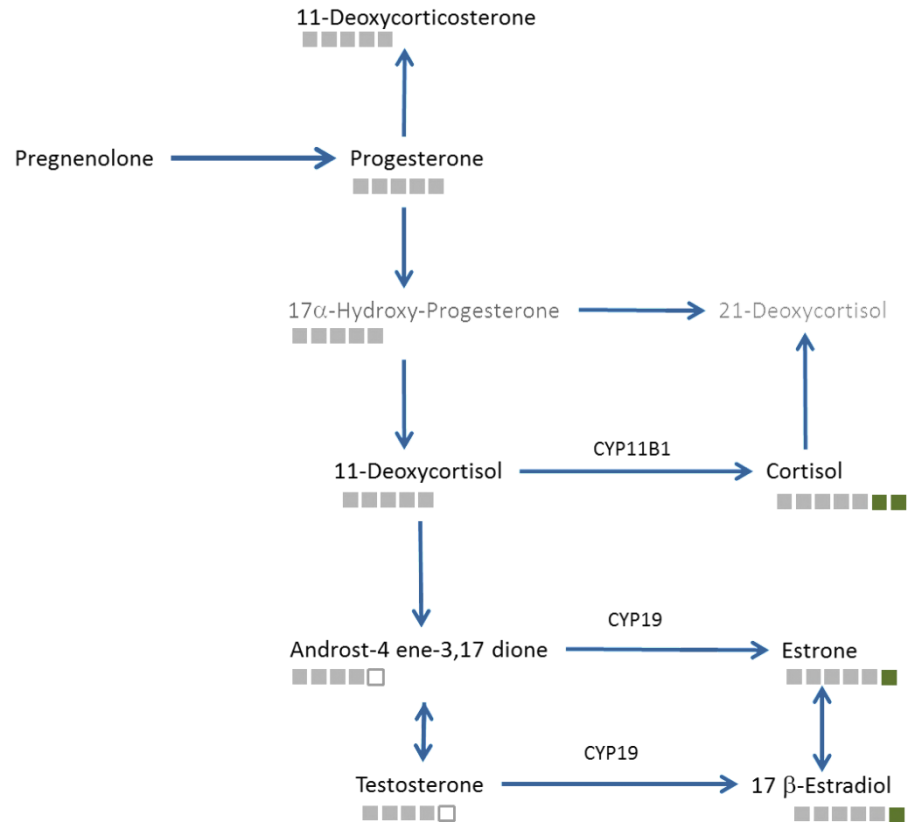
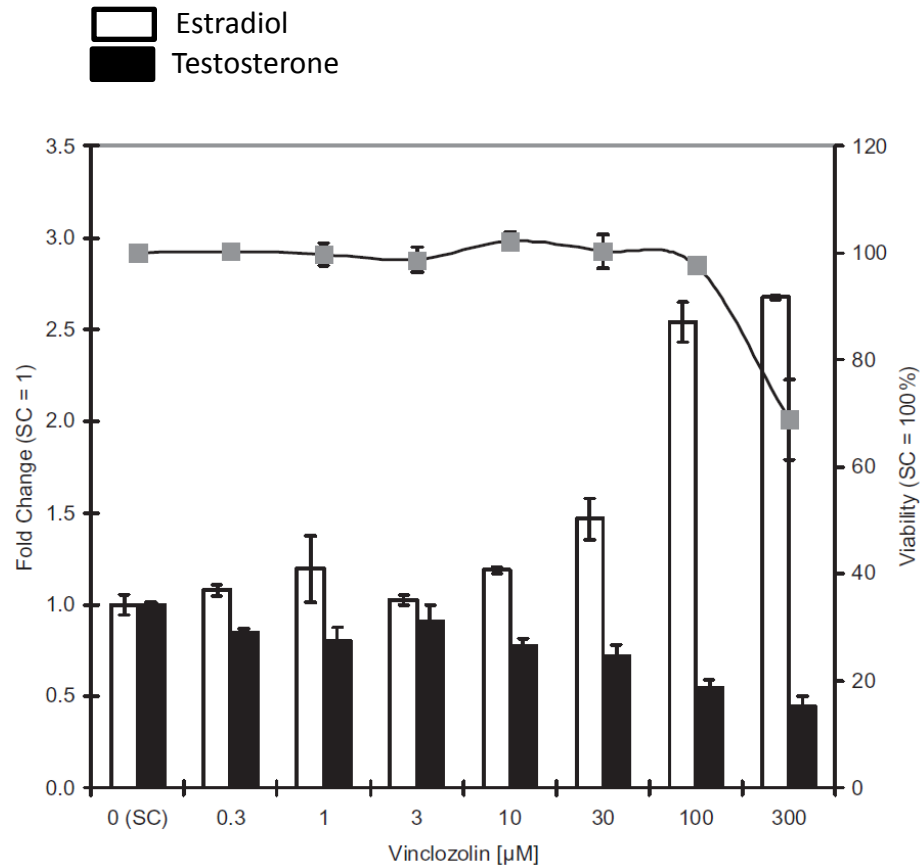
# Tier 1: Steroidogenesis assay OECD TG 456 and OPPTS 890.1550

- The objective of the steroidogenic screen assay is to detect any substance that would **disrupt estrogen and/or androgen gonadal steroid hormone production.**



**Figure 3.1:** Steroidogenic pathway in H295R cells. Enzymes are in italics, hormones are bolded and arrows indicate the direction of synthesis. Gray background indicates corticosteroid pathways/products. Sex steroid pathways/products are circled. CYP = cytochrome P450; HSD = hydroxysteroid hydrogenase.

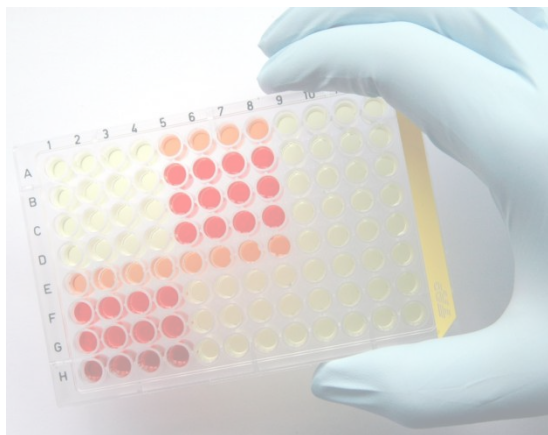
# Tier 1: Effects of vinclozolin on the levels of estradiol and testosterone



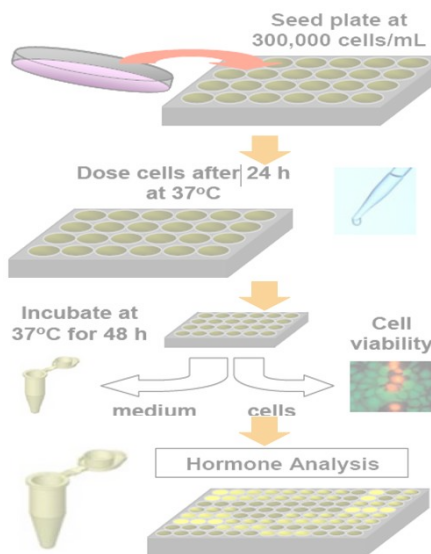
# An integrated strategy for detection of Endocrine Modes of Action under 3Rs TIER 2

*In vitro*

Receptor dependent



Hormone synthesis



Refined *in vivo*

Metabolic profiling

MetaMap\_TOX - Microsoft Internet Explorer

Selected Metabolites (Find Compounds)

Direction	Method	Grade	Metabolite	17-alpha-Ethinylestradiol (MOA13) fb7	fb14	fb28	fb7
up	GC polar	SO	Metabolite 1	1.22	1.37	1.38	1.49
up	GC polar	SO	Metabolite 2	1.44	1.39	1.45	1.46
up	GC polar	SO	Metabolite 3	1.15	1.22	1.22	1.18
down	GC polar	SO	Metabolite 4	0.7	0.74	1.09	0.5
down	GC polar	SO	Metabolite 5	0.78	0.69	0.76	0.78
down	LC polar	SO	Metabolite 6	0.95	0.68	0.31	0.6
down	LC lipid	SO	Metabolite 7	0.83	0.89	0.91	0.71
down	LC lipid	SO	Metabolite 8	0.18	0.3	0.22	0.24
down	LC lipid	SO	Metabolite 9	0.21	0.35	0.29	0.24
down	LC lipid	SO	Metabolite 10	0.12	0.27	0.24	0.2
down	LC lipid	SO	Metabolite 11	0.21	0.41	0.42	0.33
down	LC lipid	SO	Metabolite 12	0.19	0.32	0.38	0.32
down	LC lipid	SO	Metabolite 13	0.19	0.62	0.39	0.2
down	LC lipid	SO	Metabolite 14	0.15	0.34	0.37	0.15
down	LC lipid	SO	Metabolite 15	0.19	0.26	0.29	0.11
down	LC lipid	SO	Metabolite 16	0.46	0.28	0.44	0.18
up	LC lipid	SO	Metabolite 17	1.85	3.16	1.38	1.46
down	LC lipid	SO	Metabolite 18	0.39	0.51	0.65	0.38
down	LC lipid	SO	Metabolite 19	0.34	0.42	0.40	0.3
down	LC lipid	SO	Metabolite 20	0.24	0.17	0.31	0.29
down	LC lipid	SO	Metabolite 21	0.25	0.48	0.38	0.3
down	LC lipid	SO	Metabolite 22	0.28	0.42	0.5	0.38
up	LC lipid	SO	Metabolite 23	2.35	5.85	3.25	1.38
up	LC lipid	SO	Metabolite 24	1.52	2.65	1.52	1.13
down	LC catecholamine	SO	Metabolite 25	1.03	0.67	0.66	0.63

Selected Metabolites (Find Compounds)

logout

© Metanomics 2005-2008, Version 3.1 (3912) MetaMap

Assessment for Mammalian Endocrine Activity

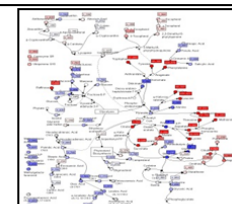
# Tier 2: *In vivo* 28 day study combined with metabolomics analysis

- Confirmation of *in vitro* findings (potency / NOAEL)
- Detection of compounds that only act *in vivo* (e.g. metabolism specific)
- OECD 407 (28 day rat study)
- Estrus cycle analysis
- Pathology of endocrine organs
- **Metabolome analysis**



## Target Metabolites

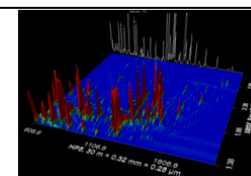
- **Currently 300** per sample matrix
- Structure ID established
- Sensitive detection at actual levels
- Absolute quantitation possible



## Metabolite Structure Identification

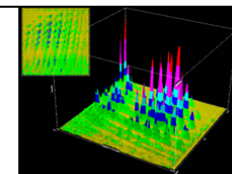
## Known-Unknown Metabolites

- Additional ca. **500** compounds
- Metabolite ID not final but indexed
- Relative concentrations reliably measured



## Total Metabolome Signature

- **Up to 9.000** analyte signals per sample
- Directly used in Data Mining
- Based on original 3D MS data sets



# Analysis of effects of 14 compounds tested *in vitro* and *in vivo*

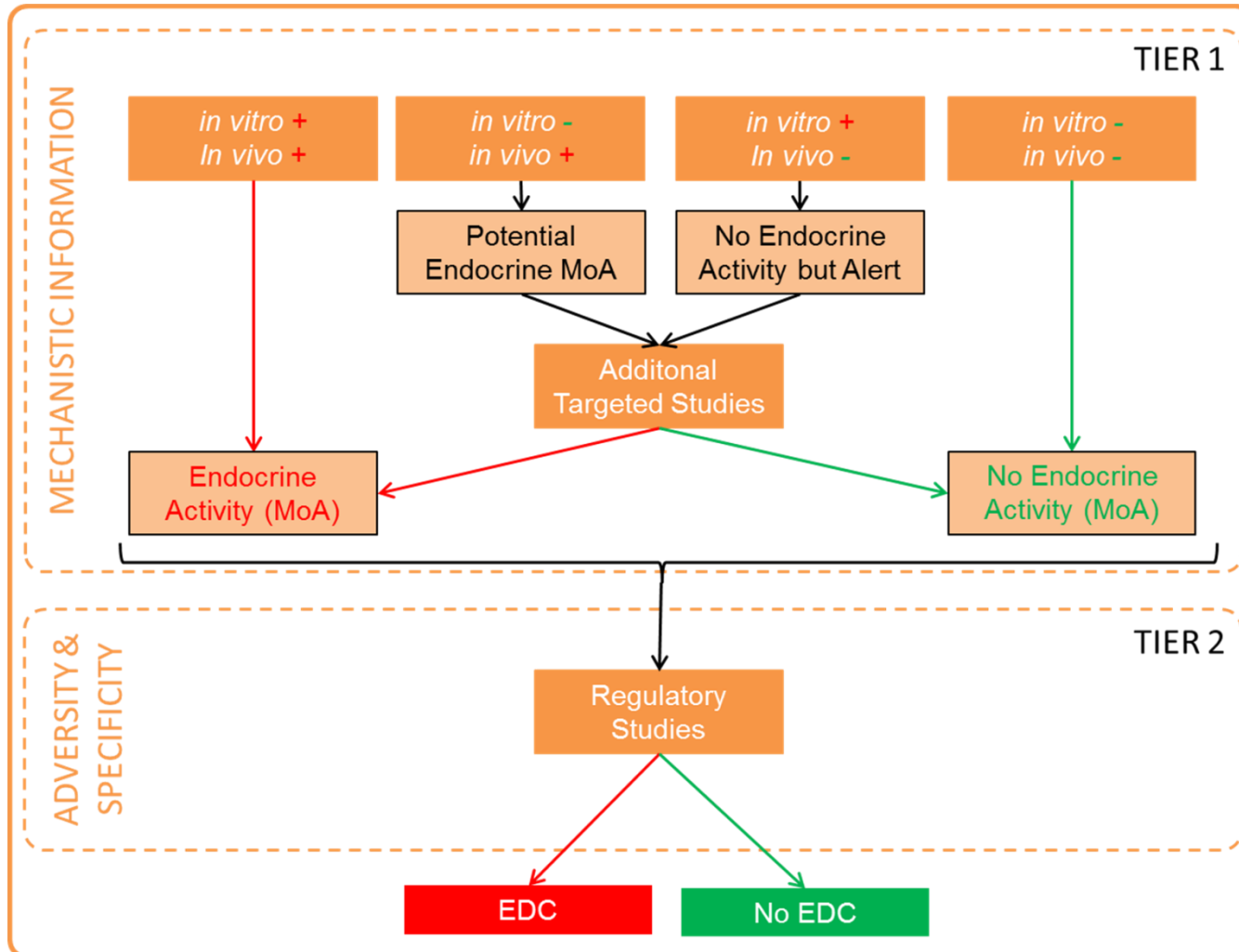
Substance	CAS No.	YES	YAS	H295R E <sub>2</sub> levels	H295R T levels	Metabolome analysis <sup>e</sup>
Bisphenol A	80-05-7	E - <sub>a</sub>	- AA	↑	↓	+/-
Caffeine	58-08-2	- -	- -	- <sub>b</sub>	- <sub>b</sub>	-
Colchicine	64-86-8	- - <sub>a</sub>	- -	- <sub>b</sub>	- <sub>b</sub>	-

■ Of the nine known EDCs all substances were determined to exert receptor mediated and/or biosynthesis related mechanisms.

■ **The effects were confirmed by *in vivo* metabolome analysis** in the OECD TG 407 study.

testosterone	80-09-7	AE	- <sub>a</sub>	↓	↓	A E
Tamoxifen	10540-29-1	E AE	- (AA)	↓	↓	A adrenal steroid synthesis inhibition <sup>f</sup>
Trenbolone	10161-33-8	E -	A - <sub>a</sub>	- <sub>d</sub>	- <sub>d</sub>	E A
Vinclozolin	50471-44-8	- - <sub>a</sub>	- AA	↑	↓	AA adrenal steroid synthesis inhibition <sup>f</sup>
Zearalenone	17924-92-4	E - <sub>a</sub>	- AA	↑	↑	E

# Assessment of mammalian endocrine effects using a combination of *in vitro* assays and *in vivo* assays

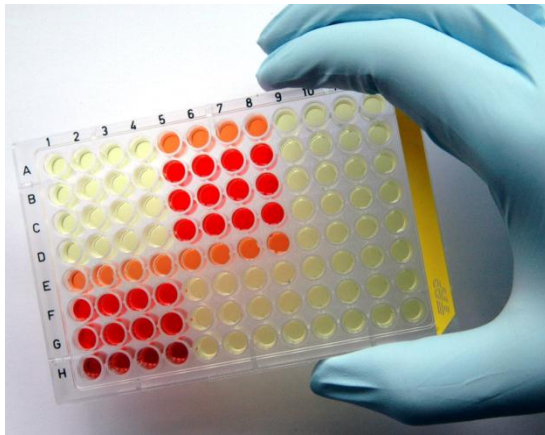




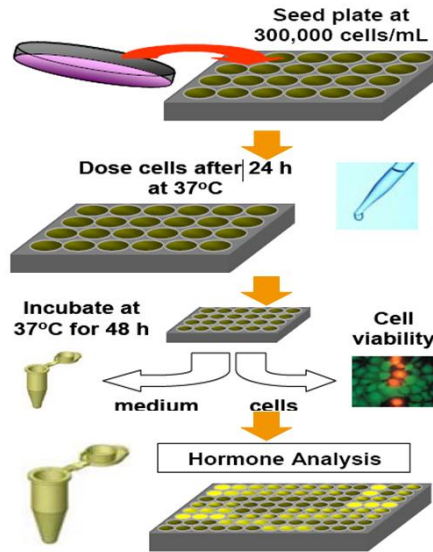
# Tiered Testing for Identification of Endocrine Disruption

## In vitro

### Receptor dependent



### Hormone synthesis



## Refined in vivo

### Metabolic profiling

Direction	Method	Grade	Metabolite	17- $\alpha$ -Ethinylestradiol (MOA13)	17-Ethinylestradiol (MOA13)		
up	GC polar	SO	Metabolite 1	1.22	1.37	1.38	1.49
up	GC polar	SO	Metabolite 2	1.44	1.39	1.45	1.46
up	GC polar	SO	Metabolite 3	1.15	1.22	1.22	1.18
down	GC polar	SO	Metabolite 4	0.7	0.74	1.09	0.5
down	GC polar	SO	Metabolite 5	0.78	0.69	0.76	0.78
down	LC lipid	SO	Metabolite 6	0.95	0.68	0.31	0.6
down	LC lipid	SO	Metabolite 7	0.83	0.89	0.91	0.71
down	LC lipid	SO	Metabolite 8	0.18	0.3	0.22	0.24
down	LC lipid	SO	Metabolite 9	0.21	0.35	0.29	0.24
down	LC lipid	SO	Metabolite 10	0.12	0.27	0.24	0.2
down	LC lipid	SO	Metabolite 11	0.21	0.41	0.42	0.33
down	LC lipid	SO	Metabolite 12	0.19	0.32	0.38	0.32
down	LC lipid	SO	Metabolite 13	0.19	0.62	0.39	0.2
down	LC lipid	SO	Metabolite 14	0.15	0.34	0.37	0.15
down	LC lipid	SO	Metabolite 15	0.19	0.26	0.29	0.11
down	LC lipid	SO	Metabolite 16	0.46	0.28	0.44	0.18
up	LC lipid	SO	Metabolite 17	1.65	3.16	1.38	1.46
down	LC lipid	SO	Metabolite 18	0.39	0.51	0.55	0.38
down	LC lipid	SO	Metabolite 19	0.34	0.42	0.48	0.3
down	LC lipid	SO	Metabolite 20	0.24	0.17	0.31	0.29
down	LC lipid	SO	Metabolite 21	0.25	0.48	0.38	0.3
down	LC lipid	SO	Metabolite 22	0.28	0.42	0.5	0.38
up	LC lipid	SO	Metabolite 23	2.35	5.65	3.25	1.38
up	LC lipid	SO	Metabolite 24	1.52	2.65	1.52	1.13
down	LC catecholamine	SO	Metabolite 25	1.03	0.67	0.56	0.63

Retrospective analysis of in house data showed that with the proposed testing strategy additional *in vivo* studies **Hershberger assay** (OECD 441), **uterotrophic assay** (OECD 440), **pubertal assays** are not needed.

# Thank you

- **Laboratory of Development of Alternative Methods**
- **Laboratory of Applied Alternative Methods**
- **BASF Experimental Toxicology and Ecology**
- **Metanomics GmbH**

