Toxicity profiling and effect-based safety assessment of chemicals and chemical mixtures as present in plastic leachates

Bart van der Burg – BioDetection Systems BV
Department of Innovation

bart.van.der.burg@bds.nl

Food packaging: safety concerns

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- **Compounds of concern:**
- **-> Bisphenol A**
- **-> Phthalates**
- **… but there may be more!**

Current legislation for food contact materials

- **Known compounds: specific migration limits (SML) in mg/kg food**

  Relevant examples:

Compound	SML (mg/kg food)
4-Cumylphenol	0.05
Benzophenone	0.6
Bisphenol A	0.6
Diisodecylphthalate	9
Butylbenzylphthalate	30

- **Unknown compounds (NIAS): overall migration limit (OML) of 60mg/kg food or 10mg/dm2 food packaging material**

Current situation: safety testing inadequate

- Leachates:
- a **complex mixture** of chemicals, metabolites and degradation products

- Current (chemical) analysis: based on monitoring **known compounds**
- Fails to detect **unknown** chemicals present in leachates: Threshold of Toxicological Concern approach, works only for relatively low potency compounds

Is this a problem?

Example: If a compound with the estrogenic potency of estradiol would leach into food at 60mg/kg food, this would equal **>300 Oral Contraceptive (OC) pills** per kg food!

Compound	OC-equivalents at 60mg/kg
17b-Estradiol	317
Zearalenone	16
4-n-amylphenol	3
Genistein	0.1
Bisphenol A	0.02
4-tert-octylphenol	0.02
4-cumylphenol	0.01
Daidzein	0.01
Bisphenol F	0.004

Problems risk assessment of mixtures

- No satisfactory risk assessment mixtures using chemical analytics
- TTC concept not suitable for high potency toxicants
- No reliable in silico methods for mixtures
- But……bioassays do give valuable information!

To avoid occurence of unknown high potency toxicants bioassays can be used, e.g. for endocrine disrupting compounds, dioxins and genotoxic agents

ERalpha CALUX is sensitive enough to detect at SMLs

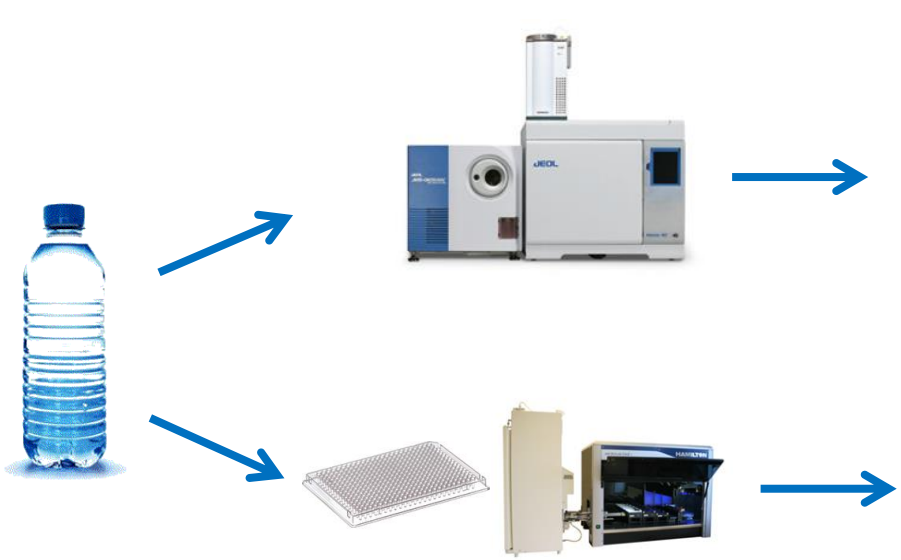
The Eralpha CALUX limit of detection (LOD) is lower than the specific migration limits (SML) set for the following compounds:

Compound	SML (mg/kg food)	ERa CALUX LOD (mg/kg food)
4-cumylphenol	0.05	0.01
Benzophenone	0.6	0.6
Bisphenol A	0.6	0.01
Diisodecylphthalate	9	9
Butylbenzylphthalate	30	0.05
17b-Estradiol	0.5/3.8E-06*	4.3E-07

*trigger value for water (mg/L)

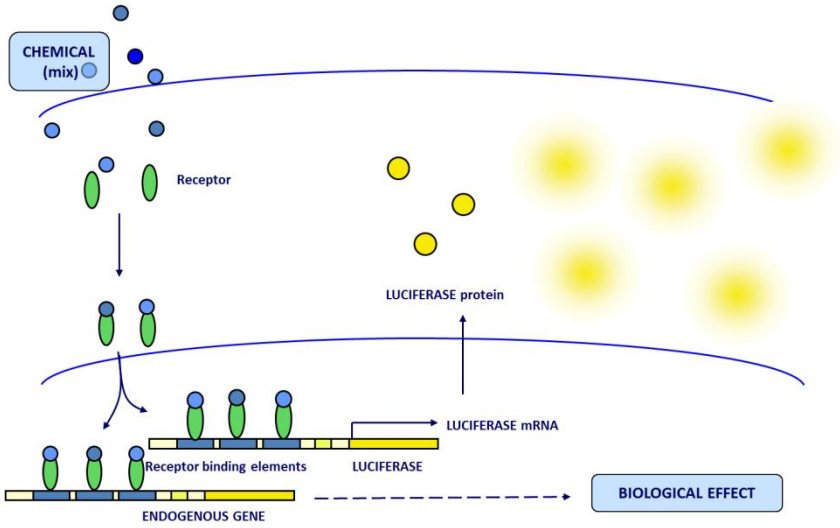
The Solution: effect-based monitoring

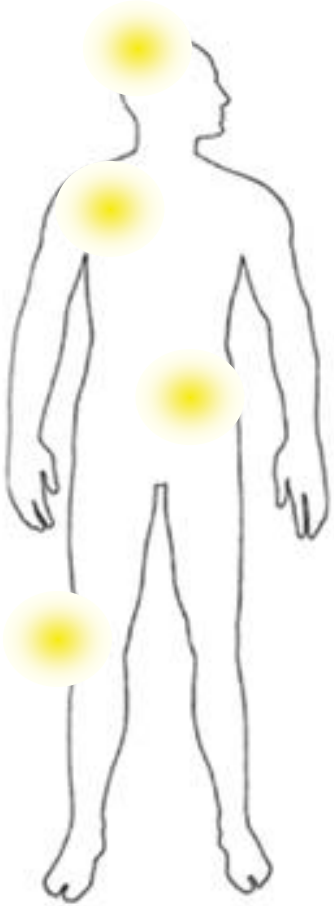
Chemical analysis: measuring the tip of the iceberg



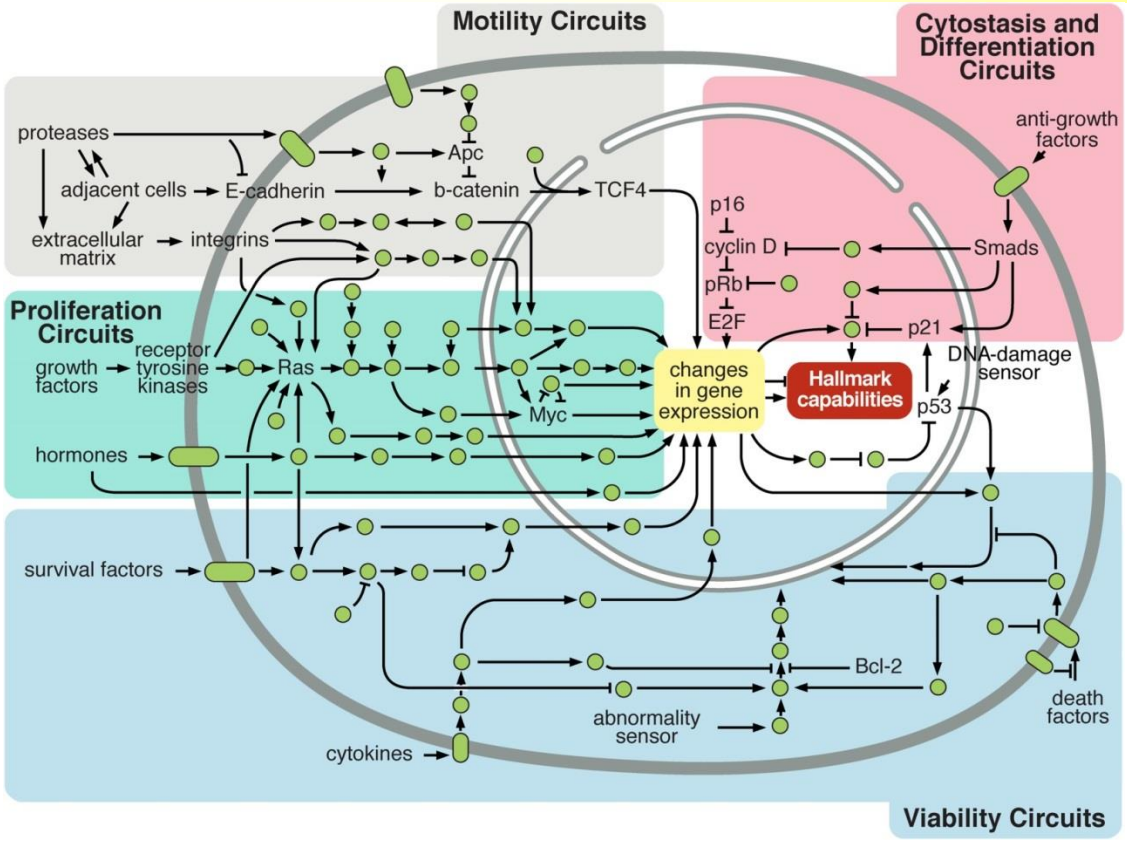
- Effect-based monitoring: measuring all **active** chemicals, including “unknowns”

Pathway-based analysis for rapid hazard identification





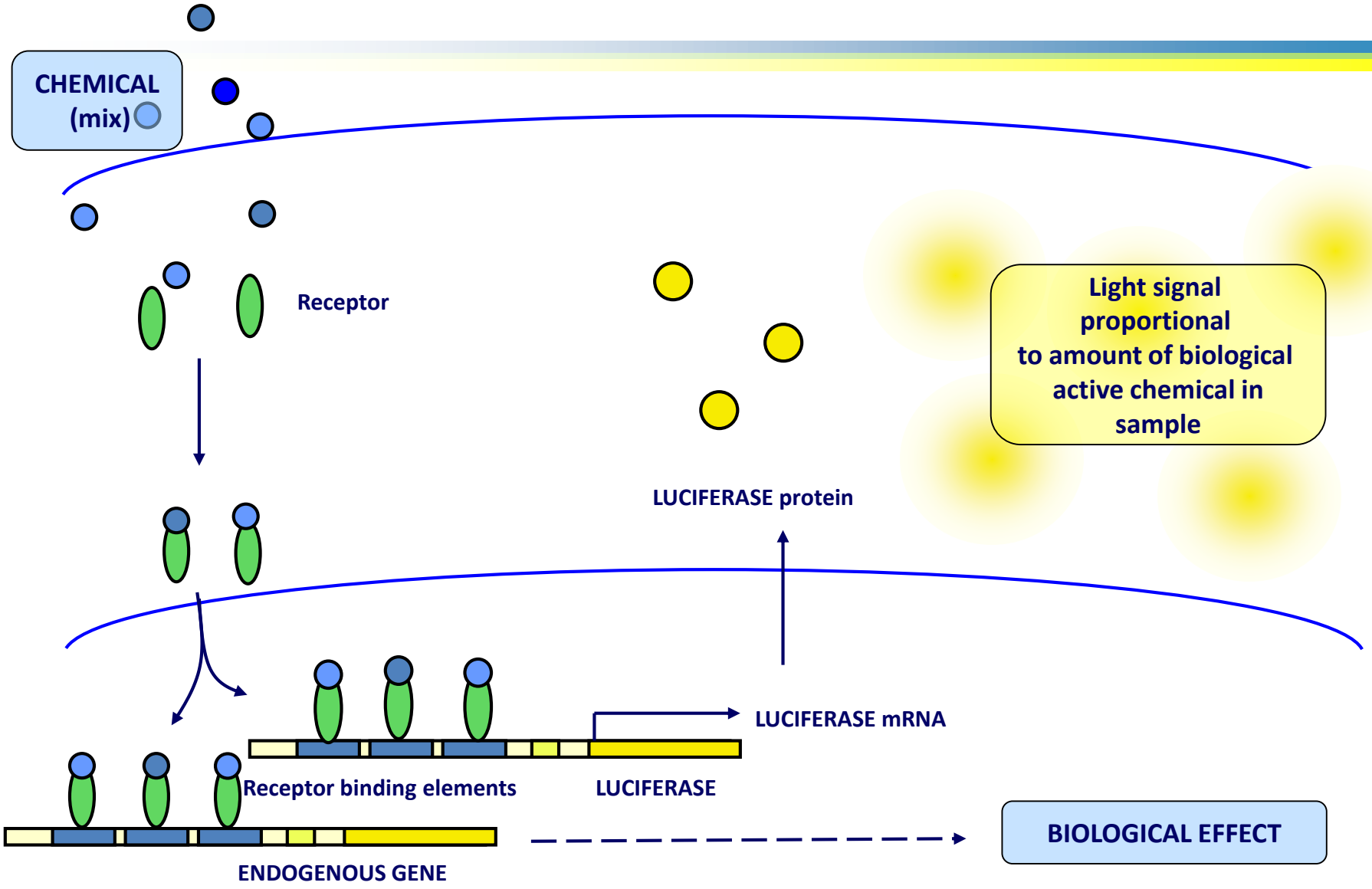
Pathway-based analysis for rapid hazard identification



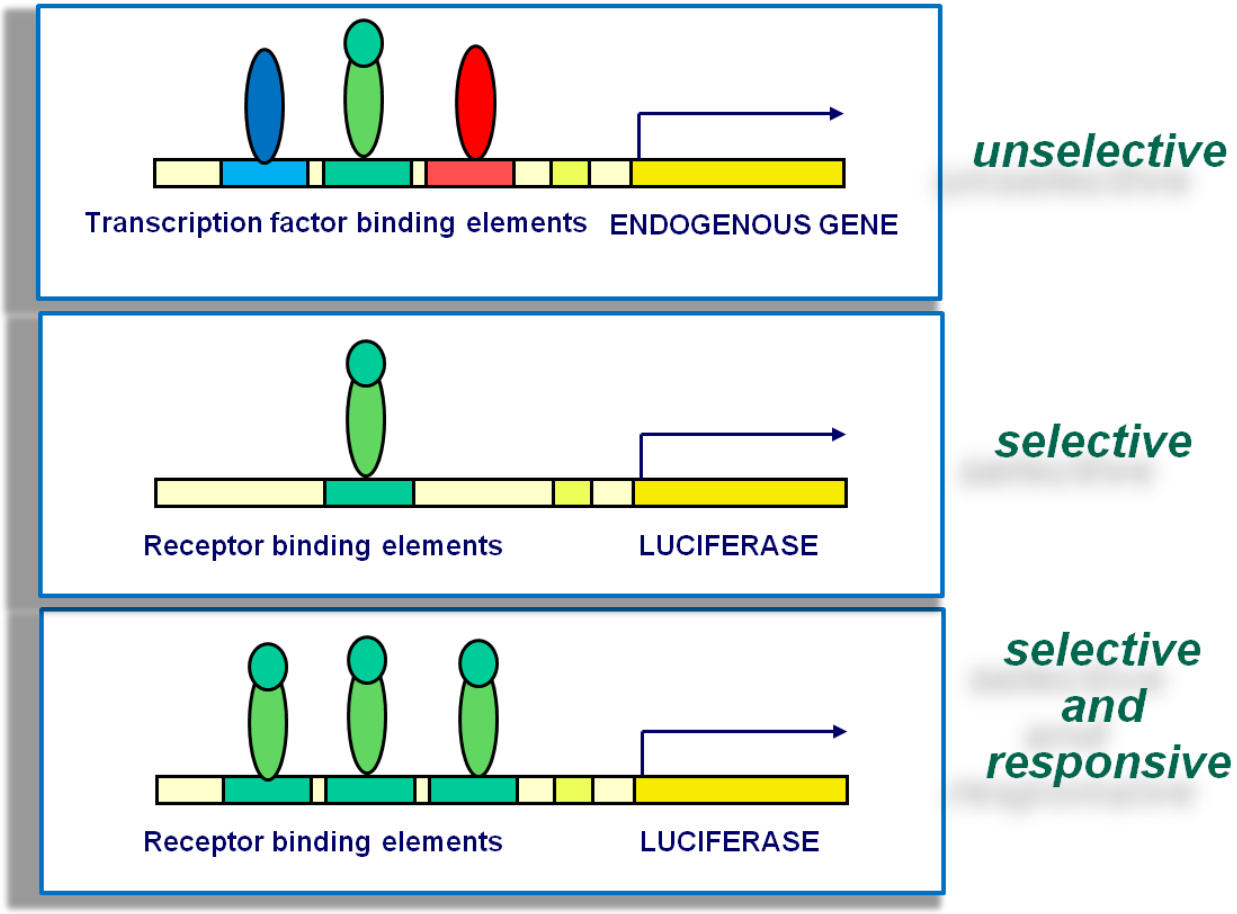
Hanahan and Weinberg 2011, Cell 144(5):646-74

- **Cancer diagnosis: pathway analysis is increasingly used in complementing and replacing conventional pathology**
- **Toxicology is next in line**

principle CALUX® pathway-based reporter gene assays



specificity CALUX® pathway-based reporter gene assays



Legler et al (1999) Toxicological Sciences 48, 55-66.

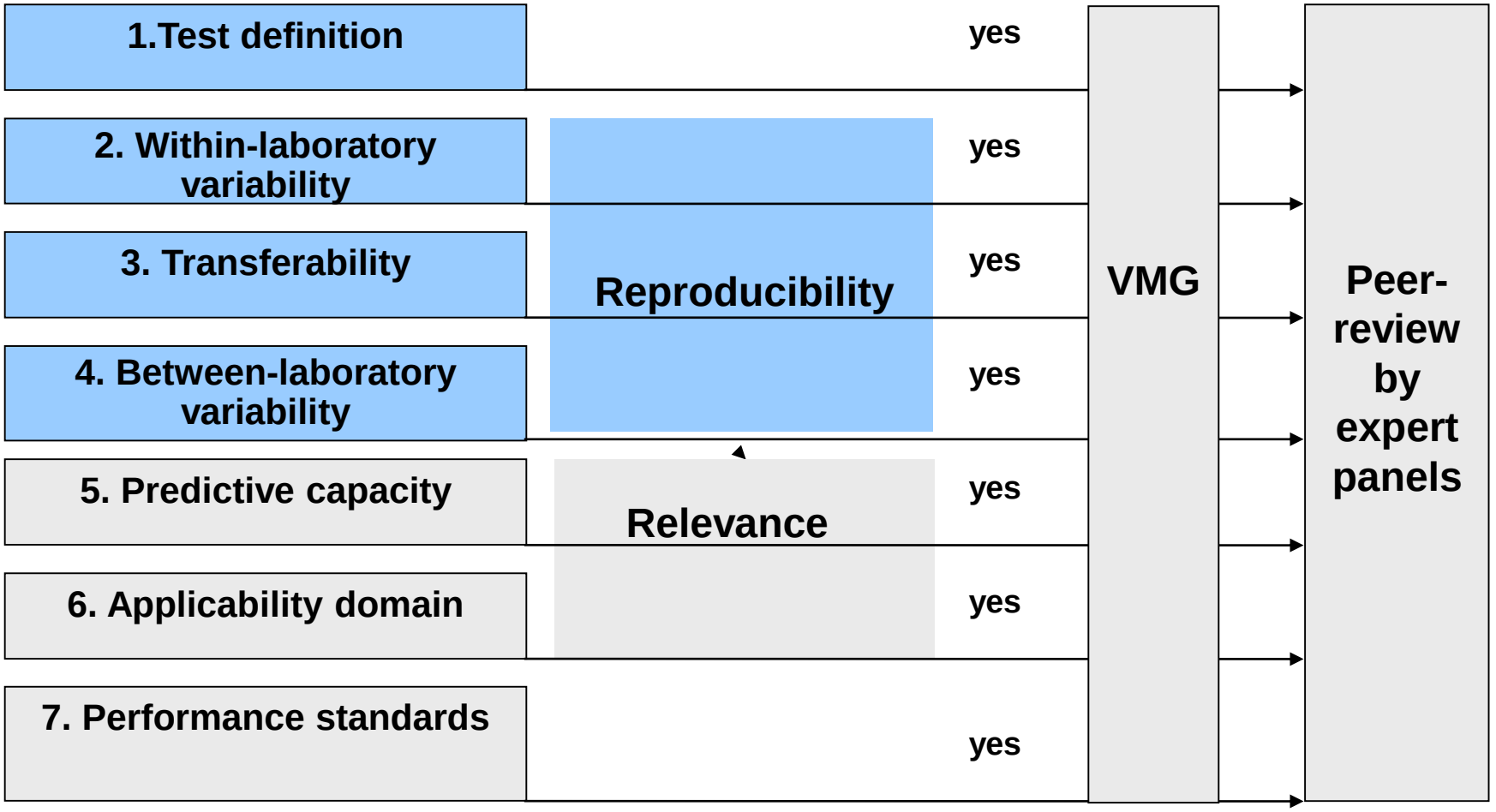
overview CALUX® pathway-based reporter gene assays

name	basal line	species	pathway	reference compound	key reference
DR CALUX	H4IIE	rat	dioxin receptor activation	2,3,7,8-TCDD	Van Vugt 2013
PAH CALUX	H4IIE	rat	dioxin receptor activation	benzo-a-pyrene	Pieterse 2013
ER CALUX	T47D	human	estrogen receptor activation	17β-estradiol	Legler 1999
ERalpha CALUX	U2OS	human	estrogen receptor α activation	17β-estradiol	Sonneveld 2005 OECD 2013
antiERalpha CALUX	U2OS	human	repression estrogen receptor α activation	tamoxifen	Van der Burg 2010a, OECD 2013
ERbeta CALUX	U2OS	human	estrogen receptor β activation	17β-estradiol	Van der Burg 2013

- **Sensitive quantitative assays for major hormonal systems and cell signalling pathways**
- **Adresses major types of toxicity (general toxicity, genotoxicity/carcinogenicity, endocrine disruption, reproduction, developmental tox., obesogens, etc)**
- **More than 50 assays**
- **Metabolism (S9, phase1 &2, steroidogenesis)**
- **Compatible with PBPK modeling**
- **Robust and specific: suitable for complex mixtures**
- **Data on ca 400 chemicals**

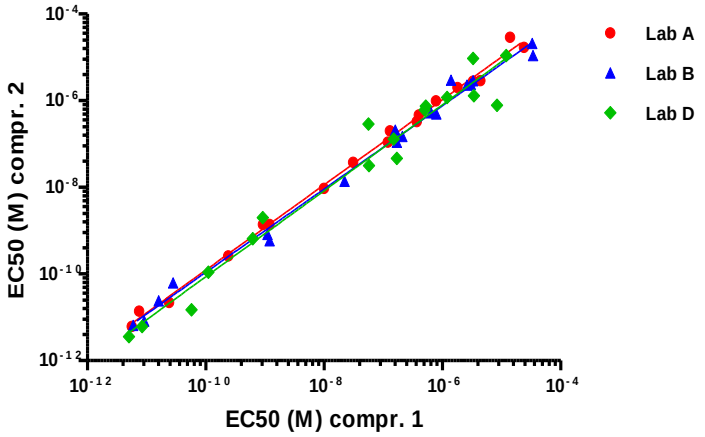
genotox CALUX	U2OS	human	p53-dependent pathway activation +/-S9	cyclophosphamide	Van der Linden 2014
TCF CALUX	U2OS	human	wnt/TCF pathway activation	lithium chloride	Piersma 2013, Van der Burg 2013
AP1 CALUX	U2OS	human	AP1 pathway activation	TPA	Piersma 2013, Van der Burg 2013
HIF1alpha CALUX	U2OS	human	Hif1alpha pathway activation	cobaltous chloride	Piersma 2013, Van der Burg 2013
ER stress CALUX	U2OS	human	ERSE activation leading to endoplasmic reticulum stress	tunicamycin	Piersma 2013, Van der Burg 2013

Modular Approach ECVAM Test validity



Hartung T., Bremer S., Casati S., Coecke S., Corvi R., Fontaner S., Gribaldo L., Halder M., Hoffmann S., Janusch Roi A., Prietro P., Sabbioni E.,
Scott L., Worth A., Zuang V. A modular Approach to the ECVAM principles on Test validity. ATLA 32, 467-472, 2004

Validation: reproducibility/transferability



Intra/interlaboratory reproducibility: coefficient of variance of active test compounds (either agonistic or antagonistic) was *less than 4%*

Fit statistics EC50 ago 1 vs ago 2

	Lab A	Lab B	Lab D
slope	0.9843	0.9615	0.9921
y-intercept	-0.0717	-0.3493	-0.1422
R-square	0.996	0.992	0.964
	y=0.984x-0.0717	y=0.962x-0.3493	y=0.992x-0.1422

Validation analysis results: concordance of classification (ERα CALUX vs ICCVAM)

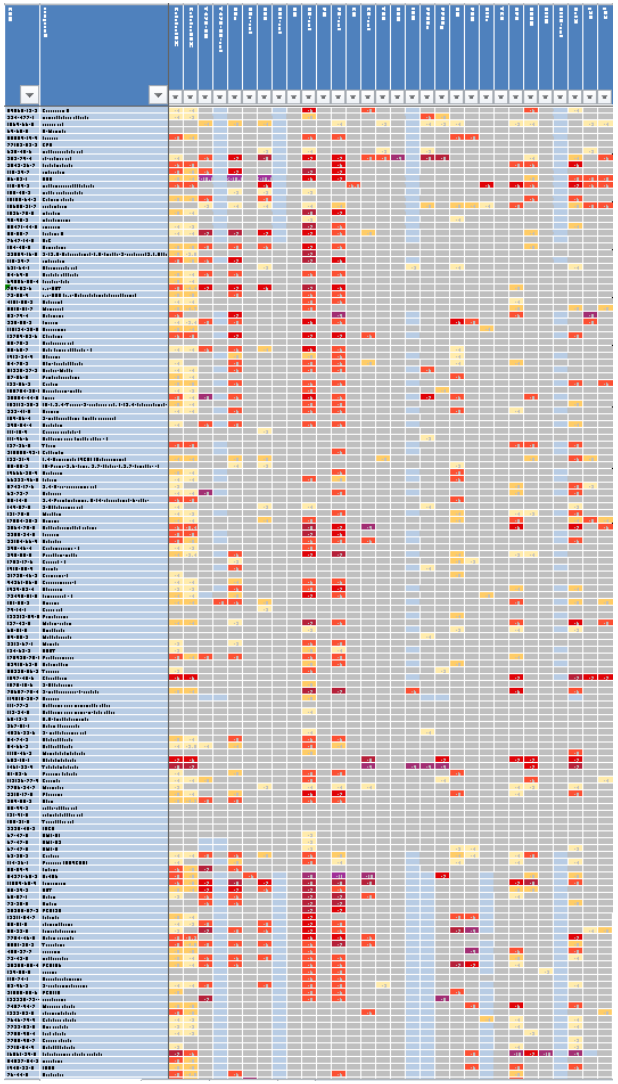
Agonism	Lab A	Lab B	Lab D
Overall accuracy (%)	96	96	100

Antagonism	Lab A	Lab B	Lab D
Overall accuracy (%)	100	100	100

Can HTS pathway-based assays be used to predict toxicity?

tests

compounds



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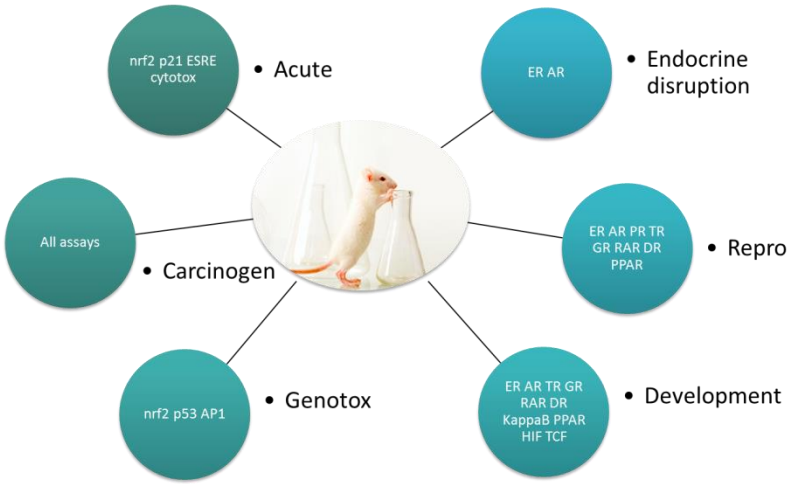
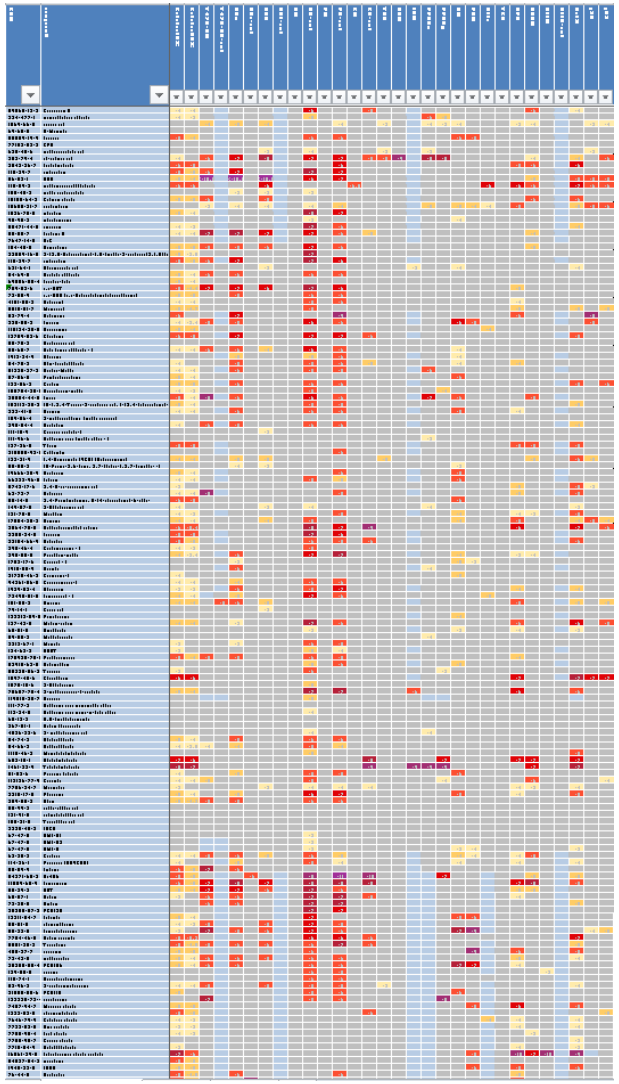


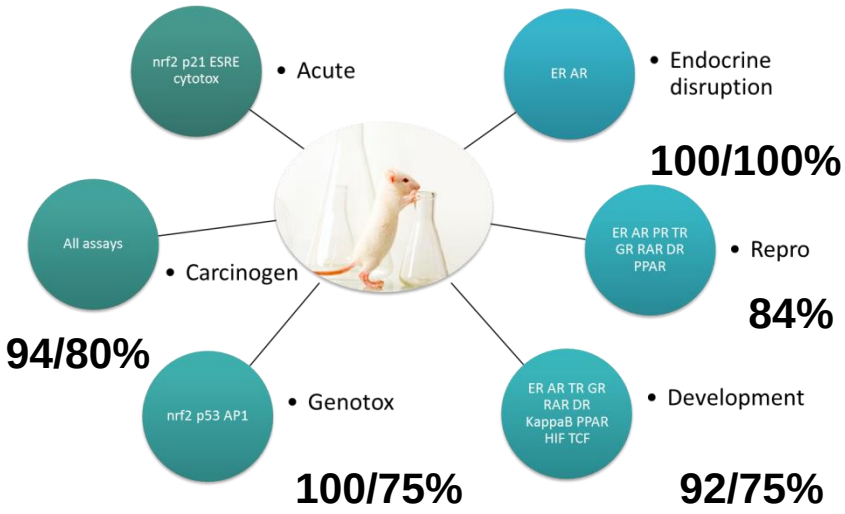
Chemscreen

Various validation studies: predictivity CALUX panel/subsets

tests

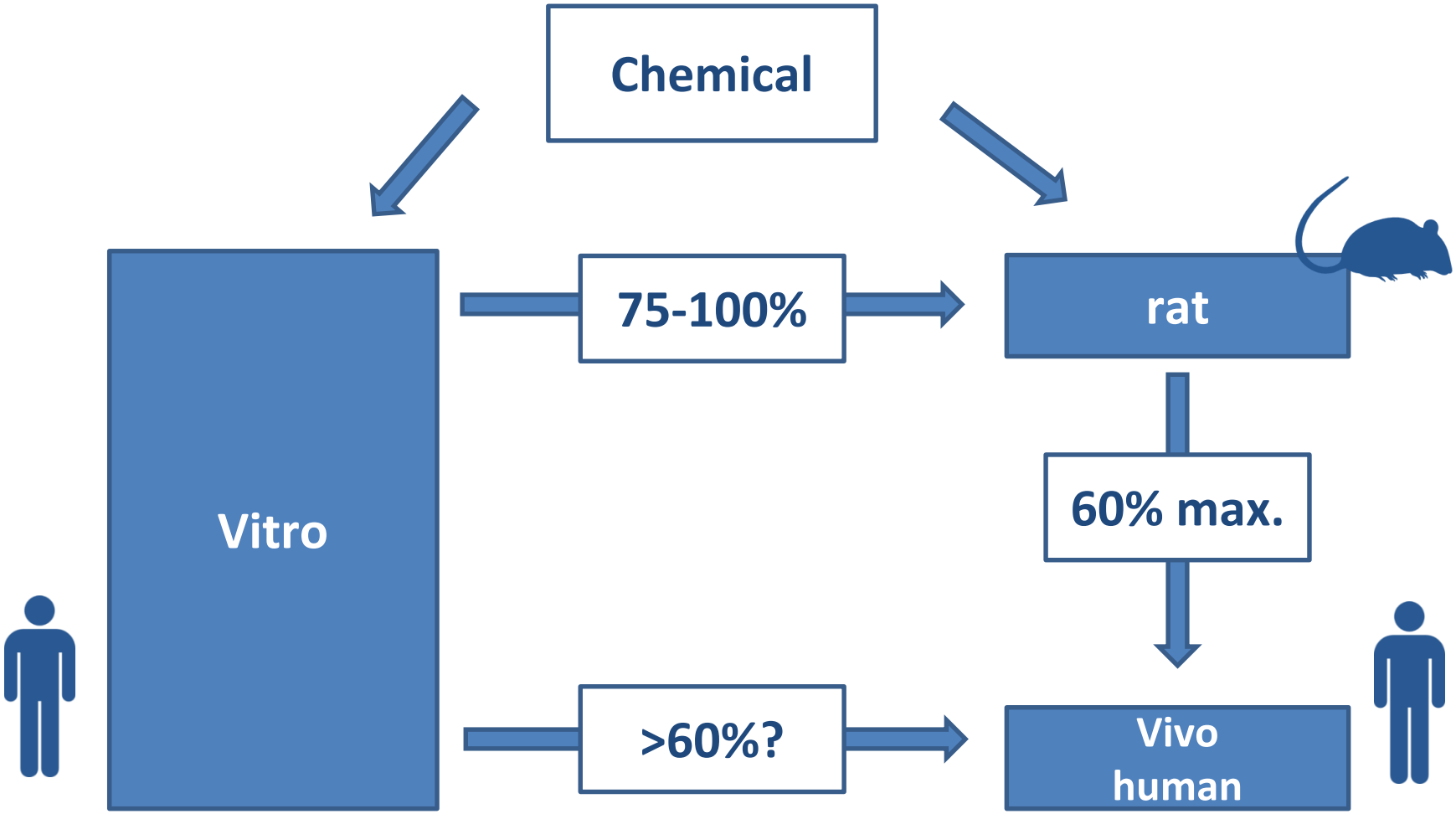
compounds



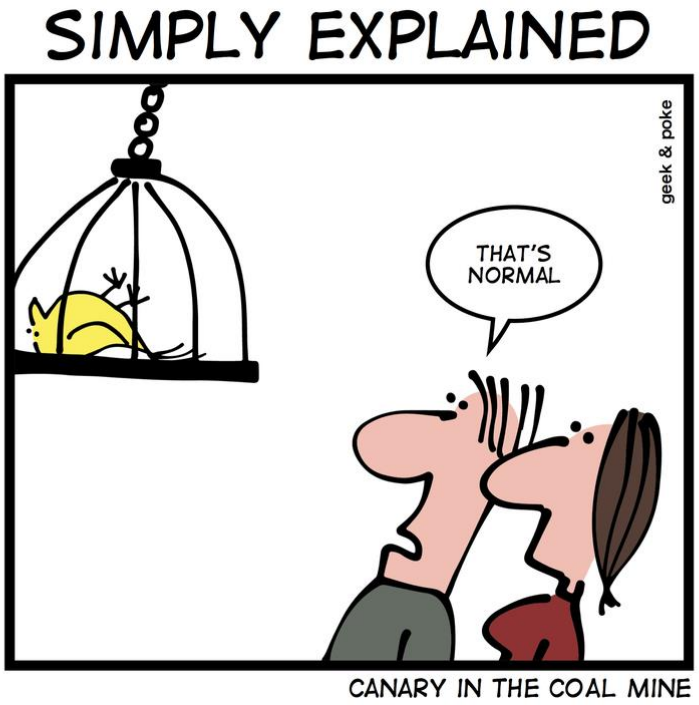


Predictions range between 75-100% (published data/in press)

Validation: what level of predictivity to expect?



Risk assessment of mixtures: extrapolation and interpretation



Mechanistic validation use Adverse Outcome Pathways to link to OECD guidelines & application

- Adverse Outcome Pathway (AOP): chain of linked **key events** at different levels of biological organisation that lead to an **adverse outcome**.
- Central elements to support chemical risk assessment based on mechanistic reasoning.

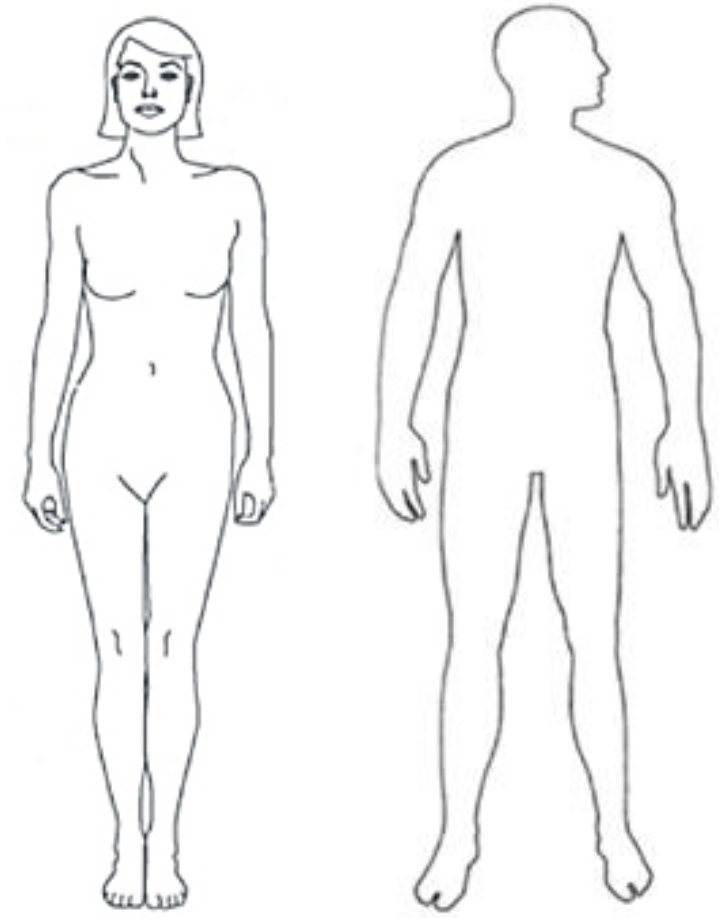
Addresses the needs of various OECD programs:

- the OECD Test Guidelines Programme for the identification of candidate *in vitro* test methods to become OECD Test Guidelines;
- The OECD QSAR Project for the identification of profilers for grouping chemicals
- the OECD Hazard Assessment activities for the development of Integrated Approaches to Testing and Assessment (IATA; ITS) for defined hazard endpoints.

http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm

Example: reproductive toxicity, particularly endocrine disruption

i.e. disturbance of hormone balance by chemicals



Major concerns:

- **Cancer**
- **Decreased fertility**
- **Obesity**

OECD; conceptual framework with many tests

OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals

Note: Document prepared by the Secretariat of the Test Guidelines Programme based on the agreement reached at the 6th Meeting of the EDTA Task Force

Level 1 Sorting & prioritization based upon existing information	•Physical & chemical properties, e.g., MW, reactivity, volatility, biodegradability •Human & environmental exposure, e.g., production volume, release, use patterns •Hazard, e.g., available toxicological data	
Level 2 *In vitro* assays providing mechanistic data	•ER, AR, TR receptor binding affinity •Transcriptional activation •Aromatase & Steroidogenesis *in vitro* •Aryl hydrocarbon receptor recognition/binding	•High Through Put Prescreens •Thyroid function •Fish hepatocyte VTG assay •QSARs; Others (as appropriate)
Level 3 *In vivo* assays providing data about single endocrine Mechanisms and effects	•Uterotrophic Assay (estrogenic related) •Hershberger Assay (androgenic related) •Non-receptor mediated hormone function	•Fish VTG assay (estrogenic related) •Others (e.g. thyroid)
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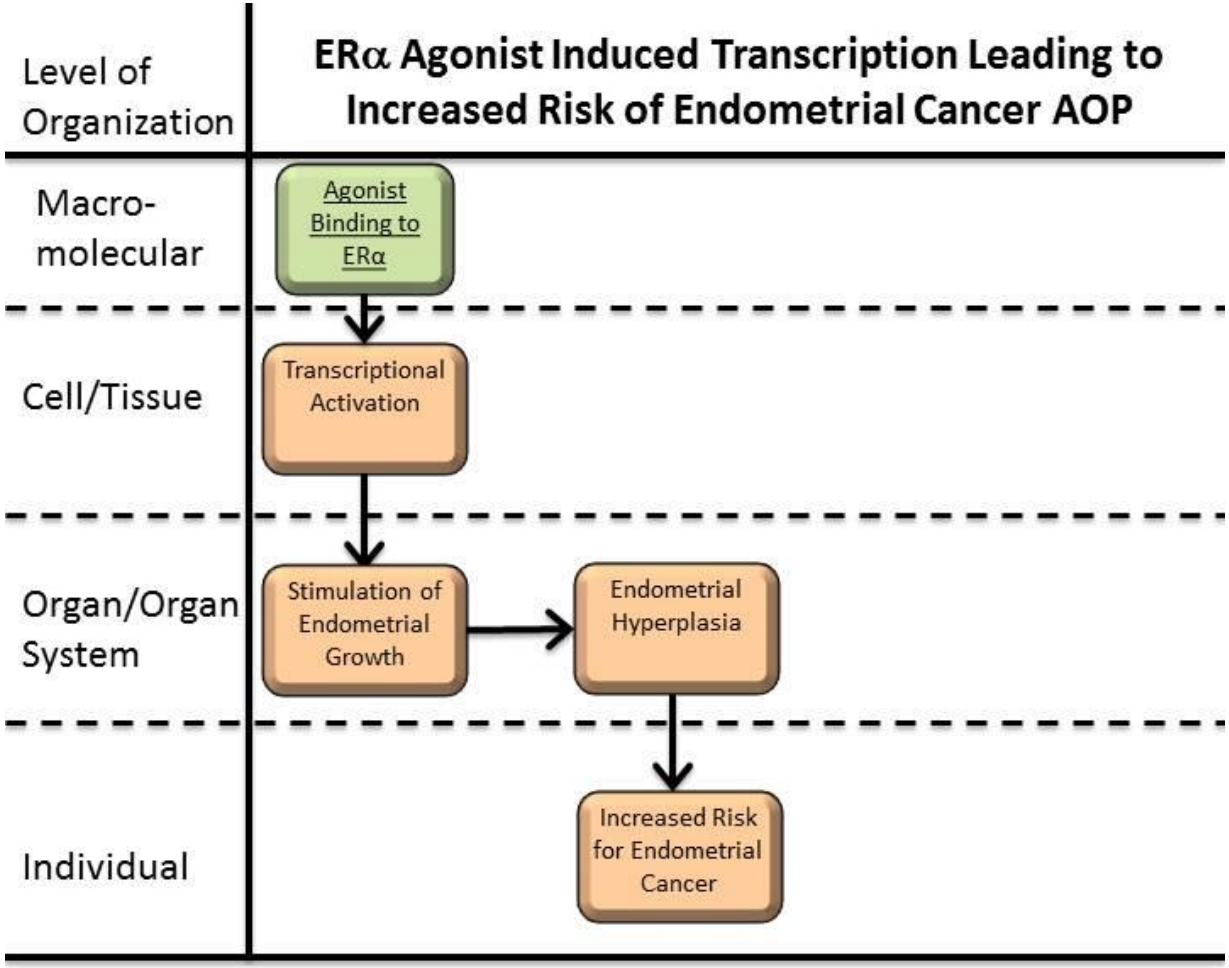
Level 5 *In vivo* assays providing data on effects from endocrine & other mechanisms	•1-generation assay (TG415 enhanced) •2-generation assay (TG416 enhanced) •Reproductive screening (TG421 enhanced) •Combined 28 day/reproduction screening test (TG 422 enhanced)	•Partial and full life cycle assays in fish, birds, amphibians & invertebrates (development & reproduction)

AOP example: adverse effects by estrogens



OECD TG455/457

Molecular initiating event

↓

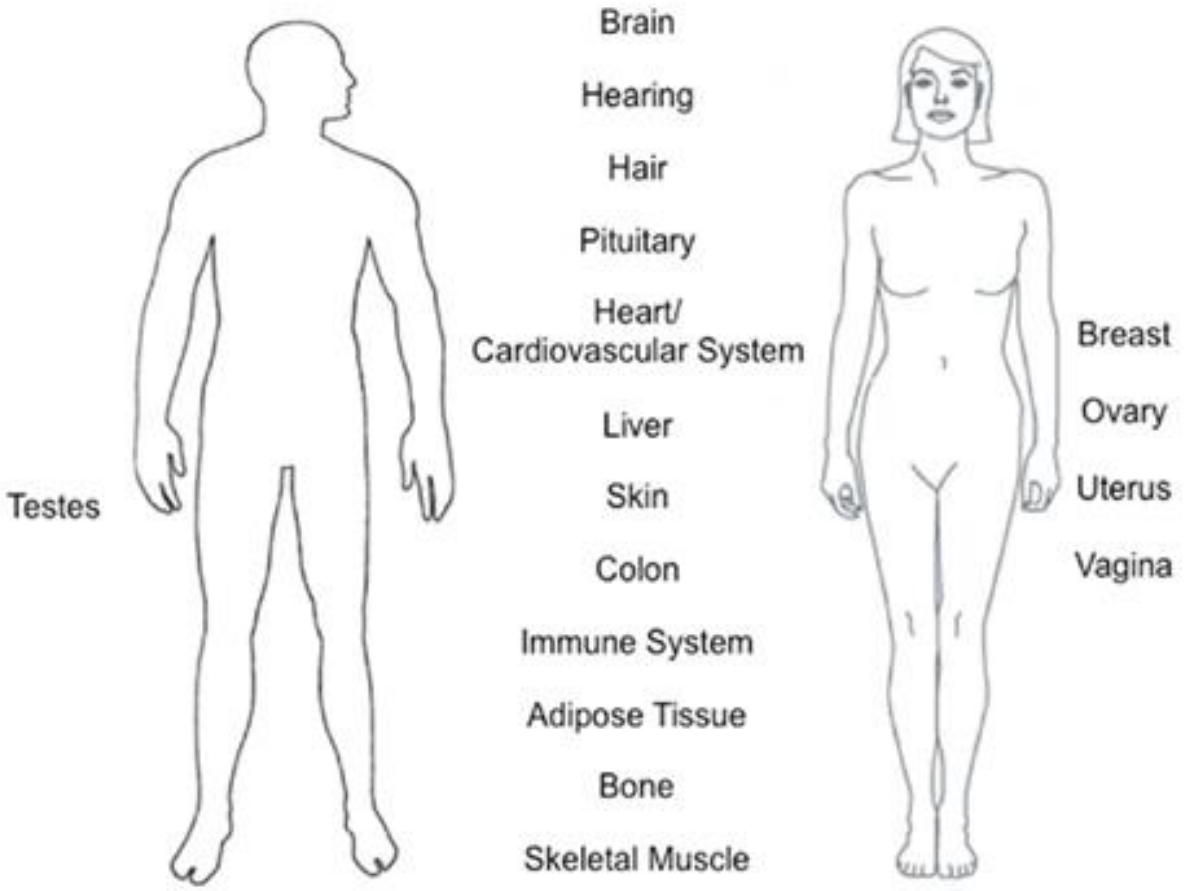
Key events

↓

Adverse Outcome

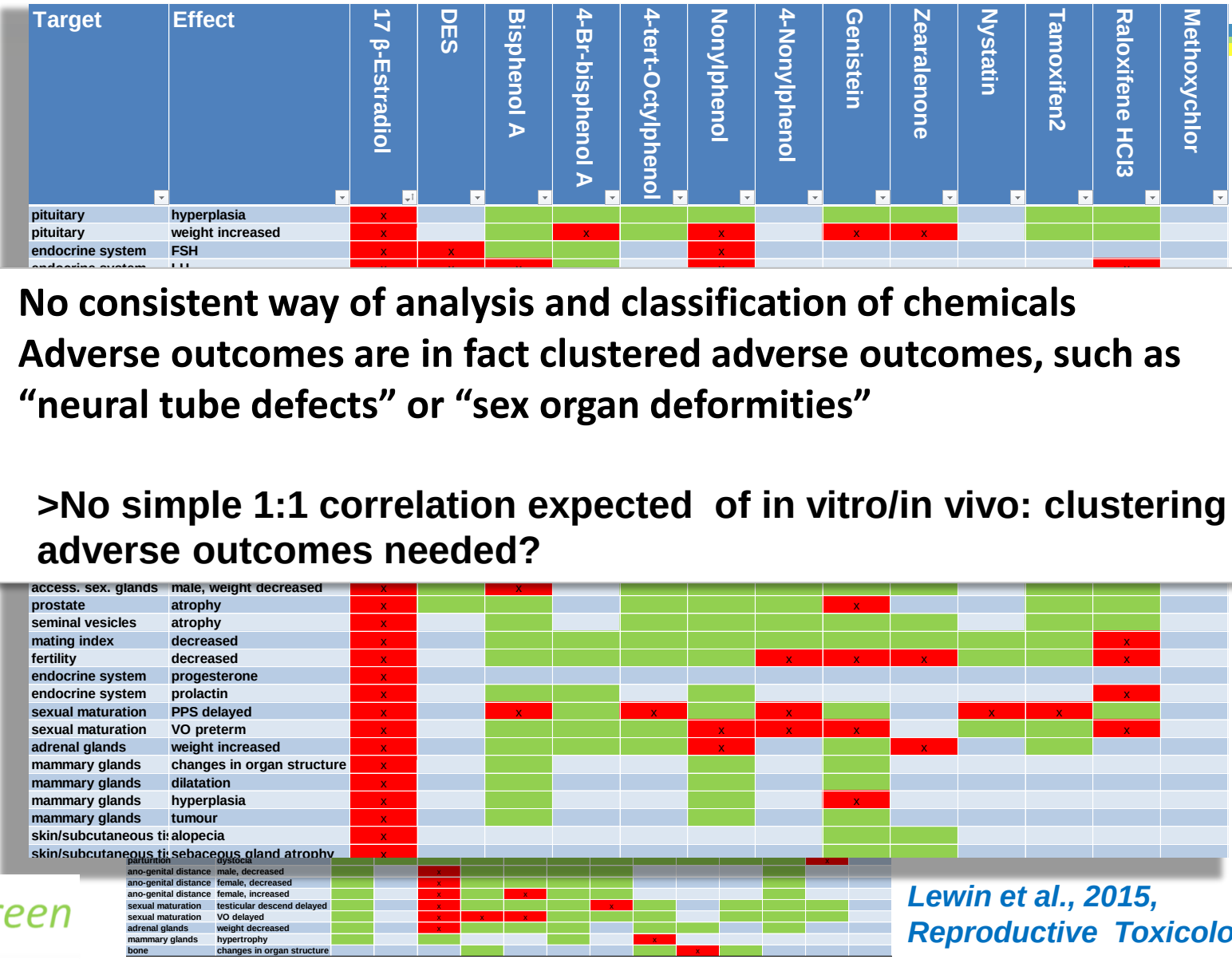
Becker et al., Regulatory Toxicology and Pharmacology , in press

Many estrogen target tissues



Dozens of AGFs needed for estrogens alone?

Adverse effects in rodents by estrogens



Target	Effect	17 β-Estradiol	DES	Bisphenol A	4-Br-bisphenol A	4-tert-Octylphenol	Nonylphenol	4-Nonylphenol	Genistein	Zearalenone	Nystatin	Tamoxifen2	Raloxifene HCl3	Methoxychlor
pituitary	hyperplasia	x												
pituitary	weight increased	x			x		x		x	x				
endocrine system	FSH	x	x				x							
access. sex. glands	male, weight decreased	x		x										
prostate	atrophy	x							x					
seminal vesicles	atrophy	x												
mating index	decreased	x											x	
fertility	decreased	x						x	x	x			x	
endocrine system	progesterone	x												
endocrine system	prolactin	x											x	
sexual maturation	PPS delayed	x		x		x		x			x	x		
sexual maturation	VO preterm	x					x	x	x				x	
adrenal glands	weight increased	x					x			x				
mammary glands	changes in organ structure	x												
mammary glands	dilatation	x												
mammary glands	hyperplasia	x							x					
mammary glands	tumour	x												
skin/subcutaneous ti	alopecia	x												
skin/subcutaneous ti	sebaceous gland atrophy	x												
parturition	dystocia												x	
ano-genital distance	male, decreased		x											
ano-genital distance	female, decreased		x											
ano-genital distance	female, increased			x										
sexual maturation	testicular descend delayed		x			x								
sexual maturation	VO delayed		x	x	x									
adrenal glands	weight decreased		x											
mammary glands	hypertrophy					x								
bone	changes in organ structure						x							

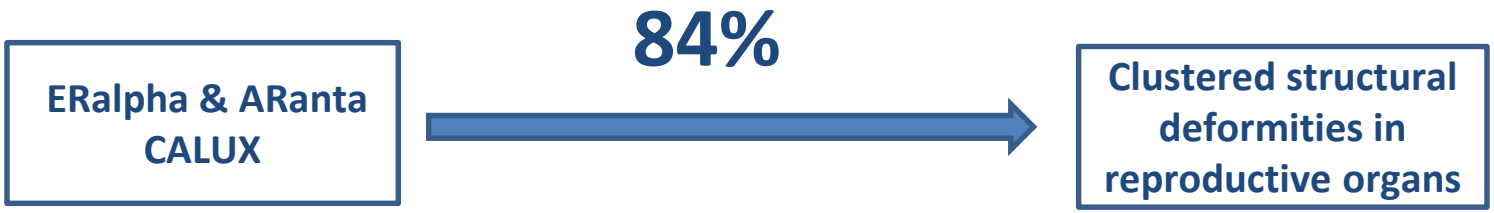
- **No consistent way of analysis and classification of chemicals**
- **Adverse outcomes are in fact clustered adverse outcomes, such as "neural tube defects" or "sex organ deformities"**

>No simple 1:1 correlation expected of in vitro/in vivo: clustering adverse outcomes needed?

Lewin et al., 2015, Reproductive Toxicology 55

Linkage to clustered AOPs

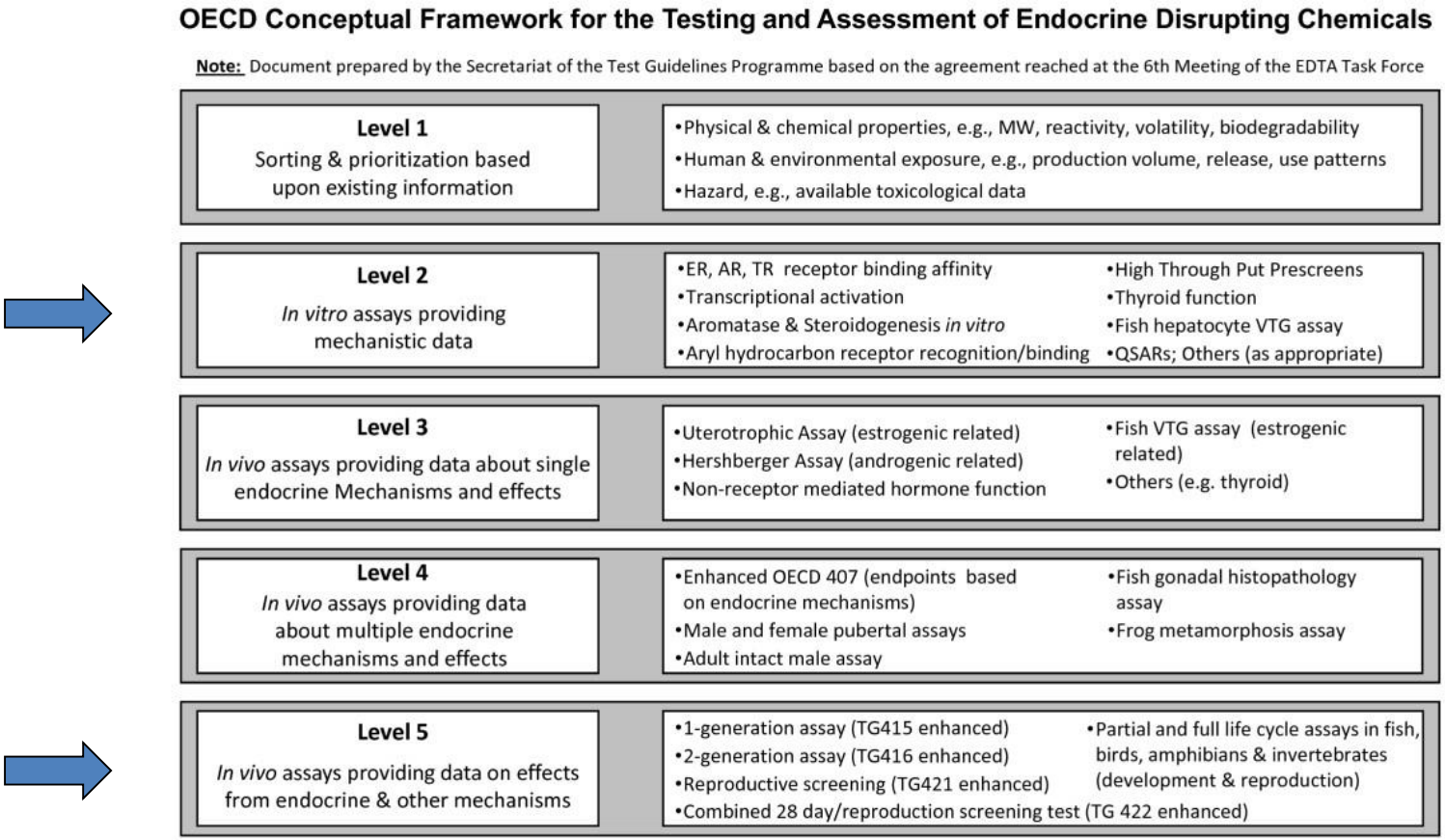
Compound groups	overall accuracy (%)	correct/total
Neural tube defects	47	9/19
Reproductive organ deformities	84	16/19



- ✓ **Chemical grouping non-informative, but biological grouping needed**
- ✓ **ER/AR balance clearly links to structural deformities in reproductive organs**

Van der Burg et al. (2015) Reproductive Toxicology 55: 95-103
Lewin et al. (2015) Reproductive Toxicology 55:81-94

A level 2 test can predict an adverse outcome (level 5)



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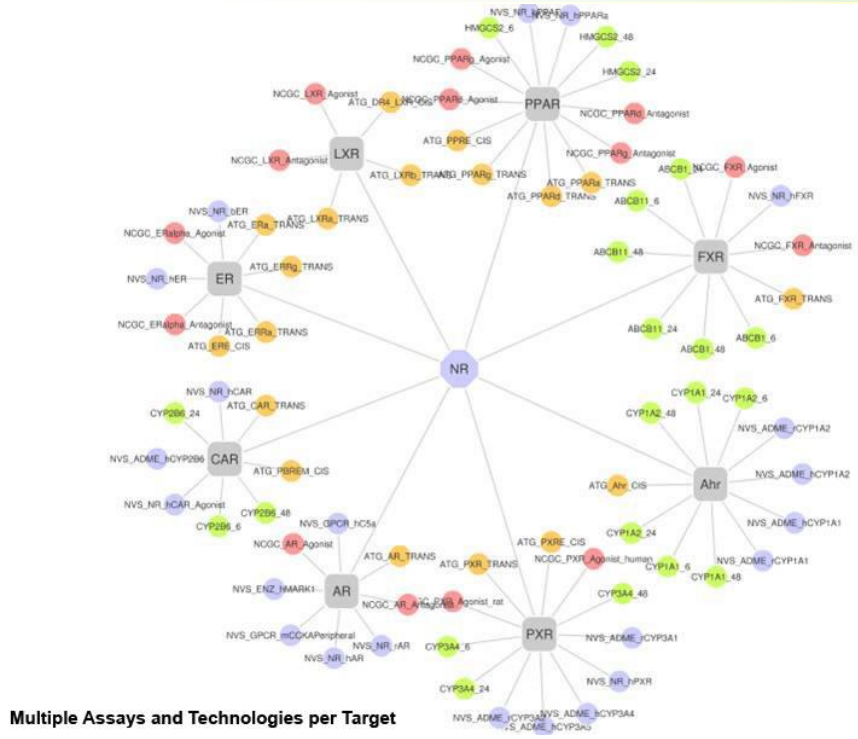
Many more HTS assays needed to predict estrogenicity?

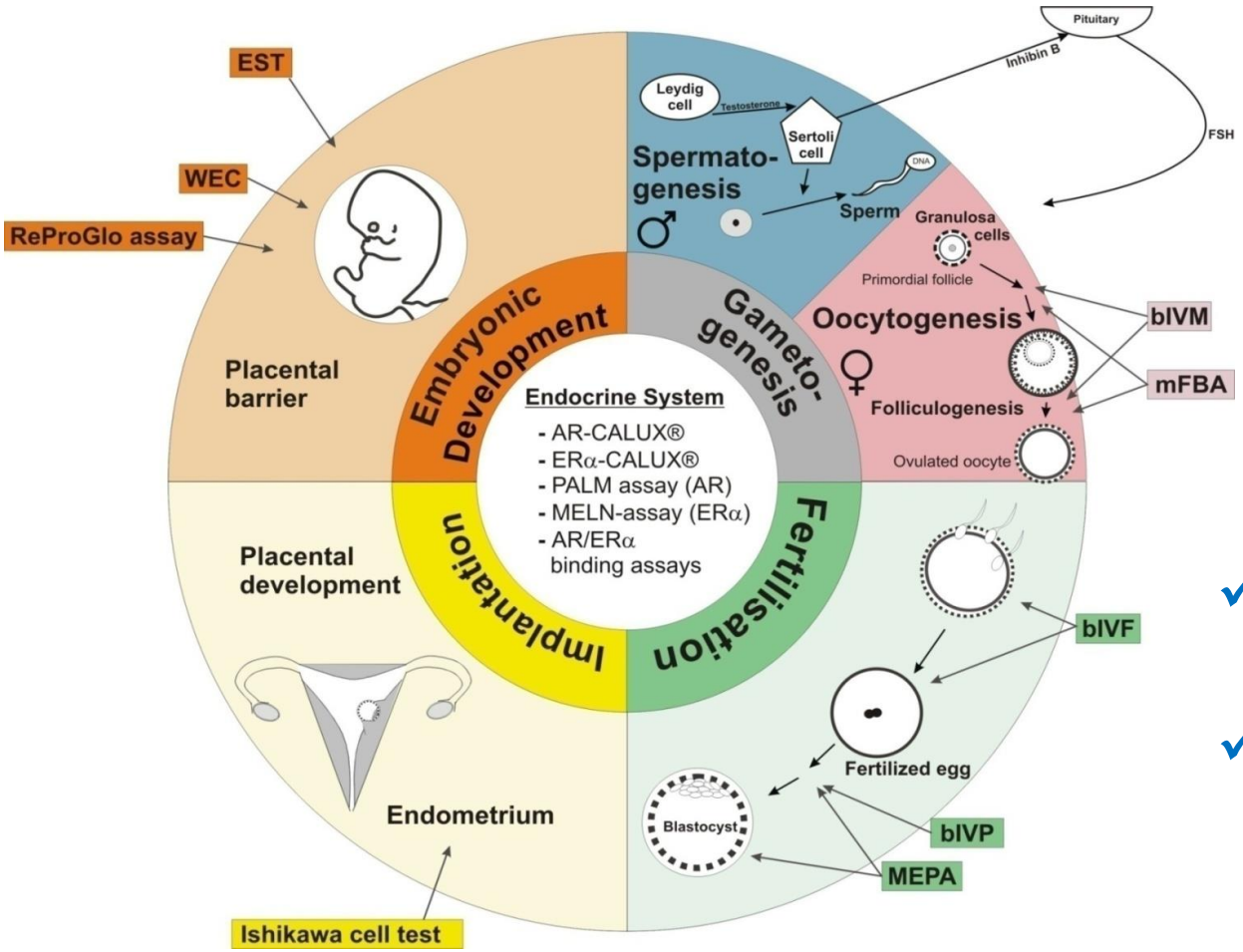


Multiple Assays and Technologies per Target

	1 CALUX	18 TOXCAST	REFERENCE
17alpha-Ethinylestradiol	-12,30	1,00	6
meso-Hexestrol	-11,70	0,99	6
17beta-Estradiol	-11,50	0,94	6
Diethylstilbestrol	-11,20	0,94	6
17alpha-Estradiol	-9,80	1,06	4
Estrone	-9,50	0,81	4
4-Octylphenol	-6,20	0,12	4
Genistein	-8,00	0,54	2
5alpha-Dihydrotestosterone	-7,50	0,40	2
Bisphenol A	-7,30	0,45	2
4-Cumylphenol	-7,00	0,38	2
o,p'-DDT	-6,90	0,39	2
Kepone	-6,60	0,17	2
Butyl benzyl phthalate	-6,40	0,18	1
Methoxychlor	-6,20	0,25	1
Kaempferol	-6,10	0,25	1
17-Methyltestosterone	-6,00	0,50	1
Fenarimol	-5,70	0,11	1
Ethylparaben	-5,40	0,09	1
p,p'-DDE	-5,30	0,07	1
Dicofol	-5,30	0,00	1
Dibutyl phthalate	-5,20	0,03	1
4-Nonylphenol	-5,10	0,09	1
Di(2-ethylhexyl) phthalate	-4,00	0,00	1
Atrazine	-4,50	0,00	0
Haloperidol	0,00	0,01	0
Spironolactone	0,00	0,00	0
Corticosterone	0,00	0,00	0
Flutamide	0,00	0,00	0
Procymidone	0,00	0,00	0
Linuron	0,00	0,00	0
Reserpine	0,00	0,00	0
Ketoconazole	0,00	0,00	0

- **Toxcast: many assays per pathway (18 assays)**
- **Our approach: One selective, validated assay with minimal false positives (ERalpha CALUX)**

Can a test battery predict animal tests results?



✓ ***ReProTect:*** *Yes*

✓ ***ChemScreen:*** *Yes*

Schenk et al. 2010 Reproductive Toxicology 30, 200-218; Piersma et al. 2013. Reproductive Toxicology

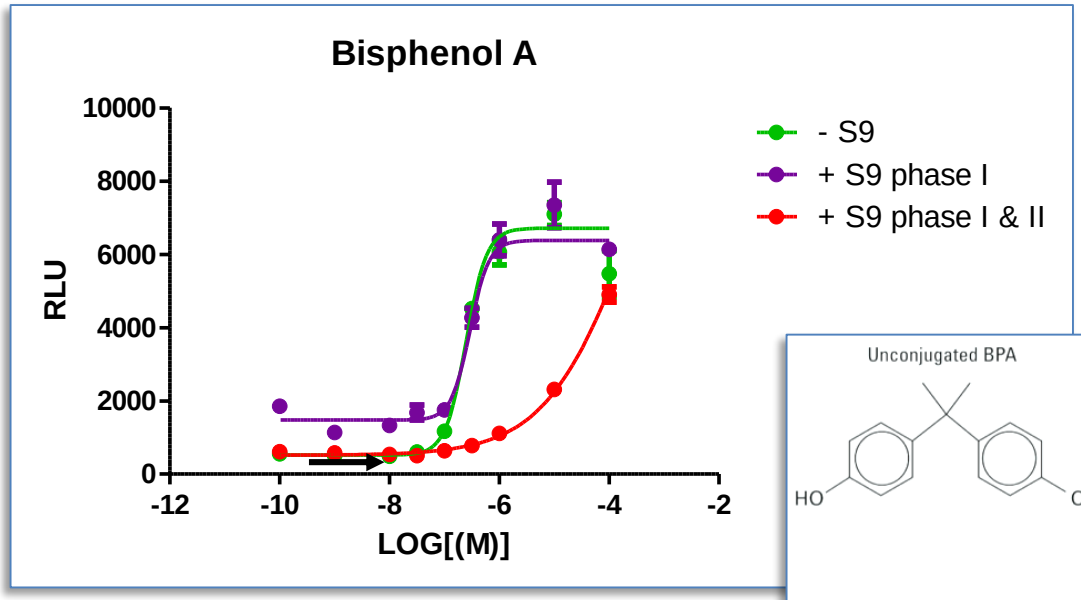
Battery performance

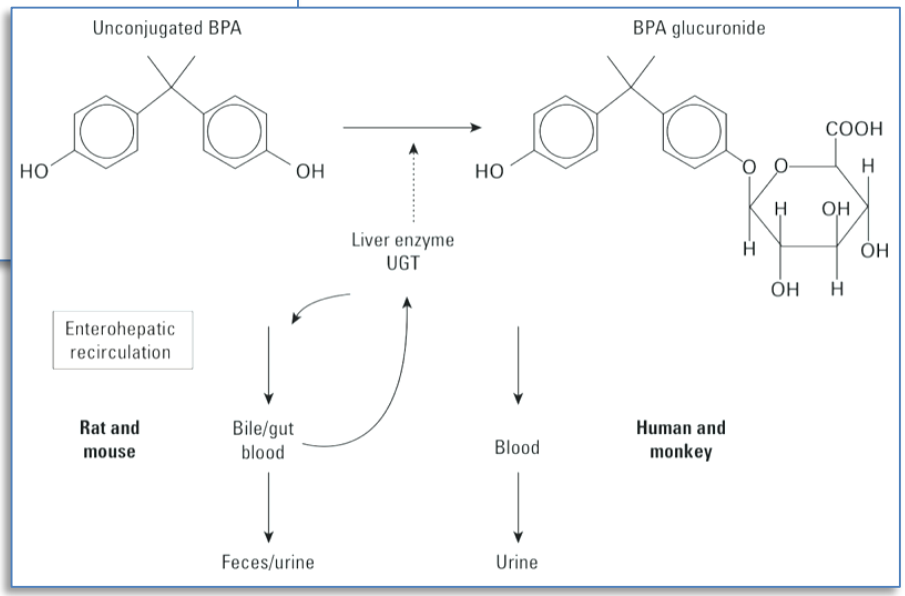
compound	Toxicity in vivo	EST	ZET	ReProGlo	Cyp17	Cyp19	CALUX	CALUX PBPK	battery
Cyclosporin A									
Monoethylhexyl phthalate									
Sodium valproate									
D-mannitol									
Flusilazole									
Glufosinate ammonium									
Methoxy acetic acid									
Retinoic acid									
Dioctyltin chloride									
Endosulfan									
Diethylstilbestrol									
Methylmercury chloride									

✓ **CALUX HTS model same prediction as complex tests (EST/ZET)**

Piersma et al. 2013 Reproductive Toxicology 38, 53

Modular metabolism methods





- Bisphenol A in ERalpha CALUX:

✓ **Not affected by Phase I metabolism**

✓ **Inactivated by phase II metabolism**

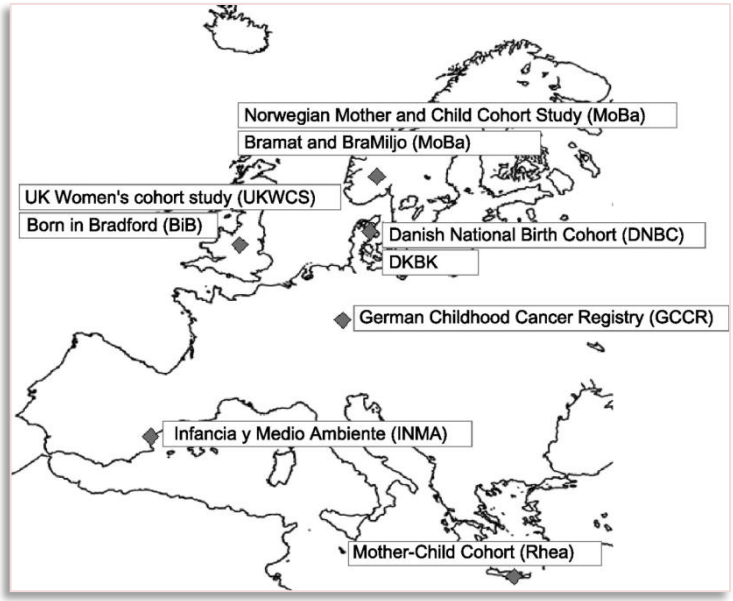
Thresholds/trigger values

- **Guideline limit values for bioassays, if available**
- **Guideline limit values chemicals: convert to bioassay threshold (underestimation)**
- **Risk assessment using reference chemicals; trigger values Brand et al (water)**
- **Bioassay-based: Biological pathway altering dose/epidemiological data showing effect in humans correlated to bioassay activation level**
- **Etc.**

	ADI/TDI reference (µg/kg bw/day)a	Safety factor ADI/TDIa	Bioavailability reference	fup reference	Bioavailability other compounds	fup other compounds	External equivalent dose	Trigger value
ERα	0.050 (E2)	100	5%	2%	50%	16%	0.625 ng E2-eq/kg bw/day	3.8 ng E2-eq/L
AR	2 (T)	1000	3.5%	2%	50%	23%	12.17 ng T-eq/kg bw/day = 1.826 ng DHT-eq/kg bw/day	11 ng DHT-eq/L
GR	0.015 (DEX)	100	70%	23%	100%	70%	3.450 ng DEX-eq/kg bw/day	21 ng DEX-eq/L
PR	30 (P4)	100	10%	2.5%	100%	10%	750 ng P4-eq/kg bw/day = 55.5 ng Org2058-eq/kg bw/day	333 ng Org2058-eq/L

Mixtures: Specificity allows to measure exposure in humans

- **Pathway specific bioassays are valuable for human monitoring**
- **E.g. associations between DR-CALUX responses and:**



- **markers of childhood leukemia**
- **low birth weight**
- **shorter gestational time**
- **changes in AGD in young boys**
- **immune system functions later in life**

➢ **Derivation of thresholds /"trigger values" possible**

NewGeneris
Newborns and Genotoxic exposure risks

Estrogenicity detected in Leachates

Food simulant 1: 20% EtOH (water, low alcohol-beverages)

Sample	Estradiol equivalents (mg/kg food simulant)
PET	< 4.3 E-07
Polypropylene	< 4.3 E-07
HD polyethylene	< 4.3 E-07
LD polyethylene	< 4.3 E-07

Food simulant 2: 50% EtOH (fruit juice, high alcohol-beverages, milk/cream/cheese)

Sample	Estradiol equivalents (mg/kg food simulant)
PET	< 4.3 E-07
Polypropylene	< 4.3 E-07
HD polyethylene	1.3 E-05
LD polyethylene	1.3 E-05

For HD- and LDPE, the detected estrogenic equivalents in mg/kg food are 3.5x higher than the trigger value in water

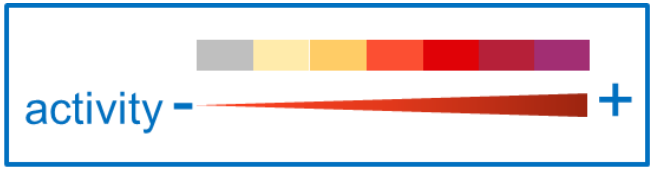
Biobased plastics: a safer alternative?

Not necessarily:

- **'Drop-in plastics': chemically identical to petrochemical counterparts… their toxicity profile will likely also be the same**
- **Biobased plastics will need similar additives… (plasticizers, UV/thermo-stabilizers, coatings, colorants)**
- **Biomass used to generate biobased plastics may vary in quality… toxicity profile of the resulting plastics may vary accordingly**

CALUX profile of plastic additives

compound	Cytotox10%	ERa	ERa+S9	ERa-anti	ERb	ERb-anti	AR	AR-anti	PR	PR-anti	GR	GR-anti	TRb	RAR	LXR	PXR	PPARa	PPARg	DR	PAH	Hif1a	TCF	AP1	ESRE	NFkB	Nrf2	p21	p53 GENTOX	p53 S9 GENTOX
Di(2-ethylhexyl)phthalate		-4.0								-5.2						-6.4													
Di-n-octyl phthalate																													
monoethylhexyl phtalate	-3.5							-4.5									-5.5	-4.7											
diisodecylphthalate		-4.4			-4.2																								
diisononylphthalate																												-3.0	
Dicyclohexylphthalate	-4.5	-5.3								-5.4		-5.1				-6.7													
Diethylphthalate	-3.5	-4.3						-5.0		-4.3																			
Diisobutyl phthalate	-4.0	-5.7						-5.3		-5.5																			
Dibutylphthalate	-4.5	-5.2						-5.5		-5.5																			
Di(n-hexyl)phthalate	-3.5	-5.0						-5.0		-5.5		-4.5							-4.0				-4.2						
Butyl benzyl phthalate	-3.9	-6.4			-4.4			-5.6		-5.5									-3.7										
di(2-ethylhexyl)adipate																													
Benzophenone	-3.5	-5.2						-6.0		-4.8																			
Etyl paraben	-3.0	-5.2			-5.2			-5.0		-4.0																		-3.5	
4-tert-octylphenol	-5.5	-7.2			-8.5			-6.4		-6.1						-6.0													
4-n-octylphenol	-4.7	-6.2						-5.6		-5.3																			
Nonylphenol	-4.9	-5.1			-5.6			-6.5		-5.5														-4.6					
4-Cumylphenol	-4.2	-7.0	-6.4		-7.0			-6.7		-6.1		-4.5																	
p-(tert-pentyl)phenol	-4.0	-7.7						-6.3		-5.9																			
Diphenyl-p-phenylenediamine	-4.0	-5.5						-5.2		-5.4																			
Bisphenol A	-4.0	-7.3			-6.8			-6.8		-5.5		-4.5												-4.3					
Bisphenol A-dimethacrylate		-6.6			-6.5			-6.0		-5.5						-5.3												-4.7	
Bisphenol F		-6.6			-6.7			-5.4		-4.8						-4.3								-4.7				-4.5	-3.3



- *Most additives are active on the endocrine assays:*

  they act as estrogens, anti-androgens and anti-progestins

First applications in read across, safe design/green chemical identification

compound	Cytotox10%	Cytotox50%	ERa	ERa-anti	ERb	ERb-anti	AR	AR-anti	PR	PR-anti	GR	GR-anti	TRb	RAR	PPARa	PPARg	DR	PAH	Hif1a	TCF	AP1	ESRE	NFkB	Nrf2	p21	p53
bisphenol A	-4	-3.7	-7.3		-6.8			-6.8		-5.5		-4.5										-4.3				
Butyl benzyl phthalate	-3.9	-3.5	-5.7		-4.4			-6.1		-5.7							-3.7									
Di(n-hexyl)phthalate	-3.5		-5					-5		-5.5		-4.5					-4				-4.2					
Dibutylphthalate	-4.5	-4	-5.2					-5.5		-5.5																
Diethylphthalate	-3.5	-3.0	-4.3					-5		-4.3																
Diisobutyl phthalate	-4.5	-4	-5.7					-6		-6																
Nonylphenol	-4.9	-4.7	-5.1		-5.6			-6.5		-5.5												-4.6				
FDCA																										

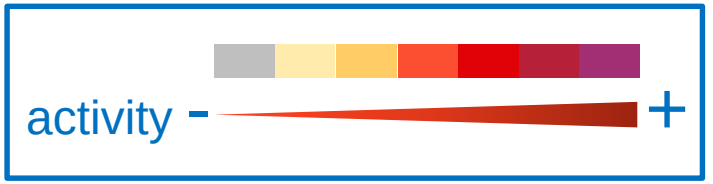
- **Case study: CALUX panel identifies FDCA as a potentially non-toxic alternative to current plastic ingredients/building blocks**
- **Cases that show applicability to different chemical classess**
- **Comparable “read-across” methods are increasingly used in chemical safety assessments; used in approx. 30% reproductive tox dossiers (100-1000TPA) in REACH (ECHA 2014)**

Kroese et al., 2015 reproductive toxicology 55; Van Vugt-Lussenburg, in preparation

Conventional vs Biobased plastic building blocks

Novel Biobased furan-ester plastic additives

Currently used phthalate-based analogues



compound	Cytotox20%	ERa	ERa+S9	ERb	AR-anti	PR-anti	GR-anti	TRb-anti	PPARa	PPARg	DR	AP1	ESRE	Nrf2	p53 GENTOX	p53 S9 GENTOX
DEHF																
DEHP		-3.9														
DIBF		-4.3	-4.0													
DIBP		-5.7			-5.6	-5.7										
DIDF																
DIDP																
DMFDCA		-3.7	-3.4													
DMTPA		-5.9	-5.6	-4.5											-4.0	
DMOPA					-4.7	-3.6										
DMIPA		-3.3			-3.1										-2.5	

- *The bio-based furan-based compounds are less active compared to their phthalate-based counterparts.*

FOOD & BIOBASED RESEARCH
WAGENINGEN UR

Plastic Migration Study

Do endocrine-active compounds leach from conventional and biobased plastics to similar extent?

Perform migration study according to EC regulation:

1. **Fill container with food simulant (depending on intended use)**
2. **Incubate for 10 days, 60° C**
3. **SPE-extraction of the 'leachate'**
4. **Analyse extract on CALUX panel (including Cytotox, ERα, anti-AR, anti-PR, Genotox)**

BioDetection Systems

Conclusions

- Specific CALUX high throughput panel of assays with good predictions in vivo effects available (e.g. endocrine disruption, reproductive toxicity, genotoxicity/carcinogenesis and acute toxicity)
- Pharmacokinetic modeling further improves predictions
- OECD/ECVAM/ISO validation, incorporation in guidelines, AOP linkage
- Test batteries can often be relatively simple when using specific assays
- Applicable for read-across, safe design/green chemistry
- Specially designed and very suitable for real-life assess safety of complex mixtures such as non-intentionally added substances and plastic leachates