EuroMix:
An assessment of mixture toxicity using in vitro analyses of liver steatosis to develop an adverse outcome pathway based strategy

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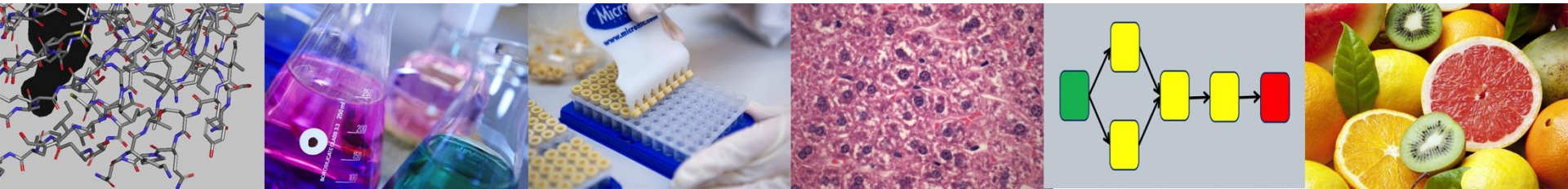
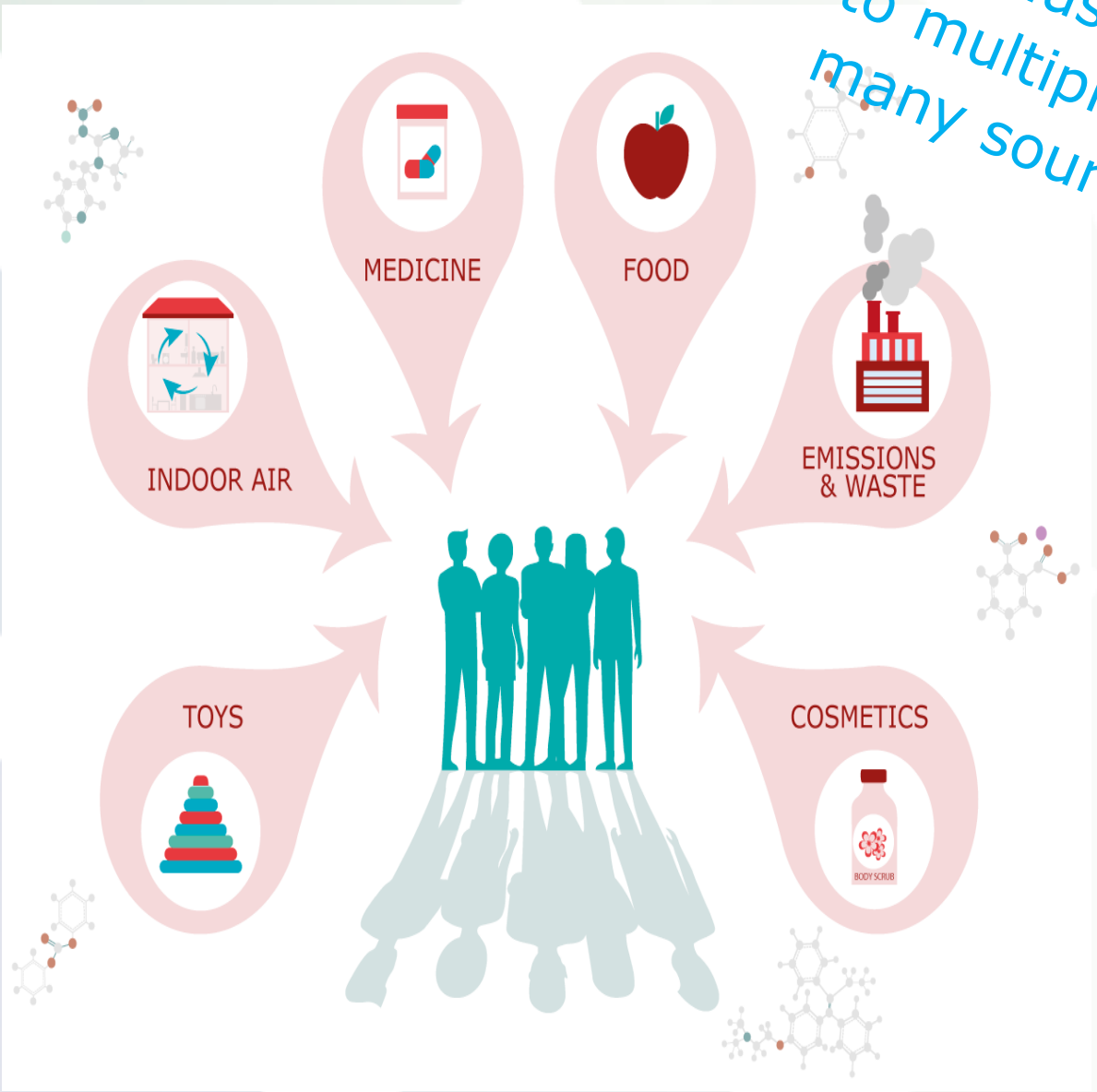


Exposure to
Why is it important?

EuroMix

...because we are exposed to multiple chemicals via many sources



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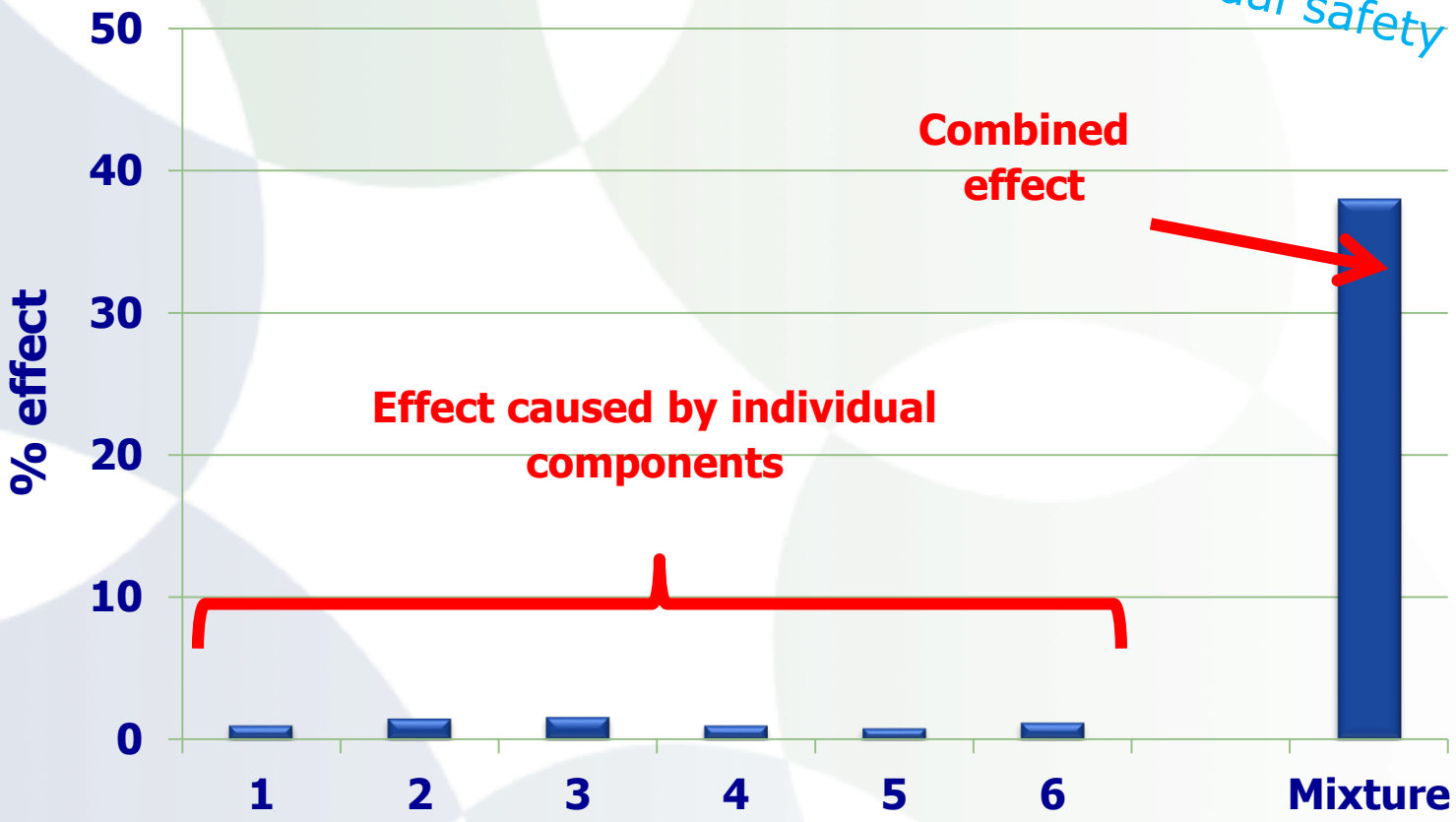
Why is it important?

…chemicals can lead to adverse effects on human health or the ecosystem, even if single substances in the mixtures are below their individual safety thresholds



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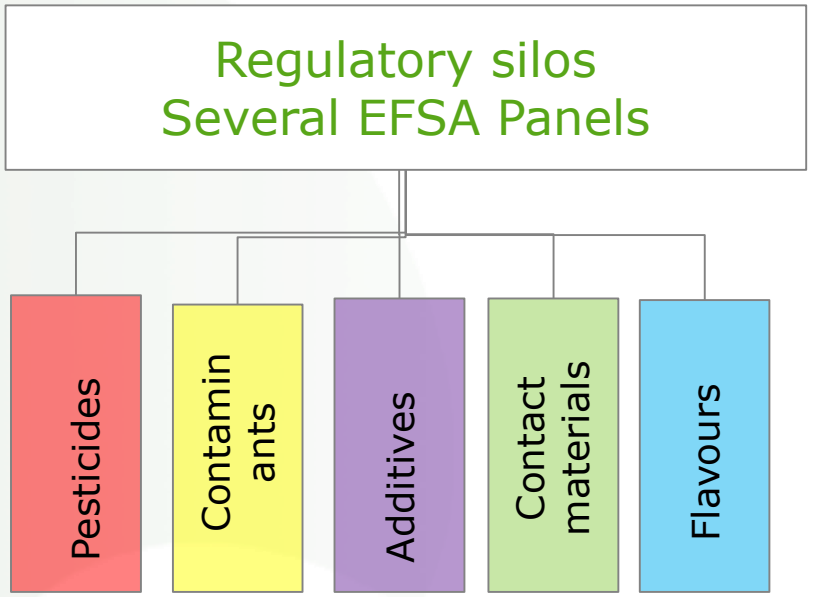
How are chemicals regulated?

REACH; not much structured data on non-dietary exposure, and no mixture testing

Pesticide Directive 1107/2009
Contaminants Directive 315/83 and 1881/2006
Additive Directives 1333/2008

Many data gaps in monitoring



Focus of hazard data and testing is on the critical effect, not on chemicals forming a mixture. Lacking of data and testing is needed.

Crit Rev Toxicol. 2018 Oct;48(9):796-814. doi: 10.1080/10408444.2018.1541964. Epub 2019 Jan 10.

Overview on legislation and scientific approaches for risk assessment of combined exposure to multiple chemicals: the potential EuroMix contribution.

Rotter S[1], Beronius A[2], Boobis AR[3], Hanberg A[2], van Klaveren J[4], Luijten M[5], Machera K[6], Nikolopoulou D[6], van der Voet H[7], Zilliacus J[2], Solecki R[1].

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Analysis EU legislation – identified problems

- Most of the reviewed European Regulations stipulate to consider potential combined effects from exposures to multiple chemicals.
- No clear legal mandates to assess the combined effects implemented in Regulations, as long as harmonised and accepted methods are lacking.
- No framework for a systematic and integrated assessment of mixture effects taking into account different routes of exposure and different product types.

MRL Regulation requires developing new methodologies for CRA.

→ **Method development is the first necessary step to implement clear legal mandates and establish guidelines for a sound risk assessment.**

EFSA requested the Scientific Committee to **develop an overarching guidance document** on the harmonisation of risk assessment methodologies **for human health and ecological risk assessment** of chemical mixtures within and across regulatory sectors (EFSA 2016).

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Combined exposure via the diet

- exposure of consumers against mixtures of:

  - food contaminants
  - pesticides

- toxicological testing and risk assesment only done for single compounds

Need:

- strategy for risk assessment of mixtures
- methods for toxicological testing of mixtures

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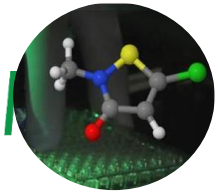
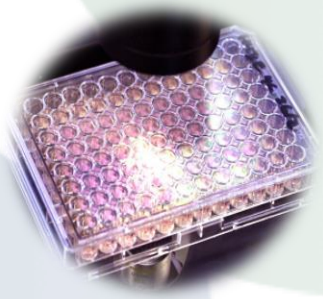
Review: Application novel tools

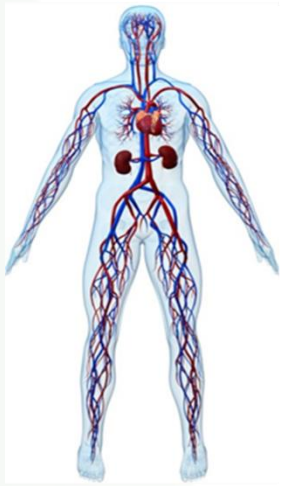


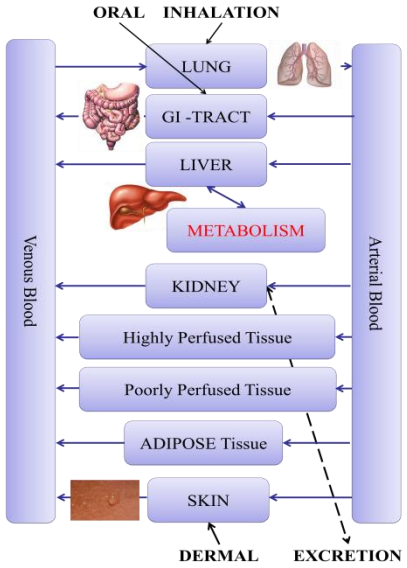
in vitro testing



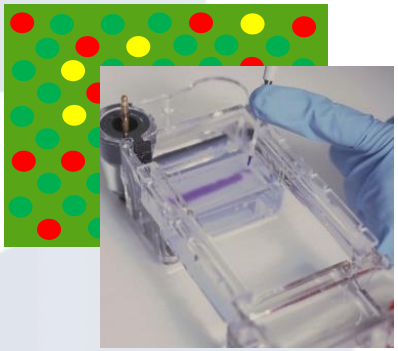
PBTK

physiologically based toxicokinetic modelling





omics

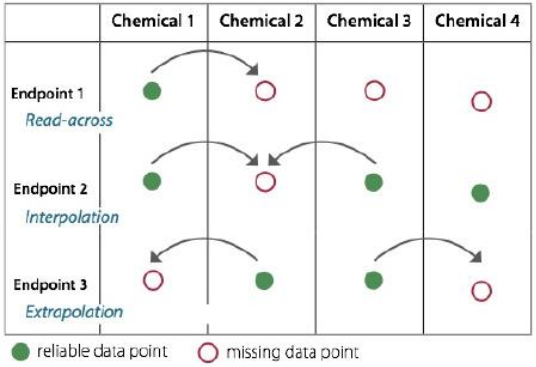


Transcriptomics
Proteomics
Metabolomics

QSAR

Quantitative Structure Activity Relationship

Read-across



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EFSA grouping pesticides into CAGs

Cumulative Assessment Groups (CAG)s

- Level 1: organ level
- Level 2: phenomenological endpoints (*outcome of animal study DAR*)
- Level 3: mode of action (*requires mechanistic data, hardly available*)
- Level 4: mechanism of action (*not feasible in Europe)*

Two EFSA opinions published in 2013

- grouping principles and CAGs for thyroid and nervous system grouping at level 2: data quality not optimal, might need refinement
- dissimilar acting pesticides should be included in the CAG

Grouping principles might be conservative due to precautionary principle in the Pesticide Act

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Grouping consideration

How should chemicals be grouped (from draft EFSA guidance)

- Common regulatory domain
- Common source
- Common functional group(s)
- Common chemical classes
- Common breakdown products
- Common ‘critical’ target organ
- Common MoA or AOP

→ **AOP wise testing** (OECD and JRC considerations), alternative test strategy moving away from animal testing

→ **Relative Potency Factors** (or reference points) as point of departure for risk assessment

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EuroMix: endpoints and CAGs

EFSA: grouping of chemicals into Cumulative Assessment Groups (CAGs)

- aligning EFSA methodology with specific endpoints in EuroMix:

EuroMix	**EFSA CAG**
liver toxicity	Liver – Fatty changes
developmental toxicity	Reproductive and developmental toxicity - Malformation
endocrine effects	Reproductive and developmental toxicity

- linking mode of action to endpoints as well as *in-vitro* data to *in-vivo* outcome
  → concept of **Adverse Outcome Pathways (AOP)**

liver toxicity	⟶	AOP for liver steatosis
developmental toxicity	⟶	AOP for cranio-facial malformation
endocrine effects	⟶	AOP for androgenic/estrogenic disruption

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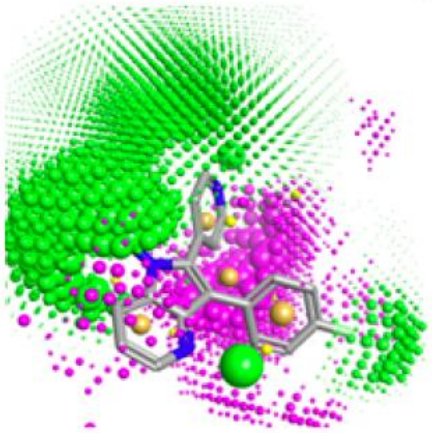
EuroMix testing strategy for mixture

EuroMix

In silico



Hazard

Co-exposure

Bioassay tool box (in vitro)

relevant mixtures

In vivo confirmation

selected mixtures

No. of chemicals

Uncertainty

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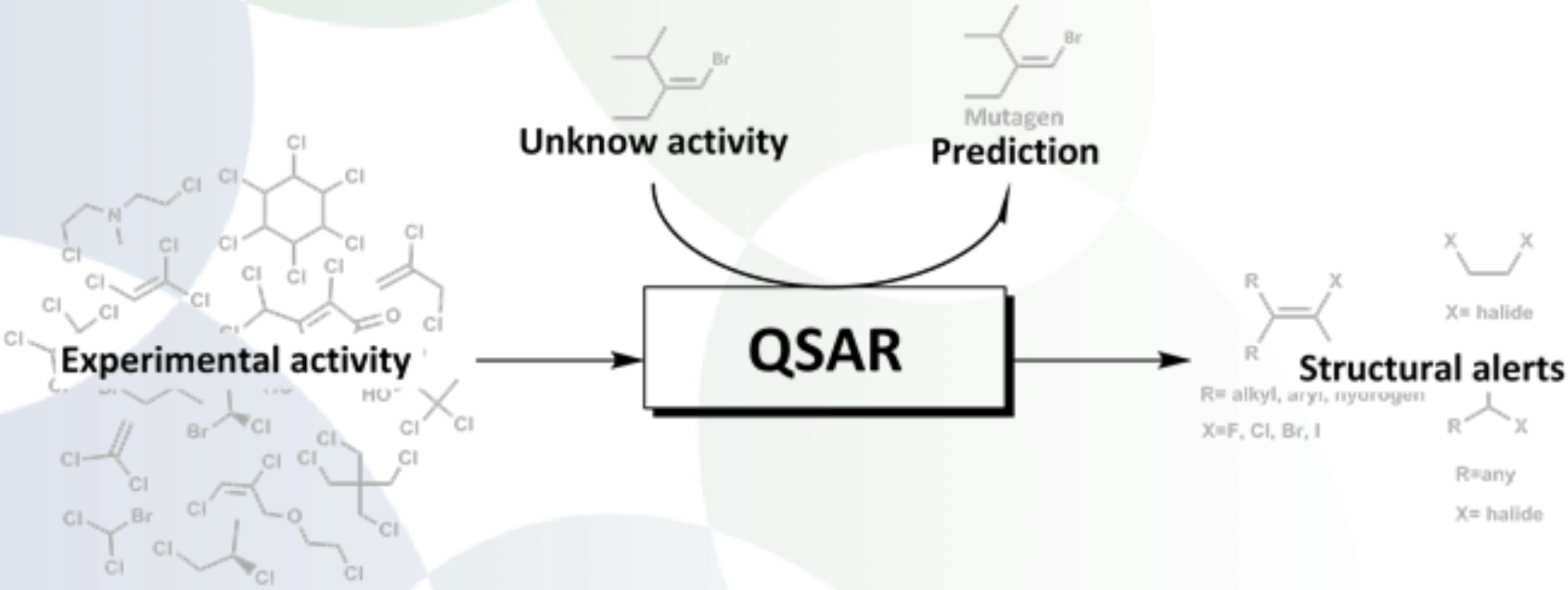
CAGs based on (Q)SARs?

SAR: alerts

QSAR: quantitative models (within an alert, or "global")



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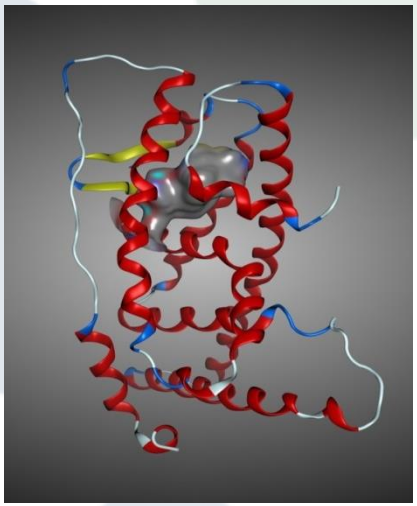
QSARs used for all kind of chemicals

Literature research and in silico modelling for 1600 chemicals in EuroMix model and data platform



Several chemical clasess are adressed

1. pesticides (558),
2. biocides (34),
3. NIAS- FCM (66),
4. mycotoxins (20),
5. alkaloids (66)
6. environmental contaminants (dioxins, PCBs, flame retardants)
7. additives (several classes)

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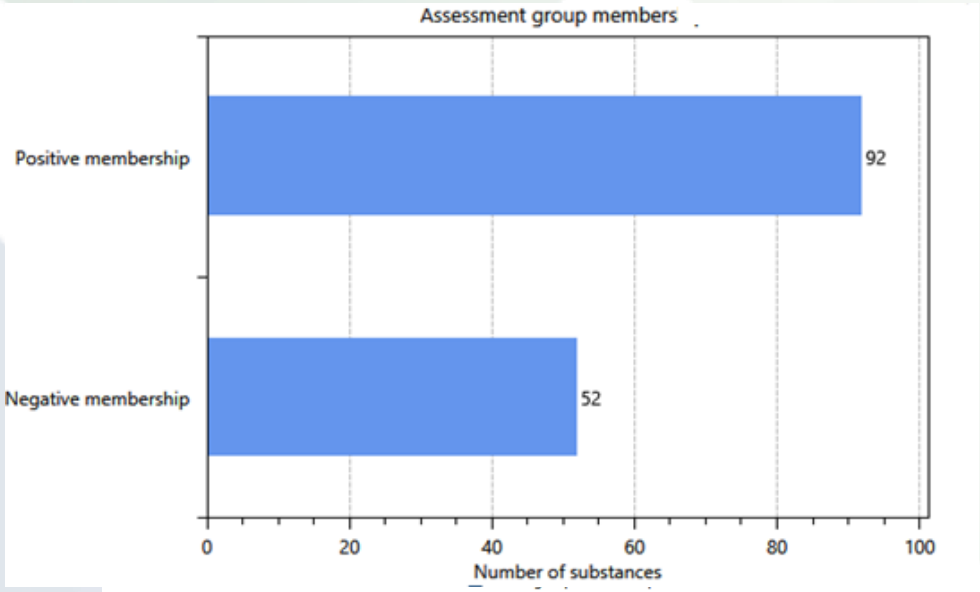
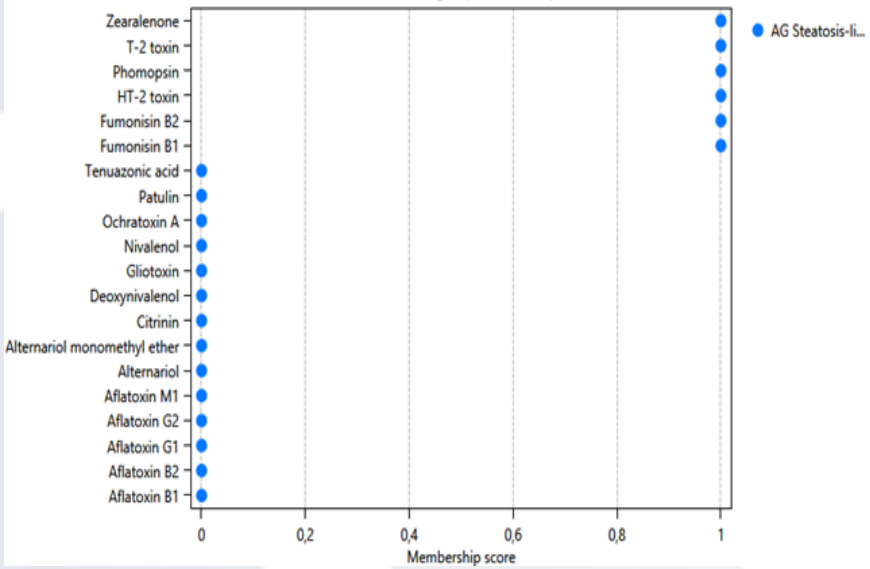
QSAR useful for grouping





144 pesticides grouped following EFSA Pesticide Unit, which still has to go through a EFSA toxicological and stakeholder review process

144 can be reduced to 92 relevant pesticides

In the meanwhile 6 mycotoxins are positive

Combined effect pesticides and mycotoxins?

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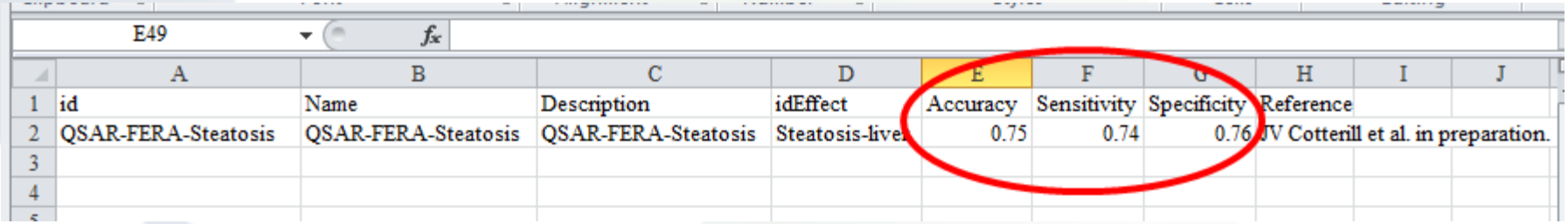
Endpoint specific QSAR

EuroMix effort

1. 29 QSARs and 21 molecular docking were tested

2. 80.000 test results inserted in the EuroMix data and model platform

3. Many QSARs performed poorly, an endpoint specific QSAR performed the best



E49

	A	B	C	D	E	F	G	H	I	J
1	id	Name	Description	idEffect	Accuracy	Sensitivity	Specificity	Reference		
2	QSAR-FERA-Steatosis	QSAR-FERA-Steatosis	QSAR-FERA-Steatosis	Steatosis-liver	0.75	0.74	0.76	JV Cotterill et al. in preparation.		
3										
4										

Recommendation:

1. Consider to continue on mixture endpoint specific QSAR
2. Need for future EFSA guidance on the use of in silico models in human risk assessment

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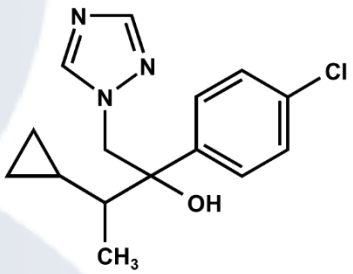
European Test and Risk Assessment Strategies for Mixtures (EuroMix)

- define a bioassay tool box to represent key elements of the AOP for liver steatosis

training compound → **Proof of Principle: AOP** → **AOP**

- test compound: cyproconazol



- Widely used fungicide
- Mode of action: Inhibition of the ergosterole synthetase (CYP51)
- Known target organs (chronic exposure): liver; endocrine system

- cellular system: HepaRG cells

- retain many characteristics of human hepatocytes
- express CYPs, Phase II enzymes, hepatic drug transporters and nuclear receptors

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Test concept: first step

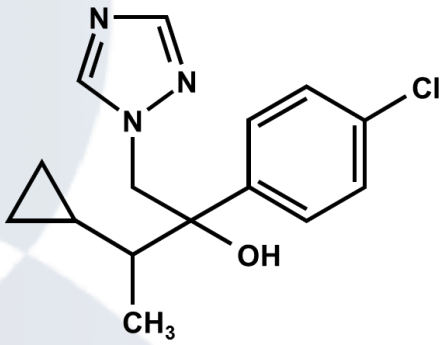
- identify optimal *in-vitro* models and define a bioassay tool box

training compound

Proof of Principle: AOP

AOP

Cyproconazole



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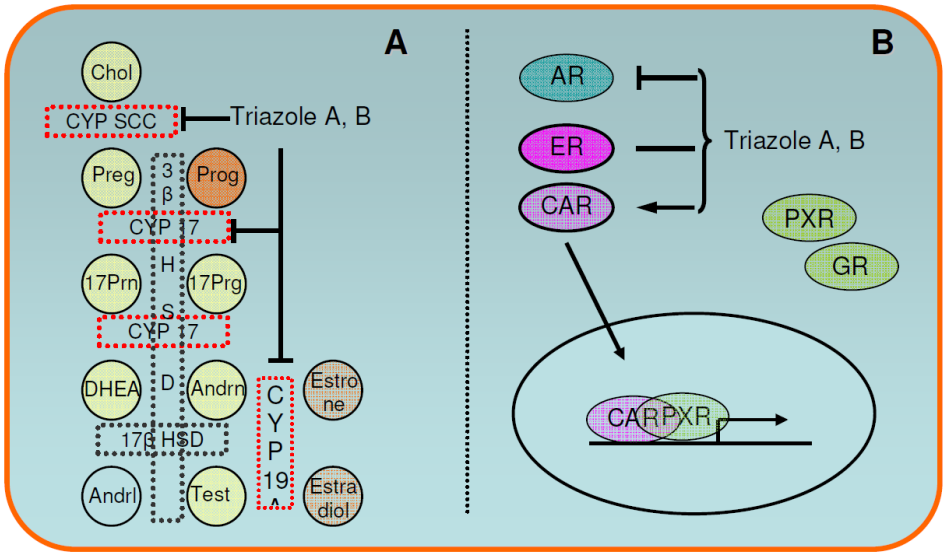

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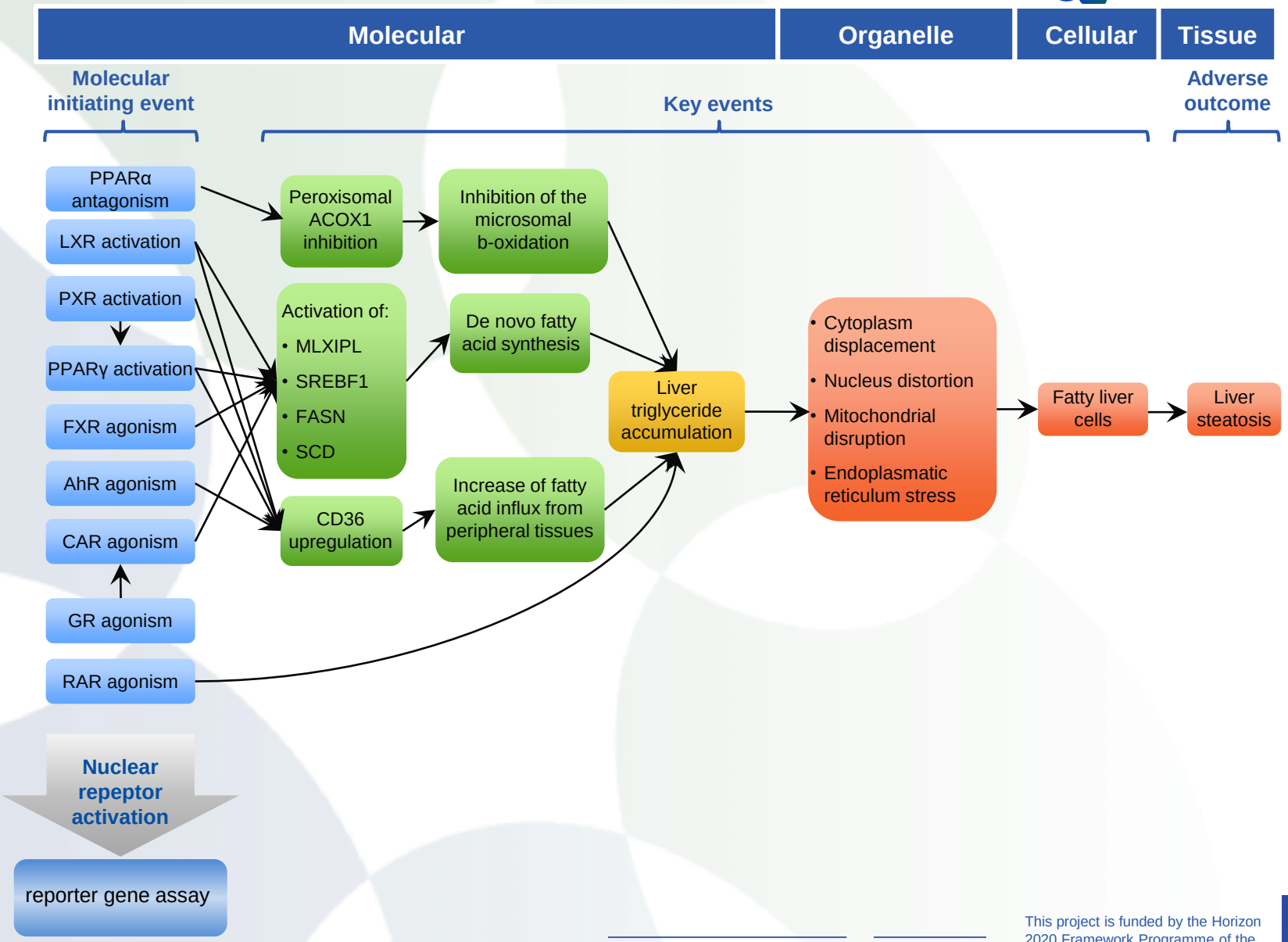
AOP for liver steatosis



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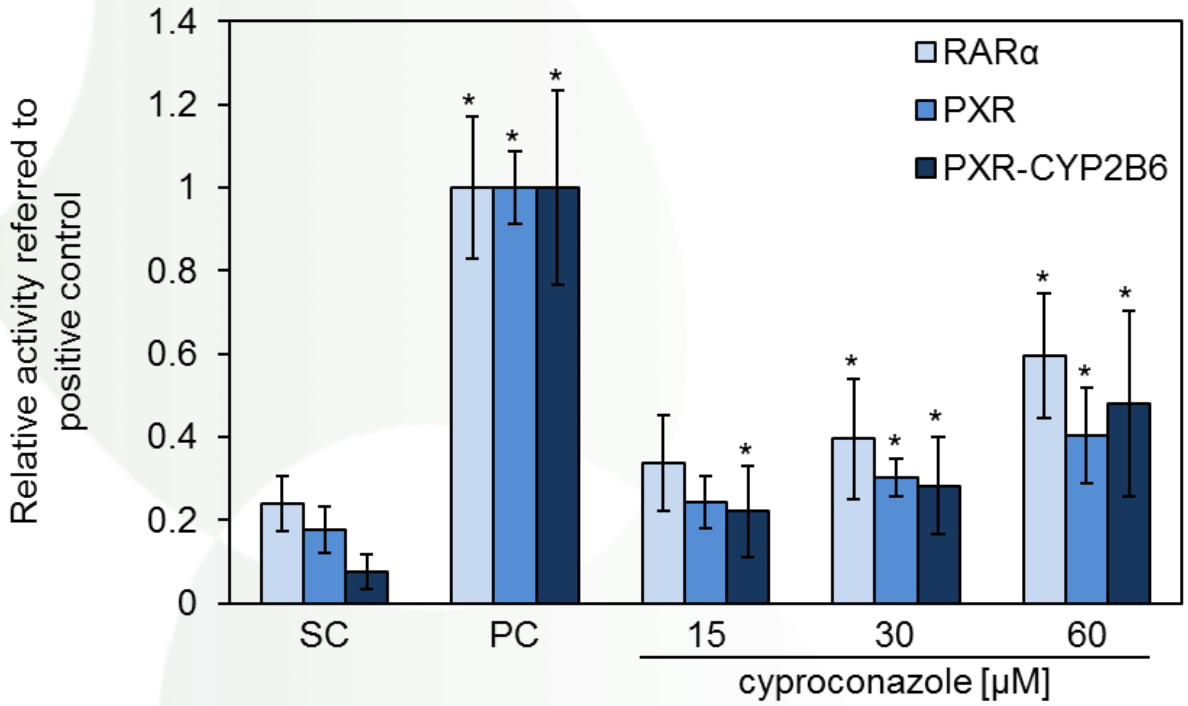
Nuclear receptor activation
→ reporter gene assays

A

Assay	Activation
AhR	-
CAR	-
CAR-CYP2B6	-
FXR	-
GR	-
LXRα	-
PPARα	-
PPARγ	-
PPARδ	-
PXR	+
PXR-CYP2B6	+
RARα	+
RXRα	-
VDR-CYP2B6	-

B



HepG2/HEK293; 24h

→ **RARα and PXR as 2 of the 9 receptors in the AOP were activated by Cyproconazole**

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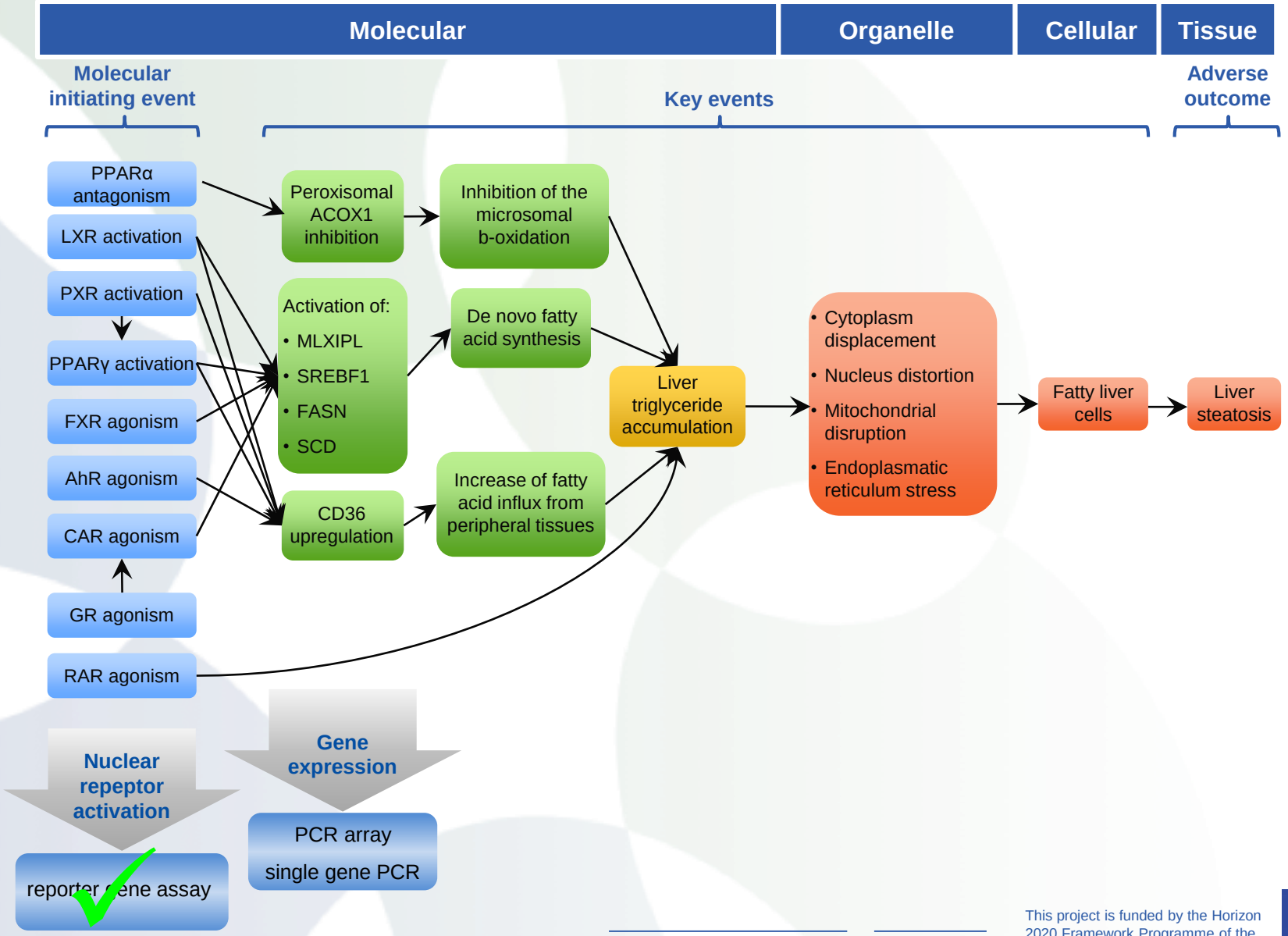
AOP for liver steatosis



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Gene expression analysis

EuroMix

A

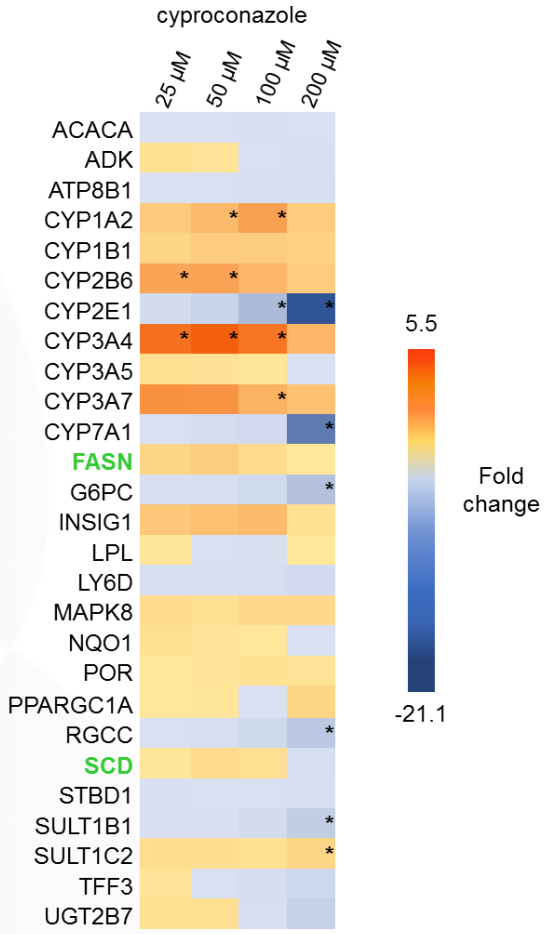
69 steatosis specific target genes

Gene	Fold change	Gene	Fold change	Gene	Fold change
ACACA	1.93	CYP3A7	6.00	MTTP	-1.30
ACOX1	1.01	CYP7A1	-2.13	NOS2	-1.18
ADK	1.90	CYP7B1	-1.25	NQO1	1.82
ALDH1A1	-1.28	DNM1	1.12	NR0B2	-1.21
ALDH2	1.50	ENO1	-1.18	PCCA	-1.29
AQP2	N.D.	FAS	1.06	PDK4	-1.70
ATP8B1	1.80	FASN	-2.59	PNPLA3	-1.50
CCL5	-1.13	FBXO32	1.37	POR	2.02
CD36	-1.22	G6PC	-1.97	PPARA	1.75
CEBPD	-1.60	G6PD	1.31	PPARGC1A	2.31
CES2	1.25	GPD1	-1.44	RETN	N.D.
COMT	1.20	HAAO	-1.23	RGCC	-2.05
CYBB	1.04	HADHB	1.62	SCD	2.14
CYP1A2	5.00	IL6	-1.60	SLCO4A1	1.21
CYP1B1	4.62	INSIG1	2.03	SREBF1	-1.38
CYP2A6	1.09	JUN	1.46	STBD1	1.80
CYP2B6	6.91	KHK	-1.28	SULT1B1	-3.40
CYP2C19	1.51	LMNA	-1.17	SULT1C2	2.63
CYP2C9	-1.17	LPL	2.31	SYT1	-1.12
CYP2D6	1.21	LY6D	-2.31	TFF3	-1.95
CYP2E1	-3.50	MAPK8	2.06	TUBB2B	1.36
CYP3A4	14.70	MLXIPL	-1.21	UGT2B7	-1.87
CYP3A5	2.45	MSMO1	1.66	VCP	-1.24

AOP-genes
up regulation
down regulation

HepaRG; 200 µM cyproconazole, 24h

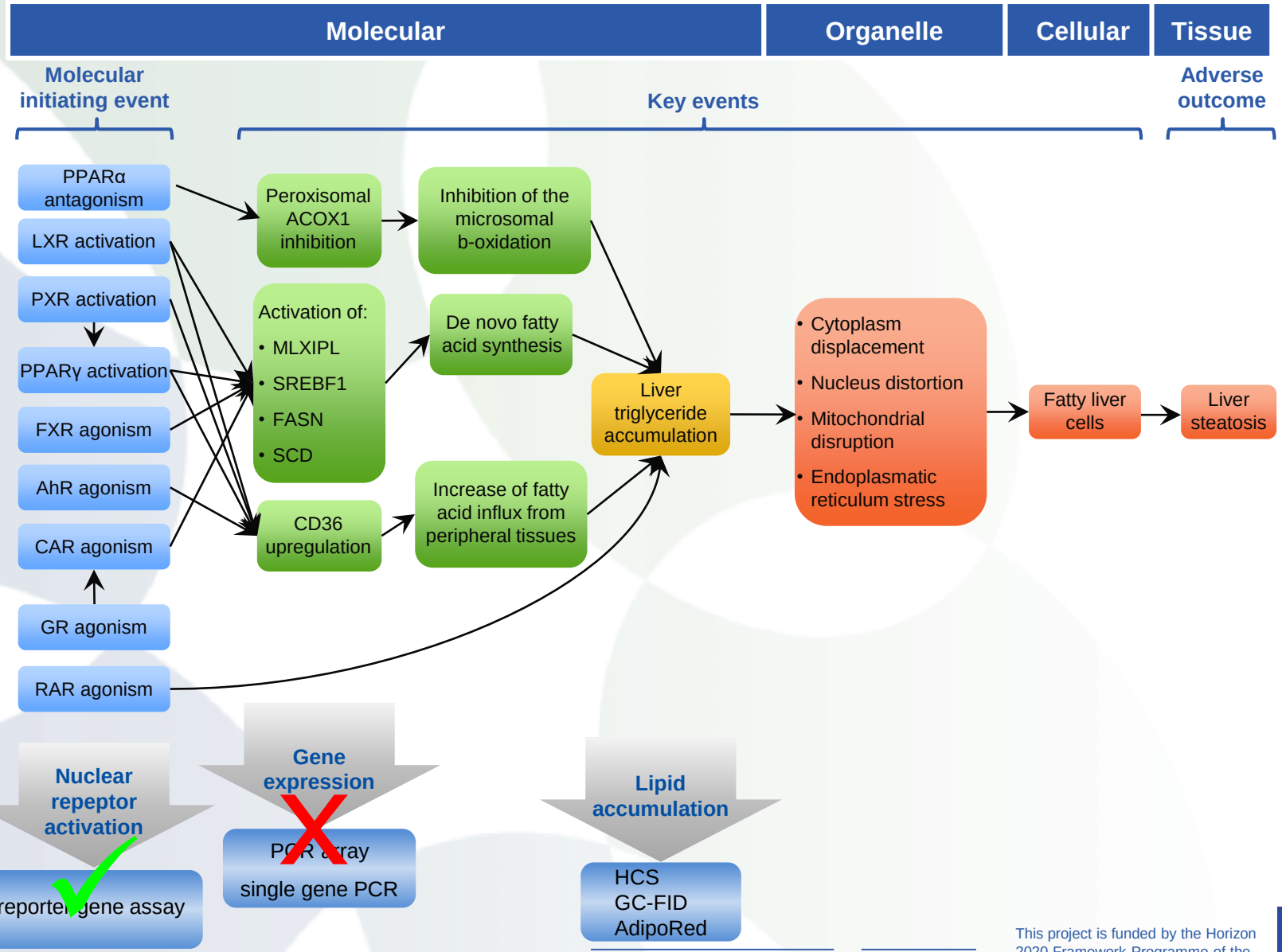
B



HepaRG; 25-200 µM cyproconazole, 24h

→ **The 6 AOP-specific genes are not deregulated by cyproconazole**
→ **Screening 69 steatosis specific target genes revealed other genes that might be involved in key events**

AOP for liver steatosis



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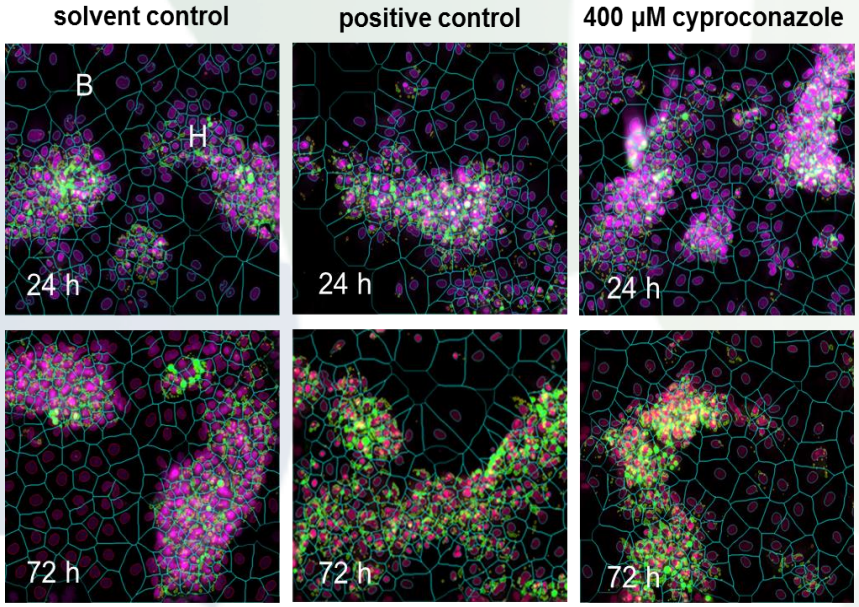
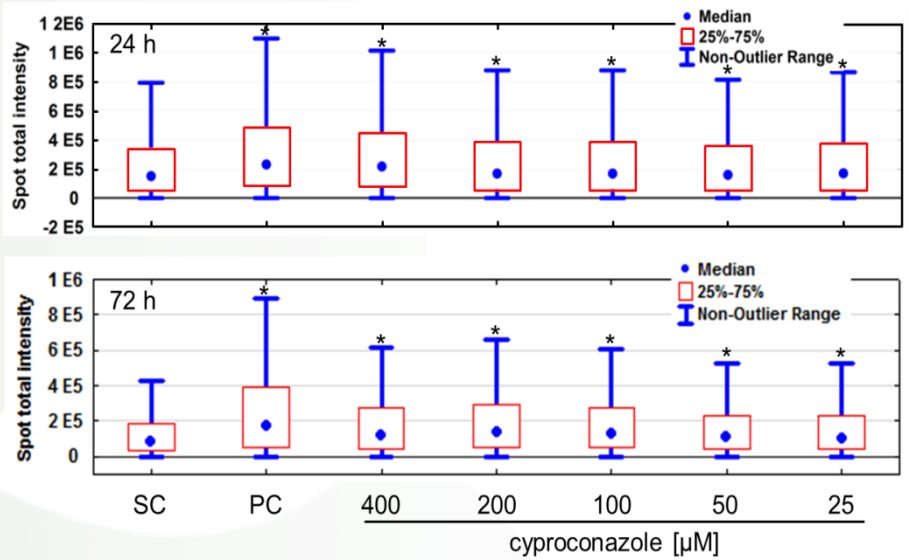
Liver triglyceride accumulation

High content screening



neutral lipids, cell nuclei



HepaRG; 25-400 µM cyproconazole, 24h, 72h

→ **Cyproconazole induces neutral lipid accumulation after 24 h and 72 h**

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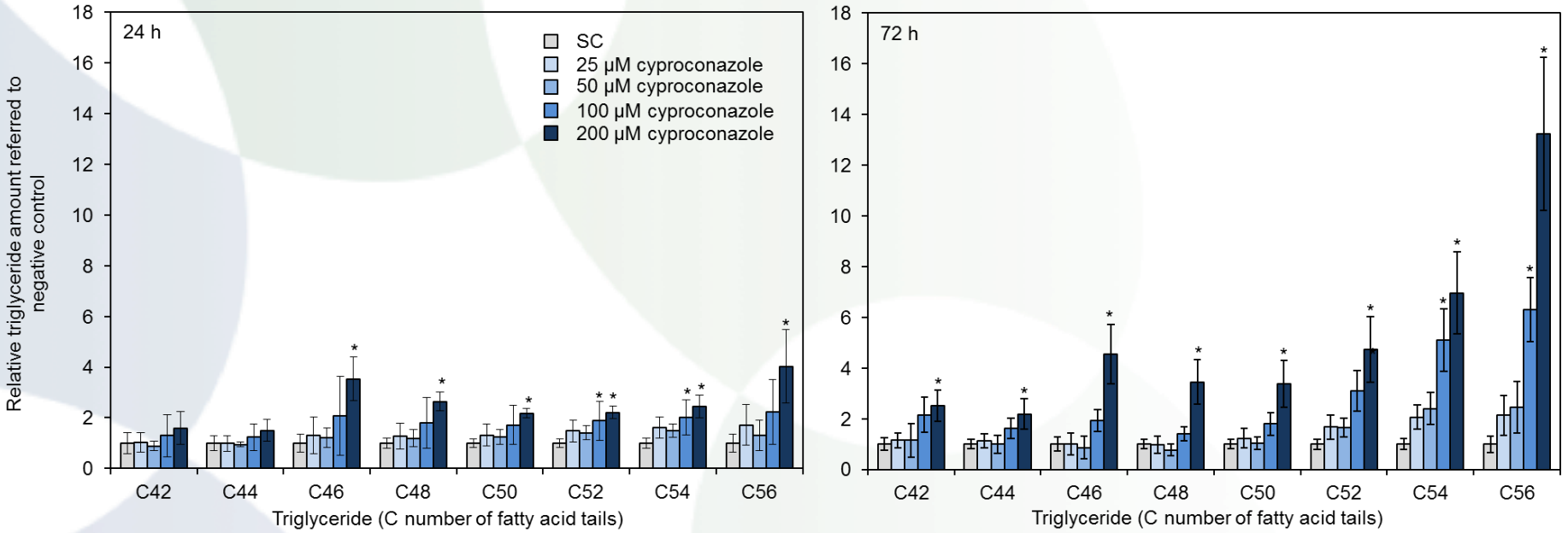
Liver triglyceride accumulation

GC-FID



HepaRG; 25-200 µM cyproconazole, 24h, 72h

→ **Cyproconazole induces neutral lipid accumulation after 24 h and 72 h**

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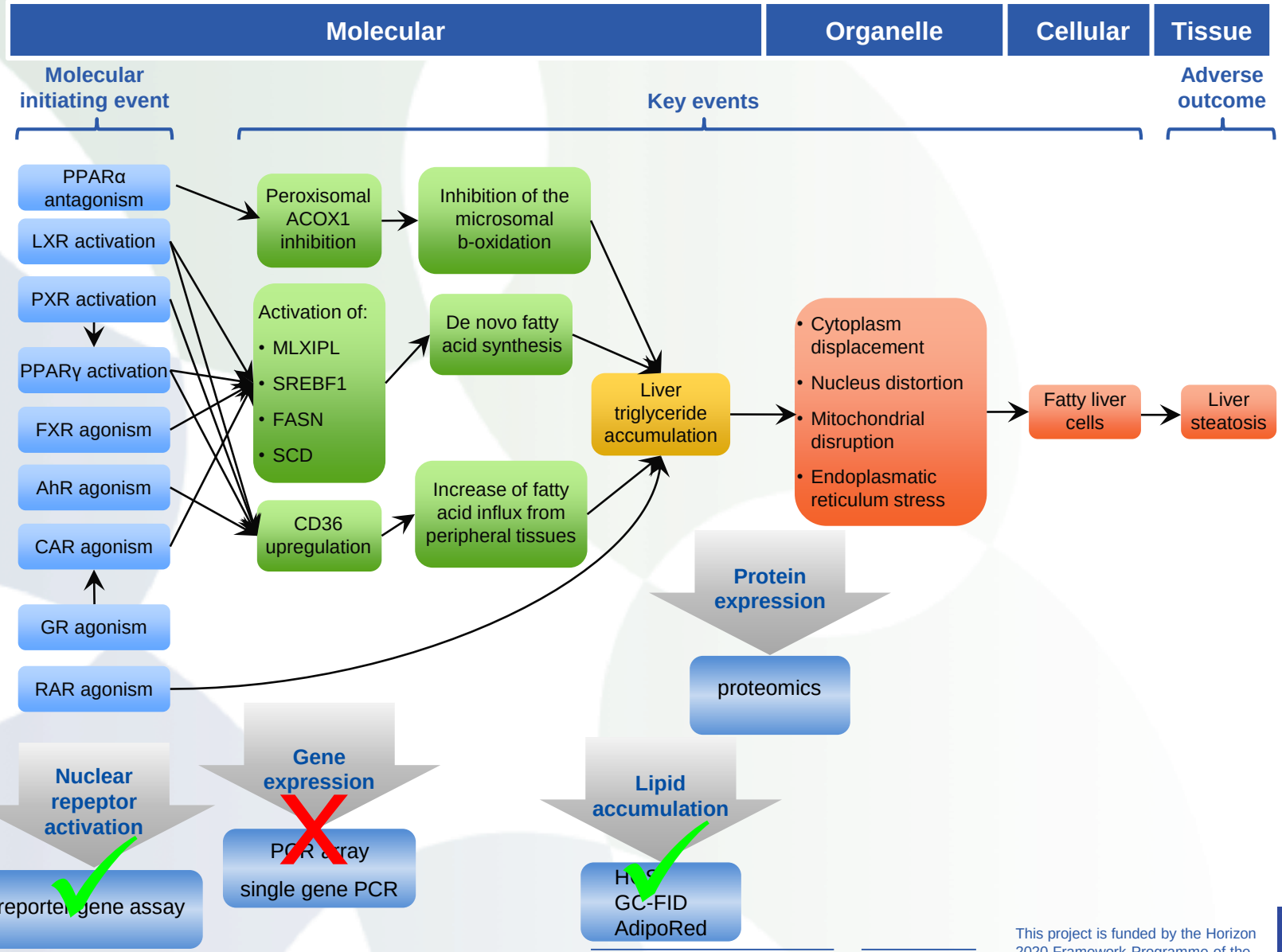
AOP for liver steatosis



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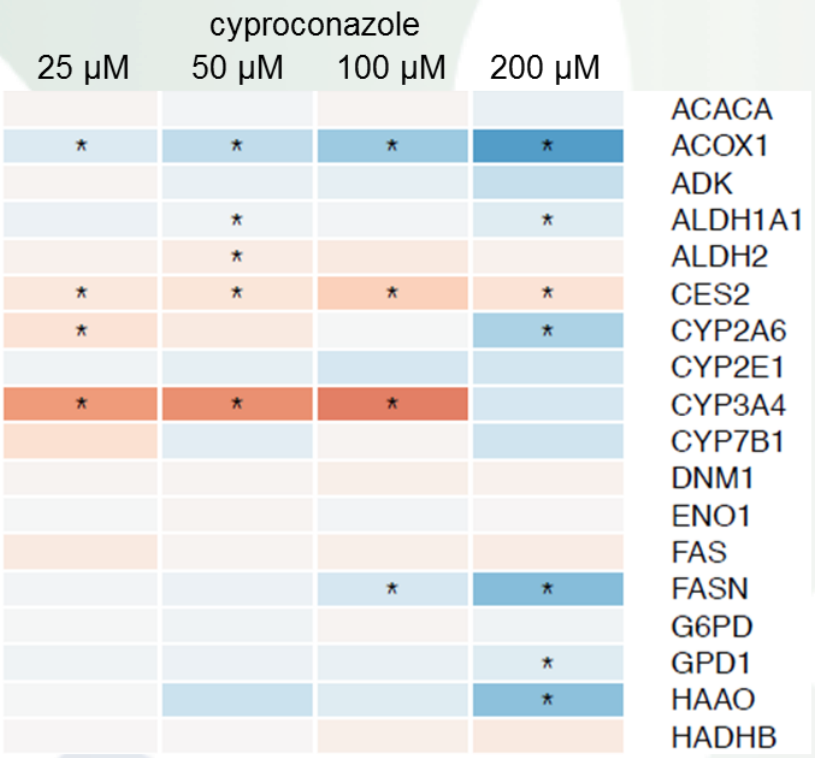
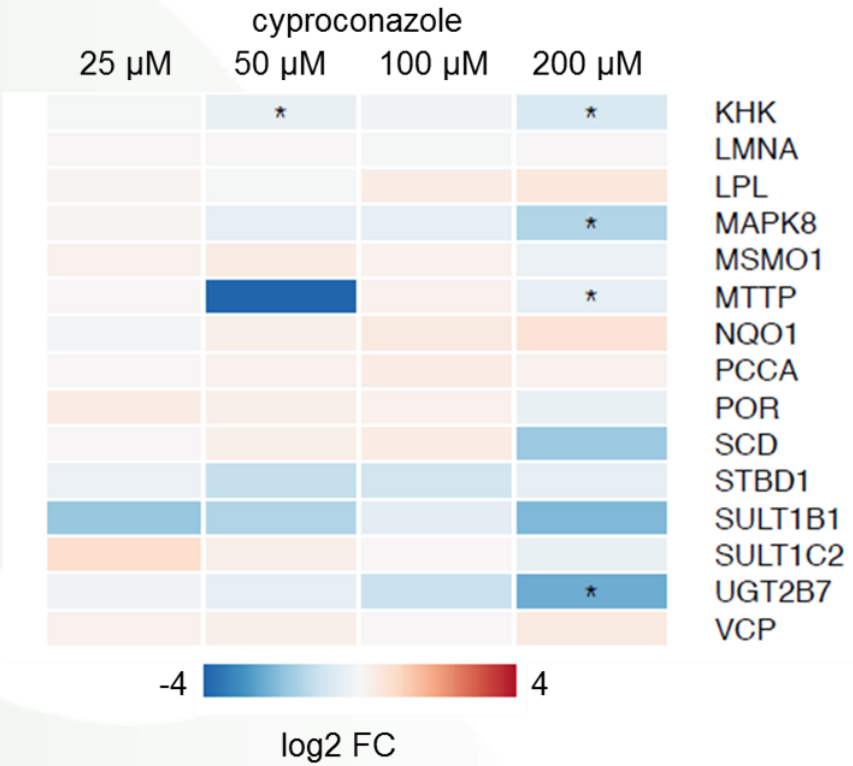
Protein abundance changes





HepaRG; 25-200 µM cyproconazole, 72h

→ **At the protein level, 72 h exposure to cyproconazole leads to deregulation of key transporters and enzymes involved in xenobiotic and lipid metabolism**

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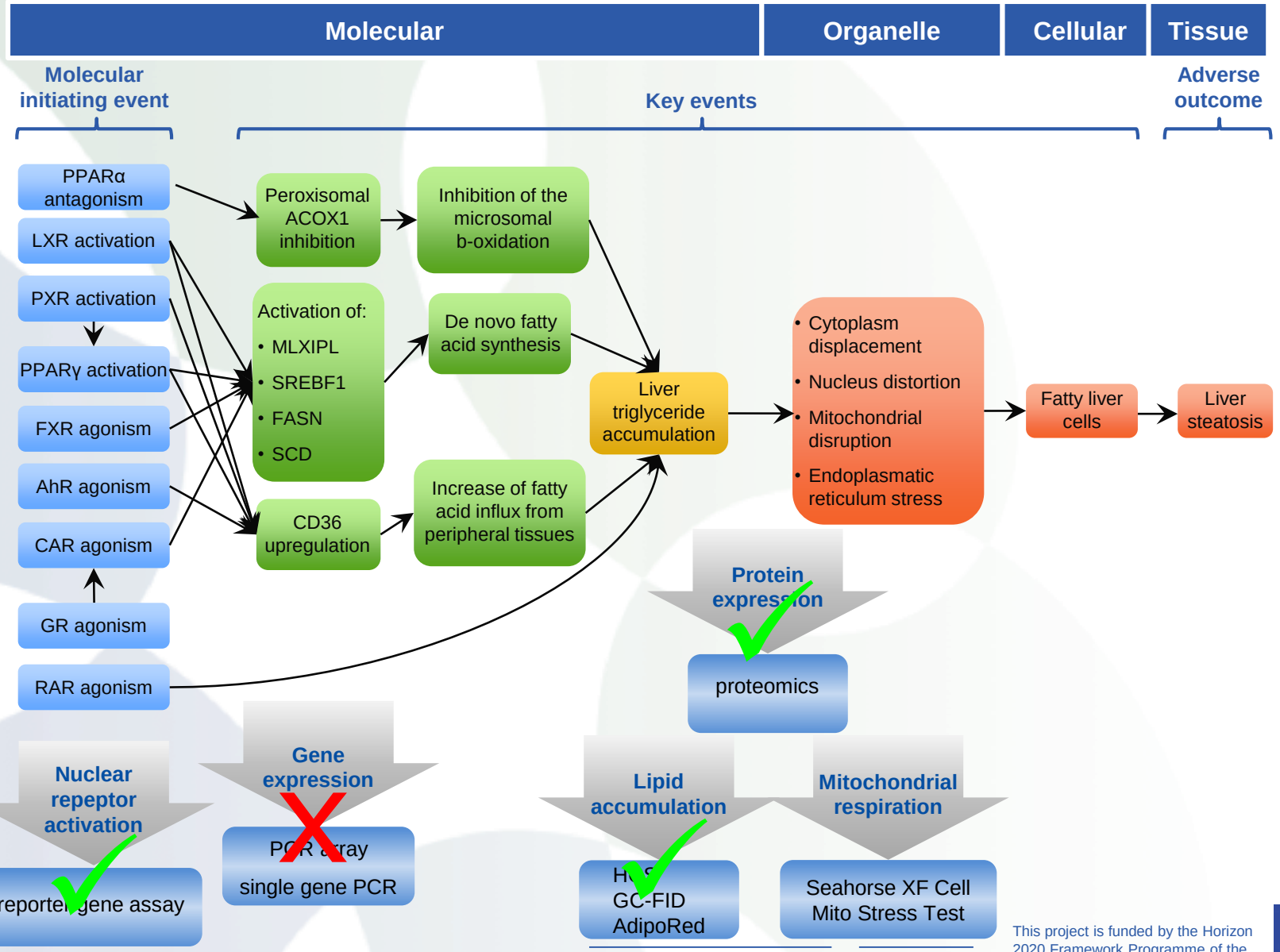
AOP for liver steatosis



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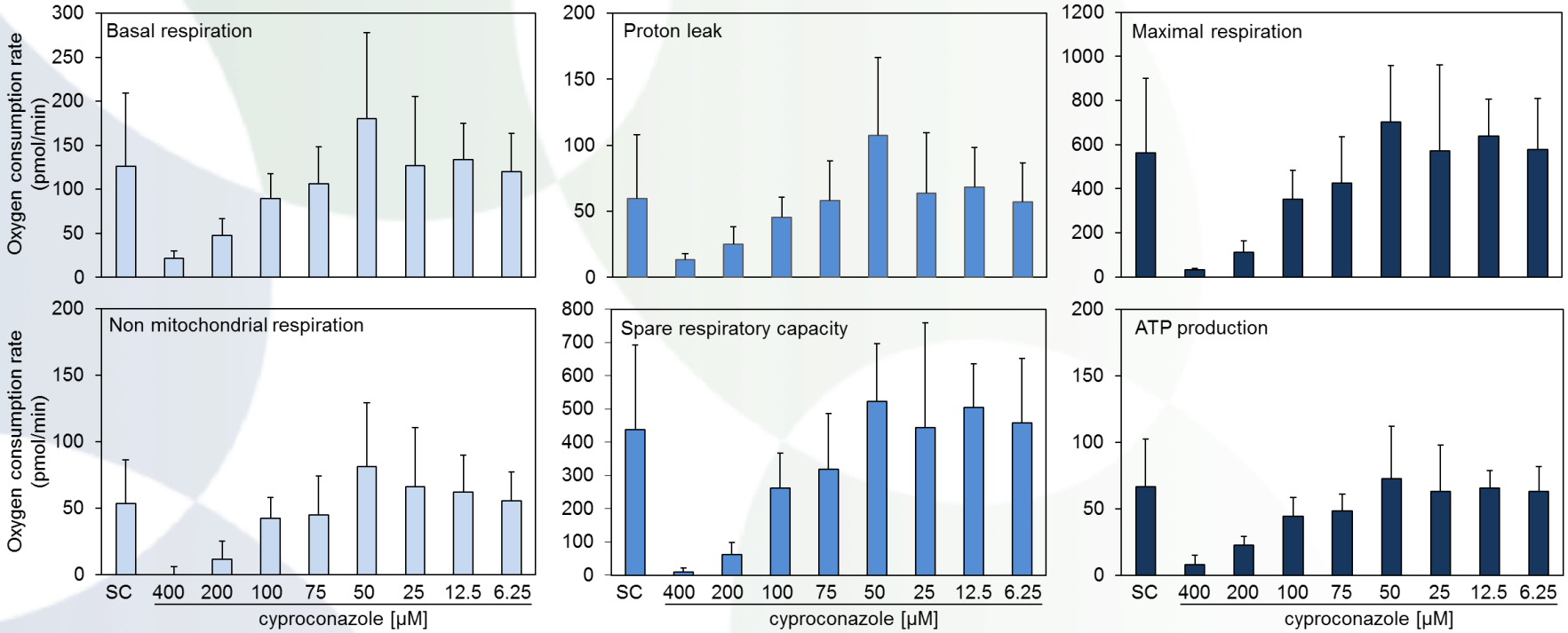
Mitochondrial disruption

Seahorse XF Cell Mito Stress Test



HepaRG; 6.25-400 µM cyproconazole, 72h

→ Cyproconazole disrupts mitochondrial respiration after 72 h

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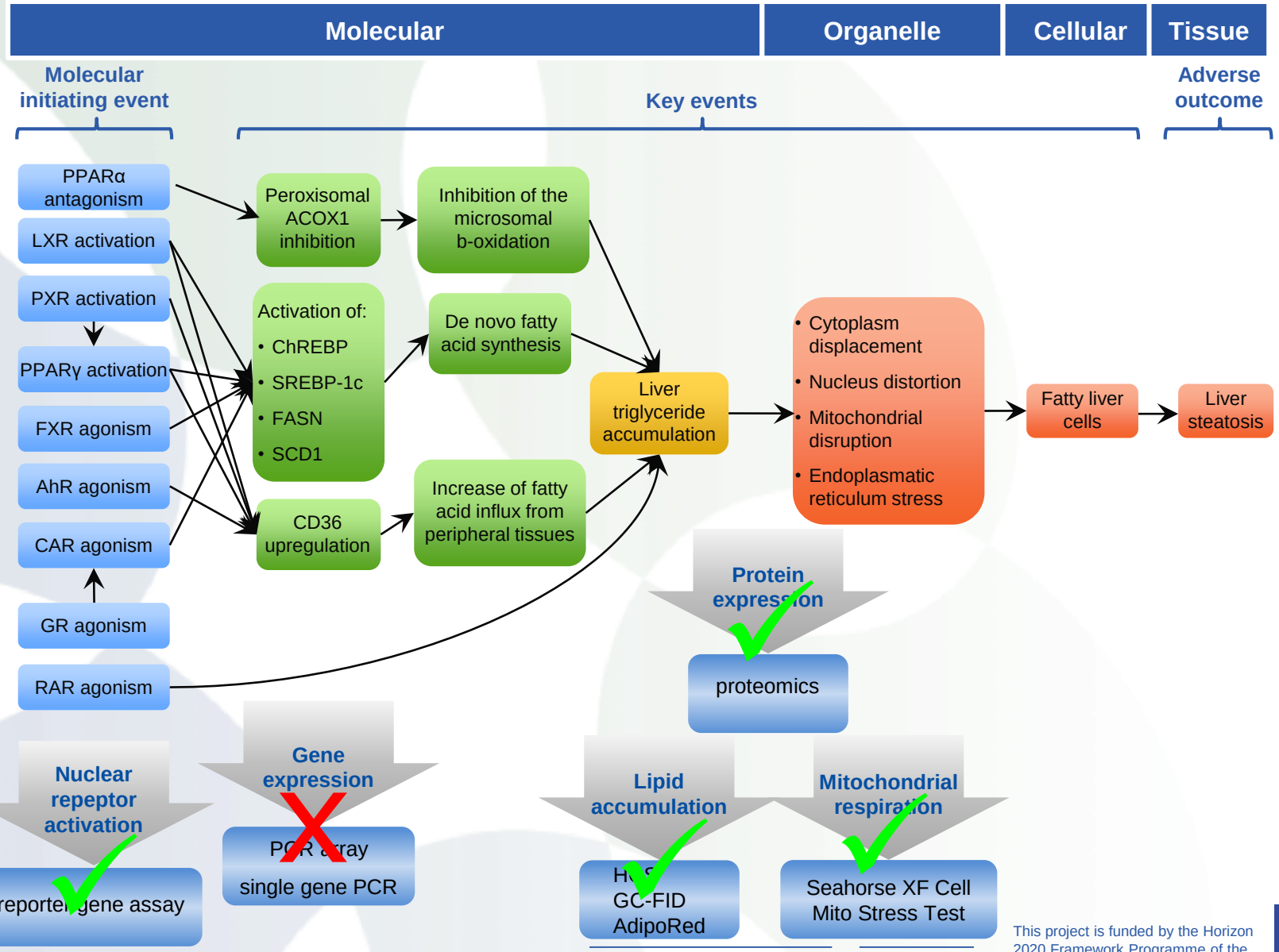
AOP for liver steatosis - summary



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Test concept: second step

- testing combined effects of compounds

Proof of Principle: AOP

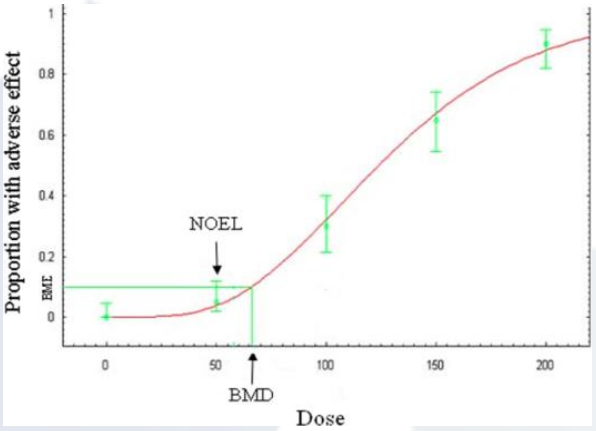
training compound → **AOP**

Mixture analysis: similar/dissimilar MoA

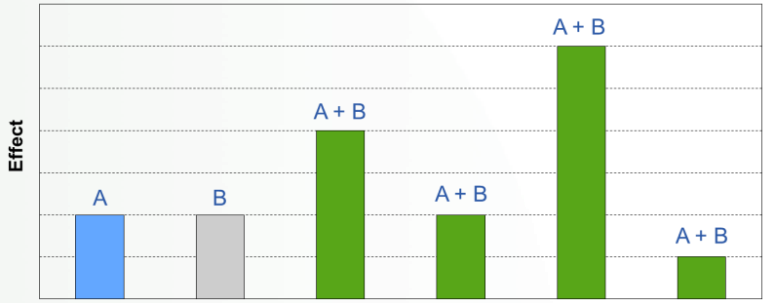
test compounds

single compounds ⟷ **combinations**

- dose response curves
- Relativ Potency Factors (RPF)



- equipotent mixtures
- dose addition?
- interactions?



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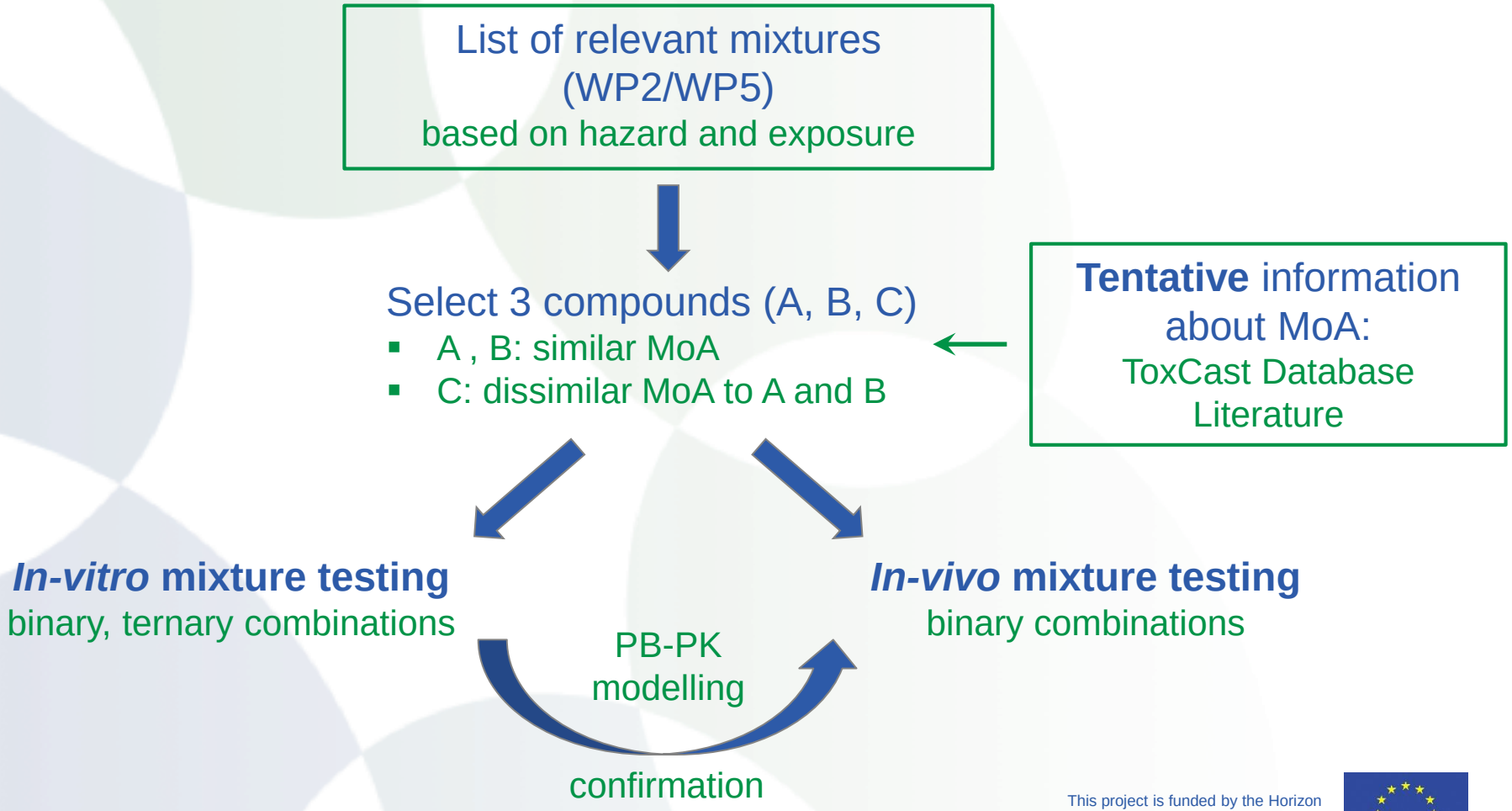
Selection of compounds for mixture experiments

Aim: Identify mixtures for *in-vitro* and *in-vivo* testing



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Selection of compounds for mixture experiments (hazard & exposure based)

Similar MoA
→ PXR activation

- Imazalil (activation of PXR, AhR, CAR, RAR)
- Thiacloprid (activation of PXR and PPARγ)
- Fenpyroximate (activation of PXR)
- T0901317 (activation of PXR, LXR)

Similar MoA
→ PPAR activation

- PHX (activation of PPARα/γ, RXR, GR, RAR)
- PHP (activation of PPARα/γ, RXR, GR, RAR)

Dissimilar MoA
→ nuclear receptor independent

- Cyclosporin A
- Clothianidin

Select 3 compounds (A, B, C)

- A, B: similar MoA
- C: dissimilar MoA to A and B

Imazalil
Thiacloprid
Clothianidin

***in vitro* and *in vivo* mixture testing**

PHX
PHP
Clothianidin

***in vitro* testing**

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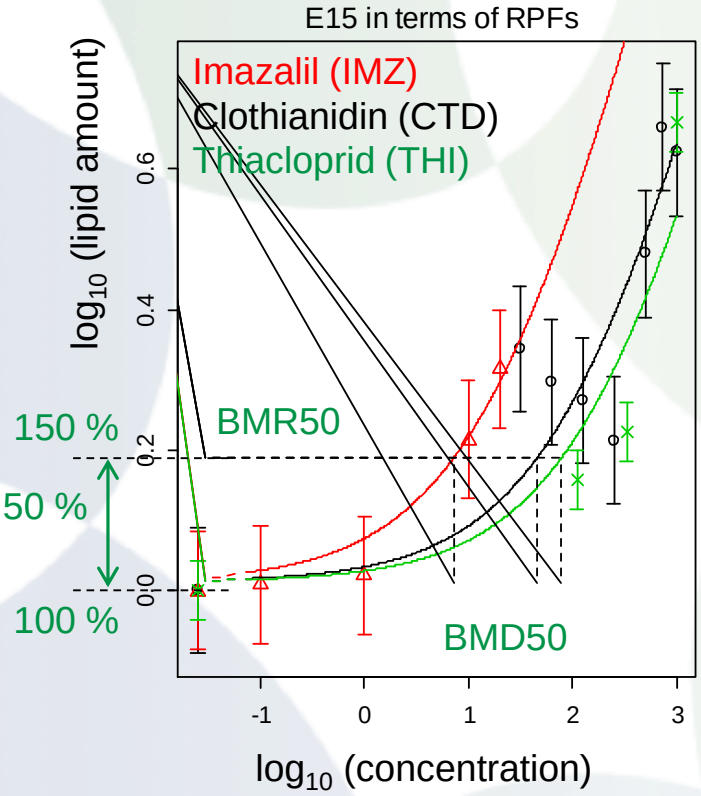
Estimation of relative potency factors (RPF) for mixture testing

- RPF was estimated from lipid analyses (GC-FID) of single compounds (BMD50)
- RPFs are calculated with dose response modelling software PROAST by comparing the whole curves of each compound :



RPF based on BMD50:
RPF-THI (reference) : 1
RPF-CTD : 1.717
RPF-IMZ : 10.87

→ Imazalil is 11 times more potent than Thiacloprid

→ epuipotent mixture testing in all toolbox assays

BMD – Bench Mark Dose

BMR – Bench Mark Response

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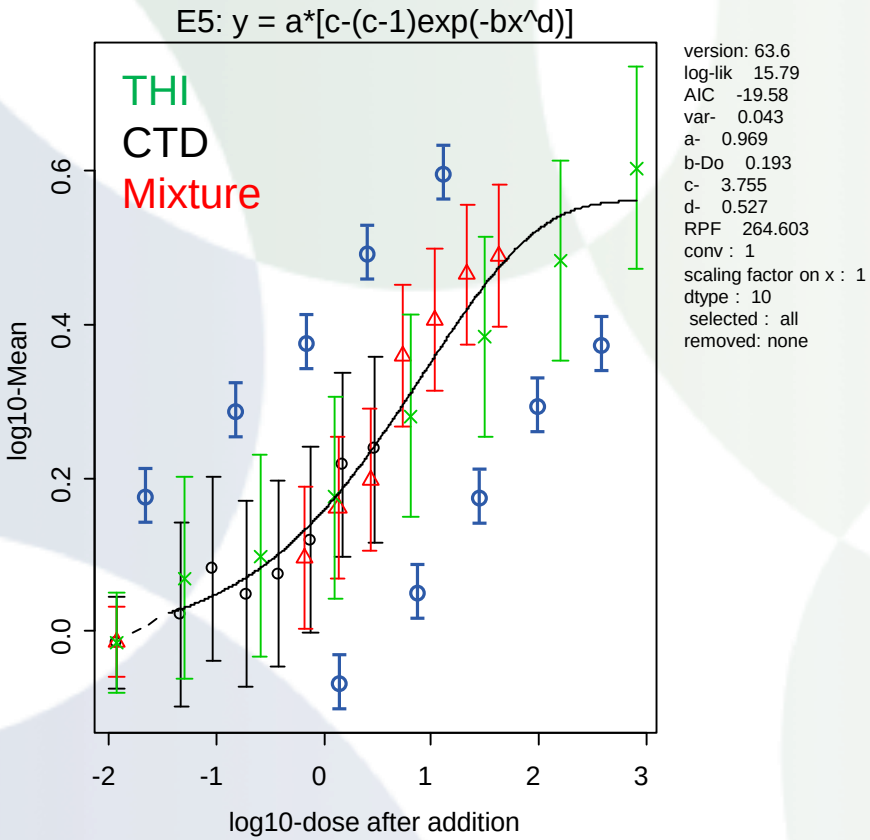
Dose addition analysis with PROAST

Adjustment of RPF and dose range



dose addition:
most confidence intervalls intersect curve

synergism:
responses of the mixtures will be shifted to the left

antagonism:
responses of the mixtures will be shifted to the right

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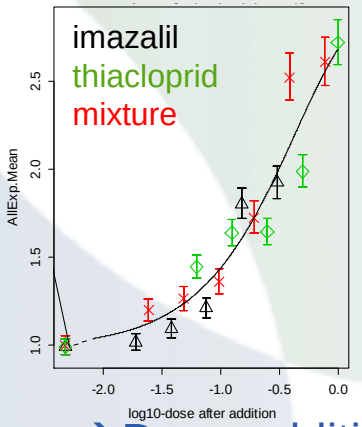
Results: Lipid accumulation
High content screening (HCS)

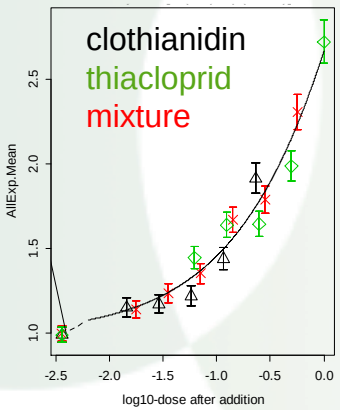
EuroMix

IMZ+THI



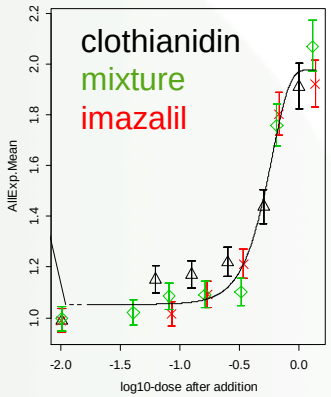
→Dose addition

CTD+THI



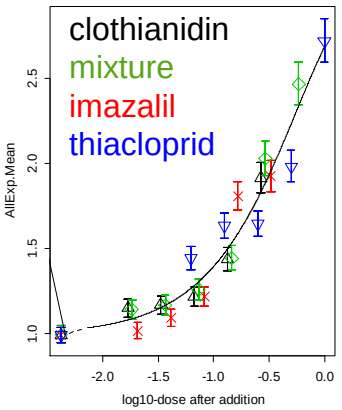
→Dose addition

CTD+IMZ

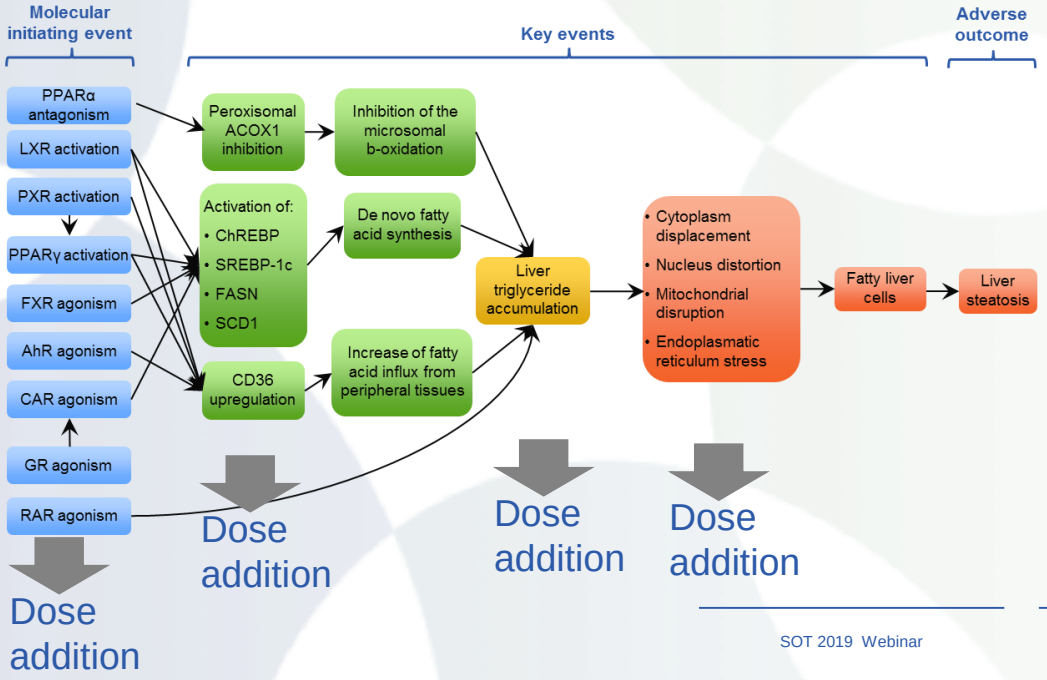


→Dose addition

CTD+IMZ+THI



→Dose addition



- All toolbox assays show similar results
- *in vitro* toolbox successfully tested for mixtures

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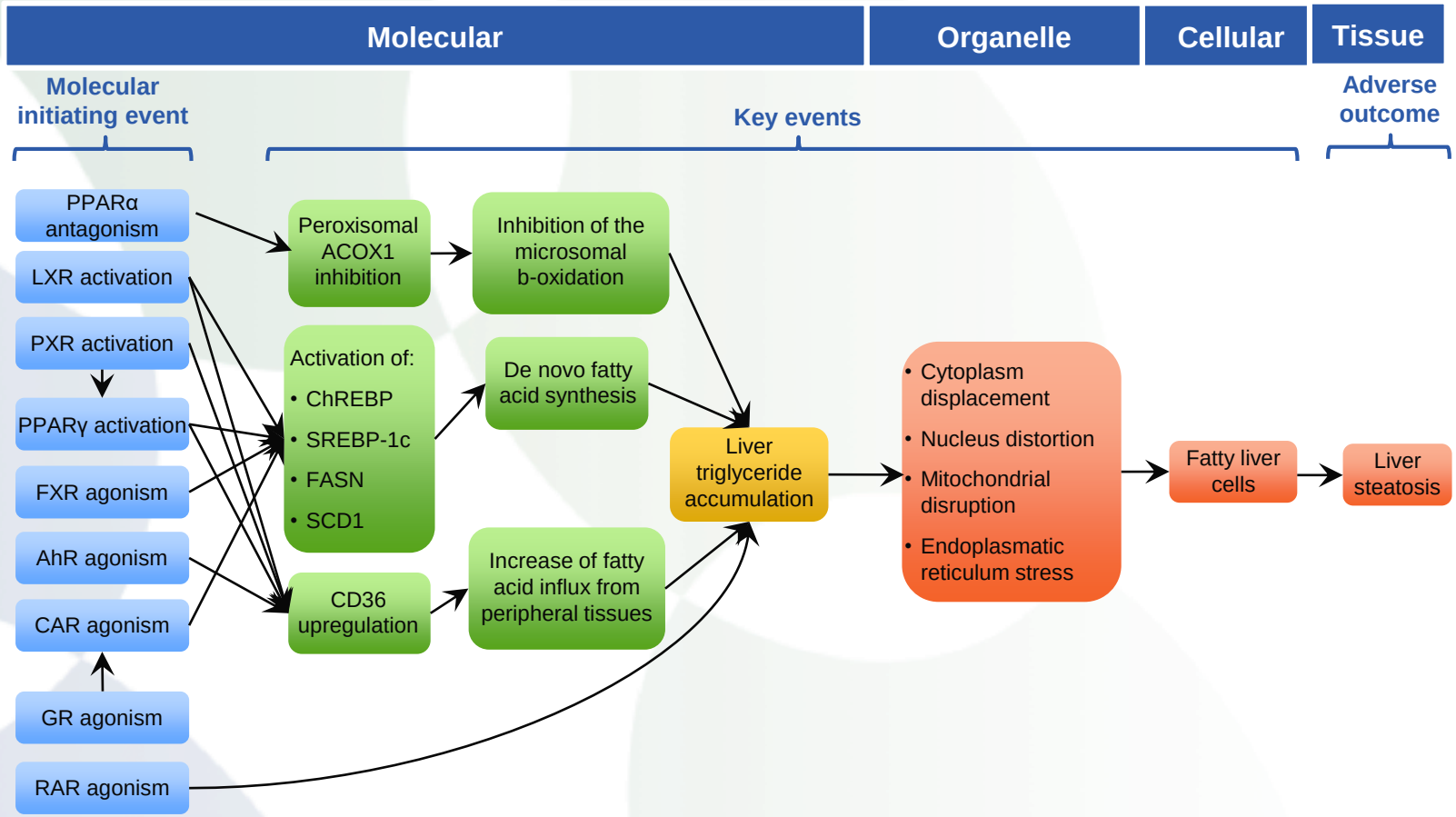
Is the MoA of Clothianidin really NR independent ???



Thiacloprid (activation of PXR and PPARγ)
Imazalil (activation of PXR, AhR, CAR,RAR)

Similar MoA
→ mainly PXR activation

Clothianidin

Dissimilar MoA
→ nuclear receptor independent

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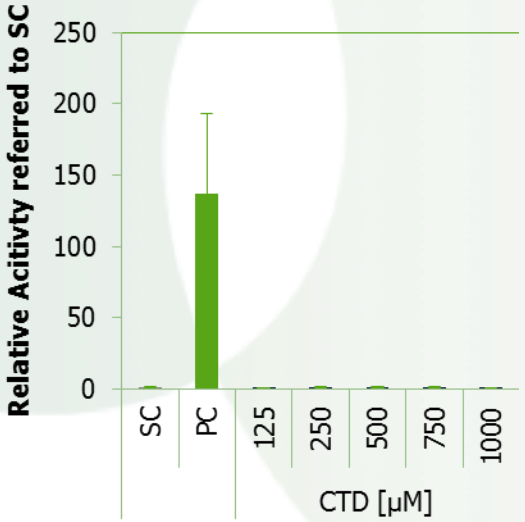
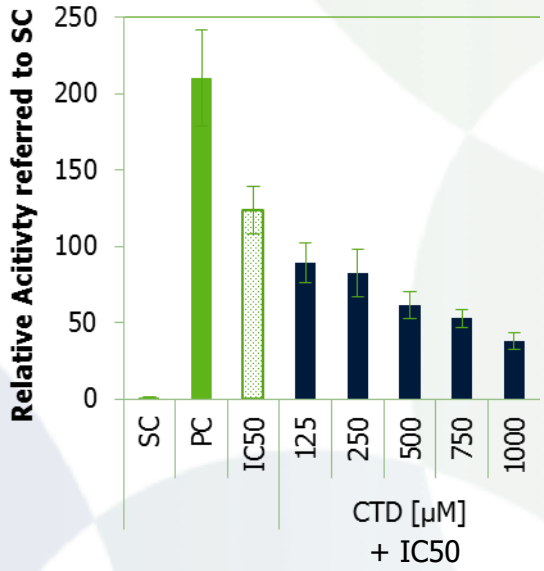
Clothianidin: PPARa antagonism?!

PPARa agonism



→ ~~PPARa agonist~~

PPARa antagonism



→ PPARa antagonist ✓

Summary E1-E3

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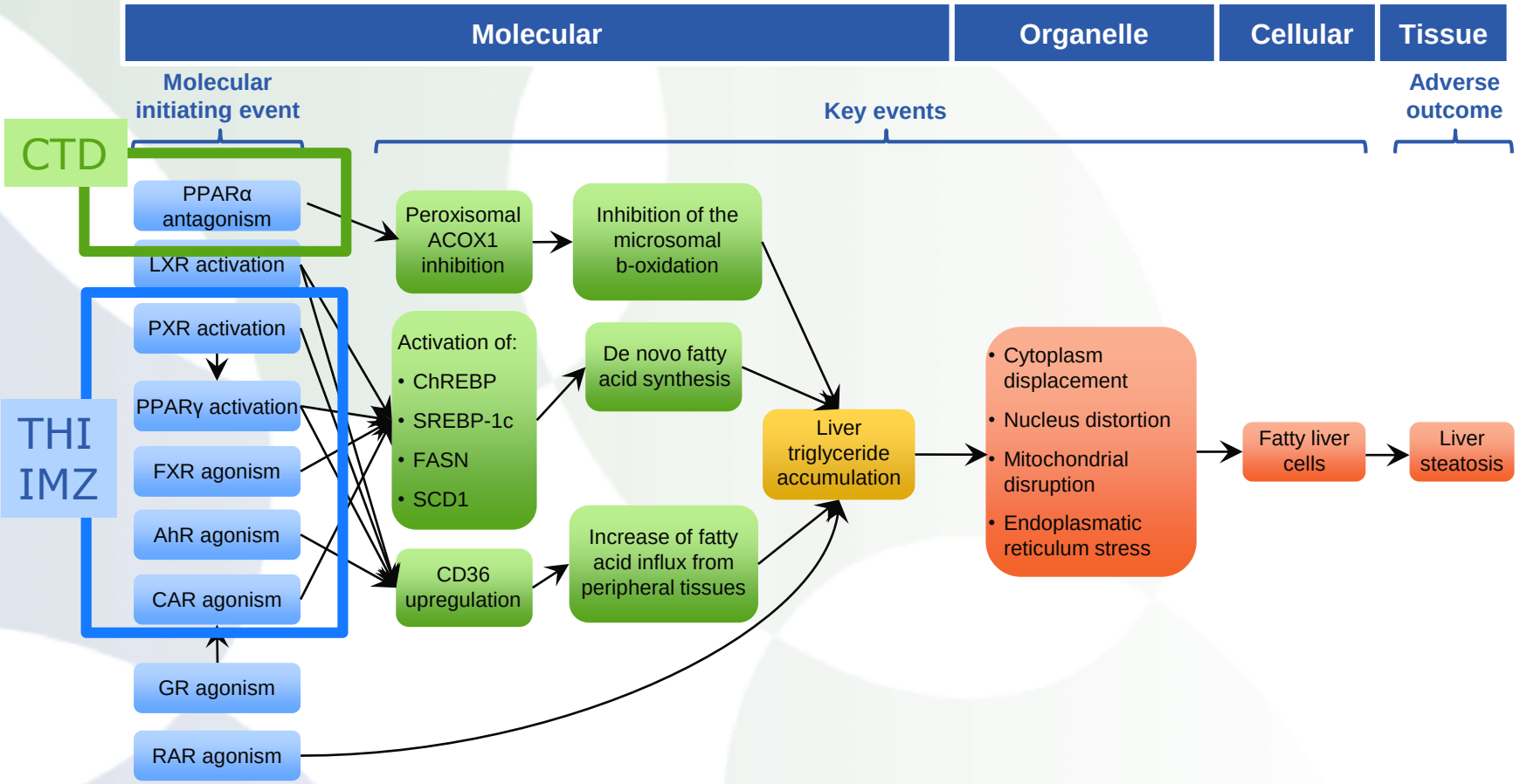
AOP for liver steatosis



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Clothianidin

Dissimilar MoA
→ ~~nuclear receptor independent~~
→ PPARa antagonist

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Summary mixture testing

Results:

1. Reliable Relative Potency Factor (RPF) for each pesticide
2. Test results for pesticides acting via similar or dissimilar MoA
3. Information whether pesticides belong to CAG or not
4. Confirmation/rejection of dose-addition assumption (mixtures)
5. Cost effective tests to generate RPFs for other chemicals (non-pesticides)
6. Implementation of all data in the EuroMix Toolbox Database

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Test concept: third step in vivo confirmation

- Compare relative potency factors generated in vitro and in vivo
- Apply in vitro-in vivo extrapolation
  - one generic PB-PK model in EuroMix toolbox
  - nine specific PB-PK model in EuroMix toolbox

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EuroMix participants

22 beneficiaries from 16 countries linked to international organisations including WHO, FAO and EFSA.
EuroMix is coordinated by RIVM.

EuroMix

Thank you for your attention

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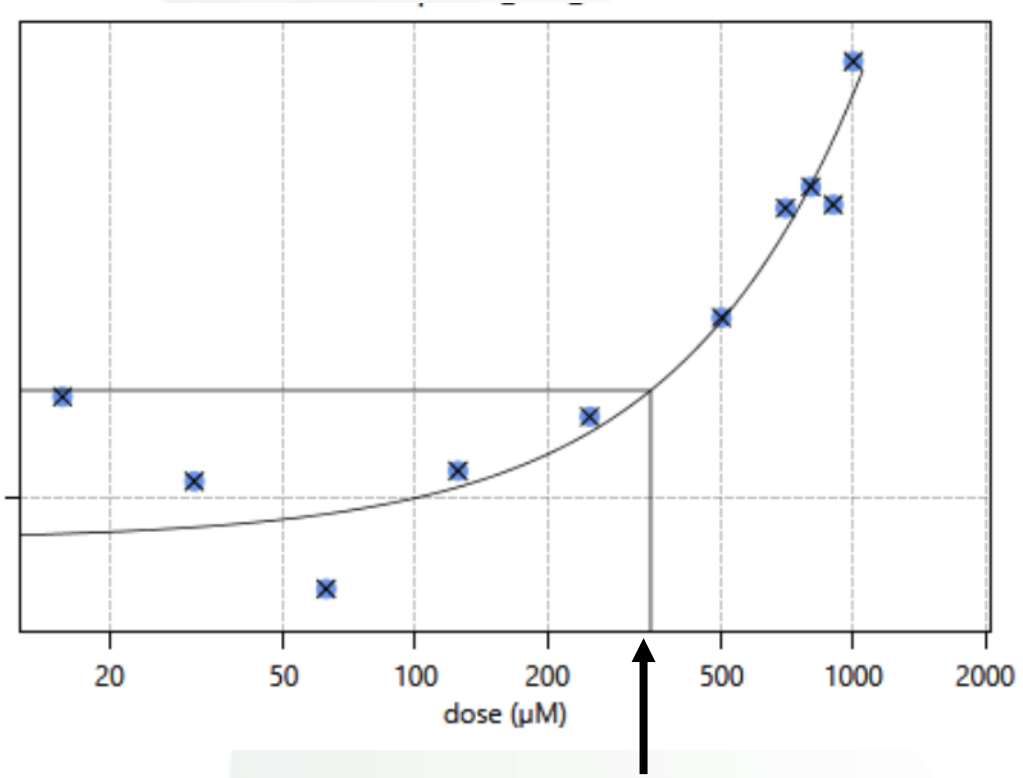
Benchmark dose response modelling

Benchmark response (BMR)



Benchmark dose (BMD)

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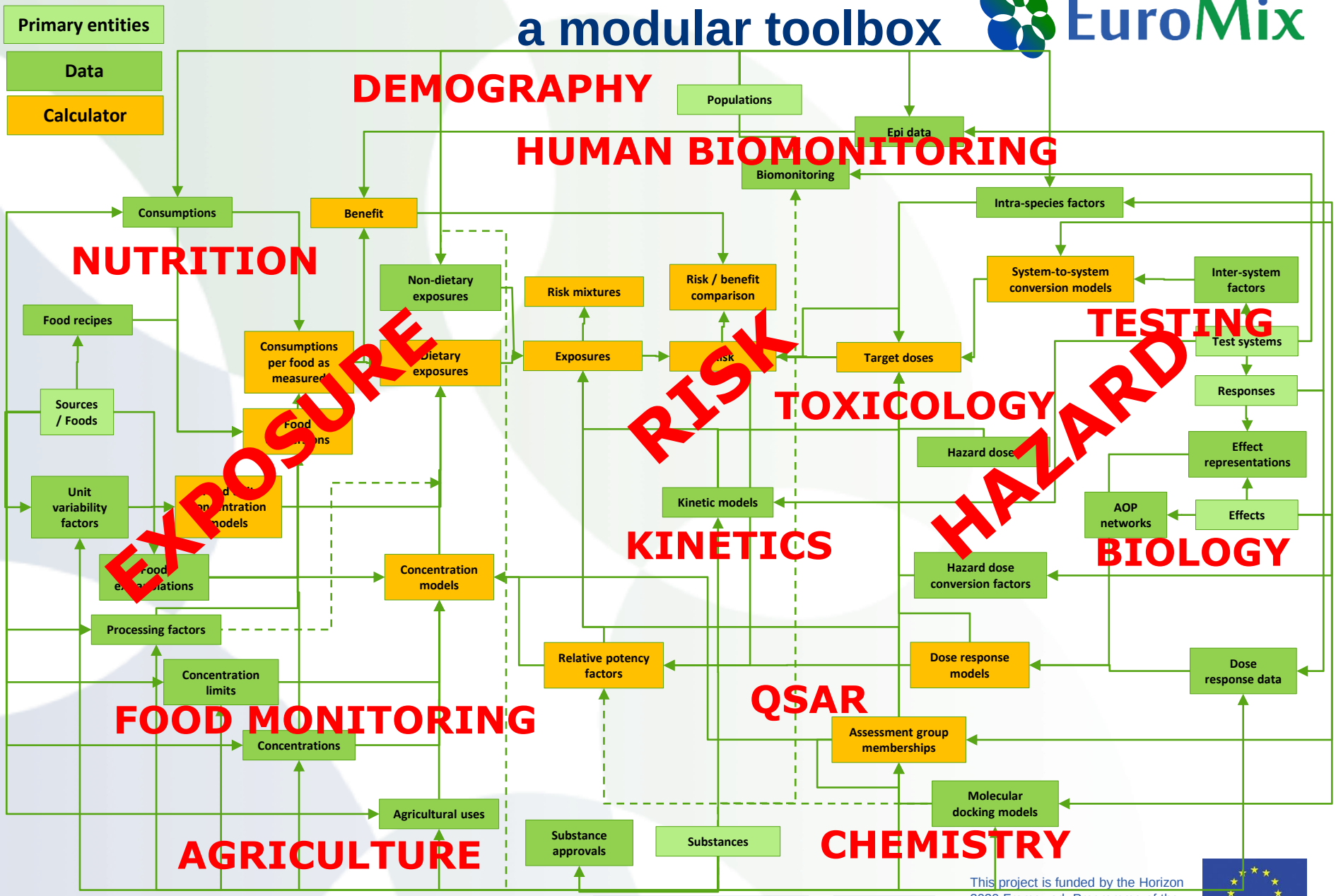
MCRA 9/ EuroMix database: a modular toolbox



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