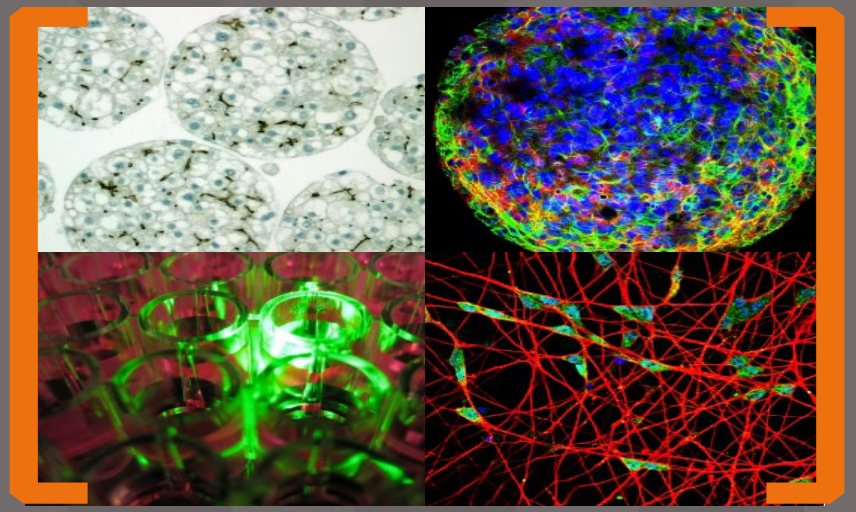
# High throughput transcriptomics (HHTr) applications in read across: EU-ToxRisk experience

Bob van de Water
Leiden University



# The vision of the EU-ToxRisk project

**The vision is to drive a paradigm shift in toxicology towards an animal-free, mechanism-based integrated approach to chemical safety assessment.**

The ultimate aim:

1) pragmatic, solid **read-across procedures** incorporating mechanistic and toxicokinetic knowledge

2) **ab initio hazard and risk assessment strategies** of chemicals with little background information

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# Topics

1. Application of HTTr for read-across of carboxylic acid (valproic analogues)

2. Application of HTTr for biological read-across of mitochondrial complex inhibitors

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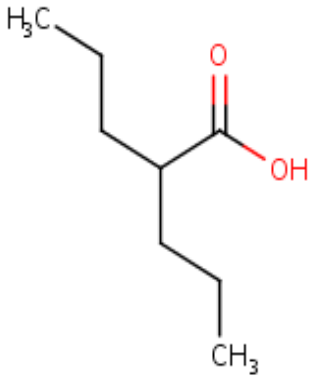
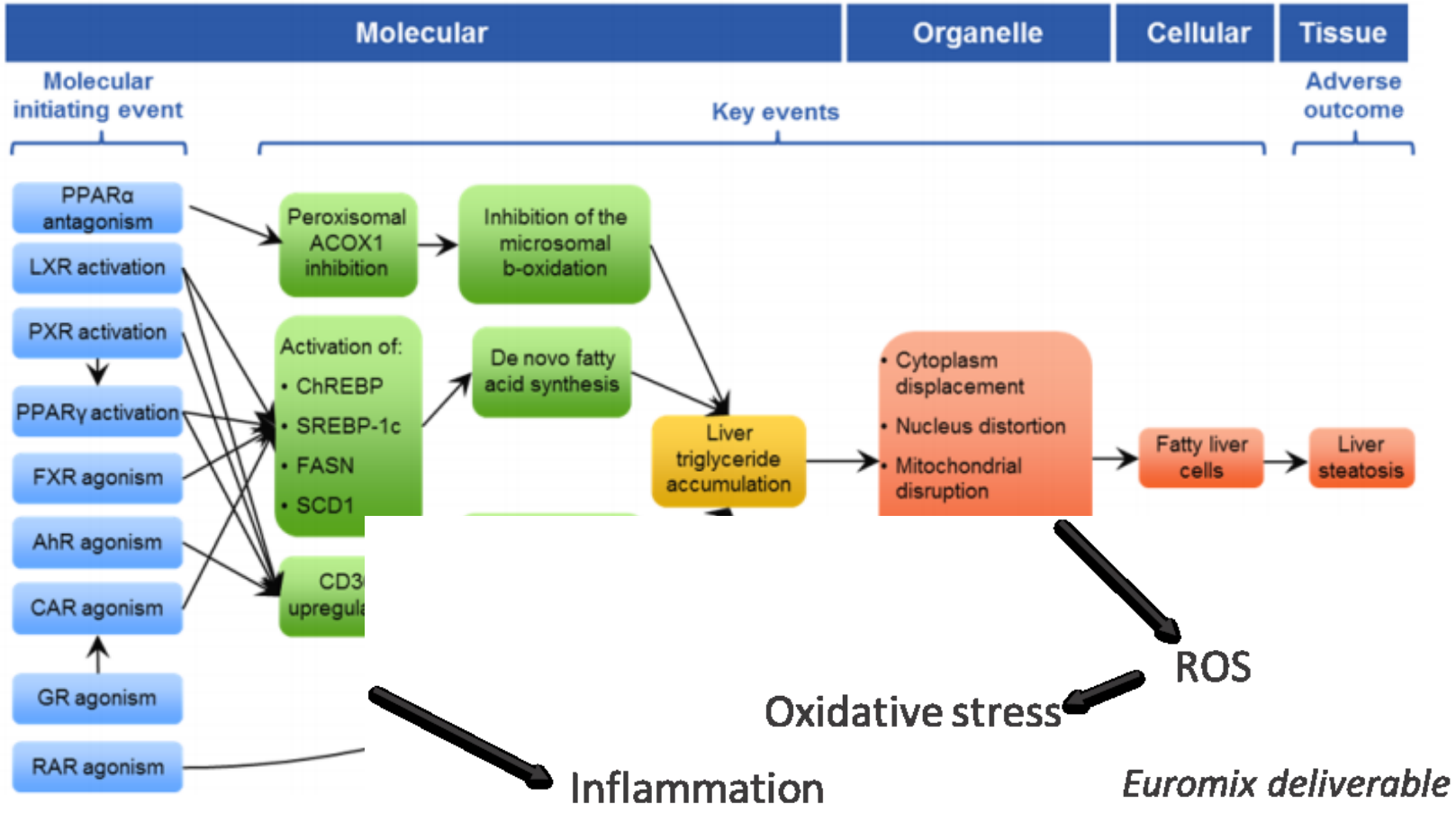
# Valproic acid and liver steatosis.




Do carboxylic acid VPA analogues have similar mode of action?

Can HHTr TempOseq support biological RAx?

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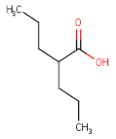
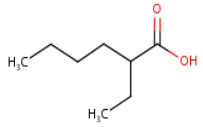
# EU-ToxRisk Case study: carboxylic acids and liver steatosis

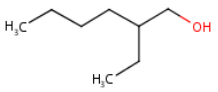
***In vivo* steatosis positive**



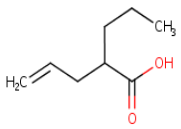
Valproic acid



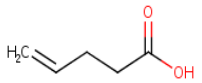
2-Ethylhexanoic acid



2-Ethyl-1-hexanol

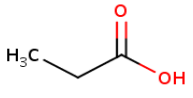


4-ene VPA

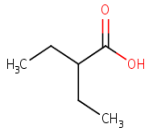


4-Pentenoic acid

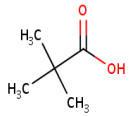
***In vivo* steatosis negative**



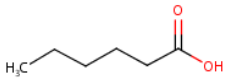
Propionic acid



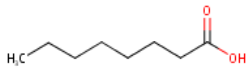
2-Ethylbutyric acid



Pivalic acid

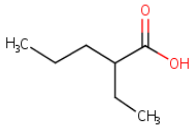


Hexanoic acid

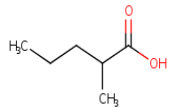


Octanoic acid

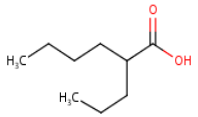
***In vivo* unknown**



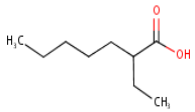
2-Ethylpentanoic acid



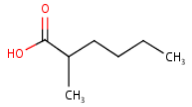
2-Methylpentanoic acid



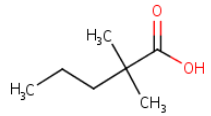
2-n-Propylhexanoic acid



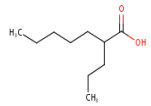
2-Ethylheptanoic acid



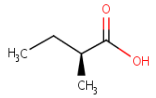
2-Methyl-hexanoic acid



2,2-Dimethylpentenoic acid



2-Propylheptanoic acid



2-Methylbutyric acid

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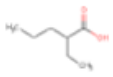
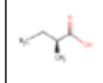
# Annotation of various carboxylic acids with different side chains (1).

| | Carboxylic acid annotations (1) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Source compound | | | | | | | |
| CAS | 31080-39-4 | 3274-29-1 | 3274-28-0 | 99-66-1 | 149-57-5 | 20225-24-5 | 88-09-5 | 1730-91-2 | 97-61-0 | 4536-23-6 | 75-98-9 |
| Name | 2-Propyl-heptanoic acid | 2-Ethyl-heptanoic acid | 2-Propyl-hexanoic acid | Valproic acid | 2-Ethyl-hexanoic acid | 2-Ethyl-pentanoic acid | 2-Ethyl-butyric acid | 2-Methyl-butyric acid | 2-Methyl-pentanoic acid (2-methylvaleric acid) | 2-Methyl-hexanoic acid | Pivalic acid |
| Abbreviation | 2-PHPA | 2-EHPA | 2-PHXA | VPA | 2-EHXA | 2-EPA | 2-EBA | 2-MBA | 2-MPA | 2-MHA | PIV |
| Structure |  |  |  |  |  |  |  |  |  |  |  |
| Structure (smiles) | CCCCCC(CCC)C(=O)O | CCCCCC(CC)C(=O)O | CCCCC(CCC)C(=O)O | CCCC(CCC)C(=O)O | CCCCC(CC)C(=O)O | CCCC(CC)C(=O)O | CCC(CC)C(=O)O | CC[C@H](C)C(=O)O | CCCC(C)C(=O)O | CCCCC(C)C(=O)O | CC(C)(C)C(=O)O |
| Structural similarity rel. to 99-66-1 | 0.97 | 0.97 | 0.97 | 1.00 | 0.97 | 0.94 | 0.73 | 0.80 | 0.88 | 0.91 | 0.53 |
| Chain length at position 2 | 5/3/0 | 5/2/0 | 4/3/0 | 3/3/0 | 4/2/0 | 3/2/0 | 2/2/0 | 2/1/0 | 3/1/0 | 4/1/0 | 1/1/1 |
| Branched | branched | branched | branched | branched | branched | branched | branched | branched | branched | branched | branched |
| Steatosis in vivo data | unknown | unknown | unknown | positive[1] | positive[2] | unknown | negative[3] | unknown | unknown[4] | unknown | negative[5] |

1 Espandiari, P. et al. (2008) Journal of Applied Toxicology. Tong, V. et al. (2005) Toxicological sciences. Sugimoto, T. et al. (1987) Epilepsia. Löscher, W. (1992) Epilepsy Research. Abdel-Dayem, M.A.(2014), Drugs RD. Knapp, A.C.(2008) J Pharm Exp Ther. Ibrahim, M.A.(2012) Life Science Journal. Patty's Toxicology(2012) Chapter Pharmaceuticals.
2 Juberg, D. R. et al. (1998) Food and Chemical Toxicology, Vol 36, 429-436. Juberg, D. R. et al. (1998) Food and Chemical Toxicology, Vol 36, 429-436. Patty's Toxicology (2012) Chapter Aliphatic Carboxylic Acids: Saturated
3 Patty's Toxicology (2012) Chapter Aliphatic Carboxylic Acids: Saturated
4 Patty's Toxicology (2012) Chapter Aliphatic Carboxylic Acids: Saturated
5 IUCLID, Shell Netherlands (1990)

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# EU-ToxRisk carboxylic acids RAx case study .

## Prediction of a 90 day repeated dose toxicity study (OECD 408) for 2-Ethylbutyric acid using a read-across approach to other branched carboxylic acids.

Authors: Sylvia E. Escher, Alice Limonciel, Barbara van Vugt, Nanette Vrijenhoek, Enrico Mombelli, Frederic Bois, Barbara Zdrazil, Annette Bitsch, Jan Hengstler, Wiebke Albrecht, Laia Tolosa, Paul Jennings, Rabea Graepel, Ulf Norinder, Regina Stoeber, Alejandro Aguayo Orozco, Richard Maclennan, Domenico Gadaleta, Thomas Exner, , Tony Long, Nazanin Golbamaki, Ciaran Fisher, Bob van de Water

### 1 Abstract / Synopsis / Executive summary

**Regulatory framework:** In this read-across we assume, that 2-Ethylbutyric acid (2-EBA) has to be registered under REACH and is produced in Europe at tonnages of more than 100 t/a. The standard REACH information requirements ask for a 90 days study with oral exposure. We use a category approach to predict the outcome of a subchronic toxicity study, according to a scenario 4. A category of branched carboxylic acids is evaluated, for which we see a consistent trend between category members with regard to the primary toxic effect, identified in the in vivo studies of analogues. New approach methodologies (NAM) like in vitro and in silico models are used in addition to in vivo data to confirm the consistent trend and for hazard characterization.

**Synopsis:** The structure of the target compound 2-EBA comprises a short chain, branched aliphatic carboxylic acid in position 2. Nine aliphatic carboxylic acids with different branched aliphatic side chains are regarded as most similar to the target compound. Beside high structural similarity the grouped compounds show a consistent trend for physico-chemical (pc) parameters, e.g. logPow and MW increases slightly with side chain length, whereas water solubility and vapour pressure decreased. The pc-parameters do however not alert for a potential bioaccumulation in vivo. Two compounds have in vivo animal studies with repeated oral exposure. 2-Ethylhexanoic acid (2-EHA) has subchronic guideline studies, in which liver hypertrophy was observed together with an increase of the relative liver weight. Valproic acid (VPA) induced liver steatosis in shorter-term subacute studies. The read-across hypothesis is therefore, that 2-EBA is a liver toxicant with special concern for steatosis. In addition to the nine structural analogue, Pivalic acid (PVA) is tested as negative control compound. PVA has a third substituent in position 2 and did not induce any liver toxicity in a subacute study up to the highest tested dose. A negative compounds is needed to judge on the accuracy of NAM data.

NAM data showed a consistent trend with regard to toxikokinetics and toxikodynamics within the grouped compounds.

**Toxikokinetics:** A rat physiology-based pharmacokinetic (PBPK) model was established, based on in vivo data, and used to calculate plasma and target organ concentrations, which guided the selection of a relevant concentration range for in vitro testing. Human PBPK models were established for all read-across compounds based on physiochemical properties and in vitro clearance data (e.g. plasma protein binding (ppb) and intrinsic hepatic clearance ($CL_{int, Hep}$). Human in vivo pharmacokinetic data for VPA was identified and verified good predictive performance based on observed plasma concentration data in humans. Based on this proof of concept IVIVE-PBPK models were used for in vitro to in vivo extrapolations for all analogues.

**Toxikodynamics:** Several adverse outcome pathways are available describing the development of liver steatosis. About 50 published signalling pathways leading to steatosis were compiled from literature and summarized in an adverse outcome pathway (AOP) network. The AOP network



- EU-ToxRisk RAx case study
- Liver steatosis AOP-based
- KE event analysis
- OECD IATA case study working group
- Official report published
- Gene expression profiling not involved

➢ **Can high throughput transcriptomics contribute to RAx of carboxylic acids**

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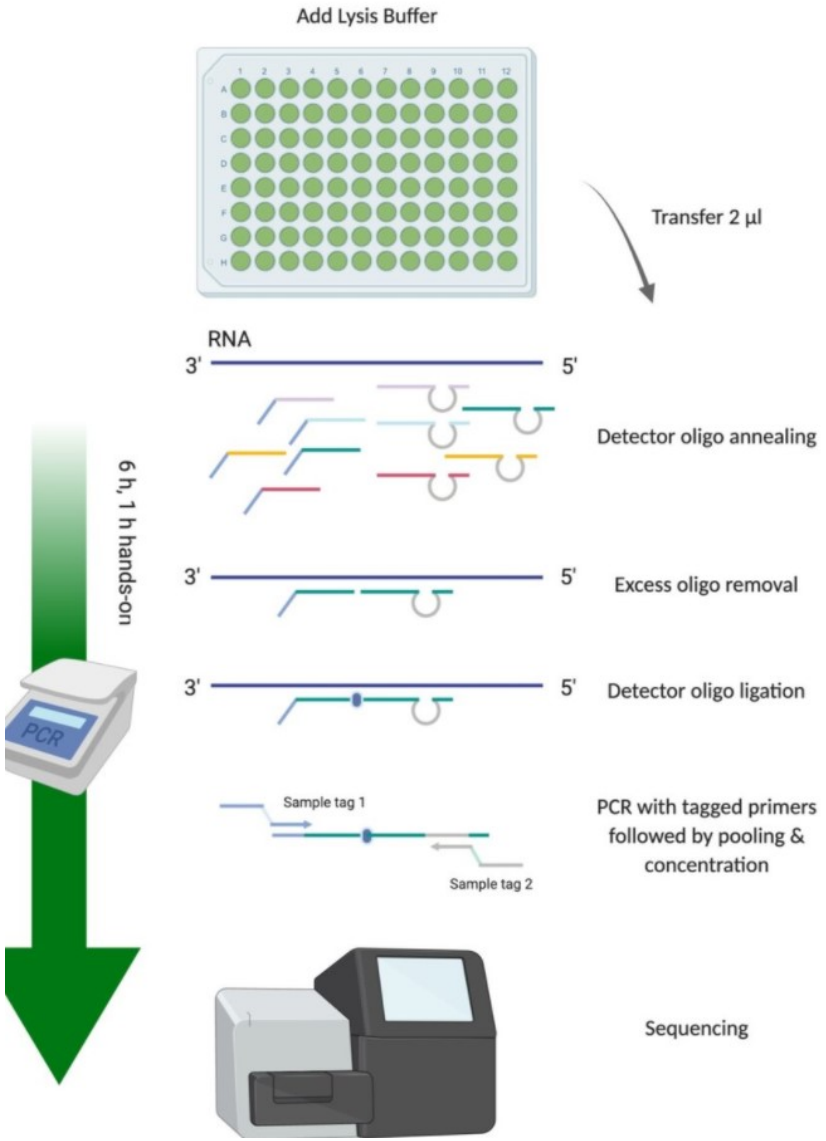
# High throughput transcriptomics – TempO-seq technology




- EU-ToxRisk gene panel (~3500 genes; NTP S1500+ plus additional selected genes)
- whole transcriptome gene panel

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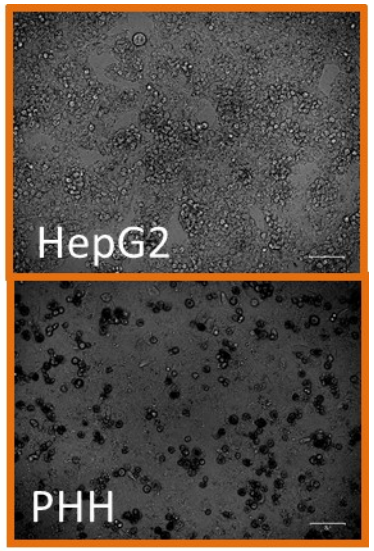
# Unravel Mode-of-Action of VPA analogues with TempOSeq in liver cells

Day 1 Plating

Day 2 Exposure

Day 3 Lysis

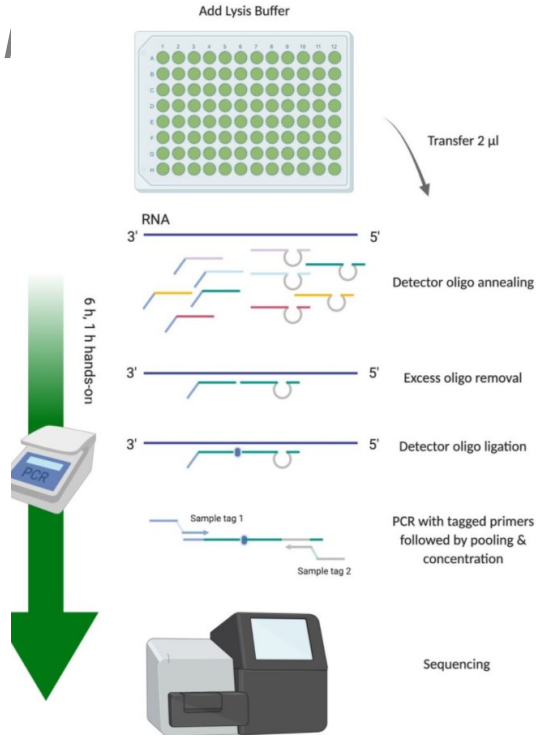
TempOSeq




10 donor pool
BioIVT

*18 analogues*
*0.2 – 16 mM (7 conc)*
*24h treatment*
*~400 samples*

*EU-ToxRisk gene*

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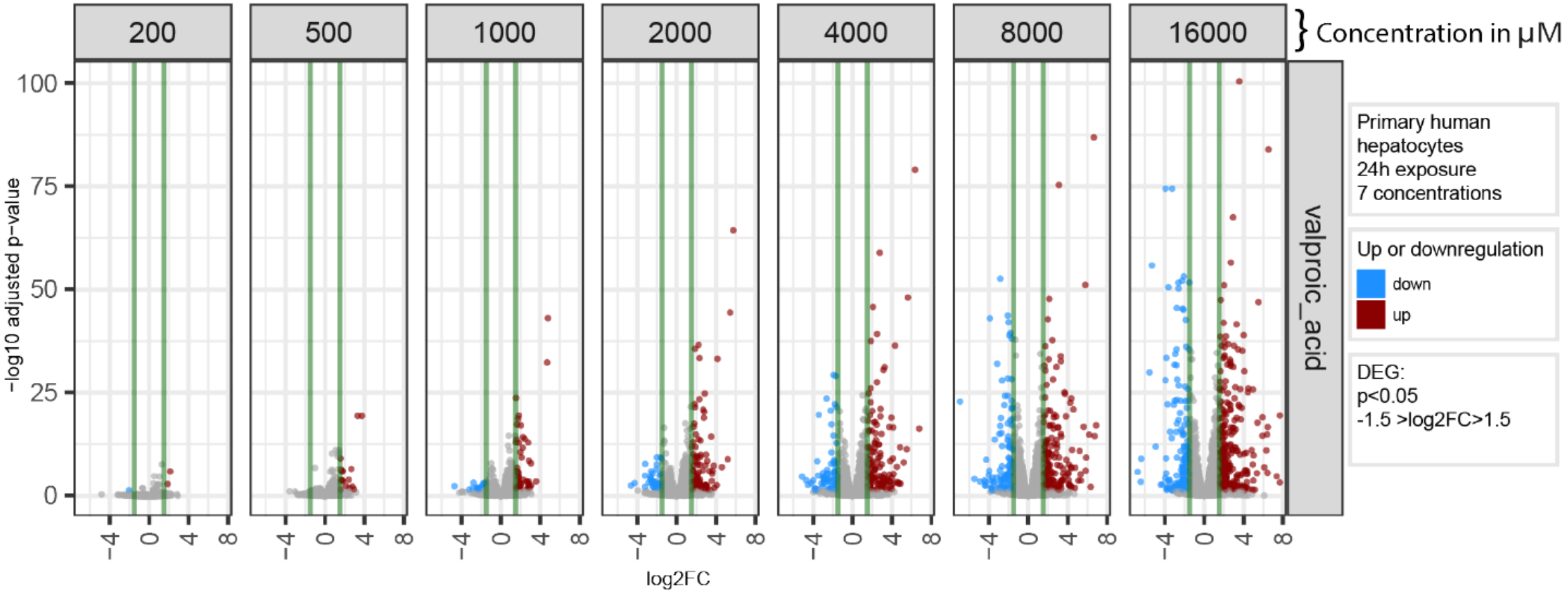
# VPA causes a dose dependent increase in differently expressed genes in primary human

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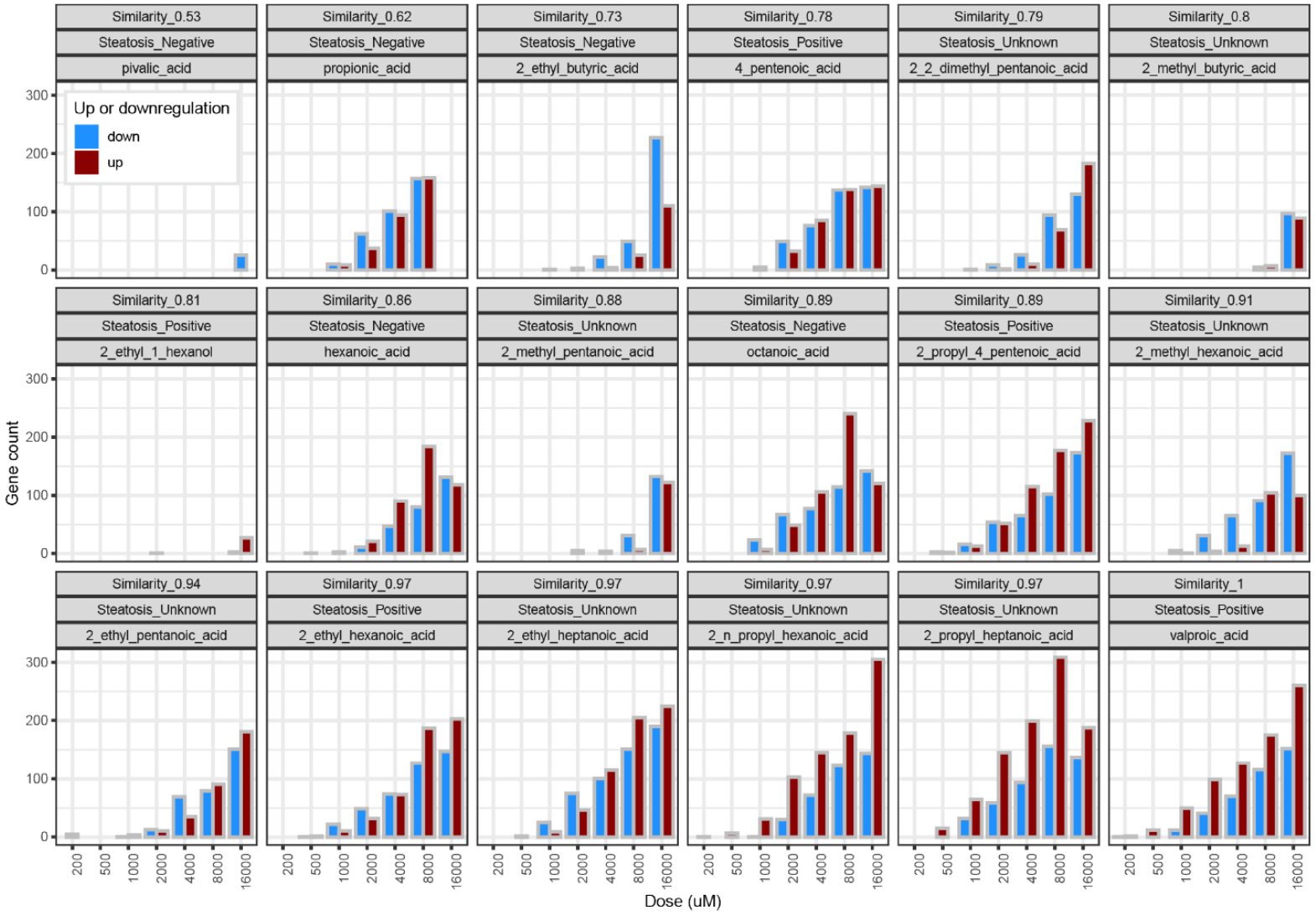
# Effect of VPA analogues on DEGs in PHHs




DEGs with cutoff: $p < 0.05$, $-1.5 < log2FC > 1.5$

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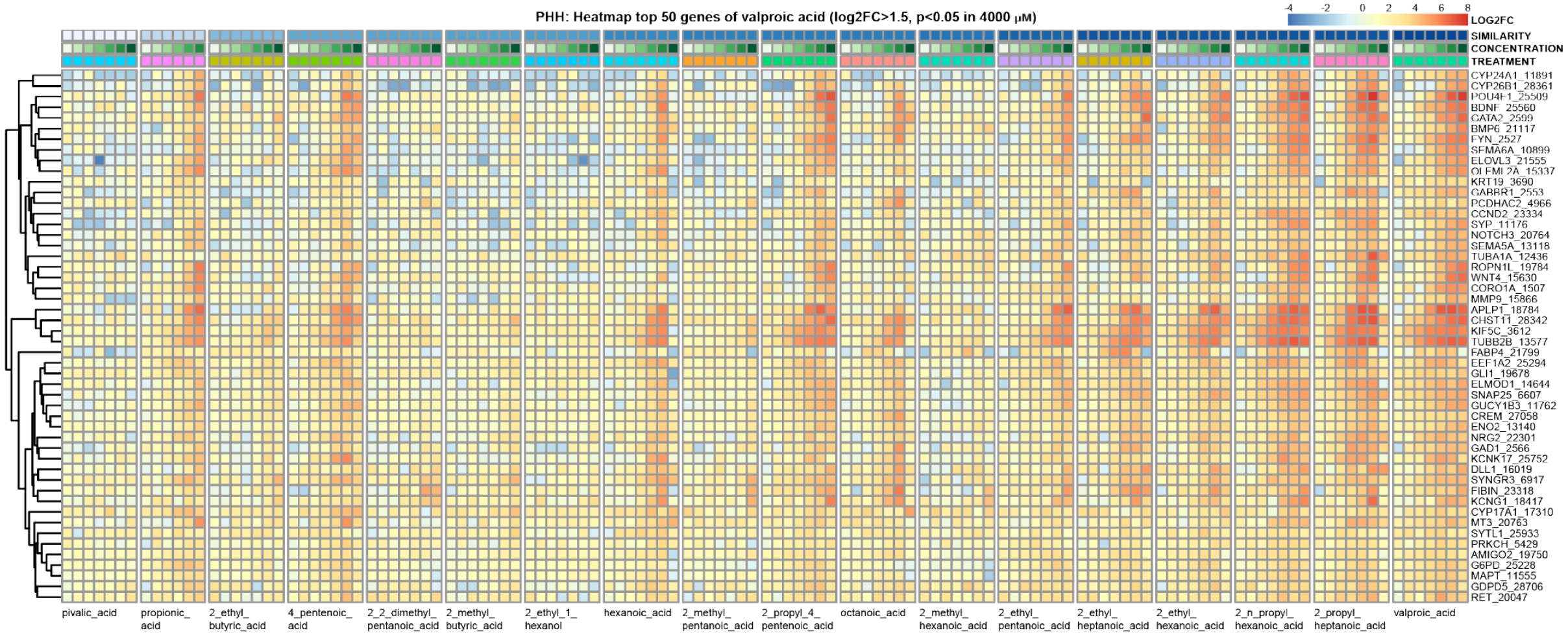
# Active VPA analogues have similar concentration-dependent transcriptomic response in PHH

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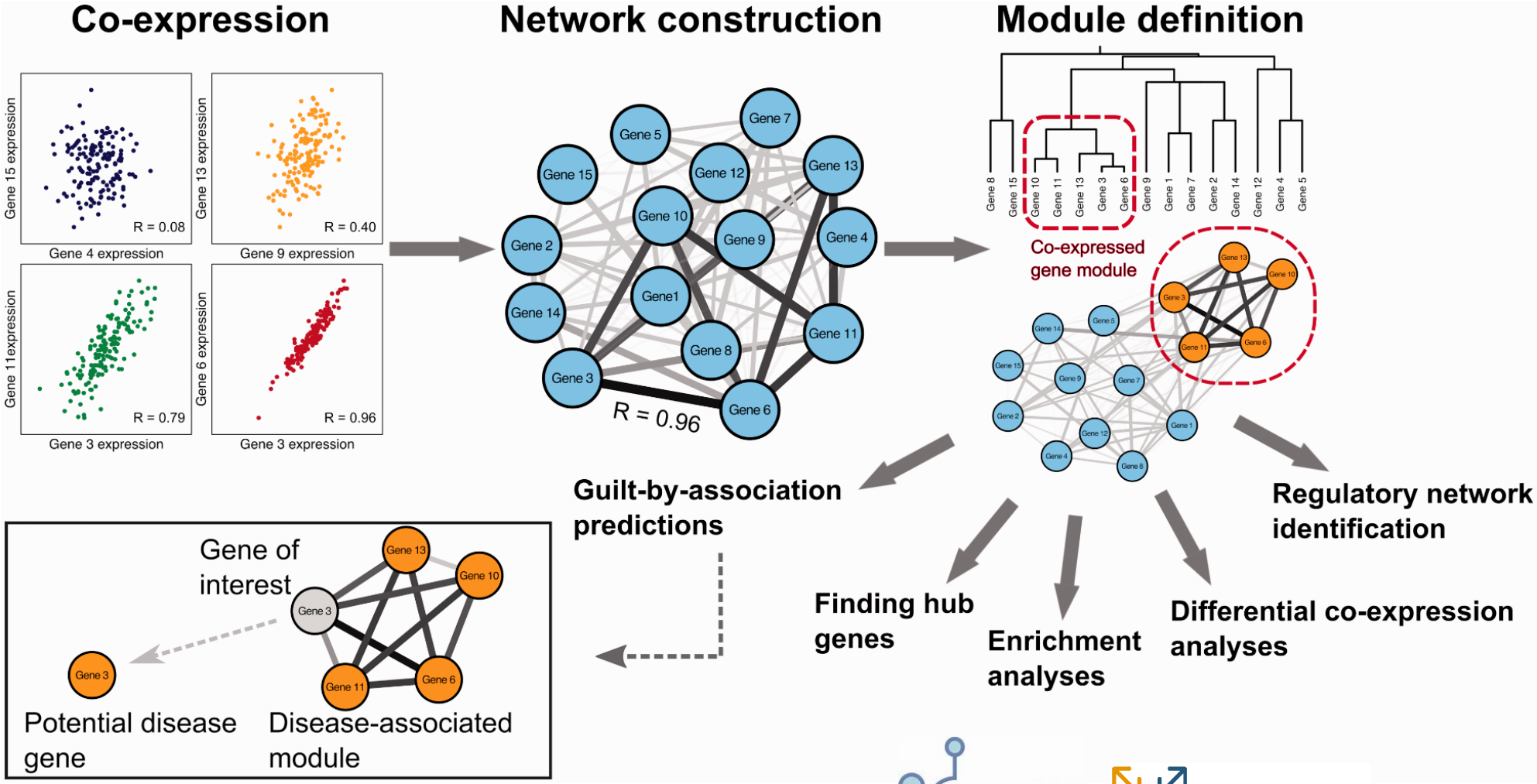
# Weighted Co-regulated Gene Network Analysis (WGCNA) to establish toxicogenomics maps of tissue cell and injury responses

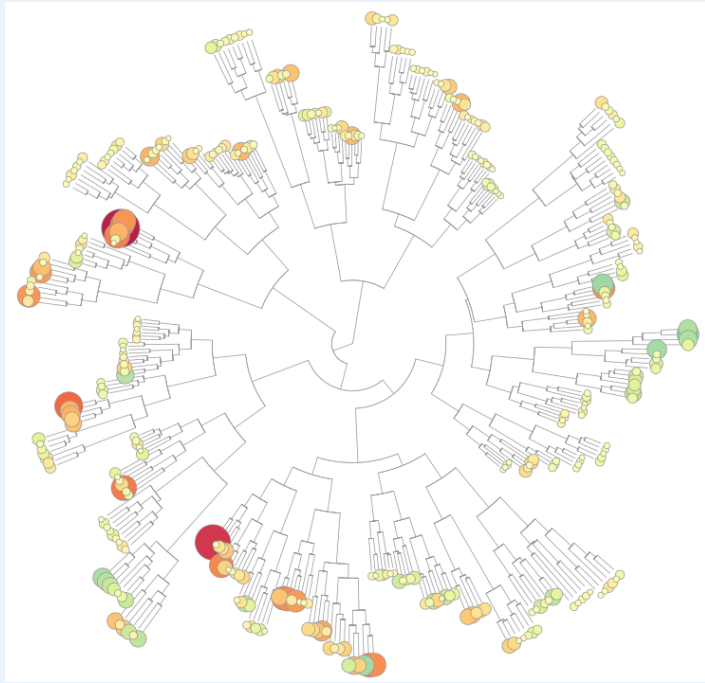



Giulia Callegaro

# TXG-MAPr tools

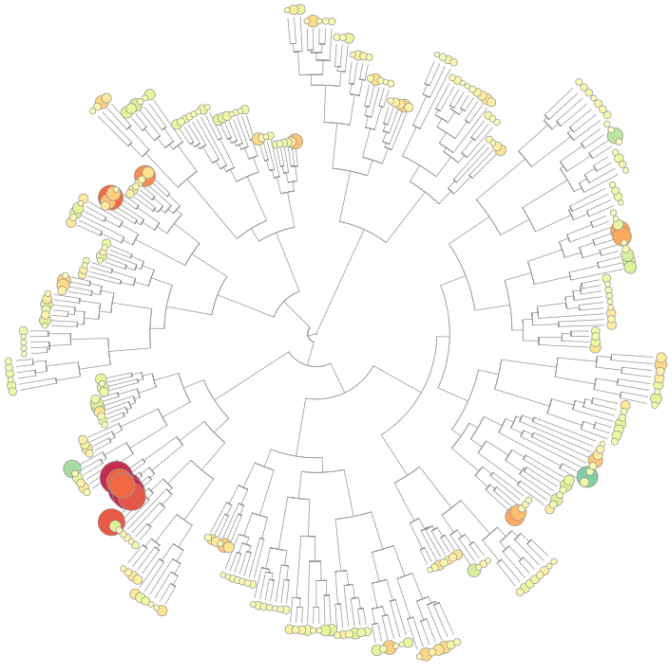
## Public domain toxicogenomics-MAP (TXG-MAP) R-shiny framework

Primary human hepatocytes



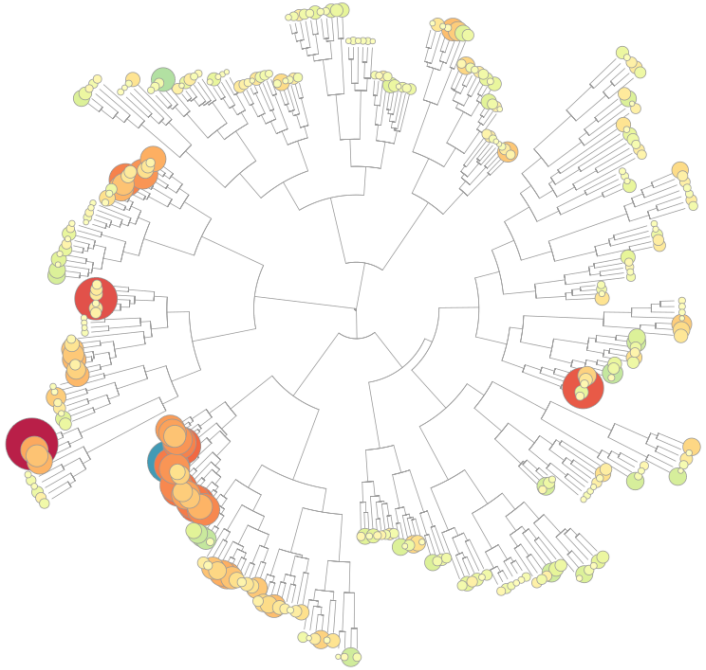
24 h 6 µM cyclosporine A

Rat Liver



24 h 300 mg/kg cyclosporine A

Rat kidney



24 h 300 mg/kg cyclosporine A

Callegaro et al. Arch Toxicol. 2021

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(99+) Bob van de Water | LinkedIn | The International Human Epigen | TXG-MAPr - WGCNA modules ba

# TXG-MAPr - WGCNA modules based on in vivo rat liver data (TG-GATEs)

LACDR - Leiden University - v 1.0

Welcome Bob

Log out

TXG Map | TXG Map enrichment | Compounds | Modules | Genes | TFs | Compound correlation | Module correlation | Pathology | Upload | Help

Visit the help section for more details

**Compound:**

ACETAMINOPHEN

**Eigengene score:**

-36 42

-36 -28 -20 -12 -4 4 12 20 28 36 42

**Time-points:**

0.125 day | 0.25 day | 0.375 day | 1 day | 4 day | 8 day | 15 day | 29 day | Other

**Dose-level:**

Low | Medium | High | Other

**Single treatment**

Callegaro et al. Arch Toxicol. 2021

Callegaro et al. Arch Toxicol. 2021

# Toxicogenomics-MAP (TXG-MAP) based on WGCNA

Weighted gene co-expression network analysis (WGCNA) based on all PHH TG-GATEs transcriptomic data

R-shiny framework for web-based interactive visualization

Module = network of co-expressed genes

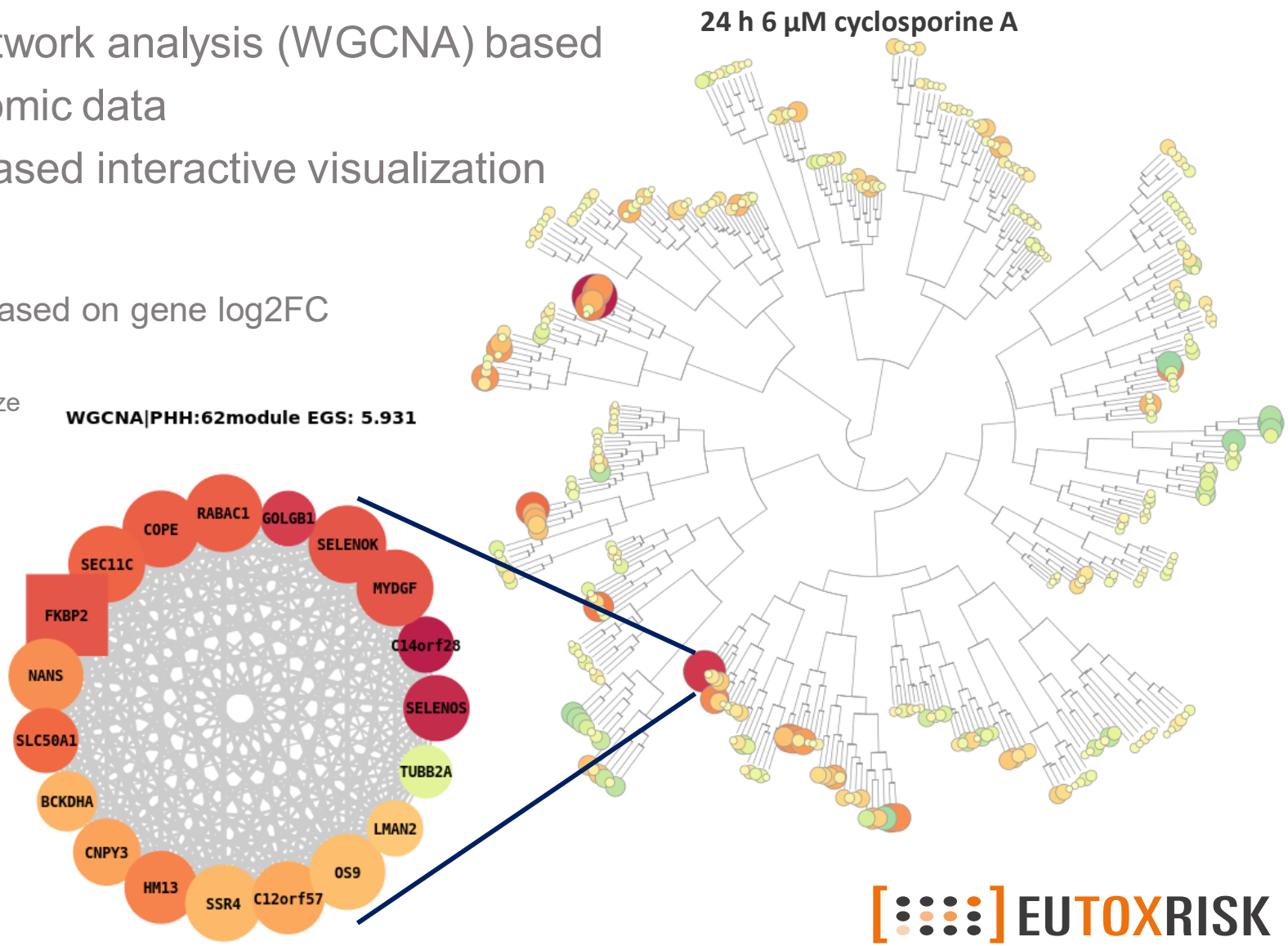
Eigengene score (EGs) = module score based on gene log2FC

TXG-MAP = Toxicogenomics-MAP

- Module responses (EGS) displayed in color and size
- Ward's hierarchical clustering of modules

## Gene-set/module enrichment

- GO-terms
- Pathways
- Transcription factors
- Interacting proteins




Callegaro et al. Arch Toxicol. 2021

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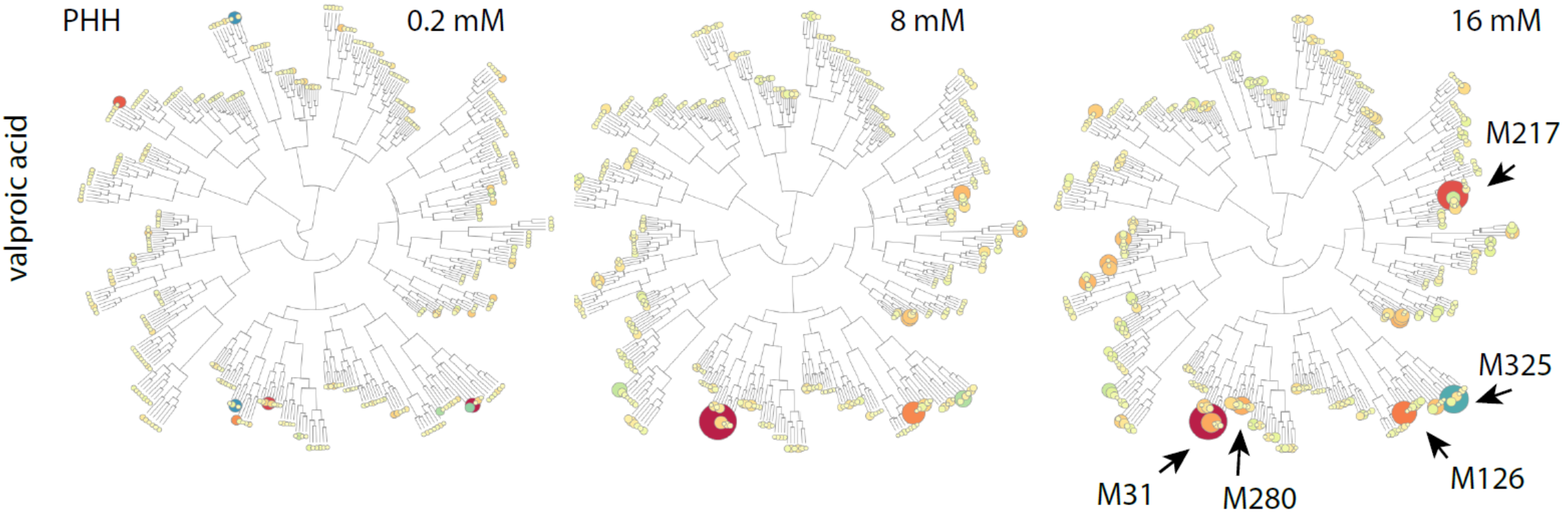
# Projection of VPA TempOseq data on PHH TXG-MAP

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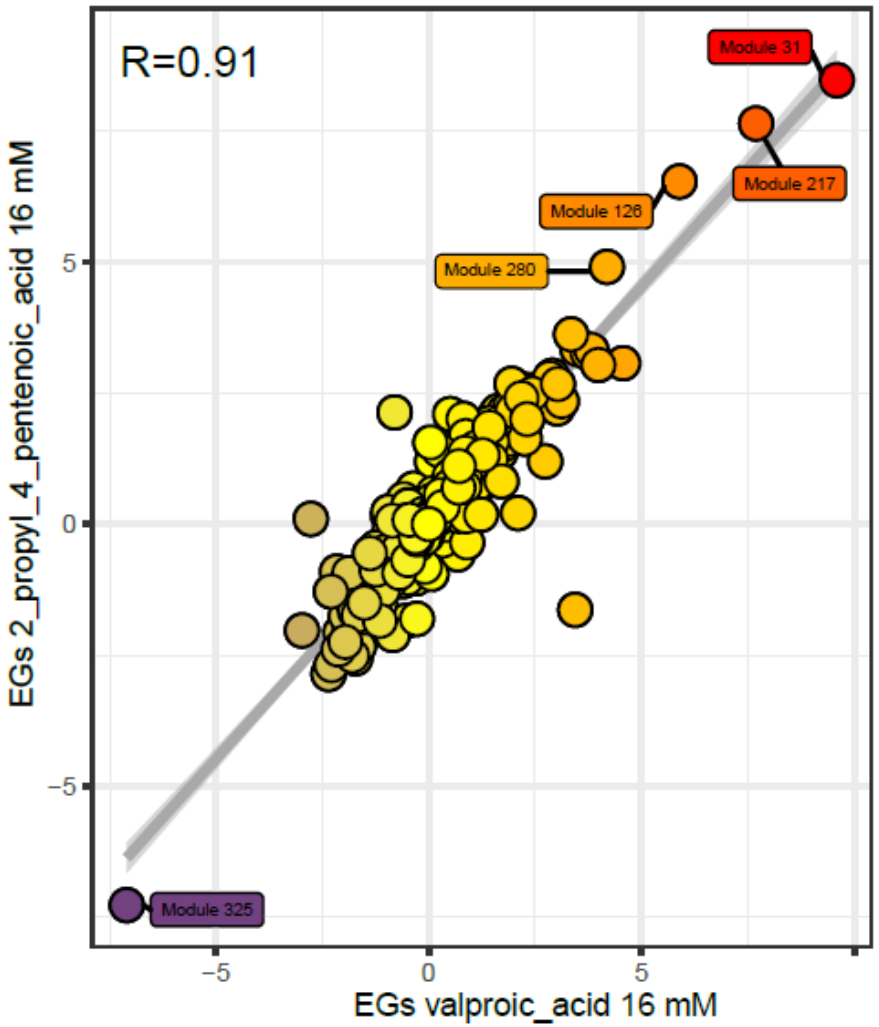
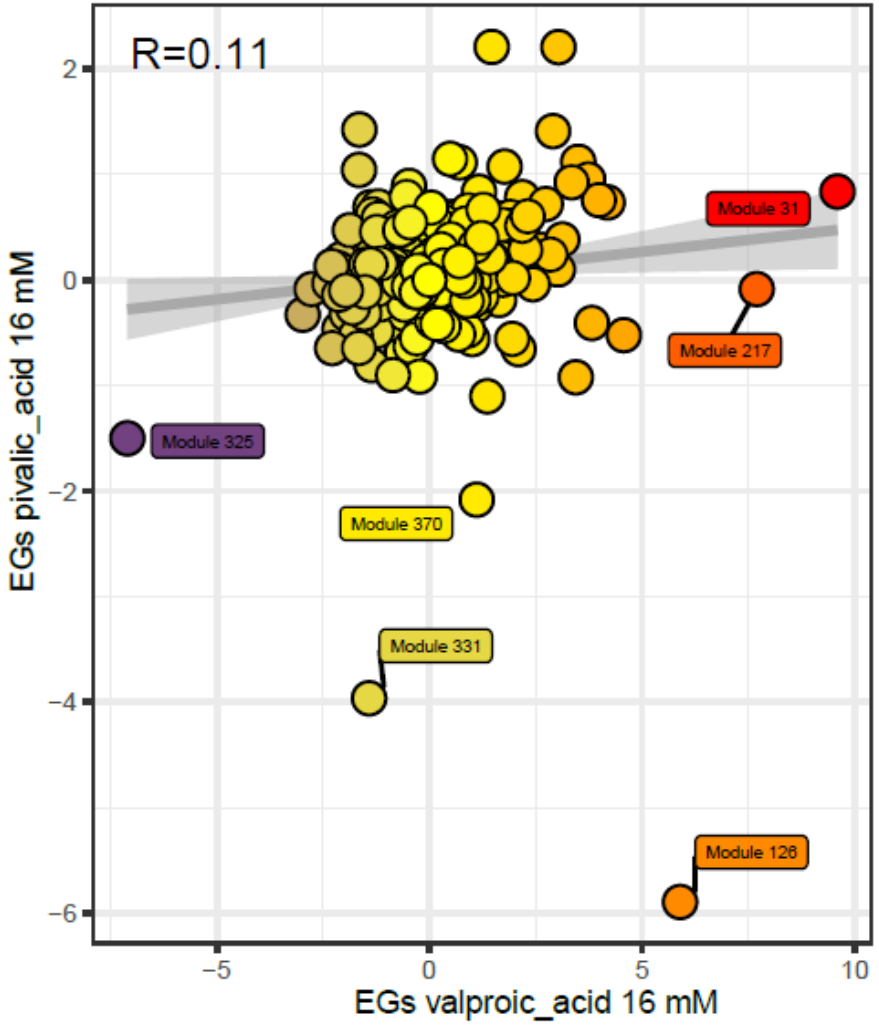
# Active VPA analogues show similar TXG-MAP module activation

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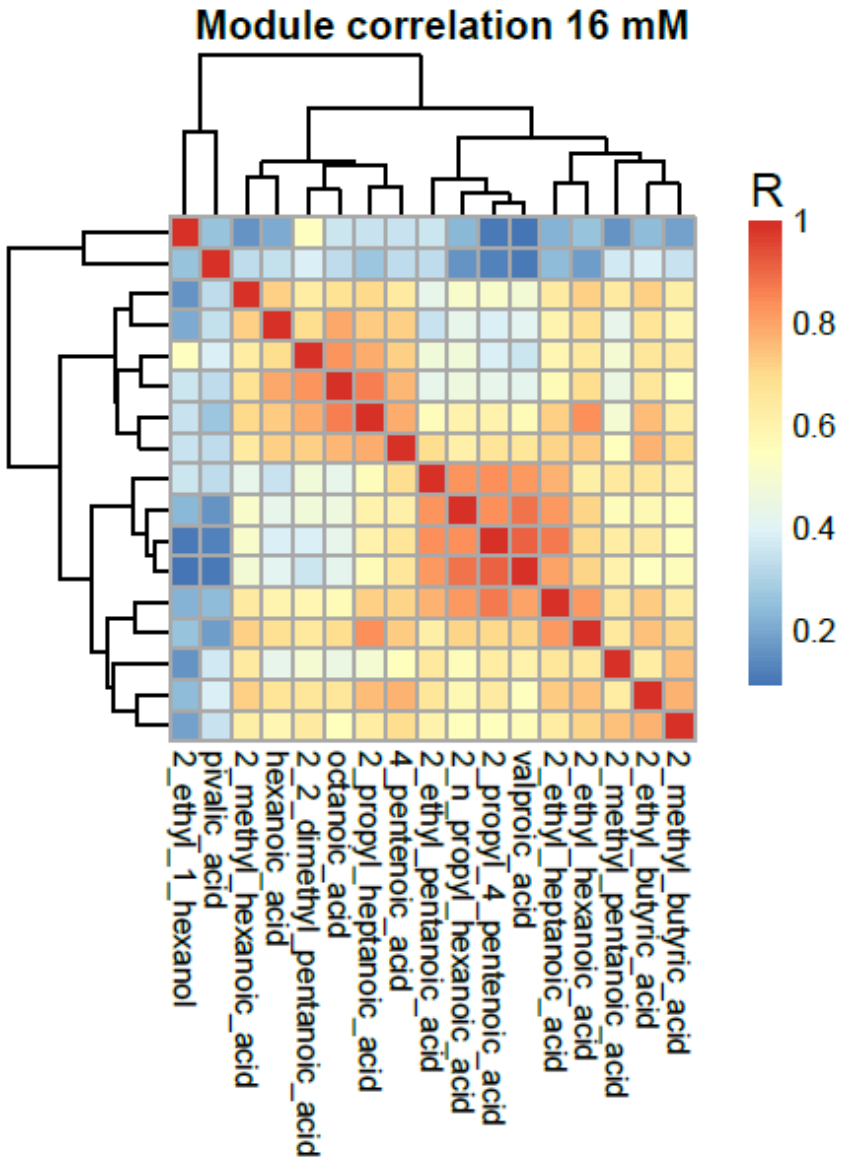
# TXG-MAP module correlation of VPA analogues for biological RAx

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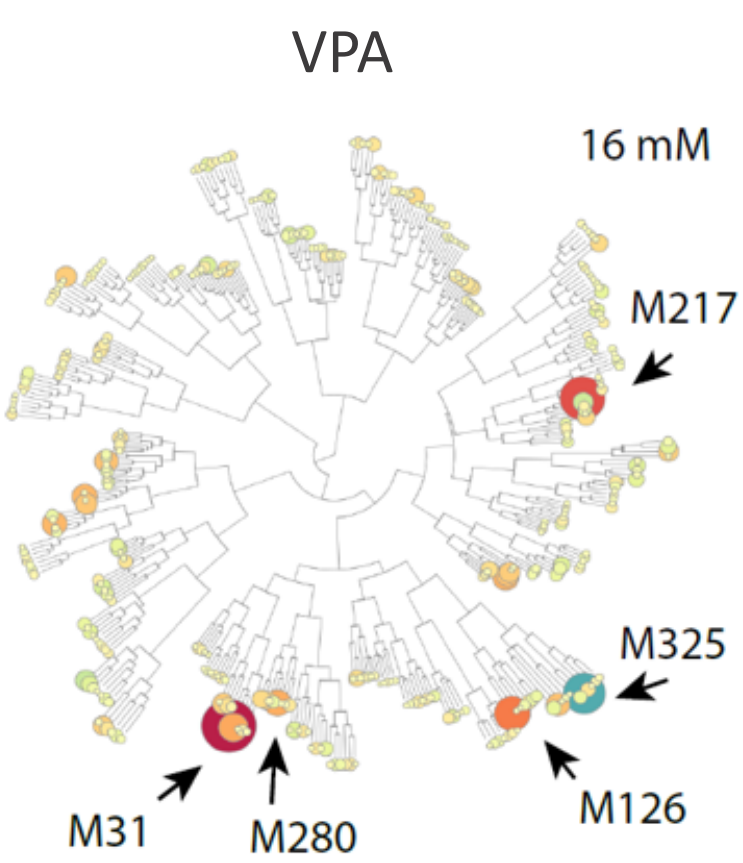
# Dose response of module activation (filtered: -2>EGS>2)

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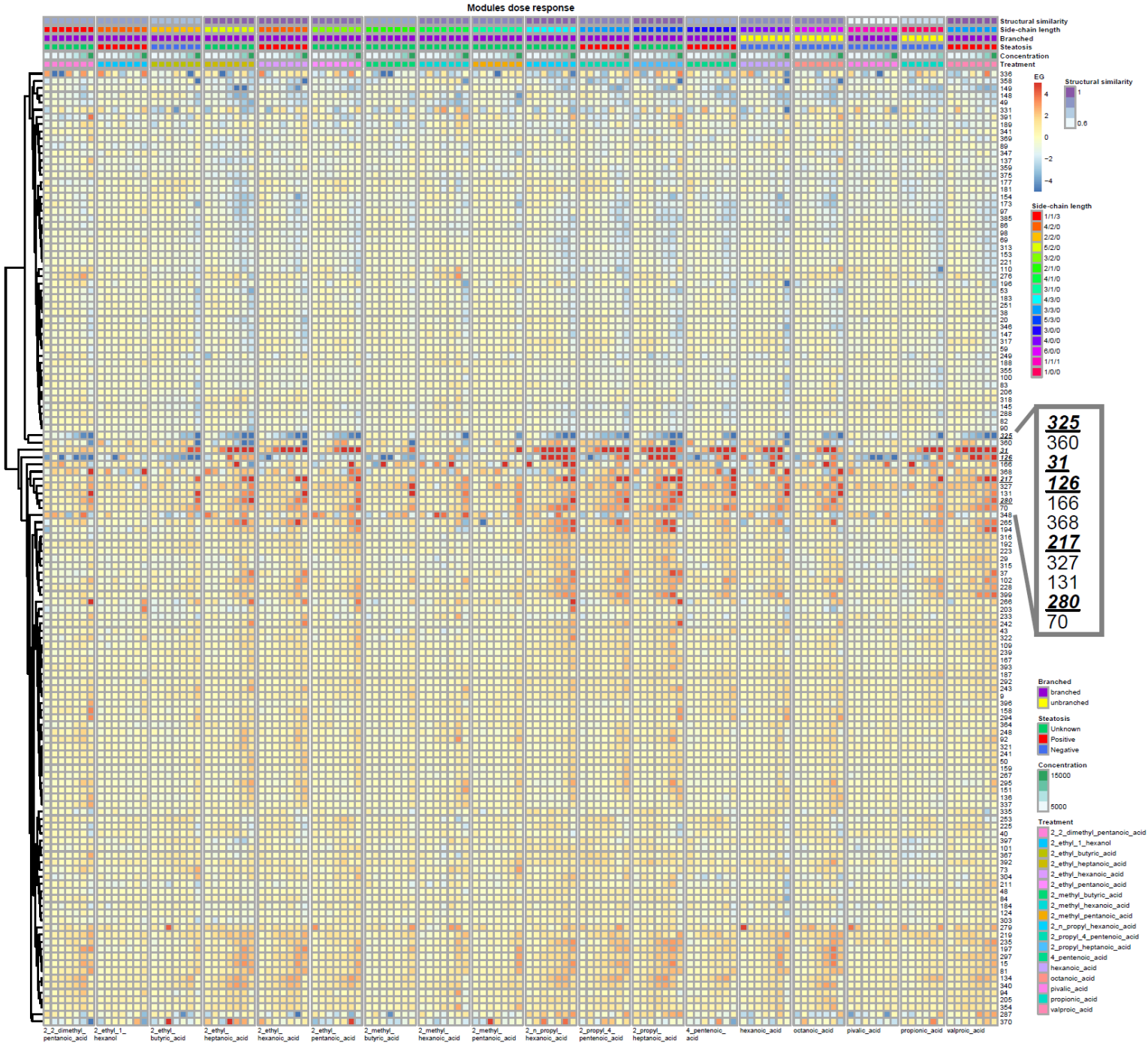
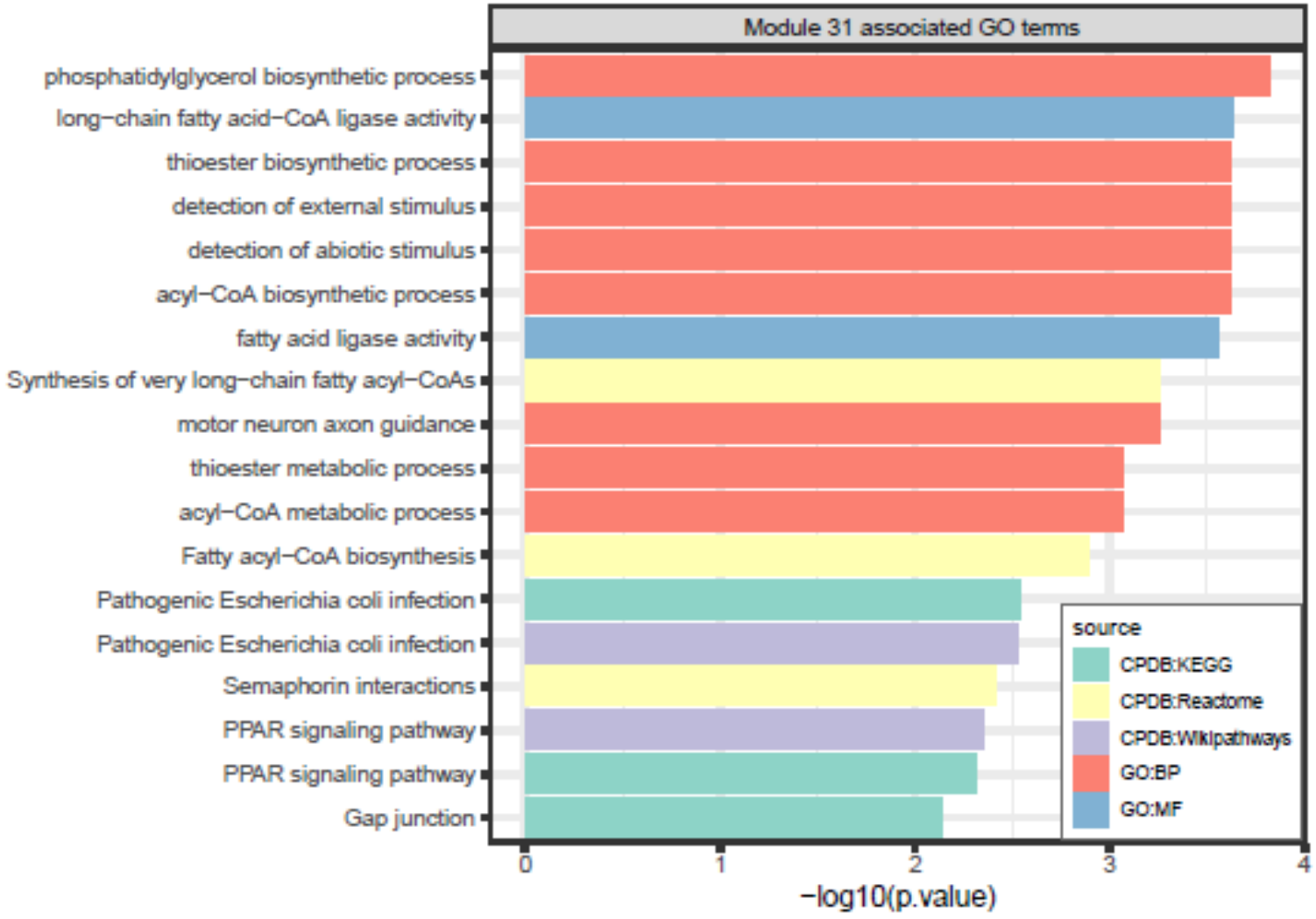


# Module 31 is associated with fatty acid related GO terms

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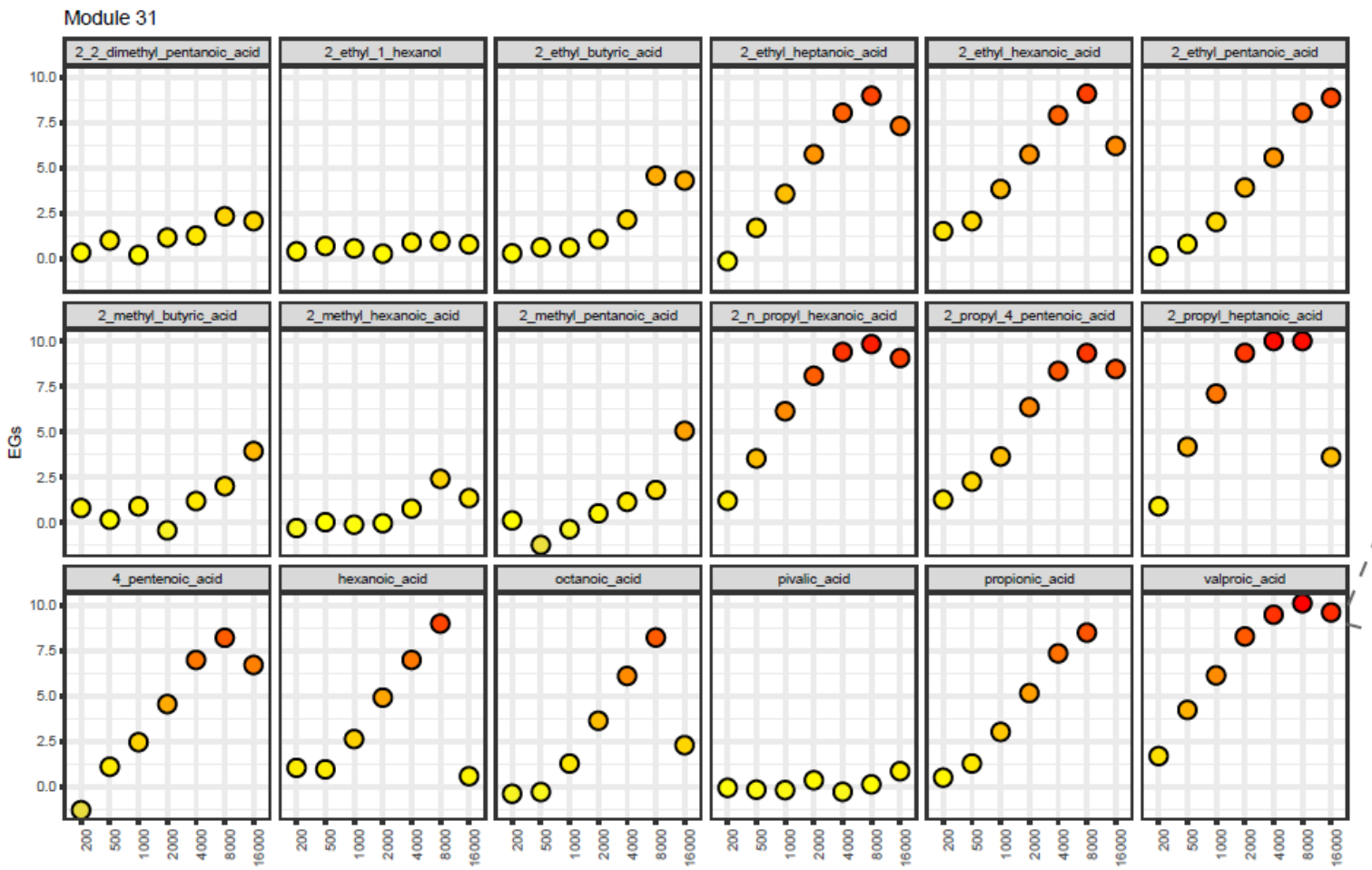
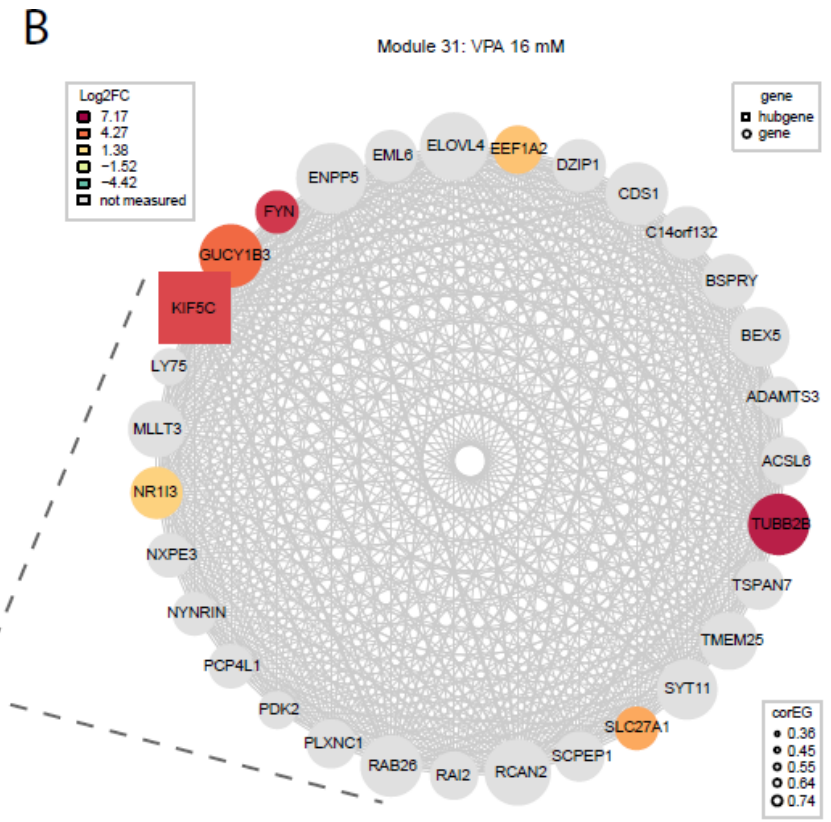
# Potency evaluation of VPA analogues:

# PHH TXG-MAPr Module 31 shows a dose response for VPA analogues

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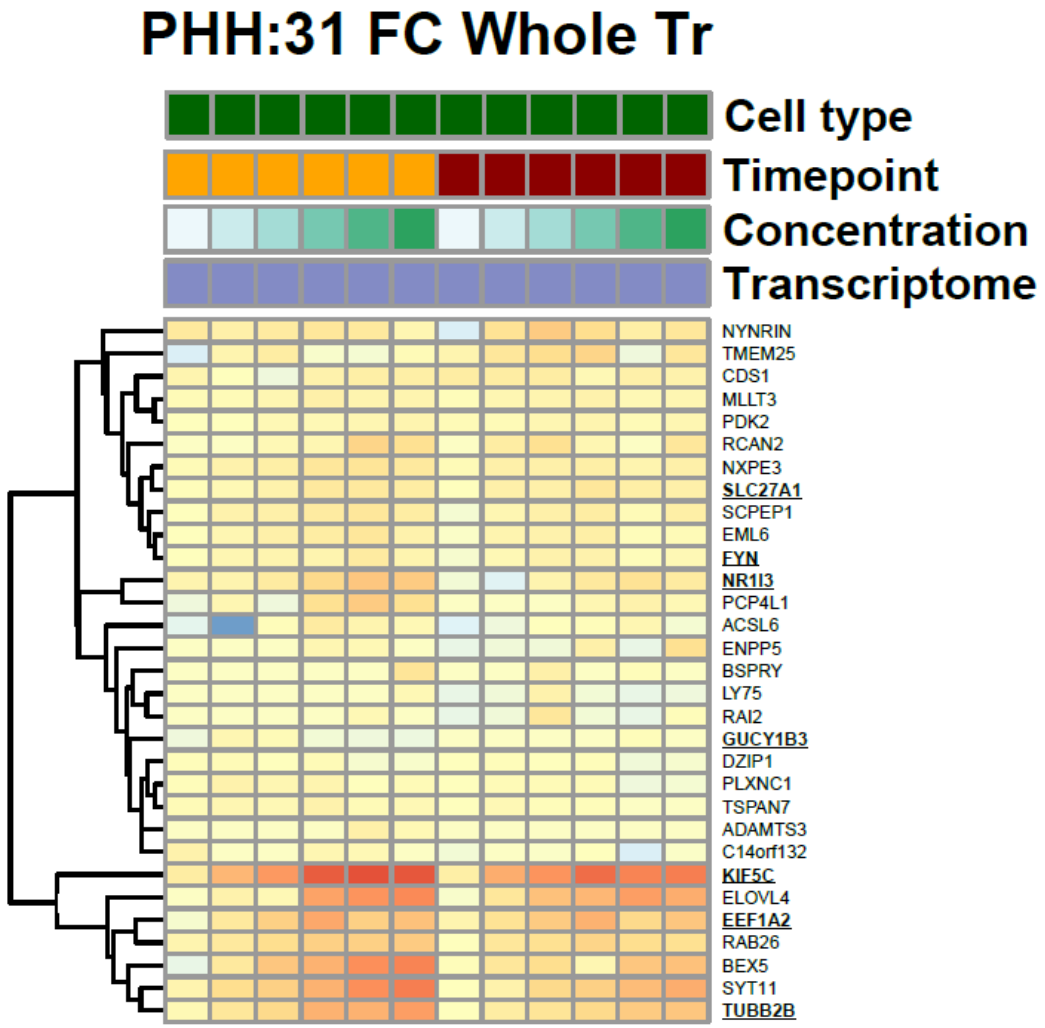
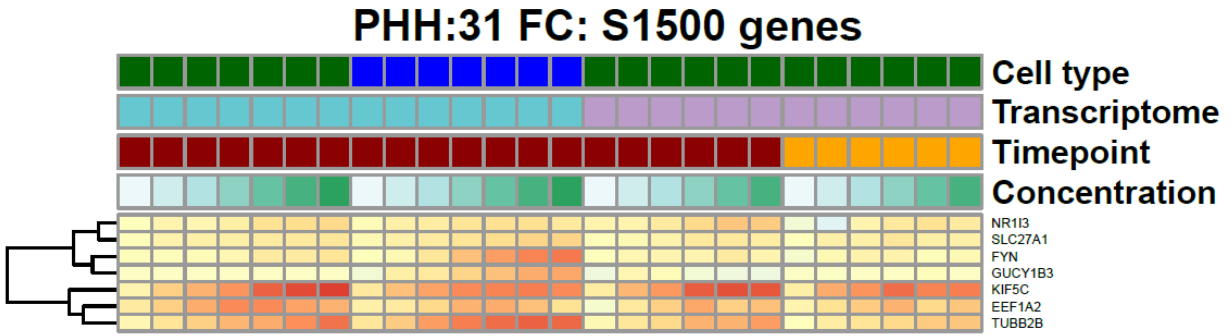
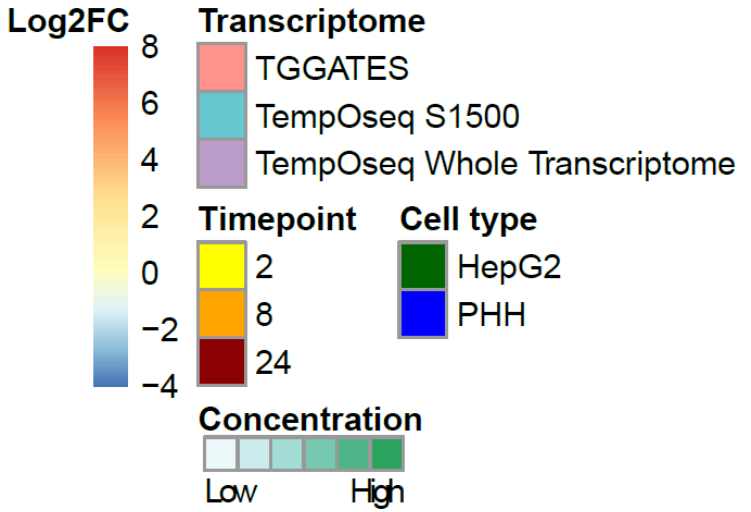
# Comparison TempOseq S1500+ with whole transcriptome, PHH and HepG2

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# Topics

1. Application of HTTr for read-across of carboxylic acid (valproic analogues)

2. Application of HTTr for biological read-across of mitochondrial complex inhibitors

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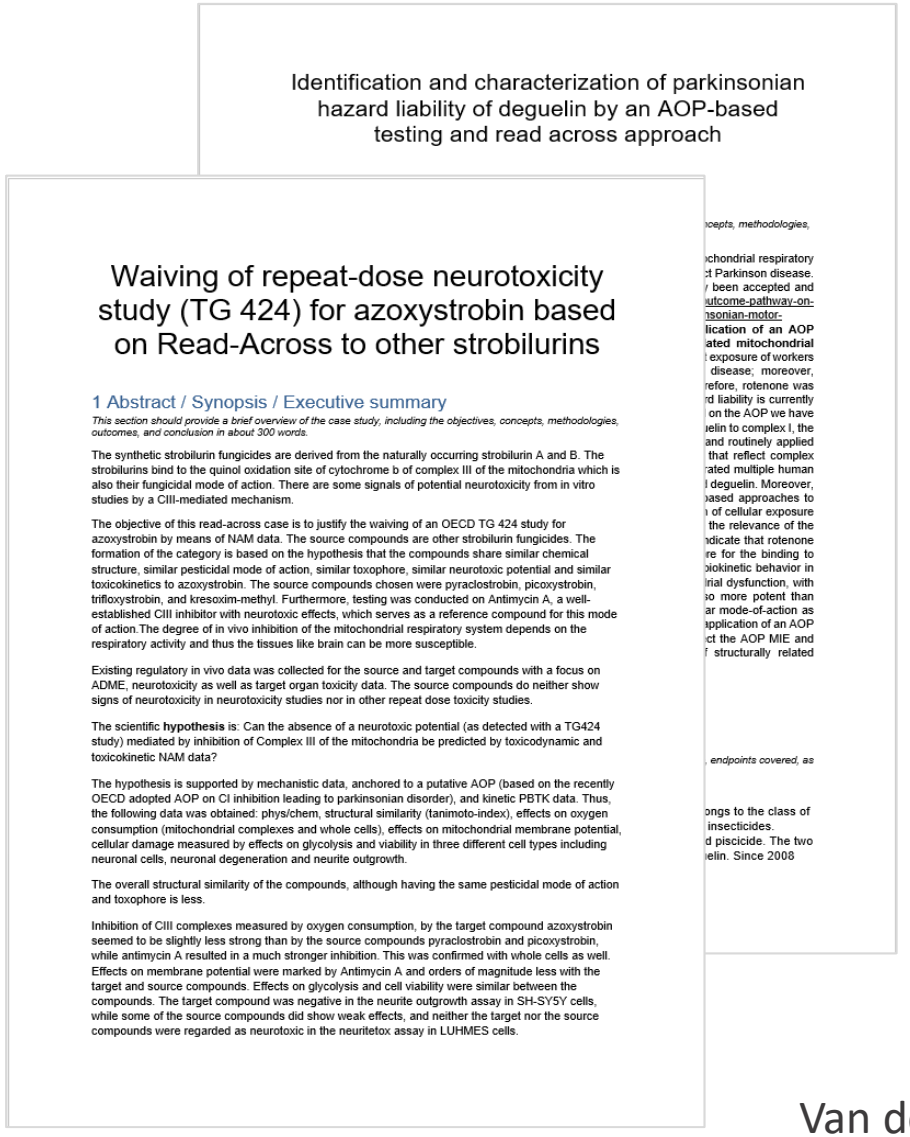
# EU-ToxRisk mitochondrial complex inhibitor biological RAx case study.



Identification and characterization of parkinsonian hazard liability of deguelin by an AOP-based testing and read across approach

Waiving of repeat-dose neurotoxicity study (TG 424) for azoxystrobin based on Read-Across to other strobilurins

1 Abstract / Synopsis / Executive summary

*This section should provide a brief overview of the case study, including the objectives, concepts, methodologies, outcomes, and conclusion in about 300 words.*

The synthetic strobilurin fungicides are derived from the naturally occurring strobilurin A and B. The strobilurins bind to the quinol oxidation site of cytochrome b of complex III of the mitochondria which is also their fungicidal mode of action. There are some signals of potential neurotoxicity from in vitro studies by a CIII-mediated mechanism.

The objective of this read-across case is to justify the waiving of an OECD TG 424 study for azoxystrobin by means of NAM data. The source compounds are other strobilurin fungicides. The formation of the category is based on the hypothesis that the compounds share similar chemical structure, similar pesticidal mode of action, similar toxophore, similar neurotoxic potential and similar toxicokinetics to azoxystrobin. The source compounds chosen were pyraclostrobin, picoxystrobin, trifloxystrobin, and kresoxim-methyl. Furthermore, testing was conducted on Antimycin A, a well-established CIII inhibitor with neurotoxic effects, which serves as a reference compound for this mode of action.The degree of in vivo inhibition of the mitochondrial respiratory system depends on the respiratory activity and thus the tissues like brain can be more susceptible.

Existing regulatory in vivo data was collected for the source and target compounds with a focus on ADME, neurotoxicity as well as target organ toxicity data. The source compounds do neither show signs of neurotoxicity in neurotoxicity studies nor in other repeat dose toxicity studies.

The scientific **hypothesis** is: Can the absence of a neurotoxic potential (as detected with a TG424 study) mediated by inhibition of Complex III of the mitochondria be predicted by toxicodynamic and toxicokinetic NAM data?

The hypothesis is supported by mechanistic data, anchored to a putative AOP (based on the recently OECD adopted AOP on CI inhibition leading to parkinsonian disorder), and kinetic PBTK data. Thus, the following data was obtained: phys/chem, structural similarity (tanimoto-index), effects on oxygen consumption (mitochondrial complexes and whole cells), effects on mitochondrial membrane potential, cellular damage measured by effects on glycolysis and viability in three different cell types including neuronal cells, neuronal degeneration and neurite outgrowth.

The overall structural similarity of the compounds, although having the same pesticidal mode of action and toxophore is less.

Inhibition of CIII complexes measured by oxygen consumption, by the target compound azoxystrobin seemed to be slightly less strong than by the source compounds pyraclostrobin and picoxystrobin, while antimycin A resulted in a much stronger inhibition. This was confirmed with whole cells as well. Effects on membrane potential were marked by Antimycin A and orders of magnitude less with the target and source compounds. Effects on glycolysis and cell viability were similar between the compounds. The target compound was negative in the neurite outgrowth assay in SH-SY5Y cells, while some of the source compounds did show weak effects, and neither the target nor the source compounds were regarded as neurotoxic in the neuritetox assay in LUHMES cells.

- EU-ToxRisk RAx case study
- AOP-based
- KE event analysis
- OECD IATA case study working group
- Official report published
- Gene expression profiling not involved

➢ **Can high throughput transcriptomics contribute to biological read across of mitochondrial complex inhibition**

Van der Stel et al. ALTEX 2021

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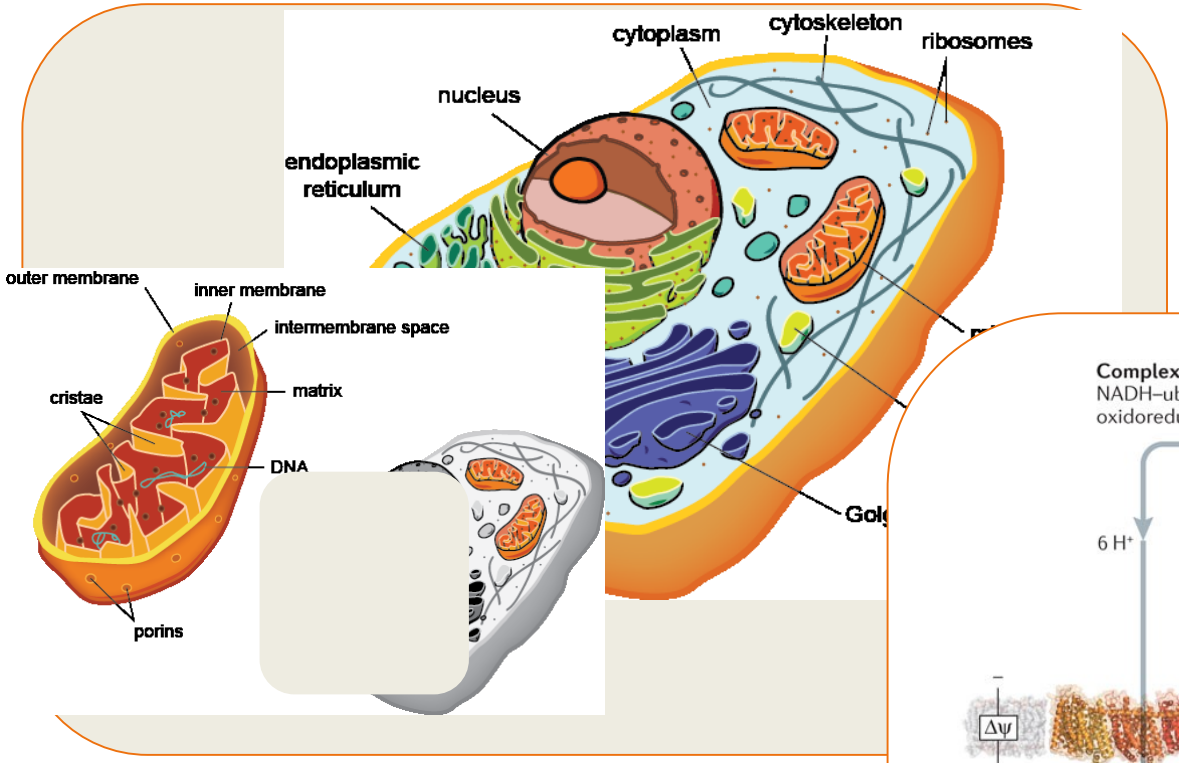
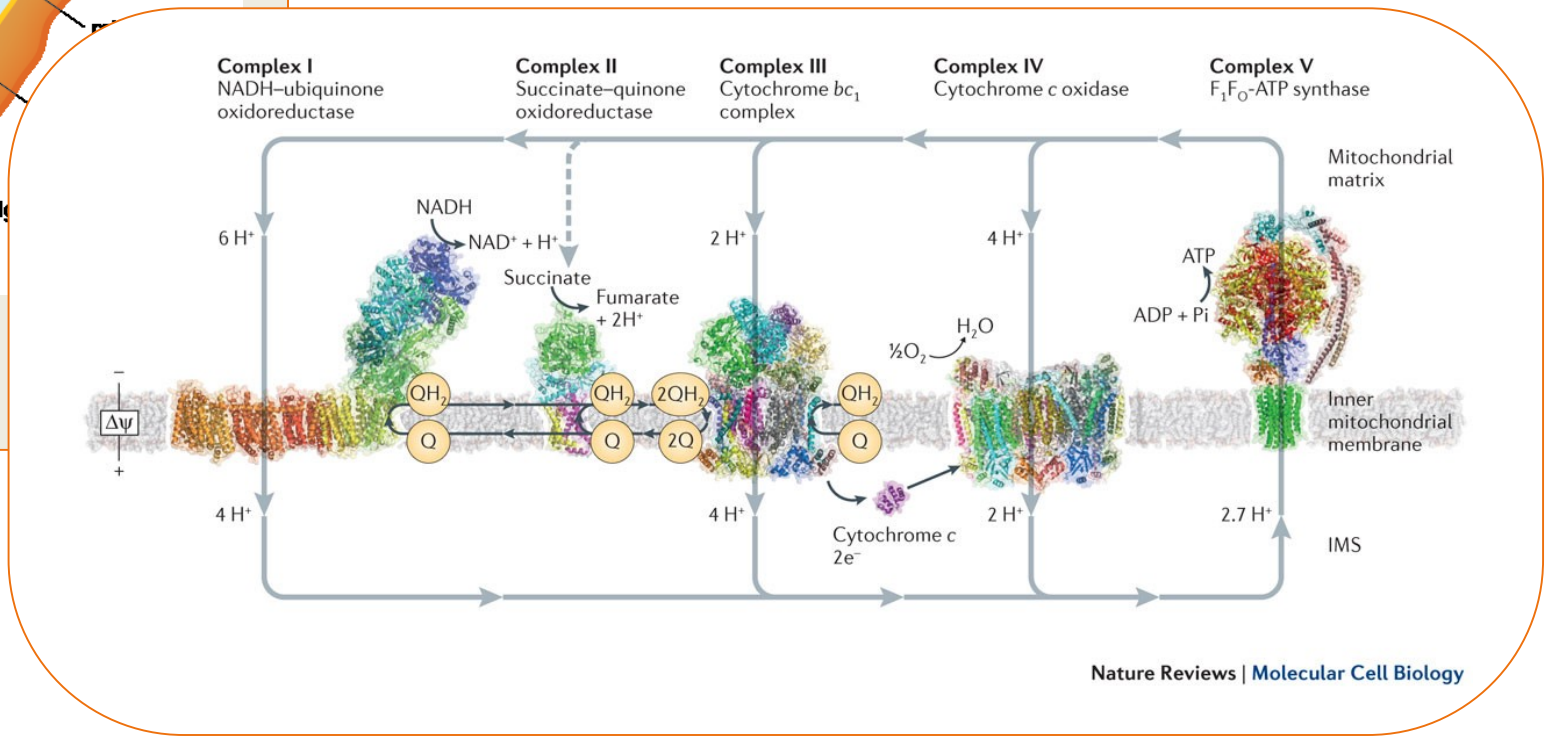
# The respiratory chain




https://www.nature.com/articles/nrm3997#f1
https://www.shmoop.com/biology-cells/most-eukaryotic-cells.html

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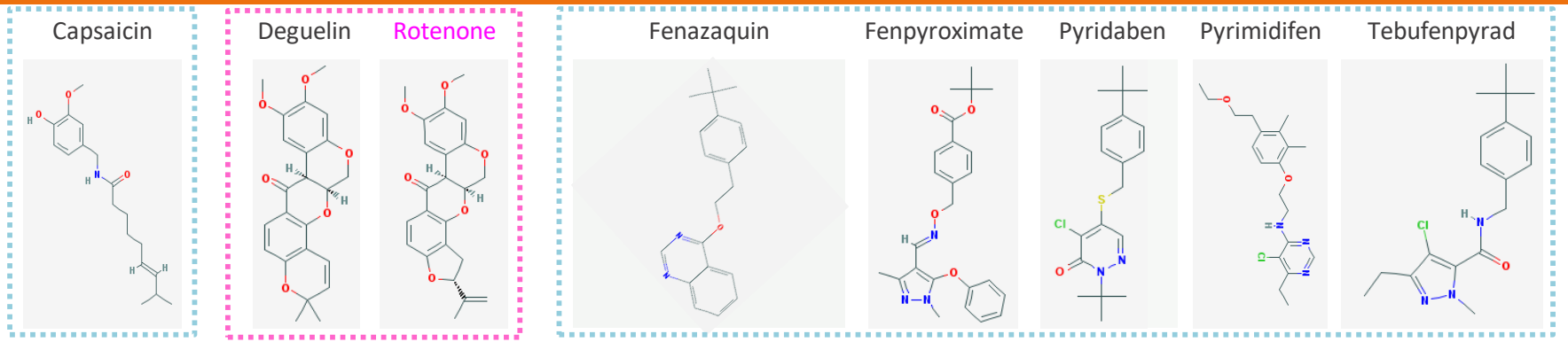
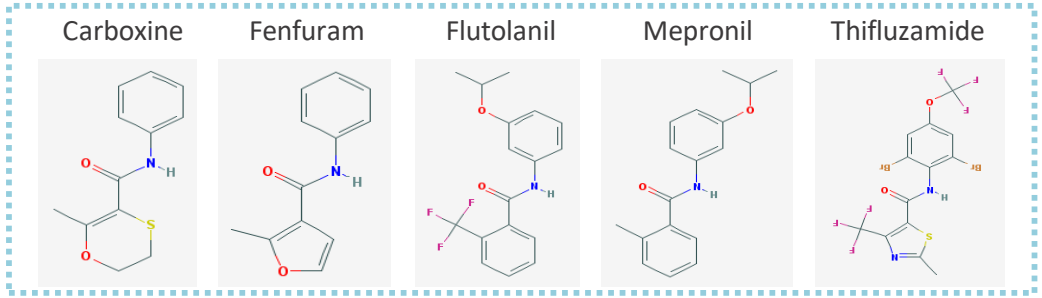
# EU-ToxRisk Case study: mitochondrial complex inhibitors – diverse agro-chemicals

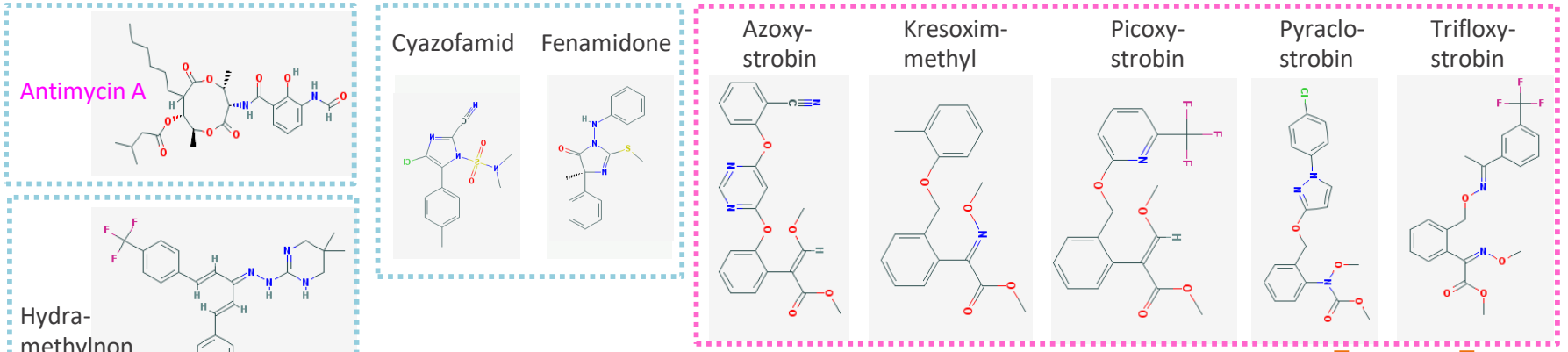
Complex I




Complex II




Complex III

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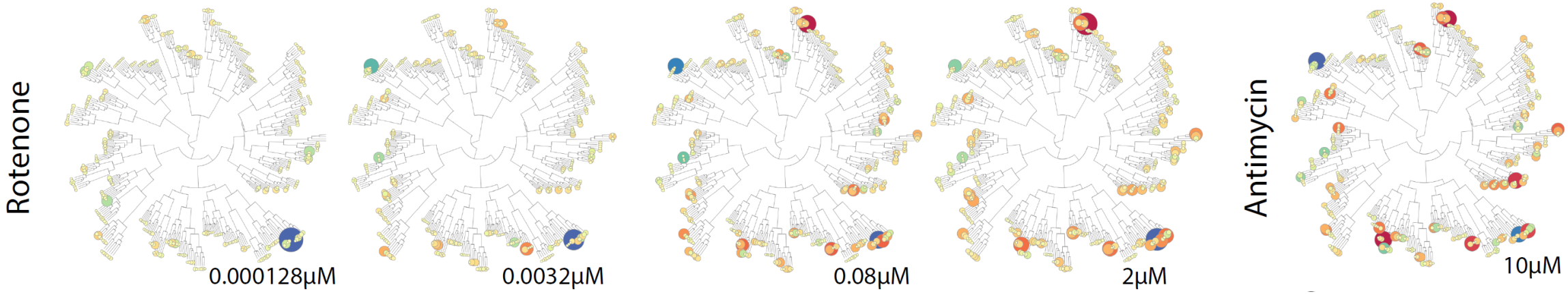
# Mapping of gene expression changes on PHH TXG-MAPr.

- HepG2 cells
- 14 MRC inhibitors (complex 1, 2 and 3)
- 8 concentrations; 3 replicates
- TempO-seq EU-ToxRisk gene panel




Van der Stel et al. Arch Toxicol. 2021

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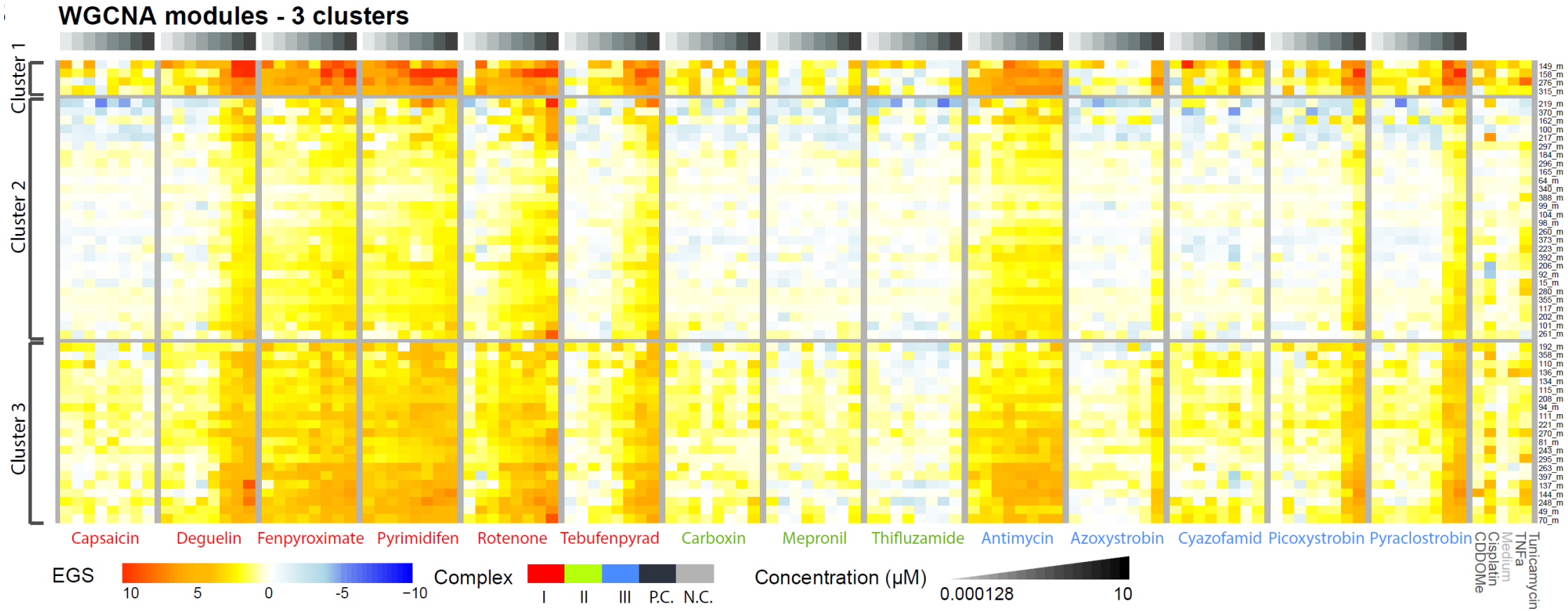
# TXG-MAPr Eigengene scores of critical modules of various mitochondrial complex inhibitors.

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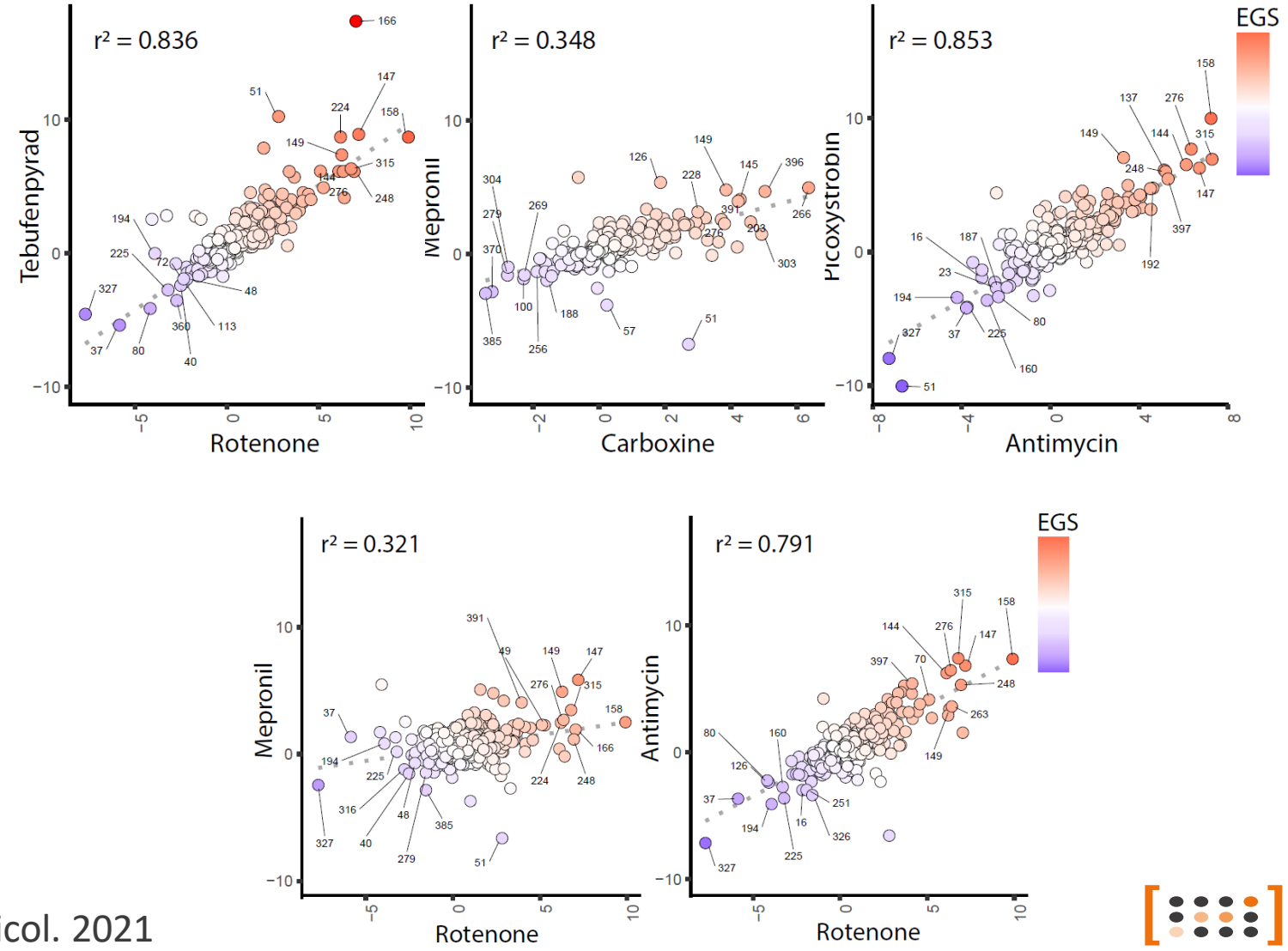
# Correlation of TXG-MAPr module activation by mitochondrial complex inhibitors.

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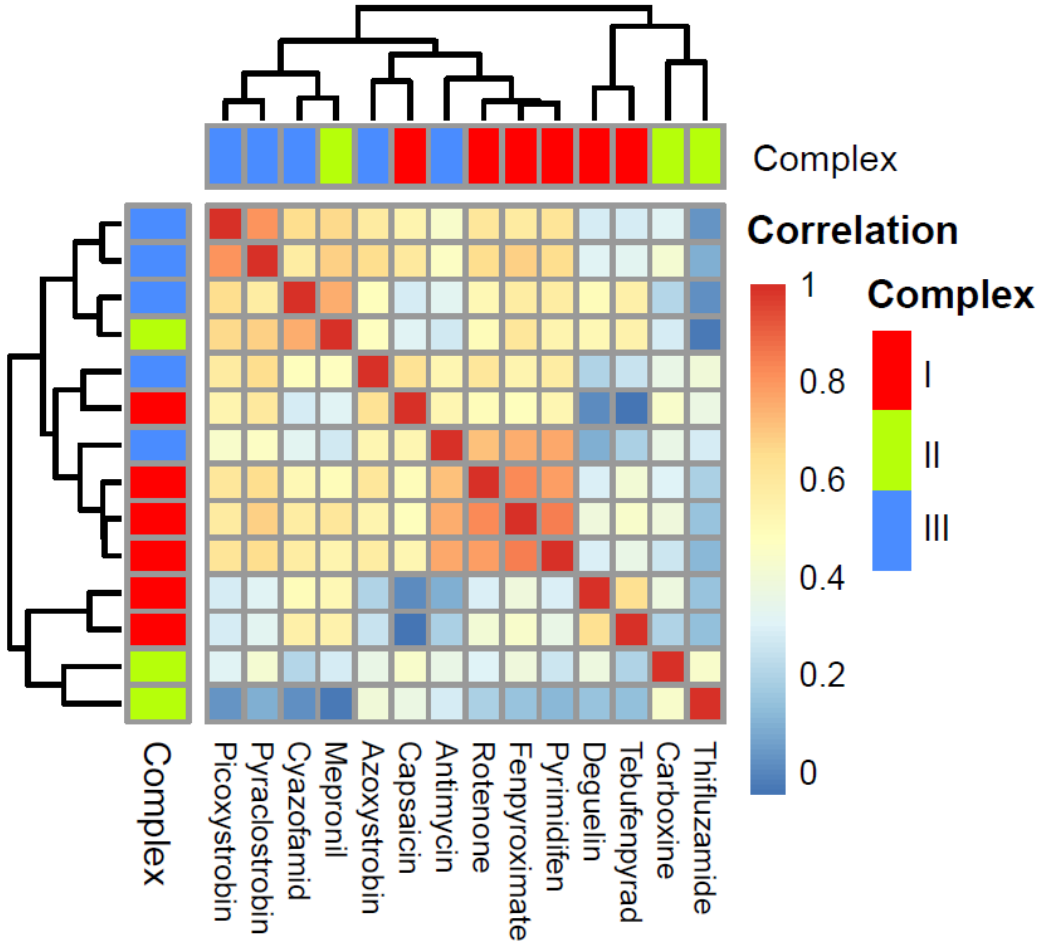
# Similarity in MoA of mitochondrial complex I and III inhibitors based on transcriptomics data.

| ETC inhibitor | Abb. | Complex | MMP BMD (µM) | Selected conc (µM) |
|---|---|---|---|---|
| Capsaicin | CAP | I | NA | 10 |
| Deguelin | DEG | I | 0.04758 | 0.016 |
| Fenpyroximate | FPX | I | 0.00067 | 0.0032 |
| Pyrmidifen | PMD | I | 0.00028 | 0.00064 |
| Rotenone | ROT | I | 0.00106 | 0.0032 |
| Tebufenpyrad | TEB | I | 0.00704 | 0.016 |
| Carboxine | CAR | II | 0.46302 | 2 |
| Mepronil | MEP | II | 2.33739 | 10 |
| Thifluzamide | THI | II | 1.76686 | 2 |
| Antimycin A | AA | III | 0.00177 | 0.0032 |
| Azoxystrobin | AZO | III | 0.44700 | 2 |
| Cyazofamid | CYA | III | 4.82265 | 10 |
| Picoxystrobin | PIC | III | 0.11466 | 0.4 |
| Pyraclostrobin | PYR | III | 0.20586 | 0.4 |

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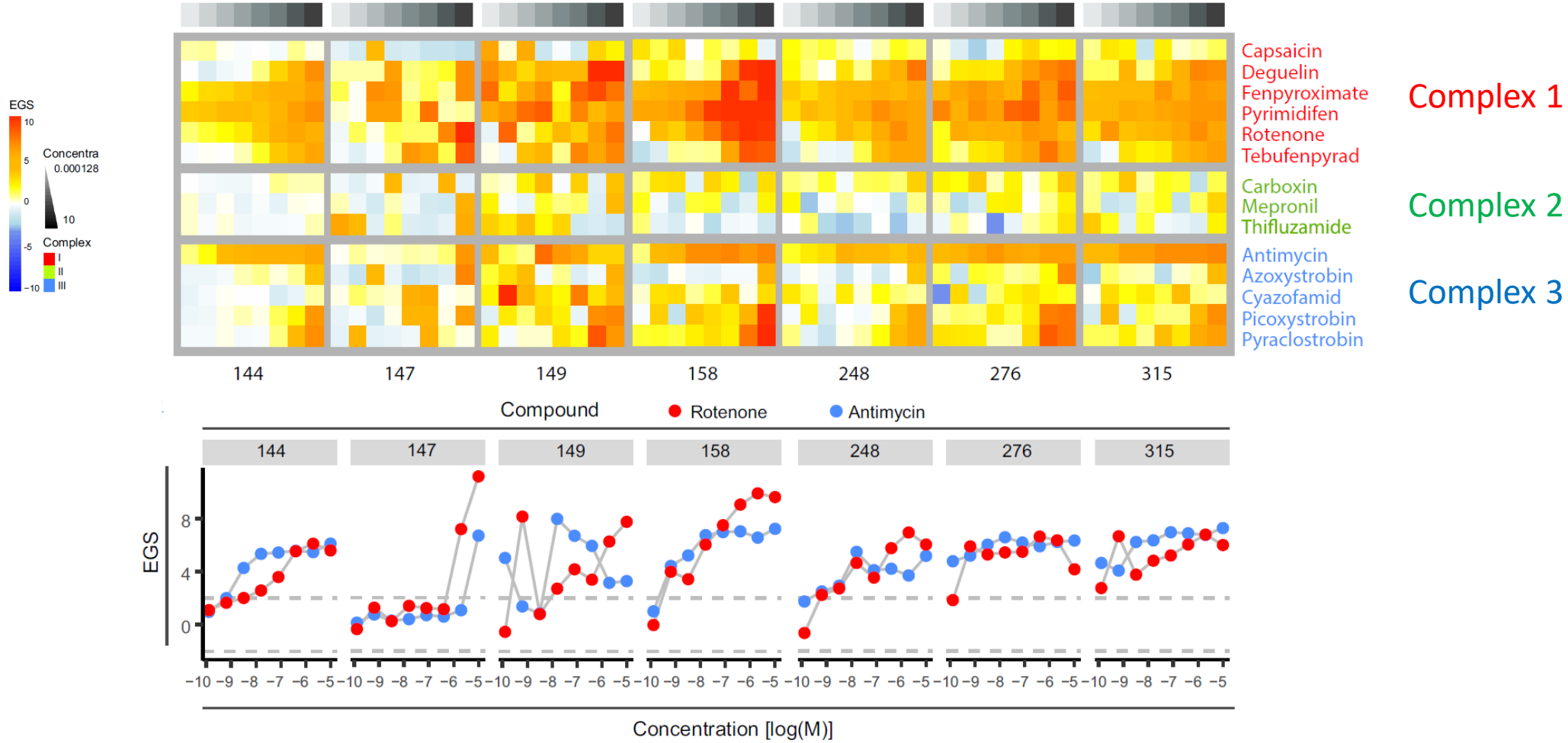
# Different potency of complex inhibitor in the activation of critical TXG-MAPr gene networks

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# Conclusions

- **HTTr can be applied for biological RAx**
- WGCNA-based **TXG-MAP module** comparison allows a **quantitative assessment** of biological similarity
- **Transcriptomics data can** directly linked to AOP outcomes
- **HHTr** can provide **mechanistic underpinning** of biological RAx
- A **surrogate TempOseq gene panel** can detect **MoA and underpin biological similarity**

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**Nanette Vrijenhoek**
**Wanda van der Stel**
**Steven Kunnen & Giulia Callegaro**
Martijn Moné
Sylvia Escher
Erik Danen
Bob van de Water

# Thank you