IMPROVED METHODS FOR ASSESSMENT OF METABOLIC EFFECTS OF ENDOCRINE DISRUPTERS

Stakeholder workshop 24 April 2024

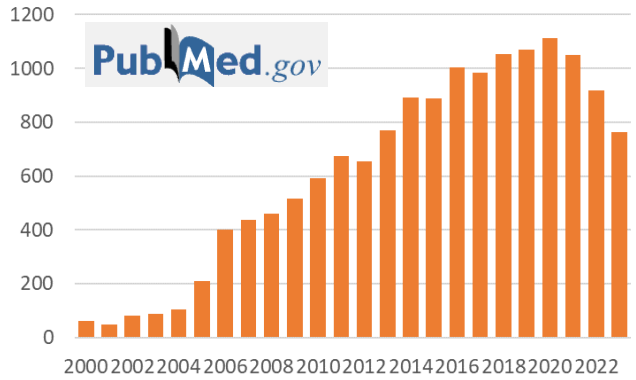
# EDCMET WP2 (PamGene)

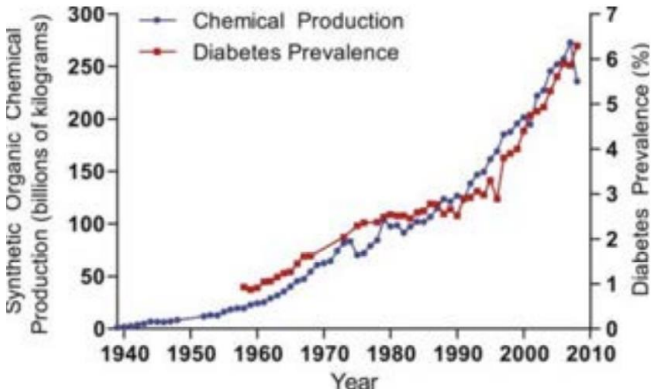
Rinie van Beuningen, PamGene

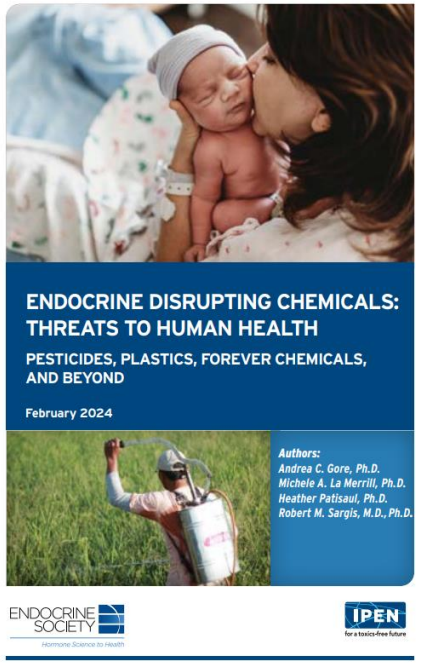
Stakeholder workshop April 24, 2024 (online)

# Endocrine Disruptors; still “a hot-topic”

- 24% of human diseases and disorders globally are attributable to environmental factors.
- About 1000 EDC papers per year
- 1 in 4 adult in the EU have a metabolic syndrome.
- Correlation between chemical production and rise in Diabetes

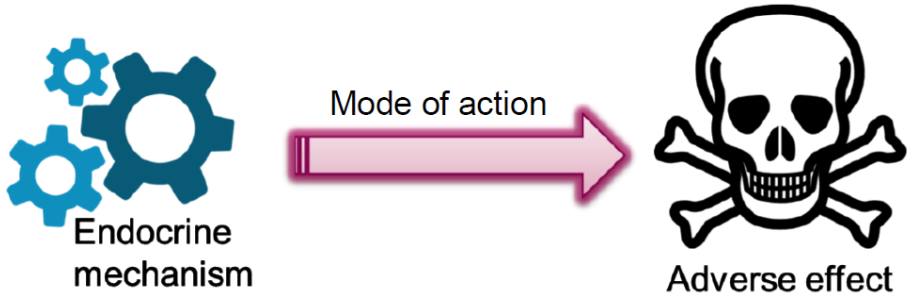


# Identification and assessment of EDs; “an EU priority”

- EDs interfere with the human hormone systems and can casue tumors, birth defects, developmental and **metabolic disorders**.



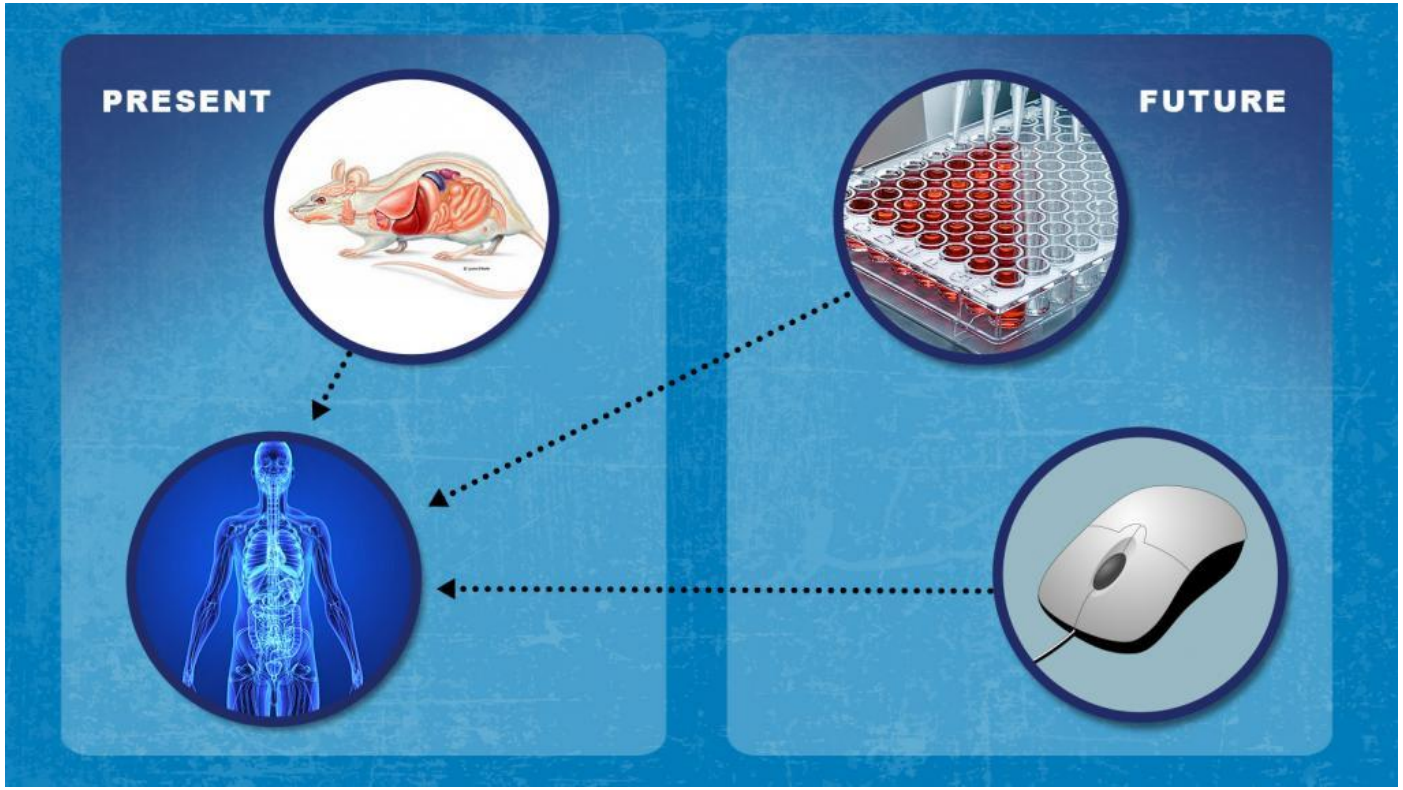

# 1000 of man-made EDs

- Examples: plastics (bisphenol A), plasticizers (phthalates), industrial solvents/lubricants, and byproducts (polychlorinated biphenyls, polybrominated biphenyls, dioxins), pesticides (methoxychlor, chlorpyrifos, dichlorodiphenyltrichloroethane), fungicides (vinclozolin) and pharmaceutical agents (diethylstilbestrol).
- Exposure: air, water, food, and consumer products.
- Accumulation: Some low (BPA, phthalates), while others can accumulate quickly like fat-dissolving EDs.
- From many substances ED properties are still unknow and requires new testing methodologies (EDCMET and other EURION)

# EDCMET objectives

- The overarching objective of the present proposal is to **develop validated in silico**, **in vitro** and **in vivo methods** assessing the **metabolic effects of EDs**. In addition, we will follow the traditional AOP paradigm to identify molecular initiating events (MIE) and predict the emergent adverse biological phenotype
- Assays & Algorithms that Predict metabolic EDs and Why

# Develop next generation ED risk assessment tools

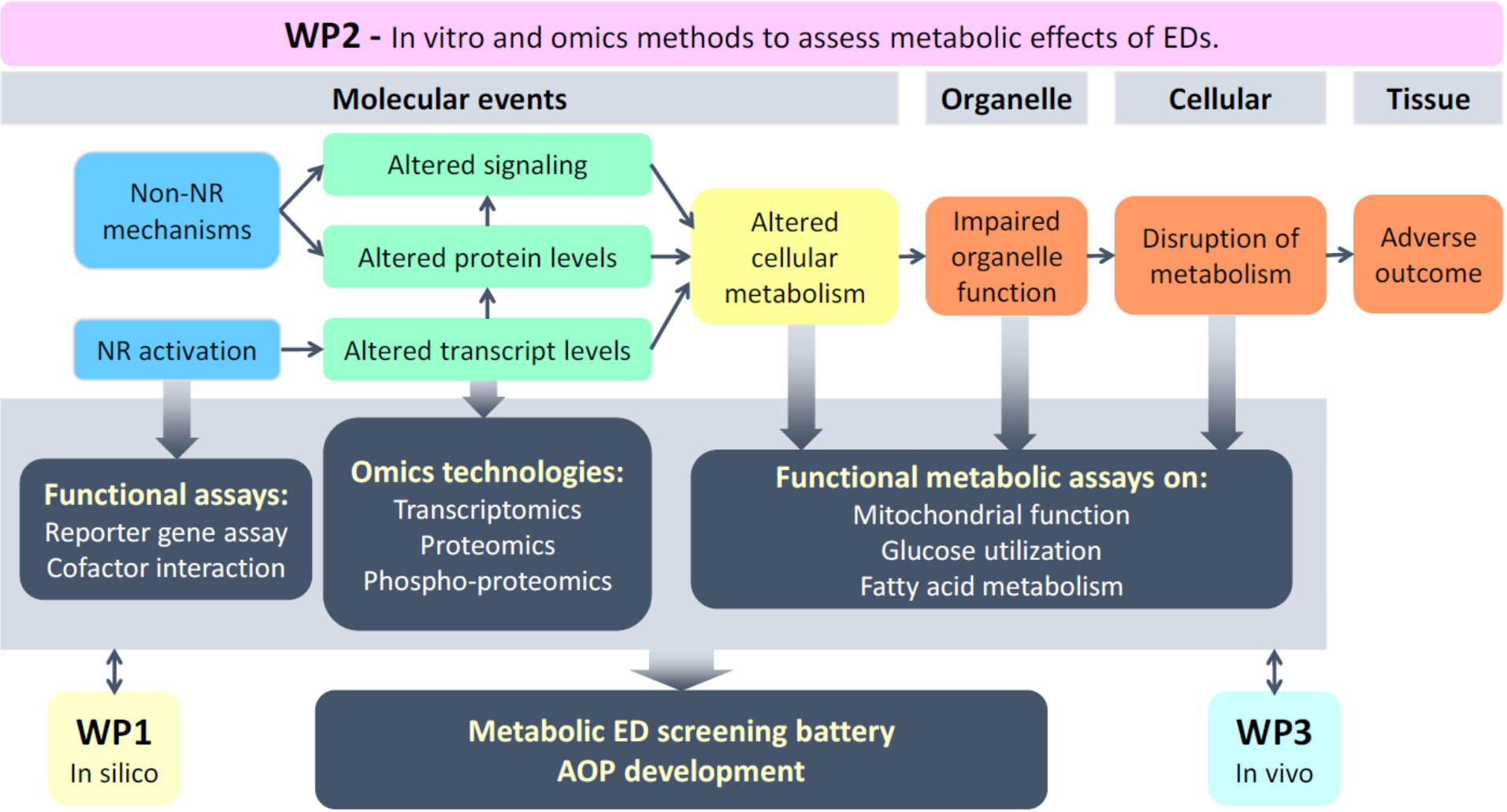



**Predicting ED effects of substances on the human body and physiology through “in-vitro” and “in-silico” models:**

In-vitro “in a pretri dish”  WP2

In-Silico “in a computer” WP1

# WP2: Structure and connections.

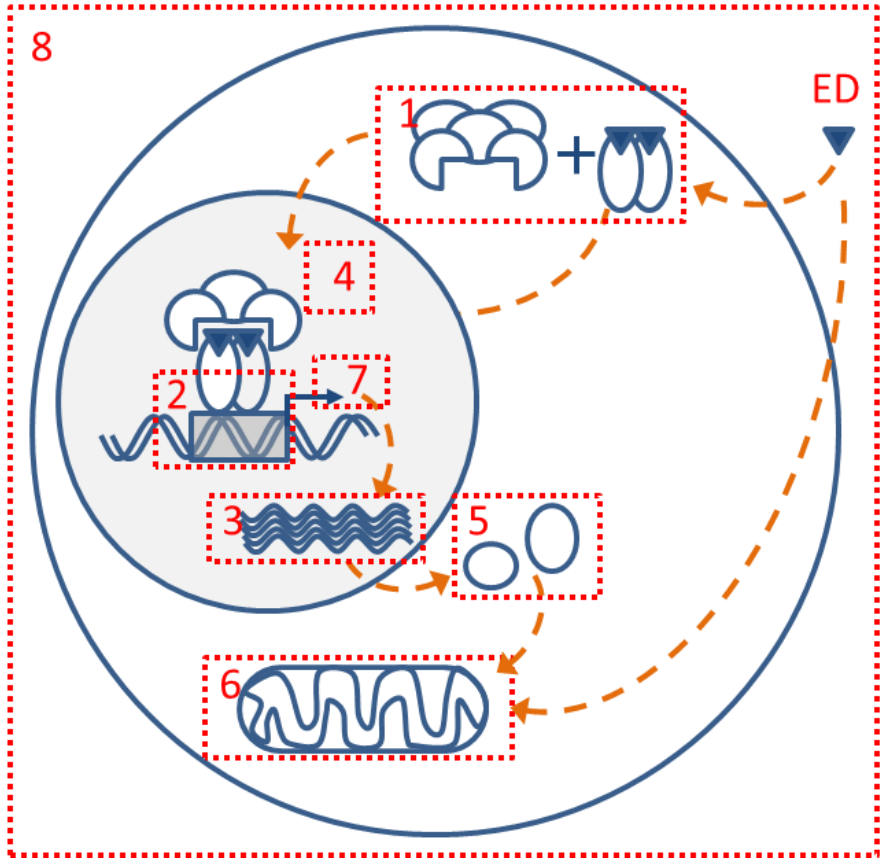


# WP2: In-vitro assay goals

- To generate in vitro **nuclear receptor-cofactor interaction** assays and **reporter assays** to assess metabolic effects of EDs mediated by nuclear receptors
- To develop **functional assays** to address the metabolic effects of EDs
- To generate entirely **novel methods** to assess metabolic effects of EDs utilizing unbiased **omics techniques**
- To work towards **implementation** of the developed test systems in an international **regulatory context**

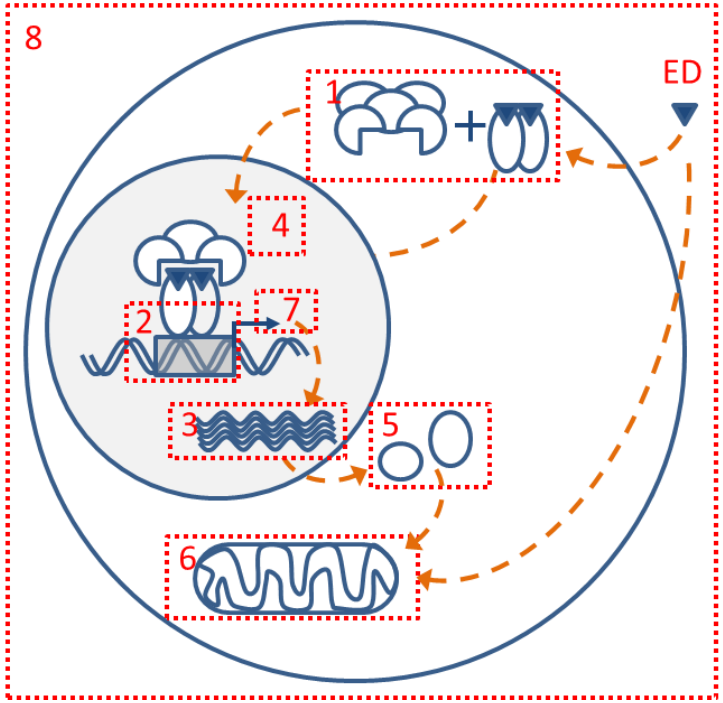
# WP2: In-vitro assays & ED mechanisms

- Developing in-vitro assays to “dissecting” how a human cell (can) react to EDs and
- Understanding the mechanism
- Understanding the downstream effects on cellular signaling.



# WP2: In-vitro assays; testing effects of EDs at the molecular level

- In vitro and omics methods to assess metabolic effects of EDs
  - 1**: Cofactor recruitment**
  - 2: ChIP-seq
  - 3: **Transcriptome profiling**
  - 4: Nuclear Translocation
  - 5: Proteomics
  - 6: **Mitochondrial assays**
  - 7: **Reporter Gene Assays**
  - 8: **Functional cell based assays**

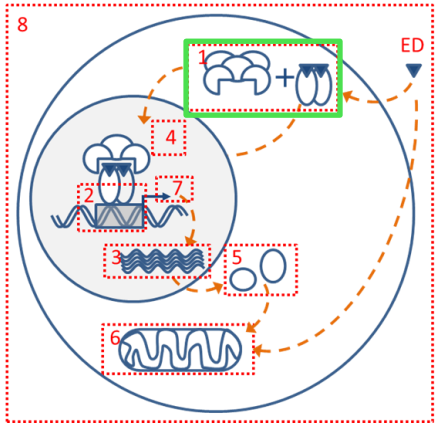


# 1) Cofactor recruitment assays

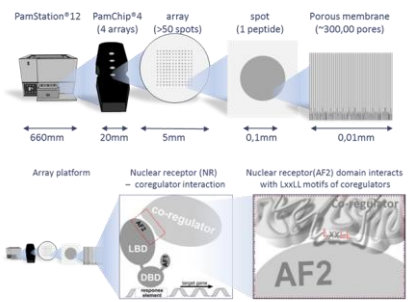
- Assessing if and by what extend compounds directly interact with the nuclear receptor machinery of a cell
- Example shows the number of interaction per compound per nuclear receptor such as the androgen receptor, estrogen receptor and others

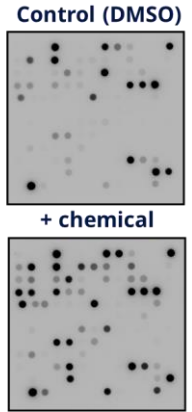
EDCs tested

| AR (LBD-GST) | CAR (LBD-GST) | ER-alpha (LBD-GST) | FXR (LBD-GST) | GR (LBD-GST) | LXR-alpha (LBD-GST) | MR-LBD-GST (ABvector) | PPAR-alpha (LBD-GST) | PPAR-gamma (LBD-GST) | PR (LBD-GST) | Receptors |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | - | - | - | - | 34 | - | - | - | - | 2 |
| - | - | - | - | 1 | 1 | - | - | - | - | 2 |
| - | - | - | - | - | - | - | - | - | - | 0 |
| 1 | 14 | 7 | 12 | - | 1 | - | 63 | - | - | 6 |
| - | 1 | - | 2 | - | - | - | - | - | - | 2 |
| - | - | 16 | - | - | - | - | - | - | - | 1 |
| - | - | - | - | - | 34 | - | - | 1 | - | 2 |
| 1 | - | - | 6 | 1 | 25 | - | - | - | - | 4 |
| - | 13 | 12 | 9 | - | - | - | - | - | - | 3 |
| 8 | - | 1 | 2 | 2 | 10 | - | 3 | - | - | 6 |
| - | 1 | 46 | 1 | - | 8 | - | - | - | - | 4 |
| - | - | 3 | 1 | - | - | - | - | 1 | - | 3 |
| - | 13 | 33 | - | - | 3 | - | 6 | - | - | 4 |
| 6 | - | - | 1 | - | - | - | - | - | 1 | 3 |
| 9 | - | - | - | 1 | 23 | - | 1 | - | - | 4 |
| 1 | - | 4 | - | - | - | - | - | 1 | - | 3 |
| - | 1 | - | 1 | - | 1 | - | 5 | - | - | 4 |
| 4 | - | - | - | - | 4 | - | - | - | - | 2 |
| 2 | - | - | 7 | - | 21 | - | - | 5 | - | 4 |
| - | - | - | 7 | - | 1 | - | - | - | - | 2 |
| 3 | - | - | - | - | - | - | - | - | - | 1 |
| 10 | 6 | 8 | 11 | 4 | 13 | - | 5 | 4 | 1 | |



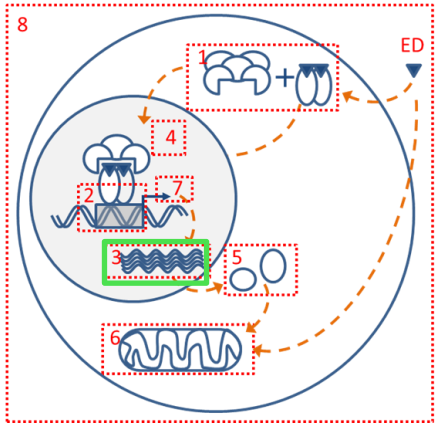

Technology outline:



# 3) Transcriptome profiling

- Identify (bio)markers using transcriptome profiling to help explain the underlying biology of endocrine disruption in human hepatocytes
- Example shows 14 gene clusters identified with the tested EDCs in liver cell lines.

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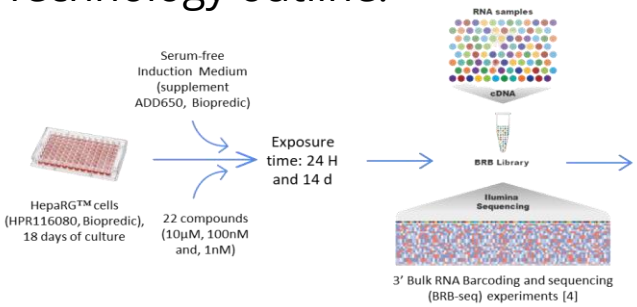
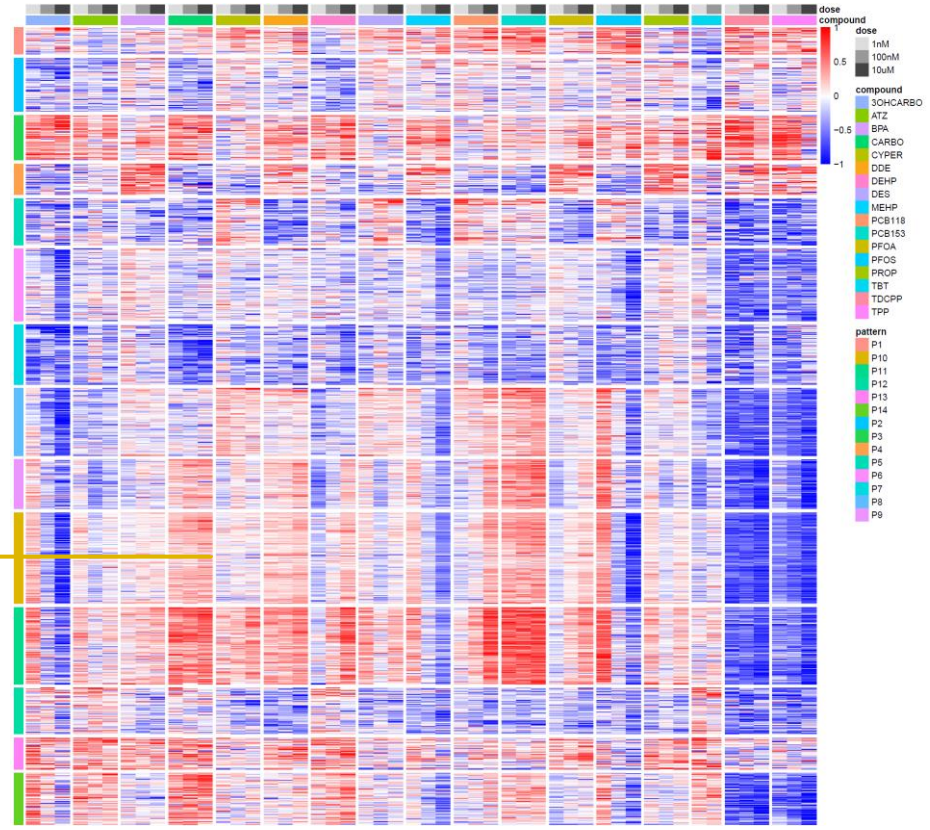



Genes involved in:

regulation of metabolic process (GO:0019222)

cellular metabolic process (GO:0044237)

14 Gene Clusters



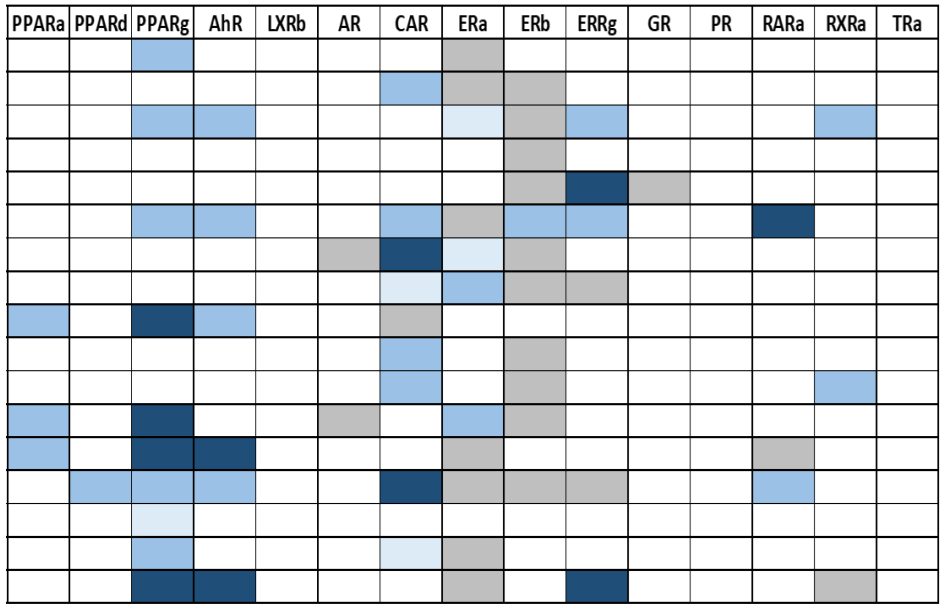

EDCs tested at 3 concentrations

# 7) Reporter Gene Assays

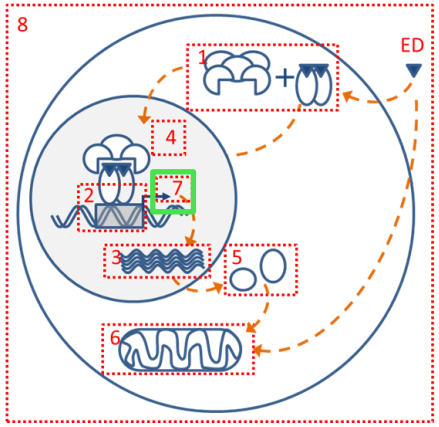
- Identify if and to what extend EDCs impact nuclear receptors in engineered cell lines.
- Example shows 15 out of 19 nuclear receptors (excluding LXRa, LXRb , PR and TRa) were activated by at least one EDC in the micromolar ranges

Nuclear Receptors tested

EDCs tested




| | |
|---|---|
| | no activity |
| | EC50 < 1 µM |
| | EC50 1-50 µM |
| | EC50 > 50 µM |
| | no dose response |

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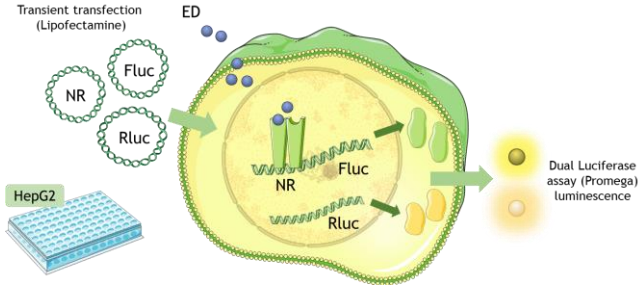
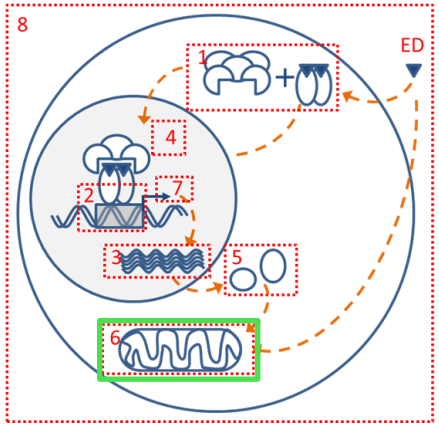


# 6) Mitochondrial assays

- Identify if EDCs lead to activation metabolism in liver cells using functional assays in mitochondria
- Example shows basal oxygen consumption and other indicators of metabolism on a number of EDCs. Effects between EDCs with similar structures or belonging to the same chemical group as well as between parent compound and metabolite vary.

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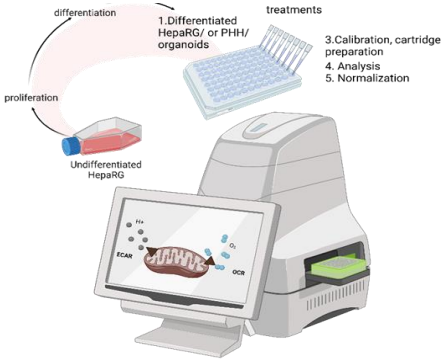
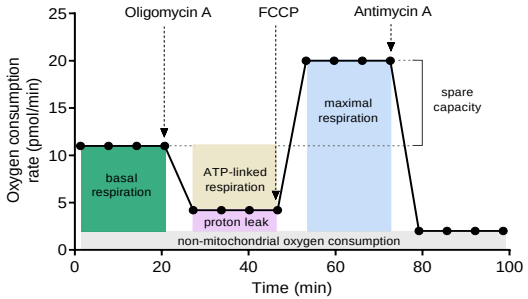
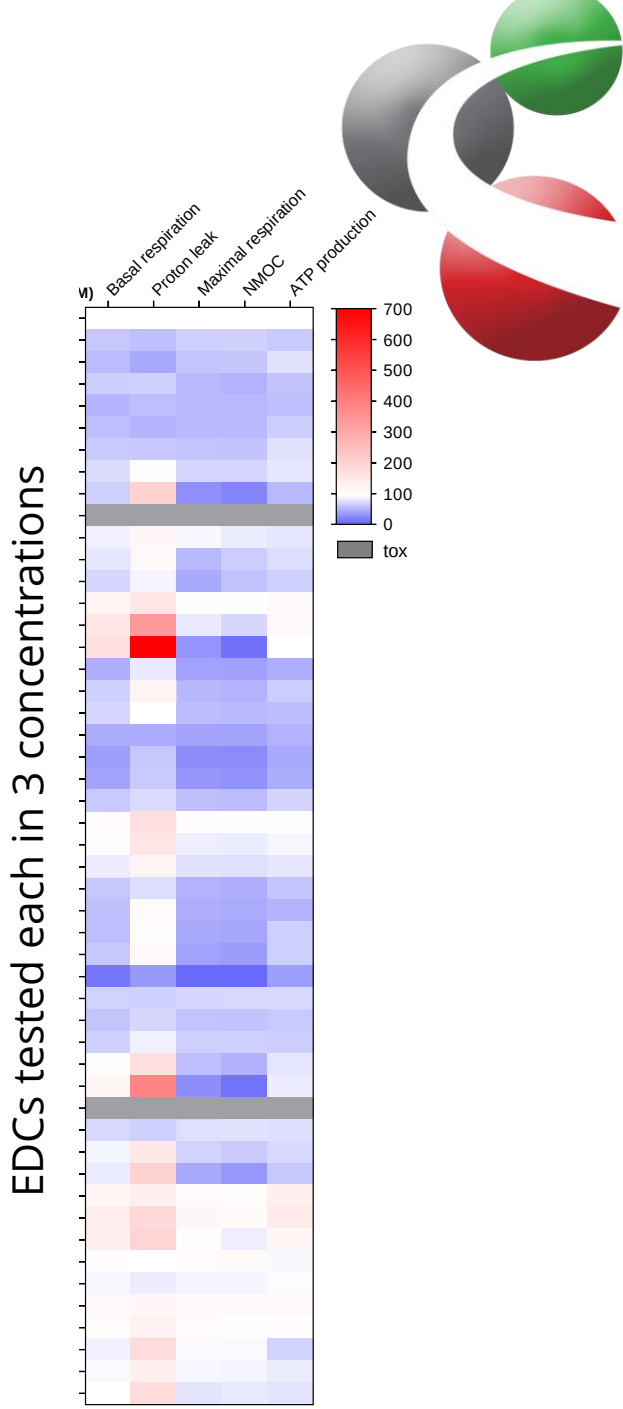


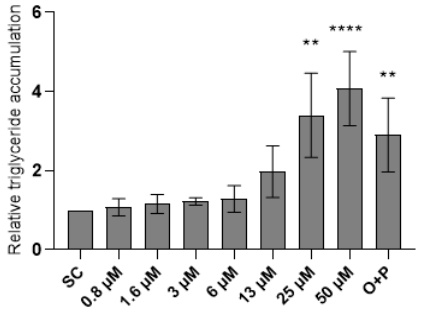



# 8) Functional cell based assays

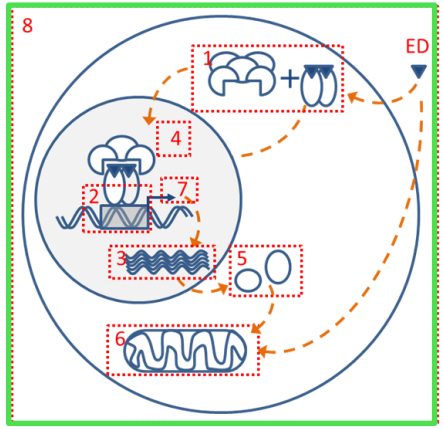
- Identify if EDCs lead to triglyceride accumulation in liver cells which is associated with metabolic diseases (Adipored assay). AdipoRed assay is the closest surrogate for in vivo steatosis.
- Example shows that 11 out of the 17 EDCs induced triglyceride accumulation.

EDCs tested in the Adipored assay

| ED | AdipoRed |
|---|---|
| 1 | +++ |
| 2 | - |
| 3 | - |
| 4 | +++ |
| 5 | - |
| 6 | +++ |
| 7 | - |
| 8 | - |
| 9 | ++ |
| 10 | ++ |
| 11 | ++++ |
| 12 | - |
| 13 | ++ |
| 14 | (++++) |
| 15 | +++++ |
| 16 | +++++ |
| 17 | (+++) |

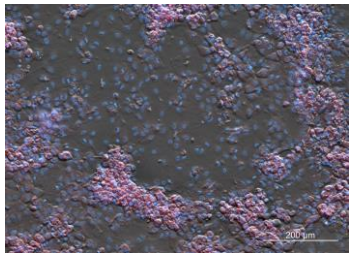



| Adipored induction | |
|---|---|
| > 2.5 | +++++ |
| > 2.25 | ++++ |
| > 2 | +++ |
| > 1.75 | ++ |
| > 1.5 | + |

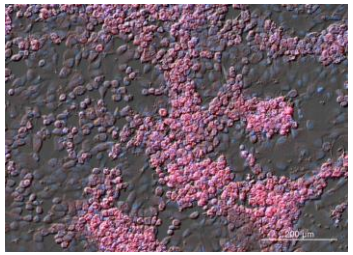



Technology outline:

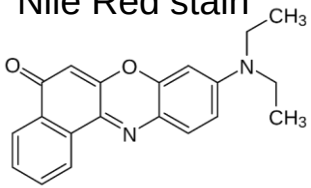
Control



EDC



Lipophilic
Nile Red stain

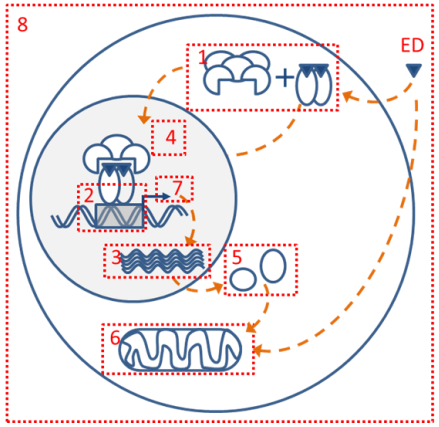


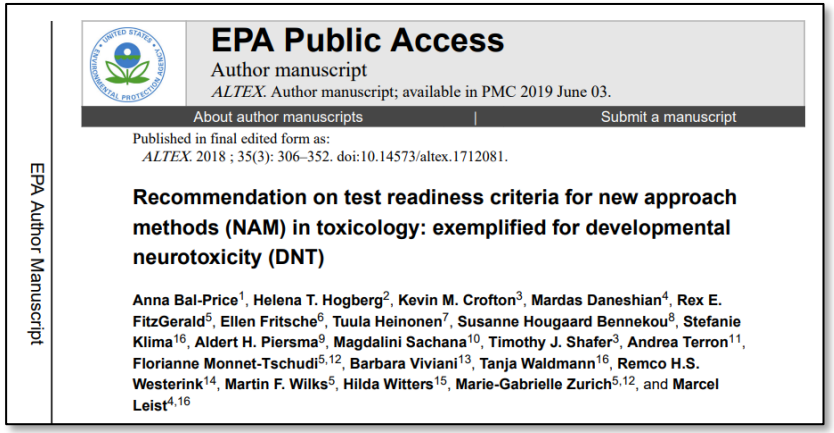
# The (AI) challenge ahead

- To validate these in-vitro assay batteries in isolation or in combination with in-silico approaches as a true predictor for any new and untested chemicals as a metabolic ED poses a real challenge.
  - Qualitative “Which test is better” using Bal-Price methodology
  - In vitro test battery validation

# Qualitative "Which test is better" using Bal-Price methodology

- Scoring of the in-vitro assays using Bal-Price model shows that:
  - All in-vitro assays need certain improvements
  - The AdipoRed assay is the best "scoring" assay
- The AdipoRed assay (no 8) is now in pre-validation via PEPPER.



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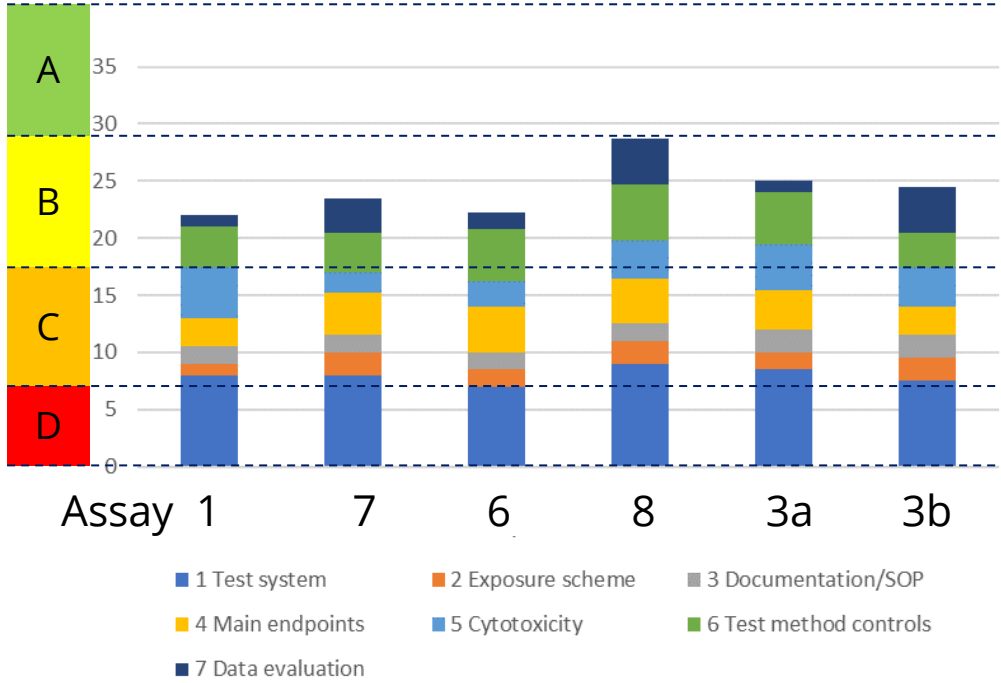
**Recommendation on test readiness criteria for new approach methods (NAM) in toxicology: exemplified for developmental neurotoxicity (DNT)**

Anna Bal-Price[1], Helena T. Hogberg[2], Kevin M. Crofton[3], Mardas Daneshian[4], Rex E. FitzGerald[5], Ellen Fritsche[6], Tuula Heinonen[7], Susanne Hougaard Bennekou[8], Stefanie Klima[16], Aldert H. Piersma[9], Magdalini Sachana[10], Timothy J. Shafer[3], Andrea Terron[11], Florianne Monnet-Tschudi[5,12], Barbara Viviani[13], Tanja Waldmann[16], Remco H.S. Westerink[14], Martin F. Wilks[5], Hilda Witters[15], Marie-Gabrielle Zurich[5,12], and Marcel Leist[4,16]




| Score | Grading | |
|---|---|---|
| < 7 | D | Not ready at all |
| 8-17 | C | Substantial improvements are required to be ready |
| 18-28 | B | Improvements are required to be ready |
| 29-35.5 | A | Test methods close to ready or ready |

# In vitro test battery validation

- Published literature on EDC-associated in vivo hepatic lipid accumulation, lipid dysregulation and/or obesity were collected and scored in three categories (0 = no evidence, 1 = contrasting evidence, 2 = positive evidence)
  - AdipoRed assay is the closest surrogate for in vivo steatosis
  - NR RGAs and mitochondrial function assay provide a second-tier assay battery
  - NR RGAs for CAR, FXR and PPARg associated with in vitro steatosis
- Further (AI) bioinformatics challenges ahead

EDCs tested

| In vivo | Adipored | CAR | PXR | FXR | LXRa | LXRb | PPARa | PPARb/d | PPARg | RXRa | RARa | TRa | VDR | ERRg | ERa | GR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 4 | 2 | 4 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 1 | 3 | 1 | 0 |
| 2 | 4 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 4 | 1 | 2 | 1 | 3 | 1 | 0 |
| 2 | 3 | 3 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 2 | 3 | 0 | 2 | 4 | 0 | 0 |
| 2 | 3 | 2 | 2 | 1 | 0 | 0 | 3 | 0 | 2 | 1 | 2 | 0 | 1 | 2 | 1 | 0 |
| 1 | 3 | 3 | 3 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 1 | 3 | 1 | 0 |
| 2 | 3 | 3 | 3 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 3 | 3 | 2 | 3 | 3 | 0 |
| 0 | 3 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 2 | 3 | 1 | 0 |
| 2 | 3 | 3 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 1 | 2 | 1 | 0 |
| 2 | 3 | 3 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 2 | 3 | 1 | 0 |
| 2 | 3 | 1 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 2 | 2 | 2 | 1 | 3 | 3 | 0 |
| 1 | 3 | 2 | 3 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 3 | 0 | 0 |
| 2 | 3 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | 3 | 0 | 3 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 2 | 1 | 0 |
| 2 | 2 | 4 | 4 | 2 | 1 | 2 | 0 | 0 | 2 | 3 | 4 | 3 | 3 | 4 | 1 | 0 |
| 2 | 2 | 0 | 3 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| 1 | 2 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 0 |
| 0 | 2 | 3 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 1 | 3 | 2 | 0 |
| 1 | 2 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 3 | 2 | 1 | 0 | 2 | 0 | 0 |
| 0 | 2 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 1 | 3 | 1 | 0 |
| 2 | 2 | 4 | 3 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 1 | 3 | 3 | 0 |
| 0 | 2 | 2 | 3 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 3 | 1 | 0 |
| 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 1 | 0 | 0 | 0 |
| 1 | 1 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 3 | 3 | 0 |
| 0 | 1 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 2 | 1 | 1 | 2 | 0 | 0 |
| 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| 1 | 1 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 1 | 0 | 0 |
| 0 | 1 | 2 | 3 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 1 | 2 | 3 | 2 | 0 |
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| 0 | 0 | 1 | 2 | 1 | 0 | 0 | 3 | 0 | 0 | 1 | 2 | 0 | 1 | 3 | 2 | 0 |
| 2 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 3 | 0 | 2 |
| 0 | 0 | 0 | 3 | 1 | 0 | 0 | 2 | 0 | 0 | 3 | 0 | 0 | 0 | 2 | 0 | 0 |
| 0 | 1 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 1 | 0 |

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- CU: Petr Pavek, Alžbeta Horvátová
- BfR: Dajana Lichtenstein, Albert Braeuning
- Eurosafe: Thomas Darde

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