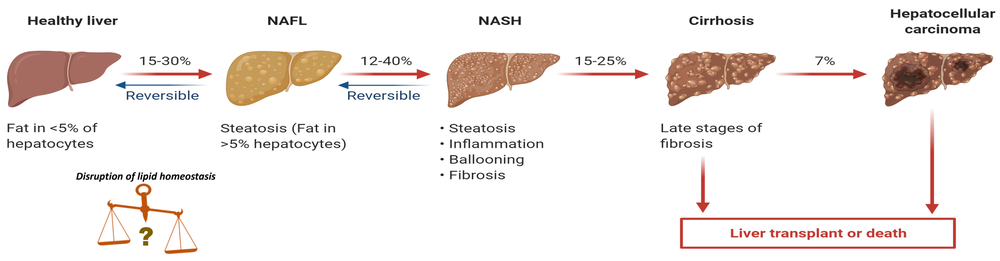
This work is funded by the European Union's Horizon 2020 research and innovation program under grant agreement no 825712 - OBERON project (https://oberon-4eu.com), that is part of the European EURION cluster (http://eurioncluster.eu/) and the Internal |Grant agency (IGA MU) student grant project MUNI/IGA/1194/2021.

Assessing Steatosis-Related Effects of Endocrine Disruptors: A Comparative Study of 2D and 3D In Vitro Approaches

Grosso, M.F., Virmani, I., Chowdhury, R. R., Řehůřková, E., Sychrová, E., Gadara, D. C., Spáčil, Z., Sovadinová, I., Babica, P.
RECETOX, Faculty of Science, Masaryk University, Brno, Czech Republic.

Overview

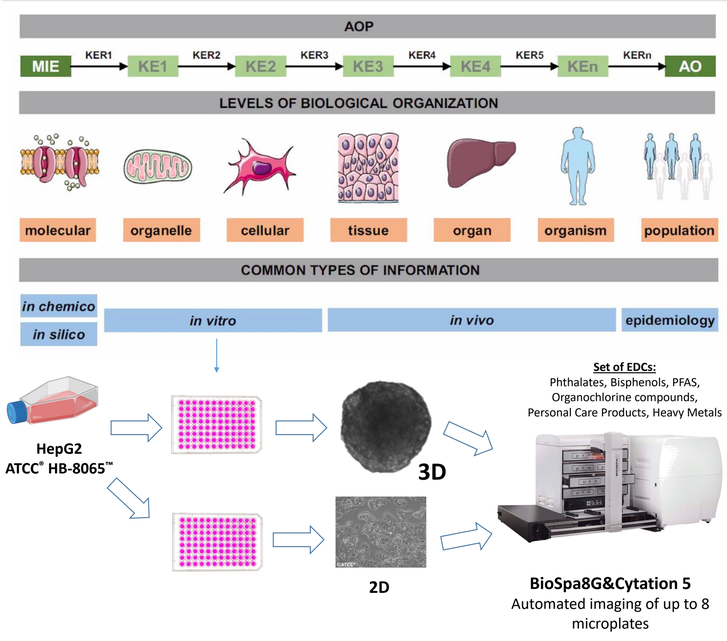
- **Endocrine-disrupting compounds (EDCs)** have been associated with adverse health outcomes, including Non-alcoholic Fatty Liver Disease (NAFLD)**. Previous studies on the association of EDCs with NAFLD are very limited.**
- There are no validated *in vitro* assays to be used to **identify and assess the potential of EDCs** to induce hepatic steatosis.

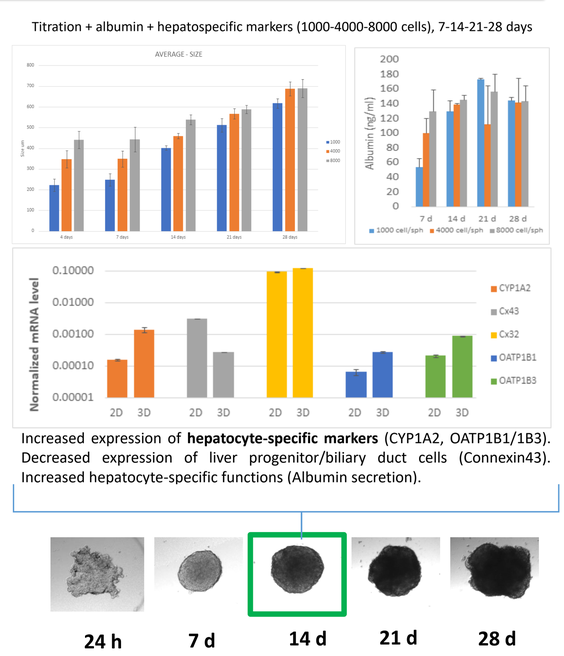


AIMS

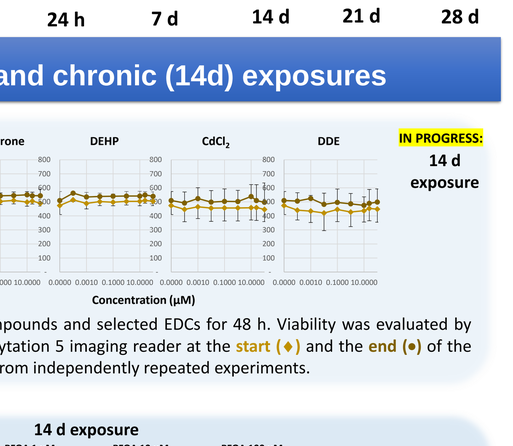
- ✓ Optimize a **simple and rapid screening assay** based on 2D and 3D *in vitro* cultures of human liver cells - **HepG2 ATCC® HB-8065™** to detect the hepatotoxic and steatogenic potential of selected food and environmental toxicants;
- ✓ **Validate its efficiency** (sensitivity, specificity, and predictive accuracy) by comparison with other human *in vitro* liver models studied within OBERON project (**HepaRG and MIHA cell lines**), additional **transcriptomic and metabolomic endpoints**, and **chronic exposures;**
- ✓ Mechanistic assessment of further molecular and cellular events and AOP key events for NAFLD/NASH.

Methods



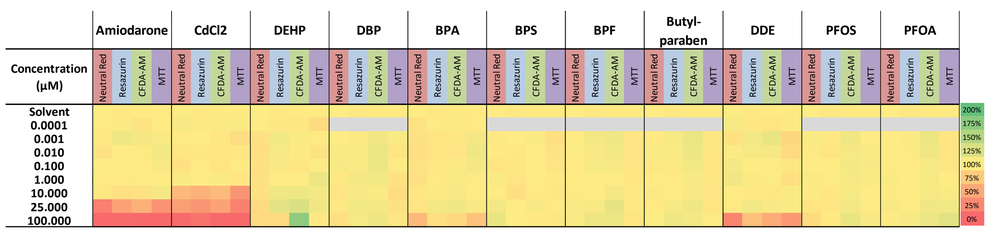


Increased expression of **hepatocyte-specific markers** (CYP1A2, OATP1B1/1B3). Decreased expression of liver progenitor/biliary duct cells (Connexin43). Increased hepatocyte-specific functions (Albumin secretion).



2D monolayers of HepG2 – Acute Exposures (48 h)

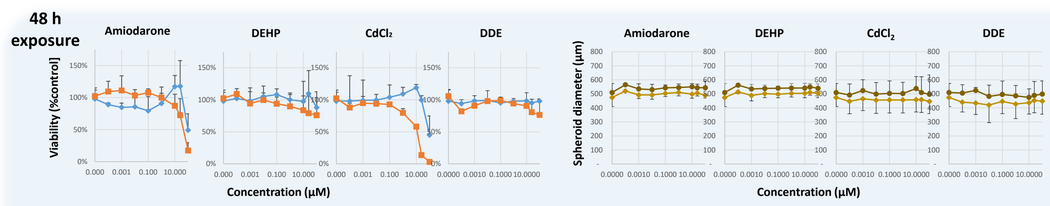
Viability



Viability assessment in 2D monolayers of HepG2 cells exposed to model compounds or EDCs for 48 h. Viability was evaluated by **Neutral red uptake**, **Resazurin**, **CFDA-AM**, and **MTT**. Data were normalized to negative control and represent means from independently repeated experiments (n≥3).

3D HepG2 spheroids – Acute (48h) and chronic (14d) exposures

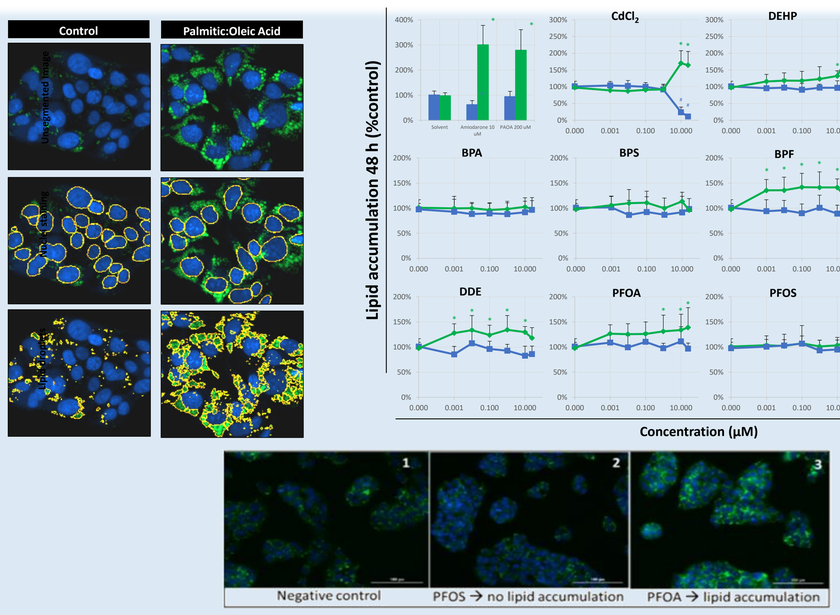
Viability



IN PROGRESS: 14 d exposure

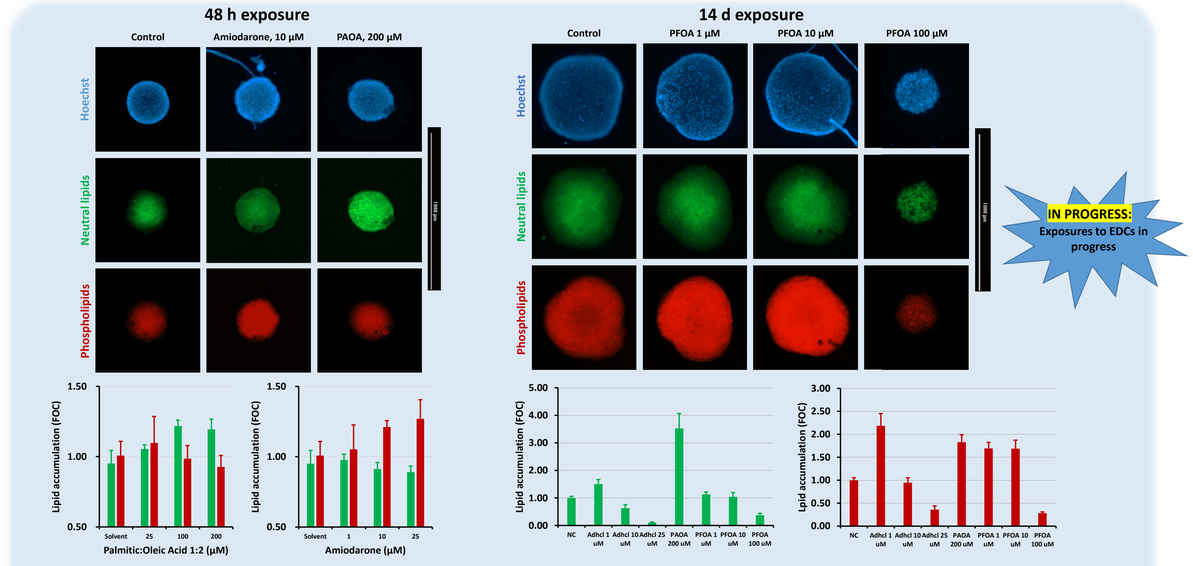
Viability and spheroid size assessment in 3D HepG2 spheroids exposed to model compounds and selected EDCs for 48 h. Viability was evaluated by Resazurin (♦) assay and ATP (■) content. Spheroid size (diameter) was evaluated by Cytation 5 imaging reader at the **start** (♦) and the **end** (●) of the exposure. Viability data were normalized to negative control and represent means ±SD from independently repeated experiments.

Lipid Accumulation



High-content imaging of lipids in 2D monolayers of HepG2 cells exposed to model compounds or EDCs for 48 h. Lipids were stained with **Bodipy 493/593**, nuclei with **Hoechst 33342**. Images were acquired using Cytation 5 imaging reader and fluorescence intensity was quantified by Gen5 software. Data were normalized to negative control and represented means±SD from independently repeated experiments (n≥4). PAOA – palmitic: oleic acid mixture (1:2).

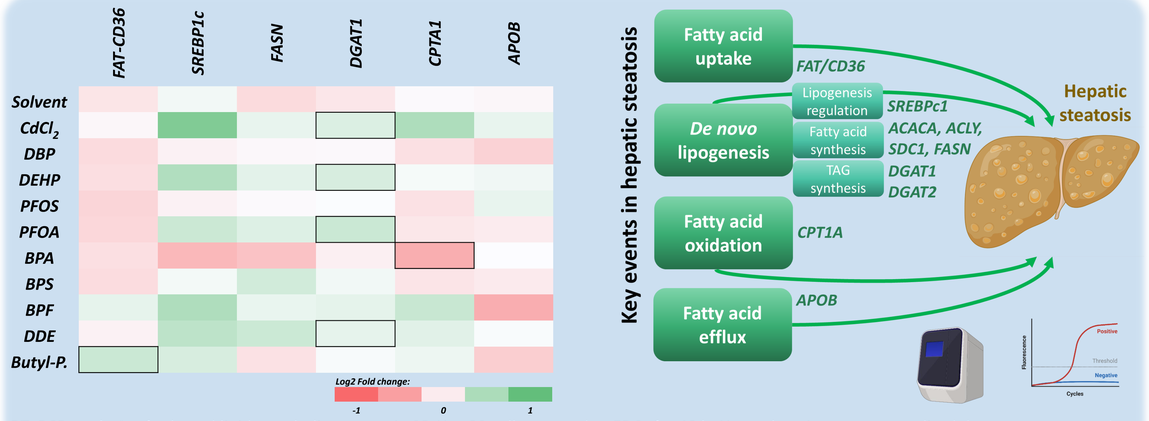
Lipid Accumulation



IN PROGRESS: Exposures to EDCs in progress

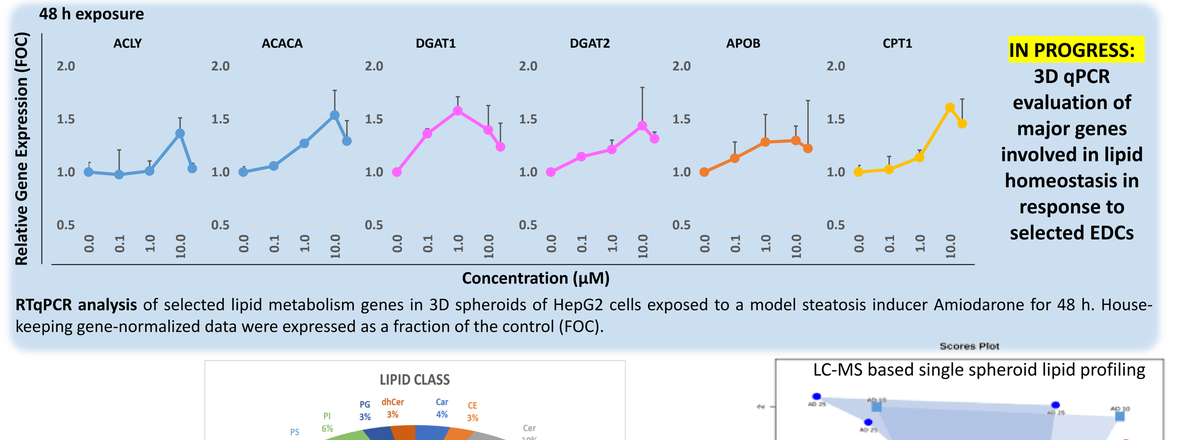
High-content imaging of lipids in 3D HepG2 spheroids exposed to model compounds. Lipids were detected with AdipoRed (ready-to-use solution of Nile Red) staining **neutral lipids (green)** as well as **phospholipids (red)**. **Hoechst 33342** was used to counterstain nuclei. Images were acquired using Cytation 5 imaging reader and fluorescence intensity quantified by Gen5 software. Lipid fluorescence was adjusted by spheroid size (Hoechst staining) and data normalized to negative control (means±SD). PAOA – palmitic:oleic acid mixture (1:2).

Lipid metabolism genes



RTqPCR analysis of selected lipid metabolism genes in 2D HepG2 cells exposed to EDCs for 48 h. House-keeping gene-normalized data were expressed as a log2 fold change compared to the control (independently repeated experiments (n≥4).

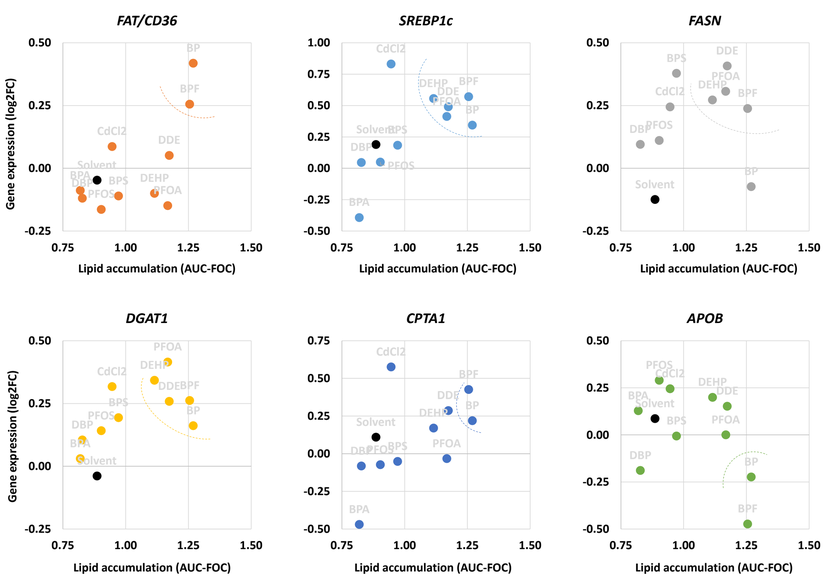
Lipid metabolism genes



IN PROGRESS: 3D qPCR evaluation of major genes involved in lipid homeostasis in response to selected EDCs

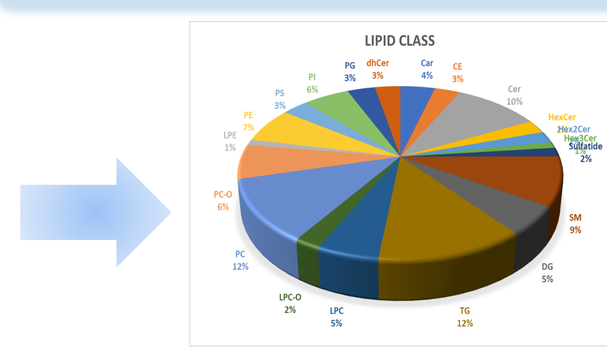
RTqPCR analysis of selected lipid metabolism genes in 3D spheroids of HepG2 cells exposed to a model steatosis inducer Amiodarone for 48 h. House-keeping gene-normalized data were expressed as a fraction of the control (FOC).

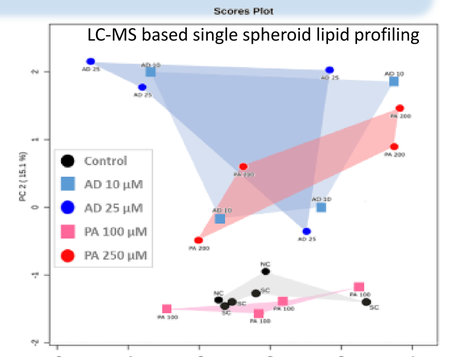
Lipidomics



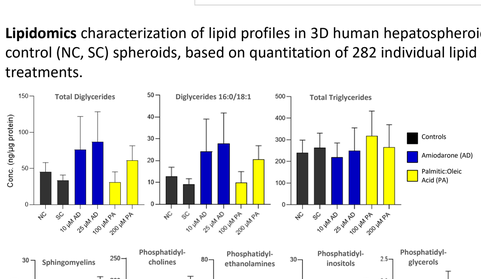
The optimized workflow allows using 2D and 3D cultures of HepG2 cells to assess key events of hepatic steatosis/NAFLD in response to EDCs with sufficient throughput and scalability, which can be further utilized for toxicity testing as well as in mechanistic studies.

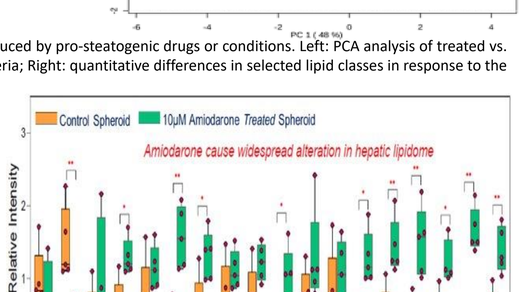
Lipidomics





Lipidomics characterization of lipid profiles in 3D human hepatospheroids, and the changes induced by pro-steatogenic drugs or conditions. Left: PCA analysis of treated vs. control (NC, SC) spheroids, based on quantitation of 282 individual lipid species passing QC criteria; Right: quantitative differences in selected lipid classes in response to the treatments.





Single-spheroid lipidomic analysis of HepG2 spheroids treated for 48 h with model steatogenic compounds: amiodarone (AD) or palmitic:oleic acid mixture (1:2).