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2D and 3D *in vitro* models of HepG2 cells to assess steatosis-relevant effects of endocrine disruptors

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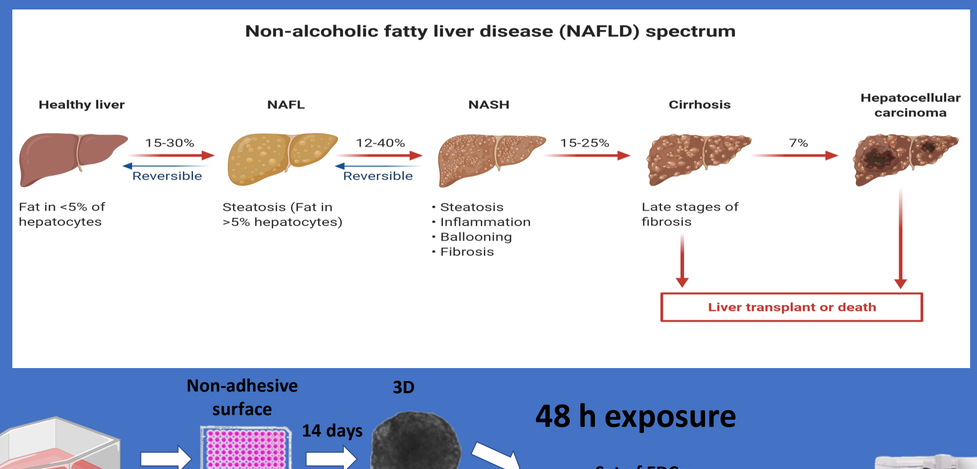
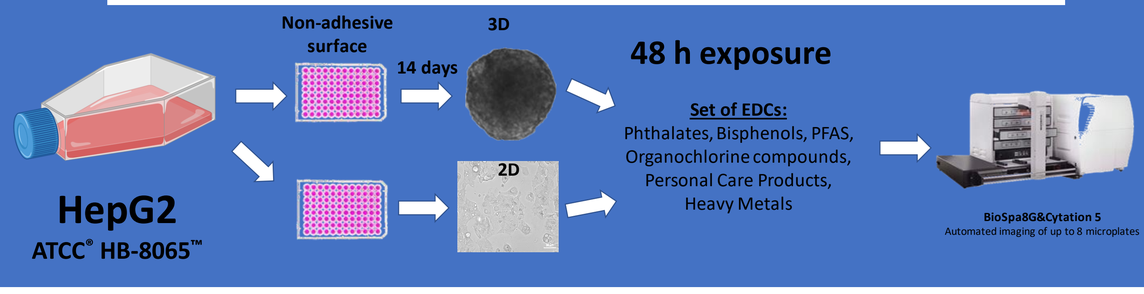
- **Endocrine disrupting compounds (EDCs)** have been associated with adverse health outcomes, including metabolic disorders such as **hepatic steatosis** (non-alcoholic fatty liver disease, **NAFLD**)
- There are no validated *in vitro* assays to be used to **identify and assess the potential of EDCs** to induce hepatic steatosis

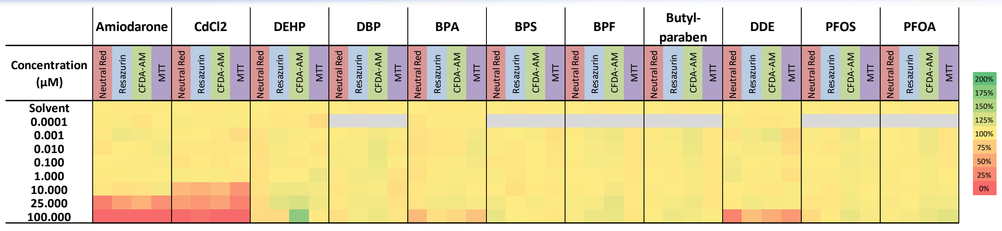
AIMS

- ✓ Optimize a **simple and rapid screening assay** based on 2D and 3D *in vitro* cultures of human **HepG2 cells** to detect hepatotoxic and steatogenic potential of selected EDCs;
- ✓ **Validate its efficiency** (sensitivity, specificity, and predicitve 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.

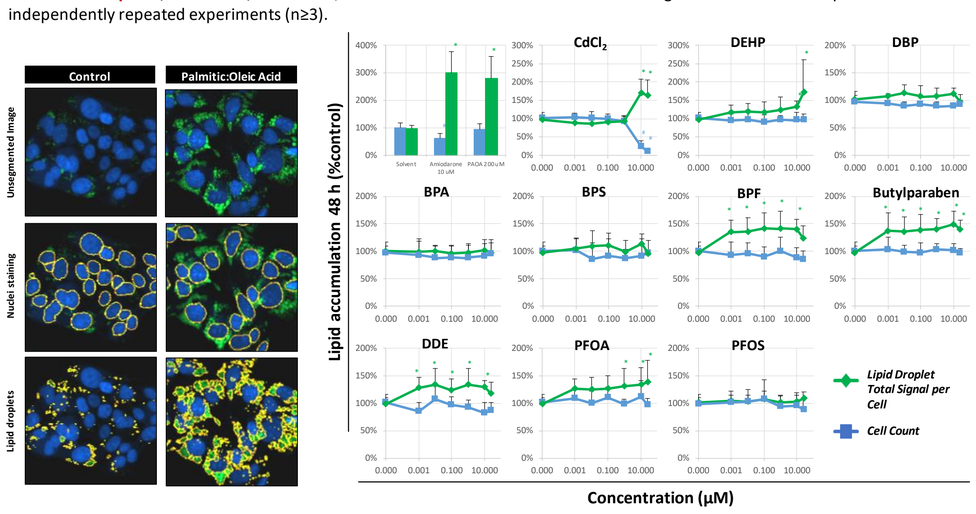




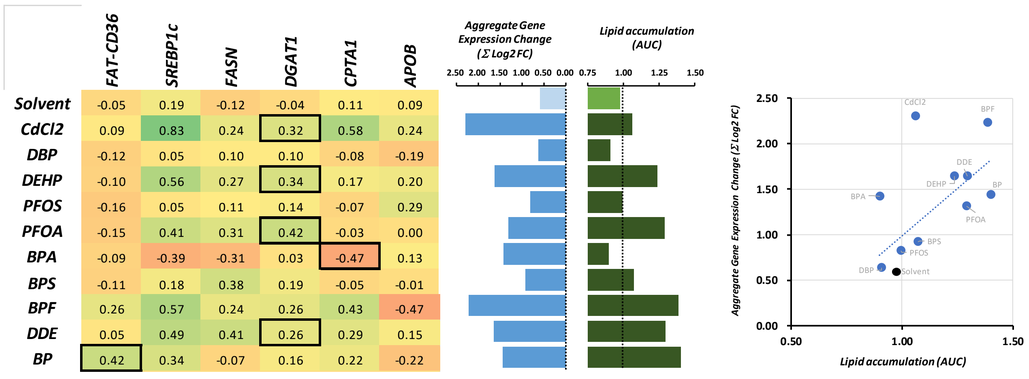
2D monolayers of HepG2



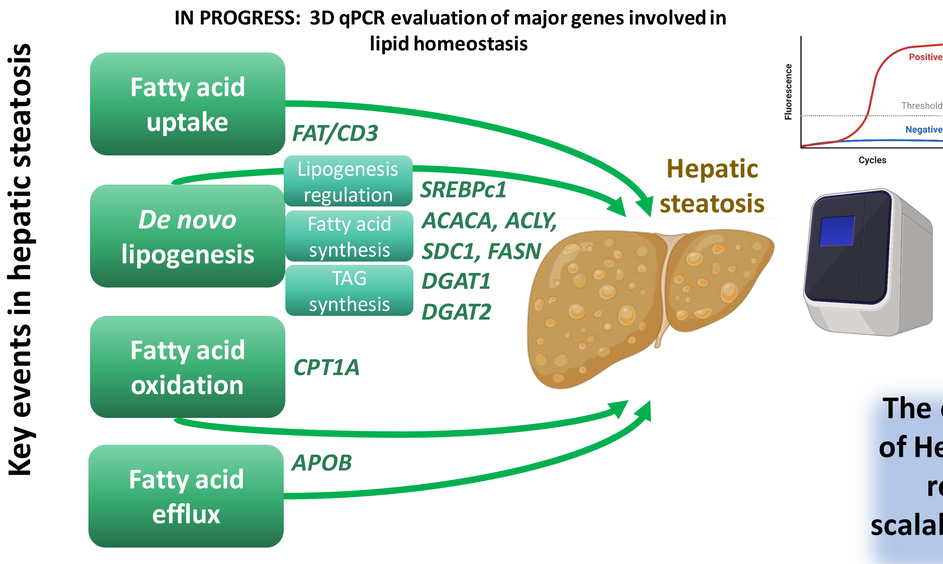
Viability assessment in 2D monolayers of HepG2 cells exposed to model compounds or EDCs for 48 h. Viability was evaluated by **Neutral red uptake**, **Resazurin**, **CFDA-AM**, and **MTT**. Data were normalized to negative control and represent means from independently repeated experiments (n≥3).



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 **DAPI**. Images were acquired using Cytation 5 imaging reader and fluorescence intensity quantified by Gen5 software. Data were normalized to negative control and represent means±SD from independently repeated experiments (n≥4). PAOA – palmitic:oleic acid mixture (1:2).



RTqPCR analysis of selected lipid metabolism genes in 2D HepG2 cells exposed to 1 µM EDCs for 48 h. Values represent house-keeping gene-normalized data expressed as Log2 fold change (n=4 independent experiments), which was also compared to lipid accumulation (Area under the curve, AUC, was calculated for lipid droplet evaluation).



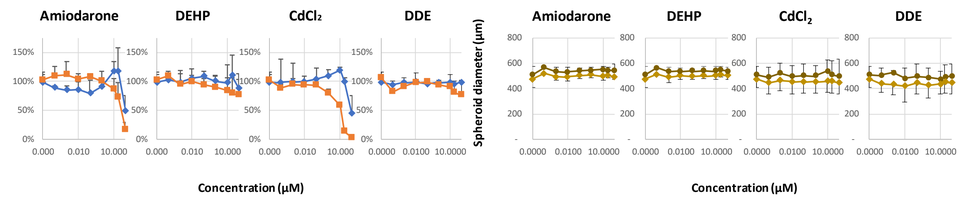
Growth, viability

Lipid accumulation

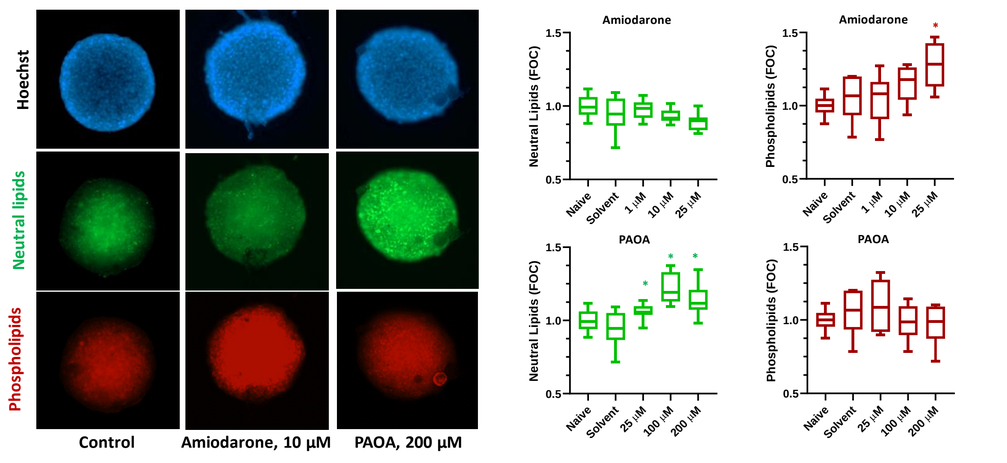
Lipid metabolism genes qPCR

Other enpoints and markers of interest: OMICs

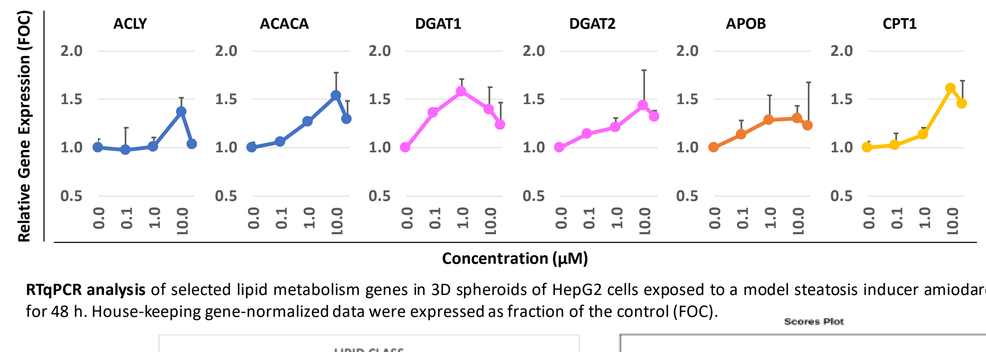
3D HepG2 spheroids



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 and image analysis (Gen 5 Prime) at the **start (♦)** and the **end (●)** of the exposure. Viability data were normalized to negative control and represent means from independently repeated experiments.

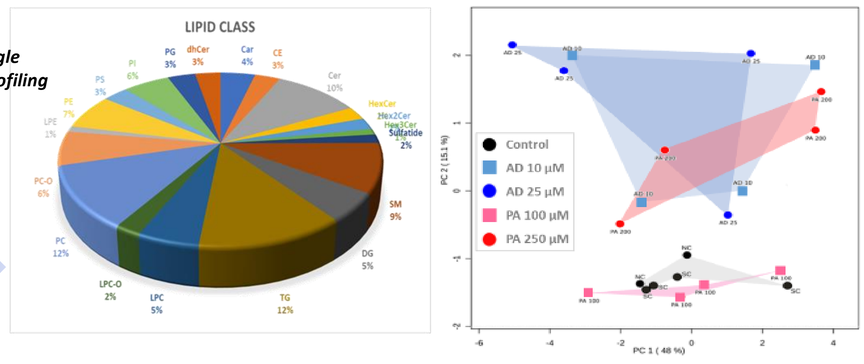


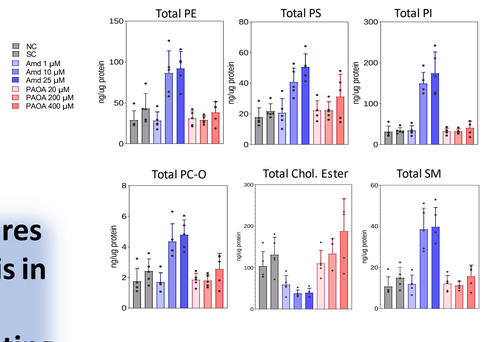
High-content imaging of lipids in 3D HepG2 spheroids exposed to model compounds for 48 h. Lipids were detected with AdipoRed (ready-to-use solution of Nile Red) staining **neutral lipids (green)** as well as **phospholipids (red)**. **Hoechst 33342** was used to counterstain nuclei. Images were acquired 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 (median/IQR/min-max of n=12 spheroids). PAOA – palmitic:oleic acid mixture (1:2).



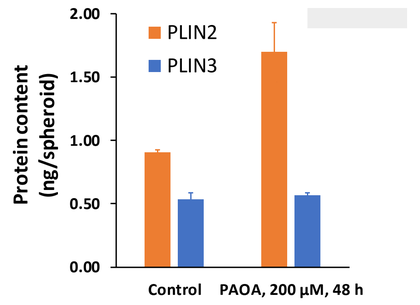
RTqPCR analysis of selected lipid metabolism genes in 3D spheroids of HepG2 cells exposed to a model steatosis inducer amiodarone for 48 h. House-keeping gene-normalized data were expressed as fraction of the control (FOC).

LC-MS based single spheroid lipid profiling





LC-MS quantification of perilipins



Lipidomics characterization of lipid profiles in 3D human hepatospheroids, and the changes induced by pro-steatogenic drugs or conditions. **Targeted Proteomics** of proteins involved in lipid droplet formation, such as perilipins (PLIN2: adipophilin; PLIN3: TIP47; PLIN1: perilipin 1 and PLIN5: OXPAT were <LOD - method optimization is ongoing)

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