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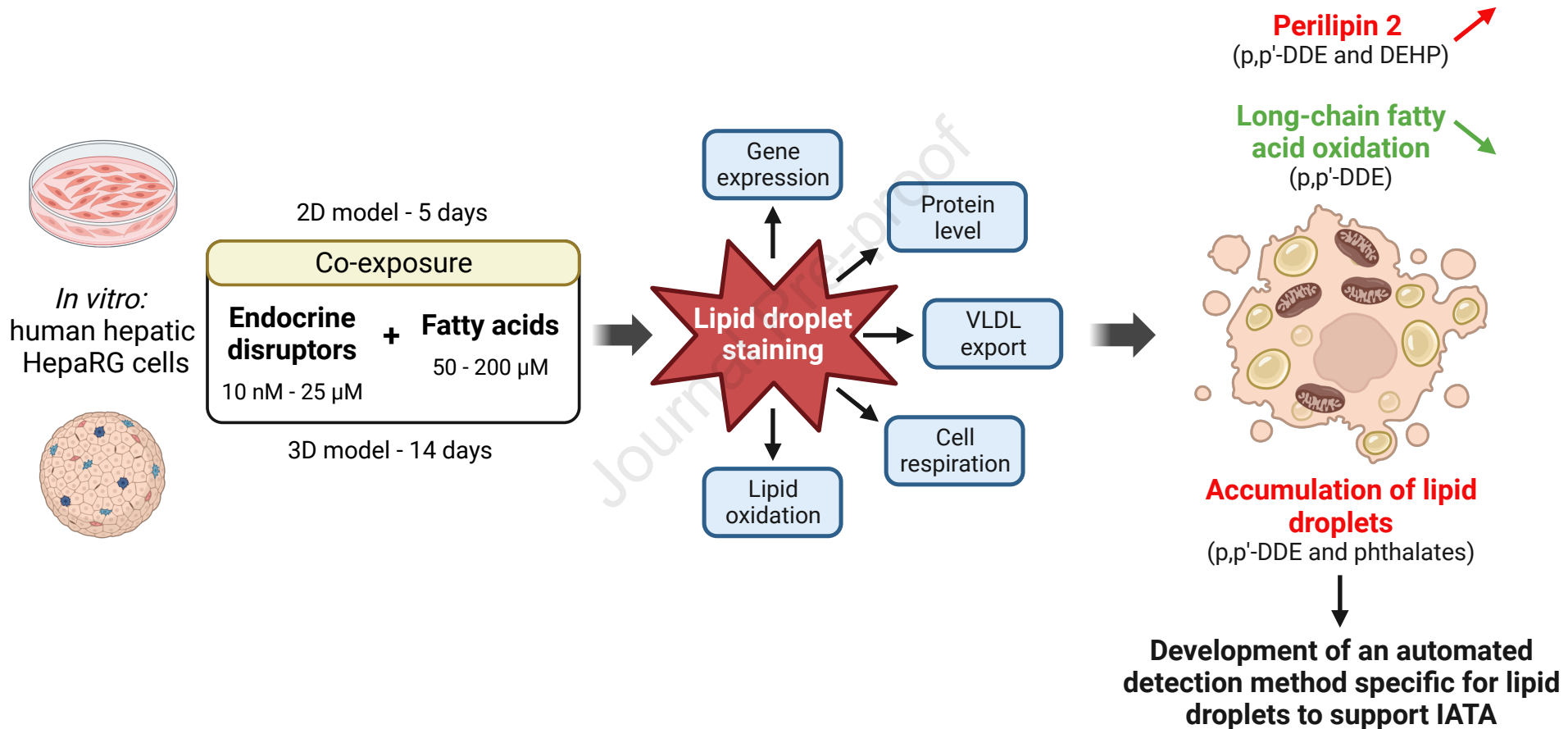
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1 **Title**

2 Assessment of endocrine disruptor impacts on lipid metabolism in a fatty acid-supplemented
3 HepaRG human hepatic cell line.

4

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6

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17 **Abstract**

18

19 The incidence of metabolic dysfunction-associated steatotic liver disease (MASLD) is
20 increasing worldwide. This disease encompasses several stages, from steatosis to steatohepatitis
21 and, eventually, to fibrosis and cirrhosis. Exposure to environmental contaminants is one of the
22 risk factors and an increasing amount of evidence points to a role for endocrine disrupting
23 compounds (EDCs). This study assesses the impact of selected EDCs on the formation of lipid
24 droplets, the marker for steatosis in a hepatic model. The mechanisms underlying this effect are
25 then explored. Ten compounds were selected according to their obesogenic properties:
26 bisphenol A, F and S, butyl-paraben, cadmium chloride, p,p'-DDE, DBP, DEHP, PFOA and
27 PFOS. Using a 2D or 3D model, HepaRG cells were exposed to the compounds with or without
28 fatty acid supplementation. Then, the formation of lipid droplets was quantified by an
29 automated fluorescence-based method. The expression of genes and proteins involved in lipid
30 metabolism and the impact on cellular respiration was analyzed. The formation of lipid droplets,
31 which is revealed or enhanced by oleic acid supplementation, was most effectively induced by
32 p,p'-DDE and DEHP. Experiments employing either 2D or 3D culture conditions gave similar
33 results. Both compounds induced the expression of PLIN2. p,p'-DDE also appears to act by
34 decreasing in fatty acid oxidation. Some EDCs were able to induce the formation of lipid
35 droplets, in HepaRG cells, an effect which was increased after supplementation of the cells with
36 oleic acid. A full understanding of the mechanisms of these effects will require further
37 investigation. The novel automated detection method described here may also be useful in the
38 future as a regulatory test for EDC risk assessment.

39 **Keywords**

40 MASLD; Endocrine disruptors; Mechanistic toxicology; Lipid metabolism; HepaRG; Risk
41 assessment

42 **1. Introduction**

43
44 Over the past decades, metabolic dysfunction-associated steatotic liver disease (MASLD),
45 formerly known as non-alcoholic fatty liver disease (NAFLD), has emerged as the most
46 prevalent liver disease worldwide. It now affects one-quarter to one-third of the general
47 population and the number of cases continues to grow (Le et al. 2022; Riazi et al. 2022;
48 Younossi et al. 2016). MASLD has been defined recently by a multi-society Delphi consensus
49 (Rinella et al. 2023). It covers a broad spectrum of liver outcomes, ranging from steatosis to
50 metabolic dysfunction-associated steatohepatitis (MASH), which can lead to fibrosis, cirrhosis
51 and ultimately to hepatocellular carcinoma (Hardy et al. 2016). MASLD is part of steatotic liver
52 disease (SLD) and encompasses patients who have hepatic steatosis and at least one of the five
53 cardiometabolic risk factors. Steatosis in individuals is defined by an excessive accumulation
54 of fat in >5 % of the hepatocytes. A steatosis without any cardiometabolic risk factors or other
55 specific SLD etiology is defined as cryptogenic SLD. MASLD is associated most often with
56 other disorders such as obesity, type 2 diabetes mellitus (T2DM) and metabolic syndrome (Fan
57 et al. 2017; Yki-Järvinen 2014; Younossi et al. 2019). Affecting several tissues (liver, adipose
58 tissue and the pancreas), MASLD is, thus, a multifactorial disease which is characterized by
59 multiple metabolic dysfunctions as a consequence of the interplay between genetic and
60 environmental factors (Lim et al. 2023). Liver functions are altered by these factors through
61 several mechanisms such as an increase in the content of hepatic triglycerides (TG),
62 mitochondrial alterations, oxidative stress, inflammation and insulin resistance (Fang et al.
63 2018).

64
65 In addition to these risk factors for MASLD, an increasing amount of evidence now indicates
66 that environmental factors and, in particular, endocrine-disrupting compounds (EDCs), are
67 involved in the development of the disease (Foulds et al. 2017; Heindel et al. 2017; Wahlang et
68 al. 2019). Multiple endocrine signaling pathways regulate liver functions and EDCs
69 legitimately impact these functions (Bernal et al. 2022). Some families of chemicals, mainly
70 used as plasticizers, are suspected to induce specific disorders of lipid homeostasis. Among
71 them, bisphenols and phthalates have been found to be associated, in the general US population,
72 with a higher risk of liver steatosis (Cai et al. 2021; Kim et al. 2019). Exposure to EDCs,
73 particularly perfluorinated alkyl substances (PFAS) which may mimic fatty acid signaling with
74 consequent adverse impacts on hepatic lipid metabolism (Sen et al. 2022), can occur also

75 through contaminated food or water. Despite these observations, few tests for metabolic
76 disruptions have been accepted or are currently under development by the Organization for
77 Economic Cooperation and Development (OECD) or the US Environmental Protection Agency
78 (EPA). This regulation is particularly deficient as to the identification and the risk assessment
79 of chemicals that can induce liver steatosis.

80

81 The European OBERON project (<https://oberon-4eu.com>) has as its goal the development of
82 an integrated testing strategy (ITS) to detect EDC-related metabolic disorders by developing or
83 improving and validating a battery of test systems. It is based on the concept of an integrated
84 approach for testing and assessment (IATA) (Audouze et al. 2020). On the basis of
85 epidemiological studies supported by human biomonitoring data which suggest an association
86 with metabolic disorders, 10 EDCs were selected for study: bisphenol A (BPA), bisphenol F
87 (BPF), bisphenol S (BPS), butylparaben (BP), cadmium chloride (CdCl_2), 1,1-dichloro-2,2-
88 bis(4-chlorophenyl)ethene (p,p'-DDE), dibutyl phthalate (DBP), bis(2-ethylhexyl) phthalate
89 (DEHP), perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS). These
90 chemicals are widely used, in large volumes, and they exhibit potential diabetogenic and
91 obesogenic properties (Heindel et al. 2022).

92

93 The present study focuses on the *in vitro* impact of these compounds on lipid metabolism,
94 effects which could lead to liver steatosis. The HepaRG cell line was used as a 2D human liver
95 model and some of the results obtained were confirmed under 3D culture configurations. This
96 cell line, composed of human hepatocytes and cholangiocytes, is a recognized mixed model for
97 the study of both drug effects and intermediary metabolism (Tascher et al. 2019). The Hepoid-
98 HepaRG 3D model exhibits enhanced hepatic differentiation and additional metabolic functions
99 (Rose et al. 2022). As liver steatosis can be induced by multiple environmental factors including
100 lipid overload or exposure to chemicals, we tested the hypothesis as to whether exposure to
101 chemicals could increase the effect of lipid overload on liver cell triglyceride accumulation. To
102 this end, the HepaRG cell line, in both 2D and 3D configurations, was exposed to EDCs along
103 with fatty acid (FA) supplementation. Cellular lipid accumulation was measured, after exposure
104 to the selected compounds, under several culture conditions. A novel technical protocol
105 employing fluorescence microscopy was used to quantify lipid droplets and overall triglyceride
106 content was determined. To decipher putative underlying mechanisms and identify biomarkers
107 of exposure and effect, the expression of several genes and proteins, known to be involved in
108 the development of steatosis, as well as physiological parameters also were analyzed.

109 **2. Materials and methods**

110 111 **2.1 Compounds and cell culture**

112 The different compounds used are listed in **Supplementary Method** (references and suppliers)
113 and EDCs were prepared in 100 % dimethyl sulfoxide (DMSO).

114 Human HepaRG hepatoma cells were obtained from Dr. Philippe Gripon and were cultured and
115 differentiated as previously described with 1.7 % DMSO to induce differentiation of the cells
116 (Aninat et al. 2006; Gripon et al. 2002; Tascher et al. 2019). Following cell differentiation and
117 five days after seeding, cells were exposed to pollutants, at various concentrations, or to the
118 vehicle with a final concentration of 1.7 % DMSO and for different time periods (cf. **figure**
119 **legends**). The protocols are presented in **Supplementary Figure 1**. Fatty acid supplementation
120 of cells was performed with several concentrations of oleic acid (OA) or a mixture of palmitic
121 and oleic acid (PA:OA) as explained in **Supplementary Method**. For Hepoid[®]-HepaRG 3-
122 dimensional (3D) culture, type I collagen was used and diluted in the culture medium as
123 previously described (Rose et al. 2022). The treatment of Hepoid-HepaRG took place 5 days
124 after seeding in collagen, for 14 days in a DMSO-free medium in the presence of 5 % fetal
125 bovine serum.

126 127 **2.2 Cell viability**

128 In order to evaluate viability after EDC exposure in the 2D cell model, differentiated HepaRG
129 cells were seeded into 96-well plates at a density of 8×10^4 cells per well prior to exposure to
130 each compound, alone or in combination with FA, at concentrations varying from 100 nM to
131 25 μ M during 3, 5 or 14 days. At the end of exposure, cell viability was assessed with a
132 microplate reader (Spark[®], Tecan) using 3 dyes: resazurin, 5-carboxyfluorescein diacetate
133 acetoxymethyl ester (CFDA-AM) and neutral red, as previously described (Raska et al. 2018).
134 For the Hepoid[®] 3D model, cell viability was assessed by measuring ATP content using the
135 CellTiter-Glo[®] 3D Assay (Promega) according to the manufacturer's protocol. Luminescence
136 was measured after 30 min of incubation using the PerkinElmer Enspire 2300 multilabel reader.

137 138 **2.3 Lipid droplet assessment**

139 Lipid droplet quantification was performed using epifluorescence microscopy by staining lipid
140 droplets and cell nuclei with Nile Red (1 μ M, 510/573 nm to assess only triglycerides) and
141 Hoechst 33342 (1 μ g/mL, 350/461 nm), respectively. Differentiated HepaRG cells were seeded

142 into 96-well plates at a density of 8×10^4 cells per well. Images were obtained and analyzed
143 using an ImageXpress Pico (Molecular Devices). A macro was programmed to select only the
144 lipid droplets in the cells based on cell size and intensity exceeding the background. To compare
145 the impact of the different treatments, a steatosis index was defined (**Supplementary Figure**
146 **2**). The results are expressed as a percentage (%) based on the mean steatosis index of the
147 control according to the exposure procedure.

148 In Hepoid-HepaRG, lipid droplets were measured after fixation with 4 % paraformaldehyde
149 containing 5 % sucrose for 10 min at room temperature. Hoechst 33342 and LipidTOX™ HCS
150 (ThermoFisher, 1/1000) staining were performed simultaneously to stain nuclei and lipid
151 droplets, respectively. Images were acquired with a Leica SP8 confocal microscope with a 40x
152 oil objective. Images (40 μ m stack) were then analyzed using ImageJ software using a specific
153 macro that detects lipid droplets and nuclei. The results are expressed as the number of lipid
154 droplets *per cell*.

155

156 **2.4 Triglyceride content**

157 Hepoid-HepaRG were treated for 14 days at a concentration of 10 μ M p,p'-DDE with and
158 without supplementation, consisting of a mixture of palmitic and oleic acid (50 μ M each). The
159 medium was renewed every 48 hours. Triglyceride concentration was measured using the
160 Triglyceride-Glo Assay (Promega) according to the manufacturer's protocol. The luminescence
161 measurement was performed using the PerkinElmer Enspire 2300 multilabel reader. Results,
162 proportional to the amount of glycerol in the sample, were then normalized to cell viability as
163 estimated by ATP measurement.

164

165 **2.5 Gene expression quantification and immunoblotting**

166 For gene expression analysis, total RNAs were extracted using the GenElute™ Mammalian
167 Total RNA Miniprep kit (#RTN350, Sigma-Aldrich). Reverse transcription was performed
168 using the high-capacity cDNA reverse transcription Kit (#4368814, Applied Biosystems®)
169 following the manufacturer's instructions on 1 μ g of RNA in 50 μ L. Then, quantitative PCR
170 was performed using 20 ng of cDNA per reaction using CFX 384 thermocycler (Bio-Rad®).
171 with Takyon SYBR® 2X qPCR Mastermix Blue (Eurogentec®) with 0.3 μ M of each primer
172 (**Supplementary Table S1**). The relative mRNA level was estimated with the delta-delta C_t
173 method using *RPL13A* and *HPRT* as reference genes (Livak and Schmittgen 2001).

174 For immunoblotting, cells were scraped into RIPA buffer (#R0278, Sigma-Aldrich). Total
175 proteins (7.5 µg) were separated by SDS-PAGE (Mini-PROTEAN TGX gels, #4561086,
176 Biorad) and transferred onto a PVDF membrane (Transblot turbo, #1704156, Biorad). The
177 membrane was saturated for 1 h at room temperature with a blocking buffer (#927-60001, LI-
178 COR Biosciences) diluted in PBS/0.2 % TWEEN (v:v) and then overnight at 4°C with the
179 primary antibody diluted in saturation buffer (references and the conditions in **Supplementary**
180 **Table S2**). After the final washes, the intensities of the bands were quantified using an Odyssey
181 infrared Imager (LI-COR Biosciences).

182

183 **2.6 Apolipoprotein B (ApoB) quantification**

184 ApoB secretion was quantified using the human apolipoprotein B ELISA PRO kit, as described
185 by the manufacturer (#3715-1HP, Mabtech). Briefly, differentiated HepaRG were seeded in 96
186 well plates and treated with the different exposure protocols. Three days after the last treatment,
187 the supernatant was collected and frozen at -20°C until quantification. Absorbance at 450 nm
188 was measured (background at 600 nm) with a microplate reader (Enspire 2300, PerkinElmer).
189 The concentration of ApoB was then extrapolated using a five-parameter logistic curve fit
190 (GraphPad Prism, 9.5.1 version). The results were then normalized to the total amount of
191 proteins.

192

193 **2.7 Measurement of oxidative respiration by Seahorse technology**

194 The oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) were
195 measured using a Seahorse XFe24 Analyzer (Agilent) to determine key parameters involved in
196 mitochondrial metabolism. After seeding into Seahorse 24 well-plates (#100777-004; Agilent)
197 at a density of 1.5×10^5 cells *per* well, differentiated HepaRG cells underwent different
198 exposure scenarios. The Mitostress protocol was performed, as described by the manufacturer.
199 At last, Hoechst 33342 (1 µg/mL final) was injected and incubated for 15 min to stain the nuclei,
200 which were counted by fluorescence microscopy analysis. The results were normalized to the
201 number of nuclei in each well.

202 To determine the part of the OCR relative to long-chain fatty acid oxidation (LCFAO), cells
203 were cultivated under the same conditions as described previously. After exposure, they were
204 washed twice and incubated for 1 h at 37°C in a CO₂ free atmosphere with DMEM XF medium
205 pH 7.4 supplemented with 2 mM L-glutamine. Three measurements were performed before and
206 after injection of etomoxir (5 µM), an inhibitor of CPT1A. Hoechst 33342 (1 µg/mL) was then

207 injected, and the cells were incubated for 15 min to stain the nuclei. The respiration relative to
208 LCFAO corresponds to the difference in OCR between the basal respiration and the respiration
209 after etomoxir injection. The results were normalized to the number of cells in each well.

210

211 **2.8 Statistical analysis**

212 The results are expressed as the mean \pm SEM of at least three independent experiments. The
213 number of replicates is given in the figure legends. Statistical analysis and graphing were
214 carried out using GraphPad Prism (10.0.2 version). A Shapiro-Wilk normality test was
215 performed to evaluate the Gaussian distribution of each dataset. In the case of a normal
216 distribution, data were analyzed by one-way or two-way ANOVA followed by a Dunnett (all
217 conditions compared to control) or Bonferroni (all conditions compared to their respective
218 control) post hoc test to assess differences between specific conditions. For datasets that did not
219 follow a normal distribution, data were analyzed using Kruskal-Wallis test followed by a
220 Dunn's correction. A p-value was used to determine sample significance with *, $p < 0.05$; **,
221 $p < 0.01$; ***, $p < 0.001$ and ****, $p < 0.0001$.

222 3. Results

223
224 The compounds used in this study were selected for their obesogenic capacities based on
225 epidemiological studies which focused on quantitative measurements of adipogenesis, body
226 mass index or waist circumference (Audouze et al. 2020). Complementary experimental studies
227 based on robust cell line models of adipogenesis were also used. For example, accumulation of
228 lipids was observed in mouse 3T3-L1 differentiated adipocytes after exposure to several
229 common or analog compounds: 25 μM PFOS or PFOA (in the presence of rosiglitazone),
230 20 μM BPA, BP or mono(2-ethylhexyl) phthalate (MEHP) – a metabolite of DEHP – and 10-
231 30 μM p,p'-DDE (Mangum et al. 2015; Modaresi et al. 2022; Taxvig et al. 2012). On the basis
232 of those results, a dose of 25 μM was, thus, selected as an upper limit and, subsequently, used
233 in this study.

234 235 3.1 Assessment of the cytotoxicity of the selected EDCs

236 When evaluating steatosis development in HepaRG cells, viability assays were performed using
237 various concentrations (from 100 nM to the upper limit of 25 μM) to be sure that the compounds
238 were used at sub-toxic concentrations. None of the compounds exhibited any toxicity on the
239 HepaRG cells in 2D culture after 72 h or 14 days (**Supplementary Table 3A**), except for 10
240 μM cadmium which seemed to alter lysosome integrity as early as 72 h.

241 The viability of Hepoid-HepaRG was assessed after exposure of the spheroids for 72 hours and
242 14 days to the various EDCs, at concentrations between 100 nM to 25 μM . No toxic effect was
243 detected irrespective of the concentration used (even at concentrations as high as 25 μM),
244 except for CdCl_2 which decreased the cellular ATP content at both 10 and 25 μM beginning at
245 72 hours (**Supplementary Table 3B**).

246 As EDCs were tested with additional exposures, such as to fatty acid supplementation (OA
247 100 μM or PA:OA 50 μM each), the cytotoxicity under these conditions also was assessed
248 (**Supplementary Figure 3**). After 5 days, no effect was observed, which was coherent with the
249 results obtained at 72 h and 14 d, except for 25 μM DEHP which non-specifically increased the
250 dehydrogenase activities (**Supplementary Figures 3A-C; Table 3A**).

251 252 3.2 Lipid droplet accumulation

253 Based on the preceding results, we used a concentration of 25 μM for all compounds except for
254 CdCl_2 (1 μM) in a first attempt to identify whether the selected EDCs could induce steatosis in

255 HepaRG 2D model. As stated above, since liver steatosis is often a consequence of a high
256 energy diet, cells were supplemented with OA (100 μ M or 200 μ M) during exposure to EDCs.
257 An exposure time of 5 days was chosen as a reference for our study, based on previous
258 experiments (Paramitha et al. 2021).

259
260 Lipid droplet formation was quantified using fluorescence microscopy images. To compare
261 treatments a specific parameter, the 'steatosis index', was defined (**Supplementary Figure 2**).
262 Without any supplementation, an increase of the steatosis index was observed only following
263 an exposure to 25 μ M DEHP, whereas exposure to CdCl₂ and PFOS produced a decrease,
264 although not statistically significant (**Figure 1A**). The quantification of total TG content also
265 showed an increase, although non-significant, only after DEHP exposure (**Supplementary**
266 **Figure 4A**). In comparison, an increase in the steatosis index was observed after exposure to
267 25 μ M of DBP (~2x), p,p'-DDE (~4x) and DEHP (~6x) supplemented with 100 μ M OA
268 (**Figure 1B**). Both the density and the size of the lipid droplets were increased (**Figure 1C**).
269 However, no increase in total TG content was observed (**Supplementary Figure 4A**). The
270 effect of p,p'-DDE, DBP and DEHP was less following supplementation with 200 μ M OA as
271 compared to 100 μ M OA, probably due to the excessive amount of lipid already present in the
272 cells, mitigating the potential (**Supplementary Figure 4B**).

273 Dose response curves for p,p'-DDE and DEHP, which were the most effective in inducing
274 steatosis following OA supplementation, were then performed. No effect on the steatosis index
275 was observed with increasing concentrations of p,p'-DDE under normal conditions of culture
276 (**Figure 2A**). However, with 100 μ M OA supplementation, the steatosis index increased with
277 increasing amounts of p,p'-DDE (significant beginning at a concentration of 10 μ M, **Figure**
278 **2A**). In contrast, for DEHP, there was an increase in the steatosis index either with or without
279 supplemental OA (**Figure 2B**).

280 Supplementation with OA increased the effects of exposure to both p,p'-DDE and DEHP by
281 about 40-fold, whereas the supplementation with OA alone increased the steatosis index only
282 12-fold (**Figure 2C**). These results could reflect a synergistic effect, or at least more than an
283 additive effect, of the combination OA and pollutants.

284 To approach a more physiological ratio of free fatty acids observed in healthy fasting humans,
285 supplementation with a mixture of OA and PA (50 μ M each) also was used (Kokotou et al.
286 2022). The effects of the pollutants, although similar, were less and were statistically significant
287 only with DEHP (**Supplementary Figure 4C**). Of note, the formation of lipid droplets in the

288 control cells exposed to fatty acids was lower after the supplementation with the mixture
289 50:50 μM PA:OA as opposed to that with 100 μM OA alone.

290 Because steatosis is a reversible process, the capacity of the EDCs to maintain this
291 pathophysiological state also was assessed (**Supplementary Figure 1C**). The release of lipids
292 was observed in the control, for which the steatosis index reverts almost to the level of the
293 control without any supplementation (2.6 % steatosis remaining) (**Supplementary Figure 4D**).
294 This release was statistically significantly decreased following exposure to DEHP (7.4 %
295 steatosis remaining).

296
297 In order to estimate the capability of pollutants to affect lipid metabolism in the 3D human
298 hepatic spheroid culture, we analyzed the impact on droplet formation of 4 prototypical
299 representatives of the different EDC families by measuring the lipids in 3D cultures. Hepoid-
300 HepaRG cells were treated with 10 μM BPA, p,p'-DDE, DEHP or PFOA for 14 days. An
301 accumulation of lipid droplets in Hepoid-HepaRG was observed after 14 days of treatment with
302 all 4 compounds tested with a statistically significant increase of 7.6-fold after exposure to p,p'-
303 DDE as compared to the control (**Figure 3A**). A 6.3-fold increase was observed after exposure
304 to DEHP ($p=0.08$). In this model, our findings also demonstrated that both the mixtures of
305 PA:OA or OA alone similarly increased TG content after 14 days (data not shown). Based on
306 these results, the accumulation of TG following exposure to 10 μM p,p'-DDE with and without
307 supplemental exposure to a FA mixture (PA:OA 50 μM each) was measured. Both p,p'-DDE
308 and FA alone caused a 2-fold increase in cellular TG content as compared to the control. The
309 combination of p,p'-DDE plus FA led to a statistically significant 3.7-fold increase in TG
310 accumulation (**Figure 3B**).

311

312 **3.3 Impact on lipid metabolism**

313 The increase in lipid droplets could be explained by several mechanisms: increase of the *de*
314 *novo* FA synthesis, decrease of lipid catabolism, increase of triglyceride and droplet formation,
315 increase of lipid uptake, or decrease of VLDL export. The expression of representative anabolic
316 genes of these pathways was measured by RT-qPCR in 2D HepaRG cells treated with either
317 p,p'-DDE, DBP or DEHP alone or with FA supplementations (**Figure 4**). Details of the qRT-
318 PCR results for each gene are given in the **Supplementary Figures 5-9**.

319

320

321 Whatever the supplementation (no fatty acid or 100 μ M OA), exposure to p,p'-DDE decreased
322 the expression of *SREBP1C*, *FASN* and *SCD1*, but did not affect *ACCI* expression. This effect
323 was not observed with PA:OA supplementation. The co-exposure of cells to DBP and FA
324 increased the expression of *SREBP1C*. The expression of the aforementioned genes were
325 unaffected upon exposure of cells to DEHP under any condition.

326
327 For the genes involved in TG synthesis, no significant effect of pollutants was observed.
328 Supplementations with FA alone decreased *DGAT2* expression. On the contrary, *PLIN2* mRNA
329 expression increased with the 100 μ M OA supplementation (1.5-fold). Co-exposure of cells to
330 both p,p'-DDE and FA led to further increases in *PLIN2* mRNA expression (2.4-fold and 1.7-
331 fold, respectively, with OA and PA:OA). An increase in the amount of PLIN2 protein was
332 observed following exposure of cells to p,p'-DDE or DEHP along with only 100 μ M OA
333 (**Figure 5A**).

334
335 No major effect on *FATP2* and *CD36* gene expression was observed under any culture
336 condition. Furthermore, no effect was observed on the level of cellular CD36 protein after EDC
337 exposures (**Figure 5B**). On the contrary, exposure to p,p'-DDE (no supplementation) decreased
338 the expression of *FABP1*. FA supplementation also decreased significantly basal *FABP1*
339 mRNA expression, which was not modified by p,p'-DDE but was suppressed by the phthalates
340 in the presence of 100 μ M OA and which was alleviated by these pollutants in the presence of
341 50 μ M PA:OA.

342 A significant decrease of *MTTP* expression was observed in the presence of FA
343 supplementations. However, no effect was observed for *APOB* expression or for APOB
344 secretion, which suggests that these pollutants did not significantly impact VLDL export
345 (**Figure 6A**).

346
347 No significant effect upon *CYP4A11* and *CPT1A* gene or protein expression was observed
348 following the different exposures (**Figure 5A**). Cell respiration is coupled with the activity of
349 the Krebs cycle which is fueled by acetyl-coA anabolism the β -oxidation of which provides a
350 significant contribution. Therefore, we measured cell respiration and showed that the basal and
351 maximal respirations were slightly decreased (although non-significantly) by p,p'-DDE either
352 in the presence of absence of additional FA (**Figure 6B-C**). To determine whether this decrease
353 was related to a disruption in lipid oxidation, we measured the long-chain fatty acid oxidation
354 (LCFAO). The LCFAO was reduced by p,p'-DDE (0.15 pmol/min/1000 cells) as compared to

355 the control (0.46 pmol/min/1000 cells), whereas no impact on this parameter was observed with
356 DEHP (0.51 pmol/min/1000 cells) (**Figure 6D**).

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357 4. Discussion

358 In the present work, we show that exposure to some EDCs along with FA promote liver steatosis
359 development. In the human hepatic cell line, HepaRG, among the 10 compounds studied,
360 exposure to DEHP increased the formation of lipid droplets in both the 2D and 3D models.
361 Furthermore, using a FA supplementation which mimics a dietary intake, the exposure to two
362 phthalates (DBP and DEHP) or one organochloride pesticide (p,p'-DDE) exacerbated the
363 formation of droplets after 5 days in the 2D HepaRG model.

364
365 Under normal culture conditions, only DEHP induced the formation of lipid droplets. This
366 effect was observed previously with the HepaRG model, after 7 days of exposure to a lower
367 concentration of DEHP, 10 nM (Franco et al. 2020). However, the 2D cell culture in that study
368 was performed in collagen-coated plates and the DMSO was reduced to 0.6 % for the duration
369 of exposure. Under those conditions, the cells might be more sensitive, since BPA (10 nM) and
370 PFOA (100 nM) also induced lipid droplet formation. Other studies have reported an increase
371 in TG content in HepaRG cells after BPA, PFOA or PFOS exposure, however these studies
372 used longer times of exposure (3 weeks of BPA, also during the proliferation phase) or higher
373 concentrations of EDCs (greater than 50 μ M of PFOS and 200 μ M of PFOA) (Bucher et al.
374 2017; Louisse et al. 2020). These results were confirmed in a 3D Hepoid-HepaRG model, in
375 which an increase in lipid droplets was observed after 14 days of treatment with 10 μ M BPA,
376 p,p'-DDE, DEHP, or PFOA. Of note, in the study by Franco and collaborators mentioned
377 above, PFOS decreased the total lipid content (but not neutral lipid content) (Franco et al. 2020).
378 Under our conditions, without FA supplementation, neutral lipid content was decreased by
379 PFOS. Together, these results highlight i) the fact that the effects of PFAS on MASLD are still
380 unclear and might depend on multiple parameters (diet, hepato-toxic compounds...), as
381 suggested by a recent meta-analysis of rodent and human studies (Costello et al. 2022) ; ii) the
382 fact that the culture conditions in different studies may affect the results obtained with the same
383 pollutant, which suggesting that studies of the exposome which integrate these multiple
384 parameters should be carried out to better understand the effect of pollutants in the context of a
385 diverse hepatic vulnerability. Moreover, when considering whole organisms, the intricate
386 relationships and dialogue between organs could also lead to different effects. Indeed, it has
387 been shown recently in zebrafish larvae that p,p'-DDE but not DEHP significantly increases
388 steatosis (Le Mentec et al. 2023). In line with this observation, differences among species
389 should also be better characterized.

390

391 A major finding of the present study is the capacity for some EDCs to induce steatosis in a
392 dose-dependent manner when supplemented with fatty acids (**Figure 2**), whereas few (DEHP)
393 or no effects (DBP and p,p'-DDE) were observed with the pollutant exposure alone. Thus, the
394 supplementation with fatty acids appears to reveal steatotic properties of compounds, probably
395 in a synergistic manner. This is in line with the "multiple-hit" theory likely involving
396 environmental pollutants (Byrne and Targher 2015; Wahlang et al. 2019). This has been
397 observed in several studies in which animals were fed a high fat diet (HFD) and exposed to
398 DEHP (rat), p,p'-DDE (rat) or other types of pollutants such as TCDD (mice) (Duval et al.
399 2017; Rodríguez-Alcalá et al. 2015; Zhang et al. 2023). However, this synergistic effect is not
400 always observed. For instance, it has been shown that a combination of HFD and a high dose
401 of p,p'-DDE (2 mg/kg) in mice leads to a decrease in the amount of lipid in liver and in
402 triglyceride content (Howell et al. 2015). The "synergistic" effect of fatty acid supplementation
403 and pollutant exposure needs to be evaluated further. Studies on different species, different
404 doses and times of exposure, carefully defined protocols and mathematical modelling (Ma and
405 Motsinger-Reif 2019) need to be combined to elucidate the mechanisms involved (common or
406 distinct.

407

408 Several protocols of FA supplementation were employed in the present study. The choice of
409 100 μ M OA was made with respect to the amounts measured in fasting individuals. The serum
410 used to supplement the cell cultures already contains TG (58 mg/dL) and probably free FA.
411 Thus, the supplementation used in the present study might be compared to a transient overload
412 of FA after a meal. Indeed, when OA was removed, the cellular lipid droplets disappeared
413 rapidly and completely. Since we observed an increase in lipid accumulation after co-exposure
414 to EDCs and FA which persisted after withdrawal of the OA, it can be assumed that cyclic
415 intake of FA would lead to a gradual increase in cellular lipid droplets, which would eventually
416 reach a steatotic steady state.

417

418 With the same total concentration of free fatty acids in the medium, fewer droplets were
419 observed following supplementation with PA:OA as compared to OA alone. PA has been
420 shown to be a poor inducer of lipid droplets in the HepG2 model (Eynaoudi et al. 2021). In our
421 study, under PA:OA supplementation, the difference in the steatosis index after EDC exposure
422 appeared to be less marked as compared to OA (100 μ M) supplementation. The pollutants seem,

423 thus, to act on processes involving OA. In the Hepoid-HepaRG 3D model, the PA:OA
424 supplementation was used, since it more accurately reflects human exposure to a combination
425 of fatty acids on a daily basis (ranging from 0.2 to 5 mmol/L), rather than focusing solely on
426 OA (Abdelmagid et al. 2015; Staiger et al. 2004). Under these conditions, the effect of exposure
427 to p,p'-DDE appeared in the PA:OA supplemented Hepoid-HepaRG 3D cell culture model
428 whereas no effect was observed in the 2D model.

429
430 In this study, the increased cellular accumulation of neutral lipids is associated with an increase
431 of PLIN2 expression following co-exposure of cells to p,p'-DDE or DEHP and OA. To our
432 knowledge, this is the first time that this effect has been observed in a human model after
433 exposure to p,p'-DDE. This is coherent with the recently characterized effect of p,p'-DDE on
434 *plin2* expression in zebrafish larvae (Le Mentec 2023). In mice, an increase in hepatic *Plin2*
435 gene expression has been observed previously following exposure to DEHP (Li et al. 2020).
436 This protein is a major component of the membrane of lipid droplets and plays a crucial role in
437 their formation and stabilization. In *Plin2* null mice, the *Plin2* deficiency led to reduced hepatic
438 TG content and prevented the induction of steatosis when mice were fed a Western-diet (Libby
439 et al. 2016; Mardani et al. 2019). Conversely, overexpression of *Plin2* in murine hepatocyte
440 AML12 cells (derived from liver of transgenic mice overexpressing transforming growth
441 factor- α) reduces the rate of TG loss by suppressing autophagy in the hepatocytes (Tsai et al.
442 2017). These studies support the idea that the increase in PLIN2 observed upon exposure to
443 p,p'-DDE or DEHP allows the maintenance of a higher level of lipid droplets in cells and helps
444 to explain their accumulation in the present model.

445
446 The increase in PLIN2 expression cannot entirely explain the increase of lipid droplets observed
447 after exposure of cells to pollutants found in the present study. Despite the variety of possible
448 mechanisms investigated, none of them were sufficient to explain the accumulation of lipid
449 droplets that developed after exposure to DEHP. In HepG2 cells, similar effects were observed
450 although with much higher concentrations of DEHP (100 μ M) and OA (500 μ M) (Zhang et al.
451 2017). The mechanism was proposed to be related to oxidative stress and to increased levels of
452 PPAR α and SREBP proteins. No change in the expression of the target genes of PPAR α
453 (*CPT1A*, *CYP4A11*) or SREBP (*FAS*, *ACCI*, *SCD1*) after DEHP exposure were observed in the
454 present study. However, DBP, another phthalate studied, has the ability to upregulate *SREBP1C*
455 expression under cellular co-exposure with FA. This might explain, partially, the formation of
456 new cellular lipid droplets but may also suggest that different phthalates may have different

457 impacts. Analysis of the level of oxidative stress in HepaRG after exposure to DEHP as has
458 been performed in several other models such as Hep3B cells or in rats (Chen et al. 2013; Ha et
459 al. 2016) might provide useful insights. *In vivo*, in the context of a HFD, DEHP (0,1 mg/kg/day
460 for 25 weeks) enhanced steatosis in the liver of obese mice which was proposed to be linked to
461 an increase in the expression of *Cd36* (Hsu et al. 2021).

462
463 Our preliminary experiments have demonstrated that exposure of cells to p,p'-DDE may impact
464 mitochondrial lipid oxidation (**Figure 6D**). Very few studies deal with the effect of p,p'-DDE
465 on lipid metabolism in hepatic cells. In the human HepG2 cell line, a decrease of basal
466 respiration by p,p'-DDE (10 ng/mL for 24 h) has been shown (Liu et al. 2017). In rodents,
467 exposure to p,p'-DDE has given contradictory results probably related to the use of different
468 protocols. On the one hand, studies have shown a decrease in lipid production or an increase of
469 carnitine palmitoyl-transferase activity and β -oxidation consistent with a role for oxidative
470 stress after exposure to p,p'-DDE for 4 weeks in rats (10 mg/kg) and in mice (2 mg/kg) (Howell
471 et al. 2015; Migliaccio et al. 2019). On the other hand, an enhanced lipogenesis along with an
472 increase of fatty acid, *de novo* synthesis proteins (*Acc*, *Fas* and *Scd1*) has been observed after
473 exposure to p,p'-DDE (8 weeks, 1 mg/kg/day everyday) (Liu et al. 2017; Morales-Prieto et al.
474 2018). In zebrafish larvae, this effect on lipogenesis has been found to be associated with an
475 upregulation of *scd1*, involved, in part, in the induction of steatosis (Le Mentec et al. 2023). In
476 the current study, the expression of *SCD1* was decreased, suggesting that the formation of TG
477 might involve mainly saturated fatty acids. In fact, the cellular lipid droplet accumulation could
478 be linked to a decrease in lipid oxidation: indeed, a decreased expression of *FASN* (**Figure 4**)
479 was observed which could lead to the accumulation of malonyl-CoA, an inhibitor of CPT1A.

480
481 Based on the European legislation concerning the characterization of EDC, three criteria are
482 required to consider a substance as an EDC: the presence of an adverse outcome, an endocrine
483 mode of action, and a link between these two elements (Andersson et al. 2018). To assess these
484 parameters, the evaluator needs reliable tools and assays. Currently, only 5 *in vitro* methods
485 have been validated by the OECD and 2 by the US-EPA (OECD 2018). It is therefore necessary
486 to provide validated methods as stated recently (Grignard et al. 2022). The method for lipid
487 droplet quantification that we describe in this paper is easy to implement, cheap, and can be
488 used in a relatively high throughput process. Moreover, a list of reference compounds for testing
489 human steatosis endpoints, *in vitro*, has been recently established and our results, obtained with
490 BPA, PFOA, p,p'-DDE, DEHP and OA, fit with those described for TG accumulation

491 (Kubickova and Jacobs 2023). Thus, we believe that our method could be part of an integrated
492 testing strategy (ITS) to identify metabolic or endocrine disruptors, in particular in line with the
493 adverse outcome pathways (AOP) under development related to steatosis (AOP 34, 36, 57, 58,
494 59, 60, 62, 232 and 318 from aopwiki.org).

495
496 In conclusion, the present study assesses the impact of EDCs on steatosis induction in a 2D
497 HepaRG cell model. Our data suggest that co-exposure with oleic acid, at a physiological
498 concentration, enhances the steatosis-inducing effects of DEHP and reveals a similar impact for
499 p,p'-DDE, and DBP, albeit to a lesser extent. This steatotic effect was confirmed for p,p'-DDE
500 and DEHP in a 3D HepaRG model without any FA supplementation. The molecular
501 mechanisms involved in the accumulation of neutral lipids after pollutant exposure are likely
502 diverse and not fully elucidated. Finally, the method described here for the detection,
503 characterization and quantification of lipid droplets could be useful as an ITS (Integrated
504 Testing Strategy) or an IATA (Integrated Approaches to Testing and Assessment) for EDC risk
505 assessment related to MASLD, or even SLD, to fill gaps in regulatory strategy to support
506 chemical safety.

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508

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519

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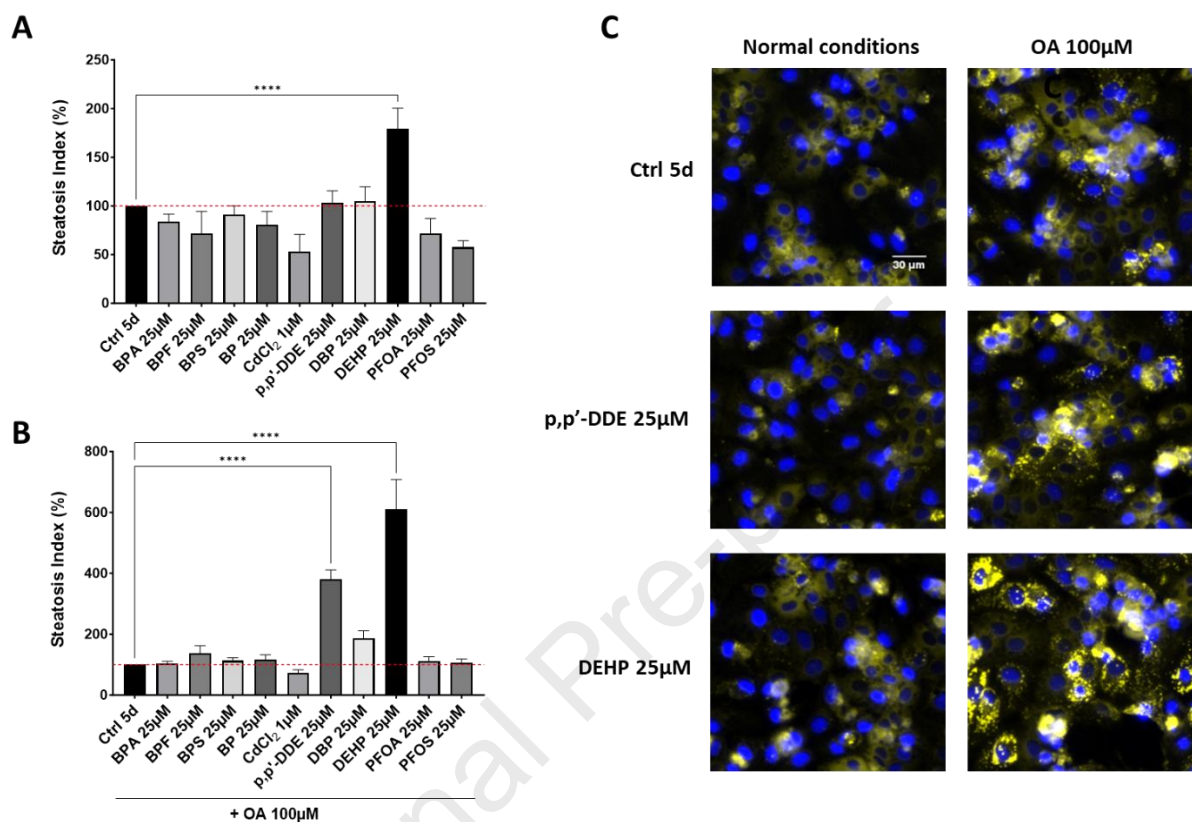
Figures

Figure 1: Assessment of steatosis index by fluorescence microscopy in 2D HepaRG cells following exposure to EDCs. Lipid accumulation was evaluated after 5 days of exposure to EDCs (**A**) in HepaRG under normal culture conditions or (**B**) under co-exposure with 100µM oleic acid (OA). To assess triglyceride accumulation, a steatosis index (cf. **Supplementary Figure 2**) was used based on Nile Red staining and analysis with an automated fluorescence microscope. Values are the mean \pm SEM relative to control (in %) from at least 4 (**A**) or 5 (**B**) independent experiments in triplicate. Statistical analysis was performed using one-way ANOVA followed by a Dunnett post-hoc correction. ****, $p < 0.0001$. (**C**) Representative fluorescence microscopy images of HepaRG cells after 5 days of exposure with pollutants alone or in combination with 100 µM OA. Cells were stained for nuclei (blue) and neutral lipids (yellow). The images were obtained at 10X magnification. Scale bar is 30 µm.

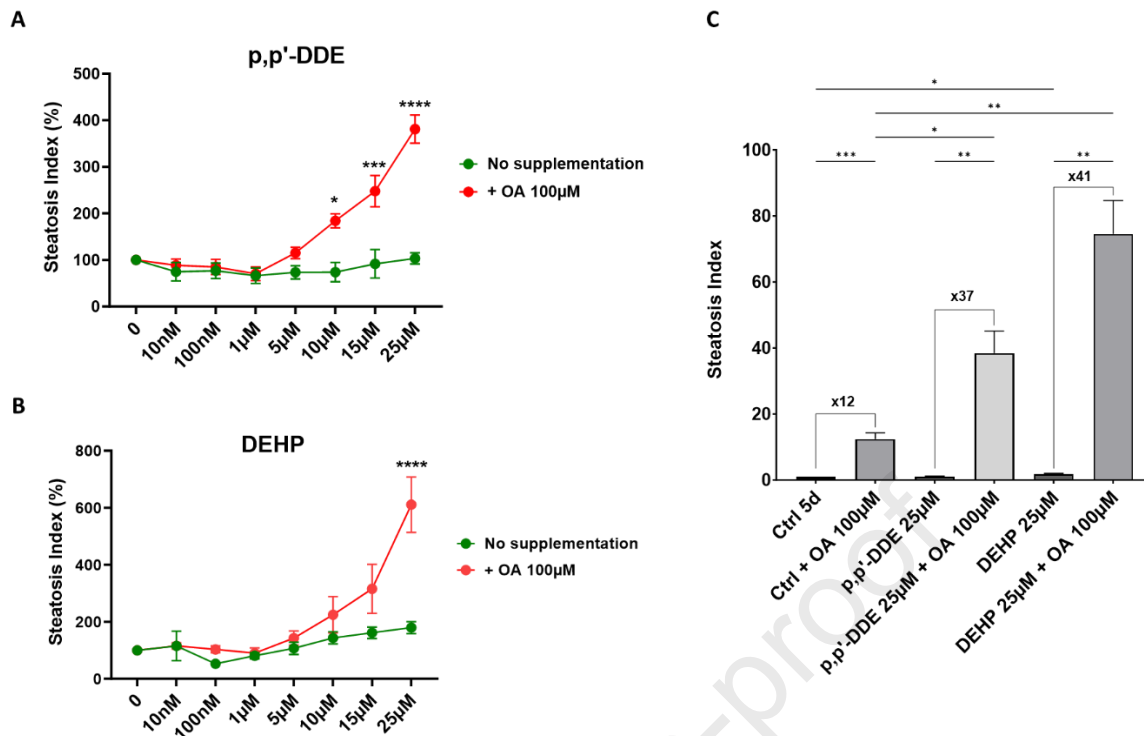


Figure 2: Impact of p,p'-DDE and DEHP exposure on steatosis in 2D HepaRG cells. Assessment by fluorescence microscopy of triglyceride accumulation in HepaRG cells following exposure to different concentrations (10 nM – 25 μM) of (A) p,p'-DDE alone (green) or in combination with 100 μM oleic acid (OA) (red); (B) DEHP alone (green) or with 100 μM OA supplementation (red). Lipid accumulation was evaluated after 5 days of exposure using a steatosis index (cf. **Supplementary Figure 2**) based on Nile Red staining and analysis with an automated fluorescence microscope. (C) Steatosis index in HepaRG cells relative to the control following exposure to EDCs alone or in combination with 100 μM OA. The values represent the average steatosis induction factor between the same condition with and without OA supplementation. Values are the mean ± SEM relative to the control (in %) from at least 3 (A, B) or 7 (C) independent experiments in triplicate. Statistical analysis was performed using one-way ANOVA followed by a Dunnett (A, B) or Bonferroni (C) post-hoc correction. Stars (A, B) represent a significant difference between exposure without supplementation and exposure with 100 μM OA supplementation. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

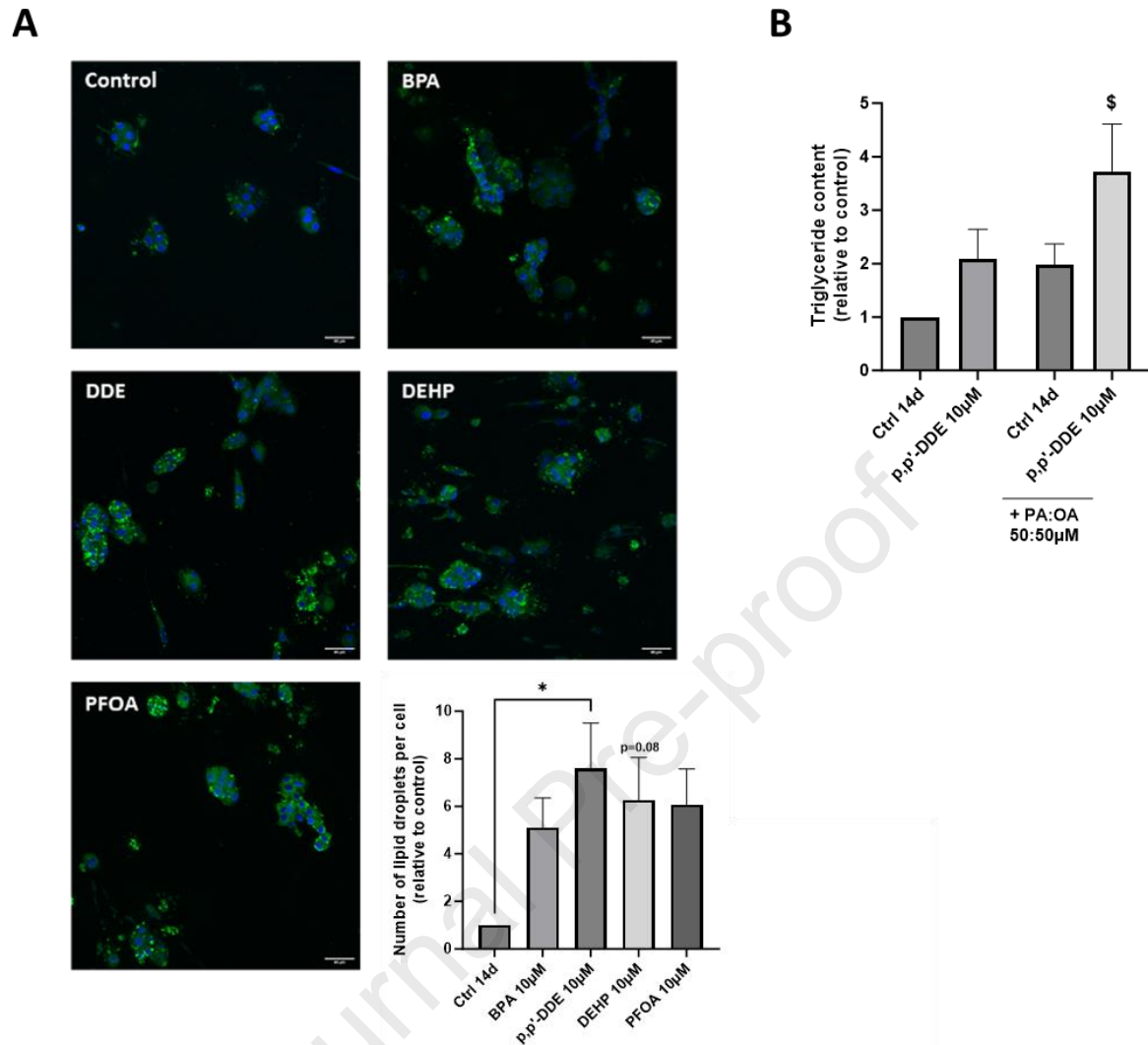


Figure 3: EDC effects on lipid metabolism in Hepoid-HepaRG. (A) Representative fluorescence microscopy pictures of Hepoid-HepaRG cells after 14 days of treatment with pollutants alone. Cells were stained for nuclei (blue) and neutral lipids (green). The images were obtained at 40X magnification. Scale bar is 40 μm . The graph shows the quantification of lipid droplets, using a specific macro, relative to control cells. (B) Assessment of triglyceride accumulation in Hepoid-HepaRG after 14 days of treatment with p,p'-DDE at 10 μM without or with supplementation with fatty acids (PA:OA, 50:50 μM). All values are the mean \pm SEM; n = 3. Statistical analysis was performed using one-way ANOVA followed by a Dunnett (A) or Bonferroni (B) post-hoc correction. *, p < 0.05; \$, p < 0.05 compared to the control at 14 days without supplementation.

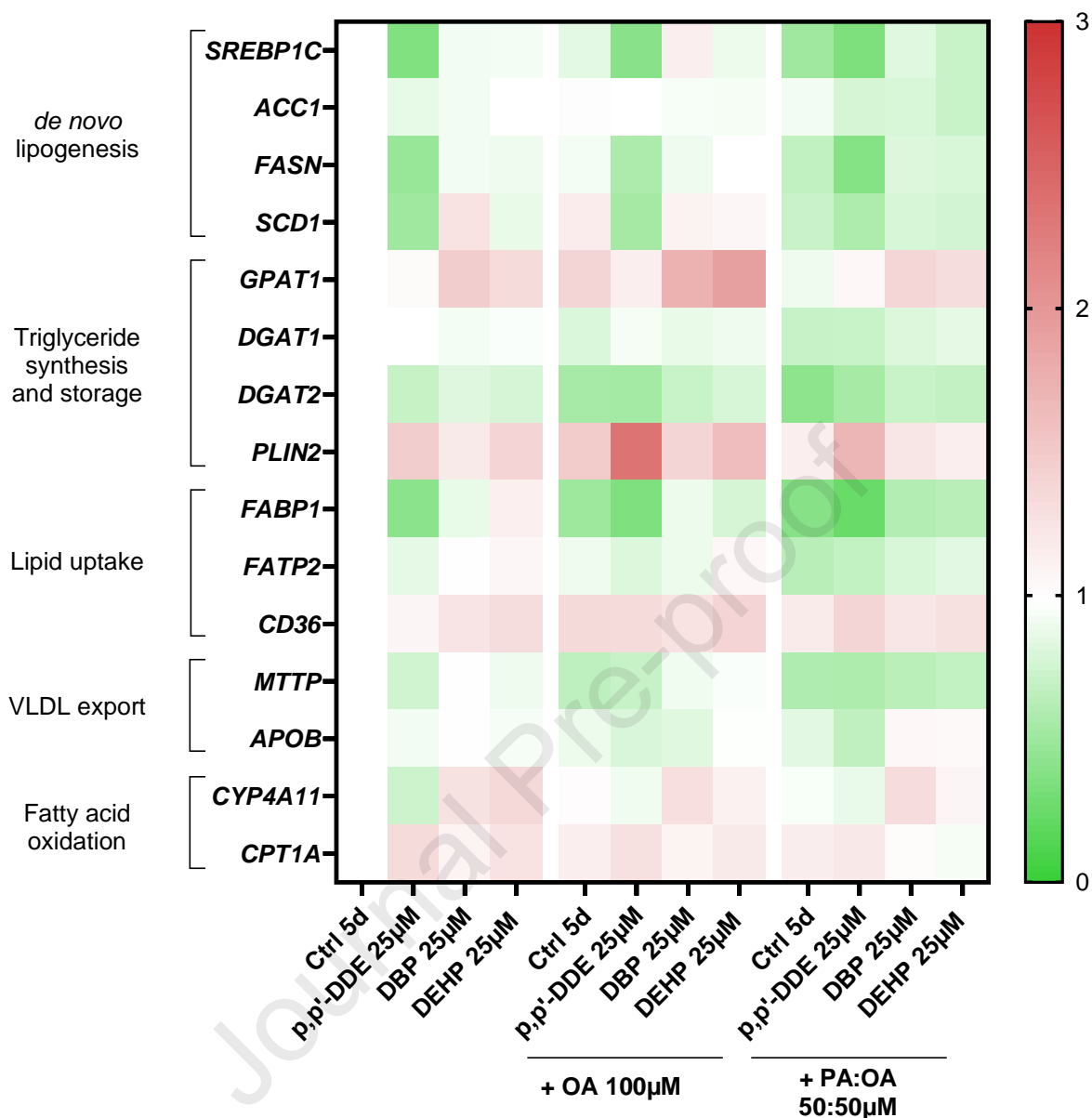


Figure 4: Heatmap of the impact of p,p'-DDE, DBP and DEHP exposure on lipid metabolism gene expression in 2D HepaRG cells. The mRNA levels of genes related to *de novo* lipogenesis, triglyceride synthesis and storage, lipid uptake, VLDL export and fatty acid oxidation were measured after 5 days of exposure to 25 µM EDCs alone or in combination with 100 µM oleic acid (OA) or a mixture of palmitic and oleic acid (PA:OA, 50:50 µM). The data are the means relative to the controls from at least 3 independent experiments. Statistical analysis was performed on treatments having the same supplementation conditions using one-way ANOVA followed by a Bonferroni correction or Kruskal-Wallis test followed by a Dunn's correction. Details of the qRT-PCR results for each gene are compiled in the **Supplementary Figures 5-9**.

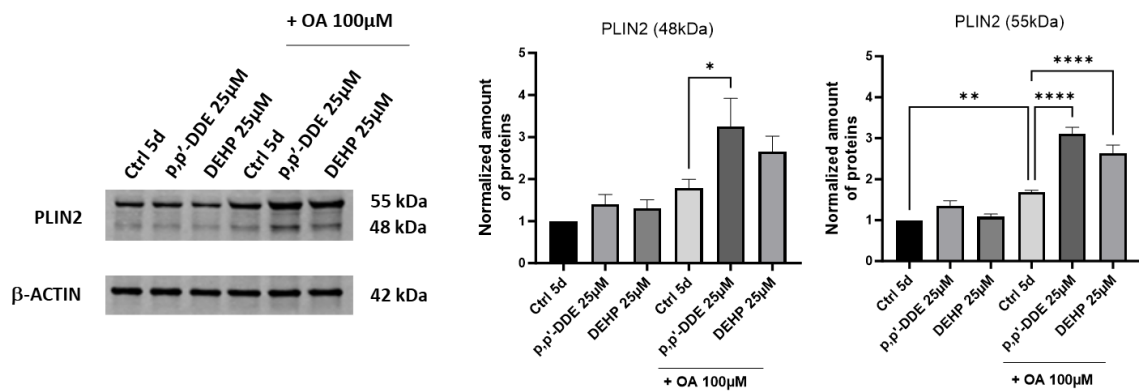
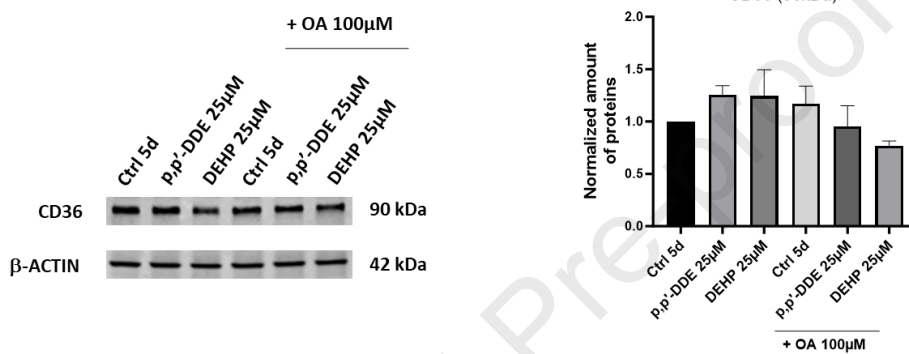
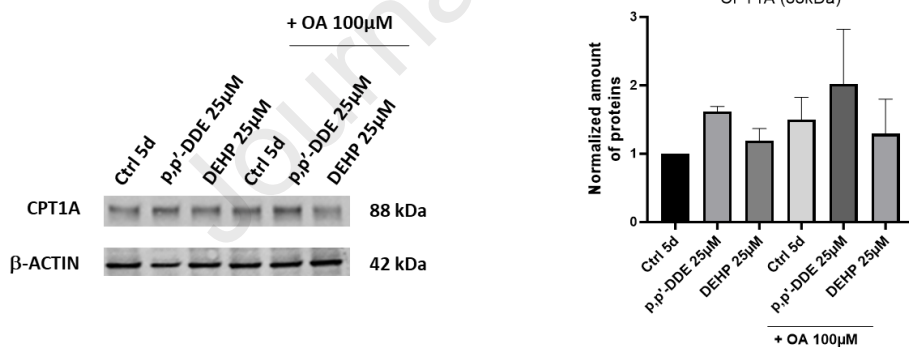
A**B****C**

Figure 5: Impact of p,p'-DDE, DBP and DEHP exposure on lipid metabolism protein levels in 2D HepaRG cells. The levels of PLIN2 (A), CD36 (B) and CPT1A (C) expression were measured after 5 days of exposure to 25 μM p,p'-DDE or DEHP alone or with an 100 μM OA supplementation. Values are the mean ± SEM relative to the control from at least 3 (B and C) or 4 (A) independent experiments. Statistical analysis was performed on treatments having the same supplementation conditions using one-way ANOVA followed by a Bonferroni correction (A, B and C). *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$.

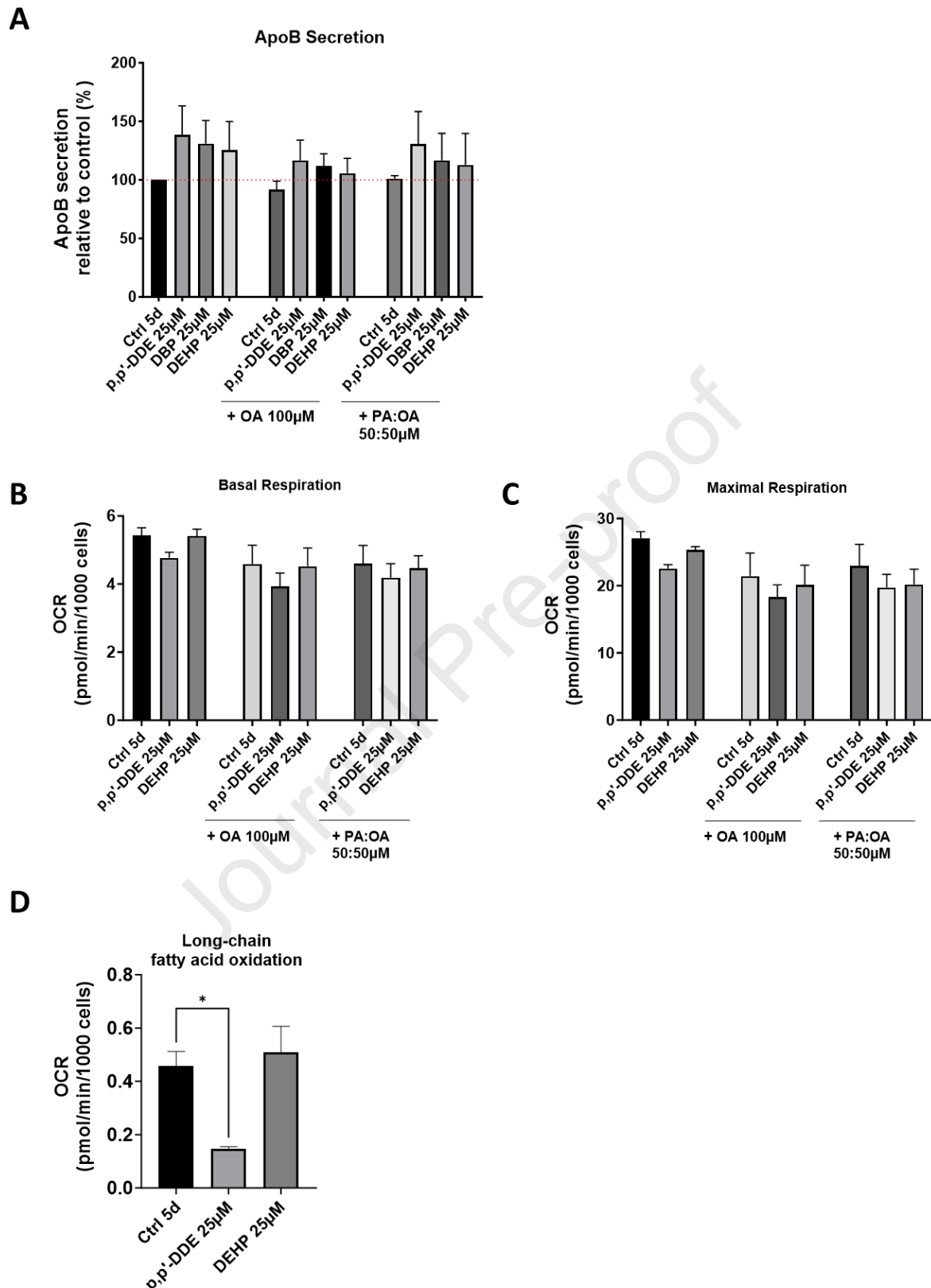


Figure 6: Impact of p,p'-DDE, DBP and DEHP exposure on physiological parameters related to lipid metabolism in 2D HepaRG cells. (A) The quantity of ApoB secreted was measured by ELISA after 5 days of exposure to 25 µM p,p'-DDE, DBP or DEHP alone or with

an OA (100 μ M) or PA:OA (50:50 μ M) supplementation. The medium was renewed after 2 days of exposure and the supernatants were collected 72h after the last treatment to assess the amount of ApoB. (**B** and **C**) The oxygen consumption rate (OCR) from mitochondrial respiration were measured after 5 days of exposure to 25 μ M EDCs alone or in combination with 100 μ M OA and a mixture of 50:50 μ M PA:OA. The maximal respiration was calculated after FCCP injection. (**D**) The OCR relative to long-chain fatty acid oxidation (LCFAO) was determined after 5 days of exposure with 25 μ M DDE and DEHP in HepaRG cells. The OCR from LCFAO was calculated as the difference between basal OCR and OCR after injection of 5 μ M etomoxir (an inhibitor of CPT1A). Values are the means \pm SEM from at least 3 (**B**, **C**, **D**) or 4 (**A**) independent experiments. Statistical analysis was performed on treatments having the same supplementation conditions using one-way ANOVA followed by a Bonferroni (**A**, **B**, **C**) or Dunnett (**D**) correction, *, $p < 0.05$.

Highlights

- DEHP induces the formation of lipid droplets in human HepaRG cells.
- Oleic acid (OA) supplementation enhances the steatotic effects of p,p'-DDE, DBP and DEHP.
- Perilipin 2 is overexpressed after co-exposure to OA and EDCs.
- p,p'-DDE exposure impairs long-chain fatty acid oxidation.
- A method developed for lipid droplet quantification could be used as a regulatory test.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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