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1 **Transcriptomic analysis in zebrafish larvae identifies iron-dependent mitochondrial**  
2 **dysfunction as a possible key event of NAFLD progression induced by**  
3 **benzo[a]pyrene/ethanol co-exposure**

4  
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26

27 **Abstract**

28 Non-alcoholic fatty liver disease (NAFLD) is a worldwide epidemic for which environmental  
29 contaminants are increasingly recognized as important etiological factors. Among them, the  
30 combination of benzo[a]pyrene (B[a]P), a potent environmental carcinogen, with ethanol, was  
31 shown to induce the transition of steatosis towards steatohepatitis. However, the underlying  
32 mechanisms involved remain to be deciphered. In this context, we used high-fat diet fed  
33 zebrafish model, in which we previously observed progression of steatosis to a  
34 steatohepatitis-like state following a 7 days-co-exposure to 43 mM ethanol and 25 nM B[a]P.  
35 Transcriptomic analysis highlighted the potent role of mitochondrial dysfunction, alterations  
36 in heme and iron homeostasis, involvement of aryl hydrocarbon receptor (AhR) signaling and  
37 oxidative stress. Most of these mRNA dysregulations were validated by RT-qPCR. Moreover,  
38 similar changes were observed using a human *in vitro* hepatocyte model, HepaRG cells. The  
39 mitochondria structural and functional alterations were confirmed by transmission electronic  
40 microscopy and Seahorse technology, respectively. Involvement of AhR signaling was  
41 evidenced by using *in vivo* an AhR antagonist, CH223191 and *in vitro* in AhR-knock-out  
42 HepaRG cells. Furthermore, as co-exposure was found to increase the levels of both heme and  
43 hemein, we investigated if mitochondrial iron could induce oxidative stress. We found that  
44 mitochondrial labile iron content was raised in toxicant-exposed larvae. This increase was  
45 prevented by the iron chelator, deferoxamine, which also inhibited liver co-exposure toxicity.  
46 Overall, these results suggest that the increase in mitochondrial iron content induced by  
47 B[a]P/ethanol co-exposure causes mitochondrial dysfunction that contributes to the  
48 pathological progression of NAFLD.

49

50 **Keyword :**

51 NAFLD, iron homeostasis, mitochondrial dysfunction, AhR, B[a]P, ethanol, zebrafish

52 **Abbreviations:**

53 AhR: Aryl hydrocarbon receptor, AhR-KO: AhR knock out , AMEN: Annotation, Mapping,  
54 Expression and Network suite of tools, ANOVA: One-way analysis of variance, B[a]P:  
55 Benzo[a]pyrene, CREEA: Comité Rennais d’Ethique en matière d’Expérimentation Animale,  
56 DMSO: Dimethyl sulfoxide, DPF: Days post-fertilization, FA: Fatty acids, FCCP: Carbonyl  
57 cyanide-p-trifluoro methoxyphenyl hydrazone, GEO: Expression Omnibus, GO: Gene ontology,  
58 GOEA: Gene ontology enrichment analysis, HES: Hematoxylin-Eosin-Safranin; HFD: High fat  
59 diet, HO1: Heme oxygenase 1, IARC: International Agency for Research on Cancer, IPA:  
60 Ingenuity pathway analysis, KEGG: Kyoto Encyclopedia of Genes and Genomes, NADP:  
61 Nicotinamide adenine dinucleotide phosphate, NAFLD: Alcoholic fatty liver disease, NaN<sub>3</sub>:  
62 Sodium azide, NASH: Non-alcoholic steatohepatitis, NOS: Reactive nitrogen species, OCR:  
63 Oxygen consumption rate, OXPHOS: Oxidative phosphorylation, PAH: Polycyclic aryl  
64 hydrocarbon, PBS: Phosphate-buffered saline, PMT: Photomultiplier tube, ROS: Reactive  
65 oxygen species, SEM: Standard error of the mean, T2DM: Type 2 Diabetes mellitus, TAFLD:  
66 Toxicant-Associated Fatty Liver Diseases, TASH: Toxicant-Associated Steatohepatitis, TCDD:  
67 2,3,7,8-Tetrachlorodibenzo-p-dioxin, TEM: Transmission electronic microscopy.

68

## 70 1. Introduction

71 Non-alcoholic fatty liver disease (NAFLD) is now well recognized as a growing  
72 worldwide epidemic, responsible for an increasing number of chronic liver diseases and  
73 consecutive mortality (Younossi 2019). NAFLD covers a large panel of liver diseases, starting  
74 from liver steatosis to its pathological progression into non-alcoholic steatohepatitis (NASH),  
75 with possible evolution towards severe and irreversible complications such as cirrhosis and/or  
76 hepatocellular carcinoma (Fazel et al. 2016). The global prevalence of NAFLD is around 25% of  
77 general population all over the world, while for NASH, it reaches between 3 to 5% (Younossi  
78 2019). It should be noted that some subpopulations are particularly affected by NAFLD,  
79 notably patients exhibiting metabolic diseases such as type 2 Diabetes mellitus (T2DM) and  
80 obesity, in which NAFLD prevalence could rise to 60 or 90% respectively (Younossi 2019).  
81 Beyond principal etiological factors of NAFLD, *i.e.* metabolic diseases like obesity and T2DM,  
82 there are several others like dietary habits (including moderate/heavy alcohol consumption),  
83 genetic polymorphisms, gender, epigenetic factors as well as environmental factors (Younossi  
84 2019). In this concern, the role of these latter factors has gained interest during last years,  
85 leading to the concept of TAFLD and TASH (Toxicant-Associated Fatty Liver Diseases and  
86 Toxicant-Associated Steatohepatitis) as proposed by Cave and co-workers (Joshi-Barve et al.  
87 2015; Wahlang et al. 2019). In line with this, several pollutants, including ligands of aryl  
88 hydrocarbon receptor (AhR), have thus been shown to induce steatosis or favor its  
89 pathological progression (Wahlang et al. 2019).

90 In this context, another challenging concern is the impact of chemical mixtures on  
91 NAFLD particularly in high-risk populations such as people already presenting fatty liver. In

92 that way, we have previously demonstrated that co-exposure to benzo[a]pyrene (B[a]P), the  
93 reference molecule of the PAH (polycyclic aryl hydrocarbon) family, in combination with a  
94 well-known hepatotoxicant, ethanol, induces the transition of steatosis towards a  
95 steatohepatitis—like state both *in vitro* and *in vivo* (Bucher et al. 2018b; Bucher et al. 2018a;  
96 Imran et al. 2018; Tête et al. 2018). B[a]P, a widespread environmental contaminant, is a  
97 potent carcinogen to human (classified in group 1 by IARC), and a strong AhR ligand (Das and  
98 Bhutia 2018; Hardonnière et al. 2017; International Agency for Research on Cancer (IARC)  
99 2012). B[a]P is a pollutant formed, like other PAHs, during incomplete combustion of organic  
100 compounds. Human exposure to B[a]P, excluding smoking or occupational exposure, is mainly  
101 food-borne notably with barbecued/grilled/broiled/smoked meats, grain and cereals (Das and  
102 Bhutia 2018; International Agency for Research on Cancer (IARC) 2012). Most of the toxic  
103 effects of B[a]P depend on its bioactivation by cytochrome P450s (CYP), which mainly occurs  
104 in liver. This thus explains the adverse effects of this pollutant on this organ through several  
105 mechanisms including oxidative stress, genotoxicity, mitochondrial dysfunction, cell death  
106 (Das and Bhutia 2018; Hardonnière et al. 2016; International Agency for Research on Cancer  
107 (IARC) 2012; Tekpli et al. 2010; Uno et al. 2018). B[a]P, as other toxicants, has been implicated  
108 in NAFLD development and progression (Wahlang et al. 2019). Regarding the impact of  
109 B[a]P/ethanol co-exposure, frequently observed in population, on steatosis progression, we  
110 recently evidenced several mechanisms using *in vitro* models such as the human hepatic cell  
111 line, HepaRG (Bucher et al. 2018a; Tête et al. 2018). However, the underlying mechanisms  
112 involved in the exacerbation of NAFLD upon such a co-exposure remain to be deciphered *in*  
113 *vivo*.

114 To this aim, we used an *in vivo* model of zebrafish larvae to have an integrative model  
115 in which the complexity and variety of cell and organ interactions are present and relevant to  
116 human NAFLD pathogenesis (Chu and Sadler 2009; Goessling and Sadler 2015; Schlegel 2012).  
117 In addition to technical advantages like small size and transparency, zebrafish larvae also  
118 present broad similarities with humans concerning liver functions and sensitivity toward  
119 xenobiotics and alcohol (metabolism, toxicity, cellular and transcriptomic responses)  
120 (Driessen et al. 2014; Driessen et al. 2013; Goessling and Sadler 2015; Goldstone et al. 2010).  
121 Besides, the full machinery for B[a]P metabolism exists in zebrafish notably with the  
122 expression of *ahr2*, the ligand-activated ortholog of AhR (Goodale et al. 2012). For the present  
123 study, we used a recently established zebrafish larvae model of high fat diet (HFD)-induced  
124 steatosis in which we observed steatosis progression following a 7 days-co-exposure to low  
125 doses of ethanol and B[a]P, respectively 43 mM and 25 nM (Bucher et al. 2018b; Imran et al.  
126 2018). In order to elucidate the mechanisms involved in the pathological progression of  
127 steatosis induced upon B[a]P/ethanol co-exposure in HFD-zebrafish larvae, a transcriptomic  
128 approach was performed in the present study. We identified and confirmed by RT-qPCR,  
129 disruptions of key processes, *i.e.* mitochondrial dysfunctions, alterations of heme and iron  
130 metabolism, AhR signaling and oxidative stress, which were then more deeply investigated.  
131 Our main results strongly suggest the involvement of a mitochondrial overload of labile iron  
132 as a key event in the liver disease progression upon B[a]P/ethanol co-exposure. Furthermore,  
133 these effects were prevented by the common iron-chelating drug, deferoxamine.

134

## 135 **2. Materials and Methods**

136

137 Details for all Materials and methods are available in supplementary material (File S1).

138

### 139 **3. Results**

#### 140 **3.1. Transcriptomic analysis identifies heme homeostasis and mitochondrial dysfunction as** 141 **potential events of liver disease progression in B[a]P/ethanol co-exposed HFD zebrafish** 142 **larvae**

143 In order to decipher the cellular mechanisms involved in the progression of liver steatosis in  
144 HFD zebrafish larvae co-exposed to B[a]P and ethanol, we performed a transcriptomic analysis  
145 based on affymetrix microarray technology (GeneChip™ Zebrafish Gene 1.0 ST Array). Briefly,  
146 HFD zebrafish larvae were chronically exposed to 43 mM ethanol and/or to 25 nM B[a]P from  
147 5 dpf until 12 dpf (doses chosen in rationale to human level exposure and already shown to  
148 induce progression of steatosis in our zebrafish model (Bucher et al. 2018b; Imran et al. 2018)).  
149 From transcriptomic analysis, 525 differentially expressed genes (DEGs) were found after co-  
150 exposure compared to control. Those genes were further partitioned into 8 expression  
151 clusters (termed P1-P8) organized in two broad expression patterns corresponding to up-  
152 regulated (315 DEGs, corresponding to P1-4) and down-regulated (210 DEGs, P5-8) genes (see  
153 supplementary Table S1 for all data) (Figure 1a and b). The functional analysis revealed that  
154 up-regulated genes were significantly associated with porphyrin metabolism and  
155 mitochondria (GO:0006787 porphyrin-containing compound catabolic process; GO:0031966  
156 mitochondrial membrane; KEGG:00860 porphyrin and chlorophyll metabolism) (Figure 1c).  
157 Interestingly, these terms were also found to be significantly enriched in the 4 up-regulated  
158 clusters (P1-4) thereby indicating that the observed alterations were not just due to one  
159 toxicant; rather both B[a]P and ethanol were effective, particularly when used in combination  
160 (Figure 1b). Regarding down-regulated genes, most of the terms appeared to be related to



161 immunity (for example: GO:0060333 IFN $\gamma$ -mediated signaling pathways; GO:0043312  
162 neutrophil degranulation or GO:0030670 phagocytic vesicle membrane) (Figure 1b). To  
163 improve relevance of findings regarding human health, we performed an Ingenuity Pathways  
164 Analysis (IPA) following conversion of the 525 zebrafish DEGs into 259 human homologs  
165 (supplementary Table S2); several terms were then presented for canonical pathways, toxicity  
166 lists and toxicity functions (summary presented in supplementary Table S3; full results are  
167 provided in supplementary Table S4). In line with the functional analysis performed in AMEN,  
168 the selected terms highlight mitochondria dysfunction and alterations in heme homeostasis,  
169 involvement of AhR signaling and oxidative stress for canonical pathways and toxicity lists  
170 (supplementary Table S3). Finally, analysis of toxicity functions markedly outlined the impact  
171 of B[a]P/ethanol co-exposure on liver diseases (supplementary Table S3) in agreement with  
172 our previous work (Bucher et al. 2018b; Imran et al. 2018; Tête et al. 2018).

173

### 174 **3.2. Validation and investigation by RT-qPCR of molecular dysregulations induced by co-** 175 **exposure**

176 In order to validate the microarray results from our zebrafish model, we performed RT-qPCR  
177 assays on zebrafish samples (Figure 2a to 2c) (Note that for all genes studied by RT-qPCR and  
178 presented by abbreviation, full names could be found in the supplementary file S5 with primer  
179 information). One of the main processes identified through the transcriptomic analysis in *in*  
180 *vivo* steatosis progression upon B[a]P/ethanol co-exposure appeared to be mitochondrial  
181 dysfunction. In fact, this was in line with our recent work realized *in vitro* in human HepaRG  
182 cell line (Bucher et al. 2018a). In order to get further insight into mitochondrial dysfunction,  
183 especially *in vivo*, we decided to perform RT-qPCR on several target genes selected either from  
184 our transcriptomic screening or according to their role in mitochondrial function (Figure 2a).

185 Most of the transcripts found to be dysregulated in our microarray (indicated by an arrow in  
186 Figure 2a to 2c), were validated by RT-qPCR with significant changes (22 transcripts out of the  
187 25 found in microarray). Among mitochondria-related genes, the expression of several  
188 transporters of metabolites (*abcg2a*, *slc25a25a*, *slc25a47a*, *slc25a48* and *tspo*) was induced,  
189 thus suggesting alterations of mitochondrial metabolism (Figure 2a). Interestingly, *abcg2a* and  
190 *tspo* are also known to be involved in heme homeostasis. Regarding genes related to electron  
191 transport chain, mitochondrial respiration and ATP production, several were found to be  
192 induced (*sdha*, *sdhaf3*, *uqcc1* and *uqcc3*), thus further pointing to alterations of mitochondrial  
193 respiration capacity (Figure 2a). In addition, expression of known regulators of mitochondrial  
194 activity (*hepb2*, *parla*, *sirt3*) was modified upon B[a]P/ethanol co-exposure; it could be noted  
195 that *hepb2* and *parla* have been implicated in cell death (Ishihara and Mihara 2017; Szigeti et  
196 al. 2006) in agreement with the deleterious effects of B[a]P/ethanol co-exposure previously  
197 reported *in vitro* (Bucher et al. 2018b; Bucher et al. 2018a; Tête et al. 2018).

198 Furthermore, another key process revealed by our microarray analysis was porphyrin  
199 metabolism, notably included in the more general term of heme metabolism (as suggested by  
200 changes of expression for *tspo* and *abcg2a*) (Figure 1c). In addition, IPA identified iron  
201 homeostasis as a new process affected by B[a]P/ethanol co-exposure, which is also closely  
202 linked to heme metabolism (supplementary Table S3). Therefore, we decided to evaluate the  
203 expression of several genes associated with heme and iron metabolism (Figure 2b). Most of  
204 the changes in gene expression, found by microarray analysis, were validated by RT-qPCR  
205 (*abcb6*, *fech*, *blvra*, *blvrb*, *tfa*) (Figure 2b). In addition, co-exposure significantly altered the  
206 expression of several genes involved in heme synthesis (*alas1*, *alas2*), in iron transport  
207 (*slc25a37* and *slc25a28* alias mitochondrial iron transporters mitoferrin 1 and 2; *slc40a1* alias  
208 ferroportin 1), and in iron storage (*fth1a* and *fthl30*, ferritin subunits; *tfa* for transferrin a)

209 (Figure 2b). Altogether, these results strongly support the idea of a perturbation in heme and  
210 iron homeostasis following B[a]P/ethanol co-exposure in our steatotic model of zebrafish  
211 larvae.

212 In addition, oxidative stress-related genes (markers of oxidative stress and/or genes involved  
213 in ROS/NOS production/elimination) were also investigated by RT-qPCR. Our data thus  
214 confirmed the changes observed in our microarray in the expression of catalase (*cat*) and  
215 peroxiredoxin 1 (*prdx1*); they also showed induction of *nos2a*, *prdx6*, *sod2* and *sod3b* (Figure  
216 2c), thus suggesting the implication of oxidative stress. Finally, potential activation of AhR  
217 signaling was explored by assessing the expression of several AhR-related genes (Figure 2c).  
218 Three genes, for which expression was increased in transcriptomic analysis, were validated by  
219 RT-qPCR (*cyp1a*, *gstp1*, *nfe2l2a* alias *nrf2*). In addition, *nqo1* (NAD(P)H dehydrogenase  
220 quinone 1, an indirect target of AhR) was also found to be significantly induced by  
221 B[a]P/ethanol co-exposure as well as *ahr2*, the ortholog of human AhR (Figure 2c).

222 Finally, in order to evaluate the relevance of the results found in zebrafish toward human  
223 NAFLD progression upon similar co-exposure, we assessed expression of several genes related  
224 to the potential mechanisms involved (mitochondrial dysfunction, alterations of heme  
225 metabolism and iron homeostasis, and AhR signaling) in HepaRG cells, an *in vitro* human  
226 model of hepatocytes, supplemented with fatty acids (FA) in which B[a]P/ethanol co-exposure  
227 was demonstrated to induce steatosis progression toward a steatohepatitis-like state (Bucher  
228 et al. 2018b) (Figure 2d). As illustrated in Figure 2d, regarding most of the studied genes, the  
229 changes in mRNA expression observed in zebrafish larvae (as indicated by white arrows) were  
230 also reproduced in steatotic HepaRG cells co-exposed to B[a]P and ethanol, even though the  
231 amplitude of changes could be less. This is particularly observable for mRNA expression  
232 changes highly significant in zebrafish, *i.e.* *abcg2a*, *tspo*, *uqcc3*, *abcb6a*, *fech*, *blvra*, *slc25a37*,

233 *slc25a28*, *nqo1*. Indeed for 7 of them, significant changes were also found in HepaRG (except  
234 for ABCB6 and SLC25A28, close to significance) (Figure 2d). Therefore, these results further  
235 validated the HFD-fed zebrafish larvae as a suitable model to study human NAFLD progression,  
236 then prompting us to more thoroughly investigate the *in vivo* role of the observed alterations.

### 237 **3.3. Assessment of mitochondrial dysfunction**

238 Due to the well-recognized role of mitochondrial dysfunction in NAFLD (Begrache et al.  
239 2013) and as it was identified in our transcriptomic analysis, we decided to evaluate  
240 mitochondrial respiration in co-exposed steatotic zebrafish larvae (Figure 3a). To this aim, we  
241 used a protocol adapted from Raftery *et al.* (2017) based on the Agilent Seahorse technology,  
242 which allows the measurement of oxygen consumption from living zebrafish larvae (Raftery  
243 et al. 2017) (described in supplementary Materials and Methods). We observed that co-  
244 exposure significantly inhibited both basal and maximal respiration without any effect on  
245 spare and non-mitochondrial respiration (Figure 3a; representative curves of OCR could be  
246 found in Figure S3). Then, in order to get further insight into mitochondrial dysfunction, we  
247 performed transmission electron microscopy (TEM) to study the ultrastructure of liver cells in  
248 co-exposed HFD zebrafish larvae (Figure 3b). Images obtained at low magnification (panel of  
249 pictures on the left) showed well-organized hepatocytes under control conditions and few  
250 biliary canaliculi. However, under B[a]P/ethanol co-exposure conditions, disorder of  
251 hepatocyte morphology was observed. Regarding more specifically the mitochondria in HFD  
252 control larvae, they appeared to represent a large part of hepatocyte surface, with a circular  
253 form and numerous cristae (central and right panels of pictures in Figure 3b) under control  
254 conditions. In contrast, after a 7-days-co-exposure to toxicants, only a few mitochondria per  
255 hepatocyte could be observed, with a smaller size and flatter form, and less observable cristae.

256 Therefore, these ultrastructure observations were in accordance with the decreased oxygen  
257 consumption induced by B[a]P/ ethanol co-exposure.

### 258 **3.4. Involvement of AhR in *in vivo* mitochondrial dysfunction and liver damages during** 259 **steatosis progression induced by B[a]P/ethanol co-exposure**

260 AhR and/or B[a]P have already been reported to be involved in mitochondrial dysfunction in  
261 diverse *in vitro* models (Bucher et al. 2018a; Hardonnière et al. 2017; Hwang et al. 2016).

262 Based upon the fact that our transcriptomic analysis outlined AhR signaling (see  
263 supplementary Table S3), we decided to investigate the role of this receptor in the

264 mitochondrial dysfunction both at a transcriptomic level and at a functional level, *i.e.*

265 mitochondrial oxygen consumption. To do so, we used the specific AhR antagonist CH223191

266 (1 µM) (Wang et al. 2019). To ensure that this antagonist did inhibit AhR activation in our

267 zebrafish model, we analyzed mRNA expression of known AhR target genes after CH223191

268 treatment of co-exposed larvae (Figure 4a). As expected, we observed an inhibition of several

269 B[a]P/ethanol-induced genes, *cyp1a*, *nqo1* and *nfe2l2* (Figure 4a). It was not the case for *gstp1*

270 mRNA expression; rather CH223191 alone induced its expression to a similar level as upon

271 toxicant co-exposure, with a further increase upon co-treatment with the three molecules

272 (Figure 4a). Thus, it probably means that the observed effect on *gstp1* expression would be a

273 secondary response linked to its role in detoxification rather than being directly targeted by

274 AhR. Looking at genes related to mitochondria (Figure 4b), we found that CH223191 was able

275 to prevent co-exposure effects on *abcg2a* and partially on *sdha* (even if, for this last gene,

276 there is no statistical difference between co-exposure conditions (BE vs CH+BE), there is no

277 more effect of co-exposure in presence of CH223191 (CH vs CH+BE)). Regarding *tspo* and

278 *uqcc3*, a significant induction was observed with the antagonist alone, but no further increase

279 occurred, when larvae were co-exposed to B[a]P/ethanol (Figure 4b). Regarding heme and

280 iron-related genes, CH223191 reversed co-exposure effect on *fech*, *slc25a28*, *slc40a1* and *tfa*  
281 but not *blvra*, *fth1a*, *fthl30* and *slc25a37* (Figure 4c). Taken together, these observations  
282 suggest that AhR activation might disturb mitochondrial activity partly through a  
283 transcriptional action. In order to further test the *in vivo* role of AhR in the steatosis  
284 progression upon co-exposure, the effects of CH223191 on the expression of mRNA markers  
285 of toxicity were analyzed by RT-qPCR (Figure 4d). We found that CH223191 inhibited the  
286 increase in mRNA expression of both *casp3* and *il6*, thus suggesting reduction of cell death  
287 and inflammation, whereas it increased *prdx1* expression. As *gstp1*, this latter increase might  
288 be associated with a secondary cell response against co-exposure-induced oxidative damages.  
289 Still trying to be relevant of human NAFLD progression upon similar co-exposure, these  
290 alterations of gene expression were evaluated in AhR-knock-out HepaRG cells (Figure 4e). By  
291 this way, we found that most of the changes induced by co-exposure in the expression of  
292 genes related to mitochondria (ABCG2, UQCC3), iron homeostasis (FECH, SLC25A28, SLC40A1,  
293 TF) or, as expected, AhR activation (NFE2L2 alias NRF2 and NQO1), were prevented (Figure  
294 4e). Finally, we investigated CH223191 effect on mitochondrial function and liver integrity  
295 (Figure 4f and g respectively). Using the Seahorse technology, we found that alterations in  
296 mitochondrial respiration induced in co-exposed HFD larvae (especially the decrease in basal  
297 respiration), were prevented by CH223191 (Figure 4f; representative curves of OCR could be  
298 found in Figure S3). We also noted that CH223191 alone could induce both maximal and non-  
299 mitochondrial respiration (Figure 4f). In addition, this AhR antagonist was able to reduce liver  
300 cell damages induced by toxicant co-exposure as shown by Hematoxylin-Eosin-Safranin (HES)  
301 staining (Figure 4g; representative pictures can be found in supplementary Figure S1). In total,  
302 these results indicate a crucial involvement of AhR in mitochondrial disruption and liver

303 injuries following B[a]P/ethanol co-exposure in agreement with *in vivo* progression of liver  
304 steatosis.

### 305 **3.5. B[a]P/ethanol co-exposure leads to disruption of heme metabolism and to an oxidative** 306 **stress involved in liver injury**

307 As our transcriptomic data clearly pointed to heme metabolism as a possible target of  
308 B[a]P/ethanol co-exposure, alterations of this process were more thoroughly analyzed  
309 through biochemical assessment. Thus, levels of heme, hemin (the oxidized and free form of  
310 heme) and bilirubin (one of the major metabolic compounds of heme degradation) were  
311 determined in our model of HFD-fed zebrafish larvae. Co-exposure was found to increase the  
312 levels of both heme and hemin (Figure 5a and b, respectively), whereas bilirubin levels  
313 remained unchanged (Figure 5c). As increased cellular heme and hemin amounts have been  
314 reported to be toxic for cells mainly *via* oxidative stress (Kumar and Bandyopadhyay 2005),  
315 we decided to test whether B[a]P/ethanol co-exposure induced such a phenomenon under  
316 our experimental conditions, and if so, whether it was involved in the related liver damages  
317 using common antioxidants, quercetin and vitamin E (Batiha et al. 2020; Burton and Ingold  
318 1989). Thus, lipid peroxidation was evaluated after co-exposure by fluorescent imaging using  
319 C11-bodipy<sup>583/591</sup> staining in liver of zebrafish larvae (Figure 5d; representative pictures can be  
320 found in supplementary Figure S4). As expected, an increase in lipid peroxidation was  
321 observed upon B[a]P/ethanol co-exposure, such an effect being prevented by quercetin (25  
322  $\mu\text{M}$ ) or vitamin E (100  $\mu\text{M}$ ) co-treatment (Figure 5d). In order to test the involvement of  
323 oxidative stress in liver disease progression, liver damages were estimated by counting the  
324 damaged cells after histological HES staining (Figure 5e; representative pictures can be found  
325 in supplementary Figure S1). As previously reported (Bucher et al. 2018b), we found that co-  
326 exposure increased the proportion of damaged cells in liver, and quercetin as vitamin E, at

327 least partly, prevented this effect. These data therefore showed the involvement of oxidative  
328 stress in the pathological progression of steatosis upon B[a]P/ethanol co-exposure of HFD-fed  
329 zebrafish larvae.

### 330 **3.6. Iron is a crucial player in the liver mitochondrial dysfunction and toxicity induced by** 331 **B[a]P/ethanol co-exposure of HFD zebrafish larva**

332 Our present results have highlighted the role of (i) mitochondrial dysfunction, (ii)  
333 dysregulation of heme homeostasis, and (iii) oxidative stress in co-exposure-induced *in vivo*  
334 steatosis progression. In addition, the transcriptomic analysis suggested changes in iron  
335 homeostasis, notably in mitochondria. In this context, it appeared necessary to further explore  
336 the involvement of iron homeostasis in mitochondrial dysfunction and liver toxicity under our  
337 experimental conditions. First, the level of labile iron in mitochondria (mostly represented by  
338 ferrous iron Fe<sup>2+</sup> (Lv and Shang 2018)) was evaluated. To do so, alive HFD-fed zebrafish larva,  
339 co-exposed or not, were loaded with the fluorescent mitochondrial Fe<sup>2+</sup>-sensitive probe, Mito-  
340 FerroGreen, followed by imaging of the liver by confocal microscopy (Figure 6a). We observed  
341 that co-exposure markedly increased the level of labile iron in mitochondria, as shown by a  
342 stronger fluorescence intensity in the liver of treated animals compared to untreated  
343 counterparts (Figure 6a, zebrafish liver is delineated by a white dot-line). In addition,  
344 quantitative analysis of liver fluorescent intensities, detected in several larvae, clearly showed  
345 the significant increase of the mitochondrial Fe<sup>2+</sup> pool in liver upon B[a]P/ethanol co-exposure  
346 (Figure 6b).

347 In order to determine how this increase of iron might affect mitochondria and thus could lead  
348 to toxicity upon co-exposure in our zebrafish model, we decided to test the potential  
349 protective effect of the iron chelator, deferoxamine (100 μM) (Figure 6c-e). We firstly  
350 evaluated the content of liver mitochondrial Fe<sup>2+</sup> using Mito-FerroGreen fluorescence



351 imaging. As expected, a co-treatment of larvae with deferoxamine totally prevented the  
352 increase of mitochondrial Fe<sup>2+</sup> content (Figure 6c). Then, the role of iron in the toxicity was  
353 evaluated by RT-qPCR assessment of genes related to mitochondria (Figure 6d), to heme and  
354 iron homeostasis (Figure 6e), and to cellular stress and toxicity (Figure 6f). Interestingly,  
355 deferoxamine inhibited some of the changes induced by co-exposure related to mitochondria  
356 (*abcg2a*, *uqcc3*) (Figure 6d), or to iron and heme homeostasis (*fech*, *slc25a28*, *slc40a1*, *tfa*)  
357 (Figure 6e). In addition, regarding the expression of the gene markers of toxicity (*i.e.* *prdx1* for  
358 oxidative stress, *casp3a* for cell death and *il6* for inflammation), deferoxamine inhibited all the  
359 changes induced by co-exposure (Figure 6f). It could be noted that deferoxamine did not  
360 prevent the effects of co-exposure on the mRNA expression of *cyp1a*, *nbfe2l2* and *nqo1*, thus  
361 confirming that the protective effect of deferoxamine was linked to iron chelation rather than  
362 an inhibition of AhR signaling (Figure 6g). Finally, the role of mitochondrial iron overload in  
363 liver toxicity was confirmed by the observation of a protective effect of deferoxamine upon  
364 liver cell damages induced by B[a]P/ethanol co-exposure (Figure 6h ; representative pictures  
365 can be found in supplementary Figure S1). Taken together, these results are in favor of the  
366 involvement of an iron overload in the mitochondrial dysfunction induced by B[a]P/ethanol  
367 co-exposure, and hence related toxicity.

368 Finally, in order to better understand the involvement of AhR signaling in the deleterious  
369 effect of B[a]P/ethanol co-exposure on the liver, we assessed the impact of AhR inhibition on  
370 liver mitochondrial iron accumulation and oxidative stress (Figure 7 and supplementary Figure  
371 S2). Interestingly, we observed that AhR inhibition was fully protective against liver  
372 mitochondrial iron accumulation (Figure 7a) and against lipid peroxidation (Figure 7b).  
373 Therefore, these results show that AhR activation is a key event on the pathological

374 progression of steatosis to steatohepatitis induced by co-exposure in our zebrafish larva  
375 model.

#### 376 **4. Discussion**

377         With the rise of NAFLD prevalence over the last decades, this liver disease has become  
378 a major health issue, due to its pathological complications like NASH, cirrhosis and cancer.  
379 Besides the well-known etiological factors of NAFLD (*i.e.* genetic factors, nutrition, obesity,  
380 diabetes), environmental toxicants have been put forward by an increasing number of studies;  
381 this has led to the emergence of the concept of TAFLD and TASH (Joshi-Barve et al. 2015;  
382 Wahlang et al. 2019; Wahlang et al. 2013). Recently we have found, both *in vitro* and *in vivo*,  
383 that co-exposure to B[a]P and ethanol, at doses not toxic in the absence of steatosis, could  
384 favor the NAFLD progression (Bucher et al. 2018b; Bucher et al. 2018a; Imran et al. 2018; Tête  
385 et al. 2018). In this context, several mechanisms have been identified *in vitro* including  
386 alterations of B[a]P and ethanol metabolism (Bucher et al. 2018b; Tête et al. 2018), NOS  
387 production (Tête et al. 2018), and mitochondrial dysfunction (Bucher et al. 2018a). However,  
388 the underlying mechanisms remained to be deciphered *in vivo*. To this aim, we used our  
389 recently developed model of HFD fed zebrafish larva, proven to be reliable to human models,  
390 in which B[a]P/ethanol co-exposure was involved in NAFLD steatosis progression toward a  
391 steatohepatitis-like state.

392         Thus, we performed a non-targeted approach by transcriptomic analysis in this model.  
393 From this, we focused on the 525 genes presenting significant changes in expression after  
394 B[a]P/ethanol co-exposure in HFD larvae. Thus, following clustering of these genes considering  
395 their expression found upon exposure to either toxicant alone or together, we performed a  
396 functional analysis based on GO enrichment (Figure 1c). Most of the annotation regarding  
397 down-regulated genes was linked to immunity. Such a result was not surprising, even in the

398 context of liver inflammation and NASH previously observed in our *in vivo* B[a]P/ethanol co-  
399 exposure model (Bucher et al. 2018b; Imran et al. 2018). Indeed each toxicant is known to  
400 induce immunosuppressive effects (Cella and Colonna 2015; Liamin et al. 2018; Szabo and  
401 Saha 2015). Interestingly, most of the immunosuppressive effects previously reported refer to  
402 host defense, which is coherent with the biological process term returned by functional  
403 analysis (Figure 1b; IFN $\gamma$ -mediated signaling pathways, neutrophil degranulation or phagocytic  
404 vesicle for example). Taken together, our results support the idea that, in the presence of  
405 NAFLD, *in vivo* exposure to toxicants might also favor infections, and doing so, potentially  
406 further aggravate liver diseases.

407         Concerning up-regulated genes, the functional analysis revealed a significant  
408 association with terms related to porphyrin/heme/iron metabolism, mitochondrial  
409 dysfunction, NAFLD/liver toxicity and AhR signaling (Figure 1 and supplementary Table S3).  
410 Some of these observations were in line with our recent work on the mechanisms involved in  
411 the *in vitro* impact of B[a]P/ethanol co-exposure on the progression of steatosis in the human  
412 hepatocarcinoma HepaRG cell line; indeed, we found a role for an AhR-dependent  
413 mitochondrial dysfunction (Bucher et al. 2018a). Based on this, we decided to further  
414 investigate these processes in our *in vivo* zebrafish model, with special emphasis on AhR  
415 signaling and mitochondrial iron/heme homeostasis.

416         Mitochondrial dysfunction is well-known to be involved in liver diseases, notably  
417 NAFLD (Grattagliano et al. 2019). From transcriptomic and RT-qPCR analysis, several  
418 alterations related to mitochondria were presently identified, with an up-regulation in the  
419 expression of several genes involved in respiratory complexe formation (*sdha*, *sdhaf3* in  
420 complex II, *upcc1* and *uqcc3* in complex III), and a down-regulation of *sdhc*, part of complex III  
421 (Figure 2a), thus suggesting disruption of mitochondrial respiration. Note that a similar change

422 in UQCC3 expression was also found in human HepaRG cells under these conditions (Figure  
423 2d). Disruption of mitochondrial respiration in B[a]P/ethanol co-exposed HFD zebrafish larvae  
424 was confirmed by the decrease in basal mitochondrial respiration (Figure 3a) prevented by  
425 AhR inhibition (Figure 4f), like in co-exposed steatotic HepaRG cells (Bucher et al. 2018a). Such  
426 functional alterations were related to changes in the ultrastructure of mitochondria (smaller  
427 and flattened, less cristae) (Figure 3b) that might be associated with more mitochondrial  
428 fission and ultimately with promotion of NAFLD, as previously reported (Li et al. 2019).  
429 Considering all these results, one might then hypothesize that the decrease in mitochondria  
430 respiration detected under our conditions could be associated with a decrease of the  
431 respiratory chain ability to produce ATP and/or a decrease of fatty acid oxidation; both  
432 potentially depending on and promoting oxidative stress in a vicious cycle (Begrache et al.  
433 2019; Li et al. 2019). With respect to respiratory chain activity and related ATP production (*i.e.*  
434 parameters related to oxidative phosphorylation [OXPHOS]), it is worth emphasizing that  
435 B[a]P and ethanol, used alone or in combination, have already been reported to decrease  
436 these parameters in steatotic human hepatocytes and other models (Begrache et al. 2019;  
437 Bucher et al. 2018b; Bucher et al. 2018a; Das and Bhutia 2018; Hardonnière et al. 2016; Tête  
438 et al. 2018). Such an alteration of OXPHOS is commonly associated with an increase of ROS  
439 production and progression of NAFLD toward NASH particularly upon exposure to xenobiotic  
440 (Wahlang et al. 2019). In line with this, a lipid peroxidation in liver was detected under our  
441 experimental conditions and was found to be involved in co-exposure toxicity (Figure 5d, e).

442 Concerning AhR regulation and mitochondrial functions, it's interesting to note that  
443 inhibition of AhR by the antagonist CH223191 had for some genes similar effect than co-  
444 exposure (BE) (Fig 4a: *gstp1*; Fig 4b: *tspo* and *uqcc3*; Fig 4c: *fech*, *blvra*, *fth1a*, *fthl30*; Fig 4d:  
445 *prdx1*). There is no clear explanation yet but such effects might rely on previously described

446 AhR mitochondrial pool and its consequences on mitochondrial function (Tappenden et al.  
447 2011). In this context, compensatory mechanisms might be hypothesized.

448 Another altered process revealed by transcriptomic analysis was heme metabolism.  
449 Interestingly, several studies have shown that heme accumulation can favor oxidative stress  
450 and NAFLD progression, and have suggested that elevation of heme catabolism (through  
451 increase in heme oxygenase 1 (HO1), for example) could be a therapeutic perspective of  
452 NAFLD (Severson et al. 2016). In the context of steatosis progression toward a steatohepatitis-  
453 like state induced by B[a]P/ethanol co-exposure, we have found for the first time that there is  
454 an accumulation of both heme and hemin while there is no change in the heme catabolic  
455 process, as visualized by the absence of increase in bilirubin level (Figure 5), despite a  
456 significant increase in *blvra* (biliverdin reductase) gene expression (Figure 2b). Previous studies  
457 have reported that the strong AhR ligand, TCDD, could alter heme homeostasis and promote  
458 NASH in mice (Fader et al. 2017). This observation, along with our present results, would thus  
459 put heme homeostasis as a potential central hub in response to cellular chemical stress during  
460 NAFLD. As the expression of several genes of heme biosynthesis was found to be elevated in  
461 zebrafish larvae (Figure 2b) or in HepaRG cells (Figure 2d), one could suggest that heme and  
462 hemin elevations would depend on a transcriptional regulation rather than a down-regulation  
463 of the catabolic pathway. In line with this, note that no change in *hmox1a* mRNA expression  
464 was detected under our conditions (Figure 2b); furthermore, the bilirubin level remained  
465 unchanged (Figure 5c). A common consequence of heme accumulation and particularly of  
466 hemin accumulation, is an increase of oxidative stress responsible for cell death (Kumar and  
467 Bandyopadhyay 2005). Thus, the presently observed biochemical effects would fit well with  
468 the increase in lipid peroxidation and its role in hepatotoxicity (Figure 5). However, several  
469 studies have also highlighted that heme and hemin are potential inducers of cellular

470 antioxidant systems and could then act to protect cells against oxidative stress (Donegan et  
471 al. 2019; Luan et al. 2017). In this context, the elucidation of the precise role of heme and  
472 hemin in our *in vivo* model of pathological progression of steatosis will require further  
473 experiments.

474         Even if the exact role of heme homeostasis remains to be determined in our model, it  
475 is well recognized that heme metabolism is closely linked to iron homeostasis, known to cause  
476 oxidative stress through Fenton reaction, and also implicated in NAFLD (Britton et al. 2016;  
477 Corradini and Pietrangelo 2012). As a mitochondrial dysfunction was presently detected, the  
478 content of labile iron contained in mitochondria was evaluated. Despite the fact that an  
479 increased deposition of iron has previously been detected in NAFLD (Corradini and Pietrangelo  
480 2012), to our knowledge, this is the first time that an increase (2-fold) in mitochondrial Fe<sup>2+</sup>  
481 pool is observed in the context of *in vivo* pathological progression of steatosis upon exposure  
482 to toxicants (Figure 6a and b). Besides an elevation of mitochondrial iron in liver, we cannot  
483 yet exclude that iron content could also be elevated in other cell compartments, notably in  
484 ferritin complex, or in blood (bound to transferrin or hemoglobin). This elevation of  
485 mitochondrial iron was actually in agreement with the up-regulation of numerous iron-related  
486 genes like ferritin (*fth1a*, *fthl30*) and mitoferrin 1 and 2 (*slc25a37*, *slc25a28*). Interestingly,  
487 most of the gene regulations regarding iron homeostasis observed under our experimental  
488 conditions were prevented *in vivo* by CH223191 or *in vitro* in human AhR-KO HepaRG cells  
489 (Figure 4c and 4e). Thus, this strongly suggested a role for AhR in the increase in iron content.  
490 This hypothesis was confirmed by the protective effect of the AhR inhibitor CH223191 against  
491 mitochondrial iron accumulation and lipid peroxidation (Figure 7). In order to estimate the  
492 role of this mitochondrial iron accumulation in our context, we used a recognized iron  
493 chelator, namely deferoxamine (Figure 6). Not only this chelator prevented the alterations in

494 mitochondrial iron pool (Figure 6c) and in mRNA expression detected for most of the genes  
495 linked to heme and iron homeostasis (Figure 6e), but it also prevented the induction of gene  
496 markers of cell death (*casp3a*), inflammation (*il6*), and oxidative stress (*prdx1*) (Figure 6f);  
497 finally, it also protected from liver injuries induced by B[a]P/ethanol co-exposure (Figure 6h).  
498 It should be noted that AhR inhibition prevented, in our *in vivo* model, the decrease in  
499 mitochondrial respiration and gene transcriptomic changes related to cell death and  
500 inflammation and liver cell damages (Figure 4). Several *in vitro* and *in vivo* studies favor a role  
501 for iron in the liver alterations induced by environmental contaminants such as TCDD and  
502 polychlorinated biphenyls (PCBs) (Fader et al. 2017; Smith et al. 1995). Especially concerning  
503 B[a]P toxicity, this PAH as well as TCDD have been reported *in vivo* to modulate expression of  
504 hepcidin, to disturb heme homeostasis and to induce liver inflammation (Fader et al. 2017;  
505 Fader and Zacharewski 2017; Wang et al. 2009). Nevertheless, a clear link between xenobiotic  
506 exposure, iron overload and liver toxicity has been, so far, only demonstrated *in vivo* for TCDD,  
507 but not for B[a]P (Fader and Zacharewski 2017). Nonetheless, B[a]P was shown *in vitro* to  
508 increase iron uptake in an hepatic cell line with consecutive increase of oxidative stress and  
509 cell death (Gorria et al. 2006b). Regarding co-exposure to B[a]P and ethanol, only one study,  
510 from our team, has previously demonstrated *in vitro* that this mixture induced an iron  
511 overload responsible for an exacerbation of oxidative stress, and hence of hepatocyte death  
512 (Collin et al. 2014). Considering the mechanisms of iron accumulation, several possibilities  
513 could be proposed and will need to be further investigated in future. First, transcriptional  
514 regulation induced by co-exposure might favor iron uptake and retention, and limit iron export  
515 from liver. This hypothesis is coherent with the induction of genes such as ferritins (*fth1a*,  
516 *fthl30*) and mitoferrin (*slc25a37*, *slc25a28*) in our co-exposed HFD-fed zebrafish larvae model,  
517 and with increase of iron uptake in F258 hepatic cells following B[a]P exposure (Gorria et al.

518 2006b). The second possibility refers to membrane remodeling, *i.e* change in membrane  
519 fluidity and/or modulation of lipid raft signaling. Indeed, such a membrane remodeling has  
520 been involved in B[a]P-induced iron elevation and apoptosis in rat hepatic epithelial F258 cells  
521 (Gorria et al. 2006a), and in labile iron increase, lysosomal membrane permeabilization,  
522 oxidative stress and cell death induced cooperatively by B[a]P and ethanol in hepatic WIF-B9  
523 cells (Collin et al. 2014). Regarding this latter point, it is worth noting that we previously  
524 demonstrated an implication of membrane remodeling in the pathological progression of  
525 steatosis in our model of HFD-fed zebrafish larvae upon B[a]P/ethanol co-exposure (Imran et  
526 al. 2018).

527         Altogether, our data strongly indicate that mitochondrial iron accumulation,  
528 dependent on AhR activation, would be largely responsible for the progression from steatosis  
529 to a steatohepatitis-like state following B[a]P/ethanol co-exposure. Iron accumulation,  
530 notably in mitochondria, is thus shown for the first time to our knowledge as a key event in  
531 toxicant-induced liver disease exacerbation in a context of NAFLD. Remarkably, these  
532 conclusions seem to be relevant of human health as iron deposition in human liver has been  
533 correlated with oxidative stress and progression toward steatohepatitis or fibrosis (Britton et  
534 al. 2016). However, the role for xenobiotic exposure, particularly in mixture, in human liver  
535 iron overload and NAFLD is still not elucidated and need to be explored. In this context, one  
536 interesting concept should be investigated deeper : the ferroptosis. Ferroptosis is a recently  
537 described specific type of non-apoptotic cell death dependant on iron and lipid peroxidation  
538 (Cao and Dixon 2016; Mou et al. 2019; Qi et al. 2020; Tsurusaki et al. 2019). Thus, ferroptosis  
539 is known to be suppressed by iron chelators as deferoxamine and with antioxidants (Mou et  
540 al. 2019; Tsurusaki et al. 2019) which is very coherent with results observed in our zebrafish  
541 model. In addition, this type of cell death has been also associated with NASH progression (Qi



542 et al. 2020; Tsurusaki et al. 2019). Under our experimental conditions, it would thus be  
543 interesting to test such an involvement of ferroptosis notably by testing the effects of a  
544 specific inhibitor, like ferrostatin-1 (Cao and Dixon 2016; Skouta et al. 2014).

545

## 546 **Conclusions**

547 On the whole, this study, using a model of HFD-fed steatotic zebrafish larva, has shed  
548 new light on the mechanisms involved in the *in vivo* transition of liver steatosis toward  
549 steatohepatitis-like state induced by B[a]P/ethanol co-exposure. Indeed, this co-exposure  
550 activates AhR, then leading to transcriptomic alterations of heme and iron homeostasis, and  
551 also of mitochondrial functions. The consequences of such alterations were notably an  
552 elevation of heme and hemin content in zebrafish larvae, probably dependent on an increase  
553 in heme synthesis, since no change in bilirubin could be observed. The role of heme and hemin  
554 are still speculative but they most likely play a role in NAFLD progression through oxidative  
555 stress. Looking at mitochondrial disruptions, the main changes impacted morphology (smaller  
556 and flattened mitochondria), mitochondrial respiration, and labile iron ( $\text{Fe}^{2+}$ ) content. We  
557 proposed that iron overload in mitochondria, possibly depending on AhR activation and likely  
558 acting *via* oxidative stress, would represent a key event in the steatosis progression induced  
559 by B[a]P/ethanol co-exposure of HFD zebrafish larvae as iron chelation has been found to be  
560 largely protective towards cell death and inflammation.

561

562

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580

581 Author contribution: Conception and design of the study (NP, MI, DLG, OS); Acquisition of data  
582 (MI, FC, BE, HLM, AD, MB, SB, AL); Analysis and interpretation of data (MI, NP, DLG, FC, LS,  
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585 Data Availability: All transcriptomic data are available in our supplemental files.

586 **Declarations**

587 Ethics approval: All animal experiments were in agreement with the European Union  
588 regulations concerning the use and protection of experimental animals (Directive  
589 2010/63/EU). All protocols were approved by local ethic committee CREEA (Comité Rennais  
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593

#### 594 **References**

595 Batiha GE-S, Beshbishy AM, Ikram M, Mulla ZS, El-Hack MEA, Taha AE, et al. The Pharmacological  
596 Activity, Biochemical Properties, and Pharmacokinetics of the Major Natural Polyphenolic Flavonoid:  
597 Quercetin. *Foods*. 2020 Mar 23;9(3):374.

598 Begriche K, Massart J, Fromenty B. Mitochondrial Dysfunction Induced by Xenobiotics: Involvement  
599 in Steatosis and Steatohepatitis. *Mitochondria Obes. Type 2 Diabetes* [Internet]. Elsevier; 2019 [cited  
600 2019 Aug 23]. p. 347–64. Available from:  
601 <https://linkinghub.elsevier.com/retrieve/pii/B9780128117521000158>

602 Begriche K, Massart J, Robin M-A, Bonnet F, Fromenty B. Mitochondrial adaptations and dysfunctions  
603 in nonalcoholic fatty liver disease. *Hepatol. Baltim. Md.* 2013 Oct;58(4):1497–507.

604 Britton LJ, Subramaniam VN, Crawford DH. Iron and non-alcoholic fatty liver disease. *World J.*  
605 *Gastroenterol.* 2016 Sep 28;22(36):8112–22.

606 Bucher S, Le Guillou D, Allard J, Pinon G, Begriche K, Tête A, et al. Possible Involvement of  
607 Mitochondrial Dysfunction and Oxidative Stress in a Cellular Model of NAFLD Progression Induced by  
608 Benzo[a]pyrene/Ethanol CoExposure. *Oxid. Med. Cell. Longev.* 2018a;2018:4396403.

609 Bucher S, Tête A, Podechard N, Liamin M, Le Guillou D, Chevanne M, et al. Co-exposure to  
610 benzo[a]pyrene and ethanol induces a pathological progression of liver steatosis in vitro and in vivo.  
611 *Sci. Rep.* 2018b Apr 13;8(1):5963.

612 Burton GW, Ingold KU. Vitamin E as an in Vitro and in Vivo Antioxidant. *Ann. N. Y. Acad. Sci.* 1989  
613 Dec;570(1 Vitamin E):7–22.

614 Cao JY, Dixon SJ. Mechanisms of ferroptosis. *Cell. Mol. Life Sci. CMLS.* 2016 Jun;73(11–12):2195–209.

615 Cella M, Colonna M. Aryl hydrocarbon receptor: Linking environment to immunity. *Semin. Immunol.*  
616 2015 Sep;27(5):310–4.

617 Chu J, Sadler KC. New school in liver development: lessons from zebrafish. *Hepatol. Baltim. Md.* 2009  
618 Nov;50(5):1656–63.

- 619 Collin A, Hardonnière K, Chevanne M, Vuillemin J, Podechard N, Burel A, et al. Cooperative  
620 interaction of benzo[a]pyrene and ethanol on plasma membrane remodeling is responsible for  
621 enhanced oxidative stress and cell death in primary rat hepatocytes. *Free Radic. Biol. Med.* 2014  
622 Jul;72:11–22.
- 623 Corradini E, Pietrangelo A. Iron and steatohepatitis. *J. Gastroenterol. Hepatol.* 2012 Mar;27 Suppl  
624 2:42–6.
- 625 Das DN, Bhutia SK. Inevitable dietary exposure of Benzo[a]pyrene: carcinogenic risk assessment an  
626 emerging issues and concerns. *Curr. Opin. Food Sci.* 2018 Dec;24:16–25.
- 627 Donegan RK, Moore CM, Hanna DA, Reddi AR. Handling heme: The mechanisms underlying the  
628 movement of heme within and between cells. *Free Radic. Biol. Med.* 2019 Mar;133:88–100.
- 629 Driessen M, Kienhuis AS, Pennings JLA, Pronk TE, van de Brandhof E-J, Roodbergen M, et al. Exploring  
630 the zebrafish embryo as an alternative model for the evaluation of liver toxicity by histopathology  
631 and expression profiling. *Arch. Toxicol.* 2013 May;87(5):807–23.
- 632 Driessen M, Kienhuis AS, Vitins AP, Pennings JLA, Pronk TE, van den Brandhof E-J, et al. Gene  
633 expression markers in the zebrafish embryo reflect a hepatotoxic response in animal models and  
634 humans. *Toxicol. Lett.* 2014 Oct 1;230(1):48–56.
- 635 Fader KA, Nault R, Kirby MP, Markous G, Matthews J, Zacharewski TR. Convergence of hepcidin  
636 deficiency, systemic iron overloading, heme accumulation, and REV-ERB $\alpha/\beta$  activation in aryl  
637 hydrocarbon receptor-elicited hepatotoxicity. *Toxicol. Appl. Pharmacol.* 2017 Apr;321:1–17.
- 638 Fader KA, Zacharewski TR. Beyond the Aryl Hydrocarbon Receptor: Pathway Interactions in the  
639 Hepatotoxicity of 2,3,7,8-Tetrachlorodibenzo-p-dioxin and Related Compounds. *Curr. Opin. Toxicol.*  
640 2017 Feb;2:36–41.
- 641 Fazel Y, Koenig AB, Sayiner M, Goodman ZD, Younossi ZM. Epidemiology and natural history of non-  
642 alcoholic fatty liver disease. *Metabolism.* 2016 Aug;65(8):1017–25.
- 643 Goessling W, Sadler KC. Zebrafish: An Important Tool for Liver Disease Research. *Gastroenterology.*  
644 2015 Nov;149(6):1361–77.
- 645 Goldstone JV, McArthur AG, Kubota A, Zanette J, Parente T, Jönsson ME, et al. Identification and  
646 developmental expression of the full complement of Cytochrome P450 genes in Zebrafish. *BMC*  
647 *Genomics.* 2010;11:643.
- 648 Goodale BC, La Du JK, Bisson WH, Janszen DB, Waters KM, Tanguay RL. AHR2 mutant reveals  
649 functional diversity of aryl hydrocarbon receptors in zebrafish. *PLoS One.* 2012;7(1):e29346.
- 650 Gorria M, Huc L, Sergent O, Rebillard A, Gaboriau F, Dimanche-Boitrel M-T, et al. Protective effect of  
651 monosialoganglioside GM1 against chemically induced apoptosis through targeting of mitochondrial  
652 function and iron transport. *Biochem. Pharmacol.* 2006a Nov;72(10):1343–53.
- 653 Gorria M, Tekpli X, Sergent O, Huc L, Gaboriau F, Rissel M, et al. Membrane fluidity changes are  
654 associated with benzo[a]pyrene-induced apoptosis in F258 cells: protection by exogenous  
655 cholesterol. *Ann. N. Y. Acad. Sci.* 2006b Dec;1090:108–12.

656 Grattagliano I, Montezinho LP, Oliveira PJ, Frühbeck G, Gómez-Ambrosi J, Montecucco F, et al.  
657 Targeting mitochondria to oppose the progression of nonalcoholic fatty liver disease. *Biochem.*  
658 *Pharmacol.* 2019 Feb;160:34–45.

659 Hardonnière K, Fernier M, Gallais I, Mograbi B, Podechard N, Le Ferrec E, et al. Role for the ATPase  
660 inhibitory factor 1 in the environmental carcinogen-induced Warburg phenotype. *Sci. Rep.* 2017  
661 15;7(1):195.

662 Hardonnière K, Saunier E, Lemarié A, Fernier M, Gallais I, Héliès-Toussaint C, et al. The environmental  
663 carcinogen benzo[a]pyrene induces a Warburg-like metabolic reprogramming dependent on NHE1  
664 and associated with cell survival. *Sci. Rep.* 2016 04;6:30776.

665 Hwang HJ, Dornbos P, Steidemann M, Dunivin TK, Rizzo M, LaPres JJ. Mitochondrial-targeted aryl  
666 hydrocarbon receptor and the impact of 2,3,7,8-tetrachlorodibenzo-p-dioxin on cellular respiration  
667 and the mitochondrial proteome. *Toxicol. Appl. Pharmacol.* 2016 01;304:121–32.

668 Imran M, Sergent O, Tête A, Gallais I, Chevanne M, Lagadic-Gossman D, et al. Membrane  
669 Remodeling as a Key Player of the Hepatotoxicity Induced by Co-Exposure to Benzo[a]pyrene and  
670 Ethanol of Obese Zebrafish Larvae. *Biomolecules.* 2018 14;8(2).

671 International Agency for Research on Cancer (IARC). Chemical Agents and Related Occupations.  
672 [Internet]. Place of publication not identified: publisher not identified; 2012 [cited 2019 Sep 3].  
673 Available from:  
674 [http://VH7QX3XE2P.search.serialssolutions.com/?V=1.0&L=VH7QX3XE2P&S=AC\\_T\\_B&C=Chemical%](http://VH7QX3XE2P.search.serialssolutions.com/?V=1.0&L=VH7QX3XE2P&S=AC_T_B&C=Chemical%20Agents%20and%20Related%20Occupations&T=marc&tab=BOOKS)  
675 [20Agents%20and%20Related%20Occupations&T=marc&tab=BOOKS](http://VH7QX3XE2P.search.serialssolutions.com/?V=1.0&L=VH7QX3XE2P&S=AC_T_B&C=Chemical%20Agents%20and%20Related%20Occupations&T=marc&tab=BOOKS)

676 Ishihara N, Mihara K. PARL paves the way to apoptosis. *Nat. Cell Biol.* 2017 31;19(4):263–5.

677 Joshi-Barve S, Kirpich I, Cave MC, Marsano LS, McClain CJ. Alcoholic, Nonalcoholic, and Toxicant-  
678 Associated Steatohepatitis: Mechanistic Similarities and Differences. *Cell. Mol. Gastroenterol.*  
679 *Hepatol.* 2015 Jul;1(4):356–67.

680 Kumar S, Bandyopadhyay U. Free heme toxicity and its detoxification systems in human. *Toxicol. Lett.*  
681 2005 Jul 4;157(3):175–88.

682 Li Z, Li Y, Zhang H-X, Guo J-R, Lam CWK, Wang C-Y, et al. Mitochondria-Mediated Pathogenesis and  
683 Therapeutics for Non-Alcoholic Fatty Liver Disease. *Mol. Nutr. Food Res.* 2019 Jun 14;e1900043.

684 Liamin M, Le Mentec H, Evrard B, Huc L, Chalmel F, Boutet-Robinet E, et al. Genome-Wide  
685 Transcriptional and Functional Analysis of Human T Lymphocytes Treated with Benzo[α]pyrene. *Int. J.*  
686 *Mol. Sci.* 2018 Nov 17;19(11).

687 Luan Y, Zhang F, Cheng Y, Liu J, Huang R, Yan M, et al. Hemin Improves Insulin Sensitivity and Lipid  
688 Metabolism in Cultured Hepatocytes and Mice Fed a High-Fat Diet. *Nutrients.* 2017 Jul 26;9(8):805.

689 Lv H, Shang P. The significance, trafficking and determination of labile iron in cytosol, mitochondria  
690 and lysosomes. *Met. Integr. Biometal Sci.* 2018 18;10(7):899–916.

691 Mou Y, Wang J, Wu J, He D, Zhang C, Duan C, et al. Ferroptosis, a new form of cell death:  
692 opportunities and challenges in cancer. *J. Hematol. Oncol. J Hematol Oncol.* 2019 Mar 29;12(1):34.

- 693 Qi J, Kim J-W, Zhou Z, Lim C-W, Kim B. Ferroptosis Affects the Progression of Nonalcoholic  
694 Steatohepatitis via the Modulation of Lipid Peroxidation-Mediated Cell Death in Mice. *Am. J. Pathol.*  
695 2020 Jan;190(1):68–81.
- 696 Raftery TD, Jayasundara N, Di Giulio RT. A bioenergetics assay for studying the effects of  
697 environmental stressors on mitochondrial function in vivo in zebrafish larvae. *Comp. Biochem.*  
698 *Physiol. Part C Toxicol. Pharmacol.* 2017 Feb;192:23–32.
- 699 Schlegel A. Studying non-alcoholic fatty liver disease with zebrafish: a confluence of optics, genetics,  
700 and physiology. *Cell. Mol. Life Sci. CMLS.* 2012 Jun 8;
- 701 Severson TJ, Besur S, Bonkovsky HL. Genetic factors that affect nonalcoholic fatty liver disease: A  
702 systematic clinical review. *World J. Gastroenterol.* 2016;22(29):6742.
- 703 Skouta R, Dixon SJ, Wang J, Dunn DE, Orman M, Shimada K, et al. Ferrostatins inhibit oxidative lipid  
704 damage and cell death in diverse disease models. *J. Am. Chem. Soc.* 2014 Mar 26;136(12):4551–6.
- 705 Smith AG, Carthew P, Clothier B, Constantin D, Francis JE, Madra S. Synergy of iron in the toxicity and  
706 carcinogenicity of polychlorinated biphenyls (PCBs) and related chemicals. *Toxicol. Lett.* 1995  
707 Dec;82–83:945–50.
- 708 Szabo G, Saha B. Alcohol’s Effect on Host Defense. *Alcohol Res. Curr. Rev.* 2015;37(2):159–70.
- 709 Szigeti A, Bellyei S, Gasz B, Boronkai A, Hocsak E, Minik O, et al. Induction of necrotic cell death and  
710 mitochondrial permeabilization by heme binding protein 2/SOUL. *FEBS Lett.* 2006 Nov  
711 27;580(27):6447–54.
- 712 Tappenden DM, Lynn SG, Crawford RB, Lee K, Vengellur A, Kaminski NE, et al. The aryl hydrocarbon  
713 receptor interacts with ATP5 $\alpha$ 1, a subunit of the ATP synthase complex, and modulates  
714 mitochondrial function. *Toxicol. Appl. Pharmacol.* 2011 Aug 1;254(3):299–310.
- 715 Tekpli X, Rissel M, Huc L, Catheline D, Sergent O, Rioux V, et al. Membrane remodeling, an early  
716 event in benzo[a]pyrene-induced apoptosis. *Toxicol. Appl. Pharmacol.* 2010 Feb 15;243(1):68–76.
- 717 Tête A, Gallais I, Imran M, Chevanne M, Liamin M, Sparfel L, et al. Mechanisms involved in the death  
718 of steatotic WIF-B9 hepatocytes co-exposed to benzo[a]pyrene and ethanol: a possible key role for  
719 xenobiotic metabolism and nitric oxide. *Free Radic. Biol. Med.* 2018;129:323–37.
- 720 Tsurusaki S, Tsuchiya Y, Koumura T, Nakasone M, Sakamoto T, Matsuoka M, et al. Hepatic ferroptosis  
721 plays an important role as the trigger for initiating inflammation in nonalcoholic steatohepatitis. *Cell*  
722 *Death Dis.* 2019 Jun 18;10(6):449.
- 723 Uno S, Nebert DW, Makishima M. Cytochrome P450 1A1 (CYP1A1) protects against nonalcoholic fatty  
724 liver disease caused by Western diet containing benzo[a]pyrene in mice. *Food Chem. Toxicol. Int. J.*  
725 *Publ. Br. Ind. Biol. Res. Assoc.* 2018 Mar;113:73–82.
- 726 Wahlang B, Beier JI, Clair HB, Bellis-Jones HJ, Falkner KC, McClain CJ, et al. Toxicant-associated  
727 steatohepatitis. *Toxicol. Pathol.* 2013 Feb;41(2):343–60.
- 728 Wahlang B, Jin J, Beier JI, Hardesty JE, Daly EF, Schnegelberger RD, et al. Mechanisms of  
729 Environmental Contributions to Fatty Liver Disease. *Curr. Environ. Health Rep.* 2019 Sep;6(3):80–94.

730 Wang E, Liu X, Tu W, Do DC, Yu H, Yang L, et al. Benzo(a)pyrene facilitates dermatophagoides group 1  
731 (Der f 1)-induced epithelial cytokine release through aryl hydrocarbon receptor in asthma. *Allergy*.  
732 2019 Sep;74(9):1675–90.

733 Wang K-J, Bo J, Yang M, Hong H-S, Wang X-H, Chen F-Y, et al. Hepcidin gene expression induced in  
734 the developmental stages of fish upon exposure to Benzo[a]pyrene (BaP). *Mar. Environ. Res.* 2009  
735 Apr;67(3):159–65.

736 Younossi ZM. Non-alcoholic fatty liver disease - A global public health perspective. *J. Hepatol.* 2019  
737 Mar;70(3):531–44.

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739

740 **Supplementary material captions:**

- 741 - **Supplementary file S1:** Details for all Materials and methods.
- 742 - **Supplementary Figure S1:** Illustrations of histological sections of zebrafish larvae to
- 743 evaluate liver damages upon B[a]P/ethanol co-exposure of steatotic zebrafish larva
- 744 with or without co-treatment with protective agents.
- 745 - **Supplementary Figure S2:** Illustrations of fluorescent staining of zebrafish larvae to
- 746 evaluate liver mitochondrial iron accumulation and lipid peroxidation upon
- 747 B[a]P/ethanol co-exposure of steatotic zebrafish larva with or without co-treatment
- 748 with AhR antagonist, CH223191.
- 749 - **Supplementary Figure S3:** Effect of B[a]P/ethanol co-exposure on oxygen
- 750 consumption rate in zebrafish larva and impact of the AhR antagonist, CH223191 (OCR
- 751 curves).
- 752 - **Supplementary Figure S4:** Illustrations of fluorescent staining of zebrafish larvae to
- 753 evaluate lipid peroxidation upon B[a]P/ethanol co-exposure of steatotic zebrafish
- 754 larvae with or without co-treatment with an antioxidant, quercetin or vitamin E.
- 755 - **Supplementary Table S1:** Excel file containing fold change data of the 525 DEGs.
- 756 - **Supplementary Table S2:** Excel file containing the table of conversion of the 525
- 757 zebrafish DEGs into 259 human homologs with associated retained fold change.
- 758 - **Supplementary Table S3:** Excel file containing the table of several terms resulted from
- 759 the Ingenuity Pathways Analysis (IPA) of the 259 human homologs of DEGs.
- 760 - **Supplementary Table S4:** Excel file containing tables of full results from the Ingenuity
- 761 Pathways Analysis (IPA) of the 259 human homologs of DEGs.
- 762 - **Supplementary Table S5:** Excel file containing tables of sequences of the tested
- 763 primers used in RT-qPCR.
- 764 - **Supplementary Table S6:** Excel file containing all compiled data and sample size
- 765 information.
- 766



767 **Figure legends:**

768 **Figure 1 Transcriptomic analyses reveal major-disrupted targets such as heme homeostasis**  
769 **and mitochondria in B[a]P/ethanol co-exposed zebrafish larvae.** Transcriptomic analyses  
770 using GeneChip™ Zebrafish gene 1.0 ST array were performed on mRNA samples from 12 dpf  
771 HFD-fed zebrafish larvae exposed to 25 nM B[a]P and/or 43 mM ethanol during 7 days (n=5).  
772 (a) A flow chart outlines transcriptomic approach and statistical analysis for gene selection  
773 and clustering. (b) A heat-map summarizes changes of expression of the 525 B[a]P/ethanol  
774 (BE)-modulated transcripts and their clustering depending on each condition. (c) Table  
775 indicate main results of gene ontology enrichment analysis using AMEN tool on GO and KEGG  
776 annotations. For each set of genes (up- and down-regulated ones in intense red and blue color,  
777 respectively; patterns [P1 to P8] in light red or blue color), significant enriched terms are given  
778 with an enrichment ratio indicating the number of annotated genes recovered out of the  
779 number of genes expected for this annotation

780

781 **Figure 2 Validation of zebrafish transcriptomic screening and relevance in human HepaRG**  
782 **cells.** (a, b, c) Zebrafish larvae were fed with HFD from 4 dpf and then treated with control  
783 vehicle (C) or exposed to 25nM B[a]P and 43 mM ethanol (BE) for seven days (from 5 to 12  
784 dpf). mRNA samples were collected from pools of 10 to 20 larvae, and mRNA expression was  
785 evaluated by quantitative reverse transcription polymerase chain reaction (RT-qPCR) for  
786 different groups of function-related genes, *i.e.* mitochondria-related genes (a) (n≥10), heme  
787 & iron-related genes (b) (n≥5), oxidative stress & AhR signaling-related genes (c) (n≥7). (d)  
788 HepaRG cells were supplemented with fatty acids for 2 days (150 μM stearic acid and 150 μM  
789 oleic acid) and co-treated (BE) or not (C) with B[a]P (2.5 μM) and ethanol (25mM) for 2 weeks  
790 (see reference (Bucher et al. 2018b) for further details) (n≥3). mRNA expression was evaluated  
791 by RT-qPCR for different groups of function-related genes. Data are expressed relative to  
792 mRNA levels found in control condition (C), set at 0 (log 2 change). Values are the mean ± SEM.  
793 \*, \*\*, \*\*\* statistically different from HFD control with respectively p<0.05; p<0.01 and  
794 p<0.001. Trends of change in gene expression found in zebrafish either in microarray (a, b, c)  
795 or by RT-qPCR (d) are indicated by white arrows when consistent with microarray or RT-qPCR  
796 observations or in grey arrows when not (by an up arrow for up-regulated genes and down  
797 arrow for down regulated genes)

798

799 **Figure 3 Evaluation of mitochondrial alterations induced by B[a]P/ethanol co-exposure in**  
800 **liver of steatotic zebrafish larva.** Zebrafish larvae were fed with HFD from 4 dpf and then  
801 treated at 5 dpf with control vehicle (C) or exposed to B[a]P and ethanol (BE) (1 μM B[a]P +  
802 173 mM ethanol for 1 day in (a), or 25 nM B[a]P + 43 mM ethanol for 7 days in (b)). (a)  
803 Measurement on Seahorse XFe24 Analyzer of mitochondrial oxygen consumption in zebrafish  
804 larva. Values are the mean of oxygen consumption rate (OCR, in pmol of O<sub>2</sub>/min) ± SEM  
805 measured from at least 7 larvae per condition. \*, \*\*, \*\*\* statistically different from HFD  
806 control with respectively p<0.05; p<0.01 and p<0.001. (b) Zebrafish liver section imaging by  
807 transmission electronic microscopy. Left panels present a large view of liver section  
808 (magnification 5000x) whereas the middle ones show images of hepatocyte (magnification  
809 8000x), with higher magnification in right panels to better evaluate mitochondria  
810 morphologies (digital magnification of previous images 3x). Images are representative of 3  
811 larvae per condition (BC, biliary canaliculi; N, nucleus; M, mitochondria; in right panel,  
812 examples of mitochondrial cristae are indicated by a red arrow head)

813

814 **Figure 4 Involvement of AhR activation in mitochondrial dysfunction and liver toxicity**  
815 **induced by B[a]P/ethanol co-exposure.** Zebrafish larvae were fed with HFD from 4 dpf and  
816 then treated at 5 dpf with control vehicle (C) or exposed to B[a]P and ethanol (BE) (25 nM  
817 B[a]P + 43 mM ethanol for 7 days in (a-d, g) or 1  $\mu$ M B[a]P + 173 mM ethanol for 1 day in (f).  
818 For these experiments, some larvae were also co-treated with 1  $\mu$ M CH223191 (CH), a specific  
819 AhR antagonist. In (e), HepaRG cells wild-type (WT) or knock-out for AhR (KO) (see reference  
820 (Bucher et al. 2018a) for details) were supplemented with fatty acids during 2 days (150  $\mu$ M  
821 stearic acid and 150  $\mu$ M oleic acid) and co-treated (BE) or not (C) with B[a]P (2.5  $\mu$ M) and  
822 ethanol (25 mM) for 2 weeks. mRNA expression was evaluated by quantitative reverse  
823 transcription polymerase chain reaction (RT-qPCR) for different groups of function-related  
824 genes in zebrafish, i.e. AhR signaling-related genes (a) (n $\geq$ 4), mitochondria-related genes (b)  
825 (n $\geq$ 4), heme & iron-related genes (c) (n $\geq$ 4), oxidative stress, cell-death and inflammation genes  
826 (d) (n $\geq$ 4) and in HepaRG cells (e) (n $\geq$ 3). Data are expressed relative to mRNA levels found in  
827 HFD untreated control larvae (C) (a to d) or in HepaRG WT control cells (e), set at 0 (log 2  
828 change). RT-qPCR values are the mean  $\pm$  SEM. (f) Measurement on Seahorse XFe24 Analyzer  
829 of mitochondrial oxygen consumption in zebrafish larva. Values are the mean of oxygen  
830 consumption rate (OCR, in pmol of O<sub>2</sub>/min)  $\pm$  SEM measured from at least 5 larvae per  
831 condition. (g) Counting of liver damaged cells was performed on histological sections of  
832 zebrafish stained by HES from at least 3 larvae per condition. Values are the mean  $\pm$  SEM. \*  
833 indicates a statistically significant effect of BE co-exposure vs control counterpart in presence  
834 or not of CH (BE vs C and CH+BE vs CH) with p<0.05 (a to g); § indicates a statistically significant  
835 effect of CH223191 inhibitor vs counterpart without CH (CH vs C and CH+BE vs BE) with p<0.05  
836 (a-d, f, g); # indicates a statistically significant interaction between co-exposure and inhibitor  
837 (a-d, f, g) or between co-exposure and AhR knock-out (e) with p<0.05

838

839 **Figure 5 Disruption of heme metabolism and involvement of oxidative stress in liver injury**  
840 **induced by B[a]P/ethanol co-exposure.** Zebrafish larvae were fed with HFD from 4 dpf and  
841 then treated with control vehicle (C) or exposed to 25 nM B[a]P and 43 mM ethanol (BE) for 7  
842 days ( from 5 to 12 dpf). Some larvae were also co-treated with 25  $\mu$ M quercetin (Que) or 100  
843  $\mu$ M vitamin E (Vit E). Levels of heme (a) (n $\geq$ 18), hemin (b) (n $\geq$ 7) and bilirubin (c) (n $\geq$ 11) were  
844 evaluated from homogenates of pools of whole larva. (d) Lipid peroxidation, marker of  
845 oxidative stress, was assessed in zebrafish larvae by quantification of fluorescence intensities  
846 in liver following staining of larvae with C11-Bodipy<sup>581/591</sup> (BC11) (n $\geq$ 6). (e) Counting of liver  
847 damaged cells was performed on histological sections of zebrafish stained by HES from at least  
848 3 larvae per condition. Values are the mean  $\pm$  SEM. \*, \*\*, \*\*\* indicate a statistically significant  
849 effect of BE co-exposure vs control counterpart in presence or not of antioxidant (BE vs C;  
850 Que+BE vs Que; Vit E+BE vs Vit E) with p<0.05, p<0.01 and p<0.001 respectively; § indicates a  
851 statistically significant effect of quercetin or vitamin E vs counterpart without antioxidant (Que  
852 vs C; Que+BE vs BE; Vit E vs C; Vit E+BE vs BE) with p<0.05 (d, e); # indicates a statistically  
853 significant interaction between co-exposure and antioxidant (d, e) with p<0.05

854

855 **Figure 6 Involvement of iron accumulation in liver mitochondrial dysfunction and toxicity**  
856 **induced by B[a]P/ethanol co-exposure of steatotic zebrafish larva.** Zebrafish larvae were fed  
857 with HFD from 4 dpf and then treated with control vehicle (C) or exposed to 25 nM B[a]P and  
858 43 mM ethanol (BE) for 7 days ( from 5 to 12 dpf). Some larvae were also co-treated with an  
859 iron chelator, 100  $\mu$ M deferoxamine (Def). (a) Imaging of free iron (Fe<sup>2+</sup>) in the liver of

860 zebrafish larvae was done by confocal microscopy after staining with Mito-FerroGreen probe.  
861 Merge of transmitted light and green fluorescent imaging are given in upper panels whereas  
862 bottom panels present enlargement of liver only in green fluorescent channel (magnification  
863 x200; dotted line outline liver). For each picture presented in bottom panels, the mean  
864 fluorescent intensity (MFI) determined is indicated. **(b)** Relative mitochondrial iron content  
865 was assessed by quantification of green fluorescent intensities, found in liver of zebrafish  
866 using previous confocal images ( $n \geq 9$ ). **(c)** Relative mitochondrial iron content was assessed by  
867 quantification of Mito-FerroGreen fluorescence intensities, detected in the liver of zebrafish  
868 using confocal images in the presence or not of Def ( $n \geq 9$ ). mRNA expression was evaluated by  
869 quantitative reverse transcription polymerase chain reaction (RT-qPCR) for different groups  
870 of function-related genes in zebrafish, *i.e.* mitochondria-related genes **(d)** ( $n \geq 3$ ), heme & iron-  
871 related genes **(e)** ( $n \geq 3$ ), oxidative stress, cell-death and inflammation genes **(f)** ( $n \geq 3$ ), and AhR  
872 signaling-related genes **(g)** ( $n \geq 4$ ) in the presence or not of Def. **(h)** Liver damaged cells counting  
873 was performed on histological sections of zebrafish stained by HES from at least 4 larvae per  
874 condition in the presence or not of Def. Values are the mean  $\pm$  SEM. \*, \*\*, \*\*\* indicate a  
875 statistically significant effect of BE co-exposure vs control counterpart in presence or not of  
876 Def (BE vs C and Def+BE vs Def) with  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  respectively; § indicates a  
877 statistically significant effect of deferoxamine vs counterpart without Def (Def vs C and Def+BE  
878 vs BE) with  $p < 0.05$  **(b to h)**; # indicates a statistically significant interaction between co-  
879 exposure and deferoxamine treatment **(b to h)** with  $p < 0.05$

880

881 **Figure 7: Involvement of AhR in liver mitochondrial iron accumulation and lipid peroxidation**  
882 **induced by B[a]P/ethanol co-exposure of steatotic zebrafish larva.** Zebrafish larvae were fed  
883 with HFD from 4 dpf and then treated with control vehicle (C) or exposed to 25 nM B[a]P and  
884 43 mM ethanol (BE) for 7 days ( from 5 to 12 dpf). Some larvae were also co-treated with a  
885 protective agent: 1  $\mu$ M CH223191 (CH). **(a)** The relative mitochondrial iron content was  
886 assessed by quantification of green fluorescence intensities in the liver using confocal images.  
887 **(b)** Lipid peroxidation, a marker of oxidative stress, was assessed in zebrafish larvae by  
888 quantification of fluorescence intensities in the liver following staining with C11-Bodipy<sup>581/591</sup>  
889 (BC11). Values are the mean  $\pm$  SEM ( $n \geq 7$ ). \*, \*\*, \*\*\* indicate a statistically significant effect of  
890 BE co-exposure vs control counterpart in presence or not of CH (BE vs C and CH+BE vs CH) with  
891  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  respectively; § indicates a statistically significant effect of CH vs  
892 counterpart without CH (CH vs C; CH+BE vs BE) with  $p < 0.05$ ; # indicates a statistically  
893 significant interaction between co-exposure and CH treatment with  $p < 0.05$ .

894

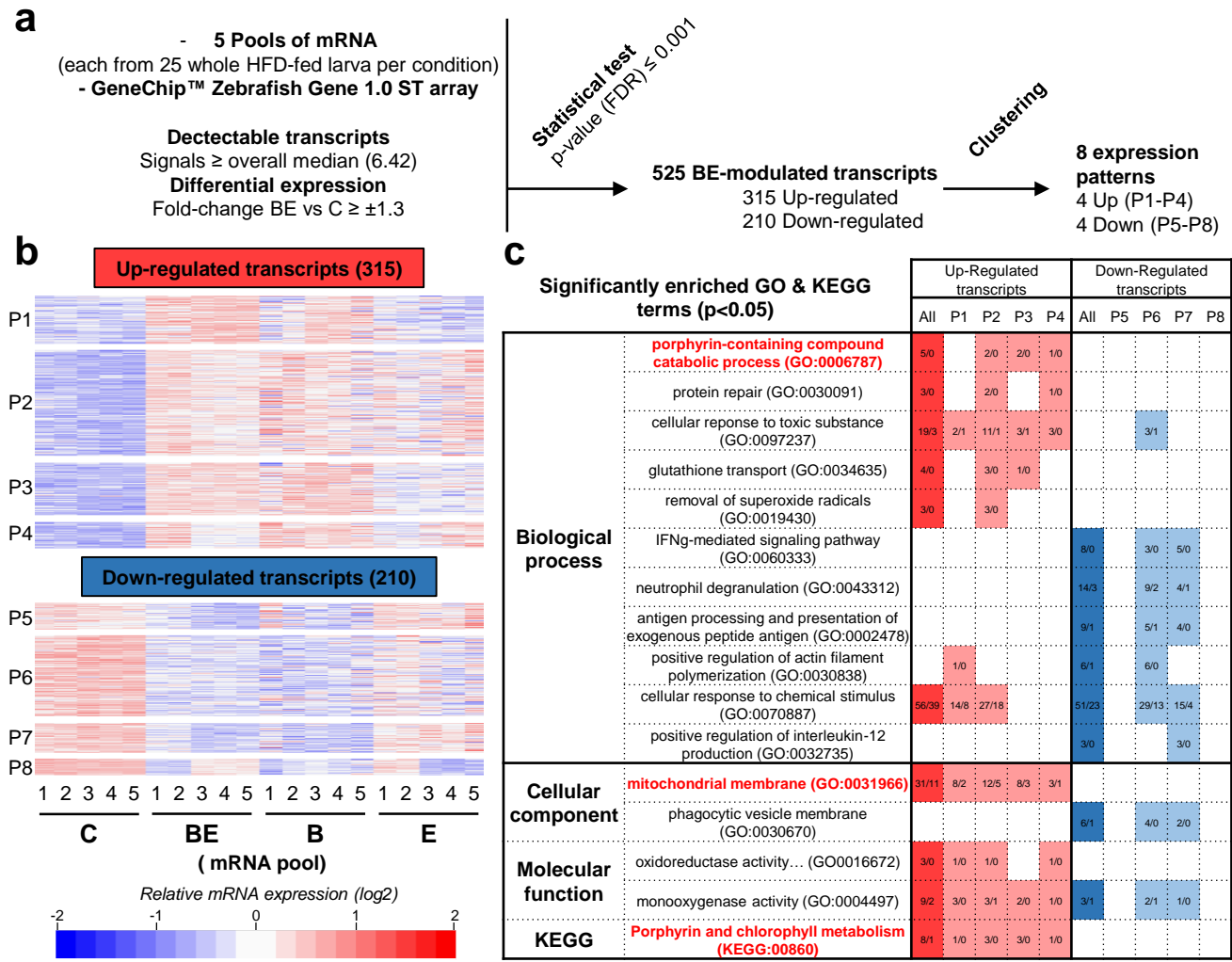


Figure 1

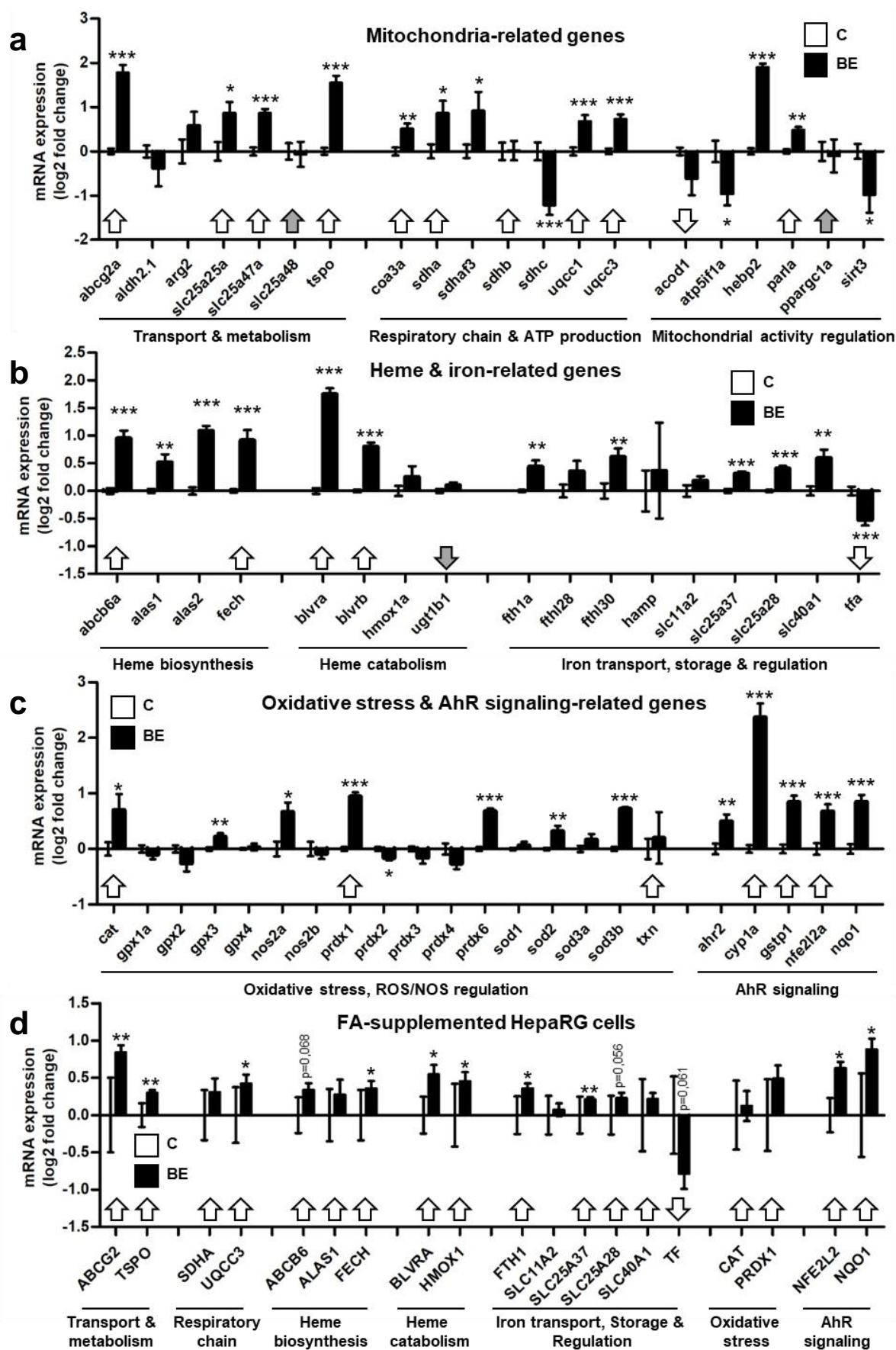


Figure 2

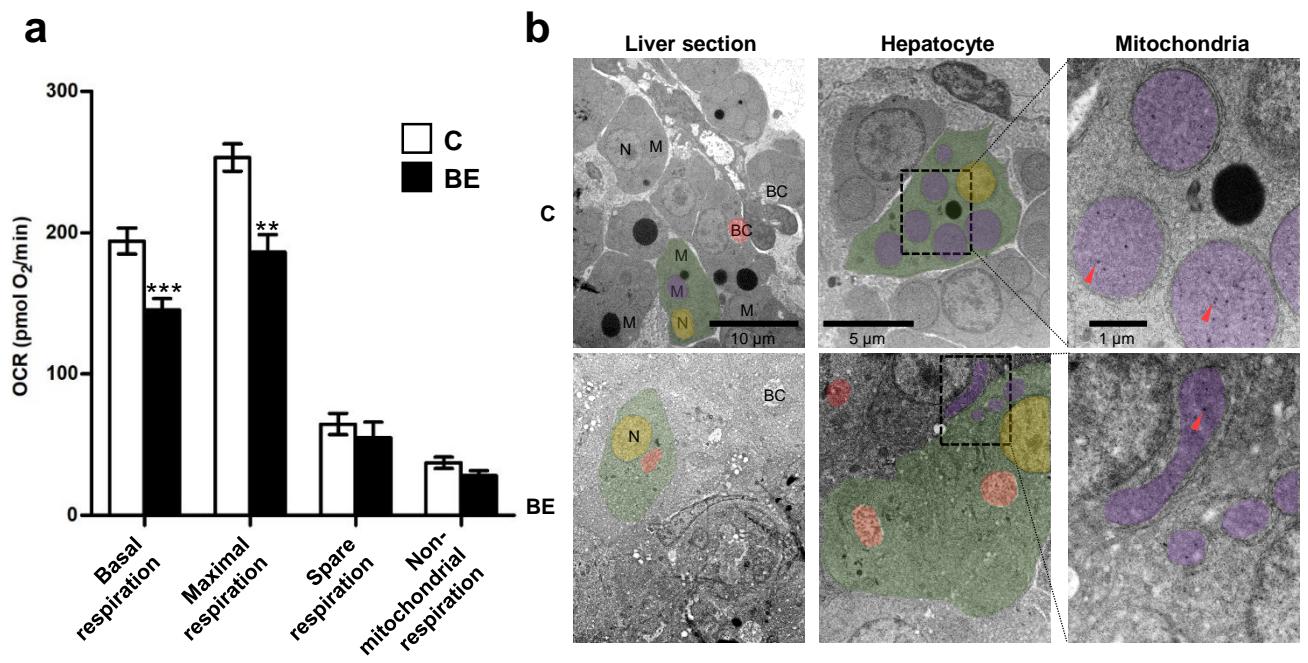


Figure 3



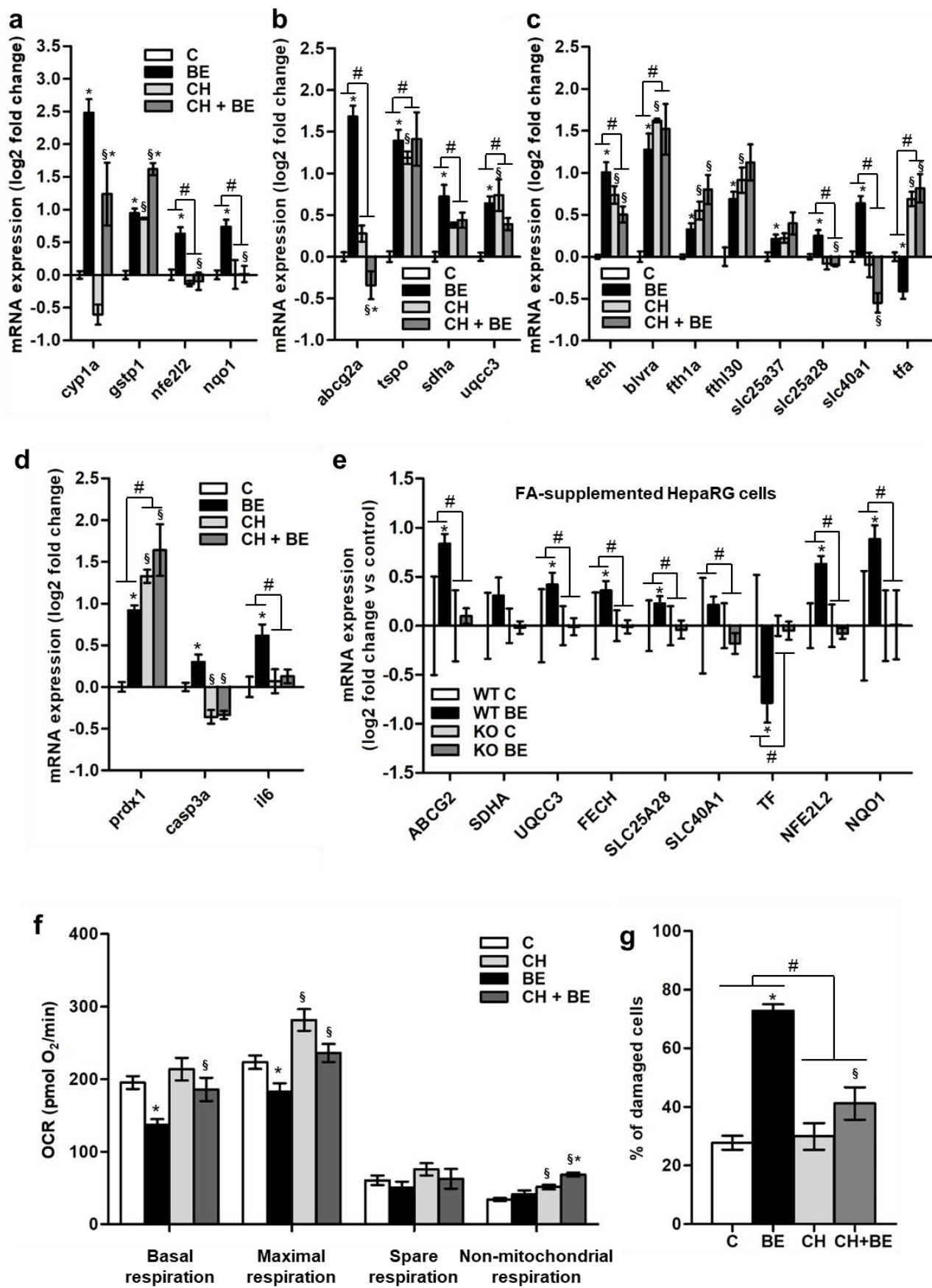


Figure 4

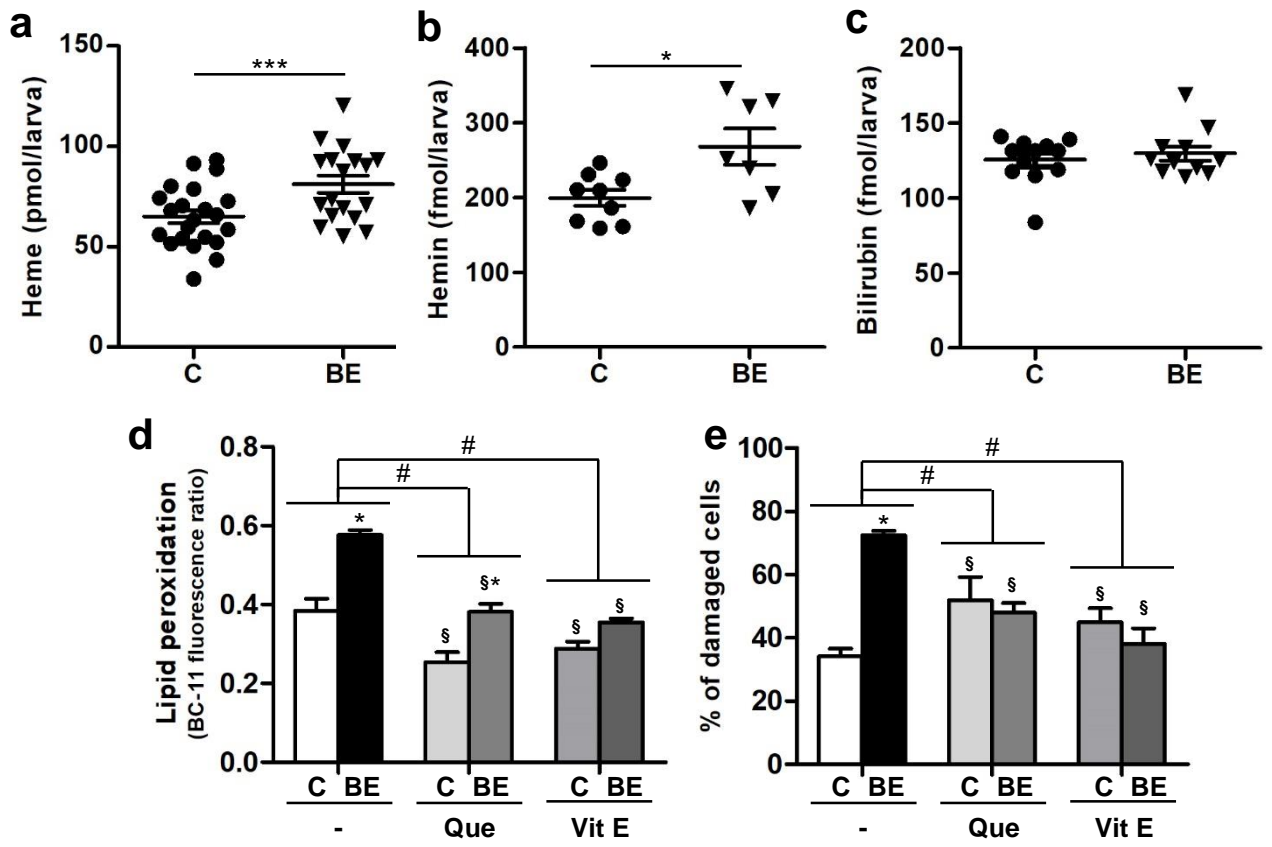


Figure 5



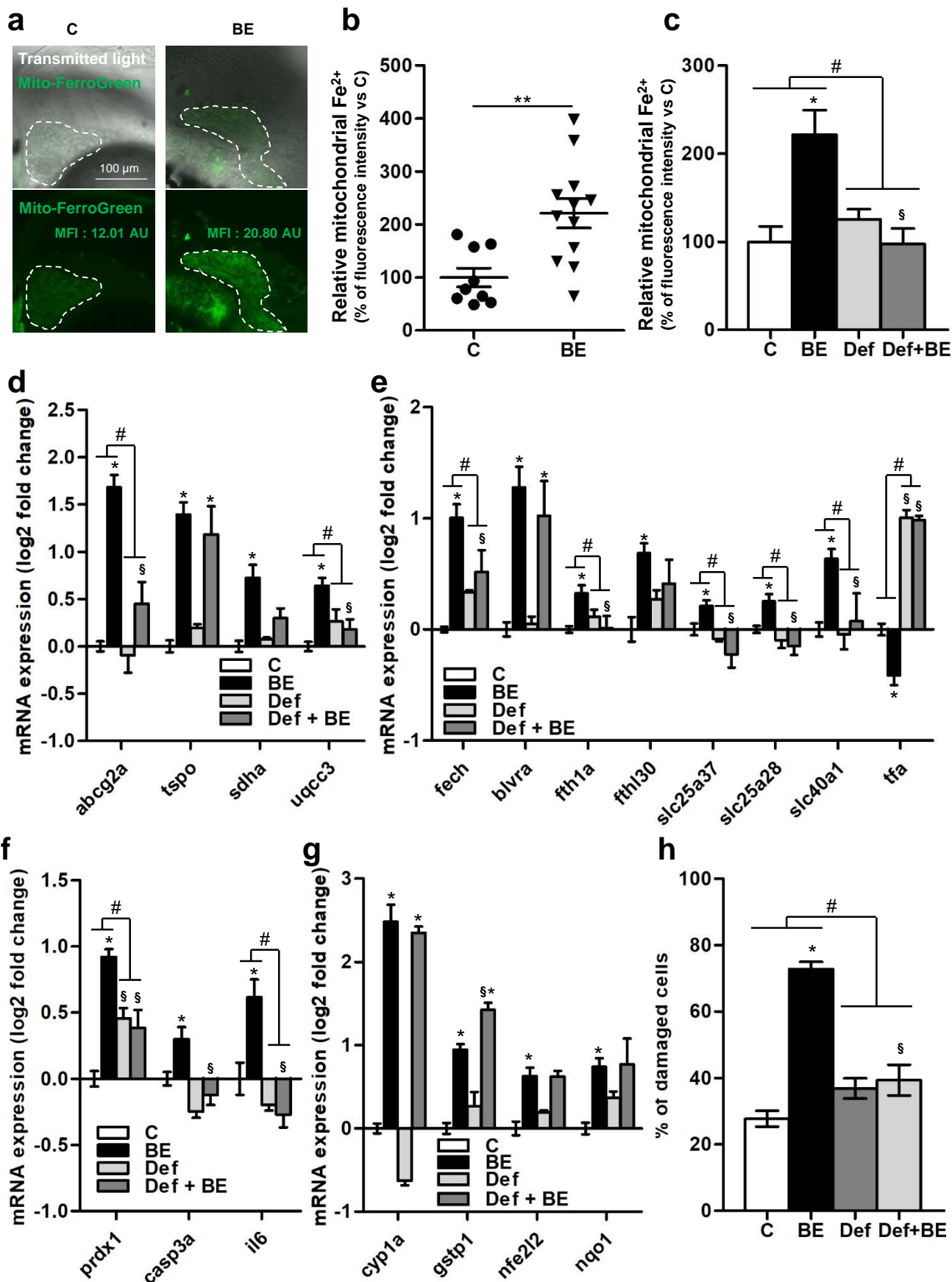


Figure 6

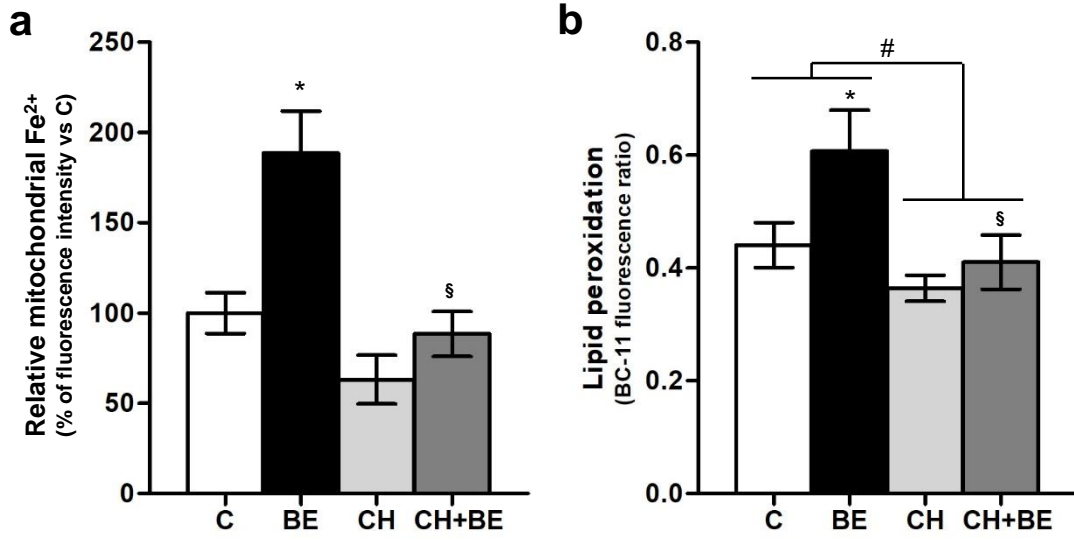
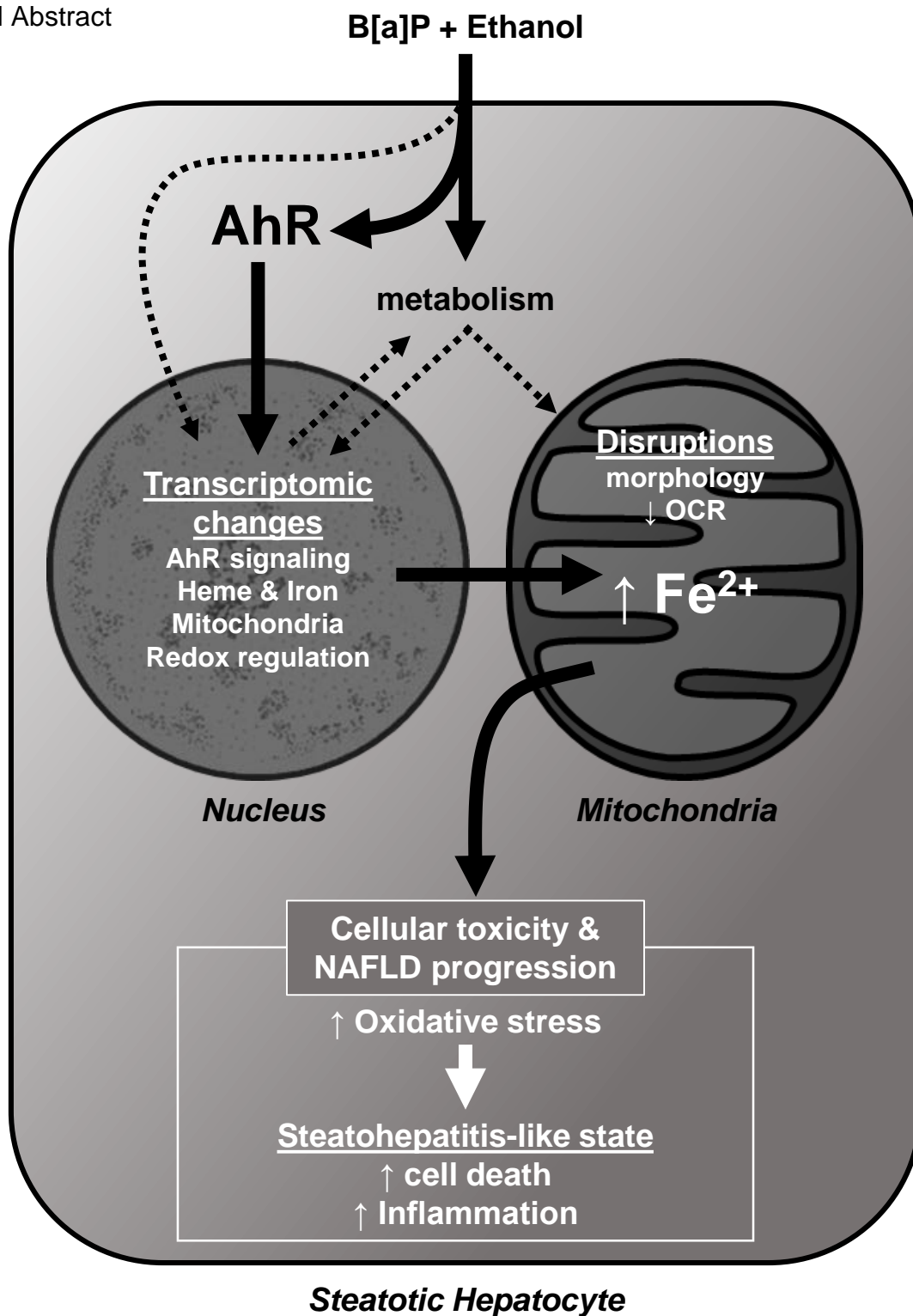


Figure 7



### Highlights

- A transcriptomic approach was used on an *in vivo* model of zebrafish larvae to reveal mechanisms of NAFLD progression induced by B[a]P/ethanol co-exposure.
- B[a]P/ethanol co-exposure aggravates NAFLD through mitochondrial dysfunction.
- AhR activation induced by B[a]P/ethanol co-exposure is responsible of mitochondrial iron overload and oxidative stress involved in toxicant-induced NAFLD progression.